Effects of water temperature on life-history traits of selected South African aquatic insects

By

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"Man is not an aquatic animal, but from the time we stand in youthful wonder beside a spring brook till we sit in old age and watch the endless roll of the sea, we feel a strong kinship with the waters of this world."

- *Hal Borland (1964)*

This thesis is dedicated to my loving parents Shirley and Trevor Ross-Gillespie and to my wife Andrea Ross-Gillespie.

Declaration

PhD thesis title: Effects of water temperature on life-history traits of selected South African aquatic insects: implications for the Ecological Reserve.

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Genetic sequencing was carried out by Dr. Tuuli Mäkinen of the South African Institute for Aquatic Biodiversity in conjunction with the Canadian Centre for DNA Barcoding (CCDB) based at the University of Guelph through the IDRC-IBOL project. GLM scripts for Matlab and R were written by Andrea Ross-Gillespie.

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Abstract

Life-history studies have informed all areas of aquatic ecological research, whilst also providing information relevant for conservation and management of aquatic systems. Given the large research gap that has existed in this regard for Southern Hemisphere lotic systems, there has been an urgent need to gather such data if effective management policies are to be implemented regionally, especially in the face of ongoing development, anthropogenic impacts, and global climate change. Furthermore, there has been a growing awareness of the need to incorporate thermal guidelines into legislation regarding environmental flows and associated water management plans. In South Africa radical new legislation introduced in 1996 resulted in rivers and aquatic ecosystems being given a right to water of their own- essentially environmental flows, required to protect the aquatic ecosystems associated with the water resource, that are determined separately for all or part of any significant water resource. This water, including both the quantity and quality, is referred to as the "Ecological Reserve." Baseline information on the relationship between temperature and life-history patterns of aquatic insects is required to inform the incorporation of thermal guidelines in the Ecological Reserve determination process.

Assuming such information can be gathered, a problem arises as to how the data can be interpreted and incorporated into management guidelines. For instance if representatives of widespread species occurring throughout a country are collected from a single location (say perhaps a single province in South Africa) and then analysed in terms of their thermal limits for growth – would these limits hold true for that same species where it occurs elsewhere? Intraspecific variability, cryptic species and broader phylogenetic constraints all influence the thermal limits of species and need to be considered when examining thermal influences on life-history patterns.

This thesis aimed to test the overarching hypothesis that while the life-history traits of aquatic insects could be constrained to some degree by their evolutionary history, they would also be impacted by thermal and hydrological regimes, inducing a degree of plasticity in their life cycles. This hypothesis was tested by examining the key life-history traits of three representative taxa of aquatic insect, namely *Lestagella penicillata* (Ephemeroptera), *Aphanicercella* spp. (Plecoptera) and *Chimarra ambulans* (Trichoptera), and how they are driven by environmental and genetic factors in six rivers situated in the south-western Cape Province of South Africa. More specifically the objectives of the thesis were to:

1. Select six study rivers that exhibit a range of environmental variability that could invoke life-history plasticity in the same widespread aquatic insect species that inhabit them. Furthermore, to investigate the interaction between flow, temperature and physicochemical variables in these selected rivers and characterise specifically their thermal and hydrological characteristics.
2. Gauge the potential effects of changes to/and variability within hydrological and thermal regimes on aquatic insects commonly used in bioassessment methods (SASS) and used as bioindicators (EPT taxa) that inhabit these six selected study rivers. This would be achieved

through the monthly collection and assessment of fundamental life-history data of the same target species occurring at each of the sites (for the period of a year), which might reveal evidence of phenotypically plastic responses (e.g. changes in voltinism and timing).

3. Gain further insight into lethal and sublethal effects of temperature on these organisms, specifically in terms of upper and lower thermal limits for egg development, time requirement for egg development, and percentage hatch success through laboratory experiments which aid the interpretation of field-collected data. Furthermore to assess nymphal growth rates, upper Lethal Temperature (LT_{50}) mortality, and timing of emergence in individuals of the same species but from different localities reared under the same laboratory conditions to test if less obvious phenotypically plastic responses are evident (e.g. differences in LT_{50} limits or growth rates)
4. Use genetic analyses to evaluate genetic divergence among the subpopulations of the selected species in order to differentiate between phenotypic plasticity and genetic determinism as the basis of life-history responses of the target aquatic species from different study sites.
5. Use these data to contribute towards the establishment of thermal guidelines for the Ecological Reserve which address the relationship between temperature and life-histoty patterns, in order to inform management of riverscapes in South Africa.

Rivers selected for the study showed range of hydrological variability, from a stable/constant and predictable hydrological regime to an unpredictable and seasonally fluctuating hydrological regime (Chapter 2). Temperature data collected during the biological sampling period revealed that the thermal regime indeed correlated well to the hydrological regime of each site –sites that exhibited more stable hydrological regimes also exhibited more stable thermal regimes. Site characterisation showed that sites differed largely in terms of the magnitude, frequency, timing and duration of the thermal and hydrological regimes but were similar in physicochemical properties. In turn this provided a suitable gradient against which to compare life-history traits of selected aquatic insects.

Results of the molecular investigation, using the CO1 gene from target species collected from each of the study sites, presented a prime example of a case where current taxonomy had overlooked cryptic species diversity (Chapter 3). More specifically the data suggested that both *L. penicillata* (maximum of 28.7% CO1 gene divergence among the six study sites and the Table Mountain site) and *C. ambulans* (maximum of 13.5% CO1 gene divergence between Table Mountain site compared to six study sites) populations showed evidence of having diverged to the point where they could be considered to be separate (sibling) species. For *Aphanicercella*, the CO1 gene was able to successfully resolve the four species identified by current taxonomy. The presence of previously undescribed morphologically cryptic species complexes, evolving under different environmental conditions (hydrological, thermal and chemical) at the sites, could account for the divergences observed. However, the effects of incomplete lineage sorting should not be ruled out. The presence of these species complexes could

substantially confound results of life-history studies and experiments of species thermal tolerance limits. In other words, variable egg development responses might be expected for *L. penicillata* populations given knowledge of the evolutionary status of the different populations.

Monthly sampling of invertebrates was carried out for the period April 2009-April 2010 in the six rivers within the Western Cape, during which target organisms collected each month were sorted, counted and measured for life-history analyses (Chapter 4). Differences in the thermal and hydrological regimes among the sites were found to indeed modulate life-history traits, where the same species was concerned, and this was more noticeable in *C. ambulans*. This species exhibited less phylogenetic constraint and more flexibility in terms of its life-history compared to *L. penicillata* and *Aphanicercella* spp. which showed greater phylogenetic constraint and greater adaptation to site-specific conditions – congruent with molecular analyses that showed higher genetic divergence among sites. Voltinism was determined in each of the target taxa: *L. penicillata* and *Aphanicercella* spp. both exhibited a slow, seasonal univoltine cycle with a single cohort easily tracked throughout the year, while *C. ambulans* showed a non-seasonal or asynchronous multivoltine life cycle with multiple generations occurring simultaneously. *C. ambulans* appeared to show a phenotypically plastic response to temperature, in that more generations (trivoltinism) were observed in warmer rivers, in comparison to univoltine populations in colder rivers. Optimal thermal ranges for growth were established through the use of GLMs, and were found to be 13-21.5°C for *L. penicillata*, <11.5°C-14.5°C for *Aphanicercella* spp.,and 14.3°C- >21.5°C for *C. ambulans*). Overall, the life-history responses of the target species assessed in this study appeared to be finely tuned to the hydrological and thermal regimes of each river studied. This could have been as a result of site specific evolution and adaptation, perhaps showing similarities on a catchment scale. However, where the same species showed differences in life-history responses (number and duration of generations) amongst rivers, the data appeared to suggest that water temperature was the most likely factor for these differences. The hydrological regime, on the other hand, was found to be the major driver in determining population size and mortality while possibly imposing a developmental time constraint for life-histories of the study taxa (especially *C. ambulans* and *Aphanicercella* spp.). The possibility that the putative effects of discharge on life-cycle and emergence might reflect synchronicity with the availability of key basal resources, or the effects of seasonal conditions on adult fitness, could however not be discounted and would require further investigation.

In order to better interpret field-collected life-history data (in terms of egg development duration, potential diapause and confirmation of size-class of first-instar nymphs), experiments investigating egg development across a range of water temperatures (5-30°C in 5°C intervals) were carried out in a controlled environment in the laboratory for each of the target taxa (Chapter 5). Water temperature effected the development of eggs of three genera quite differently. Experiments revealed that successful egg development and hatching occurred between 10-20°C for *L. penicillata*, with a high percentage

hatch (80%) at 10, 15 and 20° C treatments. For *A. scutata* successful hatching occurred also between 10-20° C but with reduced hatching success (~30%) at 20°C compared to ~80% hatching success at 10 and 15° C treatments .For *C. ambulans* successful hatching occurred at a wider range of temperatures from 10-25°C but with lower and more variable hatching success at all temperatures (average hatching success ranged from 5-20%). Thermal reaction norms in conjunction with egg hatch parameters showed that *L. penicillata* and particularly *C. ambulans* were warm adapted, while the *Aphanicercella* was cold adapted. Overall, the data presented in this chapter provided valuable information that can be used to inform the establishment of thermal guidelines for the Ecological Reserve in terms of thermal limits for egg development.

Using a novel, thermostatically controlled, flow-through system design, the sublethal effects of temperature on growth rates, body size, and the timing of emergence in conjunction with thermal tolerance limits (using a static LT_{50} experimental procedure) were assessed for two lineages of *L. penicillata* (Molenaars River and Window Stream) at a range of temperature treatments (spanning ~11 to ~27°C) in a controlled common environment (Chapter 6). Experiments evaluated in this chapter were conducted before the genetic analyses (Chapter 3) had been completed and provided insight into the degree to which genetic differences vs. phenotypic plasticity affected the traits that were monitored. Differences in growth rates, number of moults and inter-moult duration were observed when same instar larvae from the two populations were subjected to the same temperature treatment. In both populations, growth rates exhibited a phenotypically plastic response to temperature, yet optimal growth in both populations converged between 16-18°C, similar to those estimated in GLMs (Chapter 4), possibly suggesting that this is a common thermal optimum range for growth in the genus. Observed differences in growth were assumed to be as a result of genetic differences, which were in fact confirmed when molecular investigations in Chapter 3 were completed. The genetic divergence may have arisen through adaptation to differing thermal regimes in the two rivers. The population from the Molenaars River, which incurs a warmer thermal regime, had a higher thermal tolerance limit, higher growth rates and lower mortality at all temperature treatments. This finding suggested that this genus could have retained the same thermal development niche but divergent species develop at different rates (through differing inter-moult duration and or through the addition of moults) in sites exhibiting different thermal regimes.

The data presented in this thesis showed that differences in hydrological and thermal regimes of South African rivers do indeed induce a plastic response in the life-history traits of the representative aquatic insect species in habiting them – particularly in those insect taxa (e.g. *Chimarra*) exhibiting more flexible life- histories and less phylogenetic constraint. This in turn suggests that generalisations in terms of thermal guidelines made at a broad national-scale for the Ecological Reserve might be inappropriate and would at the very least need to be conceived at a regional or even local scale. Furthermore these plastic life-history trait responses should, where possible be evaluated through a

combination of laboratory experiments (common environment experiments for rearing, molecular analyses, egg development experiments) and field work in order to distinguish potential underlying genetic drivers of trait differences vs. true phenotypically plastic responses. This is particularly important as cryptic species complexes, which can often be overlooked by taxonomy, act to confound life-history trait and thermal tolerance studies. Upper and lower thermal tolerance limits for egg development derived for these representative species, in conjunction with LT_{50} values as well as the optimum temperature ranges for growth (obtained from GLM's and rearing experiments) provide fundamental information necessary in the first step of forming thermal guidelines for the Ecological Reserve.

This thesis presents a template of what data are necessary for incorporating thermal guidelines for aquatic insects into environmental flows/Ecological Reserve and how they can be collected and interpreted. The combined field and laboratory approach used in this thesis allowed for an accurate interpretation of the timing and duration of life-histories. The life-history and egg development data presented in this thesis have been used to provide one of the first approaches to modelling the effects of climate change on aquatic insects in South African rivers. Such data, currently not available for South Africa, are essential to inform decision making particularly in relation to the formulation of thermal guidelines for the Ecological Reserve, and for climate change scenario modelling exercises.

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CHAPTER 1

General introduction and thesis overview

1.1 Importance of lotic freshwater ecosystems

Streams and rivers cover only 0.8% of the Earth's surface (Dudgeon *et al.* 2006), a minute proportion when considering that these systems provide what is widely regarded as the most essential natural resource for survival - freshwater - to the human population. Humans are dependent on freshwater for drinking, irrigation water for crops and farms, fish farming, industrial processes, hydroelectric power and other power production methods, sanitation and waste disposal, as well as for recreation purposes. Yet, astoundingly, these systems also accommodate an estimated 9.5% of all animal species known to man including a third of all invertebrate species (Dudgeon *et al.* 2006, Strayer & Dudgeon 2010). The value of these systems is difficult to comprehend in economic terms and is most likely still grossly underestimated by our best attempts. However, almost 15 years ago, the value of the numerous ecosystem services that freshwater ecosystems (stream, rivers and wetlands) provide was estimated at $6.5 trillion USD per year (Costanza *et al.* 1997).

The interaction processes and flow pathways of streams, rivers and groundwaters within their catchments from source headwaters to the ocean, including the numerous human and animal impacts along the way is encompassed in the term "riverscapes" (Fausch *et al.* 2002, Wiens 2002, Stanford 2007, Tetzlaff *et al.* 2007). Riverscapes are physically complex and highly variable in space and time always operating in four dimensions (longitudinal, lateral, vertical and temporal) (Palmer & Poff 1997, Stanford 2007). Coupled with the fact that they occur in high densities over ecologically, geologically and topographically diverse landscapes, they are thus extensive and provide a wide range of habitats for plants and animals (including those that occur in the riparian zone) leading to high biological richness per unit volume (Ormerod 2003). Furthermore the organisms which account for this richness are involved in an array of ecological functions and processes which are necessary to maintain the integrity and ecosystem services provided by these systems (Malmqvist 2002, Durance & Ormerod 2007).

In this regard riverscapes also act as energy and solute transport systems. Solutes and organic matter (both autochthonous and allochthonous) are processed by specialised functional feeding groups of invertebrates which form composite species assemblages adapted to conditions in different parts of the riverscape (Vannote *et al.* 1980). Some material is not used or only partially processed by upstream assemblages. This material is transported downstream and becomes the energy source, along with local inputs, for different composite species assemblages that are specifically structured to capitalise on the inefficient processing of upstream communities (Vannote *et al.* 1980).

A major component of the invertebrate fauna which largely comprise these species assemblages is the highly diverse and ubiquitous aquatic insect fauna (Hynes 1970, Merritt & Cummins 1996). Aquatic insects provide a fundamental link in trophic interactions as they a) are essential role players in processing of organic matter (algae, leaf litter, detritus), b) are an important component themselves of

overall river productivity (Stanford 2007) and c) provide a resource for secondary consumers such as fish.

Aquatic insects are commonly used as bioindicators in biomonitoring schemes because of their general ability to exploit most lotic habitats, as evidenced by their broad distribution ranges, high abundances and diversity. Within the aquatic insects three main orders (Ephemeroptera, Plecoptera and Trichoptera- also known as the EPT taxa) comprise families that are generally more sensitive to water quality, including various types of pollution (e.g. nutrient enrichment, contamination by heavy metals), reduced discharge (in this thesis discharge is also referred to as flow) and oxygenation as well as increased temperature. Various biomonitoring metrics have therefore been developed that incorporate the abundance, richness and ratio of these specific EPT taxa relative to other more tolerant taxa such as the Chironomidae when assessing water quality (see Plafkin *et al.* 1989, Barbour *et al.* 1999, Dallas 2013). Similarly, the South African Scoring System (SASS) for the rapid assessment of water quality (Chutter 1998, Dickens & Graham 2002) utilises a metric of macroinvertebrate presence and abundance (family level). In this widely used protocol, many of the families represented within the EPT taxa are given high sensitivity weightings based on their intolerance of poor water quality conditions (Dickens & Graham 2002, Dallas 2004).

1.2 Factors effecting the integrity of lotic freshwater ecosystems

Riverscapes, while of global ecological importance and responsible for sustaining human life, are also sensitive to external variables and surrounding environmental changes (e.g. shading from riparian vegetation, runoff from surrounding land use) (Poff *et al.* 1997, Poole & Berman 2001, Caissie 2006, Strayer & Dudgeon 2010). As a result they incur a myriad of anthropogenic stressors (Ormerod *et al.* 2010) and are often highly fragmented within landscapes (largely as a result of dams). This fragmentation results in the blocking of natural migration corridors for fish and other aquatic invertebrates, making the whole riverscape vulnerable to climate change (Woodward *et al.* 2010).

The complexity and interrelatedness of environmental variables acting on riverscapes and particularly their aquatic fauna is illustrated in Fig. 1.1. This figure provides a broad template, for reference, of topics covered in this thesis.

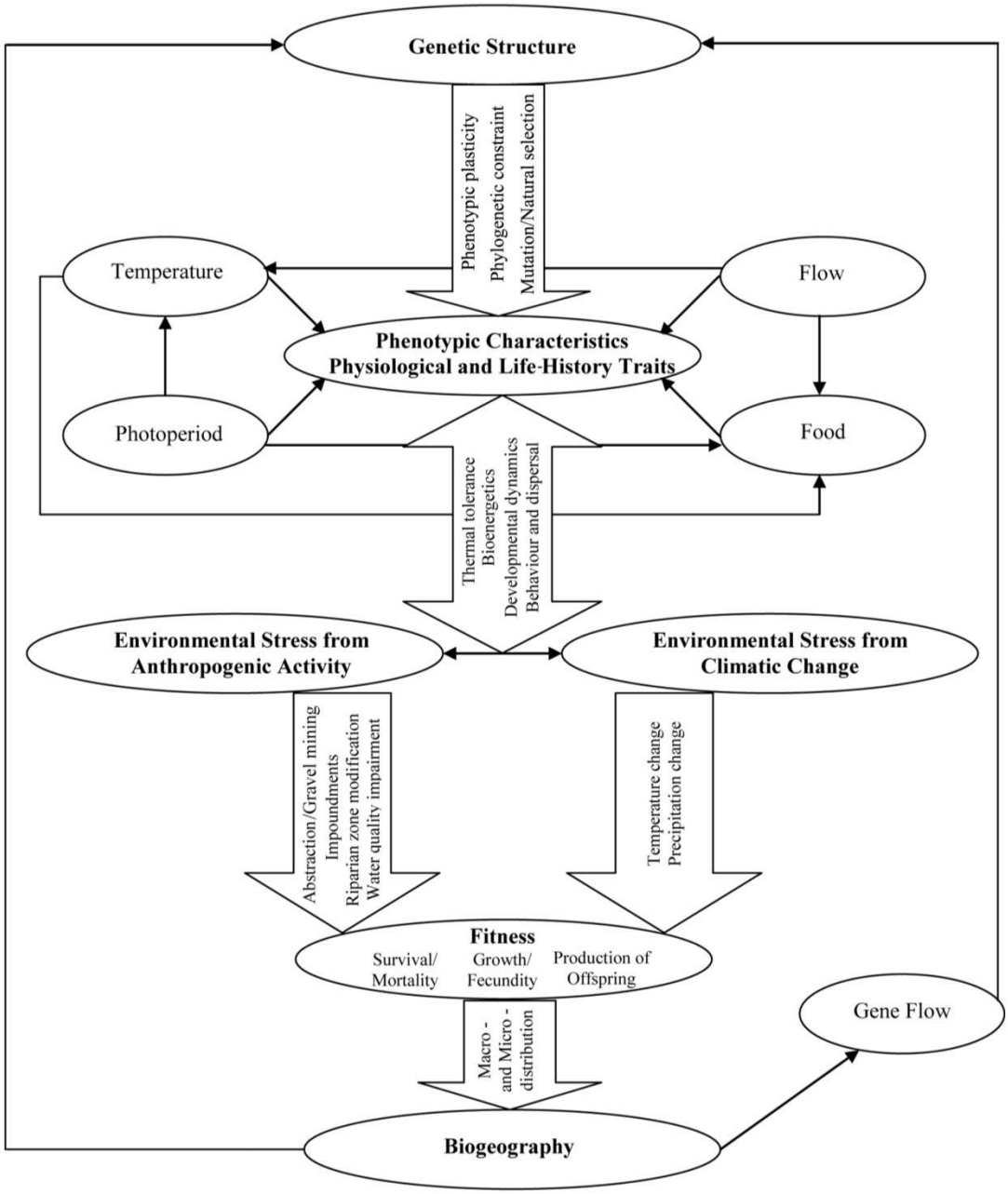

Fig. 1.1. Relationship between environmental variables acting on the biology of aquatic fauna in riverscapes. Adapted from Sweeney *et al.* (1992).

1.2.1 Environmental variables

Riverscapes are influenced by a number of environmental factors including precipitation patterns, watershed topography, underlying geology and soil composition as well as surrounding vegetation and land-use. Flow regime is influenced predominantly by precipitation patterns which influence surface, soil and groundwater inputs (Poff *et al.* 1997). Rivers in areas where precipitation is not seasonal can exhibit stable hydrographs because of the influence of hyporheic or groundwater inputs. Such rivers would be considered groundwater dominated rivers/streams (see Poff *et al.* 1997). In contrast rivers/streams in other areas can be runoff dominated, closely mirroring seasonal precipitation patterns (Poff *et al.* 1997). In certain cases where rainfall has a strong seasonal pattern, the effects of ice and

snow melt produce predictable flow patterns (Poff *et al.* 1997). Flow is responsible not only for nutrient and sediment transport downstream, but ultimately shapes the habitat and substrate properties available to plants and animals in riverscapes, affecting biotic composition and providing a template for adaptation and evolution (Southwood 1977, Poff *et al.* 1997, Bunn & Arthington 2002). In particular, five components of flow viz. the magnitude, timing, frequency, duration and rate of change regulate many ecological processes in riverscapes (e.g. disturbance, nutrient cycling, migration) and can influence abundance, distribution ranges, life-histories and biotic interactions of aquatic insects (Resh *et al.* 1988, Junk *et al.* 1989, Allan 1995, Power *et al.* 1995, Richter *et al.* 1996, Poff *et al.* 1997, Richter *et al.* 1997).

Fluctuations in water temperature on the other hand are largely influenced by incoming solar radiation (linked to latitude and subsequent changes in day length/photoperiod over the seasons) leading to diurnal variations and seasonal ranges. The amount of shading at a site (as a result of overhanging riparian vegetation or steep valley sides) as well as the ability of light to penetrate the water column (affected by turbidity/suspended material of the water) directly impact on the amount of incoming solar radiation and can significantly alter water temperature (e.g. Hynes 1970, Minshall 1978, Rutherford *et al.* 1997). Along with incoming solar radiation (both short wave and long wave), other meteorological conditions such as wind speed, air temperatures and humidity have an effect on evaporative cooling and convective heat transfer (differences between the temperature of the river and the atmosphere causing sensible heat transfer/flux via advection and diffusion) and result in temperature changes at the air/surface water interface (Caissie 2006). Water temperature is also influenced through certain hydraulic conditions acting on both the streambed/water interface and the air/surface water interface. For instance at the streambed/water interface, thermal buffering and dampening can occur from thermally stable cold groundwater inputs or heated geothermal inputs entering a stream channel (Caissie 2006). Similarly, the inflow temperature from dams or from industrial thermal effluents (e.g. heated water from power plants) can have an effect. Additionally, the flow rate, volume of water in the river channel and bottom slope and bed roughness also influence water temperature conditions and heat exchange at the both streambed/water interface and the air/surface water interface (Poole & Berman 2001, Gu & Li 2002, Caissie 2006). Water temperature in turn plays a crucial role in regulating many physical, chemical and biological characteristics of riverscapes, such as metabolic rates, physiology, thermal tolerances and life-history traits of organisms and thus it also affects nutrient cycling and productivity as well as the solubility of organic chemicals, nutrient concentrations and oxygen concentration (Vannote *et al.* 1980, Poole & Berman 2001, Gu & Li 2002, Caissie 2006, Rostgaard & Jacobsen 2005, Webb *et al.* 2008).

Water temperature together with flow are arguably the most important master variables that regulate the ecological processes, determine the overall health of riverscapes and shape the invertebrate communities within them (Coutant 1999, Bunn & Arthington 2002, Jackson *et al.* 2007). As such,

modification of these variables by human activities can have drastic effects on the ecology of riverscapes.

1.2.2 Anthropogenic impacts

The difficulty in balancing the ever increasing demand for water for basic human needs versus the need to effectively conserve and maintain the integrity of riverscapes has led to these systems becoming potentially the most endangered ecosystems on the planet (Dudgeon *et al.* 2006). This is owing to several reasons: 1) riverscapes have already faced a number of species extinctions (Master 1990, Williams & Miller 1990, Ricciardi & Rasmussen 1999, Master *et al.* 2000, DeWalt *et al.* 2005) 2) they stand to face greater declines in biodiversity than terrestrial or marine systems (Ricciardi & Rasmussen 1999, Sala *et al.* 2000), 3) large gaps exist in the knowledge of these systems particularly from parts of the world where they are most threatened (e.g. Mediterranean and tropical regions) (Filipe *et al.* 2013, Dallas 2013) and 4) current approaches and actions by freshwater ecologists and leading freshwater journals have thus far been relatively unsuccessful in spearheading effective conservation (Strayer & Dudgeon 2010).

Human activity, through facilitating access to water, has dramatically altered the natural flow regime of riverscapes. Examples of these alterations include: the construction of dams and interbasin transfers along with other engineering schemes, land cover changes and urbanization of floodplains, canalization and construction of levees, water abstraction for irrigation, as well as groundwater pumping (Poff *et al.* 1997, Poff *et al.* 2007, Vörösmarty *et al.* 2010). Concurrently, deforestation, thermally heated effluents from hydroelectric power plants, hypolimnetic and epilimnetic dam releases, water abstraction as well as channel engineering alter the natural thermal regimes of riverscapes (Poole & Berman 2001, Caissie 2006, Olden & Naiman 2010).

Furthermore, increasing trends in fishery landings from inland waters suggests overexploitation of these resources (Strayer & Dudgeon 2010), while widespread invasions of non-native species have also been facilitated through intentional stocking in such fisheries, as well as through illegal aquarium releases and international shipping (Rahel 2007, Rahel & Olden 2008). Unfortunatley, these non-native species are likely to be more successful in riverscapes that have been modified through dams and canal construction (Bunn & Arthington 2002). This represents a major problem particularly in the Mediterranean-type climate riverscapes of the Cape Floristic Region of the Western Cape (Marr *et al.* 2013). Additional human impacts include: pollution (including nutrient rich runoff from crops or effluents from instream trout farming), mortality (e.g. chemical or organic domestic and industrial effluents), and habitat degradation (e.g. excavation of sand from rivers, sediment deposition resulting from upstream deforestation, rivers running dry for parts of the year owing to water abstraction) (Dudgeon *et al.* 2006).

A recent analysis by Vörösmarty *et al.* (2010) puts into perspective the threat of human activities to global water security and biodiversity. The study yielded an alarming estimate that nearly 80% of the world's population live in threat of freshwater security. Furthermore, the impacts of humans on riverscapes are expected to be exacerbated by the current and predicted effects of global climate change (Poff *et al.* 2003, Durance & Ormerod 2007, Vörösmarty *et al.* 2010, Woodward *et al.* 2010).

1.2.3 Global climate change

Over the past two decades there has been a considerable increase in the number of journal articles addressing the predicted effects of and issues surrounding global climate change on freshwater ecosystems (Strayer & Dudgeon 2010). Riverscapes are particularly vulnerable to predicted global climate change owing to their fragmented nature, overexploitation by humans for goods and services, biological richness and the limited dispersal abilities of aquatic invertebrate fauna (Vörösmarty *et al.* 2010, Woodward *et al.* 2010). The large majority of aquatic fauna of riverscapes are ectothermic. Therefore increasing water temperatures (linked to increasing air temperatures) will have a direct impact on the physiology of ectotherms including their respiration, growth, behaviour, life-histories, bioenergetics, distribution and community structure as well as trophic linkages of these fauna (Sweeney *et al.* 1992, McKee & Atkinson 2000, Winder & Schindler 2004, Harper & Peckarsky 2006, Durance & Ormerod 2007, Ficke *et al.* 2007, Acuña *et al.* 2008, Rahel & Olden 2008, Durance & Ormerod 2009, Flenner *et al.* 2009, Woodward *et al.* 2010, Pace *et al.* 2013). Such temperature increases are expected to favour species invasions of more thermally tolerant exotic taxa, while reducing the success of native taxa and those with narrow thermal tolerance bands, which are often rare or endemic species (Rahel & Olden 2008, Domisch *et al.* 2012). Global climate change estimates predict an extreme scenario of up to 3.4°(2.0-5.4°C) rise in annual average surface temperature and a moderate scenario of up to a 2.4°C (1.4-3.8°C). These estimates are respectively the A2 and B2 model scenarios of the 4th assessment report of the Intergovernmental Panel on Climate Change (IPCC 2007). More specifically, temperature and CO_2 increases are likely to track alterations of seasonal and inter-annual precipitation patterns, leading in some areas to a greater intensity, duration and frequency of extreme climatic events (floods, typhoons, droughts and fires) (IPCC 2007). While global average runoff is expected to increase through global increases in precipitation (Goudie 2006), lower latitudes and Mediterranean-type climates will face severely reduced annual runoff, generally becoming hotter and drier (Midgley *et al.* 2005, IPCC 2007). Overall, the perceived benefits of increased runoff in certain areas will be impacted by the associated higher risks of flooding, lower water quality and shifts in water supply (IPCC 2007).

Mediterranean regions (areas around the Mediterranean Basin as well as the South West Australia, central Chile, coastal California and the south-western Cape Province of South Africa), which are centres of endemism and hotspots for species richness, are characterised by predictable seasonal disturbance events (Gasith & Resh 1999, Filipe *et al.* 2013). They are expected to be amongst the

world's regions most impacted by global climate change (Dallas 2013, Filipe *et al.* 2013). Given the numerous factors impacting riverscapes and their aquatic fauna, the logical question that follows is that of the mitigation of such impacts. An example of one such intervention is the incorporation of "environmental flows" into policies and guidelines for regulated river management both globally and in South Africa (Tharme 2003).

1.3 Balancing conservation and human resource use: the Ecological Reserve

In light of the need to maintain natural flow regimes in rivers, a substantial body of research, largely driven by the works of Poff *et al.* (1997), led to the recognition of "environmental flows." As such, altered rivers or a river earmarked for regulation, can be assessed in order to determine the volume of its original flow that should be maintained to permit ecosystem functioning (Tharme 2003, Arthington *et al.* 2010).

A more precise definition of environmental flows comes from the International Environmental Flows Conference held in Brisbane Australia in 2007 as cited in Arthington *et al.* (2010):

‘‘Environmental flows describe the quantity, timing and quality of water flows required to sustain freshwater and estuarine ecosystems and the human livelihoods and well-being that depend upon these ecosystems’’

The impetus for maintaining environmental flows was primarily the need to provide drinking water for basic human needs whilst conserving ecosystem integrity (Richter *et al.* 2003, Tharme 2003). Tharme (2003) provides a review of various methods involved in environmental flow assessments.

In South Africa the national status of rivers and aquatic ecosystems was upgraded through radical new legislature introduced in 1998, namely the National Water Act (Act No. 36 of 1998) (Republic of South Africa 1998). Through the declaration of the “Ecological Reserve,” this new legislature resulted in rivers and aquatic ecosystems being given a right to water of their own, which they previously did not have. The Ecological Reserve, similar to environmental flows, is defined as:

“...the water required to protect the aquatic ecosystems of the water resource. The Reserve refers to both the quantity and quality of the water in the resource, and will vary depending on the class of the resource. The Minister is required to determine the Reserve for all or part of any significant water resource. If a resource has not yet been classified, a preliminary determination of the Reserve may be made and later superseded by a new one. Once the Reserve is determined for a water resource, it is binding in the same way as the class and the resource quality objectives” (Republic of South Africa 1998).

The legislation recognises the determination process of the Reserve for all or part of a water resource. The Ecological Reserve and basic human needs became the only two sectors in the country which were granted a right to their own water (King *et al.* 2003).

In actually conducting environmental flow and Reserve determinations, particularly in developing countries in arid climates like South Africa, a number of challenges are of course incurred (King & Brown 2006). Some of the more common problems include the lack of data, funding and trained personnel. King & Brown (2006) further describe the major challenges as being: a) the transformation of hydrological data into an ecologically relevant format, b) the provision of quantified predictions of river responses to flow, c) the description of the impacts of river change on common-property users of the rivers, d) the provision of information in a format that decision makes could use and e) the guidance of monitoring as well as adaptive management. The above-mentioned challenges, along with the increasing number of innovative solutions arising from the advancement of technologies, have resulted in this topic becoming an active area of research with a growing body of literature today (see Poff *et al.* 2010, Arthington *et al.* 2010).

Definitions and assessments of environmental flows, including that of the Ecological Reserve, are concerned primarily with water quantity. One major shortcoming of this is that they do not take into account several components of water quality, in particular water temperature (Olden & Naiman 2010), despite its importance as a major driving variable of ecosystems (Poole & Berman 2001, Caissie 2006). More recently, however, there has been a push to incorporate thermal regimes into environmental flow assessments (globally, see Olden & Naiman 2010) and into the Ecological Reserve (in South Africa, see Dallas *et al.* 2012, Rivers-Moore *et al.* 2013a).

Olden & Naiman (2010) propose five major challenges to incorporating water temperatures into environmental flow assessments, especially with regard to regulated (dammed) rivers:

1. Advancing our understanding of dam-induced impacts to riverine thermal regimes
2. Advancing our understanding of the ecological consequences of altered thermal regimes
3. Demonstrating the availability and success of temperature management strategies
4. Incorporating thermal-criteria into environmental flow assessments
5. Designing temperature-enlightened environmental flow assessments in a changing climate

The acquisition of high resolution long-term data sets of environmental variables (rainfall, air temperature, water temperature, flow rates/discharge) is therefore essential to a) assessing the variability of individual rivers, b) understanding the link between temperature and flow in riverscapes across the country, c) understanding the role that temperature and flow play in shaping ecological processes in riverscapes, and d) investigating the effects of temperature and flow at the level of

individual organisms/communities through the collection of fundamental biological information such as life-history data and thermal tolerance limits (Olden & Naiman 2010, see also Webb *et al*. 2008).

1.4 A South African perspective

Historically, the relatively small body of work available for South African river ecosystems (in comparison to the Northern Hemisphere) has been of concern (Dallas 2008, Filipe *et al.* 2013), especially given the importance of understanding the combined impacts of temperature and flow on riverscapes in decision- and policy-making processes. While studies on flow have perhaps received more attention than those focusing on water temperature (e.g. King *et al.* 2000, Hughes & Hannart 2003, King *et al*. 2003, Tharme 2003, King & Brown 2006, Rivers-Moore *et al.* 2007) the need for relevant long and short term South African water temperature data, as well as biological data for aquatic organisms, is evident.

This said, researchers have made a considerable contribution towards addressing this data paucity through funding received from the Water Research Commission (WRC) of South Africa. Particularly noteworthy studies include:

- the provision of baseline temperature profiles for a large number of rivers across the country (Dallas *et al.* 2012),
- the investigation of links between flow, air temperature and water temperature through modelling approaches (Rivers-Moore *et al.* 2008a, 2008b, 2012),
- the development of metrics for assessing variability of ecologically important variables derived from water temperature data (similar in concept to Indicators of Hydrological Alteration (IHA) developed by Richter *et al*. 1996) (Dallas *et al.* 2012, Rivers-Moore *et al.* 2012),
- experimental determination of Incipient Lethal Upper Temperature (ILUT) limits and Critical Thermal Maxima (CTM) of a wide range of aquatic invertebrates throughout the Western Cape (Dallas & Rivers-Moore 2012, Dallas & Ketley 2011),
- the exploration of micro-scale heterogeneity in water temperature (Dallas & Rivers-Moore 2011),
- the investigation of the effects of water temperature predictability on macro-invertebrate assemblages (Eady *et al.* 2013),
- the setting of environmental water temperature guidelines for management purposes (Dallas *et al*. 2012, Rivers-Moore *et al.* 2013a) and
- practical applications in the form of utilising biological information (i.e. life-history information) in climate change scenario based modelling (Rivers-Moore *et al*. 2013b).

1.4.1 What and where are the gaps?

While this aforementioned valuable body of work provides the initial steps towards what is likely to become an important growing field of research in the short-term future (Webb *et al*. 2008), the following gaps in the knowledge-base are evident:

- High-resolution, long-term (ideally greater than five years) water temperature datasets are needed for rivers across the country that can provide reference profiles for the rivers[1].
- Further elucidation is required of the complex interactions between flow and temperature to better understand the seasonality, variability and predictability of riverscapes.
- Experimental determination of thermal tolerance limits should be conducted for a greater number of aquatic organisms from different regions across the country. Note that work on this has commenced (Dr. H. Dallas, pers. comm., University of Cape Town, 2013).
- Much work is required on the sublethal effects[2] of temperature on aquatic organisms across the country. Specifically this involves collecting data (both through field sampling and laboratory experiments) on life-history cycles, egg development, secondary productivity and bioenergetics, thermal preference ranges, thermoregulatory behaviour, growth rates and timing of specific life-history traits (i.e. hatching and emergence), and relating these to temperature.
- Further investigation is required into species assemblages, community responses (such as composition, abundance distribution ranges) and trophic interactions in relation to temperature.
- Additional taxonomic revisions, genetic analyses and phylogenetic studies are needed to aid species identification, particularly for immature stages and morphologically uniform taxa.

1.5 Research aims

Given the difficulty in setting thermal guidelines for the Ecological Reserve in South Africa owing to a) the fact that fundamental life-history data and thermal tolerance data for aquatic insects coupled with thermal and hydrological data are severely lacking in South Africa and b) that widespread species may exhibit different thermal tolerance ranges and optima in different parts of their distribution in the country thus making generalisations of suitable temperature ranges difficult, the research presented in this thesis aims to test a single overarching hypothesis. This hypothesis is: that while the life-history traits of aquatic insects could be constrained to some degree by their evolutionary history, they would also be impacted by thermal and hydrological regimes, inducing a degree of plasticity in their life cycles. In testing this hypothesis the thesis also aims to address firstly, some of the research gaps highlighted in the previous sections in order to build up local knowledge for better understanding and

[1] Long-term datasets of flow are available for many rivers across the country and do not represent a gap as such. However, continued maintenance of weirs and monitoring systems is essential, as well as upgrading the existing infrastructure with newer technologies and increasing the number of rivers monitored.

[2] Sublethal effects refer to those that do not kill a cell or organism, but usually forces adaptation for survival (McGraw-Hill 2002)

management of South African river ecosystems, and secondly to add information on environmental determinants of life-history patterns to a global wealth of knowledge from a geographical region where relatively few studies have been undertaken thus far. In order to test the aforementioned overarching hypothesis, the specific objectives of the individual chapters were:

1. Investigate the interaction between flow, temperature and physicochemical variables, through the selection of six study rivers exhibiting a range of thermal and hydrological characteristics.
2. Gauge the potential effects of changes to hydrological and thermal regimes on aquatic insects commonly used in bioassessment methods (SASS) and used as bioindicators (EPT taxa) through the collection and assessment of fundamental life-history data.
3. Gain further insight into lethal and sublethal effects of temperature on these organisms, specifically in terms of growth rates, mortality, egg development and timing of emergence, through conducting laboratory experiments (in addition to the interpretation of field data).
4. Use genetic analyses to define species boundaries and explore the possibility of extensive genetic divergence as well as potential phenotypically plastic life-history responses of the target aquatic species from the different study sites.
5. Contribute towards the establishment of thermal guidelines for the Ecological Reserve, in order to inform management of riverscapes in South Africa.

In addition to the primary aims listed above, the range of rivers selected for the study intends to provide a platform on which to investigate possible predicted effects of climate change impacts on river systems and aquatic organisms, and further to provide data that are necessary for scenario-based modelling of climate change.

1.6 Chapter outline

Chapter 1: General introduction and overview of thesis

Chapter 2: Site selection and site characteristics: selecting hydrological and thermal regime gradients as a platform for life-history trait investigations.

Flow and temperature are introduced as major drivers of the ecology of riverscapes. This chapter also provides the rationale for the study site selection procedure and gives detailed site descriptions. On the assumption that flow and water temperature are correlated, site selection prior to sampling is based on flow data as temperature data did not exist at the time. Subsequent collection of water temperature data for the six sites during the period of sampling is used to support this assumption and provide site characteristics. Analyses of flow and temperature data are undertaken using metrics and multivariate analyses to elucidate the links between these two variables, as well as to characterise the study sites in terms of seasonality, variability and predictability. The thermal and hydrological profiles of the rivers presented in this chapter form a backdrop for data interpretation for the remainder of the thesis.

Chapter 3: Molecular investigations of *Lestagella penicillata*, *Aphanicercella* spp. and *Chimarra ambulans*: genetic profiles for interpreting life-history traits.

The three target taxa (from the EPT) that form the focus of this thesis are introduced in Chapter 3. Molecular analyses are used to compare the degree of genetic divergence of target taxa amongst study sites, in order to evaluate the relative contributions of intraspecific phenotypic plasticity vs. extensive genetic divergence when interpreting data in later chapters. The molecular analyses also allow for current taxonomic work on these taxa to be evaluated as well as for species identification and taxonomic descriptions of immature stages.

Chapter 4: Environmental modulation of life-history patterns.

Life-history data collected for the target taxa are presented in this chapter and are analysed in relation to temperature, flow and water quality variables through the use of regression analyses and generalised linear modelling techniques (GLMs). The results of the analyses allowed for the timing of specific life-history traits (i.e. hatching and emergence) to be determined, and for optimal growth conditions in natural populations to be explored and contrasted for the different study sites.

Chapter 5: The role of temperature in egg development.

This chapter presents experimental data on the embryogenesis, egg developmental period and hatching success for each of the target taxa under different temperature treatments. The results are used to provide a more accurate interpretation of field collected life-history data for the same species, and to inform the sublethal effects of temperature on these taxa as defined by thermal limits and optima for egg development and hatching. Findings are discussed in the context of thermal adaptation and the evolution of life-history traits.

Chapter 6: The role of temperature in larval growth rates, survival and adult emergence of *Lestagella penicillata*

Further information on sublethal and lethal effects of temperature on one of the target taxa is provided. Growth experiments were conducted under differing temperature treatments in order to better interpret life-history data collected from the field, and to investigate differences in growth between individuals from two thermally contrasting rivers. Thermal optima for growth obtained in the laboratory experiments are used to verify those obtained in the GLM assessments. The growth experiments served the additional purpose of providing a suitable setup for conducting a concurrent long-term Lethal Temperature (LT_{50}) experiment to determine upper temperature limits for survival.

Chapter 7: General discussion and synthesis

CHAPTER 2

Site selection and site characteristics: selecting hydrological and thermal regime gradients as a platform for life-history trait investigations

Summary

Given the importance of linking and also understanding biotic responses to abiotic variables within riverine ecosystems (as highlighted by Rivers-Moore *et al.* 2010) especially in terms of developing thermal guidelines for the Ecological Reserve, this thesis aims to address a critical component necessary for this objective to be realised - specifically the link between hydrological, as well as thermal regimes and the life-histories of selected aquatic insects. This chapter served as the initial step in this process. Six study sites that exhibited a gradient in terms of their hydrological and thermal regimes were selected for biological sampling. These variables were considered to be the most important in determining the timing and nature of life-histories of aquatic insects. In the absence of historical daily temperature data, however, these sites were selected based on multivariate and time-series analyses of historical daily flow data only, on the assumption that flow and water temperature are closely related – thus a variable hydrological regime should correlate to a variable thermal regime. The subsequent collection of water temperature data at each of the selected sites over the duration of the biological sampling period (1year) were then used to test this assumption, using newly developed thermal metrics to characterise the time-series, taking into account frequency, duration and timing of thermal states. Sites were also characterised using historical flow data and water temperature data as well as physicochemical data collected during the biological sampling period. The thermal and hydrological characterisation of the study sites served as the backdrop against which to contrast the life-history traits and molecular analyses of selected aquatic insects presented in the chapters that follow. Selected sites showed range of hydrological variability, from a stable/constant and predictable hydrological regime to an unpredictable and seasonally fluctuating hydrological regime. Temperature data collected during the biological sampling period revealed that the thermal regime indeed correlated well to the hydrological regime of each site –sites that exhibited more stable hydrological regimes also exhibited more stable thermal regimes. Site characterisation showed that sites differed largely in terms of the magnitude, frequency, timing and duration of the thermal and hydrological regimes compared to physicochemical properties. In turn this provided a suitable gradient against which to compare life-history traits of selected aquatic insects.

2.1 Introduction

2.1.1 The importance of flow and temperature in characterising lotic ecosystems

Owing to constant interactions with the physical environment as it gradually changes from source to mouth, lotic ecosystems are subject to continual change, making them highly variable in space and time (Hynes 1970, Vannote *et al.* 1980, Palmer & Poff 1997, Gordon *et al.* 2004). Lotic ecosystems incur a frequency and intensity of environmental changes (i.e. high variability) that is not commonly observed in other ecosystems (Power *et al.* 1988), with the disturbance regime playing a pivotal role in structuring these systems (Fisher *et al.* 1982, Resh *et al.* 1988, Lytle *et al.* 2008). Globally, only relatively recently has the importance of maintaining variability within these systems been considered within a management context (Poff *et al*. 1997, Richter *et al.* 1997, Reynolds 1998, Arthington *et al.* 2006, Caissie 2006, Naiman *et al.* 2008)

As such, the classification and characterisation of these systems within and among broad geographic areas, ecoregions or even climates is of considerable interest and importance to freshwater biologists and managers alike and remains an area of debate (see for example Biggs *et al.* 1990, Rosgen 1994, Kondolf 1995, Thomson *et al.* 2001, Caissie 2006, Rivers-Moore *et al.* 2008a). One of the reasons for this lies in the fact that such a wide range of variables can be used to classify and characterise these systems (e.g. biological indicators, geomorphological features, flow characteristics and also water quality indicators). The question thus arises: Which variables are most useful for characterising or profiling rivers, especially in an ecological context?

Flow, water temperature, physical structure, longitudinal continuum, macro-nutrient availability and water quality are considered the most important variables in lotic ecosystems (Hynes 1970). Of these variables however, flow and water temperature have generally received the most attention in limnological research as they act master drivers of ecological and biological processes and they constitute an integral component of the variability experienced in these systems (Poff & Ward 1989, Tockner *et al.* 2000, Rivers-Moore *et al.* 2008a, 2008b). Considering the importance of understanding the link between abiotic and biotic processes for river management, as well as the need to establish and maintain "natural" ranges of variability in rivers (Dallas 2008, Rivers-Moore *et al.* 2008a), it is apparent that long-term data sets pertaining to flow rate and water temperature alone are crucial for river managers and biologists. This is owing to the fact that they can provide a) a good indication of overall river condition and or deviations from reference states, b) an indication of the aquatic communities likely to be present in the river, c) insight into the temporal variability (seasonal and annual) of the system, and d) suitable means by which rivers can be classified, characterised or profiled.

Links between flow and temperature have been demonstrated in several studies (e.g. Schlosser 1991, Arscott *et al.* 2001, Langan *et al.* 2001, Gu & Li 2002, Webb *et al.* 2003, Rivers-Moore & Jewitt 2004,

Rivers-Moore & Lorentz 2004, Rivers-Moore *et al.* 2005, Rivers-Moore *et al.* 2013a among others), where it has been found that a) low flow conditions ultimately lead to higher water temperatures with low concentrations of dissolved oxygen, b) flow rate and discharge are as significant as meteorological effects in controlling water temperature c) altered flow regimes generally lead to altered thermal regimes and d) daily maximum temperature is more sensitive to flow rate than daily mean water temperature.

Groundwater input has been shown to affect both the hydrological and thermal regime, generally producing more stable regimes in both cases when compared to rivers that are dominated by surface run-off (Sear *et al.* 1999). Groundwater inputs have also been linked to a high baseflow index[3] as well as a lower co-efficient of variation (C.V.) in flow (Vannote & Sweeney 1980, Sear *et al.* 1999). Furthermore groundwater has been shown to affect the thermal heterogeneity at river sites (Mosley 1983) and even within different habitat types at a site (e.g. riffles - see Evans & Petts 1997).

Given the importance of flow and temperature, but notwithstanding the effects of groundwater, these two variables were used as the basis for the site selection procedure presented in this chapter. While historical daily flow data (in some cases dating as far back as 1972) are readily available for many rivers across the country through the Department of Water Affairs Hydrological Information System (HIS), no high-resolution long-term data sets exist for water temperature and in this study flow had to be used as a proxy for water temperature. The collection of reliable long-term water temperature data sets (~10yrs minimum) for rivers across a broad geographical and geological scale (i.e. from as many different ecoregions in South Africa - Kleynhans *et al.* 2005) is therefore a necessity. Analysis of available flow data requires dealing with time series data which in itself poses a challenge.

2.1.2 How to deal with time series data

One method of characterising time series data of flow and water temperature is to consider the co-efficient of variation, a straightforward and robust approach especially in relation to the length of the data set, but which is sensitive to extreme values (which often tend to be present in hydrological data sets, and to a lesser degree in thermal data sets) (Poff 1996). An alternative is to consider using Colwells' (1974) indices of predictability which are less sensitive to extreme values and thus more suited for detecting subtle differences in seasonality or hidden periodicities in a range of data types (Stearns 1981, Rivers-Moore *et al.* 2008a)[4]. A combination of both of these indices, however, generally proves to be most informative, and they have been incorporated into a very useful software package developed by Richter *et al.* (1997) (Indicators of Hydrological Alteration -IHA) for analysing time series of daily flow records for rivers. Recently, similar indices (and the automated calculation thereof)

[3] A measure of the amount of flow in a river during dry or low flow periods calculated as the ratio of annual baseflow to total annual run-off.
[4]More details regarding Colwell's indices of predictability are provided in the Methods section.

were incorporated into an excel spreadsheet, using a series of macros, for the purpose of analysing time series of daily temperature records (see Rivers-Moore *et al.* 2008a). Collectively IHA and the metrics developed by Rivers-Moore *et al.* (2008a) offer the simplest means of holistically characterising system variability or for profiling South African rivers based on thermal and hydrological variables.

Another useful method for monitoring and analysing time series data is 'the 'Sequential T-test Analysis of Regime Shifts" algorithm (STARS v3.2 http://www.beringclimate.noaa.gov/regimes/: Rodionov 2004, Rodionov & Overland 2005). While being one of several methods used to identify and analyse change-points or regime shifts in time-series data (see Easterling & Peterson 1995 and Lanzante 1996 for other methods), STARS is particularly practical because a) it can be used to process data in real time or as it becomes available, b) it requires no visual inspection of the time-series, c) it can be used with no *a priori* hypotheses regarding the timing of shifts/change-points, d) can be used on data with multiple shifts/change-points, e) can be used to analyse time-series regardless of whether the input data are in the form of anomalies or absolute values, and f) is capable of detecting shifts/change-points towards the end of time-series, allowing for substantially shorter time-series to be analysed. An additional component to the STARS algorithm, termed "prewhitening" also allows for the correction of autocorrelation which can often be a problem in time-series data (Rodionov 2006). The output from such an analysis compliments time-series analyses using Colwell's predictability indices as well as measures of C.V., by allowing for a finer scale interpretation of the timing of significant changes in hydrological and thermal datasets.

Regimes shifts can be detected using STARS in both the mean values and the variances for a given data set (Rodionov & Overland 2005), and several studies have highlighted the application of STARS in detecting shifts in time-series of both environmental and biological data (see for example Marty 2008, Kamburska & Fonda-Umani 2009, Megrey *et al.* 2009). While STARS has commonly been used to detect shifts in much longer time-series (decadal), it has also been used to detect shifts over shorter time periods of only 1-3 years (see Howard *et al.* 2007) making it suitable in situations where limited data are available.

2.1.3 Aims

The aim of the site selection procedure was to select six study sites that differed in terms of their thermal and hydrological regime. Given that historical water temperature data are not available for South African rivers, the selection was based on multivariate analyses of metrics and variables derived from historical daily flow data only, under the assumption that flow and water temperature are correlated. The subsequent collection of water temperature data along with flow data for the six sites during the biological field sampling period was then to be used to verify this assumption. These data, in conjunction with analyses of historical flow data, were to provide information regarding site characteristics in terms of thermal and hydrological variability. The six rivers should exhibit different

degrees of thermal variability or predictability[5] (see Methods section for a more detailed definition) that could be used as the backdrop against which to compare biotic responses such as growth, life-history responses and timing of specific life-history traits (*viz.* hatching/emergence) in key aquatic insect taxa. Such information has been highlighted by Dallas (2008) and Rivers-Moore *et al.* (2008a) as being crucial to the initial step of determining the temperature component of the Ecological Reserve as well as developing thermal guidelines for water managers in South Africa. The aim of this study is thus twofold:

1) To select study sites (based on historical flow data) that exhibit a range of mean annual water temperatures and differing degrees of thermal variability/periodicity. Such a selection will enable the testing of the assumption, that sites exhibiting variable or stable flow regimes should similarly show variable or stable thermal regimes. This is based on findings by Rivers-Moore *et al.* (2008a, 2008b) who examined the links between thermal and hydrological data for other South African river systems.

2) To profile the selected study sites in terms of flow and temperature, using techniques such as STARS, IHA and Colwell's predictability indices, combined with additional measurements of physicochemical variables to establish a basis for further investigations of the life-histories of aquatic insects occurring at these sites (Chapter 4). In turn these investigations will help inform the establishment of thermal guidelines for the Ecological Reserve.

2.2 Methods

2.2.1 Site selection procedure

Data for site selection

A total of 11 perennial rivers in the Western Cape were pre-selected, taking into account the following considerations: occurrence within roughly the same ecoregion[6] (level 1 ecoregion as defined by Kleynhans *et al.* 2005), the presence of an active flow gauging station near the site, the presence of a previously installed (December 2008) water temperature logger (see Dallas & River-Moore 2010), a relatively natural condition as a reference state, the site accessibility and also the travelling distance to the river. Each of these 11 pre-selected rivers had active flow gauging stations between 100 and 500m downstream of the proposed sampling site. Historical daily flow/discharge data (averaged to m^3/s) for each of these rivers were obtained from the Department of Water Affairs (www.dwa.gov.za). Where possible at least 20 years' worth of historical data (from 1988-2008) were obtained for each river.

Historical flow data from the 11 pre-selected sites were subsequently analysed using *Indicators of Hydrological Alteration* (IHA) Ver. 7 software developed by Richter *et al.* (1996). This software uses

[5] Predictability is defined as complete certainty regarding a specific state for a given time.
[6] A classification/typing of an area based on several environmental factors. In South Africa, physiography, climate, geology, soils and potential natural vegetation, have been used as the delineators of Level I ecoregions.

daily flow data collected over a user-defined period of years (normally a minimum of 20 years daily flow data is suggested for Colwell's indices to be meaningful (Dr. Nick Rivers-Moore, pers. comm., University of Kwa-Zulu Natal, 2009)) to derive 33 ecologically relevant hydrological parameters based on a number of calculation procedures and threshold limits (see Table App2A.1, Appendix 2A). It also uses Colwell's indices to give an estimation of predictability and constancy for daily flow time series data.

After conducting an initial data analysis with IHA on all 11 sites, sites with large amounts of missing or interpolated flow data were removed. Additionally, sites showing similar or overlapping flow characteristics (i.e. not contributing to a wide range of flow variability) were also removed. In this manner site selection was narrowed down from 11 pre-selected sites to six sites. The results obtained from the IHA analysis relating to the 33 hydrological parameters provided the data that were used to assess these six selected sites using multivariate techniques namely Principle Coordinate Analyses (PCO or PCoA) and Cluster Analyses.

Definition of predictability

Colwell's predictability indices, owing to the fact that they are not sensitive to extreme values, have been shown to be useful tools for detecting periodicities in non-metric, ordinal, as well as nominal data and also for interpreting time-series data such as flow regimes and thermal regimes in an ecologically meaningful manner (Stearns 1981, Poff 1996, Richter *et al.* 1996, Rivers-Moore *et al.* 2008a).

Indices derived by Colwell (1974) can be used to classify temporal patterns into a measure of predictability (P) based on the degree of the two separable components, constancy (C) and contingency (M). These components have different implications for the ecology of systems (Colwell 1974). Constancy will be high when a certain state (of a measured variable) remains the same over time, while contingency is high when certain states fluctuate in a strongly cyclic or periodic manner corresponding to specific time periods. In this regard predictability can be high as a result of either high constancy (a predictably stable system) or high contingency (a predictably seasonal system). Similarly, predictability will be low for a system when both the contingency and constancy scores are low (i.e. when state is variable with no obvious seasonal trends). Predictability (P) is reported on a scale of zero to one with the two components (C) and (M) commonly reported as percentages of overall predictability using the following the equation:

$$\text{Predictability } \mathbf{(P)} = \text{Constancy } \mathbf{(C)} + \text{Contingency } \mathbf{(M)} \qquad (2.1)$$

When there is complete certainty regarding a specific state for a given time, the system is considered predictable with a predictability score of one.

Statistical analyses for site selection procedure

Multivariate techniques were employed to determine the range of hydrological variability exhibited by the six selected study sites, the similarities between the sites in terms of flow parameters, as well as the specific flow parameters responsible for the observed similarities. Prior to conducting the multivariate analyses, the 33 parameters used in IHA were assessed using a simple correlation matrix, and variables showing high multicollinearity (>0.8) were removed (see Table App2A.2, Appendix 2A).

Following the removal of correlated variables, data were then normalised and sites were analysed using a cluster analysis technique (group average relatedness) based on the Euclidean distance measure. An unconstrained ordination of multivariate data or a PCO, using the Euclidean distance resemblance measure, was then used to determine which explanatory variables contributed the most to the dissimilarity between the sites and to partition the amount of variability that is explained in the data according to these variables. All multivariate analyses were performed using PRIMER 6+PERMANOVA software package from Plymouth Marine Laboratory, UK (Clarke & Gorley 2006, Anderson *et al.* 2008).

2.2.2 Field sampling

The location of each of the six rivers selected for sampling based on the site selection procedure in section 2.2.1 is indicated in Fig. 2.1.

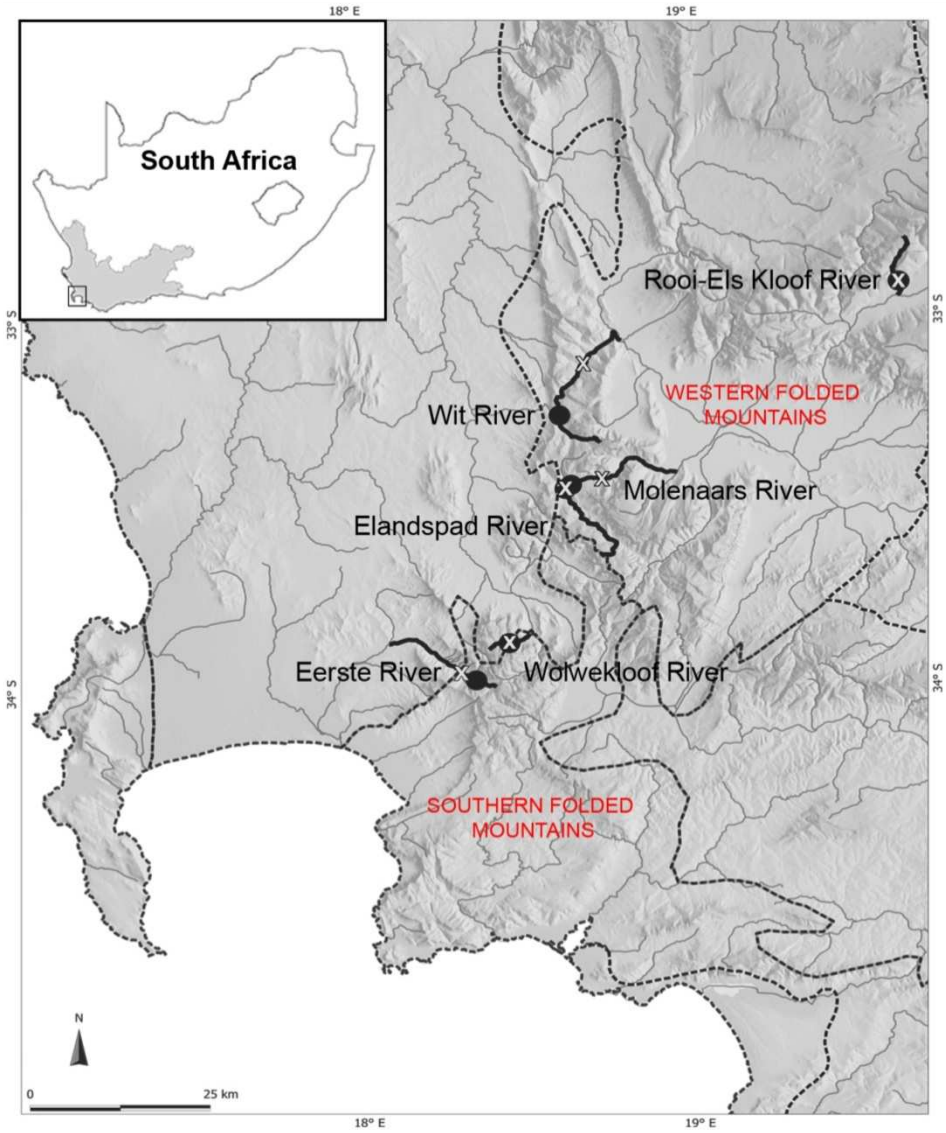

Fig. 2.1. Gauging stations (white crosses) and sites (black circles) at which monthly aquatic invertebrate samples were collected from April 2009 to April 2010. The level 1 ecoregions (Western Folded Mountains, Southern Folded Mountains) (Kleynhans *et al.* 2005) within which the sites occur are bounded by dashed lines.

Study site descriptions

The six sites selected for the study all occur within the relatively unimpacted upper reaches/mountain stream headwaters of their respective rivers. This is in line with the suggestions of Dallas (2009) to focus work of this nature in areas unaffected by anthropogenic activity. Physical, geographical and geomorphological characteristics of the sites are presented in Tables 2.1, 2.2 and 2.3. All of the rivers occur in the winter rainfall region of the Western Cape, are perennial and can be classified as oligotrophic fynbos streams. Furthermore, the rivers exhibit a single thread low sinuosity channel pattern, a relatively uniform cobble/boulder substrate and identical underlying geology (*viz.* quartzitic sandstone, subordinate shale and tillite) (Vegter 1995) but vary in terms of aspect, canopy cover and wetted surface channel width, which ranges from 2m to 10m (during summer) and from 5m to 40m (during winter high flow periods). Seasonal differences in flow experienced at each of the sites are shown in Fig 2.2.

Field sampling and data collection

Biological sampling at the six selected sites took place every month from April 2009 to April 2010. During these monthly site visits measurements of a number of physicochemical variables were also recorded.

Standard water quality variables included pH (Crison PH 25 portable pH meter), electrical conductivity (EC) (Crison CM 35 portable conductivity meter), dissolved oxygen (DO) (Crison OXI 45 portable oxygen meter) and turbidity (Eutech TN 100 Portable turbidimeter). Spot measurements of temperature were also collected on site, prior to biological sampling. Three random measurements taken at different locations along the stretch of river at each sampling site, every month, were used to calculate mean monthly values for all measured physicochemical variables. Measurements were taken at different times of the day across the sites, but the time of recording for a particular site was roughly constant for each sampling occasion.

In addition to the collection of water quality variables, wetted surface channel width (or active channel width) measurements were recorded from three fixed positions (roughly 25m apart) at each site every month. The three fixed positions were located at the downstream end, the middle, and the upstream end of each site. In months where the river at the site could not be crossed to obtain channel width measurements, observations of water levels on each bank were noted and measurements were then taken the following month once the flow had subsided sufficiently.

Tables 2.1, 2.2 and 2.3. Physical and geographical information (**2.1**), geomorphological characteristics (**2.2**) and channel morphology characteristics (**2.3**) pertaining to the six study rivers selected for life-history analyses from the Western Cape, South Africa. Information taken with permission from Dallas & Rivers-Moore (2010). Downstream is denoted by d/s.

2.1

River	Latitude	Longitude	Ecoregion I (after Kleynhans *et al.* 2005)	Longitudinal zone (after Rowntree & Wadeson 2000)	Stream order	DWA gauging station code
Eerste	-33.993776	18.975550	Southern Folded Mountains	Mountain Stream	1	G2H037
Elandspad	-33.736667	19.114722	Western Folded Mountains	Transitional	2	H1H033
Molenaars	-33.731390	19.115000	Western Folded Mountains	Transitional	2	H1H018
Rooi-Els Kloof	-33.461100	19.617860	Western Folded Mountains	Mountain stream	1	H2H005
Wit	-33.637090	19.107890	Western Folded Mountains	Transitional	1	H1H007
Wolwekloof	-33.944167	19.026389	Southern Folded Mountains	Mountain stream	1	G1H038

2.2

River	Altitude (m. a. s. l.)	Aspect (degrees)	Median groundwater levels (m) (after Colvin *et al.* 2007)	Condition of local catchment
Eerste	380	280	8	Afforestation, alien vegetation, aquaculture d/s, recreational, nature conservation
Elandspad	450	125	7	Wilderness area
Molenaars	440	110	7	Alien vegetation, roads, recreational, nature conservation
Rooi-Els Kloof	481	80	19	Agriculture (crops), wilderness area
Wit	660	180	9	Nature conservation
Wolwekloof	330	115	7	Afforestation, felled area, alien vegetation wilderness area

2.3

River	Channel modifications	Channel type	Canopy cover	Active channel width (m)
Eerste	Causeway and bridge d/s	Alluvial with dominant type: Boulder	Partially Open	5 to 10
Elandspad	Weir d/s	Mixed bedrock and alluvial: Boulder	Open	10 to 20
Molenaars	Bridge	Mixed bedrock and alluvial: Boulder	Partially Open	20 to 50
Rooi-Els Kloof	Weir d/s	Mixed bedrock and alluvial: Cobble, boulder	Partially Open	5 to 10
Wit	None	Mixed bedrock and alluvial: Boulder	Open	20 to 50
Wolwekloof	Large dam d/s, small weir d/s	Mixed bedrock and alluvial: Boulder	Open	10 to 20

Fig. 2.2. Six rivers selected for life-history studies within the Western Cape, South Africa showing (A) summer and (B) winter flow conditions. The rivers from left to right from the top are: (1) Eerste (facing upstream), (2) Elandspad (downstream), (3) Molenaars (upstream), (4) Rooi-Els Kloof (downstream), (5) Wit (upstream) and (6) Wolwekloof (downstream).

Water chemistry data for each of the study sites was obtained from the Department of Water Affairs (www.dwa.gov.za) and summarised to provide a comparison among selected sites. Median annual values and standard deviation were calculated for data sets containing monthly values for roughly 20 consecutive years, ranging in most cases from approximately 1978 to 2011.

For the duration of the sampling period, daily flow data were obtained (from the gauging stations at each site) in conjunction with hourly measurements of water temperature as well as relative humidity and air temperature (collected using Dallas I-button air temperature/relative humidity loggers installed at each site – see Rivers-Moore & Dallas 2008 and Dallas & Rivers-Moore 2010). For the collection of water temperature data, HOBO® TidbiT® v2 water temperature loggers (Onset Computer Corporation 2008) were installed at each study site in February and March 2009, prior to the commencement of sampling. These loggers were set to continuously record water temperatures every hour for the duration of the sampling period (April 2009-April 2010). Each logger was bolted into position within a rectangular galvanized-steel housing attached to a length of steel cable, in turn secured to either a large boulder or bedrock in a run section of the thalweg near the sampling area. Data from water temperature loggers were downloaded at approximately six-month intervals. Corresponding hourly (or two-hourly) air temperature and relative humidity values were recorded for the sites using Dallas I-button air temperature/relative humidity loggers. The Dallas I-button loggers, which included a solar radiation shield, were each secured to an aluminium stake approximately 1m high off the ground (see Dallas & Rivers-Moore 2010 for further information regarding the exact location of each air temperature logger and the collection of data, see also Rivers-Moore & Dallas 2008 for logger specifications). Of two water temperature data loggers installed at the Wolwekloof site (one installed in riffle habitat and another in pool habitat), the logger from the riffle habitat went missing during high flows in June 2009 and was replaced with a new logger in October 2009 after the flows had subsided. Owing to the loss of the logger, temperature data were missing at this site for the period from 5 May to 20 October 2009. Subsequently further gaps in the data came about from February 2010 until the end of the sampling period in April 2010, owing to technical difficulties with a replacement logger. Various methods were employed to model water temperatures over the period for which data was missing. Details of the methods employed as well as the results obtained are given in Appendix 2C.

Statistical analyses of study site data

Hourly water temperature data were converted to daily mean, minimum and maximum values as well as daily range and daily standard deviation values. Water temperature data were then analysed in a similar manner to those of daily flow data (with the IHA software package) but using a series of metrics developed by Rivers-Moore *et al.* (2008a, 2010). These temperature metrics perform the same function as IHA but are specifically designed for use with time series of daily water temperature data. Similar to IHA, these metrics derive a number of ecologically meaningful parameters (see Table App2A.3,

Appendix 2A) from daily water temperature data while also incorporating Colwell's indices to provide a measure of predictability and constancy in the water temperature time series data. As such, the use of these metrics allows for the thermal characteristics/regimes of different rivers to be compared in a manner similar to how hydrological characteristics were compared.

Prior to applying Colwell's predictability indices, correlations between mean annual temperature values and mean annual temperature standard deviation values for the six sites were conducted. This procedure is in accordance with the guidelines proposed by Rivers-Moore *et al.* (2008a). These guidelines highlight the fact that data used in predictability indices (Colwell 1974) of time series (especially data with fixed lower bounds) require that the mean and standard deviation be uncorrelated. While hydrological data often incur a lower bound of 0 (baseflows or zero flow days), leading to a high correlation between the mean and standard deviation, this is generally not the case for water temperature data which is not bounded by a lower limit of 0. Results of the correlations were non-significant ($p > 0.01$) and therefore no logarithmic transformation was applied to the water temperature data, prior to using Colwell's predictability indices and the corresponding thermal metrics developed by Rivers-Moore *et al.* (2008a, 2010)[7].

While study sites were initially selected using approximately 20 years of daily flow data, collection of daily water temperature data for a select number of rivers has only recently started in South Africa (see Dallas & Rivers-Moore 2010). Water temperature data was therefore only available for a period of 13 months for each of the sites included in this study. This relatively small data set limits the long-term interpretations of thermal regime that can be inferred for the sites (using time series analyses and Colwell's predictability indices), especially for sites exhibiting more variable annual thermal regimes. This is largely because a minimum of 5 years is suggested for water temperature time series data to provide meaningful results using Colwell's indices (Dr. Nick Rivers-Moore, pers. comm., University of KwaZulu Natal, 2009). A data set of 13 months can nevertheless provide important preliminary insights into the short term intra-annual thermal trends. Similar analyses using daily water temperature data for periods ranging from 12 to 32 months have yielded useful information regarding the degree to which thermal parameters differed between rivers across South Africa (Rivers-Moore *et al.* 2008a, 2008b, 2010)

For comparative purposes the same multivariate procedures (PCO and Cluster Analyses) as described in the initial site selection procedure were repeated to analyse the water temperature data collected from each river. Prior to conducting multivariate analyses, the 40 parameters used in the thermal metrics were assessed using a simple correlation matrix, and variables showing high multicollinearity ($r > 0.8$)

[7] Colwell's indices are calculated automatically for daily flow data in IHA 7.1. As such, prior correlations of annual mean and standard deviation values as well as logarithmic data transformation are not required. This is not the case, however, when using thermal metrics developed by Rivers-Moore *et al.* (2008a, 2010) in which Colwell's indices are calculated manually.

were removed (see Table App2A.4, Appendix 2A). Following the removal of correlated variables, data were then normalised and sites were analysed using a cluster analysis technique (group average relatedness) based on the Euclidean distance measure in order to assess the similarity of the sites based on water temperature data.

A PCO, using the Euclidean distance resemblance measure, was then used to ascertain which explanatory variables contributed most to the dissimilarity between the sites and to partition the amount of variability that is explained in the data according to these variables. All multivariate analyses were performed using PRIMER 6+PERMANOVA (Clarke & Gorley 2006, Anderson *et al.* 2008) software package from Plymouth Marine Laboratory, UK.

Mean daily range in water temperature and mean daily flow data collected over the 13 month sampling period were analysed using STARS v3.2 (Rodionov 2004, Rodionov & Overland 2005). STARS uses a combination of equal-weighted and weighted arithmetic means in conjunction with sequential students t-tests to statistically determine the timing and magnitude of significant shifts from one stable state (or regime) to another within time series data (Rodionov & Overland 2005, Lees *et al.* 2006). In other words each successive point in the time-series data is analysed (using student t-tests) in relation to the mean of the preceding points or regime (stable state). When a significant difference (either in the form of a decrease or increase) is detected then that point is considered to indicate the potential start of a new stable state or regime (a change-point). As data points are added, they are used to confirm or reject this hypothesis in a similar manner using the regime shift index (RSI), which is calculated as the cumulative sum of normalised deviations from the hypothetical mean level for the "new" regime. The difference between the mean level of the current regime and the RSI is tested using a t-statistic. If a negative RSI is calculated then RSI is reset to 0 and the mean for the regime is recalculated incorporating the value of the added data point. If a positive RSI is calculated throughout the cut-off length (specified by the user) for a regime to be tested, then it means a significant shift exists at that time point and an associated *p* value is reported. If a positive RSI is not calculated for the entire defined cut-off length, then the point at which the initial significant difference was observed is regarded as an outlier. If multiple variables are being analysed to determine regime shifts, then the total RSI is the sum of the RSI values from the time series for each variable for each year (see Rodionov & Overland 2005 and Rodionov 2006 for further information).

The STARS algorithm, as a statistical model, incorporates several model parameters and constants which need to be specified prior to analysing time-series, namely: a minimum value for the length of a regime (cut-off length)[8], a significance level for statistical tests and a weighting parameter for data

[8]Cut-off length is normally measured in years, where each data point in a time series represents a value for a single year. In this study however where only a single years worth of daily data has been used, the cut off length parameter specified by the user refers to length of a regime in days and not in years.

outliers (Huber parameter). The use of prewhitening[9] can be specified along with the type of parameter estimator. In this study the OLS (ordinary least squares) parameter estimator in conjunction with the prewhitening function was applied to both flow and temperature data. A significance level of 0.05, with cut-off length = 5 and Huber parameter = 1 was applied to flow data, while for temperature data, a significance level of 0.01, with cut-off length = 10 and Huber parameter = 1, was used. A subsample size setting of 6 was used for the prewhitening for both data sets.

2.3 Results

2.3.1 Site selection procedure

Flow metrics and multivariate analyses

The IHA analysis indicated that the six study sites exhibited a wide range of flow characteristics (Table 2.4). Predictability values for flow ranged from 0.32 (Wolwekloof River) to 0.56 (Rooi-Els Kloof River) and mean annual flows from 0.25m^3/s (Rooi-Els Kloof River) to 5.12m^3/s (Molenaars River). The moving average of maximum flow over a 30-day period showed a range of almost 18m^3/s among the sites, highlighting the wide range of winter flow volumes experienced across the sites. Constancy values indicated that certain sites exhibit stable flows within each year (e.g. Rooi-Els Kloof River - Constancy = 0.66) and others a more seasonal pattern (e.g. Wit River - Constancy = 0.33). A complete table of the output from the IHA analysis is presented in Table App2B.1 in Appendix 2B.

Table 2.4. Summary of annual flow characteristics for the six study sites using IHA 7.

Site	Mean annual flow (m^3/s)	Annual C.V. of flow (%)	Max. 30-day mov. ave (m^3/s)	Predictability (0-1)	Contribution of constancy to predictability (%)
Rooi-Els Kloof	0.25	1.55	0.981	0.56	66
Elandspad	2.81	3.79	9.551	0.54	50
Molenaars	5.12	2.66	18.840	0.51	46
Eerste	0.79	2.16	2.913	0.41	45
Wit	4.14	2.57	18.200	0.39	33
Wolwekloof	3.16	3.00	2.171	0.32	64

Cluster analysis (Fig. 2.3) revealed that the selected sites were each distinctive with respect to their flow variables. Some patterns of grouping or shared similarities were visible, though not totally clear. The Rooi-Els Kloof River was shown to be the most distinct, followed by the Wolwekloof River and then the Eerste River, while the Wit River showed a closer resemblance to that of the Molenaars and Elandspad rivers. The most closely related sites were the Elandspad and Molenaars rivers, which is to be expected as the former is a tributary of the latter. Study sites located on these rivers were also the

[9] Prewhitening is the process whereby unwanted autocorrelations in time series data are removed prior to performing an analysis.

closest to one another in terms of geographic distance - but these sites were nevertheless retained in order to contrast minor temperature differences that might occur in tributaries of a single drainage area.

The results of the PCO analysis using non-correlated flow parameters derived from the IHA analysis are presented in Fig. 2.4. The first and second axes (PCO1 and PCO2) accounted for 35.3% and 32.6%, respectively, of the total variance in the flow data measured across each of the study sites. Flow variables exhibiting a correlation greater than 0.6 to the first two PCO axes have been overlayed in the form of vectors. The strength and relationship of the Pearson correlations of these variables to the PCO axes are indicated by the relative length of the vector (the inner edge of the circle delineating a correlation of 1), in conjunction with the angle (e.g. vectors perpendicular to PCO axis 1 indicate a weak correlation) and direction of the vectors (the sign of the correlation is determined relative to the origin of the PCO axis in question). The similarity between sites, based on the Euclidean distance resemblance measure, is indicated by the contours surrounding sites at a set similarity distance of 3.6 and relates to the cluster analysis presented in Fig. 2.3.

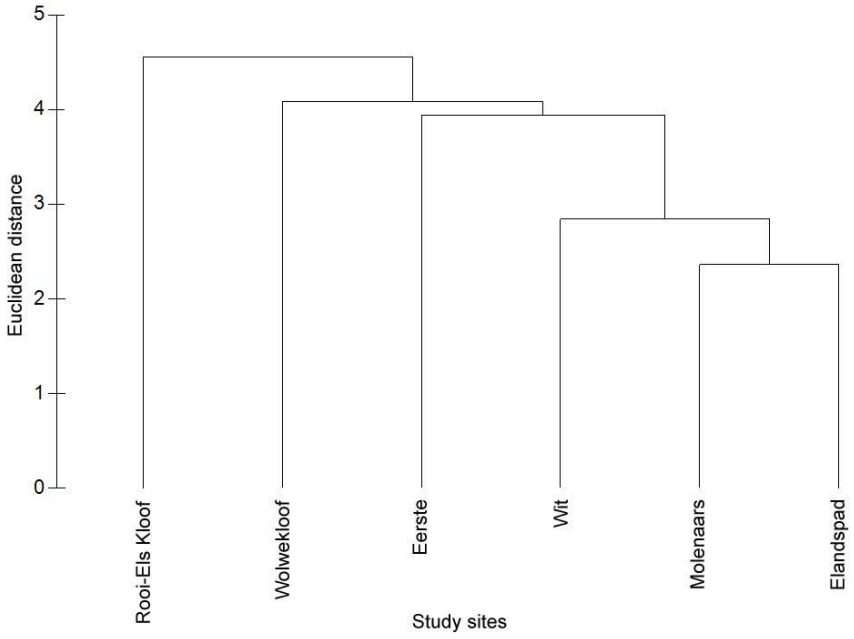

Fig. 2.3. Cluster analysis of study sites based on variables derived from historical daily flow data using the Euclidean distance measure and group average relatedness.

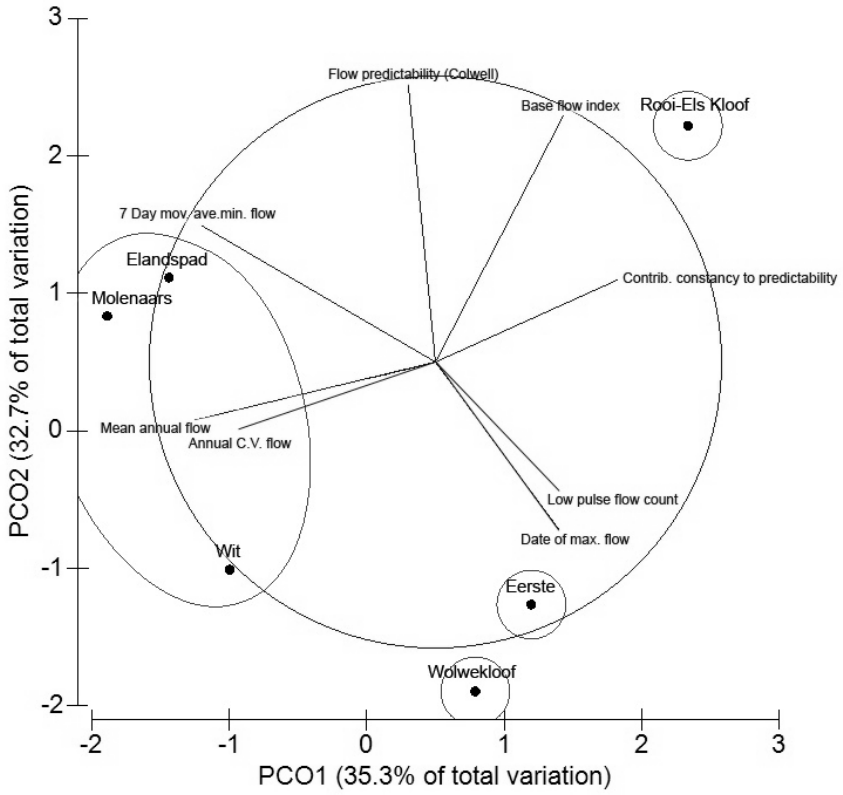

Fig. 2.4. Principle Coordinates Analysis (PCO) of study sites (black circles) using variables derived from historical daily flow data. Vectors represent variables showing correlations (r > 0.6) to the first two PCO axes (PCO1 and PCO2). The inner edge of the circle delineates a correlation of 1. Ellipses around sites indicate the similarity between sites at a Euclidean distance of 3.6.

Variables showing the highest correlation to the first PCO axis were, in order: mean annual flow, seven-day moving average of minimum flow, annual C.V. of flow and constancy as a proportion of the total predictability of flow. Variables showing the highest correlation to the second PCO axis were flow predictability, baseflow index and date of maximum flow.

2.3.2 Field sampling

Flow and water temperature

Comparisons of two-hourly air temperatures and corresponding two-hourly water temperatures are provided for each of the study sites in Fig. 2.5. Air temperatures were observed to be comparable among sites, while water temperatures were found to be markedly different, especially over the summer months from December 2009 to February 2010. Water temperatures recorded between June and August 2009 were less variable than summer water temperatures and were similar across all sites except the Wolwekloof River, for which temperatures over this period were modelled[10] and also the Rooi Els-Kloof River which exhibited markedly reduced short term variability over the entire sampling period. Modelled water temperatures in the Wolwekloof River (May to October 2009) were observed to be

[10] See Appendix 2C for further details.

distinctly more variable than both the temperatures recorded from the other sites for the same time period and also the preceeding recorded water temperatures from April to May 2009. The Wit River showed the most variable temperatures over summer in contrast to the Rooi-Els Kloof River which showed the least variability and distinctly lower water temperatures (see also Fig. 2.7). The gap in air temperatures observed for the Eerste River from October 2009 to February 2010 was as a result of a technical error with the air temperature logger that prevented data from being recorded for this period of time.

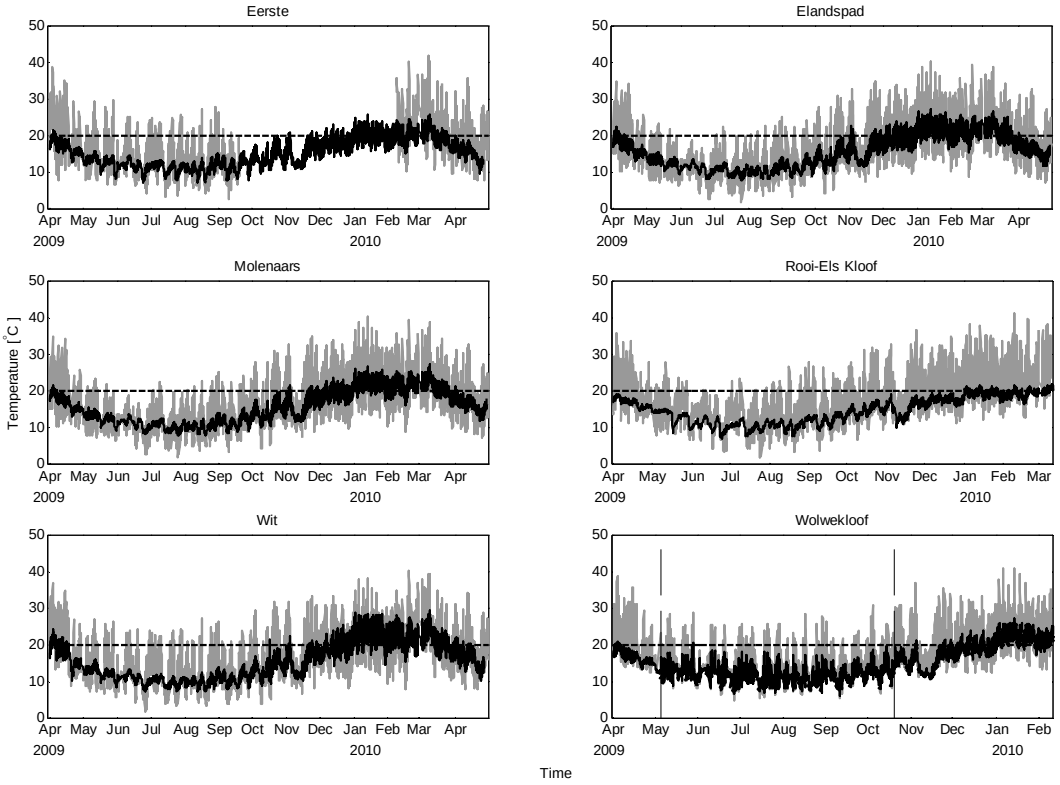

Fig. 2.5. Comparison of air (grey trace) and water temperatures (black trace) (2 hourly intervals) from the six study rivers. Dashed vertical lines for the Wolwekloof River demarcate the period for which water temperatures were modelled from air temperatures. Dashed horizontal lines indicate 20°C in each plot for comparison.

Mean monthly water temperature and mean monthly standard deviation in water temperature for the period February 2009 – April 2010 (Fig. 2.6) revealed a noticeable separation of the sites, particularly over the warmer spring and summer periods (Fig. 2.6) where the Eerste and Rooi-Els Kloof rivers maintained lower summer values.

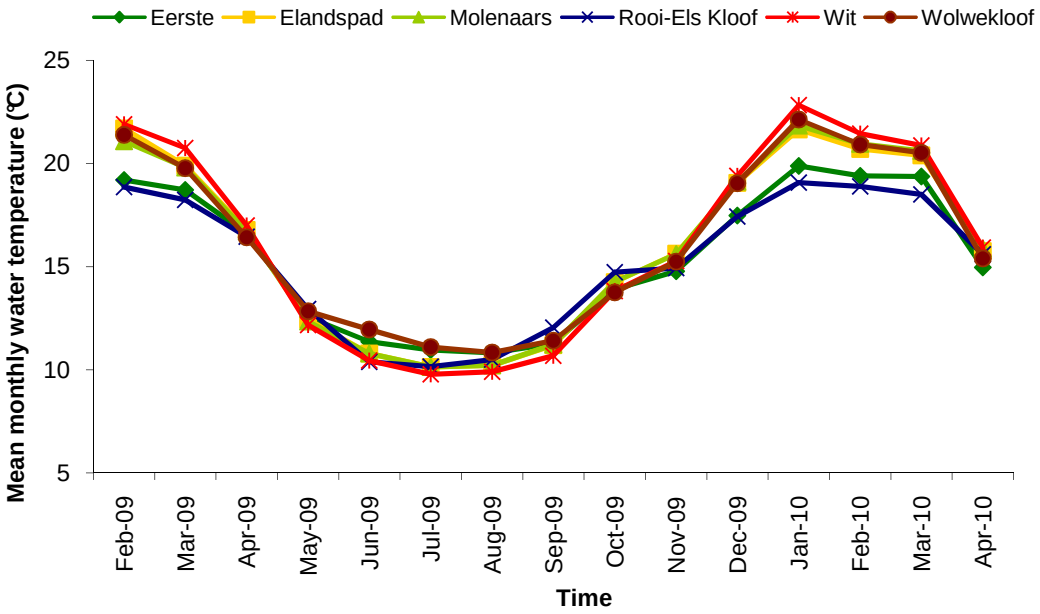

Fig. 2.6. Mean monthly water temperatures for the six study rivers for the period February 2009 – April 2010.

Similarly, high monthly standard deviation values were observed for all rivers from the onset of warmer spring conditions (mid-September) through summer to mid-March (Fig. 2.7).The Wit River consistently showed the greatest standard deviation in monthly temperature over the sampling period, in marked contrast to the Rooi-Els Kloof which showed the lowest (Fig. 2.7).

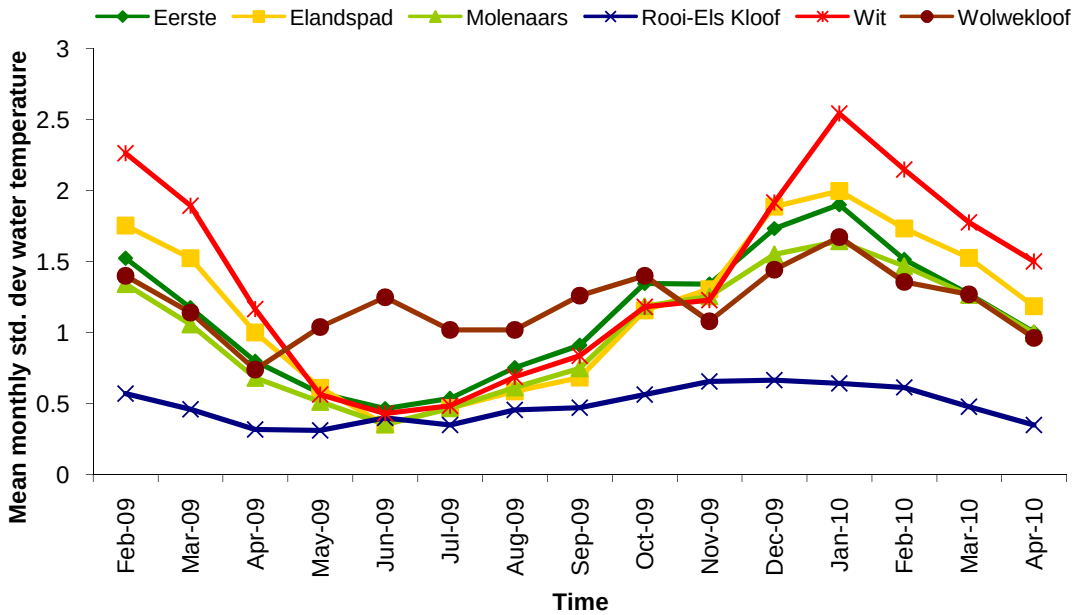

Fig. 2.7. Mean monthly standard deviation in water temperatures for the six study rivers for the period February 2009 – April 2010.

Mean monthly temperatures and mean monthly standard deviation in water temperature in all rivers dropped considerably at the onset of the first cold fronts in autumn (March/April 2009 and 2010), and subsequently remained low over the winter period, with less obvious separation of the sites (Fig. 2.7). When compared to the other sites, the Wolwekloof River showed only slightly warmer modelled mean

water temperatures over the winter period from May to mid-October, but considerably higher standard deviation values for the same period.

Accumulated maximum daily water temperatures or accumulated maximum Degree Days (DD) calculated separately for each of the six sites, for the entire period for which temperatures were recorded (23 February 2009 – 25 April 2010), indicated moderate site separation, with the Wit River having accumulated the highest number of maximum DD (7751°C) and the Rooi-Els Kloof River the lowest (6827°C) (Table 2.5). Using accumulated maximum DD, rivers were ranked for this same period (Table 2.5). Rivers were further compared over two seasonal time periods (*viz.* "Cooling period": April –September 2009 and "Warming period": October 2009– March 2010) (Table 2.5B and C). For both the entire period, and the 'warming' period, the same ranking was achieved using maximum accumulated DD.

Table 2.5. Thermal rank, accumulated mean, maximum and minimum Degree Days (DD) collected from the six study rivers for three time periods **A)** Entire period (23 February 2009 – 27 April 2010), **B)** "Cooling" period (1 April 2009 – 30 Sept 2009) and **C)** "Warming" period (1 October 09 – 31 March 2010).

A) Entire Period

River	Accumulated mean DD	Accumulated max. DD	Accumulated min. DD	Thermal rank using accumulated max. DD (warmest to coldest)
Wit	6750.5	7751.9	6011.1	1
Elandspad	6704.4	7597.5	6062.9	2
Molenaars	6724.2	7533.1	6161.3	3
Wolwekloof	6812.0	7441.1	6311.2	4
Eerste	6488.8	7389.0	5913.6	5
Rooi-Els Kloof	6415.7	6827.7	6147.8	6

B) Cooling period

River	Accumulated mean DD	Accumulated max. DD	Accumulated min. DD	Thermal rank using accumulated max. DD (warmest to coldest)
Eerste	2239.8	2512.3	2091.0	1
Wolwekloof	2306.5	2436.4	2194.9	2
Elandspad	2178.2	2401.8	2030.3	3
Molenaars	2174.4	2383.4	2038.7	4
Wit	2129.8	2377.6	1962.9	5
Rooi-Els Kloof	2207.1	2354.9	2115.5	6

C) Warming Period

River	Accumulated mean DD	Accumulated max. DD	Accumulated min. DD	Thermal rank using accumulated max. DD (warmest to coldest)
Wit	3442.3	4018.2	3014.2	1
Elandspad	3384.1	3901.7	3012.8	2
Molenaars	3405.8	3862.6	3069.9	3
Wolwekloof	3381.1	3764.9	3079.1	4
Eerste	3175.8	3697.9	2842.8	5
Rooi-Els Kloof	3137.5	3337.4	2993.8	6

Similar to the rankings of the six study sites achieved using accumulated maximum degree days, thermal duration curves produced for the study sites based on mean daily water temperatures from 1 April 2009 to 31 May 2010 (Fig. 2.8), revealed that the Wit River showed the greatest percentage time exceeded in a year for summer temperatures, followed closely by the Wolwekloof, Molenaars and Elandspad rivers, while the Eerste and the Rooi-Els Kloof rivers revealed the lowest water temperatures. Greatest separation of sites occurred in the warm end of the water temperature spectrum, essentially during the summer months of December-February (Fig. 2.8). All sites, barring the Rooi-Els Kloof, however showed convergence in terms of the percentage time exceeded in a year for which temperatures were below approximately 15°C, suggesting generally similar water temperatures durations during the winter months (June-August) (Fig. 2.8). The Rooi-Els Kloof River exhibited a slightly higher percentage time exceedance of temperatures in the middle of the water temperature spectrum, relative to the other sites, indicative of a more stable thermal regime, perhaps owing to thermal buffering from ground water inputs throughout the year (Fig. 2.8).

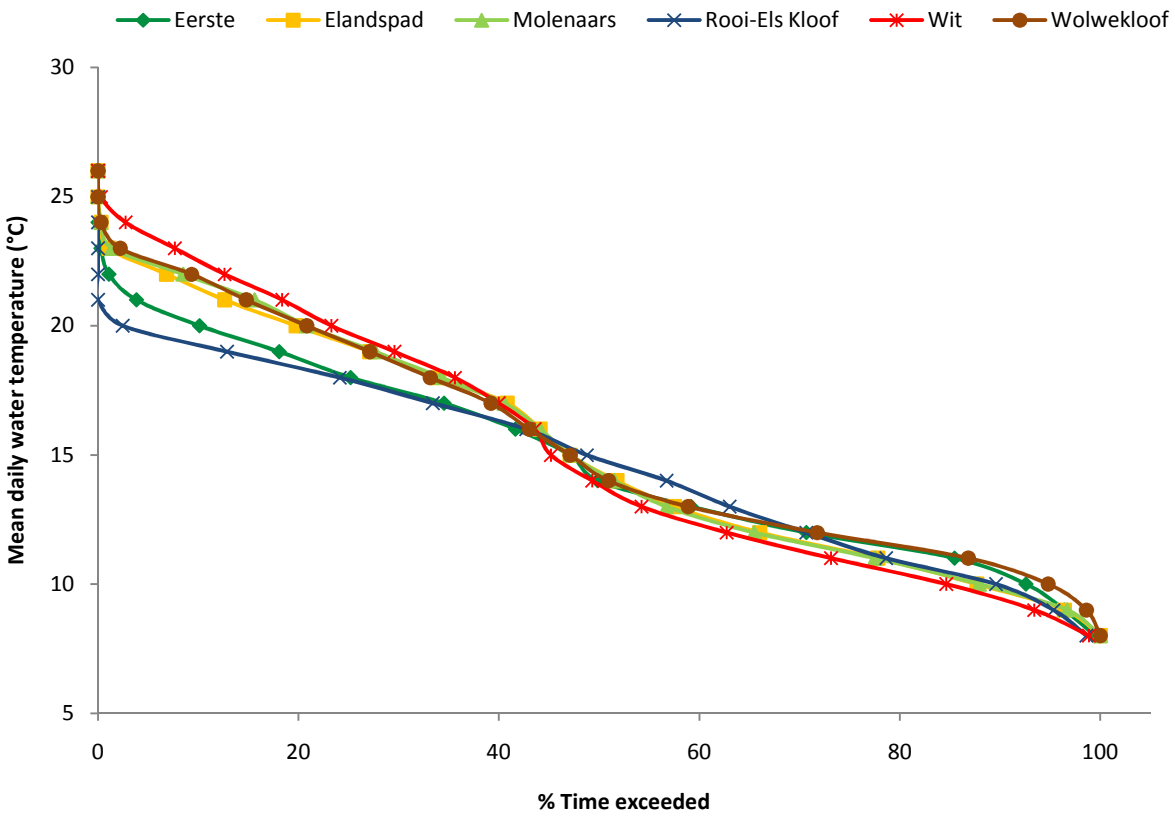

Fig. 2.8. Temperature duration curves for the six study sites for the time period 1 April 2009 - 31 March 2010.

Monthly precipitation data (calculated from daily records), for each of the sites, are presented in Fig. 2.9. While the first rainfall events of the season were recorded in early autumn (April/May) for several of the sites (coinciding with the first cold fronts passing over the Western Cape), highest precipitation values were recorded in June for all sites. The amount of rainfall experienced at each site was shown to differ somewhat, with the Rooi-Els Kloof receiving the lowest monthly precipitation and the Wolwekloof the highest. Total rainfall recorded for the period (March 2009-April 2010) at each of the sites was: Eerste - 1056mm, Elandspad – 657mm, Molenaars – 657mm, Rooi-Els Kloof – 282mm, Wit – 812mm and Wolwekloof – 1965mm.

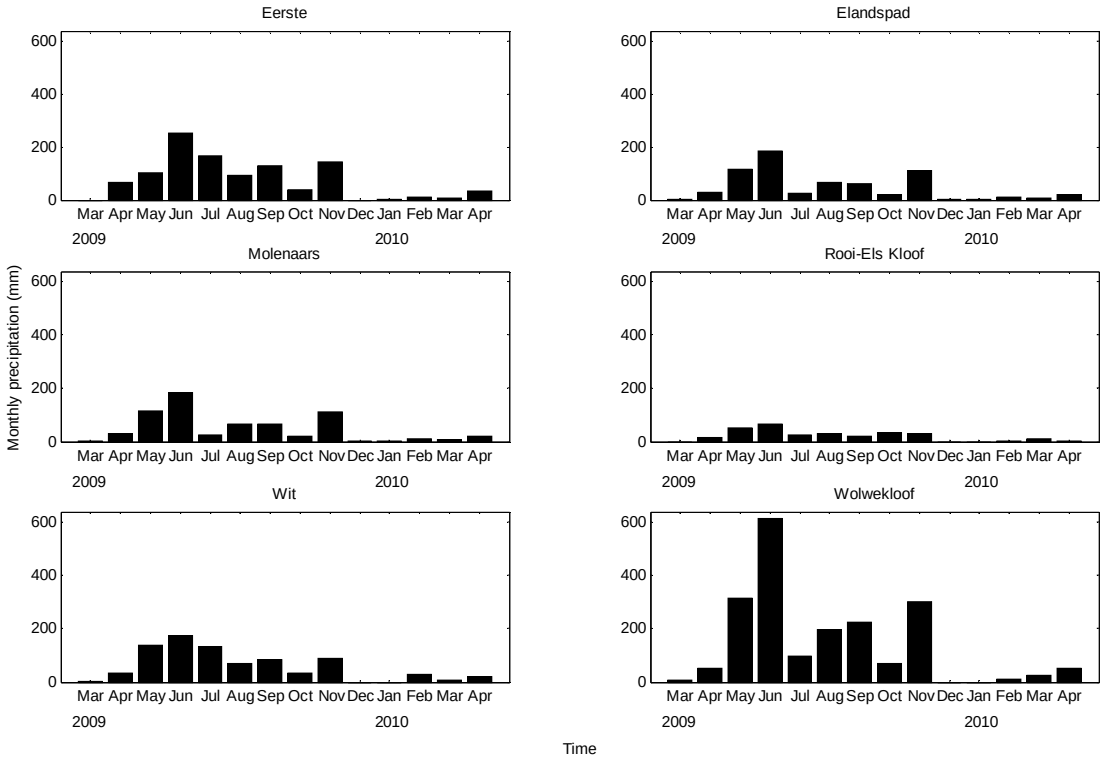

Fig. 2.9. Total monthly precipitation (calculated from daily records) from the six study rivers for the period March 2009 - April 2010. Data with permission from the South African Weather Service (SAWS).

Mean monthly flows varied considerably among sites, with certain rivers (*viz.* Molenaars, Wit, Elandspad) showing peak flows orders of magnitude greater than flows occurring over the same time periods in the remaining rivers (Eerste, Wolwekloof and Rooi-Els Kloof). All sites experienced coinciding periods of high flows or spates in the months of June and November 2009 (Figs. 2.10, 2.11). The first winter flows in each river occurred in early May 2009 (Fig. 2.10). These first flows mark the onset of the first heavy winter rains (Fig. 2.9) and more variable hydrological conditions at the sites for the general period spanning from May 2009 (autumn) through to November 2009 (spring). After the late-spring spate in November 2009, flows returned to baseflow conditions, which prevailed from December onwards through summer. Baseflows recorded for each site were: Eerste – 0.03-0.14m^3/s, Elandspad – 0.50-1.19m^3/s, Molenaars – 1.02-2.29m^3/s, Rooi-Els Kloof – 0.13-0.18m^3/s, Wit – 0.24-0.27m^3/s, and Wolwekloof – 0.01-0.12m^3/s.

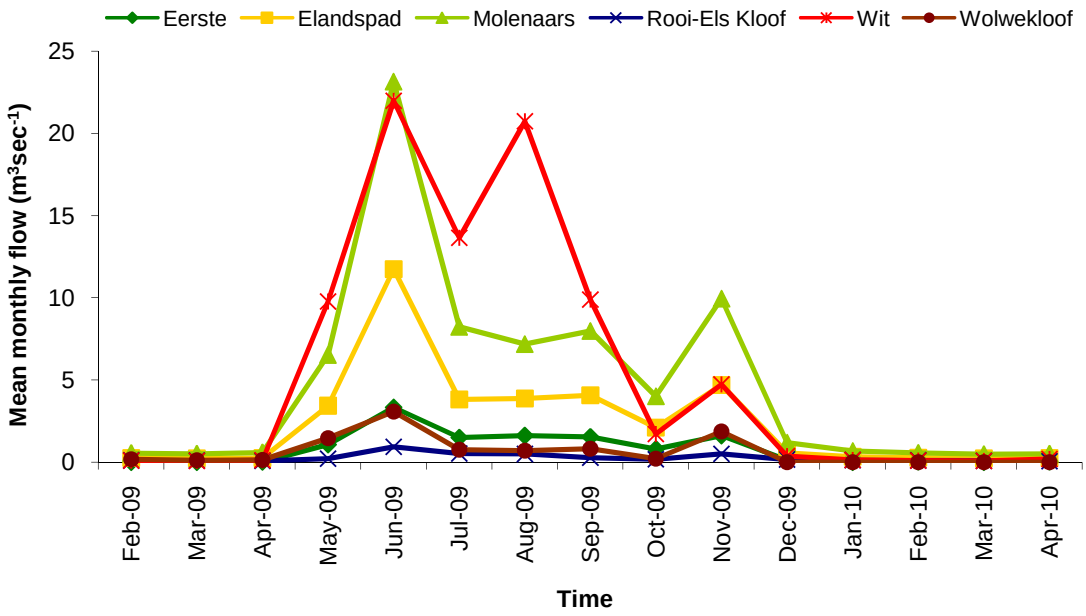

Fig. 2.10. Mean monthly flow (discharge) values collected from the six study for the period February 2009 – April 2010.

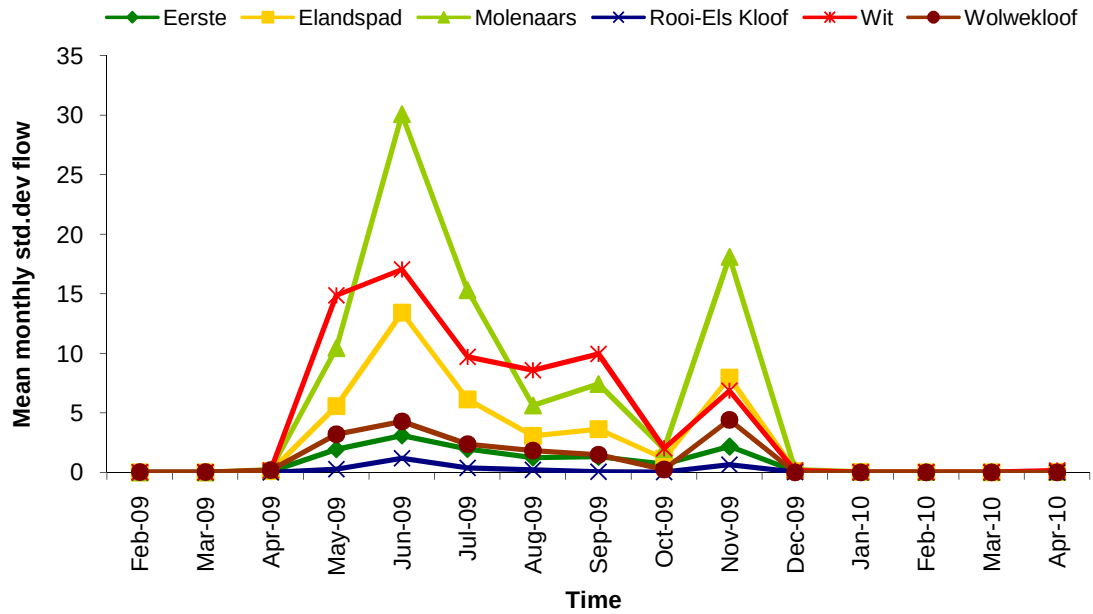

Fig. 2.11. Mean monthly standard deviation in flow (discharge) collected from the six study rivers for the period (February 2009 – April 2010).

Mean monthly standard deviation values for flow were on average highest in the Molenaars River, for the time period from May to December 2009, with variability noticeably higher than other sites during spates in June and November (Fig. 2.11). The Wit and Elandspad rivers showed similar trends in mean monthly standard deviations ranging from 3 to 15m^3/s from May to December 2009, but the Wit River showed greatest variability in flow during late winter (August to September). As with the mean monthly flows, the lowest mean monthly standard deviations in flow were exhibited by the Wolwekloof, Eerste and Rooi-Els Kloof rivers respectively, in order of decreasing variability.

Thermal metrics and multivariate analyses

The six study sites exhibited a wide range of mean temperatures and of thermal predictability and constancy values (Table 2.6). Predictability values for water temperature were generally higher than those reported for flow metrics and ranged from 0.56 (Wit River) to 0.64 (Rooi-Els Kloof River), while mean annual water temperatures ranged from 14.98 (Rooi-Els Kloof River) to 15.81°C (Wolwekloof River). The moving average of maximum water temperatures over a 30-day period showed a range of almost 7°C among the sites. Constancy values were generally lower for water temperatures than for flow, suggesting a stronger seasonal cycle of temperature within the year. However certain sites showed signs of maintaining more stable temperatures within the year (e.g. Eerste River- constancy = 0.46) in contrast to the Wit River which had the lowest constancy score (0.31). A complete table of the output from the thermal metric analysis is presented in Table App2B.2 in Appendix 2B.

Correlations of Colwell's predictability indices were calculated with respect to to several annual statistics are provided in Table 2.7. Correlations indicated several strong relationships between annual flow and water temperature statistics and variability measures as well as a significant ($p < 0.05$) link between the annual C.V. of flow and mean annual water temperature.

Table 2.6. Respective annual water temperature characteristics of study sites obtained from metrics developed by Rivers-Moore *et al.* (2008a, 2010).

Site	Mean annual water temperature (°C)	Annual C.V. of water temperature (%)	Max. 30-day mov. ave. of water temperature (°C)	Predictability (0-1)	Contribution of constancy to predictability (%)
Rooi-Els Kloof	14.98	22.82	20.39	0.64	42
Molenaars	15.66	28.09	24.49	0.61	36
Elandspad	15.60	27.83	25.06	0.60	35
Eerste	15.13	24.20	23.04	0.59	46
Wolwekloof	15.81	26.53	24.95	0.59	36
Wit	15.71	31.27	27.10	0.56	31

Table 2.7. Correlation matrix for annual descriptive statistics derived from daily hydrological and thermal data for six selected study sites in the Western Cape. Strong correlation coefficients ($r > 0.5$ and < -0.5) are marked in bold and significant correlations ($p < 0.05$) are marked with an asterisk.

	Mean annual flow	Annual C.V. of flow	Flow predictability	Contribution of flow constancy to predictability
Mean annual temp.	0.405	**0.896 ***	-0.459	0.168
Annual std. dev. temp.	**0.822**	0.402	**-0.546**	**-0.500**
Annual C.V. of temp.	**0.844**	0.265	**-0.517**	**-0.604**
Temp. predictability	-0.323	-0.100	**0.722**	**0.726**

Cluster analyses based on variables derived from daily water temperature (Fig. 2.12) revealed a separation of the sites almost identical to that observed when using daily flow data (see Fig. 2.3). In general, some shared similarities between the Molenaars, Elandspad and Wit rivers resulted in these particular sites showing some degree of grouping while for the most part the remaining sites appeared to be unique in terms of their thermal characteristics. The Rooi-Els Kloof River was shown to be most thermally distinct from the other sites, likely owing to a combination of a high thermal predictability and constancy score.

The results of the PCO analysis, using non-correlated thermal parameters derived from the thermal metrics, are presented in Fig 2.13. The first and second axes (PCO1 and PCO2) accounted for 44.9% and 30.7%, respectively, of the total variance in the temperature data measured across each of the respective study sites.

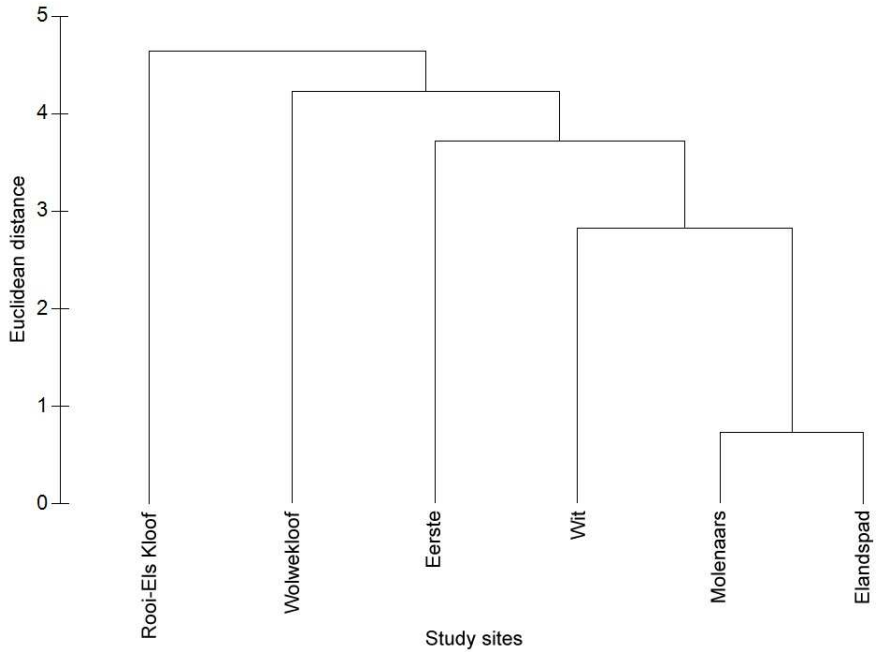

Fig. 2.12. Cluster analysis of study sites based on variables derived from daily water temperature data using the Euclidean distance measure and group average relatedness.

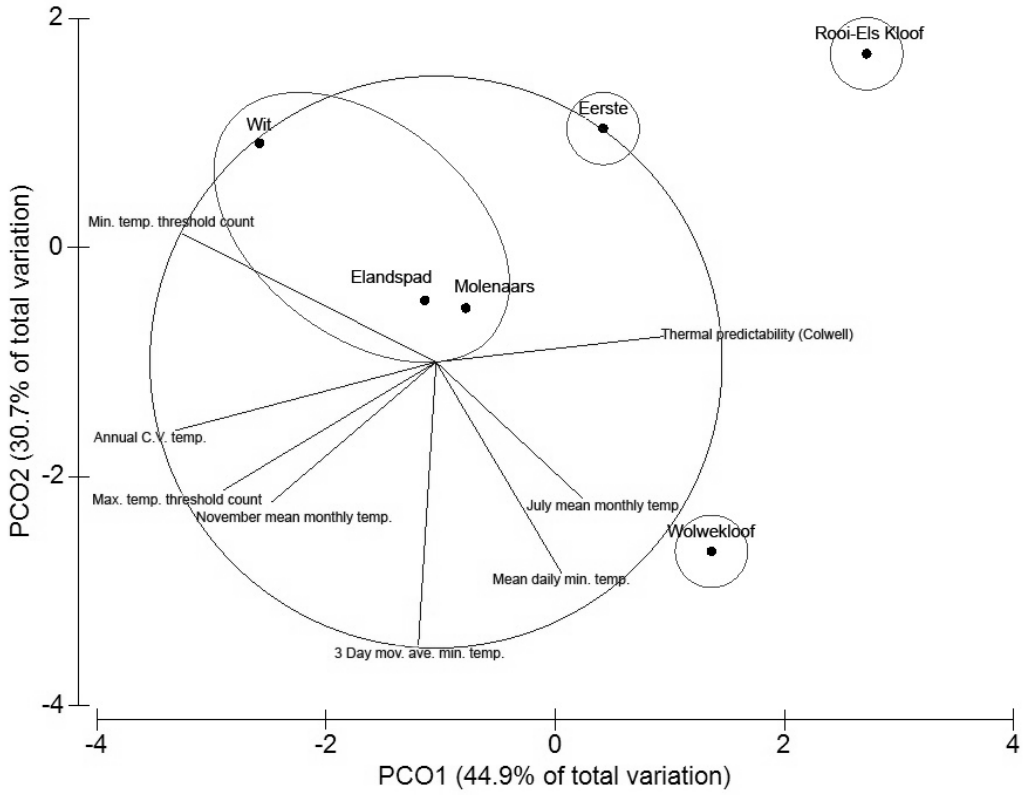

Fig. 2.13. Principle Coordinates Analysis (PCO) of study sites (black circles) using variables derived from daily water temperature data. Vectors represent variables showing correlations (r > 0.6) to the first two PCO axes (PCO1 and PCO2). The inner edge of the circle delineates a correlation of 1. Ellipses around sites indicate the similarity between sites at a Euclidean distance of 3.6.

Variables showing the highest correlation to the first PCO axis were, in order: annual C.V. of temperature, count of water temperatures below the minimum threshold and temperature predictability. Variables showing the highest correlation to the second PCO axis were: three-day moving average of minimum temperature, mean minimum temperature and mean monthly temperature for November.

Regime shift analyses

Significant differences (p < 0.05) were observed in the number, timing and magnitude of regime shifts in both temperature and flow data collected from each of the six study sites (Fig. 2.14). Tables summarizing the regime shifts for each of the rivers are presented in Appendix 2D. In Fig. 2.14, significant (p < 0.05) regime shifts are shown for the duration of the sampling period for both flow and temperature data. Given the more labile nature of the hydrological regime and separate flow events, positive regime shifts in the mean values are in most cases accompanied by negative shifts occurring shortly afterwards. Such trends were not evident in the shifts detected in the temperature data. The timing of these shifts did not always coincide across rivers and certain shifts were not evident across all rivers for these periods (Table App2D.1 in Appendix 2D).

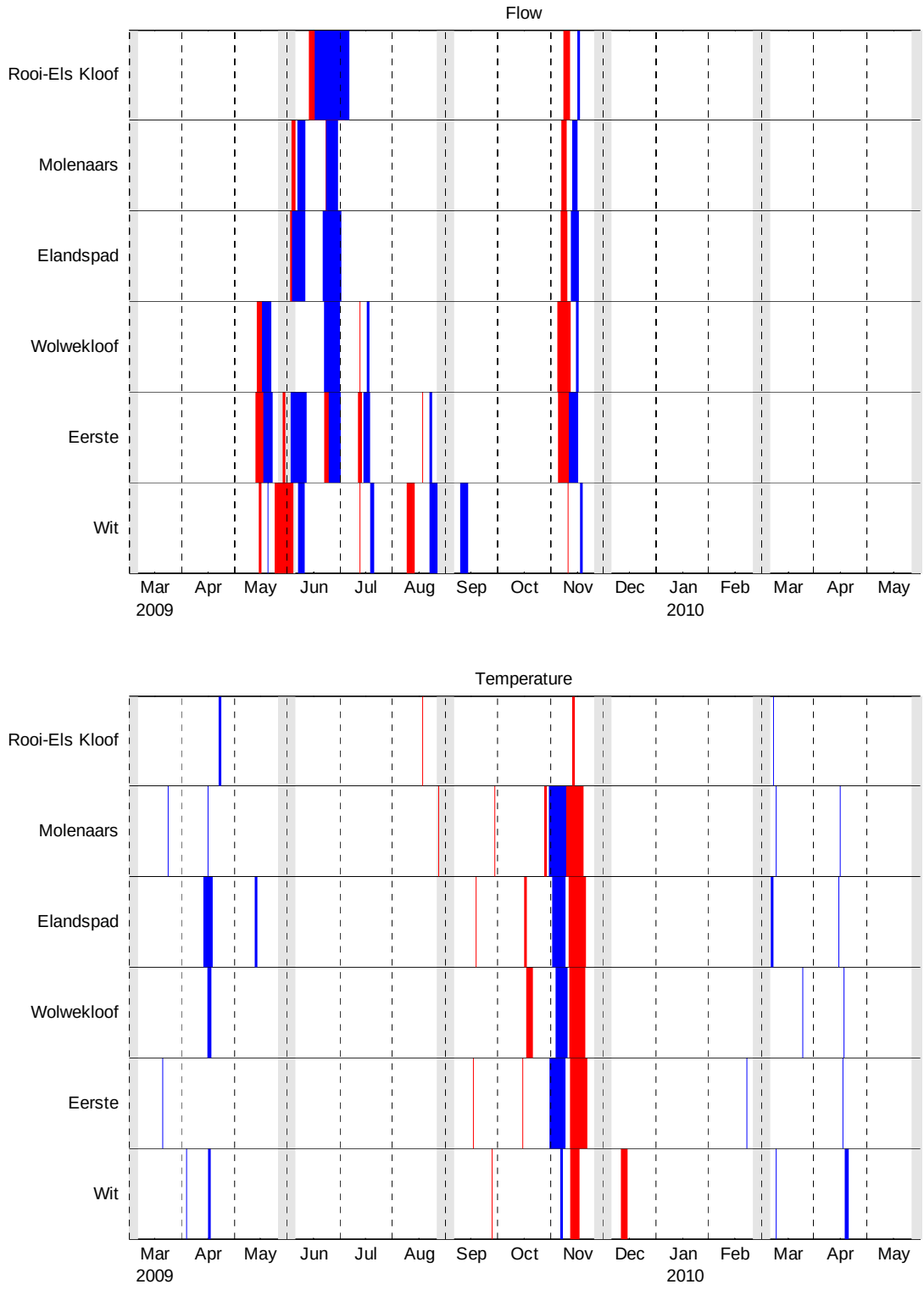

Fig. 2.14. Regime shifts in mean daily flow records and mean daily water temperature records for six rivers in the Western Cape, South Africa for the period March 2009 – May 2010. Red and blue lines indicate positive and negative regime shifts respectively. Line width represents the magnitude of shifts in terms of the Regime Shift Index (RSI) relative to previous regimes. Grey vertical bars denote the duration of the four seasons of the year based on the calculation of thermal metrics by (Rivers-Moore *et al.* 2010).

Shifts in the daily water temperature data prior to the onset of winter (mid-autumn) appeared to be largely unrelated to shifts in the flow regime. In contrast, the large negative temperature shift in late-

spring (~6 November) for all sites was clearly linked to the late spring regime shift in flow. Generally all six rivers showed similar timing of temperature shifts with the exception of the Rooi-Els Kloof, Wolwekloof and the Eerste rivers (Table App2D.2 in Appendix 2D).

The number of regime shifts experienced in the respective rivers provides an indication of both the thermal and hydrological variability/stability of the rivers over the study period The Rooi-Els Kloof River incurred only four thermal regime shifts over the study period, all of which were of low magnitude in terms of the RSI. In contrast, the Wit River experienced twice as many thermal shifts over the same period, several of which were relatively large in terms of the RSI. The first shift in the Rooi-Els Kloof River also occurred later than in other rivers, marking a delayed decrease in water temperatures towards autumn/winter. This pattern was mirrored in the Elandspad and Wolwekloof rivers. Additionally, sites experiencing a greater number of spates and late winter flow regime shifts incurred a delay in the timing of positive temperature shifts towards the onset of spring.

Physicochemical variables

Changes in DO, pH, EC and turbidity, collected at monthly intervals from the six sites for the duration of the sampling period (April 2009 – April 2010), are shown in Fig. 2.15.

DO concentration levels at all study sites increased to highest levels in August following initial winter spates in June and July in conjunction with the gradual decline in mean water temperature values (Fig. 2.15). The Rooi-Els Kloof, Wolwekloof and Elandspad rivers had the highest average DO concentrations over the sampling period of 10.07 ± 1.05, 9.92 ± 1.37 and 9.85 ± 1.05mg/l while the Wit, Eerste and Molenaars rivers showed lower values of 9.63 ± 1.44, 9.62 ± 1.04 and 9.53 ± 1.15mg/l (Fig. 2.15).

Mean EC values for all sites were low, ranging between 11 and 28µS/cm over the duration of the sampling period (Fig. 2.15). Values at all sites were highest during the warm late-summer to autumn months (February – April). High values observed in the Eerste River especially in May 2009 are likely to have been a result of large amounts of ash, silt and sediment entering the system during the first winter spates following a fire that spread through the Jonkershoek Nature Reserve in late February/early March 2009.

At all sites pH ranged between 4 and 7 (Fig. 2.15). Mean pH values in the Wolwekloof, Rooi-Els Kloof and Wit rivers were the most stable over this period, showing only slight increases over spring and summer (October to December). Mean values with standard deviations (calculated over the entire sampling period) for these rivers were 4.4 ± 0.3, 5.46 ± 0.4 and 4.7 ± 0.5. For the remaining rivers *viz.* Eerste, Elandspad and Molenaars, values were marginally higher 5.81 ± 0.8, 5.27 ± 0.6 and 5.60 ± 0.8. Values in the Eerste, Elandspad and Molenaars rivers were more variable and showed sharp declines in

June coinciding with the first winter spates, followed by peaks in July and then gradual increases over spring and summer to more stable levels.

In general low average turbidity values (0-2 NTU's or Nephelometric Turbidity Units) were recorded from all sites during the sampling period with the exception of the Eerste River (Fig. 2.15). Turbidity levels in the Eerste River were considerably affected as a result of the fire, and subsequent flushing of the system during the initial winter spates in June.

Sites showed distinct separation with respect to active channel width measurements, which correspond directly to the mean annual flow measurements for the rivers (not shown in Fig. 2.15). During the low flow summer periods, the Wit, Rooi-Els Kloof and Wolwekloof rivers had the lowest channel width measurements ranging from 3 to 5m, while in the high flow winter period, the Wit, Molenaars and Elandspad rivers were shown to have the highest values ranging between 15 and 20m. Sharp increases in channel width for all rivers were observed in June coinciding with the winter spates. Channel width in the Wit River increased approximately 10-fold during spate conditions in June with values reaching as high as 40m. Values remained elevated throughout the winter period before gradually declining in spring. As channel width can increase/decrease rapidly during spate conditions, the snapshot of channel width taken on each sampling occasion does not likely reflect the full extent of channel width changes experienced in each river

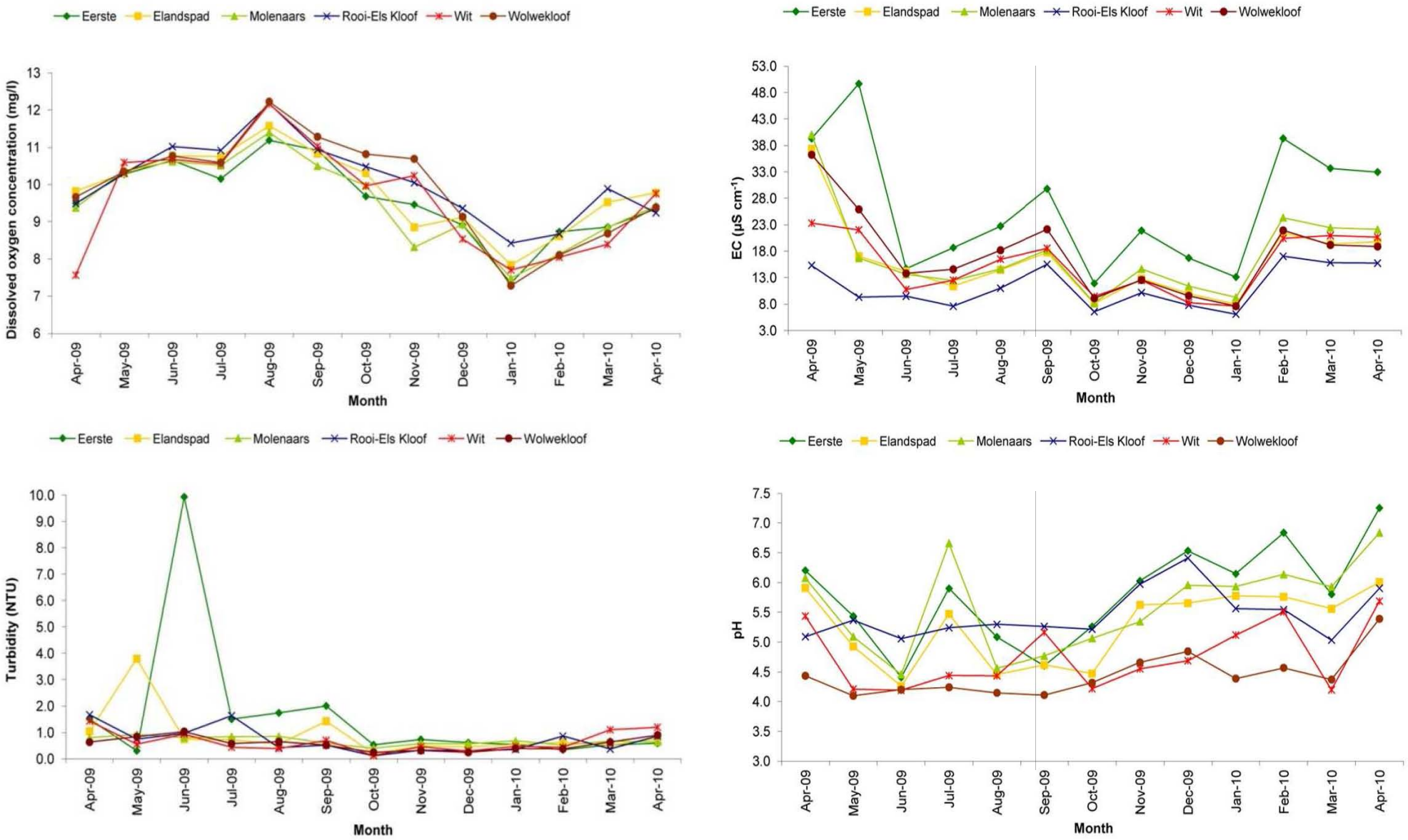

Fig. 2.15. Mean monthly measurements of four physicochemical variables collected from six rivers in the Western Cape, South Africa for the period February 2009 – April 2010

A summary of the major water chemistry variables (median values) obtained from historical records from the Department of Water Affairs for each of the six study rivers is provided in Table 2.8. Measurements in most cases, apart from pH, were similar among study sites. One exception was the Eerste River which showed marginally higher values of total dissolved solids (TDS), sodium (Na^+), alkalinity and chloride (Cl^-) than the other sites. Additionally the Rooi-Els Kloof, Wit and Wolwekloof rivers showed slightly lower Potassium (K^+) levels than the other sites while the Wit and Wolwekloof rivers also revealed sulphate levels (SO_4^{2-}) almost twice as high as the other sites. Highest nitrate and nitrite values (NO_3+ NO_2-N) were observed in the Molenaars and Elandspad rivers and were almost 2-3 times higher than other sites.

Table 2.8. Summary of historical water chemistry data for six rivers in the Western Cape, South Africa. Median annual values are reported for each variable for the period of data collection specified. Standard deviation is provided in brackets. Data with permission from Department of Water Affairs Water Management System.

Variables	Eerste	Elandspad	Molenaars	Rooi-Els Kloof	Wit	Wolwekloof
TDS (mg/L)*	27.00	21.39	23.00	19.49	21.69	20.00
Ca^{2+} (mg/L)	1.55(1.07)	1.37(1.27)	1.06(1.05)	1.08(1.02)	1.09(1.61)	1.21(1.54)
Mg^{2+} (mg/L)	0.82(0.36)	0.74(0.35)	0.65(0.34)	0.69(0.40)	0.75(0.80)	0.69(0.85)
K^+ (mg/L)	0.52(0.19)	0.49(0.52)	0.50(0.34)	0.31(0.37)	0.33(0.40)	0.28(0.35)
Na^+ (mg/L)	5.13(1.09)	3.11(0.91)	3.63(1.11)	2.57(1.33)	3.26(2.91)	2.88(3.87)
Alkalinity (mEq/l)	6.00(3.22)	5.23(3.19)	5.74(3.79)	5.72(3.90)	5.29(5.40)	5.21(5.42)
Cl^- (mg/L)	9.12(2.69)	5.89(1.84)	5.98(1.93)	4.81(2.07)	6.12(4.65)	5.98(6.22)
F^- (mg/L)	0.08(0.05)	0.08(0.06)	0.08(0.06)	0.08(0.07)	0.08(0.06)	0.08(0.06)
Silicate (mg/L)	3.07(1.00)	2.43(0.89)	3.01(1.37)	2.43(0.70)	1.80(0.95)	1.68(0.81)
SO_4^2 (mg/L) $^-$	3.78(2.00)	3.38(1.94)	3.89(2.56)	3.60(2.85)	4.40(3.14)	4.36(3.18)
NO_3+ NO_2-N (mg/L)	0.04(0.04)	0.11(0.10)	0.08(0.08)	0.03(0.03)	0.03(0.05)	0.03(0.11)
NH_4-N (mg/L)	0.02(0.01)	0.04(0.05)	0.04(0.06)	0.04(0.23)	0.05(0.24)	0.04(0.03)
PO_4^{3+}-P (mg/L)	0.02(0.03)	0.03(0.02)	0.02(0.02)	0.01(0.01)	0.02(0.08)	0.02(0.04)
pH	6.46(0.65)	6.21(0.80)	5.87(0.98)	5.77(0.86)	5.40(0.99)	5.32(0.95)
Data collection (years)	1978-1994	1995-2010	1970-2009	1973-2009	1971-2010	1983-2008

*A rough conversion from TDS to EC is provided in Dallas & Day (2004): TDS(mg/l) = EC(mS/m) x 6

2.4 Discussion

2.4.1 Site selection procedure

The site selection procedure, based on the analysis of historical flow data using the IHA software package provided a useful method for selecting sites exhibiting a wide range of hydrological characteristics including seasonal variability in flow. The study sites exhibited a range of annual flow statistics (*viz.* mean annual flow, C.V. of flow, minimum and maximum moving averages as well as Colwell's indices of predictability). It has been demonstrated that variation in flow volumes impact thermal patterns (Rivers-Moore *et al.* 2004, 2005). As such, the range in flow statistics (when compared to findings regarding several other rivers across South Africa) (Rivers-Moore *et al.* 2008a, 2008b) was

considered to be large enough to produce the desired concomitant range in thermal characteristics necessary for the purposes of examining the impact on life cycles of selected aquatic insects.

In terms of Colwell's predictability indices, thermal predictability values did not show the same range across the rivers as was observed for flow predictability. This is most likely to be an artefact of using only 13 months' worth of temperature data for calculating predictability indices (which generally require a minimum or five years data in the case of water temperature and a minimum of 20 years for flow data (Dr. Nick Rivers-Moore, pers. comm., University of KwaZulu Natal, 2009)). Thermal predictability indices were therefore unable to provide information on interannual thermal variability. This said, rivers experiencing more variable flow conditions generally exhibited higher mean annual water temperatures (Table 2.7). Furthermore rivers with higher mean annual flow exhibited a higher annual standard deviation and annual C.V. of water temperature (Table 2.7).

Water temperature metrics calculated for data from 2009-2010, while not allowing for interpretations of interannual variation, did however offer first insights into the intraannual variability of the thermal regime in the rivers. Analyses of thermal data collected for each of the study sites, when compared to the initial analyses performed on historical daily flow data, revealed a similar grouping of sites (Figs. 2.3, 2.11). A strong correlation between mean annual water temperature and annual C.V. of flow along with a strong correlation between thermal predictability and flow predictability was found (Table 2.7). This suggests that differences in flow characteristics among the sites are mirrored by differences in thermal characteristics. In turn this observation supports the contention by Ward (1985) that Southern Hemisphere rivers (including highly variable South African rivers) with extreme flow fluctuations typically exhibit concomitant extreme temperature fluctuations with marked interannual differences in thermal regimes. This in effect implies that flow regimes/flow predictability could be used as a proxy to evaluate different thermal regimes/thermal predictability and is supported by the findings in this study. Some evidence of this is provided in Rivers-Moore *et al.* (2008a, 2008b) who have shown that maximum diel water temperature range increased and thermal predictability decreased with increasing stream order (generally increasing flow volumes).

In the PCO plot of water temperatures (Fig. 2.13), the Wolwekloof River was most distinct from the other sites as a result of a combination of relatively high values for the variables mean monthly temperature for July, annual mean minimum temperature and counts of temperatures below the minimum threshold. This may be due to the effects of the modelling of water temperatures for the Wolwekloof River over the winter months. Particularly note worthy are the considerably high modelled standard deviation values for the Wolwekloof River over May-Mid October 2009. This is most likely due to the fact that the model can not accurately account for cold groundwater inputs during winter months. These groundwater inputs would likely result in additional buffering of thermal variability, thereby reducing standard deviation of water temperatures to values similar to those observed in other

rivers over winter. Though, since a) the modelled mean monthly water temperatures appeared to be consistent with the mean temperatures recorded for the other sites, and b) the mean monthly water temperatures were the primary variable used for analyses in this study (rather than standard deviation), the modelled temperatures were thus considered adequate for the purposes of this study. That said modelled standard deviation, maximum and minimum daily water temperature data for the Wolwekloof River, should be considered with caution when interpreting life-history data presented in Chapter 4.

2.4.2 Field sampling

Flow and water temperature: metrics and multivariate analyses

Daily flow and water temperature data collected over the study period confirmed the separation of sites based on the historical daily flow data used in the initial site-selection procedure. Daily flow data showed a good separation of sites, with the larger Molenaars, Elandspad and Wit rivers all showing higher sustained flows over the entire period from late autumn through winter (April-September) in comparison to the smaller Wolwekloof, Eerste and Rooi-Els Kloof rivers. These differences in flows are largely attributable to a) the relative sizes of the catchment areas feeding these streams - something which could be investigated further by reanalysing the data using specific discharge (discharge/area) which would serve to remove catchment size effects on the flow data, b) the amount of local precipitation at each site (Fig. 2.9), c) stream order (e.g. the Molenaars and Elandspad rivers are both second order streams) and d) the respective position of the site in relation to the longitudinal profile of each river. Greatest differences in water temperature among the sites were observed for summer and autumn (December to March), where water temperatures differed by as much as 7°C over a 30-day period (from roughly 20°C to 27°C). In the light of recent LT_{50} and CTM work conducted on aquatic invertebrates in South African rivers (see Dallas & Ketley 2011, Dallas & Rivers-Moore 2011), thermal differences as great as these could result in water temperature threshold limits for development and even survival of several aquatic insect species being reached or surpassed at some of the study sites. The differences in water temperatures and flows exhibited at each of the study sites thus provide a good contrast against which to compare the life-history responses and timing of traits of selected aquatic insect taxa.

The Rooi-Els Kloof River provided the best example of a river with both a high constancy score and a high predictability score for both flow and temperature. The low stable temperatures observed in this river over summer, despite the fact that air temperatures were similar to the other streams, in conjunction with the low standard deviation in both flow and temperatures as well as markedly higher baseflow index, suggest that this stream is in fact largely groundwater fed[11]. While all perennial rivers

[11] A preliminary analysis of Hydrogen and Oxygen isotope (δD and $\delta^{18}O$) samples, collected from the study sites, was also conducted but it did not yield conclusive information as to the extent to which the study rivers are fed by groundwater. The investigations suggested that the Wit River may have the lowest relative input of groundwater

in the Western Cape are likely to receive some groundwater inputs that sustain baseflows over summer (Assoc. Prof. Jenny Day, pers. comm., University of Cape Town, 2009), certain rivers are likely to receive a greater contribution of groundwater inputs than others (e.g. the Rooi-Els Kloof River). Median groundwater levels were observed at the greatest depth at this site when compared to the other sites (Table 2.2). This finding may seem counter intuitive in that deeper groundwater reserves could be thought to have less of an impact on the site. However it could also be possible that the river is strongly influenced by groundwater nonetheless through a) a presence of greater subsurface reserves of groundwater at the site and b) the fact that this deeper groundwater may be less affected by surface air temperatures than shallow groundwater reserves and is thus colder. The Eerste River showed similar thermal trends suggesting that it may too receive large inputs of groundwater, however median groundwater levels and baseflow index values did not provide sufficient support for this conjecture. Further studies such as stable isotope analyses (using monthly samples of river water in conjunction with local precipitation samples and borehole samples) are needed to confirm these speculations of groundwater inputs.

In contrast, the Wit and the Wolwekloof rivers appear to be more run-off dominated rather than groundwater dominated – as evidenced by the lowest thermal and hydrological predictability and constancy scores (arising from highly variable flow and water temperatures).

Regime shift analyses

The number of flow regime shifts at each site correlated to Colwell's predictability values based on historical flow data, while the timing and magnitude of the shifts appear to be related to both the timing and the amount of precipitation experienced at each site. The regime shift analyses indicated that shifts in the flow regime for the sampling period varied across all six rivers, but two distinct groupings were obvious, these were: Group 1, rivers with more regime shifts (i.e. more variable flow) in the sampling period (the Eerste, Wit, and Wolwekloof) and Group 2, rivers exhibiting fewer regime shifts (i.e. less variable flow) in the sampling period (Elandspad, Molenaars, Rooi-Els Kloof). Rivers in Group 1 showed the three lowest flow predictability values calculated from historical daily flow data, while rivers in Group 2 exhibited the three highest calculated predictability values.

Excluding the additional shifts that were exhibited by rivers in Group 1 (from July to September 2009) as a result of more variable flows in winter, the relative timing and duration of the shifts in the mean

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compared to the other study rivers. The Wit River was the only river to have an isotopic signature of baseflow that plotted below the Local Meteoric Water Line (LMWL), normally indicative of a) a strong evaporative effect or b) recharge from high altitude source. The evaporative effect could be a result of less groundwater input to baseflow and higher air temperatures, but since isotope data of borehole samples as well as precipitation samples from each of the sites are unavailable for comparison to the river water samples collected, the available information (in the absence of a dedicated isotope study) was not sufficient to draw concrete conclusions. As such the matter was not taken further in this study.

values were similar across all rivers. Differences in timing were often not more than two to three days apart (see Appendix 2D). This suggests that for the most part the same climatic conditions or cold fronts were responsible for the shifts in all rivers, with observed differences in the number of shifts, largely as a result of the relative amount of precipitation occurring at each of the sites (linked also to the relative size of the catchments). In general regime shift analyses of flow appear to provide a useful tool for quantifying the "flashiness" or intraannual variability of the hydrological regime in rivers.

With the exception of the spring spate that occurred simultaneously in all rivers, the timing of thermal regime shifts was shown not to be as closely related to the timing of hydrological regime shifts as was initially expected. While the sites generally showed similar timing, number and duration of thermal shifts, some subtle differences in the trends were noted. Positive spring and summer thermal shifts occurred later in the Wit, Wolwekloof and Eerste rivers. These later shifts appear to coincide with the timing and number of hydrological regime shifts observed from July to September, suggesting that spates and high flows occurring slightly later in the rainy season delay the onset of warmer spring and summer conditions in these rivers. Additionally these rivers also revealed lower predictability scores in terms of both flow and temperature.

The Rooi-Els Kloof River, which was noticeably the coldest river during the spring period, appeared to be unaffected by the cooling effect of the spring cold front (4^{th}-5^{th} November) which led to negative thermal shifts in the other rivers. This could be as a result of the higher baseflow index calculated for this river which in turn suggests a greater relative proportion of groundwater input compared to the other rivers (see Appendix 2B and Table 2.2). A large proportion of groundwater input over the year could have the effect of buffering water temperatures, in turn reducing the cooling effects of potential snow melt input and also cold fronts or related storm events (see Sear *et al.* 1999). This is most apparent in the comparison of air temperatures and water temperatures, specifically between the Wit and Rooi-Els Kloof rivers (see Fig. 2.5)

In general thermal shifts occurred seasonally and, except for spring, the shifts occurred independently of flow. Flow regime shifts occurring later in the season, from late winter to spring and affected the timing of positive thermal regime shifts. The greater number of high flows and flow regime shifts experienced over this period resulted in a delayed warming of water temperatures in early summer. Within the year over which the sampling was conducted, only two major periods of thermal regime shift were evident (see Fig. 2.14 and Appendix 2D). These shifts occurred simultaneously across all rivers and coincided closely (roughly within 2 weeks in both 2009 and 2010) with dates marking the March (autumnal) and September (spring) equinoxes (20-21 March and 22-23 September, respectively). In 2009 the daily average hours of sunlight for Cape Town, South Africa for the period between 20^{th} March and 22^{nd} September was 10.88 hours, compared to 13.13 hours between the period 22^{nd} September 2009 and 20^{th} March 2010. As such while thermal shifts were influenced to a degree by

flow, they appeared to be largely driven by photoperiod (day length changes coinciding with the dates of the March and September equinoxes).

Physicochemical variables

Generally in clear perennial mountain streams DO is seldom a limiting factor, as it very rarely drops to levels low enough to negatively affect aquatic taxa (Hynes 1970). In this study the lowest levels of oxygen saturation were recorded for the warmest river over summer, namely the Wit River (see Fig. 2.15). These levels however were still within the range of 85-90% oxygen saturation, suggesting suitable habitat conditions even during this warm and low flow period in this river.

In general, EC showed a typical inverse relationship with flow. Marked declines in EC were observed over periods of high flows (June, October), likely due to dilution from winter spates, while levels increased substantially during slower flowing warmer periods (see Fig. 2.15). Some increasing trends in EC were observed over late-winter as flows resided, but levels once again dipped as a result of dilution with spates occurring in November. The observed peaks in turbidity and EC in the Eerste River (associated with a fire in its surrounding area) are unlikely to have had any major adverse effects on the biotic responses of taxa within the river. However, high levels of Total Suspended Solids (TSS) might have reduced the amount of light penetrating the water column thereby affecting primary productivity and growth rate for certain taxa.[12].

Overall sites showed pH mean and range values similar to those presented for fynbos mountain streams in the southern and western coast water quality management area (see Dallas *et al.*1998). Sharp declines were observed in the pH levels for three rivers (*viz.* the Elandspad, Molenaars and Eerste) during the winter spates in June and August, supporting the findings of Dallas *et al.* (1998) (see Fig. 2.15). In the Rooi-Els Kloof and Wolwekloof rivers, pH levels remained largely the same for the entire winter period and showed typical increases with the onset of warmer water temperatures in spring and summer. The decreases observed from April-June for the majority of the rivers most likely relate to the fact that lower levels of pH are commonly observed following the first heavy rains of autumn and winter which result in high flows or spates (Dallas *et al.* 1998). The first spate conditions of the season usually result in the flushing of organic and humic matter that accumulates over the autumn leaf fall, which in turn leads to an increase concentration of organic acids in the water body and thus lower pH levels (Dallas *et al.* 1998). Heavy rainfall events also lower the pH of river water as a result of rain water taking up dissolved minerals and organic molecules as it percolates through soil within the catchment and eventually enters the rivers. The observed increases in pH for the Molenaars, Elandspad, Eerste rivers and, to a degree, the Wit River during July could relate to pH levels re-stabilising after initial high flow events and after flushing of natural salts from the catchment. Similar trends in the timing of leaf fall and

[12]This conclusion is based on the findings of Britton (1991) in a study looking at the impacts of fire on a river within the same catchment as the Eerste River.

the accumulation of Coarse Benthic Organic Matter (CBOM) in rivers within the Western Cape were noted by King *et al.* (1987) and Stewart & Davies (1990). The range in recorded pH values for each of the study sites can also be attributed to diel fluctuations in pH (see Dallas *et al.* 1998), as measurements were recorded at different times of the day at each site.

Sites selected for this study were all situated in either mountain stream or transitional zones, within protected (nature conservation) or wilderness areas. This was purposely chosen in order to as far as possible control for factors (such as water quality variables), which may have further confounded the analyses of biotic responses of aquatic invertebrates to flow and temperature gradients. Values calculated for all chemical constituents are well within the Target Water Quality Range (TWQR) values presented in the water quality guidelines for South Africa (Department of Water Affairs and Forestry 1996) and are representative of healthy upper reaches of mountain streams in the fynbos biome of the Western Cape (see Dallas *et al.* 1998). Annual median and standard deviation values of important water chemistry constituents provided in Table 2.8 indicated that over long time periods sites were generally similar and shared similar ranges of values, though some minor differences were observed. Most noticeable was the range in pH values among the sites as well as the elevated nitrate + nitrite values especially for the Molenaars and Elandspad rivers. While these differences cannot be ignored, in this study water chemistry constituent data such as those provided in Table 2.8 from the DWA were not measured during the sampling process, owing to cost limitations, and thus cannot be directly linked to life-history data presented in Chapter 4. This said, apart from pH and perhaps nitrate + nitrite values, it is unlikely that other chemical constituents (barring exposure to toxic levels) would have even minor influences on aquatic insect life-history traits as water temperature and flow are the major variables driving lotic ecosystems (Poff & Ward 1989, Tockner *et al.* 2000).

CHAPTER 3

Molecular investigations of *Lestagella penicillata*, *Aphanicercella* spp. and *Chimarra ambulans*: genetic profiles for interpreting life-history traits

Summary

Detailed systematics including molecular analyses are important for studies of life-history trait responses. This is particularly true for the taxa in question, in relation to environmental conditions, as they provide better insight as to whether life-history plasticity or genotypic variation underlies the observed differences. Additionally, molecular data can provide confirmation of species identification, which is crucial in life-history analyses. In this chapter molecular analyses were performed using the CO1 gene of target species of the Ephemeroptera (*Lestagella penicillata*), Plecoptera (*Aphanicercella clavata, A. flabellata, A. scutata, A. barnardi*) and Trichoptera (*Chimarra ambulans*) collected from each of the six study sites as well as an additional site on Table Mountain. The main aims of this molecular investigation were to a) assess intra- and inter-specific genetic divergence between species from each of the study sites b) detect cryptic species complexes potentially overlooked by current taxonomy and c) assess the possibility of catchment isolation. The CO1 data suggested that both *L. penicillata* (maximum of 28.7% CO1 gene divergence among the six study sites and the Table Mountain site) and *C. ambulans* (maximum of 13.5% CO1 gene divergence between Table Mountain site compared to six study sites) populations showed evidence of having diverged to the point where they could be considered to be separate (sibling) species. The presence of previously undescribed morphologically cryptic species complexes, evolving under different environmental conditions (hydrological, thermal and chemical), could account for the divergences observed, though the effects of incomplete lineage sorting, should not be ruled out. Further, morphological and molecular analyses, with more extensive sampling and the utilisation of additional genetic markers (e.g. *16S* and *PEPCK*) along with IBD analyses are suggested to resolve the systematics of these taxa. For *Aphanicercella*, the CO1 gene was able to successfully resolve the four species identified by current taxonomy. Additionally, molecular data was used in conjunction with diagnostic characters to confirm the new descriptions provided here of early-instar (including instar I) nymphs of *A. flabellata*. Results of this study presented a prime example of a case where current taxonomy had overlooked cryptic species diversity, which could substantially confound results of life-history studies and experiments of species thermal tolerance limits. More specifically, the results suggested that life-history data, egg development and rearing experiments for *L. penicillata* should be interpreted in conjunction with molecular data. In other words, variable egg development responses might be expected for *L. penicillata* populations given knowledge of the evolutionary status of the different populations. A finding that warranted further investigation was that of consistent molecular divergence observed in populations of all three study genera collected from the Window Stream site (Table Mountain) compared to sites in other mountain ranges. This suggested that thermal tolerance limits could perhaps be driven by site-specific adaptation in geographic and genetic isolation, most notably when Table Mountain (Window Stream) populations were compared to populations in other mountain ranges separated from the Peninsula. The notion of Table Mountain having acted as a refugium for stenothermic montane species of Gondwanan origin, as

evidenced by high levels of endemism in the fauna and flora of the Cape peninsula mountains was therefore one of particular relevance in this study (discussed further in Chapter 7). Overall, the molecular analyses using the CO1 gene provided a valuable contribution to the limited knowledge database of South African aquatic insects. More specifically they also provided important information with regards to the systematics of two species (*L. penicillata* and *C. ambulans*) widely used in biomonitoring regimes and which are sensitive to anthropogenic impacts on water quality and thermal change. Additionally this study has also provided a useful platform from which further morphological and molecular studies can be conducted targeting these taxa. Such studies, if conducted in conjunction with environmental investigations, could be used a) to resolve the phylogeny of the taxa and b) to determine potential drivers of genetic divergence, population gene flow, dispersal ability and species tolerances.

3.1 Introduction

Accurate and rapid species identification is fundamental not only to the basic and applied fields of aquatic research but also to numerous aspects of biological research, for example studies in evolutionary biology, conservation biology, ecophysiology, biodiversity and biomonitoring (Hebert *et al.* 2003a, Tautz *et al.* 2003, Ball *et al.* 2005, Jinbo *et al.* 2011, Hernandez-Triana *et al.* 2012). Where thorough morphological and genetic studies have been conducted for groups of taxa and these studies in turn have been accompanied by detailed taxonomic revisions, correct species identification by the end user is made possible. This however is not the case in parts of the world where exceptional diversity and a lack of taxonomic capacity meet, such as in the tropics and southern temperate zones (Stewart & New 2007, Butlin *et al.* 2009). Ironically, these less understood areas, like the Cape Floristic Region of South Africa investigated in this study, have been shown to contain numerous centres of endemism as well as biodiversity 'hotspots' (centres of endemism coinciding with areas of greatest threat) (Picker & Samways 1996, Myers *et al.* 2000), elevated levels of species richness (Platnick 1991), and generally a greater diversity than that of the better documented northern temperate regions (Stewart & New 2007). In the face of the biodiversity crisis (see Savage 1995), predicted effects of global climate change, as well as the impacts of ongoing anthropogenic activity, relatively slow progress is being made towards the documentation of biodiversity, including the rate of species description and identification (Hajibabaei *et al.* 2011). As such, our uncertainty regarding the Earth's biodiversity, in its entirety, remains a major challenge to overcome and has lead to a controversial debate emerging in the literature as to whether the existing tools and techniques utilised in traditional taxonomy are sufficient to meet this challenge.

Allozyme electrophoresis, microsatellites and DNA sequence variation have been used widely since the late 1970's to study the taxonomy, ecology and evolution of numerous taxa, including mayflies (Ephemeroptera) (Monaghan & Sartori 2009, Avise 2010). Initially the focus of these early investigations was on delimiting species in morphologically cryptic groups and, through the use of genetic markers, making links between immature and adult stages (Monaghan & Sartori 2009). What followed was an increase in population genetics studies, where dispersal was commonly inferred through measures of gene flow (Monaghan & Sartori 2009). Additionally, several key studies on freshwater invertebrates have a) provided evidence for genetic variation (allozyme variation) affecting life-history traits (Sweeney *et al.* 1986, Postma *et al.* 1995a, Miller & Hendricks 1996, Kavanaugh 1998) and b) linked genetic variability (allozyme variation) with certain environmental factors e.g. flow (Robinson *et al.* 1992), temperature (Sweeney *et al.* 1986) and depth preference (Weider 1984, Muller & Seitz 1993). Of importance too are those studies that assessed phenotypic plasticity (Blanckenhorn 1991, Peckarsky *et al.* 2002, Peckarsky *et al.* 2001) and contributed to the ever growing interest in the question of the evolution of phenotypic plasticity, its role in speciation, its ecological consequences and its developmental cost (Via & Lande 1985, Stearns & Koella 1986, Scheiner 1993, DeWitt 1998,

Pigliucci 2005, Pfennig *et al.* 2010, Foster 2013). Over the years however advances in computational technologies and sequencing (at an unprecedented rate) have lead to DNA sequencing becoming a simple, cost-effective and rapid means of providing large amounts of robust genetic data (Hajibabaei *et al.* 2007, Jinbo *et al.* 2011, Hajibabaei *et al.* 2011).

Of particular interest, and the cause of much debate among taxonomists, geneticists and ecologists alike, has been the advent of DNA barcoding. Proposed by Hebert (2003a, 2003b), DNA barcoding uses a standard primer set, applicable to all animal phyla (Folmer *et al.* 1994), to amplify a short section of an organism's genome as a molecular identifying tag. The same section of the genome is used for all organisms and is a 648-base pair (bp) region of the mitochondrial cytochrome-c oxidase subunit 1 (CO1) gene. This region (CO1) exhibits a wide range of phylogenetic signal, fast mutation rates, and a conserved sequence among conspecifics - making it suitable to discriminate between species (Hebert *et al.* 2003a, 2003b). Using a standard protocol, the initial idea behind DNA barcoding was to create a comprehensive DNA barcode library for a broad range of organisms that could be rapidly processed at large throughput facilities (Jinbo *et al.* 2011). In this manner DNA barcoding was intended to be used to a) compare the sequence data from an unidentified specimen to that of a reference library containing sequences from known (described) species in order to provide species level identification, b) detect potential morphologically cryptic species complexes, and c) to detect new species (Hajibabaei *et al.* 2007). Where many specimens or a number of additional genes have been sampled, DNA barcoding can also be used for population-level analyses and for interrogating deep phylogenetic relationships (Hajibabaei *et al.* 2007).

The DNA barcoding enterprise has received mixed reception. On the one hand it has received criticism for: using a single gene in isolation for taxonomic studies (Funk & Omland 2003, Moritz & Cicero 2004, Roe & Sperling 2007), the utilisation of distance-based methodologies (DeSalle *et al.* 2005, Meier *et al.* 2006), the effects of sampling scale (Bergsten *et al.* 2012), its feasibility and actual intended use along with its efficacy (Rubinoff 2006, Rubinoff *et al.* 2006, Dasmahapatra *et al.* 2010) and even its scientific relevance - specifically to taxonomy (Ebach & de Carvalho 2010). On the other hand however numerous studies have shown its abilities to: accurately link larval and adult stages for species in which this has previously been difficult (mayflies - Ball *et al.* 2005, butterflies -Gossner & Hausmann 2009), identify microscopic organisms (zooplankton, Bucklin *et al.* 2007), flag species complexes (Ståhls & Savolainen 2008, Lara *et al.* 2010, Hernandez-Triana *et al.* 2012), accurately resolve relationships in groups containing a large number of species (e.g. several major groups of the phylum Arthropoda - Hebert *et al.* 2003a, Aves - Hebert *et al.* 2004, Lepidoptera - Silva-Brandão *et al.* 2009), provide rapid, reliable identifications for biomonitoring programs and water quality assessment programs (Ball *et al.* 2005, Webb *et al.* 2007, Hajibabaei *et al.* 2011), build large reference libraries of barcodes (Zhou *et al.* 2009, 2011, Webb *et al.* 2012) and also to provide some novel applications (Jurado-Rivera *et al.* 2009 - insect-host plant associations, Holmes *et al.* 2009 - shark and ray fin

identification, Clare *et al.* 2009 - predator prey relations in bats). Taylor & Harris (2012), in a revision of Barcoding over the past 8 years and in considering recent advances of Next Generation Sequencing (NGS), state that "Clearly, arthropod taxonomy is a field that stands to benefit from the added clarity promised by DNA barcoding"(see also Jinbo *et al.* 2011). However, in spite of some of the praise for DNA barcoding and opinions of its ability to become the core of taxonomy ('DNA taxonomy' - Blaxter 2003, Tautz *et al.* 2003), it is important that the limitations of barcoding are acknowledged (Alexander *et al.* 2009, Pauls *et al.* 2010) and that as far as possible traditional taxonomy accompany DNA barcoding studies (Sundberg *et al.* 2010).

In southern Africa a widely available set of taxonomic keys, entitled "Guides to the Freshwater Invertebrates of Southern Africa" published by the Water Research Commission of South Africa, do exist for major groups of aquatic invertebrates, such as the Ephemeroptera, Plecoptera and Trichoptera amongst others (Barber-James & Lugo-Ortiz 2003, de Moor & Scott 2003, Stevens & Picker 2003). However, while these keys represent an excellent collation and synthesis of a large body of data into available documents, they are nevertheless still incomplete and are unable to properly address identification in immature stages for many taxa. Additionally many of the taxa regrettably rely on taxonomic works that have not been sufficiently revised for some time (e.g. the works of Barnard 1932, 1934a, 1940). This reality is evident in several of the genetic studies focusing on aquatic invertebrates from southern Africa. Although these studies are relatively limited in number, they have in most cases yielded results suggesting a need for taxonomic revision, with several discoveries of cryptic species complexes and potentially new species, highlighting a rich area of research requiring further attention (e.g. Daniels *et al.* 2001, Wishart & Hughes 2001, 2003, Stevens 2009, Pereira-da-Conceicoa *et al.* 2012). Inevitably in such a situation as this, a problem that remains is knowing where to focus limited resources and attention in future studies. With the importance of establishing thermal guidelines for the Ecological Reserve in South Africa, one area that may benefit from such focused attention is the taxonomic revision (supported by rapid genetic analyses like DNA barcoding) of thermally sensitive taxa that are also used for biomonitoring.

In the chapters that follow within this thesis, representative species from Ephemeroptera[13], Plecoptera[14] and Trichoptera[15] (EPT taxa), are investigated in terms of a) their life-history traits from six different rivers within the Western Cape of South Africa (Chapter 4) where the rivers/sites were purposely selected (Chapter 2) to represent a range of thermal and hydrological regimes, b) egg development rates at experimental temperatures (Chapter 5) and c) growth rates at experimental temperatures (Chapter 6).

[13] *Lestagella penicillata* (family Teloganodidae)
[14] Four species of Plecoptera within the *Aphanicercella barnardi* species complex, *A. barnardi, A. clavata, A. flabellata* and *A. scutata* (family Notonemouridae)
[15] *Chimarra ambulans* (family Philopotamidae)

These abundant and widespread taxa (within South Africa) are commonly used in biomonitoring procedures in South Africa (Chutter 1972). They represent taxa not only sensitive to anthropogenic impacts on water quality but also to increasing water temperature (Dallas & Ketley 2011, Dallas & Rivers-Moore 2012, Dallas *et al.* 2012, Rivers-Moore *et al.* 2013b), thus highlighting their importance as indicator species and relevance as target species in studies aimed at establishing thermal guidelines for the Ecological Reserve. Furthermore they have easily recognisable morphological characters used to define them as species. Of interest is that two of the taxa (*Lestagella* and *Aphanicercella*) are considered to be of Gondwanan origin (Day 2005, Stevens 2009) with limited distribution and potentially unique thermal requirements (stenothermy).

In the specific context of the components of this thesis that are to follow (Chapters 4-6), the aims of this chapter are to: 1) confirm species identification using available taxonomic keys in combination with molecular data, for the species of aquatic insects utilised in this thesis from seven different study site/sampling localities, 2) to investigate whether genetic differences exist between the different populations of the study species that might reflect differences in life-history traits, 3) to determine if catchment isolation has resulted in significant genetic divergence, 4) confirm species identification of immature stages of *A. clavata, A. scutata, A. flabellata* and *A. barnardi* for which no taxonomic keys are presently available, 5) interpret results with regard to the existing and available taxonomic keys as well as the limited genetic data available for aquatic insects in South Africa.

3.2 Methods

3.2.1 Collection of specimens

Larvae and adults of *L. penicillata, A. barnardi, A. clavata, A. flabellata, A. scutata,* as well as *C. ambulans* were collected in August 2011 from the six sites selected for this study (see Chapter 2) and also from an additional site from the Kirstenbosch Gardens on the slopes of Table Mountain (Window Stream) (Table 3.1). Adult *Aphanicercella* and *Chimarra* were collected off rocks using an aspirator. Adults of *L. penicillata* were collected by rearing black wingpad nymphs (nymphs at the final stage of maturity prior to emerging as adults) to maturity in an artificial laboratory setup. Upon collection, all larvae and adult specimens were preserved in 96% ethanol and stored at -20°C until molecular analyses were performed.

Table 3.1. Site locality information for specimens of *Lestagella penicillata*, *Aphanicercella* spp. and *Chimarra ambulans* collected for genetic analysis.

Site name	Latitude	Longitude	Level 1 Eco-region	Species analysed
Eerste	-33.993776	18.975550	Southern Folded Mountains	*L. penicillata, A. flabellata, C. ambulans*
Elandspad	-33.736667	19.114722	Western Folded Mountains	*L. penicillata, A. flabellata, A. scutata, C. ambulans*
Molenaars	-33.731390	19.115000	Western Folded Mountains	*L. penicillata, A. flabellata, C. ambulans*
Rooi-Els Kloof	-33.461100	19.617860	Western Folded Mountains	*L. penicillata, A. barnardi, C. ambulans*
Window	-33.983351	18.430928	Southern Folded Mountains	*L. penicillata, A. clavata, C. ambulans*
Wit	-33.637090	19.107890	Western Folded Mountains	*L penicillata, C. ambulans*
Wolwekloof	-33.944167	19.026389	Southern Folded Mountains	*L. penicillata, A. scutata, A. flabellata, C. ambulans*

3.2.2 Identification and preparation of specimens

Collected specimens of *L. penicillata* were identified using the most recent revision of the family Teloganodidae (McCafferty & Wang 1997) in conjunction with original descriptions by Barnard (1932, 1940). For the identification of species of *Aphanicercella*, taxonomic descriptions of the nymphs produced by Picker & Stevens (1997) and Stevens & Picker (1999) were used together with original descriptions by Barnard (1934b, 1940). Additional taxonomic notes and drawings regarding morphological descriptions for both immature and mature nymphs of the species of *Aphanicercella* collected in this study are presented in Appendix 3A. Included in the appendix are descriptions of abdominal setal patterns of the four species, which were compared to those provided by Stevens (2009) (see Table App3A.1 in Appendix 3A). Descriptions of early-instar nymphs of *A. barnardi*, *A. flabellata* and *A. scutata* are also provided in Appendix 3A (Fig. App3A.2), as they are not available in current taxonomic keys and were necessary for the identification of nymphs analysed in the study of life-histories (Chapter 4). A key to the families of Trichoptera for the Afrotropical region produced by Scott (1978), together with descriptions by Barnard (1934a, 1940) were used to identify *C. ambulans*. Collected and identified specimens that were to be sequenced were photographed using a Leica EZ 4 dissecting microscope fitted with a digital camera (Appendix 3B).

3.2.3 Extraction, PCR Amplification and sequencing

Samples were sent to the Canadian Centre for DNA Barcoding (CCDB)[16] for molecular analyses, via the South African Institute for Aquatic Biodiversity (SAIAB)[17], a partner organisation of Consortium for the Barcode of Life (CBOL). DNA extraction, PCR amplification and sequencing took place at the CCDB and followed the CCDB protocols (Ivanova *et al.* 2006 - www.dnabarcoding.ca). A summary of

[16] The CCDB is a high throughput genetic facility based in the Institute of Biodiversity (BIO) at the University of Guelph, Canada.

[17] Initial DNA sequences for *L. penicillata* from the Rooi-Els Kloof and Molenaars rivers and Window Stream as well as *C. ambulans* from the Eerste River were obtained by Dr. Tuuli Makinen at the South African Institute for Aquatic Biodiversity (SAIAB). The Primers LepF1/LepR1 were used for specimens of *Chimarra* sequenced at SAIAB.

the methods employed at the CCDB for DNA extraction, PCR amplification and sequencing are provided below.

Extractions were carried out using a Biomek® FXP robotic liquid handling station (Keckman Coulter Inc.) with a 96 multichannel head and a Thermo Cytomat hotel. Polymerase Chain Reaction primers developed by Folmer *et al.* (1994), namely LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'), along with polymerase from Invitrogen™ (Platinum® *Taq* DNA Polymerase), were used in a first attempt to amplify the full DNA barcode region: a *c*. 648bp long target region 58–705 from the 5_0-end of the *cytochrome c oxidase 1* gene (CO1). PCR amplification was carried out following the PCR conditions of Hebert *et al.* (2003a). These primers (HCO2198 and LCO1490) are commonly used by the CCDB as they have been shown to successfully amplify this target gene region from a wide range of invertebrate taxa (Hebert *et al.* 2003a).

An Applied Biosystems 3730xl DNA analyser along with BigDye™ Terminator (version 3.1) were used for the sequencing procedure (bi-directional), while a semi-automated AutoDTR™ method from EdgeBio® was used for post-cycle sequencing clean-up. Information regarding the DNA extraction, PCR amplification and sequencing procedures as well as laboratory equipment used at the CCDB is available at the following internet addresses: www.dnabarcoding.ca/CCDB_DNA_Extraction.pdf, www.dnabarcoding.ca/CCDB_Amplification.pdf, www.dnabarcoding.ca/CCDB_sequencing.pdf and www.dnabarcoding.ca/CCDB_Equipment_Infrastructure. Following sequencing, detailed specimen records including locality information, digital images and sequence information were uploaded to the Barcode of Life Database (BOLD—http://www.boldsystems.org). Information pertaining to these specimen records can be accessed through BOLD under the project file "South African freshwater invertebrates" (SAAIN).

3.2.4 Sequence analysis

One taxonomically identified species from the genus *Lestagella* (*L. penicillata*) obtained from seven different localities (2-3 specimens from each locality) in the Western Cape was used in the analysis of the Ephemeroptera. In total 17 separate sequences (including a single sequence for the outgroup) were aligned. For the analysis of the Plecoptera, five taxonomically identified species of the genus *Aphanicercella* (*A. clavata, A. barnardi, A. flabellata and A. scutata*) obtained from six different localities were used (1-2 specimens of each species), giving rise to a total of 14 separate aligned sequences (including a single sequence for the outgroup). For the analysis of the Trichoptera, one taxonomically identified species from the genus *Chimarra* (*C. ambulans*) obtained from seven different localities (1-2 specimens from each locality), was used. The total number of aligned sequences used in the analysis of *Chimarra* was also 14.

Sequence data (CO1 gene) for the outgroups included in the phylogenetic analysis were obtained from GenBank at the National Centre for Biotechnology Information (NCBI) using the BLASTn search engine (www.ncbi.nlm.nih.gove/genbank). From the BLASTn search results, only sequences of specimens in related taxa that exhibited similar nucleotide sequences (compared to those of single representatives of each of the three genera) and which were positively identified were used as outgroups. The outgroup taxa selected were *Eurylophella temporalis* (Ephemerellidae), *Aphanicerca capensis* (Notonemouridae) and *Dolophilodes distinctus* (Philopotamidae). GenBank accession numbers for the outgroup taxa are provided in Appendix 3C. Sequence data for all specimens were aligned and analysed using Molecular Evolutionary Genetics Analysis (MEGA) version 5.0 software (Tamura *et al.* 2011), following recommendations by Cywinska *et al.* (2006) and Hebert *et al.* (2003a). The MUSCLE alignment method (Edgar 2004), with Neighbour Joining (NJ) selected as clustering method 1 and UPGMB selected as clustering method 2, was used to align sequence data for all specimens. MUSCLE, while not being the fastest alignment algorithm currently available, has been shown to provide accurate and reliable results in moderate processing times (see Nuin *et al.* 2006). It has also been shown to be more accurate than several other alignment programs (viz. CLUSTAL W, Dialign2.2, POA, Dialign-T and Kalign) (see Nuin *et al.* 2006) and has the added benefit of being freely available with an easy to use interface in MEGA 5. Following sequence alignment, separate tests utilising a maximum likelihood approach for finding the best DNA nucleotide substitution models were conducted in order to determine the most likely model of evolution in the nucleotide sequence data for each genus. Saturation of the sequence data was visualised using plots of transitions and transversions with the F84 distance parameter and tested for each genus separately using DAMBE (Xia & Xie 2001, Xia *et al.* 2003, Xia & Lemey 2009). Nucleotide composition as well as Guanine+Cytosine (GC) percentage composition was calculated from sequence data pertaining to each genus.

3.2.5 Phylogenetic analysis

Based on the outcome of the maximum likelihood test for the best fit model of nucleotide substitution, the model with the highest Bayesian Information Criterion (BIC) score employed in MEGA 5[18] was used to construct pairwise genetic distance matrices and Maximum Likelihood (ML) phylograms for each of the study genera in order to visualise the clustering pattern of specimens and to assess levels of genetic divergence. To check for congruence in phylogenetic tree construction methods for each genus, two distance-based trees, one utilising a clustering-based method algorithm, namely NJ, and the other utilising an optimality-based method algorithm, namely Minimum Evolution (ME), were compared. In

[18] MEGA 5 allows for a limited number of models to be used for constructing phylogenetic trees using the NJ, ME methods. These being: No. of differences, p-distance, Jukes-Cantor, Kimura 2-parameter, Tajima-Nei, Tamura 3-parameter, Tamura-Nei, Maximum Composite Likelihood (MCL), LogDet (Tamura-Kumar) - Gamma distributions describing the rate of substitutions among sites can be specified for each of these models, while the proportion of invariant sites cannot be specified. For this reason where the model with the best fit (based on the maximum likelihood test for best fit model of nucleotide substitution) could not be used to construct the phylogenetic tree the model with the next highest BIC score employed in MEGA 5 was used.

addition to these distance-based trees, separate Maximum Parsimony (MP) cladograms were also constructed. In order to assess phylogenetic tree construction reliability in all phylogenetic trees, bootstrapping methods[19] (1000 replicates) were used. Bootstrap support for nodes was compared across all phylogenetic tree construction methods and is indicated on the ML trees for each genus. In ME trees, the heuristic method used was the Close-Neighbour-Interchange (CNI), with initial trees obtained using an NJ algorithm. The MP trees were obtained using bootstrapping methods (1000 replicates) as well as the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). For constructing ML phylogenetic trees, the heuristic method used was the Nearest Neighbour Interchange (NNI) with initial tree(s) for the heuristic search obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. For the construction of all phylogenetic trees (using both distance-based and optimality-based methods), codon positions included were 1st+2nd+3rd+Noncoding. Transitions and transversions were included as substitutions and all positions containing gaps and missing data were eliminated using the complete deletion option. Uniform rates of nucleotide substitution were assumed among sites.

Phylogenetic signal was assessed using the Consistency Index and Retention Index (CI and RI, respectively) for MP trees. These indices provide, respectively, a relative measure of homoplasy in the cladogram, and the proportion of synapomorphy expected from a data set that is retained on a tree within the sequence data. Phylogenetic tree construction, analyses of reliability and tests for nucleotide evolution models were all performed in MEGA 5 (Tamura *et al.* 2011).

3.3 Results

3.3.1 Data characteristics

Information regarding sequence characteristics as well as summary information from the parsimony analysis and maximum likelihood analysis of nucleotide substitution for the sequences of specimens of each of the genera sequenced are given in Table 3.2.

[19] Bootstrapping analysis allows confidence values to be derived for the groupings of sequences in a tree. Random samples of sites from the original alignment are used to redraw trees (one tree per resample). The percentage of trees from 1000 resampling replicates which resolve the same groupings from the original tree are then presented by a number above the nodes.

Table 3.2. Data characteristics and analysis summaries of the CO1 DNA barcoding region of specimens from three genera of aquatic insect (*Lestagella*, *Aphanicercella* and *Chimarra*) from different localities in the Western Cape, South Africa. The number of taxonomically identified species (# Spp.), number of sequences obtained for each genus (# Seq.), length of aligned sequences in terms of base pairs (Length), percentage Guanine-Cytosine content (GC), number of variable sites (# Var.) number of parsimony informative sites (# Pi) are given. The parsimony search summary comprises the number of trees retained (# Trees), tree length (Score), consistency index (CI) and retention index (RI). The nucleotide substitution analysis summary comprises the best fit model following a maximum likelihood analysis in MEGA 5 with the associated Bayesian Information Criterion score (Model (BIC)) and the log likelihood score (lnL).

	CO1 sequence characters				**Parsimony analysis**						**Nucleotide substitution**			
Taxa	**# Spp.**	**# Seq.**	**Length**	**GC**	**# Var. (%)**	**# Pi (%)**	**# Trees**	**Score**	**CI**	**RI**	**Best model used for ML tree (BIC)**	**lnL**	**Model used for NJ, ME and MP trees (BIC)**	**lnL**
Lestagella	1	17	641	45.6	209 (32.61)	164 (25.59)	7	326	0.829	0.938	TN93+I (4825.27)	-2240.68	T92+G (4854.61)	-2269.29
Aphanicercella	4	14	629	37.0	129 (20.51)	58 (9.22)	8	166	0.819	0.915	T92+G (3507.70)	-1626.69	T92+G (3507.70)	-1626.69
Chimarra	1	14	525	35.8	142 (27.05)	50 (9.52)	3	179	0.782	0.853	HKY+G (3147.94)	-1440.43	T92+G (3152.66)	-1451.70

The consistency and retention indices revealed low relative amounts of homoplasy in the sequence data for each genus, providing support for the results obtained from MP phylogenetic tree construction. Average Guanine-Cytosine (GC) content was highest in *L. penicillata* (45.6%) along with the total number of variable and parsimony informative sites (32.6 and 25.83 % respectively) when compared with the Plecoptera and Trichoptera. Tests for sequence saturation using DAMBE (Xia *et al.* 2003, Xia & Lemey 2009) revealed no significant results, therefore suggesting no significant effect of saturation on the phylogenetic inference of each genus.

Maximum likelihood fit analyses of 24 different models of nucleotide substitution for the Ephemeroptera, Plecoptera and Trichoptera studied revealed that the models with the best fit based on BIC scores were, in order, the TN93+I (Tamura-Nei model) (Tamura & Nei 1993) with invariant sites, the T92+G (Tamura 3 parameter model) (Tamura 1992) with a gamma distribution, and the HKY+G (Hasegawa, Kishino, Yano model) (Hasegawa *et al.* 1985) with a gamma distribution (Table 3.2). Complete outputs of the results of the maximum likelihood fit analysis of nucleotide substitution models for each genus are provided in Appendix 3D.

3.3.2 Phylogenetic analyses

Ephemeroptera

For the Ephemeroptera, while all samples were identified via morphological features (using larvae) as belonging to a single species *L. penicillata*, the ML, NJ and ME phylograms unexpectedly revealed two major clades separated by long branch lengths (Fig. 3.1). The percentage sequence divergence between samples on these two major clades was exceptionally high (maximum of 28.7%) (Table 3.3). Specimens

from the Molenaars, Elandspad and Rooi-Els Kloof rivers, along with specimens from the Window Stream and Eerste River sites collectively comprised the first of the two major clades in the phylogram (Fig. 3.1). Bootstrap values for the node supporting this clade were high (97-99%), while smaller clades were separated by branch lengths indicating divergences as high as 10.6% (e.g. between sequences of Win Lp2 and Roo Lp2) (Fig. 3.1). Individual specimens from the Eerste River formed a distinct clade, as did those from the Window Stream. Both of these clades exhibited high bootstrap values for the respective supporting nodes (97-100%) and average within-group divergences of 0.01% and 0.0% respectively. Specimens from the Molenaars and Elandspad rivers showed high similarity and occurred in the same clade as a single divergent specimen from the Rooi-Els Kloof River (Roo Lp2); this clade had an average within-group divergence of 0.30% (Table 3.3). The node supporting this clade also exhibited high bootsrap values (98-100%) in all tree construction methods (Fig. 3.1). The node supporting the respective clades containing specimens from both the Window Stream and Eerste River sites should however be inferred with caution as low bootstrap values (<75%) were observed in all phylograms construction methods. The second major clade (with node boostrap support of 100% in all tree contruction methods) comprised two smaller clades separated by branch lengths indicating approximately 6.0% divergence (Fig. 3.1). The first of these two smaller clades contained two closely related individuals from the Rooi-Els Kloof River (Roo Lp1 and Roo Lp3) and the second comprised specimens from the Wolwekloof and Wit River sites which showed high similarity (0.2% within-group divergence) (Table 3.3). Each of the aforementioned smaller clades exhibited high boostrap values for the supporting nodes (96-100%) and were also separated by branch lengths greater than 3% divergence (Fig. 3.1).

The different phylogenetic tree construction methods used exhibited congruence in terms of bootstrap support values for nodes presented on the ML phylogram in (Fig. 3.1). No bootstrap support was found, however, for the basal node (node circled in Fig. 3.1) in the ML phylogram of *L. penicillata*. The same holds true for the basal node in the phylograms of the other genera studied.

Variable levels of sequence divergence were observed across the sequences of *L. penicillata*. Generally individuals collected from the same site exhibited very small or zero divergence values. An exception to this was observed in the specimens collected from the Rooi-Els Kloof River, which were split into two separate clades. Two specimens from this site (Roo Lp1 and Roo Lp3) revealed 0% divergence, while the third specimen (Roo Lp2) exhibited divergence as high as 25.6% from the other two (Fig. 3.1, Table 3.3).

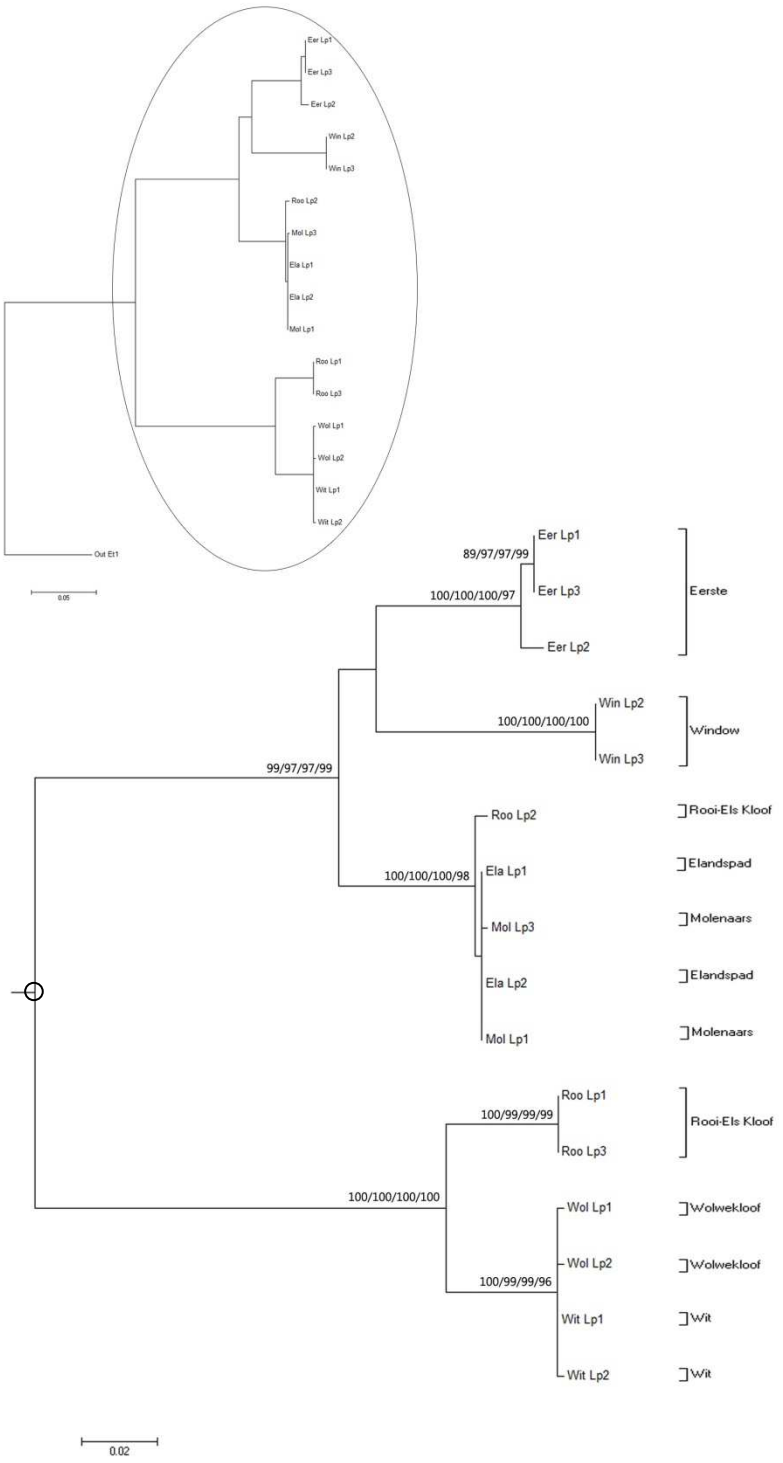


Fig. 3.1. Maximum likelihood phylogram for specimens of *Lestagella penicillata* based on the Tamura-Nei model (Tamura & Nei 1993). The tree with the highest log likelihood (-2329.9047) is shown. Bootstrap support (1000 replicates) with values > 80% for MP/NJ/ME/ML methods are shown above branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.0010% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 641 positions in the final dataset. The basal node is circled.

Table 3.3. Estimates of evolutionary divergence between sequences of *Lestagella penicillata*, measuring the proportion of nucleotide differences between each pair of sequences. Analyses were conducted using the Tamura-Nei model (Tamura & Nei 1993). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.0010% sites). The analysis involved 16 nucleotide sequences excluding the outgroup. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

	Eer Lp1	**Eer Lp2**	**Eer Lp3**	**Ela Lp1**	**Ela Lp2**	**Mol Lp1**	**Mol Lp3**	**Roo Lp2**	**Roo Lp1**	**Roo Lp3**	**Win Lp2**	**Win Lp3**	**Wit Lp1**	**Wit Lp2**	**Wol Lp1**	**Wol Lp2**
Eer Lp1																
Eer Lp2	0.009															
Eer Lp3	0.004	0.000														
Ela Lp1	0.089	0.091	0.089													
Ela Lp2	0.089	0.091	0.089	0.000												
Mol Lp1	0.089	0.091	0.089	0.000	0.000											
Mol Lp3	0.090	0.093	0.090	0.002	0.002	0.002										
Roo Lp2	0.092	0.094	0.092	0.005	0.005	0.005	0.006									
Roo Lp1	0.269	0.271	0.269	0.255	0.255	0.255	0.256	0.256								
Roo Lp3	0.269	0.131	0.269	0.255	0.255	0.255	0.256	0.256	0.000							
Win Lp2	0.099	0.101	0.099	0.105	0.105	0.105	0.106	0.106	0.285	0.285						
Win Lp3	0.099	0.101	0.099	0.105	0.105	0.105	0.106	0.106	0.285	0.285	0.000					
Wit Lp1	0.269	0.271	0.269	0.255	0.255	0.255	0.256	0.256	0.059	0.059	0.285	0.285				
Wit Lp2	0.270	0.273	0.270	0.256	0.256	0.256	0.258	0.258	0.060	0.060	0.287	0.287	0.002			
Wol Lp1	0.270	0.273	0.270	0.256	0.256	0.256	0.258	0.258	0.060	0.060	0.287	0.287	0.002	0.003		
Wol Lp2	0.270	0.273	0.270	0.256	0.256	0.256	0.258	0.258	0.060	0.060	0.287	0.287	0.002	0.003	0.003	

Plecoptera

Four species of Plecoptera (*Aphanicercella*), mostly nymphal, were identified based on morphology. The ML, NJ and ME phylograms all revealed the separation of these species into three distinct major clades separated with moderate branch lengths, indicating divergence greater than 3% between species (Fig. 3.2). The first major clade comprised two smaller clades and a single related branch. The two smaller clades consisted of *A. scutata* samples from both the Elandspad and Wolwekloof River sites. Divergence between *A. scutata* individuals on these clades was low (maximum of 0.488%) along with negligible average intraspecific divergence (0.26%) (Fig. 3.2, Table 3.4). The single branch consisting of a specimen of *A. barnardi* from the Rooi-Els Kloof River was found to have a sequence divergence of approximately 3.72% from *A. scutata* individuals (Fig. 3.2, Table 3.4). The second unresolved major clade comprised specimens of *A. clavata* from the Window Stream with an intraspecific divergence of 0.84%, while the third major clade comprised three smaller clades consisting of specimens of *A. flabellata* from the Wolwekloof, Eerste and Molenaars River sites (Fig. 3.2, Table 3.4).

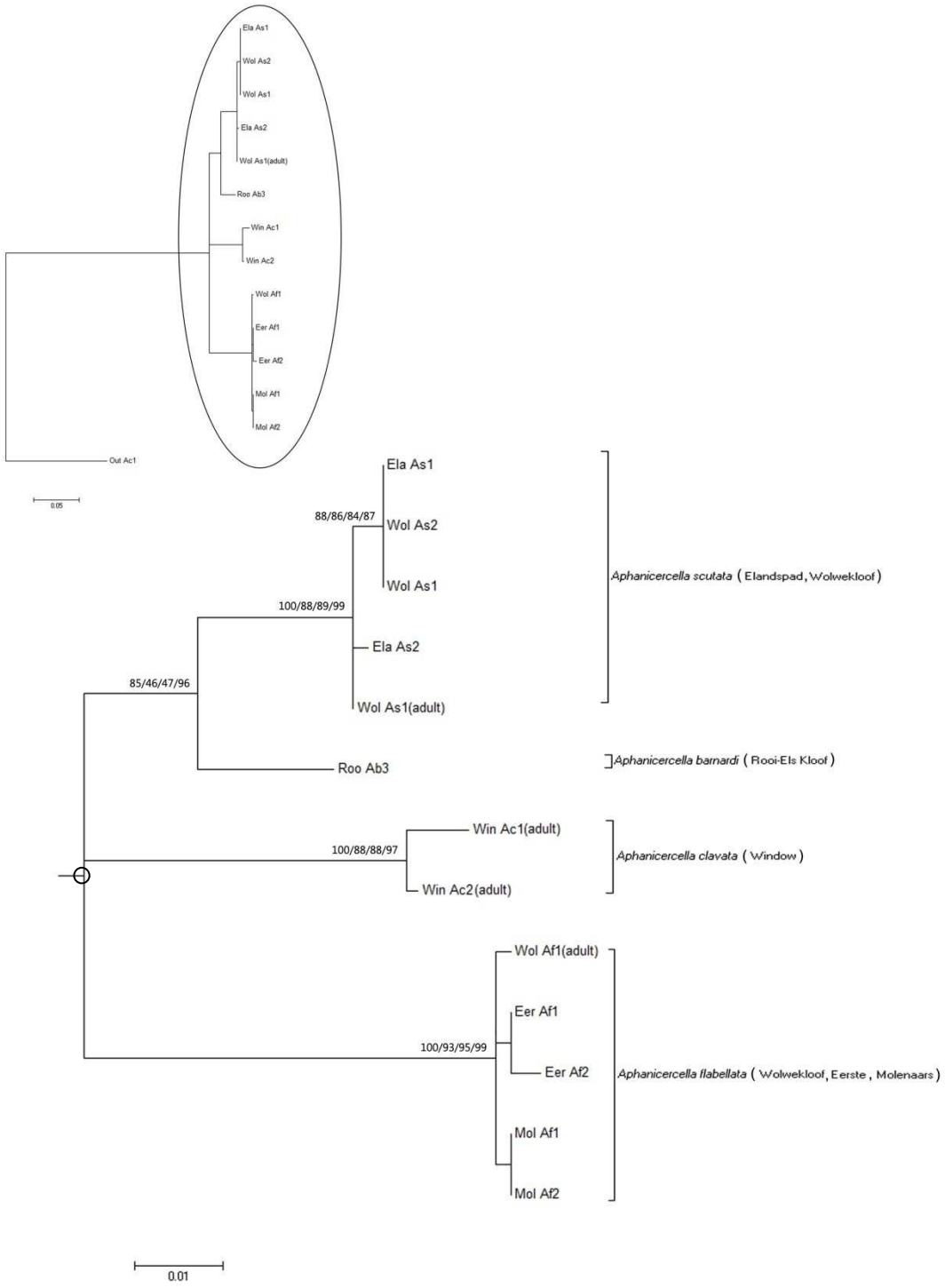


Fig. 3.2. Maximum likelihood phylogram for specimens of four species of *Aphanicercella* spp.based on the Tamura 3-parameter model (Tamura 1992). The tree with the highest log likelihood (-1631.7577) is shown. Bootstrap support (1000 replicates) with values > 80% for MP/NJ/ME/ML methods are shown above branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3088)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 629 positions in the final dataset. The basal node is circled.

This entire clade revealed an intraspecific divergence of 0.33%. Average intraspecific divergence for the genus *Aphanicerella* was 0.47%, suggestive of little population divergence, while average interspecific divergence was high (7.9%) with a maximum of 10.2% observed between *A. clavata* and *A. flabellata* (Table 3.4).

High bootstrap values were shown to support the nodes of each of the three major clades, except the node of the unresolved clade comprising *A. clavata* in each of the different phylogenetic tree construction methods (Fig. 3.2). However the ancestral relationship between the clades was found to differ in the ML phylogram compared to the NJ, ME, MP phylogenetic trees, and since no bootstrap support was exhibited for the basal node in any of the phylogenetic trees, no confident inferences can be made regarding the ancestral relationships of these species. The NJ, ME and MP trees, while not shown, were however congruent[20] in inferring specimens of *A. clavata* as comprising a clade distantly related to the clade comprising *A. scutata* with the *A. barnardi* branch and also the clade comprised of *A. flabellata*. Thus the NJ, ME and MP trees revealed an alternative topology in this regard to the ML phylogram. Bootstrap support for the supporting node in this aforementioned alternative topology however, was found to be low (<75%) for the three different construction methods, thus not allowing for confident inferences to be made.

Table 3.4. Estimates of evolutionary divergence between sequences of *Aphanicercella* spp., measuring the proportion of nucleotide differences between each pair of sequences. Analyses were conducted using the Tamura 3-parameter model (Tamura 1992). The rate variation among sites was modelled with a gamma distribution (shape parameter = 0.31). The analysis involved 13 nucleotide sequences excluding the outgroup. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

	Ela As1	**Ela As2**	**Wol As1 (adult)**	**Wol As1**	**Wol As2**	**Eer Af1**	**Eer Af2**	**Mol Af1**	**Mol Af2**	**Wol Af1 (adult)**	**Roo Ab3**	**Win Ac1 (adult)**	**Win Ac2 (adult)**
Ela As1													
Ela As2	0.005												
Wol As1 (adult)	0.003	0.002											
Wol As1	0.000	0.005	0.003										
Wol As2	0.000	0.005	0.003	0.000									
Eer Af1	0.078	0.076	0.073	0.078	0.078								
Eer Af2	0.083	0.081	0.078	0.083	0.083	0.003							
Mol Af1	0.078	0.076	0.073	0.078	0.078	0.003	0.003						
Mol Af2	0.078	0.076	0.073	0.078	0.078	0.003	0.003	0.000					
Wol Af1 (adult)	0.078	0.076	0.073	0.078	0.078	0.003	0.007	0.003	0.003				
Roo Ab3	0.035	0.034	0.032	0.035	0.035	0.073	0.073	0.068	0.068	0.073			
Win Ac1 (adult)	0.070	0.073	0.070	0.070	0.070	0.082	0.087	0.082	0.082	0.082	0.070		
Win Ac2 (adult)	0.067	0.066	0.063	0.067	0.067	0.085	0.090	0.085	0.085	0.085	0.063	0.008	

[20] The first node (basal node) also shows no boot strap support in the NJ, ME and MP methods. However, the second node from which *A. clavata* stems is shown to be a group related to the other species. This was observed in the NJ, ME and MP trees, with 43, 41 and 75% bootstrap support values respectively.

Trichoptera

Only a single species of Trichoptera, *C. ambulans* was identified morphologically using larvae from collected samples. However, phylogenetic analyses in the form of a NJ, ME and ML phylograms, unexpectedly revealed two distinct major clades separated by long branch lengths (approximately 10.7%) (Fig. 3.3). The first major clade consisted of multiple smaller clades comprising specimens from the Wit, Rooi-Els-Kloof, Molenaars, Wolwekloof, Elandspad and the Eerste River sites. Between-group divergence among the sequences on this major clade averaged 1.83%, while divergence within the sites (for sites with more than a single individual) averaged approximately 1.2% (Table 3.5). Sequences from the Wolwekloof River showed the highest within-group divergence of 2.82% (Table 3.5). The second major clade comprised specimens collected solely from the Window Stream site. Divergence between specimens from this clade and those on the first major clade was as high as 13.9%[21] (Fig. 3.3, Table 3.5). Within group divergence among sequences from the Window Stream site were however low, on average 0.38% (Table 3.5). Bootstrap support for the nodes on each of the two major clades was high (>90%) in the ML phylogram and MP cladogram but were not supported in the NJ and ME methods (Fig. 3.3). The major difference between the NJ and ME trees compared to that of the ML tree was the placement of a single specimen from the Wolwekloof River site Wol Ca1. Within group divergence between the sequences from this site were quite high (2.82%) and as a result the sample Wol Ca1 in the NJ and ME trees was subsequently placed as the most basal specimen after the outgroup. This sample was most closely related to a single clade comprising smaller clades from all other sites including the Window Stream site. The inferred phylogeny, produced by both the NJ and ME methods, should however be interpreted with caution as the bootstrap support values were all very low (<70%) (Fig. 3.3). Highest bootstrap support values of 53 and 52% for the NJ and ME methods respectively were observed for a clade consisting solely of specimens from the Window Stream site. Apart from these values bootstrap support for NJ and ME methods were all very low and thus not congruent with the MP and ML methods.

The MP tree for *Chimarra* produced a phylogeny almost identical to that of the ML tree with the only exception being that of the relationship between the samples Ela Ca2, Wol Ca2 and Mol Ca1 (Fig. 3.1). The MP cladogram, like the ML phylogram also showed high bootstrap support values for several nodes (Fig. 3.3).

[21] Comparing Wit Ca1 with Win Ca2(adult)

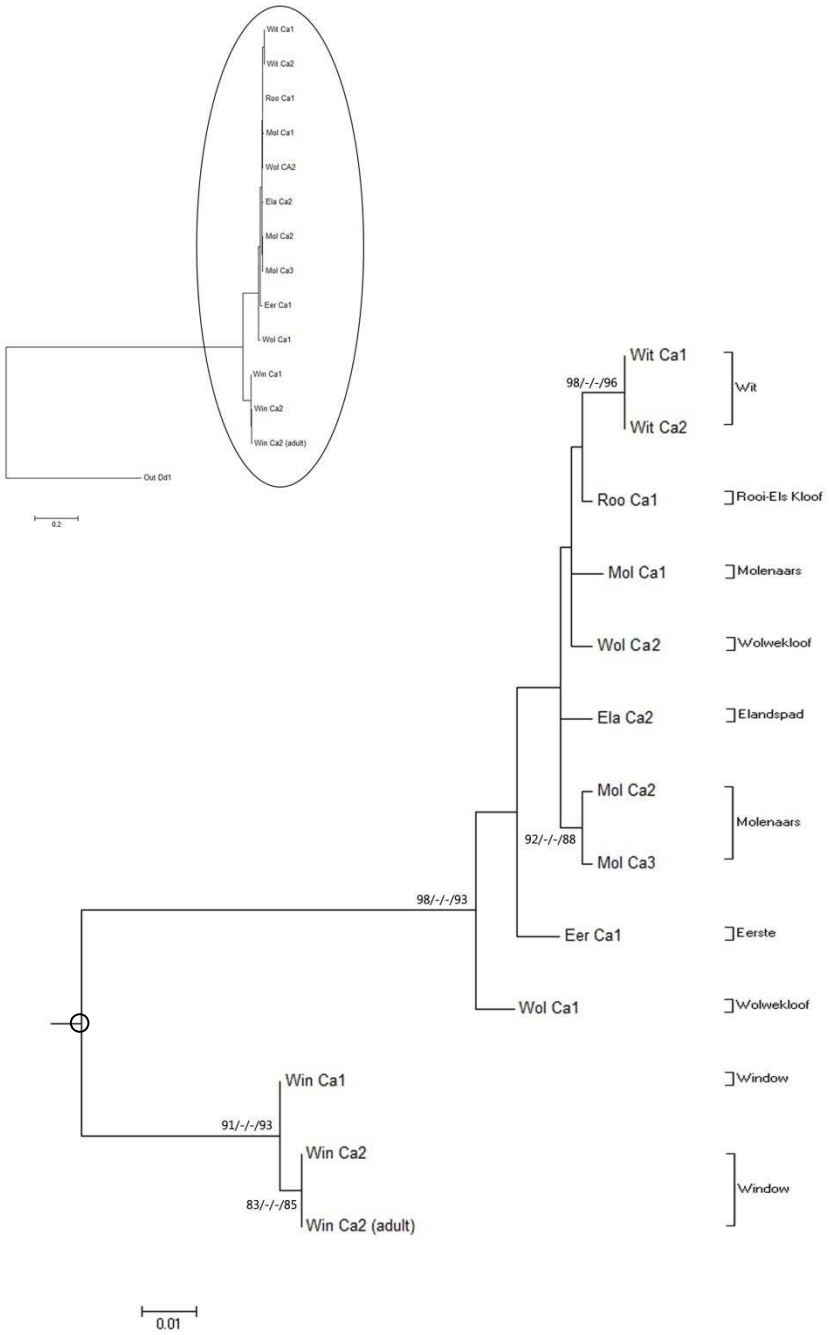


Fig. 3.3. Maximum likelihood phylogram for specimens of *Chimarra ambulans* based on the Hasegawa-Kishino-Yano model (Hasegawa *et al.* 1985). The tree with the highest log likelihood (-1468.5788) is shown. Bootstrap support (1000 replicates) with values > 80% for MP/NJ/ME/ML methods are shown above branches. Branches not supported by a particular tree construction method are indicated by "-". Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4172)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 525 positions in the final dataset. The basal node is circled.

Table 3.5. Estimates of evolutionary divergence between sequences of *Chimarra ambulans*, measuring the proportion of nucleotide differences between each pair of sequences. Analyses were conducted using the Hasegawa-Kishino-Yano model (Hasegawa *et al.* 1985). The rate variation among sites was modelled with a gamma distribution (shape parameter = 0.42). The analysis involved 13 nucleotide sequences excluding the outgroup. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

	Eer Ca1	**Ela Ca2**	**Mol Ca1**	**Mol Ca2**	**Mol Ca3**	**Roo Ca1**	**Win Ca1**	**Win Ca2**	**Win Ca2 (adult)**	**Wit Ca1**	**Wit Ca2**	**Wol Ca1**	**Wol Ca2**
Eer Ca1	0.000												
Ela Ca2	0.021	0.000											
Mol Ca1	0.023	0.014	0.000										
Mol Ca2	0.021	0.011	0.013	0.000									
Mol Ca3	0.021	0.011	0.013	0.004	0.000								
Roo Ca1	0.021	0.011	0.010	0.012	0.011	0.000							
Win Ca1	0.123	0.129	0.131	0.129	0.129	0.129	0.000						
Win Ca2	0.127	0.133	0.135	0.133	0.133	0.133	0.004	0.000					
Win Ca2 (adult)	0.127	0.133	0.135	0.133	0.133	0.133	0.004	0.000	0.000				
Wit Ca1	0.035	0.017	0.015	0.017	0.017	0.010	0.135	0.139	0.139	0.000			
Wit Ca2	0.035	0.017	0.015	0.017	0.017	0.010	0.135	0.139	0.139	0.000	0.000		
Wol Ca1	0.015	0.028	0.030	0.028	0.028	0.028	0.115	0.119	0.119	0.034	0.034	0.000	
Wol Ca2	0.021	0.019	0.010	0.011	0.011	0.008	0.129	0.133	0.133	0.014	0.014	0.028	0.000

3.4 Discussion

The phylogenetic analyses (using the CO1 barcoding gene region) presented in this study, provided a preliminary examination of the molecular differences between samples of *L. penicillata*, four species of *Aphanicercella* (*A. clavata, A. barnardi, A. flabellata* and *A. scutata*) and *C. ambulans* from several localities in the Western Cape. To my knowledge the data represent the some of the first recorded molecular data, using the CO1 gene region, for these particular taxa from a number of localities in southern Africa.

3.4.1 Ephemeroptera

L. penicillata sequences revealed a range of intraspecific divergences in the CO1 gene region, inferred from the maximum likelihood phylogram, from 0% (between conspecific samples collected from the same site) to a maximum of 28.7% (between samples on two distinct clades - Win Lp3 and Wit Lp2). In light of *a priori* taxonomic identification of specimens from all populations showing little apparent morphological difference and thus belonging to a single species, this range of genetic divergence was unexpected. It was assumed that samples from each locality would generally show low average intraspecific divergence values in concordance with values commonly observed for conspecific samples (0-1.5%) (see for e.g. Hebert *et al.* 2003, Ball *et al.* 2005, Webb *et al.* 2012). The high levels of intraspecific divergence obtained in this study for a single taxonomically identified species *L. penicillata* are however not uncommon and are somewhat similar to values recorded in phylogenetic

analyses of other mayfly species from the Northern Hemisphere, in which the CO1 gene region was also used (e.g. Ball *et al.* 2005, Alexander *et al.* 2009, Webb *et al.* 2012).

Ball *et al.* (2005), in a study using the CO1 gene to create a reference library of sequences from 80 mayfly species in the northeastern United States, found that the intraspecific sequence divergence (using the Kimura 2 parameter) was as high as 20% for several taxa, with a maximum of 25.8% recorded for species within the genus *Tricorythodes*. In another genus *Maccerfertium,* representative samples of the species *Maccerfertium modestum* were found to have genetic divergences as high as 13.7%, which was attributed to a potentially undescribed representative of a cryptic species complex in which phenotypic variation is likely to have existed.

In a similar study Webb *et al.* (2012) gathered DNA barcodes from the CO1 gene region from 4165 specimens representing 264 nominal and 90 provisional species of mayfly from Canada, Mexico and the United States. Results from the study indicated that nearly 20% of the species included two or three haplotype clusters with intraspecific sequence divergence (Kimura 2 parameter) greater than 5%, with 14 species exhibiting intraspecific divergence greater than 20%. The maximum recorded intraspecific divergence for a single species of *Fallceon quilleri* (Baetidae) was as high as 26.7%. Again, the authors suggested that high intraspecific divergences were as a result of several unidentified species complexes, polyphyletic species and potentially incorrectly synonymised species.

High intraspecific divergences were also noted in a study on the mayfly genus *Ephemerella* (Alexander *et al.* 2009), where CO1 genetic divergences (Kimura 2 parameter) between populations of the same species were 12.9% and 18.6% for the species *Ephemerella dorothea* and *Ephemerella excrucians* respectively. Cases in this study where high levels of intraspecific divergence overlapped with levels of interspecific divergence (appearing in the trees as polyphyletic and paraphyletic species) were attributed to incomplete lineage sorting[22] in emerging or closely related, cryptic species. A similar range of intraspecific divergence was found between *Baetis rhodani* populations (Williams *et al.* 2006), where seven distinct haplogroups were found exhibiting CO1 divergence between 8-19%. The authors suggested that the haplogroups represented several evolutionary lineages but concluded that their species status remained uncertain. Such a conclusion, while simple, is appropriate when considering that without additional thorough morphological and taxonomic revision in conjunction with multi loci genetic analyses, barcoding is unable to comprehensively delimit new species. Barcoding *is* however able to provide hypotheses for new species and to guide taxonomic efforts to specific taxa/groups where genetic uncertainties are evident.

[22] Incomplete lineage sorting refers to the failure of two or more lineages to coalesce such that the most recent common ancestral gene copy does not occur in the most recent common ancestral population where the lineages co-occur (for further explanation see Degnan & Rosenberg 2009).

In the context of this study, the ML phylogeny created for *L. penicillata* resolved the specimens into two major clades with a total of five terminal clusters all with strong support. Generally each of the terminal clusters represented different populations in each of the study rivers except for the occurrence of sympatric species in the Wit and Wolwekloof rivers as well as the Molenaars and Elandspad rivers. Each of these terminal clusters were separated by branch lengths greater than the 2-3% threshold commonly used to delimit species in barcoding studies (see Hebert *et al.* 2003a, 2003b and Meyer & Paulay 2005). Therefore they potentially represent putative species under the phylogenetic species concept.

The clustering pattern shown in the ML phylogram could suggest a common species existing in both the Wit and Wolwekloof sites and unique species existing in the Eerste and Window sites. The Rooi-Els Kloof site appears potentially to have two sympatric species, approximately 25.6% divergent, one of which is very closely related to or conspecific with the common species occurring at the Elandspad and Molenaars River sites.

In light of the studies already discussed by Ball *et al.* (2005), Alexander *et al.* (2009) and Webb *et al.* (2012) as well as others (Williams *et al.* 2006, Ståhls & Savolainen 2008, Pereira-da-Conceicoa *et al.* 2012), the most probable explanation for the observed clustering patterns is that the distinct clusters or evolutionary lineages represent a number of undescribed species which collectively constitute a morphologically cryptic species complex of what is currently considered *L. penicillata.* The possibility of recent divergence and incomplete lineage sorting having an effect on observed divergences, cannot be ruled out however, and should be further investigated. These lineages could have evolved in response to catchment isolation and selection responses to differing environmental conditions experienced over the range of localities, specifically thermal and hydrological variables in conjunction with chemical variables such as pH (see for e.g. Pereira-da-Conceicoa *et al.* 2012) or EC. The differences in these environmental variables among the sample localities (excluding the Window Stream, which was included later in the study as an additional sampling site) are discussed in detail in Chapter 2. Regardless of these interpretations, what is clearly evident from the cladograms is that a taxonomic review of the family Teloganodidae and specifically the genus *Lestagella* is required to confirm the findings presented here and properly resolve the phylogeny of the group. Such a review would benefit from 1) a detailed morphological study of the genus *Lestagella* investigating morphological characters that have not been previously studied (e.g. the mouthparts and relative gill size and features of the imago) and 2) further genetic analyses with more extensive sampling incorporating both the CO1 gene and additional markers such as *small subunit ribosomal 16S rDNA* (*16S*) and *phosphoenolpyruvate carboxykinase (PEPCK*). Given more extensive sampling over a wider geographic area, gene flow could be established between populations and an investigation into isolation by distance (IBD) could prove useful in determining ancestry and evolution of the group. Based on the findings of Pereira-da-Conceicoa *et al.* (2012), who investigated the phylogeny of another widely

distributed South African mayfly species, *Baetis harrisoni*, and found five distinct lineages corresponding to differences in pH, it seems likely that *L. penicillata* has similarly evolved in rivers spread across the Western Cape that experience markedly different environmental conditions, specifically different thermal and hydrological regimes, including pH (Chapter 2). As such it would be ideal to conduct a similar analysis in an attempt to correlate environmental variables to genetic variation observed between the clusters of *L. penicillata*. This said, until the suggested analyses can be conducted to confirm these findings, conclusions similar to those provided by Williams *et al.* (2006) are probably most appropriate - this being that the status of *L. penicillata* populations remains uncertain, although levels of divergence suggest that one is dealing with a species complex.

3.4.2 Plecoptera

The results of the phylogenetic analyses of the four species of *Aphanicercella* (*A. barnardi*, *A. clavata*, *A. flabellata* and *A. scutata*) revealed a clustering pattern and genetic divergences that were consistent with current taxonomic species designations. The ML phylogram showed strong support (along with NJ, ME and MP tree constructions) for four separate lineages (>2-3% divergence), each representing known species that occur across the study sites (some occurring in sympatry e.g. *A. flabellata and A. scutata*). Similar findings have also been observed by Stevens (2009) who used the CO1 gene to investigate the systematics of the Notonemouridae of southern Africa, including amongst others the same species of *Aphanicercella* studied here. In the ML tree, the basal node exhibited a polytomy (i.e. a division into more than two clades), suggesting an unresolved relationship potentially owing to lack of information. This is most likely due to the fact that the fast-evolving COI gene is not always able to provide a good indication of deeper phylogeny. Possibly for the same reason, the basal nodes in the ML phylograms for each of the three genera studied do not yield good support either.

In general low intraspecific divergences (range of 0.3-0.8%) were recorded for species of *Aphanicercella* (where more than one population was analysed for each species) while interspecific divergences were markedly higher and ranged from 3.4% (*A. barnardi* to *A. scutata*) to 8.5% (*A. clavata* to *A. flabellata*). These divergences, inferred from the ML phylogram are consistent with a) morphological differences observed among species within the genus (Stevens & Picker 1999 - although no measures of divergence were provided in their study), b) findings from other phylogenetic studies on stoneflies also using the CO1 gene region where measures of sequence divergence were reported (e.g. Zhou *et al.* 2010 and Mynott *et al.* 2011) and c) also indicate the presence of a "bar-coding gap[23]" from 0.47% (mean intraspecific divergence) to 3.2% (minimum interspecific divergence) (see Hebert *et al.* 2003, Meyer & Paulay 2005). In contrast, Zhou *et al.* (2010), in their study of the EPT taxa of

[23] If a bar-coding gap is present, then the mean intraspecific distance is smaller than the minimum inter-specific distance between specimens of a group under study. Initially it was suggested that a mean interspecific distance of about 10 times the mean intraspecific distance was a conservative threshold for delimiting new species where taxonomy is uncertain (Hebert *et al.* 2003a).

Manitoba, report an average intraspecific divergence (K2P) and an average interspecific divergence or distance to nearest neighbour (K2P) for 19 species of Plecoptera as 0.35% and 11.56%. Mynott *et al.* (2011), in a study of an alpine stonefly genus *Riekoperla*, reported minimum interspecific divergences from 7.2-19.5% and maximum intraspecific divergences ranging from 0.6-5.8%.

The ML phylogeny presented in this study provides molecular evidence to confirm species identification of nymphs based on existing taxonomy for the genus *Aphanicercella* (see Picker & Stevens 1997 and Stevens & Picker 1999). In their taxonomic study, apart from mate choice experiments, as well as shapes and relative size of the subgenital plate in females, the pattern of hairs and setae present on the abdominal tergites and sternites of the larvae was a diagnostic character used to denote the species within the genus. In the study presented here, by using mature black wingpad nymphs of the four species of *Aphanicercella* in which the same diagnostic setal patterns were evident and the adult genitalia were visible through the cuticle, nymphal identification was possible and setal patterns were able to be traced back to early-instar nymphs for each species. Thus descriptions are provided (see Appendix 3B) of setal patterns in both mature nymphs of *A. barnardi, A. clavata, A. flabellata* and *A. scutata* and in early-instar nymphs of *A. flabellata, A. barnardi* and *A. scutata*. Descriptions of early-instar nymphs (currently impossible using current taxonomic keys) were required for life-history analyses involving these species (Chapter 4) and are further supported by the molecular data presented in this study. The descriptions of setal patterns in mature nymphs of *A. flabellata* along with descriptions in early-instar nymphs of *A. flabellata, A. barnardi* and *A. scutata* have not previously been reported on and therefore provide a useful identification reference for ecological studies.

Species of *Aphanicercella* within the *A. barnardi* species complex that are examined in this study are regarded as sibling species, distinguished by minor differences in the genitalia (Stevens & Picker 1999) and also in the setal patterns (this study). Occurring largely allopatrically (with an exception being the sympatric occurrence of *A. scutata* and *A. flabellata*) throughout the diverse topography and varying environments of perennial mountain streams of the Western Cape, they are also considered to have far more localised geographic distributions, which in turn suggests that 1) they have speciated relatively recently and have undergone little range expansion (Stevens & Picker 1999) or 2) that historically they had larger ranges which subsequently became contracted owing to environmental changes. The fact that the endemic species *A. clavata* was shown to exhibit the greatest interspecific divergence within the group is congruent with findings for *L. penicillata* and other organisms (Picker & Samways 1996, Daniels *et al.* 2013) occurring on Table Mountain. This isolation of montane species is proposed to have been caused by periodic flooding of the Cape Flats during the mid-Miocene and early Pleistocene periods, which in turn separated Table Mountain from the nearest mountain range, the Hottentots Holland (Stevens & Picker 1999, Daniels *et al.* 2013). Such an isolation event is likely to be responsible for the high divergence also observed between populations of the mayfly *L. penicillata* on Table Mountain (Window Stream site) compared to those at the Wit River. Major insights into the deeper

phylogeny and evolution of this genus would be gained through further genetic studies specifically investigating IBD, dispersal ability and population genetics or gene flow.

3.4.3 Trichoptera

The ML phylogram of *C. ambulans* unexpectedly revealed two lineages, essentially separating the specimens from the Window site from the rest. Comparisons of specimens occurring in these two lineages yielded divergences as high as 13.9%. Levels of intraspecific divergence thus ranged from 0 to 13.9%. In comparing divergences of specimens comprising these lineages, the specimens from the Window Stream site yielded within-site divergences ranging from 0 to 0.4%, while specimens from the remaining sites yielded within-site divergences ranging from 0 to 3.5%.

Values similar to these have been reported for other species of Trichoptera and some closely related species in particular. Pauls *et al.* (2010) found a maximum intraspecific divergence of 5.9% and a minimum interspecific divergence of 8.05% for uncorrected percentage differences in sequences of Chilean Trichoptera belonging to the family Hydropsychidae. Whereas in a study using the CO1 DNA barcoding gene region to facilitate the identification of Trichoptera in the Tigris River, Iraq, Geraci *et al.* (2011) reported a range of intraspecific divergence from 0 to 1.7% with interspecific divergence ranging from 1.9% to 31%. Only one species pair (Hydropsychidae) showed low interspecific divergence of 1.9% and these were considered closely related species yet were morphologically distinct. Zhou *et al.* (2010) in a study of the subarctic EPT taxa of Manitoba, Canada, reported mean intra and interspecific divergences of 0.34% and 12.21% respectively for a total of 68 species of Trichoptera. Values for intraspecific divergence ranged from 0 to 5.5%, while values for interspecific divergence ranged from 3.45% to 25.79%. In a similar study also by Zhou *et al.* (2011), focusing on establishing a barcode reference library for Trichoptera of the Great Smoky Mountains, CO1 sequences were analysed from 209 species. Their analyses revealed mean intraspecific divergence ranging from 0 to 10.2% with an overall mean of 1.7%. Of the species studied 11% showed a maximum intraspecific divergence greater than 8% with two of them, *Dolophilodes distincta* and *Polycentropus cinerus,* showing exceptionally high intraspecific divergences of 14% and 9.9% respectively. These high intraspecific divergences were considered to almost certainly represent species complexes. Furthermore closely related species to those analysed in this study, also within the genus *Chimarra, viz. C. atterima, C. augusta, C. obscura and C. socia*, were found to have maximum intraspecific divergences (K2P distances calculated from the Trichoptera Barcode of Life Database Library) of 13.6, 2.7, 8.0 and 8.7%.

In this study, as all the collected larval specimens are seemingly morphologically identical, two possibilities exist for interpreting the results obtained from phylogenetic analyses of *C. ambulans*: 1) Considering the proposed threshold of roughly 3% divergence for invertebrate species delimitation (Hebert *et al.* 2003a) one could consider it possible that in fact the specimens from the Window Stream site (diverging as much as 13%) represent a previously undescribed morphologically cryptic species of

what is currently considered to be *C. ambulans*. Similarly specimens from the other sites (diverging only as much as 3.5% maximum) collectively represent a single species within this cryptic species complex. 2) In light of high intraspecific divergences recorded for other species of *Chimarra* (Zhou *et al.* 2011), specimens from the Window Stream site could still be considered representatives of *C. ambulans*, even though they show greater genetic divergence possibly owing to geographic isolation. It is the opinion of the author that the first possibility is more feasible and most parsimonious and that the Window Stream population of *Chimarra ambulans* indeed fulfills the criteria for being a distinct species. What is clear is that the current taxonomy for the genus *Chimarra* in South Africa requires some investigation, as it currently does not account for the molecular diversity observed. As larvae are less easy to identify and distinguish morphologically than adults, morphological studies of adults may perhaps reveal taxonomic differences that have been missed thus far.

CHAPTER 4

Environmental modulation of life-history patterns

Summary

Gathering, examining and understanding information on life-history patterns, through a combination of both field and laboratory work is of fundamental importance for virtually all ecological studies of freshwater invertebrates. Through the collection and assessment of this life-history data the potential effects of changes to hydrological and thermal regimes on aquatic insects commonly used in bioassessment methods (SASS) and used as bioindicators (EPT taxa) can be gauged. Life-history information gathered for the same species from multiple locations, where different environmental conditions prevail, help to determine the degree to which life-history-traits are moderated by these site specific environmental conditions versus being constrained by phylogenetic history. The aims of this chapter were 1) to present detailed life-history data (investigating life-history traits such as growth, voltinism, emergence period and hatching period) for selected indicator species from the EPT taxa (namely *L. penicillata, A. barnardi, A. flabellata, A. scutata* and *C. ambulans*), 2) to contrast life-histories exhibited by these species in a set of pre-selected rivers that experience a range of hydrological and thermal regimes (data presented in Chapter 2), and 3) to determine tolerances and optima for growth and to interpret the life-history data in relation to the major environmental variables affecting life-history traits (flow, water temperature and water quality; data presented in Chapter 2) and also in relation to genetic analyses (data presented in Chapter 3). Monthly sampling of invertebrates was carried out for the period April 2009-April 2010 in the six rivers within the Western Cape. All target organisms collected each month were sorted, counted and measured. Field-collected data were analysed using size frequency histograms for assessing life-history patterns of the study taxa. Linear regression analyses and GLM techniques were then used to assess and interpret life-history data in relation to environmental data (flow, water temperature, physicochemical variables) and to determine tolerances and optima for growth. Voltinism was determined in each of the target taxa: *L. penicillata* and *Aphanicercella* spp. both exhibited a slow, seasonal univoltine cycle with a single cohort easily tracked throughout the year, while *C. ambulans* showed a non-seasonal or asynchronous multivoltine life cycle with multiple generations occurring simultaneously. *C. ambulans* appeared to show a phenotypically plastic response to temperature, in that more generations (trivoltinism) were observed in warmer rivers, in comparison to univoltine populations observed to occur in colder rivers. *C. ambulans* showed no recruitment during periods of high flow, while individuals of the *Aphanicercella* spp. emerged as adults during high flow periods. Thus for these two taxa the life cycle appeared to be timed such that larvae and nymphs avoided unfavourable high flow conditions in winter – either through undergoing a pupal stage in the case of the former or emerging as adults in the case of the latter. The effect of physicochemical variables on life-history patterns, however, remained somewhat unclear. Optimal thermal ranges for growth were established through the use of GLMs, and were found to be 13-21.5°C for *L. penicillata*, <11.5°C-14.5°C for *Aphanicercella* spp.,14.3°C- >21.5°C for *C. ambulans*). Differences in the thermal and hydrological regimes among the sites were found to indeed impact and

modulate life-history traits, where the same species was concerned, and this was more noticeable in *C. ambulans.* This species exhibited less phylogenetic constraint and more flexibility in terms of its life-history compared to *L. penicillata* and *Aphanicercella* spp. which showed greater phylogenetic constraint and greater adaptation to site-specific conditions – congruent with molecular analyses that showed higher genetic divergence among sites. Overall, the life-history responses of the target species assessed in this study appeared to be finely tuned to the hydrological and thermal regimes of each river studied. This could have been as a result of site specific evolution and adaptation, perhaps showing similarities on a catchment scale. However, where the same species showed differences in life-history responses (number and duration of generations) amongst rivers, the data appeared to suggest that water temperature was the most likely factor for these differences. The hydrological regime, on the other hand, was found to be the major driver in determining population size and mortality while possibly imposing a developmental time constraint for life-histories of the study taxa (especially *C. ambulans* and *Aphanicercella* spp.). The possibility that the putative effects of discharge on life-cycle and emergence might reflect synchronicity with the availability of key basal resources, or the effects of seasonal conditions on adult fitness, could however not be discounted and would require further investigation.

4.1 Introduction

The invertebrate benthos present in lotic ecosystems of temperate climates and particularly within small streams exhibit very clear life-history patterns (Hynes, 1970). Throughout the year, at different intervals, many species appear to come and go as larvae and adults as they complete their development and are subsequently replaced by other species. Yet these species are always present in some stage of their life-history during the course of a year, whether in the egg stage, pupal stage or adult stage, often overlapping with the presence of other species. Gathering, examining and understanding information on these apparent life-history patterns has been stated by Butler (1984) as "being of fundamental importance for virtually all ecological studies of freshwater invertebrates." With regard to the gathering of life-history data, Hynes (1970) placed strong emphasis on field work being combined with laboratory studies, as field work leaves many gaps in the knowledge, for instance the fate and or diapause of eggs and the timing of the hatching of first-instar nymphs/larvae. Ultimately, a combination of field work and laboratory studies is able to yield much more information than either method conducted alone. Only once information about an organism's life-history has been obtained, both through field sampling and laboratory experiments, can one begin to understand and predict the response of that organism to variation and change within lotic ecosystems and the factors that induce such change (Power *et al.* 1988).

Aquatic insects, being ectothermic, are sensitive to changes in water temperature. Additionally, temperature influences the solubility of dissolved oxygen and other gasses, affects reaction rates and can also increase the toxicity effects of ammonia and certain metals (Dallas & Day 2004). Temperature has also been shown to directly affect metabolism, growth, development, emergence, reproduction of aquatic insects, thus exerting influence on almost every aspect of life-history and distribution (e.g. Anderson & Cummins 1979, Vannote & Sweeney 1980, Ward & Stanford 1982, Sweeney 1984, Rosillon 1988, Ward 1992). Diversity, abundance and distribution patterns of stream biota over elevation gradients in both lentic and lotic waters, however, appear to be influenced by a *combination* of temperature and flow variability (Statzner *et al.* 1988, Poff & Ward 1989, Ward 1992). In this regard, the contrasting life-history strategies that have been observed for aquatic insects in high latitudes of the Northern Hemisphere (synchronous and seasonal, often with strict univoltinism or semivoltinism with staggered growth and or diapause) versus those from the Southern Hemisphere and the tropics (largely asynchronous and non seasonal with bi-, tri- or multivoltine life cycles) have been directly attributed to climatic variation: primarily differences in water temperature coupled with flow regime and to a lesser degree photoperiod as well as food availability (Khoo 1964, Hynes 1970, Hynes & Hynes 1975, Poff & Ward 1989, Huryn 1996, McKie *et al*. 2004, Danks 2007).

To understand the way in which temperature, and by proxy flow, influence the evolution of life-history strategies and particularly seasonal patterns of emergence, one can refer to the "seasonal time constraint" theory developed by Rowe & Ludwig (1991). Using the Ephemeroptera as a study group,

this theory essentially suggests that when unfavourable conditions (e.g. limited food availability, temperature extremes – summer drought or winter freeze) limit the time available to aquatic insects to reproduce, then depending on its current state (e.g. body size, fat reserves) and time remaining in the season to reproduce, the insect switches priority to investment in development (maturing sexually) rather than growth (biomass gain). In this manner overall development time is shortened and individuals therefore tend to stay small and emerge earlier as smaller adults (Verberk *et al.* 2008). This switching of investment priority from growth to development and *vice versa* allows insects to maximise relative fitness (Rowe & Ludwig 1991). Thus it dictates and directly shapes the life-history cycles observed in insects. Verberk *et al.* (2008) point out that relatively long development times, small body sizes or both are therefore primary characteristics of species able to cope with adverse conditions. Examples of this are given by Sweeney & Vannote (1978) and Sweeney (1978, 1984). In general their studies indicated that at elevated temperatures within a non-lethal range, insects generally grow faster and have larger body size at emergence compared to those growing at colder temperatures.

Insects that develop under more variable temperature regimes, however, may grow at the same, faster or slower rate than those at constant temperatures depending on site specific conditions (Beck 1983, Sweeney 1984). For three mayfly species (*Drunella grandis*, *Ephemerella infrequens* and *Baetis tricaudatus*) Rader & Ward (1990) showed that seasonal growth changed at three sites with similar elevations but different temperature regimes (but see also Delucchi & Peckarsky 1989 and Mendez & Resh 2008). Site 1 experienced a colder but more variable thermal regime than the other sites as well as rapid seasonal changes, including a short summer and long freezing winter. At this site growth in all species was slow during summer-autumn, with no growth over winter, and rapid growth during spring-summer. In contrast, at site 2 which exhibited a more constant thermal regime with gradual seasonal temperature changes as well as warm winter and cool summer temperatures, growth was continuous throughout the year even in winter. Site 3, however, had the highest maximum and mean annual temperatures and also a more constant thermal regime in comparison to site 1. Intermediate rates of seasonal change and buffered winter temperatures were experienced at site 3 and growth was either continuous or rapid over the spring-summer and differed between the species. Robinson *et al.* (1992) reported a greater genetic variability in a species of Plecoptera, *Hesperoperla pacifica,* occurring in a stream with more variable flow (seasonally cyclic) compared to the same species occurring in a stream with more constant flow (seasonally constant). Additionally a more recent study by Franken *et al.* (2008) showed that the stonefly *Nemoura cinerea* exhibited adaptive phenotypic plasticity with regard growth in response to different hydraulic and substrate conditions. These conditions are ultimately controlled by flow within a stream. This example therefore illustrates the manner in which insect life-histories can be influenced on a fine scale through the interaction of various factors relating to a single variable (e.g. flow). All of the aforementioned studies illustrate collectively the flexibility of life-histories in species in response to flow variability and thermal differences between habitats (see also

Ward & Stanford 1982, Poff & Ward 1989, Schlosser 1992, Miller & Golladay 1996, Lytle 2002, Anderson *et al.* 2006).

Various studies (e.g. Campbell 1986, Huryn 1996, McKie *et al.* 2004) have shown that aquatic insects within the Southern Hemisphere tend to exhibit more flexible life cycles than those in the Northern Hemisphere. Both Hynes & Hynes (1975) and Hart (1985) suggested that these flexible and opportunistic life-histories were a selective response or adaptation to unpredictable/variable environmental conditions. Highlighting this variability and unpredictability in flow and climate, Poff *et al.* (2006a), showed that on a global scale South Africa and Australia have the highest interannual and intraannual variation in flow when compared to Europe, the United States and New Zealand.

Studies from South Africa assessing aquatic insect life-histories in relation to temperature and/or flow are however scarce (with exceptions being King 1981, 1982, King *et al.* 1988, Ractliffe 2009) and as a result only broad trends have been inferred. These include: larval abundance peaks occurring in spring to late summer (King *et al.* 1988), winter life cycles being synchronised with the first winter rains (King *et al.* 1988), seasonal changes occurring at a predictable time on an annual scale (King 1981), and autumn to summer life cycles with high rates of insect emergence occurring in summer (King 1981, 1982). Ractliffe (2009) further categorised life-history responses of several aquatic insect species from the Molenaars River as follows: those exhibiting summer development life-histories, winter resistant/summer intolerant life-histories, temperature and flow resistant life-histories and high resilience unsynchronised life-histories. Those exhibiting summer development life-histories (*Aprionyx* spp., *Adenophlebia peringueyella, Cheumatopsyche afra, Chimarra* spp.) had large, dominant and fast developing summer generations, with either a second smaller generation or over-wintering portion of the initial generation. The life-histories of species in this category were assumed to be timed to avoid winter flood disturbance. Species exhibiting winter resistant/summer intolerant life-histories (*Elporia uniradius, Lithogloea harrisoni, L. penicillata,* as well as all Plecoptera collected – *Desmonemoura pulchellum, Aphanicerca* spp.) showed moderate to high levels of flood resistance, with some species showing maximization of larval development in winter (e.g. *Elporia*). Some species with temperature and flow resistant cycles (i.e. high levels of flood resistance and tolerance to maximum summer temperatures), showed slow univoltine cycles (*Euthralus elegans*) while others exhibited fast seasonal bivoltine life-histories (*Demoreptus capensis, Agapetus agilis, Athripsoides bergensis*). *Baetis* spp., *Simulium spp*. and *Orthocladinae* are examples of taxa exhibiting high flood resilience and unsynchronised life-histories as they revealed non-significant decreases in density and strong recruitment after floods. With only these few studies available, the links between hydrological and thermal variability in natural systems are only starting to be investigated in South Africa, and currently only few data exist regarding the thermal regimes of South African rivers (Dallas 2009, Dallas & Rivers-Moore 2012, Dallas *et al.* 2012 and Chapter 2 in this thesis).

Given the large gap in knowledge and literature that exists with regard to Southern Hemisphere and specifically southern African aquatic insects and their life-histories, the augmentation of such knowledge is vital if our ecosystems are to be effectively conserved and managed, especially in the face of ongoing development, anthropogenic impacts and global climate change. A better understanding of the life-history responses of invertebrates to thermal and hydrological regimes, as well as patterns of change in these regimes, will allow for consultants and researchers alike to a) provide more informed guidance to future development and impoundment projects and b) to begin to establish thermal guidelines for the Ecological Reserve (see Rivers-Moore *et al.* 2013a). Such information is also invaluable to the growing research interests in thermal modelling and global climate change scenarios.

The aims of this chapter are thus 1) to present detailed life-history data (investigating life-history traits such as growth, voltinism, emergence period and hatching period) for selected indicator species from the EPT taxa (namely *L. penicillata, A. barnardi, A. flabellata, A. scutata* and *C. ambulans*), 2) to contrast life-histories exhibited by these species in a set of pre-selected rivers that experience a range of hydrological and thermal regimes (data presented in Chapter 2), and 3) to determine tolerances and optima for growth and to interpret the life-history data in relation to the major environmental variables affecting life-history traits (flow, water temperature and water quality; data presented in Chapter 2) and also in relation to genetic analyses (data presented in Chapter 3).

In order to better interpret the life-history data presented in this chapter, laboratory experiments were conducted to determine the developmental period and thermal tolerance limits of eggs collected from species representing each genus (see Chapter 5). Additionally laboratory experiments were conducted to determine growth rates of representatives of *L. penicillata* occurring from two different sites under different thermal conditions (see Chapter 6).

4.2 Methods

4.2.1 Study site summary information

Detailed study site descriptions, analyses of environmental variables pertaining to each of the study sites as well as information regarding the process of selecting the specific study sites are provided in Chapter 2. In order to highlight some of the predominant differences among the selected study sites in terms of environmental variables, a brief summary of some of the key environmental and physicochemical variables (*viz.* hydrological and thermal variables) associated with each of the study sites is provided in Table 4.1. It should be noted that the variables shown in Table 4.1 represent only a small subset of the entire data-set of variables considered for inclusion in the statistical analyses presented in this chapter.

Table 4.1. Summary of important environmental and physicochemical variables associated with each of the study sites. Values in brackets indicate C.V. as a percentage for all variables except flow predictability and temperature predictability where values in brackets indicate the percentage constancy score. For accumulated Degree Days (DD) values in brackets indicate the accumulated DD based on maximum daily water temperature as opposed to mean daily water temperature.

Site	Mean annual flow (m^3/s)	Max. 30-day mov. ave. flow (m^3/s)	Flow predictability (0-1) and (% constancy)	Mean annual water temp. (°C)	Max. 30-day mov. ave. temp. (°C)	Temp. predictability (0-1) and (% constancy)	Accum. mean DD (max.)	# flow regime shifts	# temp. regime shifts	Mean pH	Mean EC (µS/cm)	Mean turbidity (NTU)	Mean chan. width (m)	Mean DO (%)	Mean nitrate conc. (mg/l)	Mean nitrite conc. (mg/l)	Mean phosphate conc. (mg/l)
Eerste	0.79 (2.16)	2.913	0.41 (45)	15.13 (24.20)	23.04	0.59 (46)	6488.8 (7389.0)	13	7	5.81 (14.39)	26.49 (44.85)	1.60 (160.26)	7.86 (25.50)	98.33 (4.54)	0.04	0.02	0.01
Elandspad	2.81 (3.79)	9.551	0.54 (50)	15.60 (27.83)	25.06	0.60 (35)	6704.4 (7597.5)	6	8	5.27 (11.90)	16.27 (47.45)	0.91 (100.86)	13.49 (17.48)	99.51 (3.82)	0.05	0.06	0.03
Molenaars	5.12 (2.66)	18.840	0.51 (46)	15.66 (28.09)	24.49	0.61 (36)	6724.2 (7533.1)	6	9	5.60 (13.84)	17.54 (47.99)	0.68 (22.87)	14.29 (23.92)	97.33 (4.33)	0.04	0.06	0.02
Rooi-Els Kloof	0.25 (1.55)	0.981	0.56 (66)	14.98 (22.82)	20.39	0.64 (42)	6415.7 (6827.7)	4	4	5.46 (7.56)	11.33 (35.22)	0.70 (70.07)	4.01 (11.74)	98.38 (3.72)	0.02	0.02	0.01
Wit	4.14 (2.57)	18.200	0.39 (33)	15.71 (31.27)	27.10	0.56 (31)	6750.5 (7751.9)	11	8	4.75 (11.62)	15.64 (36.22)	0.65 (59.94)	9.24 (98.22)	93.93 (5.49)	0.02	0.02	0.02
Wolwekloof	3.16 (3.00)	2.171	0.32 (64)	15.81 (26.53)	24.95	0.59 (36)	6812.0 (7441.1)	8	5	4.44 (8.12)	17.66 (44.67)	0.56 (44.88)	7.07 (26.89)	97.95 (4.43)	0.03	0.02	0.01

4.2.2 Field sampling

Monthly samples were collected for the period April 2009-April 2010 from each of the six study sites. On each sampling occasion, five separate riffle sections at each site were selected for sampling. Only riffle habitats were targeted for sampling, as these habitats are generally considered a) to contain the taxa most sensitive to anthropogenic impacts including alterations to water quality (Chutter 1971), b) contain high abundances, biomass and diversity of taxa, (particularly the Ephemeroptera, Plecoptera and Trichoptera - EPT taxa - commonly used as biomonitoring taxa) (Brown & Brussock 1991, Grubaugh *et al.* 1996, Rolls *et al.* 2012), and c) to be particularly sensitive to changes in hydrological and thermal regimes as well as water quality impacts arising from anthropogenic activities (e.g. dams, land cover changes), seasonal cycles (Roy *et al.* 2003, Bonada *et al.* 2006, Rolls *et al.* 2012) and therefore also global climate change.

Prior to sampling the biota with a net, the average velocity in the water column as well as a depth measurement were recorded from each riffle using a Global Water FP101 Flow Probe. Care was taken not to disturb upstream sections of the site or the riffle sections themselves during this process, in an attempt to prevent any unwanted downstream drift of aquatic macroinvertebrates.

Following the recording of *in situ* measurements of flow and depth, the riffles (starting from the farthest downstream and moving upstream) were sampled by gently picking up, shaking and brushing the surfaces of cobbles within the riffle as well as stirring the sediment underneath and surrounding these cobbles, whilst an assistant held a standard square frame kick net (30cm x 30cm x 60cm), fitted with a 80μm mesh in place, no more than two meters downstream. Over allocated and timed periods of one minute per riffle, as many cobbles as possible within the separate riffles were sampled in this manner. As far as possible, sampling effort was kept consistent for all rivers over each sampling time unit (1 minute), and each month, by utilising the same field assistants and sampling operator (VR-G). Samples were semi-quantitative since the population size distribution in each of the rivers was the parameter of primary interest, rather than absolute abundance or measures of density. However, in each riffle a total area of approximately no more than $1.5m^2$ was sampled on each occasion. For information regarding the collection of data pertaining to all environmental variables see Chapter 2.

After each replicate sample was taken, the collected invertebrates were emptied into separate 500ml plastic ball jars, preserved in 70% ethanol and then returned to the laboratory where they were stored at -20°C to optimize preservation until they could be processed (soft-bodied invertebrates gradually degrade in 70% alcohol). In addition to the replicate macroinvertebrate samples collected from riffles, approximately twenty minutes on each sampling occasion was set aside for the collection of sub-imagos and imagos. Adults were collected for the most part from the underside of stones and debris and also from riparian vegetation by hand using a combination of sweep netting and an aspirator.

4.2.3 Laboratory methods

Obtaining nymphal size measurements

Stored samples were subject to a primary sort under a dissecting microscope (20x) into the EPT taxa using a salt flotation technique (see Hauer & Resh 1996). Following this initial sort, specimens were then identified to the species level using the taxonomic keys listed in Chapter 3.

In this study, in order to assess cohort development/progression and growth, different hardened or sclerotised body parts were measured for each of the selected species. Body length measurements were not recorded as a) there are often fairly high levels of variability in this measure (frequently as a result of the effects of preserving agents distorting or dehydrating the organism, b) specimens often exhibit telescopic retraction of the abdomen and c) certain samples were of low quality containing specimens with damaged softened body parts. Collectively these factors can make it difficult to determine the relationship between size and age or instar using body length measurements. For *L. penicillata*, *Aphanicercella* spp. and *C. ambulans*, the following body parts, in order, were selected for measurement: interocular distance (IOD) (the narrowest distance between the inner edges of the eyes), head capsule width (HCW) (measured via straight line through the eyes to the edges of the head), and head capsule length (HCL) (measured via straight line from the base of the mandible through the eye to the lateral indent along the posterior margin of head capsule) (see Fig. 4.1). It was not possible to use the same body part for measurement for each of the three species owing to marked differences in morphology.

Fig. 4.1. Size measurements recorded for life-history analyses for target species A) interocular distance for *Lestagella penicillata* (Ephemeroptera), B) head capsule width for *Aphanicercella scutata* (Plecoptera) and C) head capsule length *Chimarra ambulans* (Trichoptera). Bars indicate the position of the respective measurement lines in the three taxa.

Measurements of the selected body parts of the target species were obtained from captured photographs of all of the individuals in each sample, using a calibrated Leica EZ 4 dissecting microscope fitted with a digital camera in conjunction with the Live Measurement and Analysis Module in the Leica Application Suite V3.0[24]. All individuals of each target species in each monthly sample were counted in order to provide a measure of relative abundance after being photographed and measured. A total of 16300 *Lestagella,* 1725 *Aphanicercella* and 5229 individuals of *Chimarra* were measured.

Analysis of size measurements

Using the respective measurements of hardened body parts as a measure of size increase, size frequency histograms were produced for each target species for all of the rivers. These size frequency histograms were then used to infer voltinism and as far as possible to track cohorts through time.

The numbers of black wingpad individuals in each sample were noted for *L. penicillata and Aphanicercella* spp. This provided an indication of the timing for the completion of the life cycle in these species and was also used in conjunction with the presence of sub-imagos and imagos (adults) collected each month for all species to estimate the onset and duration of the emergence period.

In order to follow cohort progression through time, mean monthly values of body measurements were analysed using linear regression in species where a single cohort or generation could be tracked (i.e. univoltine species). However, for species exhibiting overlapping cohorts or multiple cohorts (i.e. multivoltine species) these linear regression techniques could not be successfully applied. Where applicable, the slopes of the regression analyses provided an initial indication of growth rate of the target species (steeper slopes suggesting faster growth).

For species exhibiting univoltinism (*Lestagella* and *Aphanicercella*), hatch dates were estimated using backward linear regression analyses of the mean monthly size measurements. The respective intercept points for these backward linear regression analyses were set to the smallest size measurements recorded for each species. These smallest size classes were assumed to represent first-instar individuals (confirmation of the size measurements of first-instar individuals of each species was obtained in egg development experiments conducted in Chapter 5).

[24] Photographs are calibrated to the magnification level of the microscope at the time the photograph is taken such that measurements can be obtained from the photograph at a later stage using the Leica Application Suite Software. Manual calibration of the microscope was also carried out prior to taking photographs of specimens, by taking an image of a microscope graticule and confirming measurement accuracy from the photograph. Groups of 15-20 specimens were placed on a glass microscope slide and photographed at a time. In order to standardise the photographs and subsequent measurements, a glass cover slip was placed over the group of specimens to be photographed in order to flatten them and minimise distortion.

4.2.4 Statistical analyses

In order to assess life-history data for the study species in relation to the various physicochemical variables measured, both general linear models (GLM's) and multivariate methods (see Appendix 4A) were employed. Separate GLM's were used to investigate the effects of environmental variables on a measure of growth of each species. Multivariate techniques were also employed to provide an alternative and perhaps less conventional way of investigating the life-history data. In the various multivariate analyses performed, the response variable "stage compositional data" (defined as the numbers of individuals of each genus of a certain age in a given sampling month) was related to the same set of environmental variables. For the purposes of this study only the results of the GLM's are reported in full, while the multivariate analyses are provided in Appendix 4A purely for the consideration of the interested reader.

General linear modelling

A measure of growth was calculated for each study genus and, although slightly different methods were used in each case, the generic term 'growth' will be used in the main text for simplicity sake. For *L. penicillata* and *Aphanicercella* spp., growth was calculated as size increase since an estimated hatch date. For *C. ambulans* growth was an estimate of size increase per larval instar. The details of the growth calculations for each study genus and justifications for applied transformations of this response variable are given in Appendix 4B.

GLM's were used to take into account a variety of environmental variables that could influence the growth of each of the study genera (for example water temperature or flow conditions in which the samples were collected), in order to estimate the effect of these variables on growth. The GLM structure has three main properties: 1) the distribution or error structure of the response variable, 2) the systematic component or structure of the model which relates observed values to predicted values emerging from the sum of the linear effects of the explanatory variables and 3) the link function which relates the mean values of the response variable to the explanatory variables (Crawley 2007, Zuur *et al.* 2009). The GLM is a flexible and useful tool given that it can cater for a number of non-normal distributions (e.g. Poisson, negative binomial, binomial, geometric) and that it can incorporate normally distributed dependent variables along with both categorical or continuous independent variables (Zuur *et al.* 2009). In this case the response variable growth (as calculated from IOD, HCW and HCL - see Appendix 4B) was continuous while the independent predictor variables were converted to categorical factors[25]. A separate GLM was performed for each species, and in each case a Gaussian or normal

[25] The use of continuous variables makes the assumption that the response variable has a linear relationship with the predictor variables. Since this has not been established in this study it was considered better to use categorical data (Dr. Anabela Brandão, pers. comm., University of Cape Town, 2013). In other words categorical data allow for an investigation of trends in growth, as a response to changing levels of predictor variables, rather than expecting a linear response to these predictor variables.

distribution with an "Identity" link function was used. A summary of the GLM, the factors, factor levels and the reference levels used for "corner-point" parameterisation for each species are provided in Tables 4.2, 4.3 and 4.4. All environmental variables were checked for collinearity using a correlation matrix, and where two or more variables showed appreciable collinearity ($r \geq 0.8$), only one of these variables was included in the final GLM.

The equation describing each of the GLM's is given below:

$$Growth = \mu + \alpha_{River} + \beta_{Temp} + \gamma_{Std.temp} + \delta_{Flow} + \varepsilon_{D.O.} + \zeta_{E.C.} + \eta_{pH} + \theta_{Turbidity} + \iota_{Channelwidth} \quad (4.1)$$

where growth is the response variable (see Appendix 4B), μ is the intercept and the remaining symbols correspond to categorical factors explained in Tables 4.2, 4.3 and 4.4.

It should be noted that bin ranges for each of the categorical factors were chosen so that as far as possible the bins of each factor contained a similar number of data points. Since each species had a different data set with differing numbers of observations for each factor level, different bin ranges were selected for each species.

As far as possible the GLM's were standardised for the effect of time by using an estimate of age (days since hatch for *Lestagella* and *Aphanicercella* and instar number for *Chimarra*). GLM's and multivariate analyses provide information relating to the genus level only and were not used to try and differentiate between either known species of *Aphanicercella* or potential lineages of *Lestagella* as evidenced from genetic analyses (see Chapter 3). Genetic differences (notably for *Lestagella*) may however be related to the results of the GLM by assessing the effect of the variable "River", which essentially is a variable that could account for the effects on growth of additional environmental effects that were not incorporated into the model (e.g. differences in food availability, solar radiation or other differences such as genetic differences). It should be noted that inferences made in this manner are postulates and should be considered with caution.

Table 4.2. Table summarising various factors as well as respective reference levels selected for corner point parameterisation in the GLM run for *Lestagella penicillata.*

Predictor variable/factor		No. factor levels (levels)	Reference level
α_{River}	River	6 (Eerste, Elandspad, Molenaars, Rooi-Els Kloof, Wit, Wolwekloof)	4 (Rooi-Els Kloof)
β_{Temp}	Mean monthly average of water temperature (°C)	9 (<11, 11-12.5, 12.5-14, 14-15.5, 15.5-17, 17-18.5, 18.5-20, 20-21.5, >21.5)	7 (18.5-20)
$\gamma_{Std.temp}$	Mean monthly std. deviation of water temperature	5 (<1.1, 1.1-1.3, 1.3-1.5,1.5-1.7, >1.7)	1 (<1.1)
δ_{Flow}	Mean monthly flow (m^3/s)	7 (<0.05, 0.05-0.125, 0.125-0.313, 0.313-0.781, 0.781-1.953, 1.953-4.883, >4.883)	1 (<0.05)
$\varepsilon_{D.O.}$	Mean monthly dissolved oxygen (% saturation)	5 (<97.5, 97.5-99, 99-100.5, 100.5-102, >102)	1 (<97.5)
$\zeta_{E.C.}$	Mean monthly electrical conductivity (µS/cm)	5 (<13, 13-16, 16-19, 19-22, >22)	5 (>22)
η_{pH}	Mean monthly pH	5 (<4.6, 4.6-5.1, 5.1-5.6, 5.6-6.1, >6.1)	4 (5.6-6.1)
$\theta_{Turbidity}$	Mean monthly turbidity (NTU)	5 (<0.4, 0.4-0.6, 0.6-0.8, 0.8-1, >1)	4 (0.8-1)
$\iota_{Channelwidth}$	Mean monthly channel width (m)	5 (<4, 4-6, 6-8, 8-12, >12)	2 (4-6)

Table 4.3. Table summarising various factors as well as respective reference levels selected for corner point parameterisation in the GLM run for *Aphanicercella* spp.

Predictor variable/factor		No. factor levels (levels)	Reference level
α_{River}	River	6 (Eerste, Elandspad, Molenaars, Rooi-Els Kloof, Wit, Wolwekloof)	5 (Wit)
β_{Temp}	Mean monthly average of water temperature (°C)	6 (<11.5, 11.5-13, 13-14.5, 14.5-16, 16-17.5, 17.5-19, 19-20.5, 20.5-22, >22)	9 (>22)
$\gamma_{Std.temp}$	Mean monthly std. deviation of water temperature	5 (<1.1, 1.1-1.3, 1.3-1.5, 1.5-1.7, >1.7)	4 (1.5-1.7)
δ_{Flow}	Mean monthly flow (m^3/s)	8 (<0.02, 0.02-0.08, 0.08-0.1, 0.1-0.22, 0.22-0.6, 0.6-1, 1-5, >5)	1 (<0.02)
$\varepsilon_{D.O.}$	Mean monthly dissolved oxygen (% saturation)	5 (<94, 94-96, 96-98, 98-100, >100)	1 (<94)
$\zeta_{E.C.}$	Mean monthly electrical conductivity (µS/cm)	5 (<12, 12-15, 15-18, 18-21, >21)	4 (18-21)
η_{pH}	Mean monthly pH	5 (<4.6, 4.6-5.1, 5.1-5.6, 5.6-6.1, >6.1)	3 (5.1-5.6)
$\theta_{Turbidity}$	Mean monthly turbidity (NTU)	5 (<0.4, 0.4-0.6, 0.6-0.8, 0.8-1, >1)	2 (0.4-0.6)
$\iota_{Channelwidth}$	Mean monthly channel width (m)	5 (<4, 4-5.5, 5.5-7, 7-8.5, >8.5)	2 (4-5.5)

Table 4.4. Table summarising various factors as well as respective reference levels selected for corner point parameterisation in the GLM run for *Chimarra ambulans*

Predictor variable/factor		No. factor Levels (levels)	Reference level
α_{River}	River	6 (Eerste, Elandspad, Molenaars, Rooi-Els Kloof, Wit, Wolwekloof)	2 (Elandspad)
β_{Temp}	Mean monthly average of water temperature (°C)	8 (<14.3, 14.3-15.5, 15.5-16.7, 16.7-17.9, 17.9-19.1, 19.1-20.3, 20.3-21.5, >21.5)	3 (15.5-16.7)
$\gamma_{Std.temp}$	Mean monthly std. deviation of water temperature	5 (<1.4, 1.4-1.55, 1.55-1.7, 1.7-1.85, >1.85)	4 (>1.7)
δ_{Flow}	Mean monthly flow (m^3/s)	8 (<0.05, 0.05-0.15, 0.15-0.25, 0.25-0.45, 0.45-0.65, 0.65-0.85, 0.85-1.05, >1.05)	4 (0.25-0.45)
$\varepsilon_{D.O.}$	Mean monthly dissolved oxygen (% saturation)	5 (<97.5, 97.5-99, 99-100.5, 100.5-102, >102)	4 (100.5-102)
$\zeta_{E.C.}$	Mean monthly electrical conductivity (µS/cm)	5 (<13, 13-16, 16-19, 19-22, >22)	3 (16-19)
η_{pH}	Mean monthly pH	5 (<4.6, 4.6-5.1, 5.1-5.6, 5.6-6.1, >6.1)	2 (4.6-5.1)
$\theta_{Turbidity}$	Mean monthly turbidity (NTU)	5 (<0.5, 0.5-0.7, 0.7-0.9, 0.9-1.1, >1.1)	5 (>1.1)
$\iota_{Channel\,width}$	Mean monthly channel width (m)	5 (<4, 4-7, 7-10, 10-13, >13)	4 (10-13)

Validating GLM assumptions

After the optimal models were constructed for the three study genera, they were validated and assessed in terms of their adherence to underlying statistical assumptions according to the guidelines proposed by Zuur *et al.* (2009). For each model, homogeneity was assessed by plotting residuals against fitted values. Normality of errors was checked by plotting a histogram of the residuals along with a QQ-plot of the standardised deviance residuals against theoretical quantiles. In order to determine if there were trends in the residuals of the model and to ascertain if they were identically and independently distributed, Scale-Location plots were constructed. Residuals were also plotted against each explanatory variable used in the model, to check for any prevailing patterns in the residuals. Since no trends were observed, these results were not reported here. Additionally, mean residuals for each river were separately checked for autocorrelation through time using ACF plots. The mean monthly residuals aggregated across all rivers were calculated and also checked for autocorrelation using ACF plots. Residuals vs. leverage plots, which use the Cook distance function, were used to assess the model for any influential observations or outliers. Plots used to assess the underlying statistical assumptions for each of the three separate GLM's are shown in Appendix 4C together with relevant interpretations.

Statistical analyses and plots, specifically those of the GLM, were performed and constructed using R (R Core Team 2012) as well as MATLAB 2008a. All multivariate statistical analyses employed in

Appendix 4A were conducted using PRIMER 6+PERMANOVA software package from Plymouth Marine Laboratory, UK (Clarke & Gorley 2006, Anderson *et al.* 2008).

4.3 Results

4.3.1 Life-histories - *Lestagella*

Voltinism

Life-history plots for *L. penicillata* from each of the study sites, constructed using monthly size frequency histograms of IOD measurements, are shown in Fig. 4.2. Total development time for this species was shown to be between 12-13 months. It was found that the sampling period in this study spanned two generations, an older generation that had hatched in the year prior to sampling (2008) and a new generation that hatched during the year of sampling (2009). Smallest individual measurements of IOD recorded from field samples were consistently ~0.08mm. The highest mean monthly values of IOD recorded from each of the rivers are shown in Table 4.5. These values provide an indication of the mean size of individuals at the time of emergence.

Table 4.5. Highest mean monthly values of interocular distance recorded for *Lestagella penicillata* in six rivers in the Western Cape, South Africa. Mean monthly values are presented in boldface with standard deviation in brackets along with total numbers of individuals (N) comprising informing each mean.

River	Highest mean monthly interocular distance	N	Month
Elandspad	**0.726** (0.096)	144	October
Molenaars	**0.724** (0.117)	56	October
Wit	**0.698** (0.125)	10	October
Rooi-Els Kloof	**0.633** (0.052)	72	November
Eerste	**0.625** (0.128)	54	November
Wolwekloof	**0.613** (0.099)	509	October

The presence and relative abundance of black wingpad nymphs in the samples indicated that in all streams the emergence period began in the month of October and extended for differing periods of time in each of the rivers (Table 4.6). Estimated peak emergence times appeared to be earliest in the Elandspad and Wit rivers (occurring in October) and latest in the Rooi-Els Kloof River (occurring in December). Emergence periods were longest in the Rooi-Els Kloof, Eerste and Molenaars rivers, spanning a period of three months from October to December. In contrast the individuals from the Wolwekloof and Elandspad rivers had shorter emergence periods spanning two months each, while individuals from the Wit River exhibited an emergence period of only one month[26].

[26] The estimates relating to emergence periods cannot be determined to a finer scale than the sampling interval, in this case, one month.

Table 4.6. The timing of key events in the life cycles of *Lestagella penicillata* from six streams in the Western Cape, South Africa. Asterisks denote estimates based on low numbers of individuals.

River	Start of emergence	Peak emergence	Duration of emergence period (months)	First presence of next generation nymphs
Wit	October *	October*	1*	December
Elandspad	October	October	2	December
Wolwekloof	October	November	2	December
Eerste	October	November	3	January
Molenaars	October	November	3	December
Rooi-Els Kloof	October	December	3	January

Recruitment and hatching appeared to occur during the summer months of December and January but were variable amongst the study sites. In the Molenaars, Wolwekloof and Wit rivers, a period of overlap (~one month) was observed between the larger emerging nymphs of the previous year's generation and the hatchlings from the new year's generation (Fig. 4.2). Hatching periods were latest in the Eerste and Rooi-Els Kloof rivers occurring between late December and early January. Hatching in the remaining rivers appeared to occur between late November and early December.

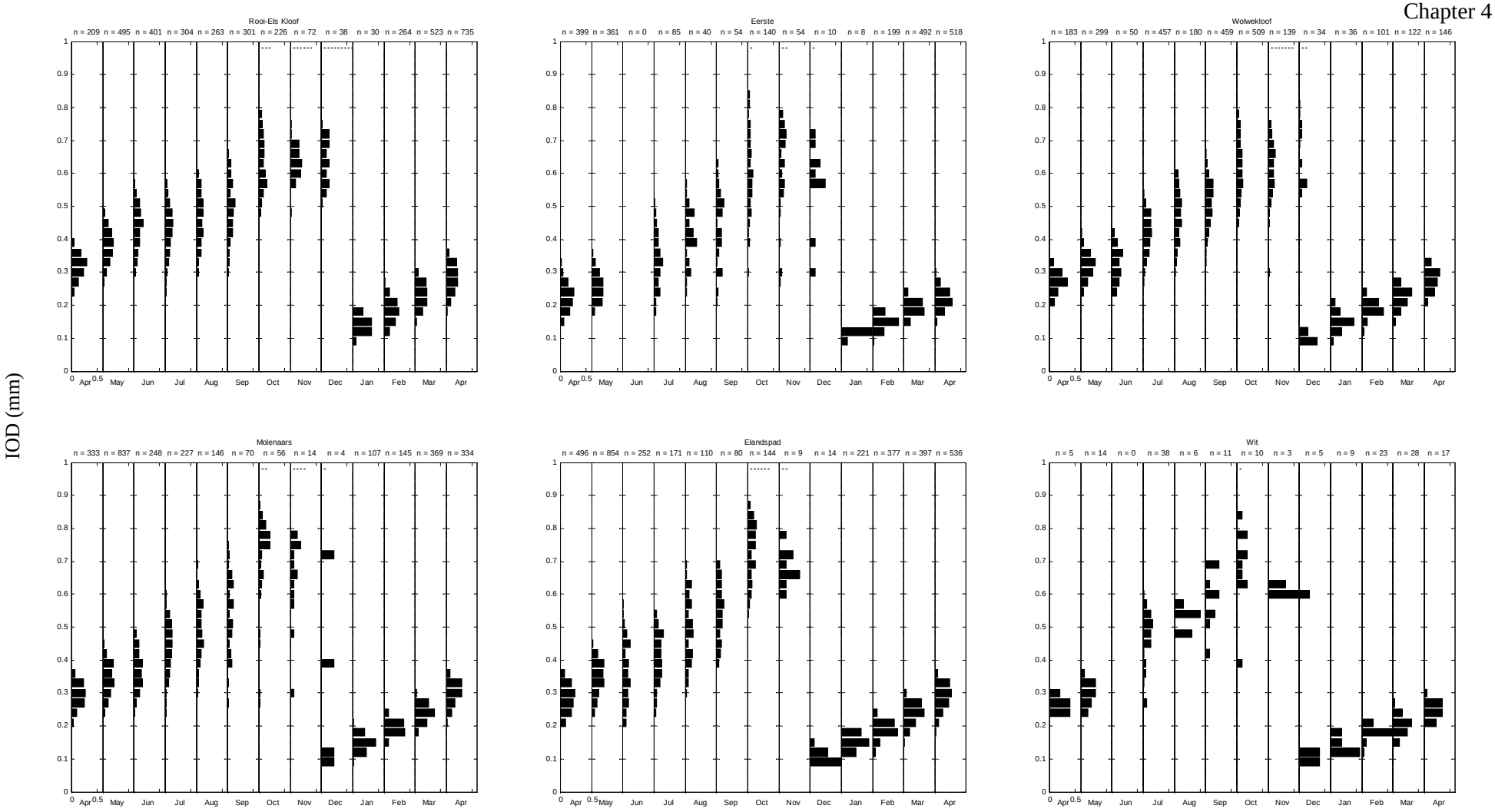


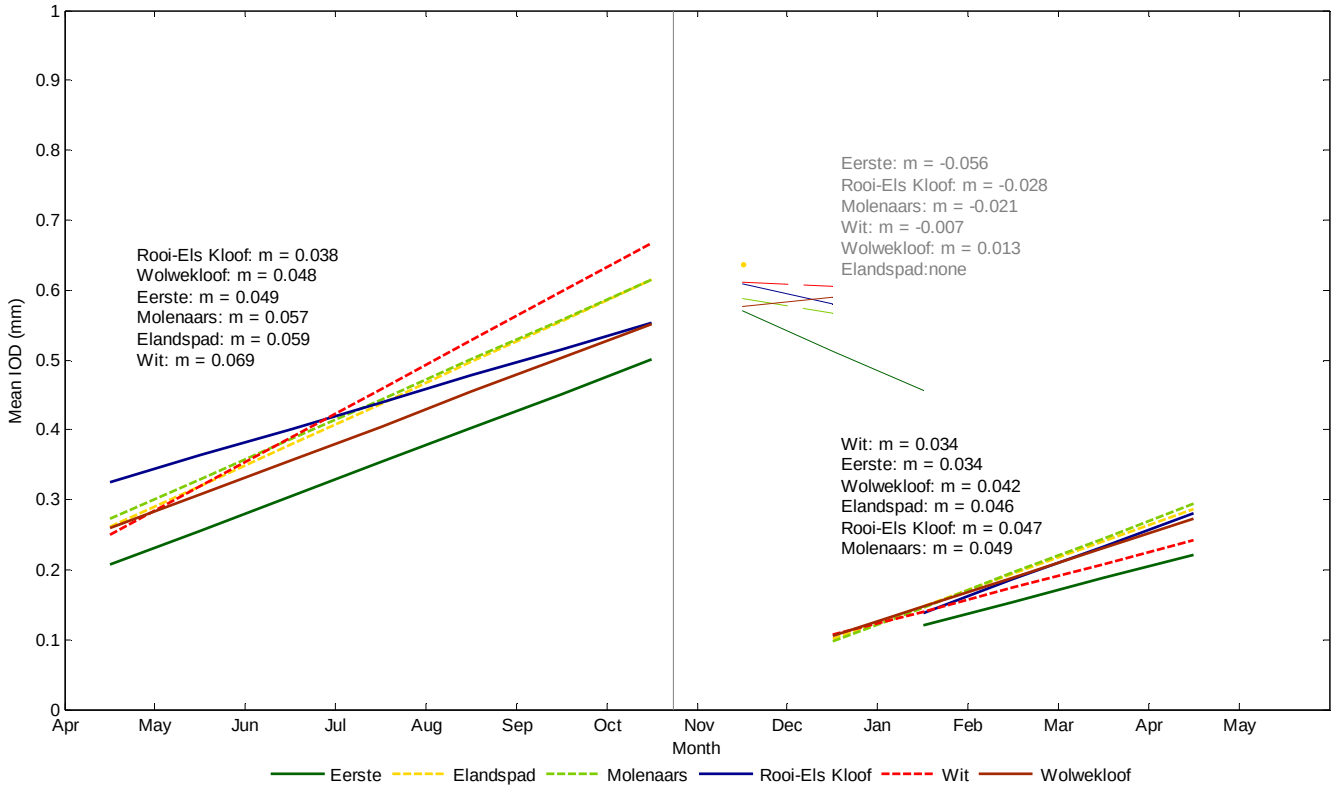
Fig. 4.2. Size frequency histograms of interocular distance measurements (mm) from individuals of *Lestagella penicillata* collected monthly from six streams in the Western Cape, South Africa for the period April 2009-April 2010. Bar length indicates relative numbers of individuals for a given size class in each month. Asterisks represent the absolute numbers of black wingpad nymphs collected in each month. Total number of individuals in the sample for each month are given at the top.

Cohort analysis

Regression analyses of mean monthly IOD measurements of *L. penicillata* plotted against time, for both the previous year's generation and the new generation, are shown for the sampling period and each study site in Fig. 4.3. Males collected from August –December 2009 in each river were excluded from these analyses as the development of the eyes obscured measures of IOD (see Appendix 4D). Respective R^2 and significance values (95% confidence level) are also presented in Fig. 4.3. Periods towards the end of the life cycle in which the mean monthly measurements of IOD were found to decrease were excluded from the regression analysis of each individual generation[27].

All estimated slopes exhibited high R^2 values. In general the regression analyses for the first generation were found not to be significant, this owing primarily to increased variation in monthly IOD measurements coincident with increasing size of individuals measured for this period. Steepest gradients were observed in the Wit, Elandspad and Molenaars rivers in contrast to the gentler gradients observed in the Rooi-Els Kloof, Wolwekloof and Eerste rivers. The steepest gradient value was observed in the Wit River (m = 0.069) and the lowest in the Rooi-Els Kloof River (m = 0.038).

[27] Such a decrease is commonly observed in life-history analyses of aquatic insects as a period of emergence is experienced towards the end of the life cycle. During this emergence period larger nymphs leave the river in order to undergo their final moult into the adult stage. In their absence only smaller individuals that remain in the river are sampled and measured and the average size of the individuals (for the period immediately following emergence) thus appears to decrease.



	First generation			
	m	***c***	***R²***	***p***
Rooi-Els Kloof	0.038	0.154	0.86	0.249
Eerste	0.049	-0.013	0.95	0.488
Wolwekloof	0.048	0.042	0.97	0.425
Molenaars	0.057	0.017	0.93	0.778
Elandspad	0.059	-0.003	0.90	0.479
Wit	0.069	-0.061	0.99	0.053

	Second generation			
	m	***c***	***R²***	***p***
Wit	0.034	-0.316	0.99	<0.001
Eerste	0.034	-0.338	0.99	<0.001
Wolwekloof	0.042	-0.421	0.99	<0.001
Elandspad	0.046	-0.475	0.99	<0.001
Rooi-Els Kloof	0.047	-0.501	0.99	<0.001
Molenaars	0.049	-0.517	0.99	<0.001

Fig. 4.3. Regression slopes of mean monthly interocular distance measurements (mm) for two separate generations of *Lestagella penicillata* collected monthly from six streams in the Western Cape, South Africa over the period April 2009-April 2010. Regression slope values in grey represent negative slopes as a result of emergence.

4.3.2 Life-histories - Aphanicercella

Voltinism

Three species of stonefly from the genus *Aphanicercella* (*A. scutata, A. flabellata and A. barnardi*) were collected during the sampling period. The three species were not all present at each of the study sites and in general overall monthly abundances were considerably lower when compared to *L. penicillata*. In some rivers two of three species were found to co-occur, but where this was the case the species were often present in different relative abundances, with one of the species usually being predominant throughout the sampling period. *A. scutata* was widespread and was recorded in the Wit, Wolwekloof, Molenaars and Elandspad rivers. In the Wit River, *A. scutata* was the only species in the genus present, while in the Wolwekloof, Molenaars and Elandspad it was found to co-occur with *A. flabellata.* Samples revealed that *A. flabellata* was the only species of this genus recorded in the Eerste River and that it occurred in marginally higher abundances than *A. scutata* in the Molenaars and Elandspad rivers. In contrast *A. scutata* exhibited slightly higher numbers of individuals than *A. flabellata* in the Wolwekloof River. Within the Rooi-Els Kloof River the only species of *Aphanicercella* present in the samples was that of *A. barnardi,* and this species also occurred only in the Rooi-Els Kloof River. Based on distribution records (Stevens & Picker 1999, 2003, Stevens 2009) *A.barnardi* appears to have a more northerly distribution in the Western Cape.

Life-history plots constructed using size frequency histograms of monthly measurements of HCW for each species of *Aphanicercella* from the respective study sites are shown in Figs. 4.4a. and 4.4b. As was the case for *L. penicillata,* the sampling period in this study spanned two generations of each of the three species of *Aphanicercella*: an older generation that had hatched in the year prior to sampling (2008) and a new generation that hatched during the year of sampling (2009). In general total development time for the species within the genus *Aphanicercella* were estimated to vary between 11 and 14 months.

Smallest individual measurements of HCW recorded from field samples for each of the three species were consistently ~0.135mm. In contrast however, HCW measurements of the largest recorded wingpad individuals of the genus were observed to differ not only among the three species but also for the same species among the six rivers sampled in this study (Table 4.7).

Table 4.7. Highest absolute monthly and mean monthly recorded measurements of head capsule width (mm) for three species of Plecoptera in the genus *Aphanicercella* in monthly samples collected from six rivers in the Western Cape, South Africa over the period April 2009–April 2010. Numbers of individuals (parentheses) river names and month of collection are indicated.

A. barnardi	***A. flabellata***	***A. scutata***
0.790(11) Rooi-Els Kloof, Jul	0.910(1) Eerste, Jul	0.843(2) Wolwekloof, Jul
	0.929(1) Elandspad, Jul	0.855(1) Molenaars, Aug
	0.755(13) Molenaars, Jun	0.821(20) Wit, Aug
	0.700(4) Wolwekloof, May	0.521(5) Elandspad, Jun

In general the three species of *Aphanicercella* revealed extended emergence occurring roughly over a period from May to September 2009. Estimated peak emergence times appeared to be latest in *A. scutata* in the Wit River followed by *A. barnardi* in the Rooi-Els Kloof River. Extended emergence periods of up to five months and four months were recorded in the Rooi-Els Kloof and Wit rivers respectively. In contrast, species occurring in the Wolwekloof and Elandspad and Molenaars rivers had shorter emergence periods spanning 1-2 months each.

Details regarding the timing of key events in the life cycles of each species are provided in Table 4.8. Low numbers of individuals were observed of *A. flabellata* and *A. scutata* in the Molenaars and Elandspad rivers limiting the interpretation and accuracy of life-history plots from these rivers. The Elandspad River is a tributary of the Molenaars River, with sites on each of the rivers occurring ~2km apart. Both rivers share similar species composition, in conjunction with only subtle differences in thermal and hydrological regimes. It was expected therefore that the same species observed to occur in these rivers would exhibit similar life-history trends – as was observed for *L. penicillata.* As such the low numbers of individuals of *A. scutata* and *A. flabellata* from these rivers (Elandspad and Molenaars) were grouped together each month to form single life-history plots for these species. Inferences were drawn from these grouped plots as they provide a more complete representation of life cycles (Table 4.8).

Table 4.8. The timing of key events in the life cycle of three species of Plecoptera of the genus *Aphanicercella* from six rivers in the Western Cape, South Africa. Asterisks denote estimates based on low numbers of individuals.

River	Species	Start of emergence	Peak emergence	Duration of emergence period (months)	First presence of next generation nymphs
Rooi-Els Kloof	*A. barnardi*	May	June / July	5	August
Eerste	*A. flabellata*	July	July	1*	September
Elandspad & Molenaars	*A. flabellata*	May	June	4	October
Wolwekloof	*A. flabellata*	June	June	2*	October
Wolwekloof	*A. scutata*	May*	May*	1*	October
Elandspad & Molenaars	*A. scutata*	June*	June*	3*	September
Wit	*A. scutata*	June	August	4	September

The recruitment and hatching appeared to occur towards the end of winter (August) in the Rooi-Els Kloof River for *A. barnardi* and over the spring months of September and October for the remaining species. Life-history plots showed recruitment periods roughly similar in duration to the observed emergence periods of adults. Recruitment periods, were taken as being the length of time from the estimated start of emergence to the date of first presence of next generation nymphs. Overlap of the two generations sampled is visible only in the month of September in *A. scutata* from the Wit River (Fig. 4.4b). For the remaining species a period of between one to two and a half months is observed between final emergence of the previous generation and the first presence of hatchlings from the new generation.

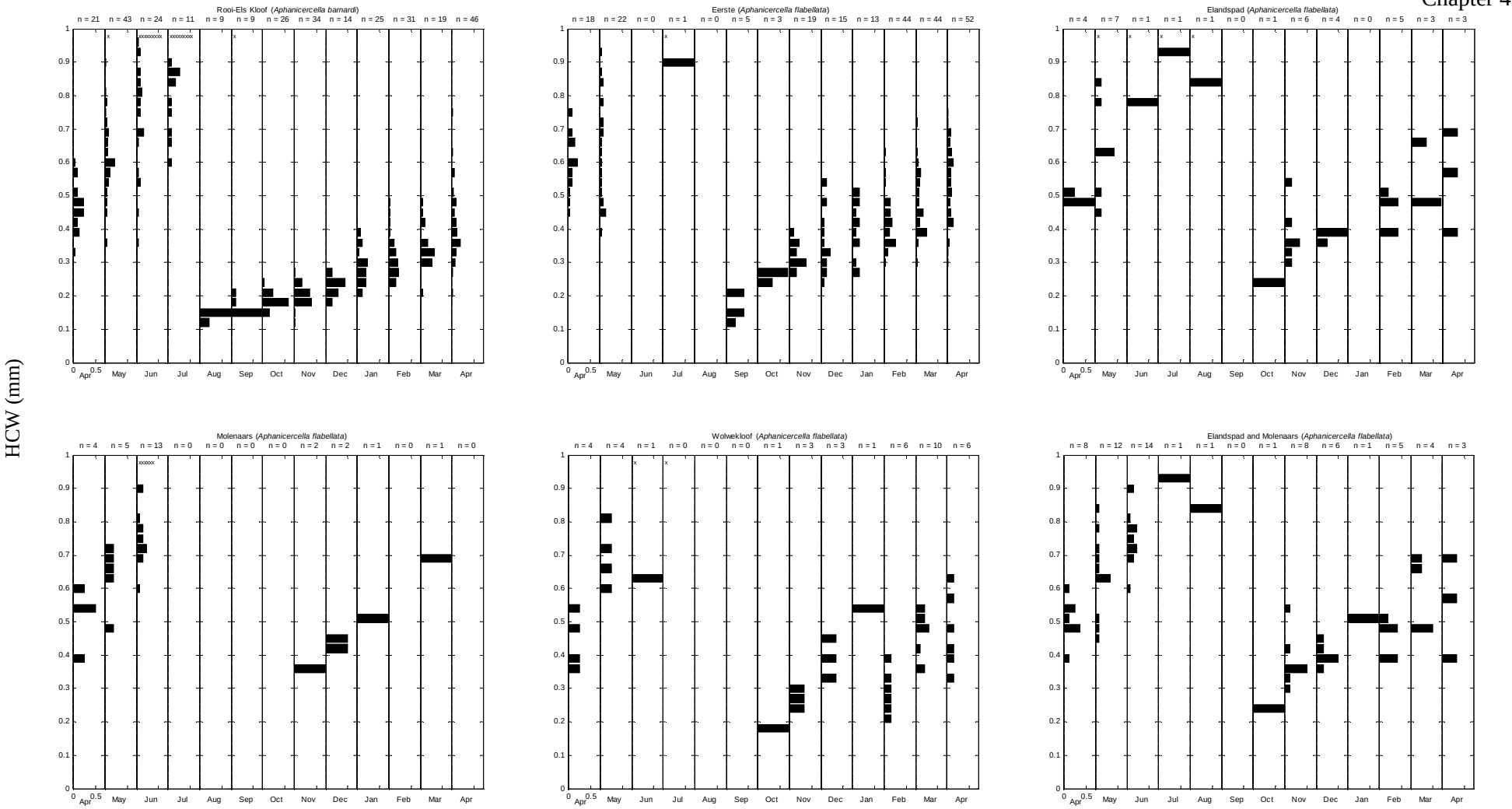


Fig. 4.4a. Size frequency histograms of head capsule width measurements (mm) of *Aphanicercella barnardi* and *Aphanicercella flabellata* collected monthly from five rivers in the Western Cape, South Africa for the period April 2009-April 2010. Bar length indicates relative numbers of individuals for a given size class in each month. Asterisks represent the absolute numbers of black wingpad nymphs collected in each month.

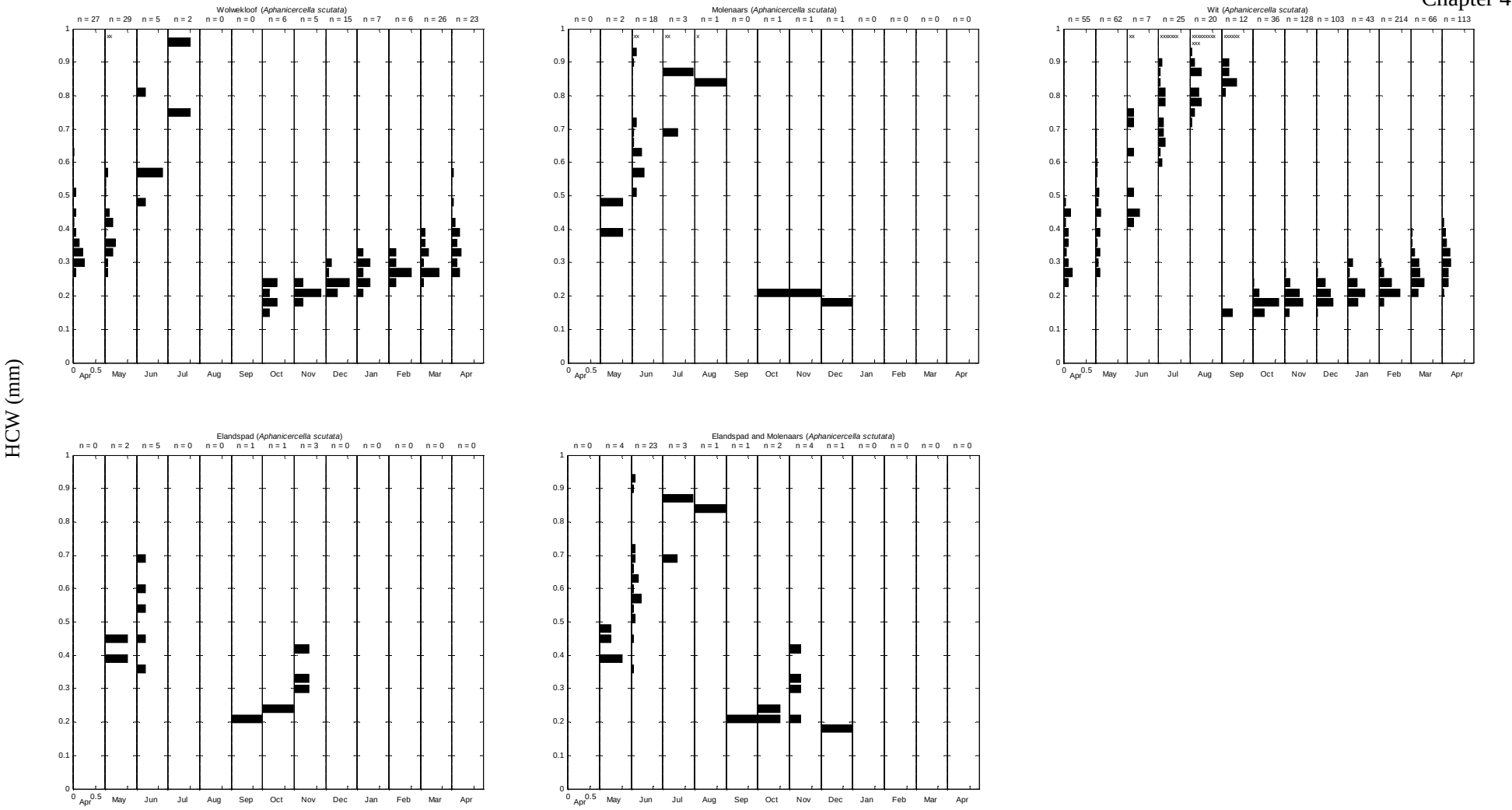


Fig. 4.4b. Size frequency histograms of head capsule width measurements (mm) of *Aphanicercella scutata* collected monthly from four rivers in the Western Cape, South Africa for the period April 2009-April 2010. Bar length indicates relative numbers of individuals for a given size class in each month. Asterisks represent the absolute numbers of black wingpad nymphs collected in each month. Total number of individuals in the sample each month are indicated by the *n* values at the top.

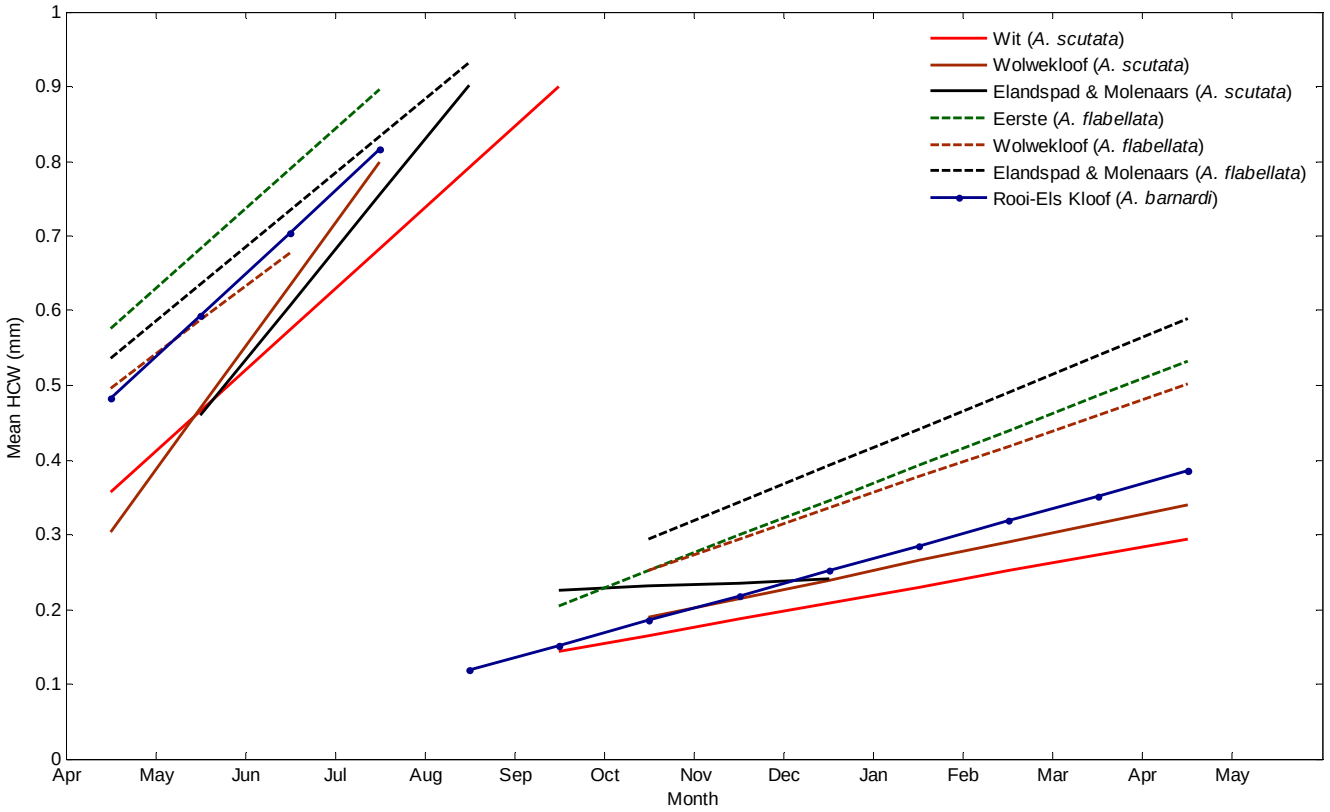
Cohort analysis

Regression analyses of the mean monthly HCW measurements of the three species of *Aphanicercella*, namely *A. barnardi*, *A. flabellata* and *A. scutata*, plotted against time, are shown in Fig. 4.5. Additionally respective R^2 and significance values (95% confidence level) for regressions conducted on both the first and second generation are indicated (Fig. 4.5). Mean monthly HCW measurements were combined for the Molenaars and Elandspad rivers for the two species *A. flabellata* and *A. scutata* to provide a more accurate indication of size increases with time.

Regression gradient values for the two generations differed markedly, with steeper gradients being observed in the older individuals of the first generation for all species and all sites when compared to the younger individuals in the second generation (Fig. 4.5). All regressions fitted to the measurements from the first generation, with the exception of the Wolwekloof River ($p = 0.050$) were found to be non-significant but with generally high R^2 values > 0.5 (Fig. 4.5). Non-significant results are not unexpected for this generation over the period from April – September as a result of a) increased variation in head capsule measurements owing to sexually dimorphic growth, b) variation in mean monthly values as a result of emergence, and c) variation in measurements coincident with increasing size.

Within the earlier instars of the second generation, all regression gradients were highly significant, and gradients showed substantial groupings according to species and more subtle differences in slope according to sites (Fig. 4.5). Clear separation was evident between the species *A. scutata* and *A. flabellata*, while *A. barnardi* from the Rooi-Els Kloof showed a gradient similar to that observed for *A. flabellata*. *A scutata* exhibited slower increases in size over time than *A. flabellata*, with the lowest gradient values observed for this species recorded in the Wit River ($m = 0.021$) followed by the Wolwekloof River ($m = 0.025$) (Fig. 4.5). *A barnardi* exhibited a slightly steeper regression gradients of 0.033, while *A. flabellata* from the Wolwekloof, Eerste and both the Elandspad and Molenaars rivers combined showed values of 0.041, 0.047 and 0.049 respectively (Fig. 4.5). Too few individuals of *A. scutata* were recorded from the Elandspad and Molenaars rivers to infer size increases with time.

Differences in the gradient values observed between the two generations (essentially differences between early and late instars) were observed to be greatest for *A. scutata* from the Wolwekloof River, followed by the same species in the Wit River. *A. flabellata* from the Molenaars and Elandspad rivers showed the smallest difference followed by the same species in the Wolwekloof and Eerste rivers respectively.



	First generation			
	m	***c***	***R^2***	***P***
Wit *(A. scutata)*	0.109	-0.133	0.97	0.058
Wolwekloof *(A. scutata)*	0.166	-0.442	0.92	0.050
Combined *(A. scutata)*	0.148	-0.353	0.94	0.083
Eerste *(A. flabellata)*	0.107	0.097	0.95	0.756
Wolwekloof *(A. flabellata)*	0.091	0.087	0.46	0.584
Combined *(A. flabellata)*	0.098	0.092	0.84	0.888
Rooi-Els Kloof *(A. barnardi)*	0.111	-0.019	0.97	0.679

	Second generation			
	m	***c***	***R^2***	***p***
Wit *(A. scutata)*	0.021	-0.060	0.93	<0.001
Wolwekloof *(A. scutata)*	0.025	-0.074	0.96	0.014
Combined *(A. scutata)*	0.005	0.179	0.01	0.123
Eerste *(A. flabellata)*	0.047	-0.239	0.96	<0.001
Wolwekloof *(A. flabellata)*	0.041	-0.182	0.47	0.008
Combined *(A. flabellata)*	0.049	-0.221	0.83	0.012
Rooi-Els Kloof *(A. barnardi)*	0.033	-0.166	0.98	<0.001

Fig. 4.5. Regression slopes of mean monthly head capsule width measurements (mm) for two separate generations of three different species of Plecoptera (*Aphanicercella*). Measurements were collected monthly from six rivers in the Western Cape, South Africa over the period April 2009 -April 2010. "Combined" - refers to the individuals of two rivers (the Elandspad and Molenaars) which were pooled together.

4.3.3 Life-histories - Chimarra

Voltinism

In contrast to *L. penicillata* and the three species of *Aphanicercella*, mean monthly size frequency histograms of HCL measurements for *C. ambulans* revealed in general the presence of more than one generation with multiple overlapping cohorts (Fig. 4.6). Individuals from a range of size classes were present for the majority of the sampling period with the number of cohorts appearing to vary across the six study rivers (Fig. 4.6). A histogram of the sizes recorded for all individuals of this species revealed a consistent grouping of HCL measurements into five discrete size classes (see Appendix 4B, Fig. App4B.1). These discrete size classes represent the total number of larval instars exhibited by this species. The range of HCL measurements within these instars across all rivers are indicated in Table 4.9. Differences in the relative numbers of the larvae present in these size classes were observed from each of the rivers over the duration of the sampling period. Relative numbers of larvae present in these size classes, observed each month over the duration of the sampling period, helped guide the interpretation of cohort progression through time and the estimation of the numbers of generations in each study river.

Table 4.9. Range in absolute head capsule length measurements observed for the five larval instar stages of *Chimarra ambulans* from six rivers in the Western Cape, South Africa.

Larval instar	Range in absolute head capsule length measurements (mm)
1	0.12 – 0.24
2	0.25 – 0.48
3	0.49 – 0.80
4	0.81 – 1.22
5	1.23 – 1.70

The smallest individual measurements of HCL recorded from field samples were consistently ~0.12mm. Mean HCL measurements recorded for the larval instars of *C. ambulans* separately in each of the study rivers are presented Table 4.10. The time lag between first-instar larvae hatching after the winter period to the appearance of fifth-instar larvae and adults suggests a total development time for this species to be between 1-2 months. This estimate would need to be confirmed through rearing experiments.

Table 4.10. Mean head capsule length measurements (mm) (in boldface) for the five larval instars of *Chimarra ambulans* in six rivers in the Western Cape, South Africa. Standard deviation (in brackets) and total numbers of individuals informing each mean are indicated.

Instar	Eerste	Elandspad	Molenaars	Rooi-Els Kloof	Wit	Wolwekloof
1	**0.192** (± 0.012) 176	**0.182** (± 0.018) 438	**0.182** (± 0.018) 653	**0.182** (± 0.018) 680	**0.182** (± 0.016) 1019	**0.182** (± 0.016) 1095
2	**0.366** (± 0.036) 158	**0.358** (± 0.029) 927	**0.358** (± 0.028) 1345	**0.358** (± 0.028) 1390	**0.360** (± 0.029) 1616	**0.360** (± 0.029) 1709
3	**0.635** (± 0.059) 41	**0.637** (± 0.047) 702	**0.636** (± 0.047) 1034	**0.635** (± 0.047) 1111	**0.637** (± 0.048) 1283	**0.636** (± 0.048) 1358
4	**1.016** (± 0.053) 12	**1.015** (± 0.070) 309	**1.007** (± 0.076) 551	**1.006** (± 0.078) 627	**1.007** (± 0.078) 694	**1.005** (± 0.078) 725
5	**1.314** (± 0.158) 2	**1.420** (± 0.103) 94	**1.393** (± 0.103) 235	**1.396** (± 0.102) 272	**1.393** (± 0.099) 325	**1.392** (± 0.099) 342

Adults collected and observed on a monthly basis indicated that the emergence period differed in all streams (Table 4.11). Additionally differences were observed in the timing, number and duration of generations as well as the appearance of first-instar larvae between the study rivers Table 4.11. Adults were present for 7-8 months (roughly October – May) of the sampling period in the Molenaars and Elandspad rivers respectively. In the Rooi-Els Kloof River, adults were recorded in only two months of the year (October 2009 and April 2010) (Table 4.11).

Based on comparisons of the relative numbers of individuals in each size class, in conjunction with the timing of emergence periods, the number of generations (and their respective duration) exhibited by this species in each river could only be estimated. As the exact development time is unknown for this species and individual cohorts could not easily be tracked through time owing to overlapping cohorts and extended emergence periods, estimates of numbers of generations should therefore be considered with caution.

Table 4.11. The timing of key events in the life cycles of *Chimarra ambulans* from six rivers in the Western Cape, South Africa.

River	Est. duration of recruitment	Presence of adults	Est. number of generations	Timing of first hatching after winter (for n >1)
Rooi-Els Kloof	Feb - May	Oct, Apr	1	February
Eerste	Mar - May	Nov, Dec, Feb - Apr	1	March
Wolwekloof	Dec - May	Dec, Feb - Apr	2	November
Molenaars	Nov - Feb	Oct, Dec - May	2	November
Elandspad	Dec – May/June	Sept, Oct, Dec, Feb - Apr	2	December
Wit	Nov – May/June	Oct, Dec - Apr	3	November

Recruitment and hatching periods varied in timing by a month or two across the rivers (which is coincident with different timing of thermal and hydrological regime shifts) but in general peak recruitment appeared to span the warmer months of spring and summer (October to May).

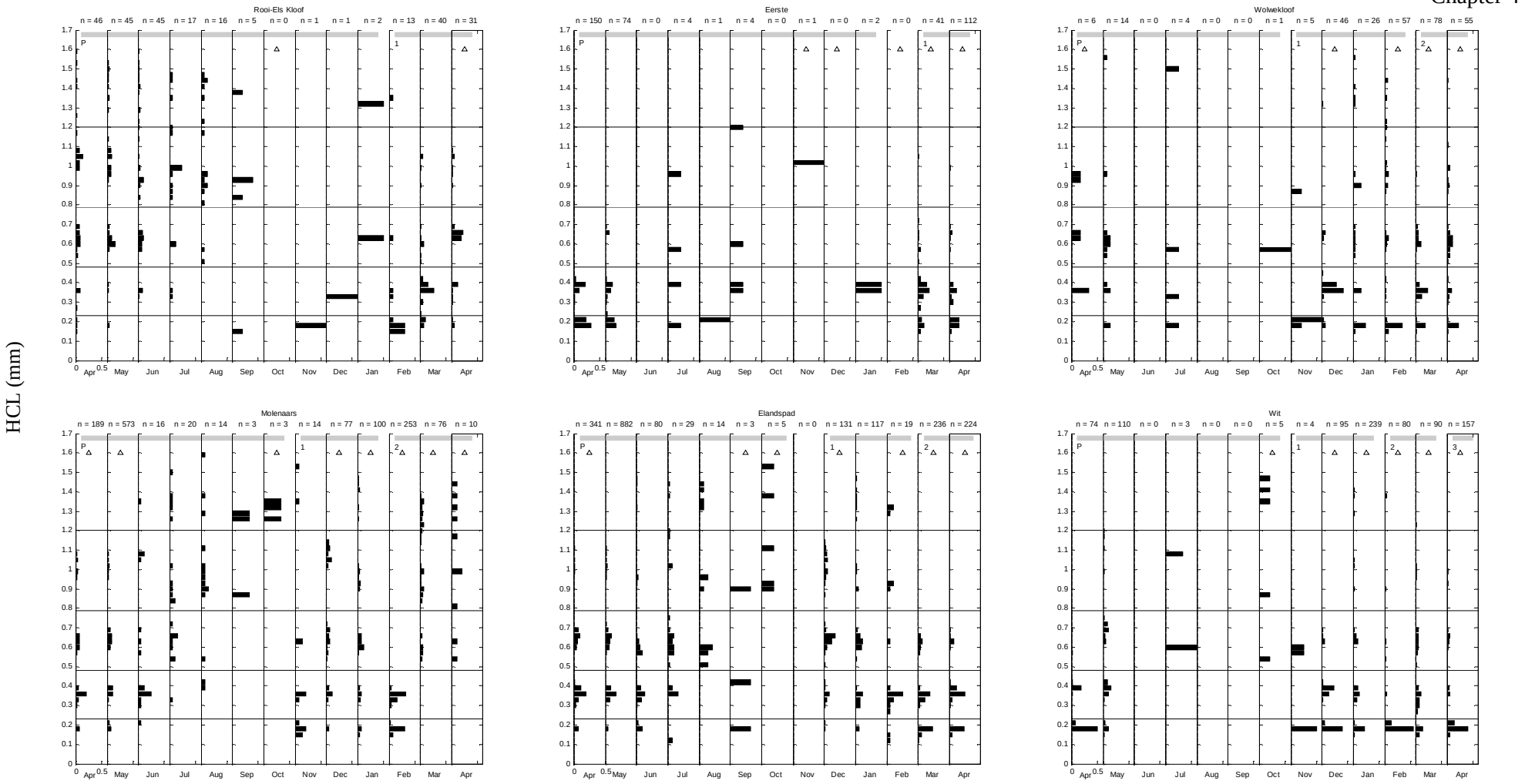


Fig. 4.6. Size frequency histograms of head capsule length measurements (mm) from individuals of *Chimarra ambulans* collected monthly from six rivers in the Western Cape, South Africa for the period April 2009-April 2010. Bar length indicates relative numbers of individuals for a given size class in each month. Dashed horizontal lines indicate separation of larval instars. Grey bars with corresponding numbers 1, 2, or 3 indicate the number and estimated duration of multiple generations. P denotes a generation from the previous year. Triangles represent months in which adults were present. Total number of individuals in the sample each month are given by the *n* values at the top.

Cohort analysis

Analysis of individual cohorts in this species was made difficult owing to asynchronous growth and presence of individuals from all larval instars in most months. Cohorts of this sort are termed indiscernible cohorts. Further estimation of generation time for this species was beyond the scope of this study and as such only basic inferences (considered suitable for the purpose of this study) were made from the available data. Multiple generations were observed for certain rivers and in most cases recruitment period appeared to be extended (occurring over several months). Individual cohorts within the sampling period exhibited both slow and fast life cycles. Slow life cycles were observed in cohorts recruited in autumn from adults of the previous year's generation. Individuals from these cohorts appeared to remain either as large larvae or pupae in the stream overwinter and showed slower progression in size during these colder and higher flow conditions. Additionally they were considered to have led to the emergence observed in most rivers around early spring (September - October 2009). Fast life cycles seemed apparent in cohorts recruited over the warmer period from early spring through summer. The estimated number of generations for each river was based largely on the counts of fast and slow life cycles. Fast life cycles were observed to range in length from 1-3 months, while slow life cycles took as long as 4 months to complete. More accurate predictions of the length of these life cycles need to be obtained through either rearing experiments or sampling over shorter intervals (a minimum of 2 weeks). No clear evidence of sexual dimorphism was found in the larvae for this species as they could not be separated according to gender.

4.3.4 Estimated hatch dates

The estimated mean dates of hatching in *L. penicillata* based on backward linear regression of mean monthly IOD measurements (from the second generation only) are shown in Fig. 4.7. Hatching in all rivers was estimated to occur in the summer months from late November to early December. Earliest hatching was estimated in the Wit River (21 November) roughly three weeks earlier than hatching in the Eerste River (10 December).

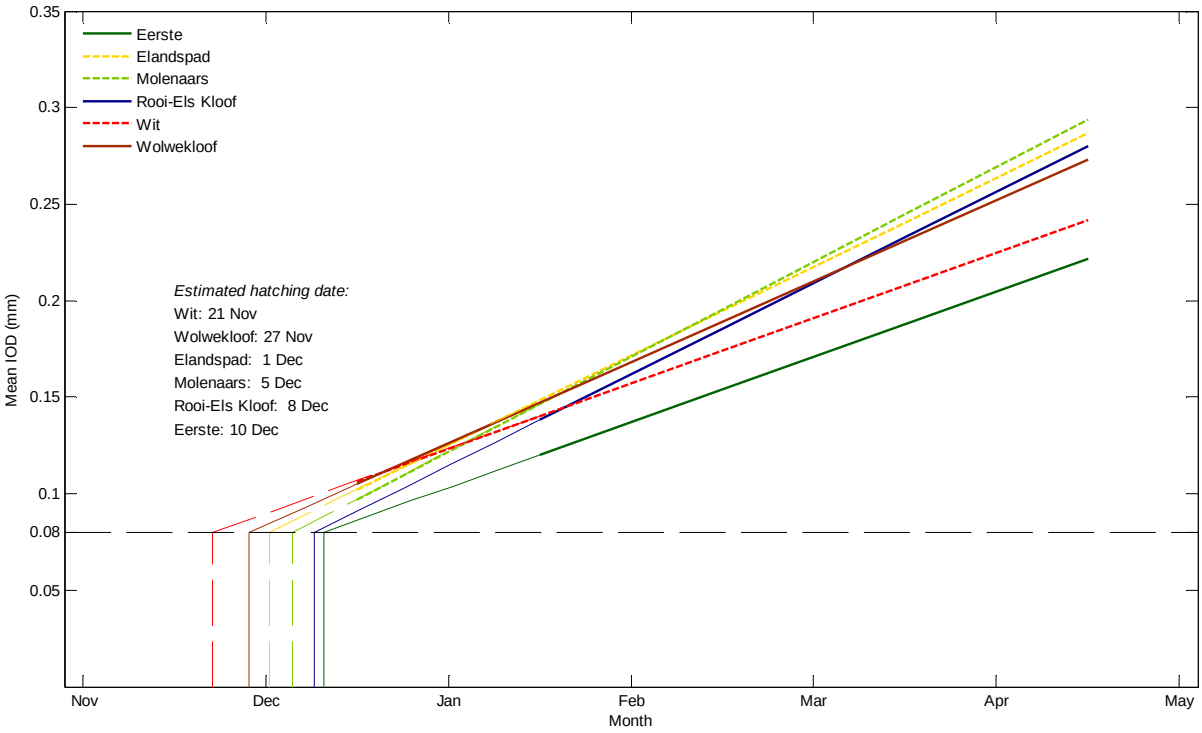


Fig. 4.7. Estimated dates of hatching in *Lestagella penicillata* based on backward regressions of mean monthly interocular distance measurements collected for single cohorts from six rivers in the Western Cape, South Africa over the period November 2009 – May 2010. Dashed and solid lines represent groupings of rivers based on similarities in thermal and hydrological regimes. The large dashed horizontal line denotes the head capsule width of first-instar hatchlings (0.08mm).

On the other hand the estimated mean dates of hatching for *Aphanicercella* spp. were found to span a longer period of time from early-July to late-August (Fig. 4.8). Hatch dates for *A. flabellata* were estimated to occur from 5 July (Elandspad and Molenaars rivers) to 27 July (Eerste River), while hatching for *A. scutata* occurred later from 4 August (Wolwekloof River) to 26 August (Wit River). The estimated hatch date for *A. barnardi* (Rooi Els Kloof River) was likewise the 26 August. Owing to low sample numbers, hatch dates could not be estimated for *A. scutata* from the Molenaars or Elandspad Rivers.

Since no clear cohorts were evident for *C. ambulans*, regression methods could not be used to estimate hatch dates in the same manner as for *L. penicillata* and *Aphanicercella* spp.

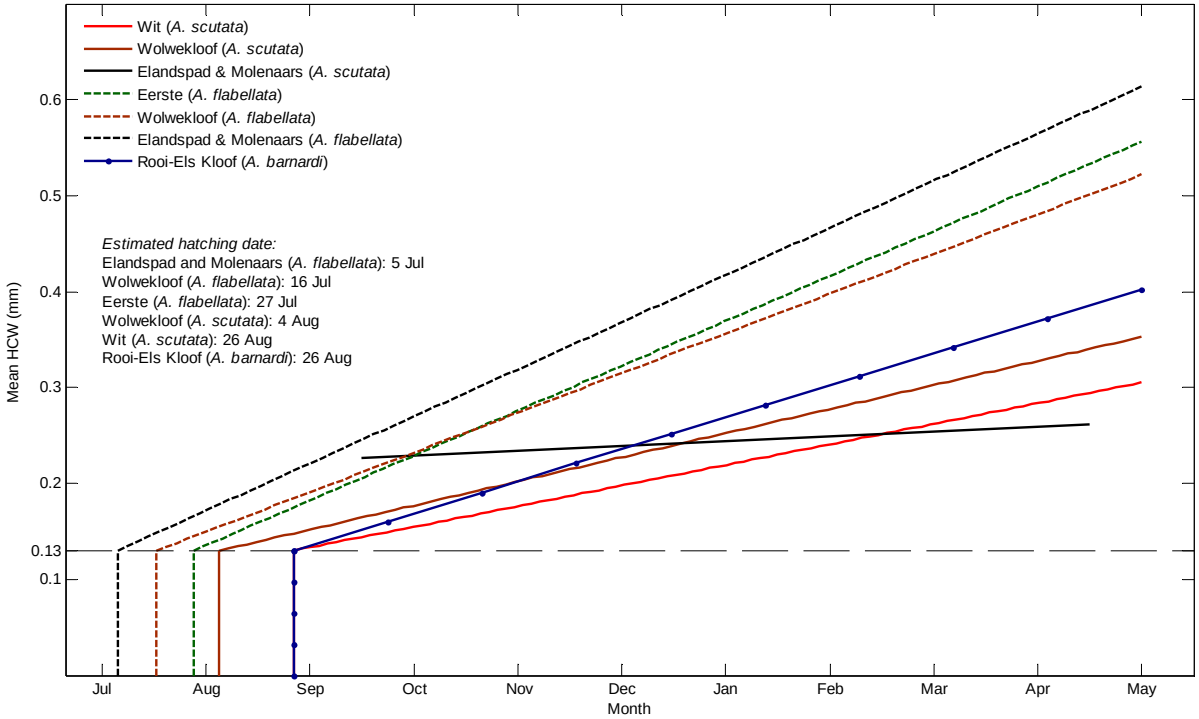


Fig. 4.8. Estimated dates of hatching in three species of Plecoptera (genus *Aphanicercella*) based on backward regressions of mean monthly head capsule width measurements collected for single cohorts from six rivers in the, Western Cape, South Africa over the period July 2009 – January 2010. Dashed, solid and marked lines represent the species *Aphanicercella flabellata*, *Aphanicercella scutata* and *Aphanicercella barnardi* respectively in each of the rivers. The large dashed horizontal line denotes the head capsule width of first-instar hatchlings (0.13mm).

4.3.5 Linking abiotic data to life-histories

GLM approach

Plots of the estimated effects of environmental variables on growth, generated from the GLM's carried out for *Lestagella*, *Aphanicercella* and *Chimarra*, are shown in Figs. 4.9, 4.10 and 4.11. Complete outputs of the estimated effects of each variable in the GLM's are provided in Appendix 4E. The respective study genera revealed contrasting growth responses, particularly with regard to hydrological and thermal parameters which showed the most marked effects on growth. Physicochemical parameters exhibited less obvious effects on growth. Some noteworthy trends are that *L. penicillata* showed optimal growth at intermediate temperatures (13-21.5°C), relatively low pH levels (4.6-5.1), low EC (<13µS/cm), channel widths greater than 10m and at moderate flows between 1.95 and 4.89m^3/s. In contrast *Aphanicercella* spp. revealed highest growth at lowest temperatures (<11.5-14.5°C) and moderate flows (~5m^3/s). *C. ambulans* exhibited fairly constant growth between instars at the widest range of temperatures (14.3°C to temperatures exceeding 21.3°C) and highest growth at low flows (0.65-0.85m^3/ s).

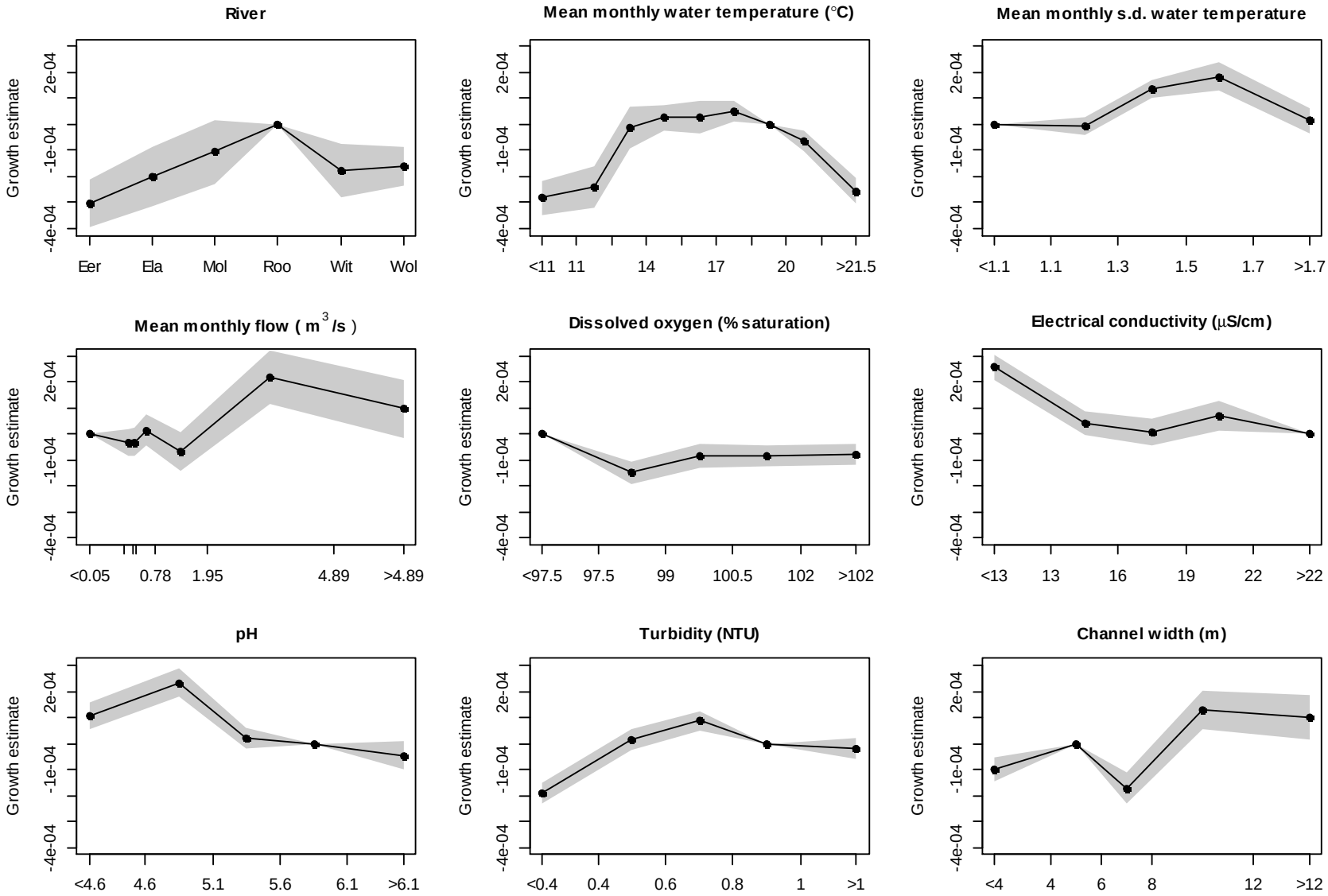


Fig 4.9. Plot illustrating the effect of factor levels of several environmnetal predictor variables on the growth coefficient of *Lestagella penicillata* as estimated by the GLM. Data used in the GLM were colleceted from monthly samples taken from six rivers in the Western Cape, South Africa (April 2009-April 2010). Site codes for the variable "River" as follows: Eer = Eerste, Ela = Elandspad, Mol = Molenaars, Roo = Rooi-Els Kloof, Wit = Wit, Wol = Wolwekloof. Grey shading represents the 95% confidence interval.

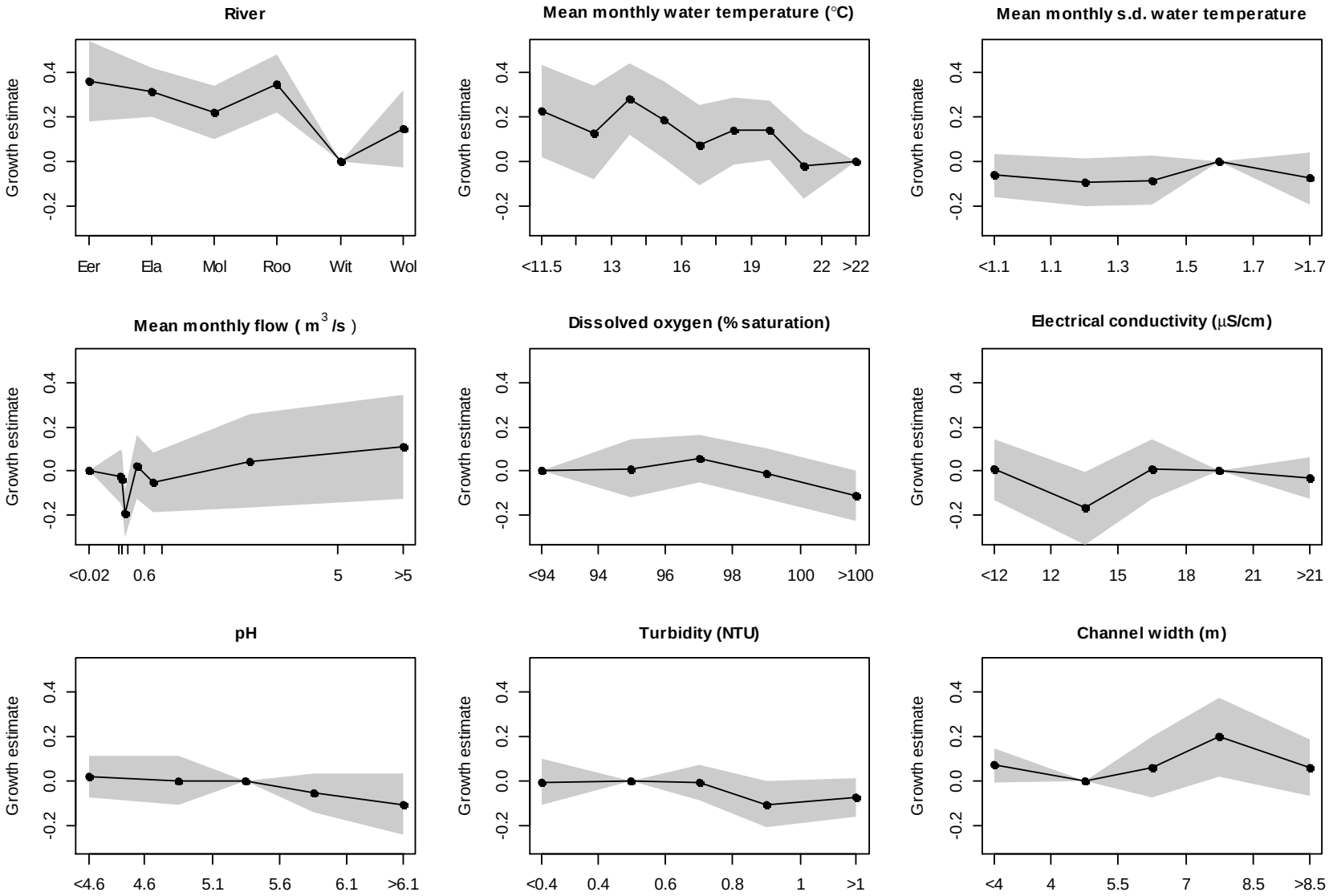


Fig 4.10. Plot illustrating the effect of factor levels of several environmnetal predictor variables on the growth coefficient of *Aphanicercella* spp. as estimated by the GLM. Data used in the GLM were collecetd from monthly samples taken from six rivers in the Western Cape, South Africa (April 2009-April 2010). Site codes for the variable "River" as follows: Eer = Eerste, Ela = Elandspad, Mol = Molenaars, Roo = Rooi-Els Kloof, Wit = Wit, Wol = Wolwekloof. Grey shading represents the 95% confidence interval.

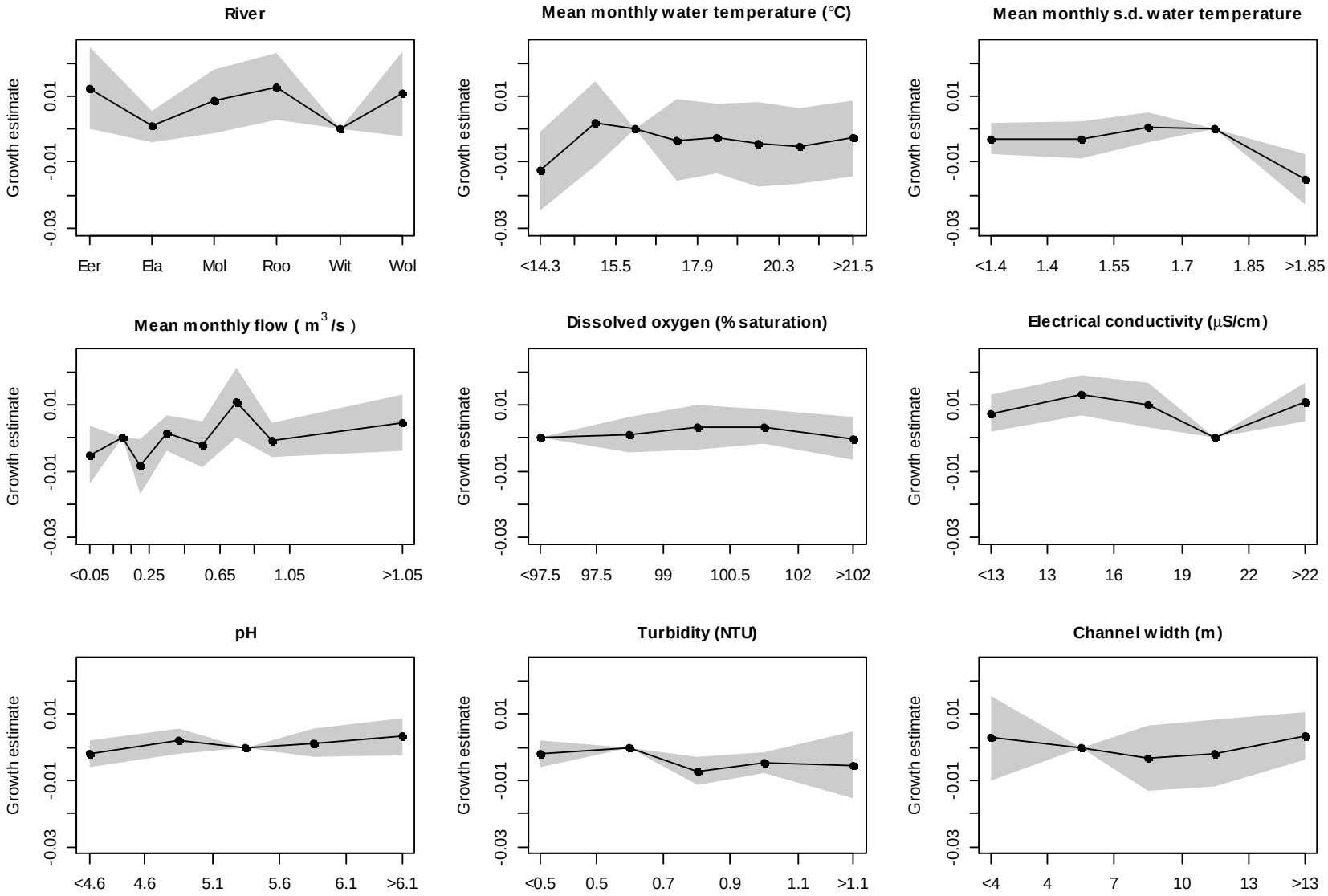


Fig 4.11. Plot illustrating the effect of factor levels of several environmnetal predictor variables on the growth coefficient of *Chimarra ambulans* as estimated by the GLM. Data used in the GLM were collecetd from monthly samples taken from six rivers in the Western Cape, South Africa (April 2009-April 2010). Site codes for the variable "River" as follows: Eer = Eerste, Ela = Elandspad, Mol = Molenaars, Roo = Rooi-Els Kloof, Wit = Wit, Wol = Wolwekloof. Grey shading represents the 95% confidence interval.

4.4 Discussion

Life-history studies inform numerous aspects of ecological and evolutionary research (Greene 2005). More so, they are necessary if accurate interpretations of community and species level responses to environmental and anthropogenic factors are to be made. While greatly valuable, such studies are time consuming (in some cases requiring multiple years of data collection), and generally funding for life-history research is limited (Resh & Rosenberg 2010). In a recent review of trends in life-history research, Resh & Rosenberg (2010) provide evidence for what they suggest is a general decline in the number of descriptive and life-history studies being published. Elliott (2009) suggests that in 25 years since an initial review on life-histories (Butler 1984), studies from egg to adult have remained rare. This is especially true in South Africa, in which no such studies on aquatic insects, such as that presented here, have ever been undertaken. Additionally, it is perturbing that while there is a noted decline in life-history studies in the Northern Hemisphere, such research is still in its infancy in the Southern Hemisphere and has to a large degree not even begun in South Africa. As Resh & Rosenberg (2010) point out, it is likely that descriptive research, such as life-history and organismal studies, have been deemed unworthy and old-fashioned when compared to more applied experimental research.

In contrast to this perceived view, life-history and descriptive studies offer unique research opportunities for hypothesis-based investigations as well as experimental studies (Resh & Rosenberg 2010). They can be predictive or applied and importantly they have the potential to expose new areas for research (Resh & Rosenberg 2010). Data generated during such studies can contribute to the generation of target water temperature criteria for South Africa. Such criteria are a useful management tool for maintaining stream temperatures, thereby ensuring the protection of aquatic ecosystems.

Target species analysed in this study exhibited markedly different life-history responses, not only in terms of voltinism, but also in the timing of several traits. These responses also differed amongst the rivers studied, in accordance to the major site groupings of the rivers (using flow and water temperature data) (Chapter 2). As such, each of the target species analysed in this chapter will be discussed separately.

Regression analyses of size measurements of different cohorts of *Aphanicercella* spp. and *L. penicillata* from the study sites allowed for comparisons of growth rate, size at maturity, emergence period and estimated hatch dates to be made across the sites and at different stages of the life cycle. These estimates (in the case of *L. penicillata*) showed that hatching occured earlier in rivers exhibiting higher average water temperatures (e.g. Wit River) than those exhibiting lower average water temperatures (e.g. Eerste River). Clear differences in growth rate were observed between the different generations (early and late instars) of both *Aphanicercella* spp. and *L. penicillata* suggesting possible ontogenetic shifts in growth rate (related to optimal thermal conditions or inherent genetic differences, see Brittain 1976a, Sweeney & Vannote 1978, Clifford *et al.* 1979), sampling error and sexually dimorphic growth.

Such differences in growth rate would likely have been responsible for differences in the timing of emergence period and overall life cycle length. The degree to which phenotypic plasticity might further influence life-history traits is explored further for *Lestagella* (Chapter 6). Additionally, the potential for phylogenetic constraint to be differentially affecting the flexibility and expression of life-history traits in each of the species is discussed in Chapter 7.

The GLM's provided a useful way of visualising and also quantifying the effect of each of the considered environmental variables on the growth of the study taxa across all sites. In particular the GLM's allowed for inferences to be made regarding optimum conditions for growth. Size measurements recorded from individuals occurring across the range of study sites allowed for a full spectrum of growth responses to be assessed for each species in relation to different ranges of environmental conditions.

It should however be noted that GLM's are just models and while no model is perfect, the approach adopted in this study provides a novel and useful tool for both informing and establishing preliminary thermal guidelines for these genera for the Ecological Reserve based on field data. Emphasis is however placed on the fact that GLM's can and should be used in conjunction with laboratory experiments as the GLM's provide an indication of optima rather than lethal limits. Egg development experiments (such as those presented in Chapter 5) give an idea of thermal thresholds or limits for successful recruitment/hatching, and CTM and LT_{50} experiments (such as those produced by Dallas 2009, Dallas & Ketley 2011, Dallas & Rivers-Moore 2012) provide insight into thermal limits for larvae/nymphs. Considering the differences observed between the life-histories of the study taxa from the different sites, the life-histories of each of the study taxa will be discussed separately.

4.4.1 Lestagella penicillata

The life-history of *L. penicillata* in all six study rivers was found to be that of a slow seasonal cycle (see Hynes 1970) with a single cohort or generation evident within a given year- i.e. univoltine. This cycle is not uncommon in Ephemeroptera and has been observed in several genera occurring both in the Southern and Northern Hemispheres (see Hynes 1970, Merritt & Cummins 1996). This finding was also in agreement with observations for the same species from different sites at the Eerste River (King (1982) and the Molenaars River (Ractliffe 2009). Representatives of this species exhibited subtle differences in the timing of life-history traits (*viz.* emergence, hatching, recruitment, mortality and growth) amongst the rivers studied - lending ecological support for the genetic analyses (see Chapter 3) which revealed considerable divergence between the various populations. These life-history traits will be discussed individually.

Emergence

The emergence period for this species appeared to be well defined, having a duration of one to three months[28]. The onset of the emergence period in this species was most likely cued to the warming of water temperatures in the respective rivers (see Chapter 2). First adult emergence occurred in October in all but one study river, namely the Wolwekloof River, in which it occurred almost a month later in November[29]. Data suggest that this is a direct result of the delayed onset of spring warming (owing to late and persistent winter flows) in this river, which is in line with the observations of Nebeker (1971a) as well as Vannote & Sweeney (1980), who reported earlier onset of emergence in both stoneflies and mayflies as a result of experimentally increased water temperatures. Emergence patterns also appeared to be timed to coincide with optimal conditions for adult flight. Mayflies mate in flight and females have been recorded to fly significant distances upstream before ovipositing (Brittain 1990). In order to maximize advantages for mating and also successfully oviposition in the correct location, warm air temperatures for flight are needed (Brittain 1990). In colder conditions these insects remain inactive as they cannot attain a suitable body temperature for flight. As such, warmer spring and early summer conditions presented suitably warm periods for flight.

Emergence periods were observed to be longer in the two coldest rivers (Rooi-Els Kloof and Eerste), with larger late-instar nymphs remaining in rivers well into December. In contrast large nymphs were absent from the warmer rivers (barring four individual stragglers in the Molenaars) during the mid-summer heat experienced in December. Similar observations were made by Ractliffe (2009) who observed that large nymphs were absent from the Molenaars River over December. This finding could be attributed to the possibility that nymphs avoid warm water temperatures over the heat of mid-summer, by emerging as adults prior to the increase in water temperatures.

Hatching

The timing of hatching in *L. penicillata* was largely informed by laboratory experiments assessing the development of eggs at a range of water temperatures (see Chapter 5). Field data however offered some valuable insights into the timing of this trait in this species. The presence of first-instar nymphs in the rivers is largely consistent with the onset and duration of the adult emergence period exhibited by the species in each of the rivers. In warmer rivers it appears that shorter adult emergence periods would most likely have resulted in more synchronized oviposition, earlier hatching and thus shorter periods of

[28] Adults that emerged from holding tanks in the laboratory at 10°C (air and water temperature) were observed to survive for up to two weeks. This unexpectedly long survival period may be a result of the cold temperature and the fact that the adults remained largely inactive (i.e. did not mate) and is therefore unlikely to reflect the natural survival time of an adult.

[29] The mean sizes of the nymphs collected from the Wolwekloof River were greatest during the month of October (Table 4.5) and showed a comparable size to those in the other rivers for the same period. This suggested that they were also nearing emergence at the time of sampling around the 17th October and most likely would have emerged shortly after sampling. Exact timing of first emergence however cannot be confirmed owing to the sampling accuracy of one month.

recruitment. In colder rivers first-instar nymphs of the new generation were noticed approximately a month later than in warmer rivers. Owing to the presence of some overlap between the new hatchlings and large nymphs of the previous generation, it was suspected that development started immediately after oviposition without diapause. Egg development experiments confirmed these observations (Chapter 5). The implications of the developmental temperature thresholds for egg development are considered to be a key factor in the control of population sizes and mortality (as a result of hatching failure) of this species and are discussed in further detail in Chapter 5.

Using life-history plots and confirmed IOD sizes of first-instar hatchlings (confirmed through egg hatching – Chapter 5), informed estimates of the timing of egg hatching could be made according to each river using backward regression analyses (Fig. 4.3). The observed presence of first-instar nymphs in field samples confirmed the validity of these estimates. Importantly these findings highlight an oversight in the detection of the first-instar nymphs in the sampling process and consequently a potential misinterpretation of recruitment periods in the studies by Ractliffe (2009) and King (1982) who both worked on *L. penicillata.* These studies used coarser mesh sizes (125µm and 600µm respectively) which would have resulted in early-instar nymphs being undetected.

Hatching in early summer could suggest a) that larger nymphs of this species cue the timing of emergence to avoid mid-summer water temperatures and b) that oviposition is timed so that eggs do not incur mid-summer water temperatures above the egg developmental threshold temperature (see Chapter 5).

Growth

Studies by Sweeney & Vannote (1978) and Sweeney (1984) have shown that growth in insects, particularly Ephemeroptera, is faster at higher temperatures, provided that these are within a non-lethal range, and as a result they exhibit larger body size at emergence than individuals growing at colder temperatures. This was clearly shown to be the case for *L. penicillata* in this study as a) cohort analyses showed that the monthly incremental increase in mean IOD (i.e. a measure of growth rate) was highest on average for warmer rivers (Fig. 4.3) in accordance with site groupings based on temperature and flow (see Chapter 2), and b) warmer rivers showed a higher mean IOD in the month of emergence (Table 4.5).

It is interesting to note that nymphs from the Wit River, while exhibiting the highest monthly incremental size increase (Fig. 4.3) in accordance with it being the warmest river, did not yield the highest mean IOD in the month of emergence. A possible explanation for this apparent anomaly is that the Wit River experienced suboptimal conditions (high water temperatures)[30] during the recruitment and

[30] High water temperatures close to the developmental threshold of 25°C, which correspond closely to critical thermal and lethal temperatures (estimated 240 hour LT_{50} = 25.9°C) proposed by Dallas (2009)

growth period of early-instar nymphs over summer. Exposure to high summer temperatures would presumably act to retard growth (see Brittain 1982), which was apparent in the lower regression gradient of the second generation in Fig. 4.3[31]. Additionally, these suboptimal high temperatures were followed by the lowest winter temperatures (generally also suboptimal for growth (Hynes 1970)) of all study rivers (~8°C). These two periods of suboptimal conditions could account for the lower observed mean monthly IOD in the month of emergence. It should be noted here however, that samples sizes from the Wit River were also generally small and as such this postulation would need to be confirmed through further studies.

While it is widely accepted that growth rates are observed to decline with increased body size (Angilletta *et al.* 2004), it has also been shown that ontogenetic shifts in the potential for growth exist (Strong & Daborn 1980), whereby organisms that started development at maximal growth rates ended development with sub-maximal rates and *vice versa*. Exposure to temperatures *both above and below* thermal optima ranges for growth has also been shown to affect aquatic ectotherms (Sweeney & Vannote 1978), by causing reduced adult body size and reduced fecundity at maturity. Such changes in growth at different stages of the life-history, during which different environmental conditions are experienced, appears to hold true for the life cycles exhibited by *L. penicillata* across the study sites. Slightly lower growth rates were observed for early-instar nymphs compared to late-instar nymphs of this species (Fig. 4.3) – possibly because of high temperatures experienced by juveniles in certain months being above the thermal optimum estimated for growth in the GLM. Additionally, a peak in growth was observed in the month of September. It is possible that the high growth rates observed in this month for all rivers represent resynchronization of growth prior to emergence. Synchronisation involves the faster developmental rate of lagging individuals, so that they can catch up to the rest of the population to ensure well timed emergence with females and thus incur a greater chance of mating. Synchronisation in this case would be important for a short-lived species such as *L. penicillata* in order to achieve competitive advantage for successful mating (Verberk *et al.* 2008). Since photoperiod is known to be an important factor relating to the timing of traits, specifically synchronization of emergence (Nylin & Gotthard 1998, Verberk *et al.* 2008), a likely cue for this synchronization would be the September equinox (occurring on the 22 September). This date marks a change in day length and subsequent increase in water temperatures (see Chapter 2).

GLM's revealed a temperature-dependent growth pattern for representatives of *L. penicillata.* Growth rates were observed to be lowest at water temperatures below 12.5°C and above 21.5°C whereas optimal growth rates were observed for temperatures between 13 and 21.5°C. This optimal range is quite a lot lower than the 96 h LT_{50} (29.5°C) and CTM limits (33.2°C) reported by Dallas & Ketley

[31] In contrast specimens from the Rooi-Els Kloof River showed little difference between the regression gradients of the first and second generation suggesting stable growth throughout the year, possibly as a result of more stable thermal and hydrological conditions

(2011) for representatives of the same species. This discrepancy suggests that while substantially higher water temperatures can be tolerated by this species, growth rate under such conditions would be reduced/suboptimal. Evidence of such suboptimal growth was observed in the Wit River where 30-day moving average of maximum water temperatures were as high as 27.10°C. The optimal range of 13-21.5°C might help to explain the lower growth rate exhibited by the second generation compared to the first generation of *L. penicillata* within the Wit River shown in Fig. 4.3. The first generation appears to experience optimal water temperatures for growth in the months of April to May (17 to 12°C respectively), whereafter winter water temperatures experienced from June-September (9.8-10.6°C) yield lower growth. From October to December, flows reduce and water temperatures increase once again to optimal conditions (13.69-19.25°C) allowing for a spurt of growth or synchronisation of growth before emergence. Eggs are oviposited by late November and approximately two weeks later the second generation of first-instar nymphs is present. These early nymphs incur the warmest water temperatures exhibited in the Wit River over the months of January to March, where water temperatures were shown to exceed the optimal range (>21.5°C). Thus growth rate is lower compared to the first generation and also to the second generation of nymphs present at other sites that incur slightly cooler water temperatures over the same period (e.g. Rooi-Els Kloof River).

While temperature was shown to have the most apparent effect on growth in the GLM's, some subtle trends were evident for some of the remaining physicochemical variables. The increase in growth rate observed at relatively greater flow levels and channel widths could be linked to food availability (in this case, algae). One might expect primary production to be greater in larger, higher order, open-canopied streams/rivers that receive more solar radiation. Increased growth rates at lower EC and pH levels, however, cannot easily be explained and might be related to species- and site-specific responses.

Abundance/mortality

The total abundances of individuals of *L. penicillata* recorded from each of the study sites were generally comparable, except for that of the Wit River. The greatest numbers of individuals in all rivers, with exception of the Wolwekloof and Wit rivers, were observed during the autumn months of April and May 2009, after which substantial declines in numbers occurred with onset of winter spates in June most likely resulting in organisms moving downstream. Numbers generally showed a continual decline through the rest of the months of winter and spring, despite spate conditions abating to more stable flows towards mid-September. This gradual decline in numbers could represent either true natural mortality (including predation and disease) or an unavailability of individuals for sampling as a result of the organisms hiding deep in the substrate during periods of unfavourable flow conditions (e.g. winter spates in June). For example, particularly low numbers of individuals (as low as 0) were recorded in the month of June for the Eerste, Wit and Wolwekloof rivers. This was most likely as a result of flood

conditions both preventing adequate sampling and also forcing organisms downstream or into the rhithron.

Total abundances were found to relate largely to flow predictability scores and the number of flow regime shifts for each river, rather than thermal parameters. A high number of flow regime shifts indicated by low predictability scores for flow, correlated with rivers exhibiting the lowest total numbers as well as lowest numbers of individuals each month. The Wit River showed the lowest total number of individuals (n = 171), the second highest number of regime shifts in flow (numbering 11) and also the second lowest flow predictability score (0.39). In contrast the Rooi-Els Kloof River had the greatest numbers of individuals (n = 3861) in total and per month, while also exhibiting the highest flow predictability score (0.56) and the lowest number of flow regime shifts (only 4).

The considerably lower numbers of individuals observed in the Wit River, however, suggest that this river experiences less favourable conditions for this species, relative to the other rivers. Based on the thermal and hydrological regimes observed for this river, it seems apparent that combinations of these two parameters are having a substantial impact on the population. Eggs laid in this river outside the suitable window for development (which occurs between early-October and mid-November) would experience mid to late summer temperatures near the critical thermal limit for egg development (this limit lies between 20 and 25°C) severely reducing hatching success and recruitment. Additionally if smaller nymphs were susceptible to estimated critical thermal limits (estimated at 25.9°C in 240 hour LT_{50} experiments for slightly larger nymphs of this species, Dallas 2009), then the first-instar nymphs hatched from eggs oviposited earlier in summer would be exposed to temperatures in late summer above these limits. The combined effects of low percentage hatching success, mortality in hatchling/early-instar nymphs, and overall thermal stress could likely produce a smaller initial population. Assuming this small population survived through summer and autumn, it would then be faced with the onset of sustained high flows, floods, and suboptimal temperatures for growth for most of winter[32]. The additional mortality experienced over winter would mean that a substantially smaller population would survive to the adult stage to produce the next generation. This cycle, if repeated in consecutive years, would severely affect the total population size of this species in this river and could therefore explain the consistently low numbers observed.

Without prior genetic analyses one might potentially interpret the differences in life-history exhibited by the same species (e.g. *L. penicillata*) across the range of sites to possibly be as a result of phenotypic plasticity in response to differing environmental conditions (e.g. water temperature). The results of the

[32] Conditions during winter are worsened by the fact that this river has a shallow cobble substrate underlain by large areas of bedrock – stones are susceptible to overturning during floods (V. Ross-Gillespie, pers. obs., 2010), as such fewer suitable refugia would be available and individuals would additionally not be able to move deep enough within the substrate to avoid high flows.

genetic analyses however, when interpreted in conjunction with the life-history patterns, tell a different story.

Genetic analyses of samples of *Lestagella* suggest that apart from the Molenaars and Elandspad River sites a different lineage (potentially undescribed species) of *Lestagella* occurred in each of the study sites. The results suggested large amounts of genetic differentiation between samples from the sites). While the status of the species would need to be reviewed using additional genetic and taxonomic work, the preliminary results suggest that *Lestagella* lineages have evolved life-history responses to the range of thermal and hydrological differences incurred in the respective study sites, such that subtle changes in the timing of specific life-history traits (e.g. emergence and hatching period) have allowed them to maximise fitness growth and fitness given the range of environmental conditions experienced in each of the sites.

It would valuable to test this assertion by conducting egg development experiments on *Lestagella* collected from each of the study sites separately and check whether the respective lineages have also evolved different thermal thresholds for egg development.

4.4.2 Aphanicercella species

The life-histories exhibited by each of three species of *Aphanicercella* analysed in this study (*viz. A. barnardi, A. flabellata* and *A. scutata*) were all that of a univoltine, slow seasonal cycle (see Hynes 1970). This cycle, along with a semivoltine cycle (taking two years to complete development), is the most common among the Plecoptera recorded from the temperate areas of Europe and North America (Merritt & Cummins 1996). In general the species of *Aphanicercella* in this study showed growth of a single cohort from late-winter (August/September) through the summer with adults reaching maturity towards the end of autumn extending well into winter (May-September). Species traits were also found to differ for these species in the six rivers studied. However comparisons of species between rivers were only possible for *A. flabellata* and *A. scutata*. In the Rooi-Els Kloof River only *A. barnardi* was found to occur. The data obtained in this study regarding the life-history of these species of *Aphanicercella* serve as the only existing reference in South Africa. Therefore, while the slight differences in life-history traits of the three species may be purely as a result of genotypic differences (Chapter 3), they cannot be confidently attributed to environmental conditions until additional data have been collected for this species elsewhere.

Emergence

Owing to low numbers of individuals collected in conjunction with low numbers of black wingpad nymphs, estimates of emergence period are quite preliminary and further confirmation will be required. In general the timing and duration of emergence periods were found to be similar for the three species. Emergence periods were less well defined than those of *L. penicillata* and extended for longer periods

(3-5 months roughly May - September). Adult emergence dates were found to coincide with those detected in other species from this genus (Stevens 2009). Emergence dates for the species appeared to coincide closely with the onset of higher flows and spates (first regime shifts detected in flow for May) experienced in each of the rivers. The observed timing of emergence could suggest that the nymphs (most likely smaller sized nymphs) of the species within *Aphanicercella* are susceptible to mortality induced from high flows or spate conditions and thus life-histories appear to have evolved such that nymphs avoid these harsher conditions by a) the timing of adult emergence over winter or b) over-wintering in the egg stage. Additionally in contrast to the Ephemeroptera, adult plecopterans stay in close proximity to the larval habitat and mate on solid substrate (Brittain 1990). As little flying is required for mating and also oviposition, colder air temperatures (such as those experienced in winter June – August) provide suitable environmental conditions for the adults (Brittain 1990).

Hatching

The identification of first-instar nymphs in field samples was confirmed through comparing measurements recorded from first-instar hatchlings obtained in egg development experiments (Chapter 5). First hatching in the *Aphanicercella* was observed to occur in August and September following a period of between 1 to 3 months since the first and last emergence of adults was noted. Developmental time of the egg could therefore be estimated from the life-history plots to similarly be anywhere between 1-3 months. This observation corresponded well to findings obtained in the egg development experiments for this species (Chapter 5) which showed that eggs took approximately 37 days (350-370 DD) to hatch at a constant temperature of 10°C[33]..

Using backward linear regression analyses on the second generation cohorts of the species of *Aphanicercella*, the mean dates of hatching could be estimated (i.e. the month where the mean HCW was that of a first-instar nymph). These dates were found to differ according first to species and then to site groupings. *A. scutata* from the Wolwekloof River was estimated to hatch ~22 days earlier than the same species in the Wit River on the 4th August. This discrepancy might be owing to the fact that none of the smallest size class of individuals were obtained in samples for both August and September from the Wolwekloof River in which they should have been present - suggesting a sampling artefact. *A. flabellata* on the other hand showed overall earlier estimated hatching dates compared to *A. scutata*, in July, possibly as a result of marginally earlier emergence owing to faster growth rates observed in this species. Earliest hatching for this species was estimated in the Molenaars and Elandspad rivers (5 July), while the latest hatching occurred in the Wolwekloof and Eerste rivers (16 July and 27 July respectively). These discrepancies could also be as a result of sampling artefacts effecting the

[33] If one takes an average water temperature from the onset of emergence in the Wit River (approximately 15 June) to the month prior to the first observed first-instar nymphs (approximately 15 August) an average of 9.7°C ±0.531°C is calculated. As such 10°C is a good estimate for determining egg development time in the winter months when oviposition was observed.

confidence of the regression analyses or alternatively as a result of colder water temperatures incurred in the Elandspad and Molenaars rivers compared to the Eerste and Wolwekloof rivers leading to slightly shorter egg development times and therefore marginally earlier hatching dates (see Chapter 5)

Growth

Using regression gradients of the second generation cohort, the rate of incremental size increases in HCW could be compared between species and sites for *Aphanicercella* (Fig. 4.5). Differential rates of size increase were noted first according to species, with *A. flabellata* exhibiting a faster rate of increase in comparison to both, *A. scutata* and *A. barnardi*, the latter two species revealing similar rates of increase. Where *A. scutata* or *A. flabellata* occurred in contrasting sites, colder rivers showed marginally faster rates of growth (Fig. 4.5) in contrast to what was observed for *L. penicillata*. Maximum head capsule sizes at emergence were generally similar for each species and across rivers, but on the whole the mean HCW measured for each species from each of the rivers showed considerable variation from the months of April onwards (Table 4.7). It is suspected that this variation is as a result of both a dramatic increase in growth and also differential growth exhibited by males and females of each species. The effects of sampling error (low sample sizes) and larger females owing to sexually dimorphic growth might have biased calculations of monthly means. Sexual dimorphism in the adults of several species of *Aphanicercella* has been reported by Stevens (2009). This sexually dimorphic growth appears to coincide with the onset of a) the first negative thermal regime shift in the year (March) - linked to changes in photoperiod/shorter day length as a result of the March equinox in the Southern Hemisphere (approximately 20 March) (see Nebeker 1971a, 1971b, Shama & Robinson 2006), b) increased food supply from autumnal leaf fall (see King *et al.* 1987, Stewart & Davies 1990) and c) winter high flows and spates.

The differences observed between the regression gradients of the first and second generation cohorts (Fig. 4.5) were considerable. Regression gradients for the first generation (growing over winter: April to September) were five to seven times steeper in the case of the *A. scutata* both in the Wit and Wolwekloof rivers than that of the second generation (growing over spring, summer and early autumn: September to April). Similarly, the regression gradients of the first generation were three times as steep in the case of *A. barnardi* in the Rooi-Els Kloof River and twice as steep in the case of *A. flabellata* from the Eerste, Wolwekloof and the combined Molenaars and Elandspad rivers, than that of the second generation for these species respectively. This marked difference between the rates of size increase suggested that growth was slower over the warmer periods of the year following hatching and that with the onset of colder temperatures and various additional cues, the rate of growth increased rapidly until emergence. This is pattern is somewhat similar to the growth recorded by Brittain (1973) for a fairly closely related species from the Northern Hemisphere, namely *Nemoura avicularis*. The numbers of *A.*

scutata in the samples collected from both the Molenaars and Elandspad rivers were too few to provide meaningful regression results.

GLM's revealed a relationship of growth and temperature in stark contrast to that observed for *L. penicillata*. For *Aphanicercella* spp. the trend in growth rate suggested that highest growth occurred at colder water temperatures (<11.5-14.5°C) and lowest growth at higher water temperatures (>20.5°C), with generally good growth observed for temperatures less than 16°C. This pattern typifies the broad labeling of this insect order as "stenothermic" (McKie *et al.* 2004). More specifically, it lends further ecological support to the notion that this genus originated in the cooler climates of Gondwanaland and has possibly retained ancestral ambient temperature requirements, thus remaining in cooler montane refugia, (McKie *et al.* 2004, Day 2005, Stevens 2009). The growth patterns in relation to temperature evident in the GLM outputs are consistent with the disparity observed in the regression gradients of the first and second generations in Fig. 4.5. As growth is higher at colder temperatures associated with the onset of autumn and winter along with higher winter flows and increased food supply, it makes sense that regression gradients measured for larger sized nymphs for this part of the year are markedly higher and also more variable. Conversely smaller regression gradients resulted from slower growth of small individuals at higher suboptimal temperatures experienced from roughly spring through to autumn (September -April).

Lowest growth rates were observed at temperatures above 20.5°C, which could suggest an upper temperature tolerance of approximately 21-22°C for *Aphanicercella*. This estimate links closely with the critical thermal maximum and lethal temperatures presented by Dallas (2009) (estimated 240hour LT_{50} = 20.7°C for a closely related species *Aphanicerca capensis*) and also with the results of the egg development experiments presented in Chapter 5 - which showed a decrease in the percentage hatch of eggs of *A. scutata* at water temperatures of just 15° and 20°C when compared to that recorded at 10°C. Brittain (1973) also showed in laboratory experiments with Nemoura avicularis that temperatures of 20°C and above for any length of time were unfavourable.

Similar to results observed for *L. penicillata* of the variables included in the GLM, water temperature was shown to have the most obvious effect on growth, with the effect of flow on growth showing only a weak trend of marginally better growth at higher flows. No distinct trends in growth were observed for the remaining physicochemical variables apart from a) DO concentration, which interestingly showed a slight decrease in growth at super saturation levels (>100%) probably owing to the fact that these concentrations only occur during spate conditions and b) relatively small channel width for which highest growth was observed at intermediate channel widths of 7-8.5m more common to upper reaches of rivers and mountain stream zones.

Abundance/mortality

Numbers of individuals from the three species of *Aphanicercella* were considerably lower in all streams than those observed for *L. penicillata*. In rivers where more than one species occurred (e.g. the Wolwekloof River where both *A. scutata* and *A. flabellata* occurred), often one species dominated and occurred in higher numbers than the other. In such circumstances too little data were collected or were available to make inferences about the possibility of interspecies competition, or functional feeding groups in relation to resource partitioning and the availability of food resources in the respective rivers.

Measures of abundances of *Aphanicercella* were confounded by a prolonged period of recruitment (~ three months in the case of the *A. scutata* in the Wit River and *A. barnardi* in the Rooi-Els Kloof River) of early-instar individuals into the populations in each river, possibly as a result of the relatively long emergence periods exhibited (up to four months) by the different species. For this reason monthly abundances appeared to increase for a large period of the total life cycle, while on the whole compared to *L. penicillata* and *C. ambulans*, low numbers were collected each month

An interesting finding was that highest total numbers of individuals of *Aphanicercella* were present in the samples from the Wit River (these being individuals of *A. scutata*). This was in contrast to *L. penicillata* which occurred in lowest numbers in this river. A possible explanation for this apparent anomaly lies in the fact that *L. penicillata* could have faced lower hatching success of eggs at higher temperatures experienced over the summer months in the Wit River. This in conjunction with subsequent mortality, owing to the exposure of surviving nymphs to spate conditions, could result in an overall decreased population size. In contrast *A. scutata* a) had an extended emergence and prolonged recruitment period over an entirely different part of the year and as such eggs would not have all been oviposited into the same (potentially unfavourable) conditions, thereby reducing the mortality of early-instars nymphs, and b) avoided unfavourable flow conditions and spates associated with winter by emerging as adults over this period and surviving as eggs attached to stones in the stream (Chapter 5). Additionally as the Wit River was in fact the coldest river over the winter months, such cold conditions would have in fact been more favourable for growth and also hatching success of eggs of *Aphanicercella* (see Chapter 5).

4.4.3 Chimarra ambulans

In contrast to the life cycles exhibited by *L. penicillata* and the three species of *Aphanicercella*, the trichopteran *C. ambulans* showed a non-seasonal or asynchronous cycle, with multiple, overlapping generations occurring within the period of a year. The voltinism or number of generations produced in a year by this species was found to vary amongst the study rivers in accordance to site groupings based on flow and temperature. Data suggested that univoltine, bivoltine and even trivoltine life cycles were exhibited by representatives of what has been shown genetically to appear to be a single species (see

Chapter 3). The life cycle characteristics in this study are in accordance with the preliminary life cycle observations of Ractliffe (2009) for an unidentified species of *Chimarra* in the Molenaars River (most likely *C. ambulans*). They also correspond with observations made by Bowles & Allen (1992) of bivoltinism and potentially trivoltinism for two closely related species, also within the genus *Chimarra*, namely *C. aterrima* and *C. obscura.* Univoltinism has been recorded for other species of *Chimarra* from the northern latitudes (Williams & Hynes 1973, Parker & Voshell 1983), while bivoltinism has been observed in parts of southern North America (Benke 1984, Parker & Voshell 1982).

What appeared to be univoltine cycles were observed in the coldest rivers namely the Rooi-Els Kloof and Eerste, while potentially bivoltine cycles were exhibited in the warmer Molenaars, Elandspad and Wolwekloof rivers. In the warmest river, the Wit, however, an additional generation over summer was thought to be observed and a trivoltine cycle was suspected. While caution should be taken when interpreting voltinism and the degree to which it is affected by factors (such as temperature, flow, photoperiod and food), several examples exist where flexibility in the number of generations per year for certain species have been attributed mainly to thermal differences (Ward & Stanford 1982). Determining the degree to which each of these factors affects voltinism is difficult but remains necessary in life-history studies concerning the Trichoptera (Bowles & Allen 1992). Based on the findings of this study, differences in thermal regime seemed the most likely and obvious factor affecting voltinism in this species. Nevertheless it should be emphasised that the interpretation of the life-history data and voltinism for this species in particular remains speculative, as the growth of cohorts could not be followed individually.

Monthly measurements of HCL revealed the clear and consistent separation of individuals into five mean size classes, within monthly samples from each of the six study rivers. These five size classes were interpreted to represent five larval instars for this species. While no data exist in South Africa regarding the life-history or number of larval instars of this species, these findings are also similar to those made by Bowles & Allen (1992) for two other species of *Chimarra*, *C. atterima* and *C. obscura*, for which five larval instars were also reported.

Emergence

Larvae were observed to decline rapidly in numbers with the onset of winter spates and the first flow regimes shifts. This response appeared more closely linked to changes in flow as opposed to regime shifts in temperature, which occurred earlier in March. In the rivers that experienced a higher number of regime shifts in flow over winter, larvae were largely absent from samples, barring a few individuals of different instars. In the Molenaars, Elandspad and Rooi-Els Kloof rivers which experienced fewer flow regime shifts, few larvae from a range of instars were present over late winter to early spring (August – October). While the majority of these larvae were in their later or final fifth-instar stages, these findings suggest a) the possibility of a quiescent or pupal stage over winter in order to avoid unfavourable flow

and thermal conditions and b) that this species shows very little larval growth over winter but that the growth of larvae does nevertheless occur (see Hynes 1970, Bowles & Allen 1992). During these periods larvae recruited late in the season remain in the stream, experiencing slow growth in their late-instar stages. Some larvae of several trichopteran species remain in this fifth-instar stage within a pupal casing over winter and emerge only with the onset of warmer water temperatures in spring. In this study evidence of a pupal stage during winter in this species was observed (visual observations made during sites visits) but pupae were not collected or quantified for life-history analyses.

Following unfavourable winter conditions, the first adults emerged towards early spring (September-October) in the Molenaars, Elandspad and Rooi-Els Kloof rivers. These were assumed to be the adults of the slow growing or quiescent larvae that remained in the rivers over winter. In rivers that experienced a later thermal regime shift in spring (September, October) owing to higher flows over winter (namely the Eerste, Wit and Wolwekloof rivers), first emergence of adults was also noted later (in November, October, and December respectively). Thus emergence of adults after the winter quiescent/pupal stage appears to be thermally cued.

Hatching

Hatching in this species appeared to be continuous, extending over much of the emergence periods for which adults were observed even over the warmest months of summer. As such, no egg diapause was suspected in this species and thermal thresholds for egg developments were suspected to be higher than those expected of *L. penicillata* and *Aphanicercella*. These observations were confirmed through egg development experiments (Chapter 5). In general hatching was observed from late-spring to as late as early-autumn. The specific onset of hatching in each river however was closely cued to a combination of the abating of winter/spring flows and the onset of warmer water temperatures (>18°C) following winter. Hatching occurred later in the colder Rooi-Els Kloof and Eerste rivers and earlier in the warmer Molenaars, Elandspad, Wit and Wolwekloof rivers. This is likely as a result of the fact that a) overwintering larvae would take longer to develop and emerge in these colder rivers thus giving rise to population increases later towards late-spring and summer and b) that eggs developing at lower water temperatures were shown to take longer to hatch (Chapter 5). Similar observations were made by Cudney & Wallace (1980) for a Northern Hemisphere species of the same genus, *C. mosleyi*. Additionally the recruitment period was observed to closely track both water temperature and flow. The duration of the recruitment period appeared to be governed to a larger degree by flow as opposed to water temperature. This was suspected because the first negative thermal regime shifts in March were not observed to halt recruitment, but rather recruitment was halted later in May and June with the first flow regime shifts. As *C. ambulans* are net spinning caddisflies, high flows and spate conditions would prove detrimental to feeding as the flows would be too fast for net structures to remain in position for any given period of time.

Growth

Growth with regards to *Chimarra* refers to the size increase between instars, as individual cohorts could not be tracked through time. GLM's revealed that growth between successive instars remained fairly constant at temperatures above 14.3°C to temperatures measured above 21.5°C (max. 7-day moving average of 27.8°C recorded for the Wit River). These data suggest a wider optimal thermal range for growth of *C. ambulans* when compared to both *L. penicillata* and *Aphanicercella* spp. Having a less conservative or phylogenetically constrained life-history involving noticeably shorter generation time and fewer larval instars, *C. ambulans*, may have been able to adapt more readily to warmer water habitats over time (this is discussed further in the following chapter and Chapter 7) when compared to the other two more conservative study species.

Additionally the optimal thermal range for *C. ambulans* encompasses substantially higher water temperatures, suggesting preferential growth in warmer waters. This is emphasised by the fact that growth between instars was shown to decrease below temperatures of just 14.3°C - a temperature at which *L. penicillata* and *Aphanicercella* both show good growth.

The higher range of temperatures observed for optimum growth ties in with results of the egg development experiments which showed successful hatching occurring at a wide range of temperatures from 10°C to as high as 25°C. These findings are somewhat in accordance with the results obtained by Dallas (2009) and Dallas & Ketley (2011) regarding the LT_{50} and CTmax limits for this species. Their studies revealed a 96 h LT_{50} temperature of 25.5°C and a median CTmax value of 32°C, while the extrapolated 240 h LT_{50} was estimated to be just 19°C. The estimated 96 h LT_{50} is just higher than the temperature at which hatching, though a low percentage, still occurred in this species (Chapter 5). The 240 h LT_{50} value on the other hand is well within the range of temperatures observed for optimal growth in the species. Collectively the GLM results and egg development experiment results presented in this thesis appear to contradict the extrapolated 240 h LT_{50} of 19°C, calculated by Dallas & Ketley (2011) for *C. ambulans.* Findings in this thesis suggest that the results of the 240 h LT_{50} are in fact an underestimation, with the results of the 96 h LT_{50} seeming more probable. Further long-term LT_{50} experimentation such as that presented in Chapter 6 would however be needed to confirm this notion.

GLM's revealed that while mean monthly flow was shown to have little effect on growth, low flows of between 0.65-0.85m^3/s yielded the highest growth estimates coinciding with warmer months of summer. These flows might also reflect an optimum range for net construction, where higher flows would be destructive. It should be noted that the range in mean monthly flow values incorporated into the GLM are quite a lot lower than the range included for *L. penicillata* and *Aphanicercella*. This is owing to the fact that *C. ambulans* was largely absent from samples collected over the winter months (perhaps also related to net construction and thermal optima for growth), providing no growth data for

the higher flow conditions included for the other species. Growth estimates were largely unaffected by the remaining physicochemical variables.

What is not addressed by the GLM is the rate at which this species progresses through the larval instars from egg to adult in the respective study sites and this rate may change significantly with temperature.

Abundance/mortality

The total abundances of individuals of *Chimarra* in each of the study rivers appeared to relate to the grouping of sites based on both thermal and flow data. Colder rivers (Rooi-Els Kloof, Eerste, and Wolwekloof), regardless of the magnitude and number flow regime shifts, were associated with lower total abundances and fewer generations within the year. As lower temperatures persist for longer periods in these rivers, the development of eggs as well as the growth of larvae would presumably be slowed leading to a slower rate of adults emerging, mating and laying more eggs. Smaller populations could therefore be expected.

Highest numbers were observed in April and May for most rivers, this could represent an accumulation of individuals over the recruitment period in combination with several environmental factors, such as optimal flow and thermal conditions for growth as well as food availability (this following the influx of leaf litter, suspended particles as well as ultra fine benthic organic matter (UFBOM) in late summer/autumn).

The monthly abundances show sharp declines coinciding with the onset of high flows and spates. As flows abated in each of the rivers and higher temperatures allowed for rapid development, recruitment was initiated and abundances rapidly increased as several generations began to develop.

4.4.4 Sampling limitations

A sampling interval of one month was used in this study. This interval was decided upon *a priori* with little knowledge of the ecology of the study organisms (e.g. hatching/ recruitment periods, larval instars) and also owing to logistic limitations (sample processing time, cost of sampling trips). Estimates unless stated otherwise were therefore accurate to one month only. As mentioned several times in this study, mortality rates and monthly abundances should be interpreted with caution as they indicate only the numbers of animals on the surface of the substrate and it is likely that this would have been influenced by timing of sampling relative to spate conditions. This is especially relevant for samples collected in winter where abundances were shown to vary greatly between species and rivers. It is possible that certain species took refuge in deeper substrate during these periods to avoid unfavorable conditions. For instance, a study by Schael & King (2005) utilizing a different sampling technique (kick sampling using a 250µm mesh kick net that samples the substrate to a greater degree) revealed much higher numbers of Plecoptera, specifically *Aphanicercella,* being sampled from riffles for the Eerste

River than was obtained in this study. Conversely though, far fewer numbers of other taxa were obtained by them, including the trichopteran, *Chimarra* spp. and the mayfly *L. penicillata*. As such each sampling method has its advantages and disadvantages, and in this study a modified method was used that was considered to be practical, time efficient, standardized across all streams, allowed for the successful collection of first-instar nymphs of all species studied, and was shown to provide suitable numbers of each target taxa to produce meaningful life-history plots.

Differences in the numbers of individuals collected between sites of certain species were therefore not considered to be primarily an artefact of sampling (except with the possible exception of June where spate conditions affected sampling protocol) but rather a true representation of differential abundances at each of the sites. This does not discount the patchy distribution of aquatic organisms commonly observed in rivers, both along longitudinal gradients and within river reaches and even within a specific habitat. It is possible therefore that sampling conducted at different locations at each of the study rivers may yield contrasting results to those presented here. But it should be emphasized here that the scope of this study was aimed only at investigating and contrasting the life-histories of these species, in a comparable manner, in a number of rivers incurring different thermal and hydrological regimes. It was not the aim to assess community responses or life-history responses within differing zones of each river. It is for this reason also that the sampling of only the riffle habitat was undertaken in each river.

A problem relating to the sampling period was incurred in that the data collected for the *Aphanicercella* spp and *L. penicillata* covered only portions of each of the two generations found in the samples and as such two separate generations were sampled. It is recommended that future studies that intend to focus on the ecology of these species utilise data from this study, so that sampling commencement date will coincide with the emergence or hatching of the species. This will allow for a more complete picture of growth over time for a single generation to be obtained.

Collection methods implemented for collecting winged adults were found to be suboptimal. While emergence traps were initially designed, constructed and installed at each site for this study, they were soon found to be ineffective for passive sampling and were consequently damaged by high flows (and gale force winds), as well as curious fishermen and baboons. As such these emergence traps were not used any further and are not reported on in this study. Winged adults were not collected for *L. penicillata* during the sampling period. These adults were scarce and only one historical record of collection from the wild exists (Barnard 1932). For other species numbers of individuals collected by sweep netting and active searching yielded low numbers. Additionally the numbers of black wingpad nymphs in samples were generally low and while they roughly indicated the onset of emergence, the relative numbers did not provide the best estimate of the timing of peak emergence.

CHAPTER 5

The role of temperature in egg development

Summary

Data relating to the egg stage are often overlooked in life-history studies of aquatic insects, yet this stage comprises a crucial component of the life-history cycle and is suitable for experimental testing in laboratory conditions. An understanding of the egg stage, including egg development time and hatching success, is necessary for the correct interpretation of total life cycle duration, timing of emergence and hatching, population size and also environmental limits for successful development. Where such information can be collected through experimental testing in the laboratory, in conjunction with field experiments and life-history data it can provide a wealth of fundamental information necessary for the establishment of conservation guidelines, while forming a platform for further studies on aquatic ecosystems. In this chapter, the sublethal effects of water temperature on the egg stage of each of the target genera (specifically egg development time, length of the hatching period and hatching success) were investigated via experiments conducted under controlled environment conditions in the laboratory. Eggs of each genus were collected and monitored daily at six incubation temperature treatments from 5-30° C. Total development time required for 50% of the eggs to hatch, hatch success, length of the hatching period, upper and lower thermal limits for development, as well as thermal reaction norms were calculated for each of the study genera. Photographs of the eggs taken every five days via a digital camera mounted on a compound microscope also allowed for visual comparisons of embryogenesis between the study genera to be made. Water temperature effected the development of eggs of three genera quite differently. Experiments revealed that successful egg development and hatching occurred between 10-20°C for *L. penicillata*, with a high percentage hatch (80%) at 10, 15 and 20° C treatments. For *A. scutata* successful hatching occurred also between 10-20° C but with reduced hatching success (~30%) at 20°C compared to ~80% hatching success at 10 and 15° C treatments. For *C. ambulans*, successful hatching occurred at a wider range of temperatures from 10-25°C but with lower and more variable hatching success at all temperatures (average hatching success ranged from 5-20%). Thermal reaction norms in conjunction with egg hatch parameters showed that *L. penicillata* and particularly *C. ambulans* were warm adapted, while the *Aphanicercella* was cold adapted. Overall, the data presented in this chapter allowed for a more accurate interpretation of the life-history data presented for *L. penicillata*, *A. scutata* and *C. ambulans* from each study site (Chapter 4). Lethal thermal limits for egg development along with the sublethal effects of temperature on hatch success provided valuable information that can be used to inform the establishment of thermal guidelines for the Ecological Reserve. Thermal reaction norms for egg development in conjunction with morphological descriptions of the eggs provided useful insights into the evolutionary history (discussed further in Chapter 7), thermal origins/preferences as well as the oviposition behaviour of each species. Descriptions of first-instar nymphs were also used to confirm the presence of first-instar nymphs in samples collected from the field that informed life-history data (Chapter 4).

5.1 Introduction

Studies concerning the life-histories of aquatic insects commonly employ the regular sampling of larvae over the period of a year, to estimate parameters such as total development time, growth, recruitment/mortality, the number of generations per year (voltinism), emergence date/flight period of adults, the date of oviposition and also the date of hatching (giving an indication of dormancy or diapause period of eggs - a resting stage providing a mechanism for evading unfavourable conditions) (Hynes 1970, Jackson & Sweeney 1995). While this approach is useful in providing a general overview of the factors influencing phenology and voltinism, it is however often, as a result of fairly coarse sampling intervals used (e.g. monthly sampling), only able to provide a rough estimate of the total egg development period, egg dormancy and also subsequent hatch dates of first-instar larvae. In some cases a coarse or irregular sampling interval can lead to an artificial hiatus being observed in life-history data between the emergence of adults and the occurrence of early-instar individuals, which in turn can lead to an inaccurate interpretation of the egg development period.

Additionally, many life-history studies suffer from inaccurate predictions of egg diapause and the inability to verify the number of larval instars. In some cases inadequate sampling methods for smaller early-instar nymphs (e.g. utilising a collecting net with a large mesh size) can lead to a major source of error (Suter & Bishop 1980), where the absence of nymphs from samples can be incorrectly interpreted as egg diapause, when actually there may be immediate hatching. In such scenarios the newly hatched nymphs may in fact hide deep within the substrate escaping normal sampling methods (Brittain 1982), resulting in misleading interpretations of life cycles. Elliott (2009) highlights this fact and suggests that more quantitative data on the egg stage and nymphal instars are required in life-history studies.

The egg development stage, the least understood component of life-history stages (Clifford 1982), is commonly excluded from studies of life-histories (Hynes 1970). Yet knowledge of the egg stage is the key to a more accurate interpretation of life-histories as it plays a major role in a) the regulation of voltinism and synchronisation of life cycles (Brittain 1990), b) the timing and length of recruitment periods of new cohorts (Butler 1984), c) controlling population size and distribution as a result of hatch success under different environmental conditions (Elliott 1988, Brittain & Campbell 1991), d) determining the distribution of closely related species in relation to niche differentiation and resource partitioning (Elliott 1988, Lillehammer *et al.* 1989) and e) the ability to relate the cues for important life-history traits to specific environmental conditions.

Water temperature has been established as being the most important factor determining egg development time (Brittain 1982, Butler 1984, Humpesch 1984, Sweeney 1984, Lillehammer *et al.* 1989, Pritchard *et al.* 1996). Threshold or critical temperatures determine hatching success and in some cases the onset and breaking of diapause as well as the length of the egg incubation period (Elliott 1972, Humpesch 1980a, 1980b, Ward & Stanford 1982, Lillehammer *et al.* 1989). Critical thermal limits for

egg development give a good indication of the time of year suitable for oviposition and egg development and effectively predict a range of temperatures suitable for the growth of nymphs.

Generally lower temperatures result in longer developmental periods and can lead to a longer or extended hatching duration or diapause, while the effect of photoperiod in the majority of studies has been recorded to have no effect on egg development (Humpesch 1980a, 1980b, Brittain 1982, Suter & Bishop 1990). For the Ephemeroptera, the overall range of suitable developmental temperatures for the eggs of several species is wide, extending only between 5 and 10°C in some species, while in others hatching is observed over a broad range from 12°C to temperatures as high as 36°C. Moderate to high percentage hatches are commonly observed at temperatures as high as 15-25°C but Brittain (1982) states that, in general, eggs of Ephemeroptera (data mostly from the Northern Hemisphere) hatch at an optimum between 3°-21°C. Similar ranges for successful hatching of eggs have been proposed for the Plecoptera (also largely from the Northern Hemisphere) (Harper 1973, Brittain 1990). However the eggs of many plecopteran species show low percentage hatches at temperatures of 20°C and above, with high percentage hatches observed at temperatures as low as 2 to 10°C and optima estimated to occur between 10 and 15°C (Brittain 1990). Less information is available for Trichoptera but a few studies on univoltine species from Europe (Wagner 1986, Hildrew & Wagner 1992, Enders & Wagner 1996) have indicated a range of temperatures (between 2-20°C) for successful egg development, similar to those observed for the Ephemeroptera and Plecoptera.

For most aquatic insects, the hatching of eggs at optimum conditions generally occurs over a short period, while for several species, egg diapause has been noted during suboptimal conditions in both summer and winter periods (Harper & Hynes 1970, Hynes 1970, Brittain 1975, Pritchard *et al.* 1996). In many species of aquatic insects the relationship between water temperature and the developmental time requirement to hatching (commonly measured in Degree Days (DD)) can be described by a power equation (Brittain 1982, Humpesch 1980a, 1980b, 1984, Pritchard *et al.* 1996). Using these equations as well as inferences from regression analyses of these equations, namely the slope of the average reaction norm, egg development parameters of species worldwide can be compared, providing an index of adaptation (Pritchard *et al.* 1996). This in turn allows for interpretation within an evolutionary context.

Relatively limited work has been carried out globally on the egg development stage of aquatic insects (Brittain 1982, Butler 1984, Elliott 2009). While more information is available for several Northern Hemisphere species of Ephemeroptera and Plecoptera and to a lesser degree the Trichoptera, information from the Southern Hemisphere is still sparse, with works emanating predominantly from Australia and New Zealand (Brittain 1995). Only a handful of studies have been carried out on the life-histories of aquatic insects in South Africa (King 1982, Palmer 1997, Ractliffe 2009). Moreover an extremely limited number of articles (Begemann 1980, de Moor 1982, de Moor 1989) have been published on egg development times of South African aquatic insects (mostly Simuliidae). Moreover, A

broad overview of seasonality in some Southern Hemisphere aquatic invertebrates was provided by Hart (1985). Chapter 4 of this thesis compared life-histories of the same representative aquatic insects across six rivers in the Western Cape - each of which exhibit differing thermal and hydrological regimes.

Eggs collected from populations of aquatic insect that inhabit sites which exhibit differences in summer temperature (i.e. differences over the period of oviposition and egg development) are likely to experience subtle differences in the DD or time requirement for egg development and subsequent hatch date. In turn these differences in egg development, which may not have been detectable in the life-history data (Chapter 4) owing to the relatively coarse sampling regime utilised in the collection of the data, may be responsible for the site-specific differences observed in the life-history data for the same species.

The aims of the present study are to: 1) present data on egg development times, with notes on the progression of the embryological stages for three species of aquatic insect from South Africa; 2) provide experimental data on egg development times under different temperature treatments to enable a more accurate interpretation of life-history data collected for the same insect species from several different rivers (where each experiences differing thermal and hydrological regimes); 3) to detect thermal limits to egg development and 4) to interpret these egg development data within the context of thermal adaptation and evolution of life-history traits of aquatic insects.

5.2 Methods

5.2.1 Field sites

Eggs of *L. penicillata* were collected from females ovipositing in the middle reaches of Window Stream in the Table Mountain National Park, South Africa (Fig. 5.1). This site was not used for the collection of life-history data but its proximity to the University of Cape Town and the abundance of *L. penillicata* at the site made it a suitable site for the collection of eggs. Window Stream is a first-order perennial mountain stream rising on the south-eastern slopes of Table Mountain. Much of the upper and middle reaches of this stream have a dense canopy cover provided by patches of indigenous forest. The stream, along with several others emanating from the slopes of Table Mountain, is largely groundwater-fed (see Harris *et al.* 1999), is unregulated and undisturbed apart from minor water extraction, and experiences fast cascading flows during the winter rainfall months of June-August (in contrast to very low flows in the summer months of December-February). Over the peak of summer, flow may be limited to the rhithron or subsurface regions which often give rise to and feed several small pools. The substrate is comprised primarily of medium to large cobbles, with patches of bedrock and gravel.

Low numbers of eggs of *C. ambulans* were initially obtained from adults collected from the Window Stream site (see Appendix 5A for details of egg collection) but the eggs used in the experiments were

instead obtained from adults collected from the Elandspad River (Fig. 5.1). See Chapter 2 for a detailed description of the Elandspad River site.

Eggs of *A. scutata* were collected from gravid females in the field from the upper reaches of the Wit River near Wellington, South Africa (Fig. 5.1). See Chapter 2 for a detailed description of the Wit River site.

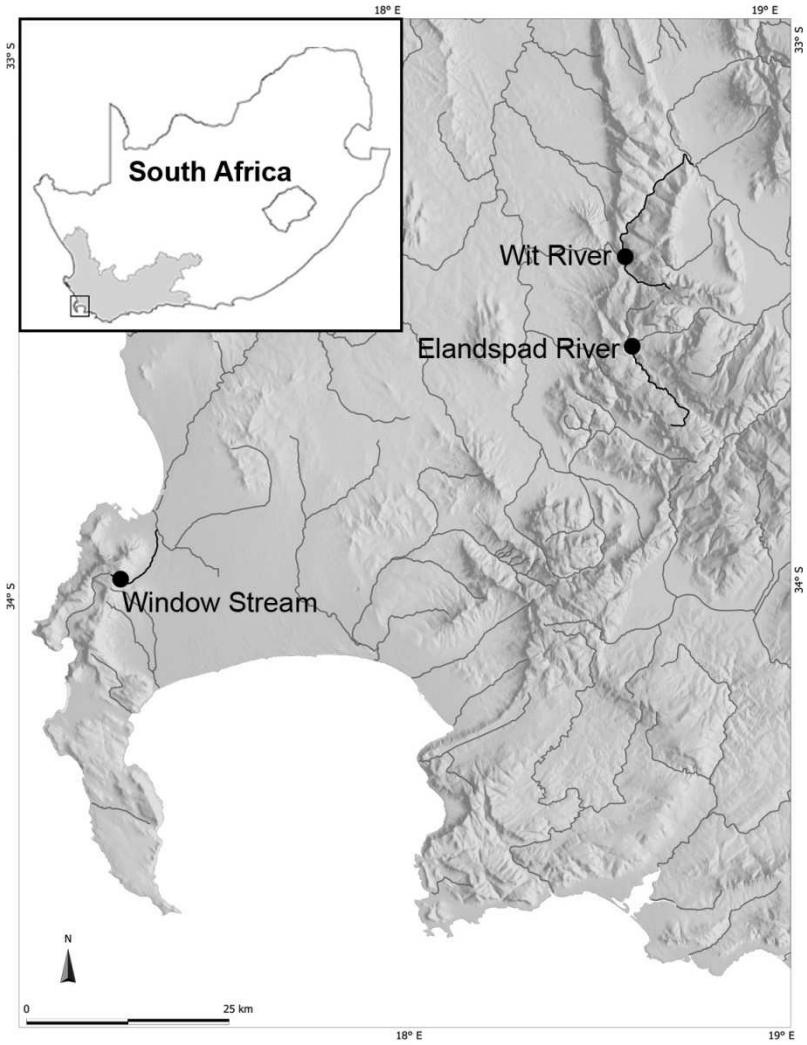


Fig. 5.1. Map showing a portion of the Western Cape of South Africa indicating the location of rivers (black lines) and collection sites (closed black circles) from which adults and eggs of the three study species were obtained.

5.2.2 Egg collection

Eggs of *L. penicillata* (Ephemeroptera), *A. scutata* (Plecoptera) and *C. ambulans* (Trichoptera) were collected from wild-inseminated females using a variety of methods detailed in Appendix 5A. Unsuccessful attempts were also made to obtain fertilised eggs of *L. penicillata*, through artificial fertilisation procedures (see Appendix 5B).

5.2.3 Laboratory procedures

For each species eggs were sorted in the laboratory under a dissecting microscope, counted and separated into 24 petri dishes. These were subjected to constant temperatures of 5, 10, 15, 20, 25 and 30°C with four replicate dishes at each temperature treatment (Fig. 5.2). Petri dishes containing eggs (50-200 for *L. penicillata*, 150-600 for *A. scutata*, and 100-500 for *C. ambulans* – see Appendix 5C, 5D, 5E for details) were filled with approximately 50ml of filtered (60µm) and aerated tap water that had circulated for at least 48h through a reservoir system with a biological filter at the University of Cape Town. The water in the reservoir system was circulated continuously and was only topped up with tap water about once every two weeks, allowing any traces of fluorine and chlorine that might be present in the water to be bubbled off prior to being used to fill petri dishes containing eggs.

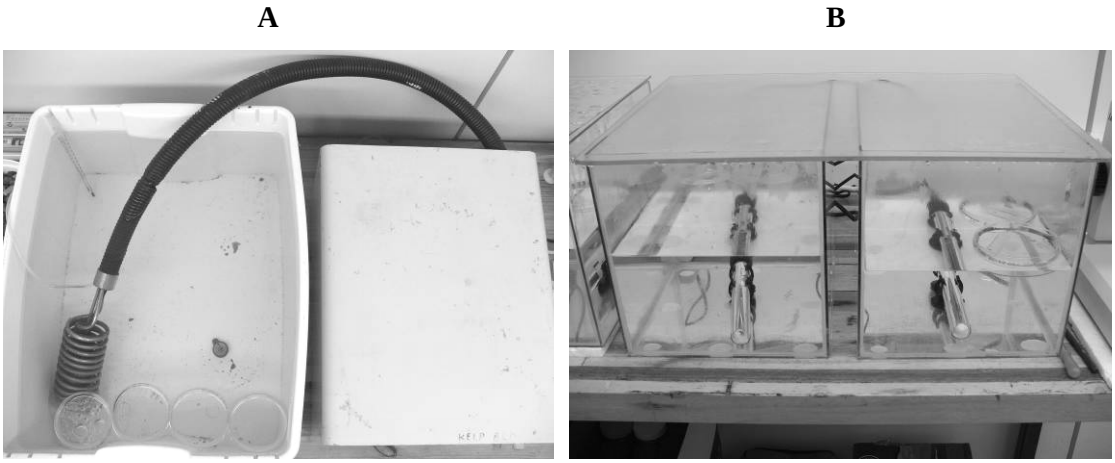


Fig. 5.2. Experimental setup showing A) replicate petri dishes containing fertilized eggs and filtered water floating in water cooled by a Haake glycol water cooler to 5° and B) replicate dishes floating in water heated by calibrated aquarium heaters.

A Controlled Environment room (CE room), air cooled to 12°C (± 1°C) and fitted with full spectrum fluorescent lights on a 12:12 h light cycle was used to maintain a constant water temperature for replicates at approximately 10°C. Within this same CE room, the remaining replicate petri dishes were floated in glass aquaria containing water heated by calibrated aquarium heaters set at temperatures of 15, 20, 25 and 30°C respectively. The water in these tanks was gently circulated using airstones linked to a compressed air supply system to ensure the even distribution of heated water. For the experimental replicates at 5°C, petri dishes were floated in a large crate containing approximately 30 litres of water that was thermostatically controlled by means of a calibrated Haake Glycol Water cooler. The water in this crate was also circulated and aerated using an airstone linked to compressed air supply system. Replicates at 5°C were also housed within the same CE room along with the other replicates.

Prior to the commencement of the experiment, the temperatures of the constant temperature experimental treatments were assessed and calibrated using HOBO® TidbiT® v2 water temperature

loggers (Onset Computer Corporation 2008). Calibration figures for these temperature treatments are shown in Table 5.1. The water in each replicate dish was replaced daily for the duration of the experiment, so as to prevent as far as possible the build-up of unwanted or harmful algae and fungi.

Table 5.1. Summary of water temperature data collected in each of the six constant temperature experimental treatments. Water temperature data were collected at 15 minute intervals for a period of 25 hours for calibration (n = 1500).

	Experimental incubation temperature treatment					
	5°C (Regulated by Haake glycol water cooler)	**10°C (Regulated by air temperature of CE room)**	**15°C (Regulated by calibrated aquarium heaters)**	**20°C (Regulated by calibrated aquarium heaters)**	**25°C (Regulated by calibrated aquarium heaters)**	**30°C (Regulated by calibrated aquarium heaters)**
Mean	5.38	10.22	15.52	20.50	24.74	29.52
Std. dev.	0.06	0.11	0.05	0.05	0.03	0.12
Min.	5.28	9.99	15.41	20.39	24.67	29.27
Max.	5.51	10.39	15.61	20.58	24.79	29.94
Range	0.23	0.39	0.19	0.19	0.12	0.68

Reference photographs as well as length measurements of major and minor axes were recorded for the eggs of each species (n = 100 for each species) prior to being placed in incubation temperature treatment tanks. The measurements of the longest and shortest axes of the eggs were then used to calculate an estimate of the volume, using the formula:

$$V = (4\pi/3)a^2c \approx 4.19a^2c \quad (5.1)$$

where V is volume, a is the equatorial diameter or minor axis and c is the polar diameter or major axis. This equation assumes that the egg is indeed symmetrical and neither dorso-ventrally flattened nor ellipsical.

Eggs were monitored daily for the duration of the experiment. A Leica DM700 digital compound microscope was used to take reference photographs of eggs (~100-200 eggs) every five days from the start of the incubation period until hatching in order to monitor changes in embryogenesis. After the first five days the number of unfertilized eggs (if present) in each replicate dish were identified and counted (unfertilized eggs appeared an opaque milky white colour with no signs of development in contrast to the translucent and more yellow colour of eggs showing signs of development).

The incubation time (in days), cumulative percentage of hatched eggs (excluding unfertilized eggs) as well as length of hatch (in days) and number of hatched eggs per day of hatching were recorded. Additionally, the eggs at each temperature treatment (combined across all four replicates) for each species were also categorized according to the following groupings; unfertilised eggs, eggs that hatched,

eggs that showed no signs of development and did not hatch, eggs that showed signs of only partial development but did not hatch, eggs that showed full development but did not hatch and lastly eggs that showed deformed development. These numbers were recorded as proportions of total number of eggs, including the unfertilised eggs.

After hatching, a small sample (n = 12) of first-instars of each species were photographed and measured with respect to 1) body length and 2) an additional hardened (sclerotised) body part. For *L. penicillata* IOD was measured, for *A. scutata* HCW and for *C. ambulans* HCL[34]. Different sclerotised body parts were measured for each taxon owing to vast differences in morphology preventing the same measure from easily being recorded from each taxon – these were the same measures used to collect life-history information in Chapter 4. The number of antennal and cercal segments in the case of *L. penicillata* and *A. scutata* were counted.

The number of DD > 0°C (i.e. the temperature of the incubation treatment multiplied by incubation time in days) until mean hatch[35] was observed in each replicate in the respective incubation temperature treatments and was recorded for all species. Equations were then calculated that best represented the relationship between these values and the incubation temperature treatments.

5.2.4 Statistical analyses

It is well established in the literature (see Ward and Stanford 1982, Humpesch 1984, Sweeney 1984, Lillehammer *et al.* 1989, Brittain 1990, Pritchard *et al.* 1996) that for aquatic insects that are not within a state of diapause, the time (*D*) in days required to complete certain stages of development (in this case the egg development stage) decreases with increasing temperature (*T*). Note that this relies on the underlying assumption that the temperature remains within the physiological limits of the insect. The relationship of this time requirement to temperature is in most cases well represented by a power function:

$$\boldsymbol{D = aT^{b}} \qquad (5.2)$$

Where *a* is the average development time at 1°C and the exponent *b* is the rate of change in development with temperature changes ($b < 0$ for insect development). Aquatic insects that have evolved different life-history responses in relation to thermal characteristics of their local environments, or as a result of phylogenetic constraints are expected to show a shift in the average reaction norm represented by equation 5.2 (Pritchard *et al.* 1996).

[34] Body parts were measured in order to relate the size of first-instar hatchlings obtained in the laboratory to the smallest individuals of the same species collected in monthly field samples so as to verify the presence of first-instar individuals in the life-history analyses of these species (Chapter 4).
[35] This refers to when 50% of the eggs that successfully hatched at a given temperature treatment had hatched.

In equation 5.2, the time for development (days) can be replaced with a more biologically meaningful measure commonly referred to as Degree Days (DD), essentially a thermal sum relating to physiological time. DD can be explained as the cumulative temperature experienced by an organism above a certain threshold temperature for development (in cases where this developmental threshold is not known for a specific organism, cumulative temperature above 0°C is normally measured). When development time (*D*) above 0°C is measured at a constant temperature treatment, as is the case in this study, the DD or thermal sum is expressed as *DT*. Equation 5.2 therefore becomes:

$$\boldsymbol{DT = aT^{b}} \qquad (5.3)$$

In order to use linear regression to analyse this relation one can employ a logarithmic transformation such that equation 5.3 is now written as:

$$\boldsymbol{\ln(DT) = \ln a + b \ln(T)} \qquad (5.4)$$

Using the above regression method, Pritchard *et al.* (1996) analysed the slope of the population average reaction norm for egg development in 95 species (115 populations) of Plecoptera, Odonata, Ephemeroptera and Diptera. Importantly though, these authors point out that at temperatures where DD or days required to complete development, begin a consistent increase (after having shown a decreasing trend with increasing temperature), such temperatures should be excluded from these analyses as they are considered to be above the thermal maximum of development for these individual species. Following this method, Pritchard *et al.* (1996) used the slope of the reaction norm as an index of adaptation, where positive slopes indicated cold adapted species, negative slopes indicated warm-adapted species and slopes of close to zero indicated generalist species. This method was employed to characterize and compare the egg development of the different orders represented in this study to those elsewhere.

Data relating to several hatch parameters (i.e. the DD requirement to mean hatch, the percentage hatch and the length of the hatch period), for each species across the range of experimental incubation temperature treatments were summarised to obtain mean and standard deviation values. Wilks Shapiro tests for normality and Bartlett tests for homogeneity of variances showed these data to be non-normally distributed, with unequal variances. As such, non- parametric Kruskal Wallis single factor analysis of variance by ranks tests (K-W tests) were performed to detect differences in the hatch parameters for each species across the experimental incubation temperature treatments. In cases where significant results were obtained from K-W tests, ad-hoc Tukey-type multiple comparisons, using the Nemenyi-

Damico-Wolfe-Dunn test (Nemenyi test), were employed[36] to ascertain significant differences in hatch parameters with temperature. All analyses were performed in R (R Core Team 2012).

5.3 Results

5.3.1 Egg morphology

The eggs of the trichopteran *C. ambulans* were the largest (approximately five times the volume of eggs of *L. penicillata*) followed by *A. scutata* and *L. penicillata* respectively (Table 5.2)

Table 5.2. Average length (µm) of the major and minor axes, and volume (mm^3) of freshly oviposited, and fertilised eggs of three species of South African aquatic insect (n = 100).

Species	Ave. length of major axis (± std. dev.)	Ave. length of minor axis (± std. dev.)	Ave. volume (± std. dev.)
Lestagella penicillata	173.20 (4.20)	98.40 (3.13)	$7.03x10^{-3}$ ($0.46x10^{-3}$)
Aphanicercella scutata	195.39 (11.82)	161.12 (16.19)	$21.62x10^{-3}$($5.38x10^{-3}$)
Chimarra ambulans	274.79 (8.66)	186.37 (6.18)	$40.01x10^{-3}$($2.61x10^{-3}$)

Viewed under a compound microscope, the eggs of *L. penicillata* and *C. ambulans* had more of a prolate spheroid shape than those of *A. scutata,* which were almost spherical (Fig. 5.3). With phase contrast lighting, eggs of *L. penicillata* showed no signs of an encapsulating substance. In contrast, a gelatinous substance was clearly visible on the external surface of eggs of both *A. scutata* and *C. ambulans* (Fig. 5.3- B and C). No attachment threads were visible on any of the eggs.

[36]The use of pairwise Wilcoxon rank sum tests with a Bonferroni p-value correction (to control for family wise error rate) was also a potential option for performing multiple comparisons following significant K-W tests. However in view of the trade-offs when using an approach as conservative as the Bonferroni correction (increasing the risk of inducing a Type II error versus reducing the risk of inducing a Type II error versus reducing the risk of inducing a Type I error) a slightly less conservative Tukey-type multiple comparison was employed. This method was deemed more appropriate especially in light of the small sample sizes in question, the presence of tied ranks and the low number of comparisons to be made.

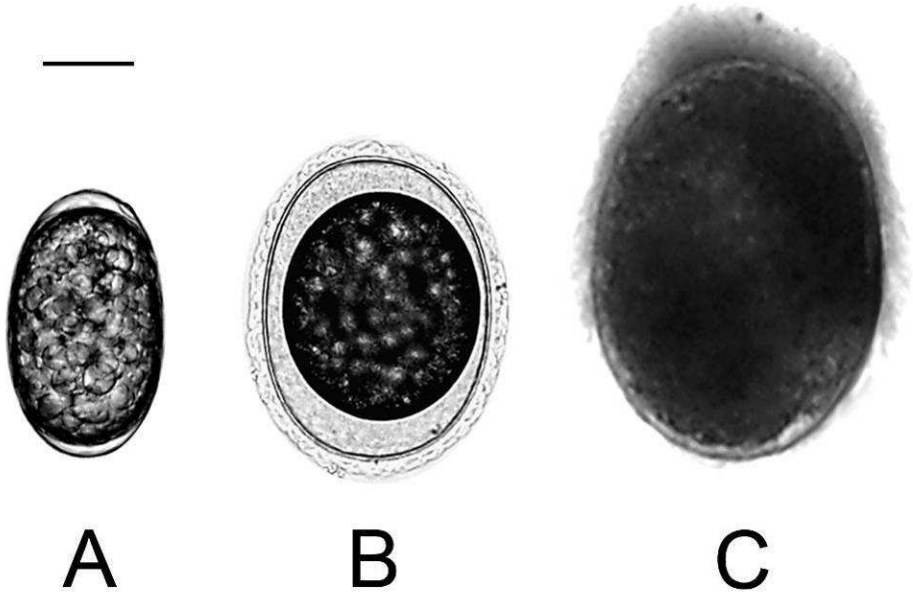


Fig. 5.3. Comparison of the size and morphology of eggs of A) *Lestagella penicillata*, B) *Aphanicercella scutata* and C) *Chimarra ambulans.* Images were taken immediately after oviposition. Scale bar = 60µm.

In the case of *A. scutata,* the hyaline gelatinous membrane that covered the entire surface of the egg appeared to enable the eggs to adhere onto surfaces, including glass and perspex. Following the initial contact with water and subsequent submersion, this substance was observed to increase in size, after which it began to dissolve and eventually disappear - though the egg remained fastened in place. In the eggs of *C. ambulans,* it was observed that a glue-like and more opaque substance was secreted by the female during oviposition. The substance appeared to cover the entire egg mass of several hundred eggs in a single layer forming an oval shape, holding it securely in place.

5.3.2 Embryogenesis

Changes in the visible appearance of the eggs of each species, recorded over the duration of the hatch experiment, for each incubation temperature treatment, are presented in Figs. 5.4.-5.6[37]. The images clearly show the successive stages of embryonic development over time, while giving an approximate indication of the period (in days) from ovipositing to hatching.

[37]In each figure, two representative eggs from each temperature treatment are shown side by side at the specified interval of days since oviposition.

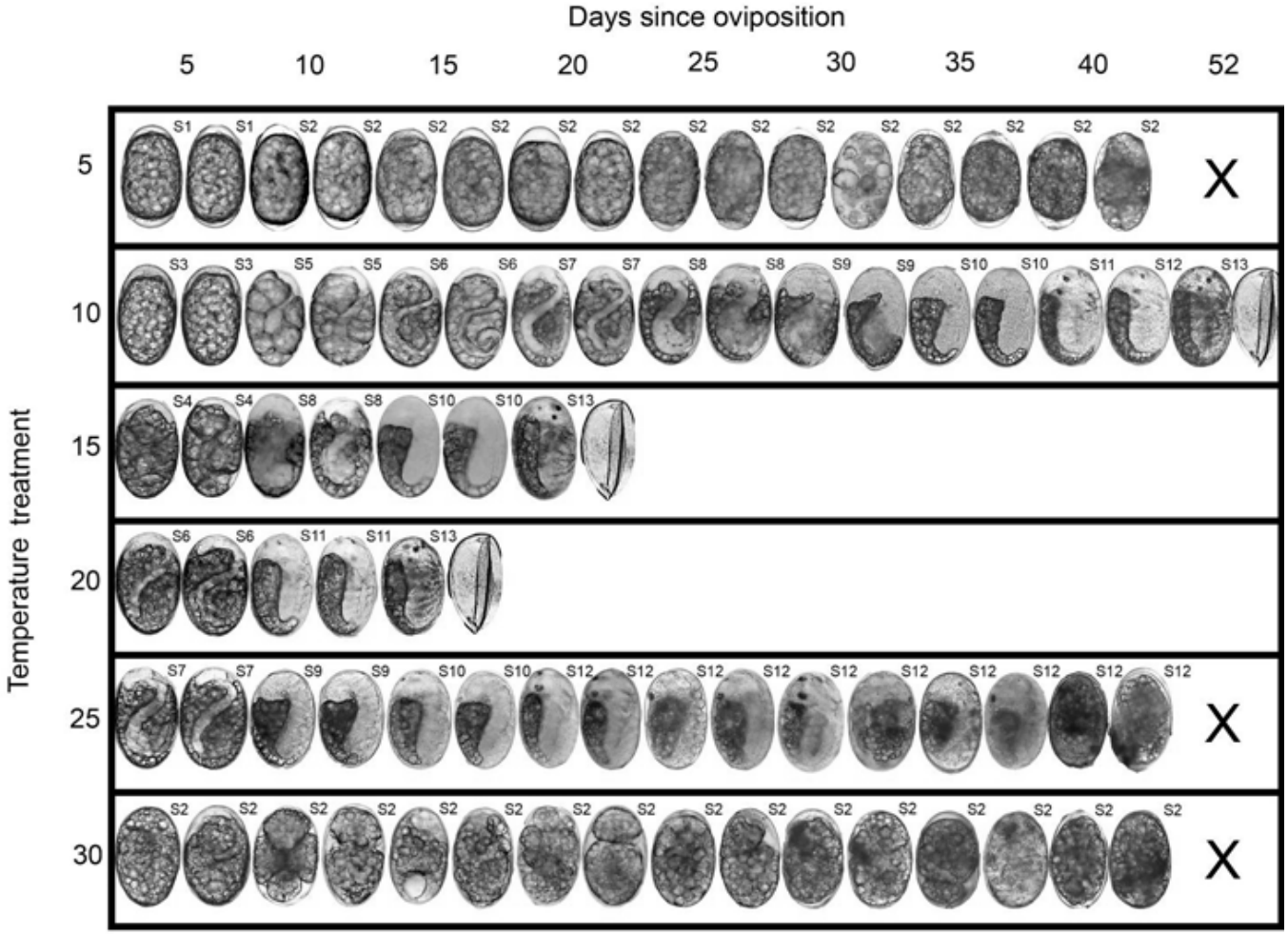


Fig. 5.4. Successive stages of embryological development at different temperature treatments (°C) for *Lestagella penicillata*. Lateral views. S1-S13 denote developmental stages as defined by Tojo & Machida (1997) for *Ephemera japonica* (Ephemeridae). X denotes no recorded hatch and an empty egg shell a successful hatch. The 13 sequential stages (S) are: S1-Egg Cleavage, S2-Blastoderm formation, S3-Germ disc formation, S4-Pear-shaped embryo, S5-Start of invagination of germ band (Anatrepsis I), S6-S-shaped embryo (Anatrepsis III), S7-Longest embryo, S8-Segmentation of embryo, S9-Proctodaeum formation, S10-Revolution (Katatrepsis), S11-Postrevolution I, S12-Postrevolution II and S13-Postrevolution III.

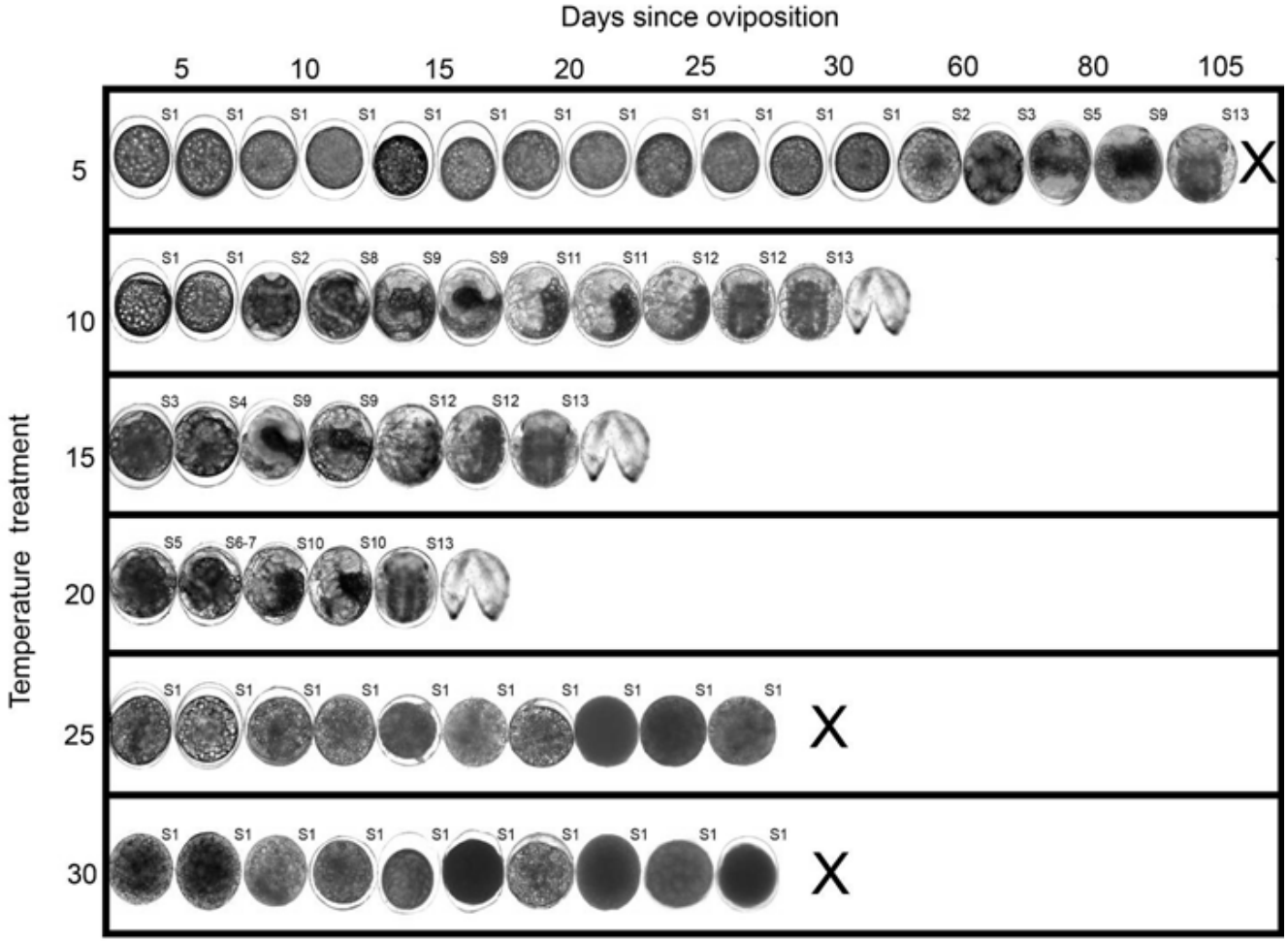


Fig. 5.5. Successive stages of embryological development at different temperature treatments (°C) for *Aphanicercella scutata.* Lateral views. S1-S13 denote developmental stages as defined by Kishimoto & Ando (1985) for *Kamimuria tibialis* (Perlidae). X denotes no recorded hatch and an empty egg shell a successful hatch. The 12 sequential stages (S) are:S1-Germ disc formation. S2-Formation of sac-like embryonic rudiment. S3-Differentiation of protocephalon and protocorm. Pear-shaped embryo. S4-Development of protocephalon and protocorm. Segmentation of embryo. S5 and S6-Embryo sinks in the yolk and increases in length.Caudal loop becomes prominent. S7-S-shaped embryo with segmentation visible. Prominent head lobe. S8-Katatrepsis occurs rapidly. Head of embryo sinks in yolk. S9-When katatrepsis is half finished embryo appears U-shaped and lies entirely on the surface of the egg. S10-Embryo enlarges occupying two thirds of egg volume. Dorsal closure almost complete and egg tooth formation on frons of head. S11-Legs and antennae (nine segments) visible. S12-Dorsal closure complete with full grown embryo occupying entire egg. Pigmentation of eyes.

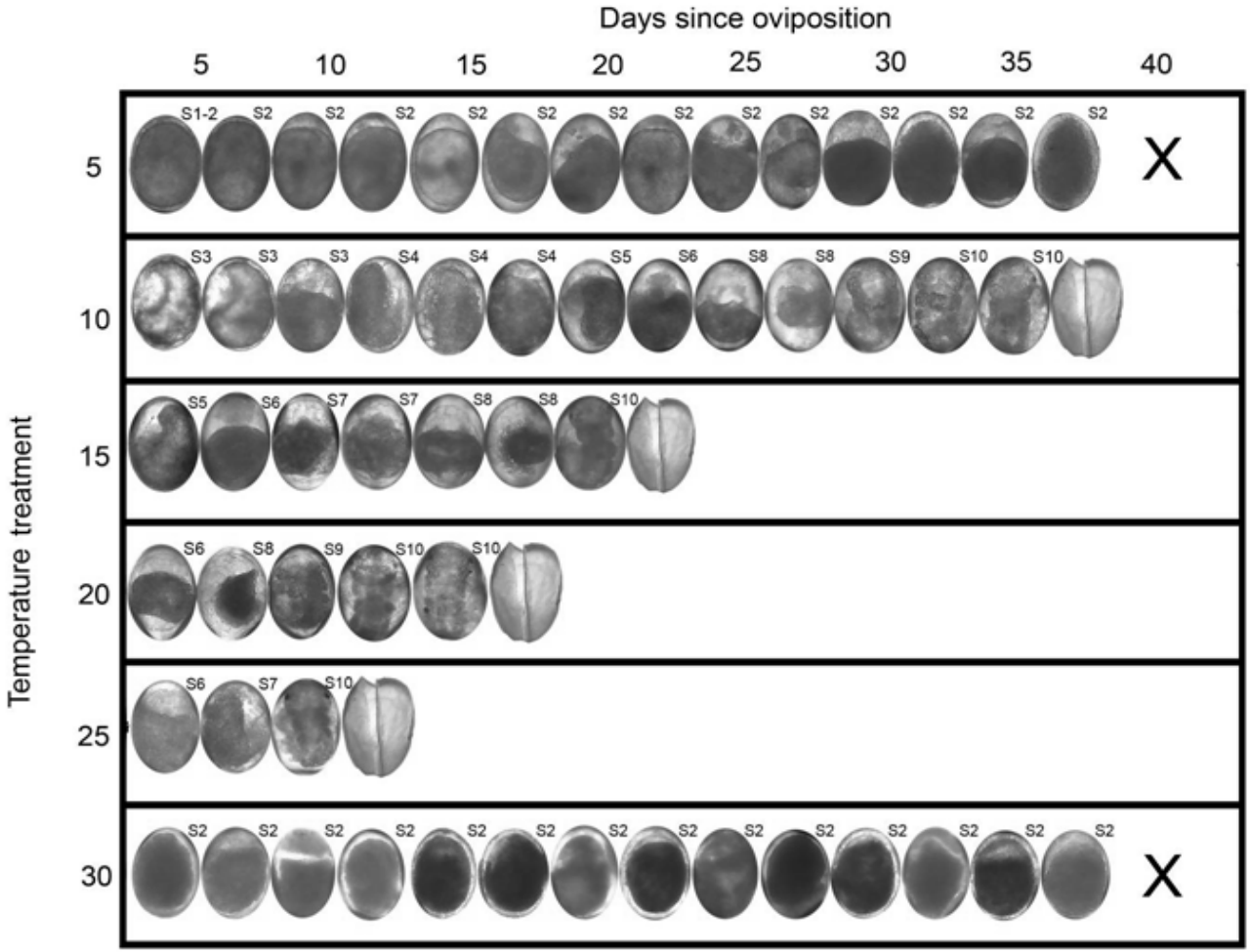


Fig. 5.6. Successive stages of embryological development at different temperature treatments (°C) for *Chimarra ambulans.* Lateral views. S1-S10 denote developmental stages as defined by Miyakawa (1973) for *Stenopsyche griseipennis* (Stenopsychidae). X denotes no recorded hatch and an empty egg shell a successful hatch. The 10 sequential stages (S) are: S1-Maturation, fertilisation and cleavage. S2-Blastoderm formation. S3-Germ disc formation to embryo formation. S4-Formation of protocephalon. Beginning of metamerism in protocorm. S5-Embryo longest in pre-revolution stage. Abdominal segments visible. Formation of telson and appendages. S6-Embryo widest in pre-revolution stage. Abdomen consisting of 10 segments. Appearance of neuropile. Formation of proctodaeum, Beginning of mid-gut epithelium formation. Differentiation of fat body, mid-gut musculature, and sub-oesophageal body. S7-Beginning of morphogenetic movement of cephalo-gnathal region. Formation of central body of tentorium and corpra allata. Differentiation of oenocytes. Formation of genital ridges. S8-Beginning of revolution of embryo. Separate labral rudiments fuse. Eyes and egg-tooth visible. Degeneration of amnion. S9-Completion of embryo revolution. Dorsal closure. Formation of rectal gills. Degeneration of serosa, tentorium, and pleuropodia. Posterior abdominal ganglia move forwards; 8^{th} (final) ganglion to 7^{th} abdominal segment. S10-Completion of head capsule up to hatching.

While detailed descriptions of the successive stages of embryogenesis in *L. penicillata, A. scutata* and *C. ambulans* do not exist, descriptions of the successive embryonic stages for different species within the same representative orders have however been given by Tojo & Machida (1997), Kishimoto & Ando (1985) and Miyakawa (1973)[38].

Tojo & Machida (1997) described the embryogenesis/external embryonic development of the mayfly *Ephemera japonica* McLachlan (Ephemeridae) as consisting of 13 stages. These stages were visible for *L. penicillata* and are indicated in Fig. 5.4. At extreme temperature treatments of 5 and 30°C only the first two stages of egg development occurred after which development ceased. At 25°C development of many eggs progressed rapidly (though at a rate slightly slower than that observed at 20°C) to the penultimate stage (S12) but failed to reach S13 and hatch. Other eggs at 25°C either ceased developing at even earlier stages or were deformed embryos.

For *A. scutata*, the changes in the embryo followed closely the patterns described by Kishimoto & Ando (1985) for a Japanese species of stonefly *Kamimuria tibialis* (Pictet) (Perlidae). In total, 12 stages of embryogenesis were described for *K. tibialis* (Kishimoto & Ando 1985). These stages are mirrored for *A. scutata* (Fig. 5.5). All 12 stages were visible amongst eggs that successfully hatched at treatments of 10, 15 and 20°C. In the 5°C treatment, eggs showed very slow development; a few eggs reached (S12) but then failed to hatch. At treatments of 25 and 30°C no development beyond stage S1 was observed and in most eggs even this initial developmental stage was not visible.

In *C. ambulans*, the successive stages of embryogenesis followed the progression described by Miyakawa (1973) for the trichopteran *Stenopsyche griseipennis* McLachlan (Stenopsychidae). The 10 stages, detailed by Miyakawa (1973), are indicated for *C. ambulans* (Fig. 5.6). Each of the successive developmental stages were observed amongst eggs that successfully hatched at temperature treatments of 10, 15, 20 and 25°C, while no development beyond the early stage S2 was observed at 5 and 30°C.

5.3.3 Egg hatch parameters

Full data sets pertaining to egg hatch parameters for *L. penicillata*, *A. scutata* and *C. ambulans* are presented in tabulated format in Appendices 5C-E respectively. In all species successful hatching of eggs was observed at the 10, 15 and 20°C incubation temperature treatments, with no hatching recorded at the 5 and 30°C treatments. For *C. ambulans* successful hatching also occurred at the 25°C treatment, although this was not observed for *L. penicillata* and *A. scutata*. In all species the incubation period, in days, decreased with increasing temperature.

[38] Not all of the sequential stages described by these relevant authors for the three orders indicated in Figs. 5.4-5-6 can be seen in sequence for a single temperature treatment. This is owing to the difference in the frequency of observations made by these respective authors (hourly) compared to those in this chapter (every 5 days for each species). Thus some stages were missed and are not shown.

Incubation temperature was found to have a significant effect ($p < 0.01$, Kruskal Wallis test) on the DD requirement to mean hatch for all three species (see Tables 5.3, 5.4).

Table 5.3. Summary of the average Degree Days (DD) required to mean hatch at several temperature treatments for three species of South African aquatic insect. No hatch is indicated by –.

Species	5°C	10°C	15°C	20°C	25°C	30°C
Lestagella penicillata	-	646.8	340.3	279.8	-	-
Aphanicercella scutata	-	367.8	387.6	408.5	-	-
Chimarra ambulans	-	345.4	275.9	252.1	263.3	-

Table 5.4. Summary of Kruskal-Wallis test results for differences in Degree Days (DD) to mean hatch, percentage hatch and length of hatch (days) for three species of South African aquatic insect among six different incubation temperature treatments (5, 10, 15, 20, 25, 30).

Species	DD to mean hatch	% Hatch	Length of hatch period
Lestagella penicillata	$\chi^2_{(2, N = 12)} = 9.8$, **$p < 0.01$****	$\chi^2_{(2, N = 12)} = 0.9$, $p > 0.05$	$\chi^2_{(2, N = 12)} = 10.2$, **$p < 0.01$****
Aphanicercella scutata	$\chi^2_{(2, N = 12)} = 9.9$, **$p < 0.01$****	$\chi^2_{(2, N = 12)} = 8$, **$p < 0.05$***	$\chi^2_{(2, N = 12)} = 10.1$, **$p < 0.01$****
Chimarra ambulans	$\chi^2_{(3, N = 14)} = 8.7$, **$p < 0.05$***	$\chi^2_{(3, N = 16)} = 0.2$, $p > 0.05$	$\chi^2_{(3, N = 14)} = 1.1$, $p > 0.05$

The DD requirement to mean hatch for *L. penicillata* and *C. ambulans* decreased with increasing temperatures from 10 to 20°C (Table 5.3, Fig. 5.7). In the 25°C treatment, however, the DD requirement for eggs of *C. ambulans* started increasing (263.3 DD), being on average greater than that observed at in 20°C treatment (252.1 DD). For eggs of *A. scutata*, on the other hand, the DD requirement increased with increasing temperatures (Fig. 5.7). Post-hoc multiple comparisons using the Nemenyi test ($\alpha = 0.01$) revealed that the highest DD requirements to mean hatch were observed at the lowest temperature treatments for *L. penicillata* and *C. ambulans*, while the converse held true for *A. scutata*. For all species significant differences ($p < 0.01$, Nemenyi test) in DD requirements were only observed for temperature treatments at 20°C compared to those at 10°C. The relationship between DD requirement and temperature for each species was best explained using a power equation (see Fig. 5.9). Generally speaking, at 15°C treatments, eggs of each of the species took approximately 20 days to develop and hatch, with signs of first hatch in the 15°C treatment recorded for *L. penicillata*, *A. scutata* and *C. ambulans*, after 19, 17 and 16 days. At 10°C first hatches were observed after 52-63, 33-35 and 31 days for the three species, while at 20°C these figures were 13, 15 and 10-14 days.

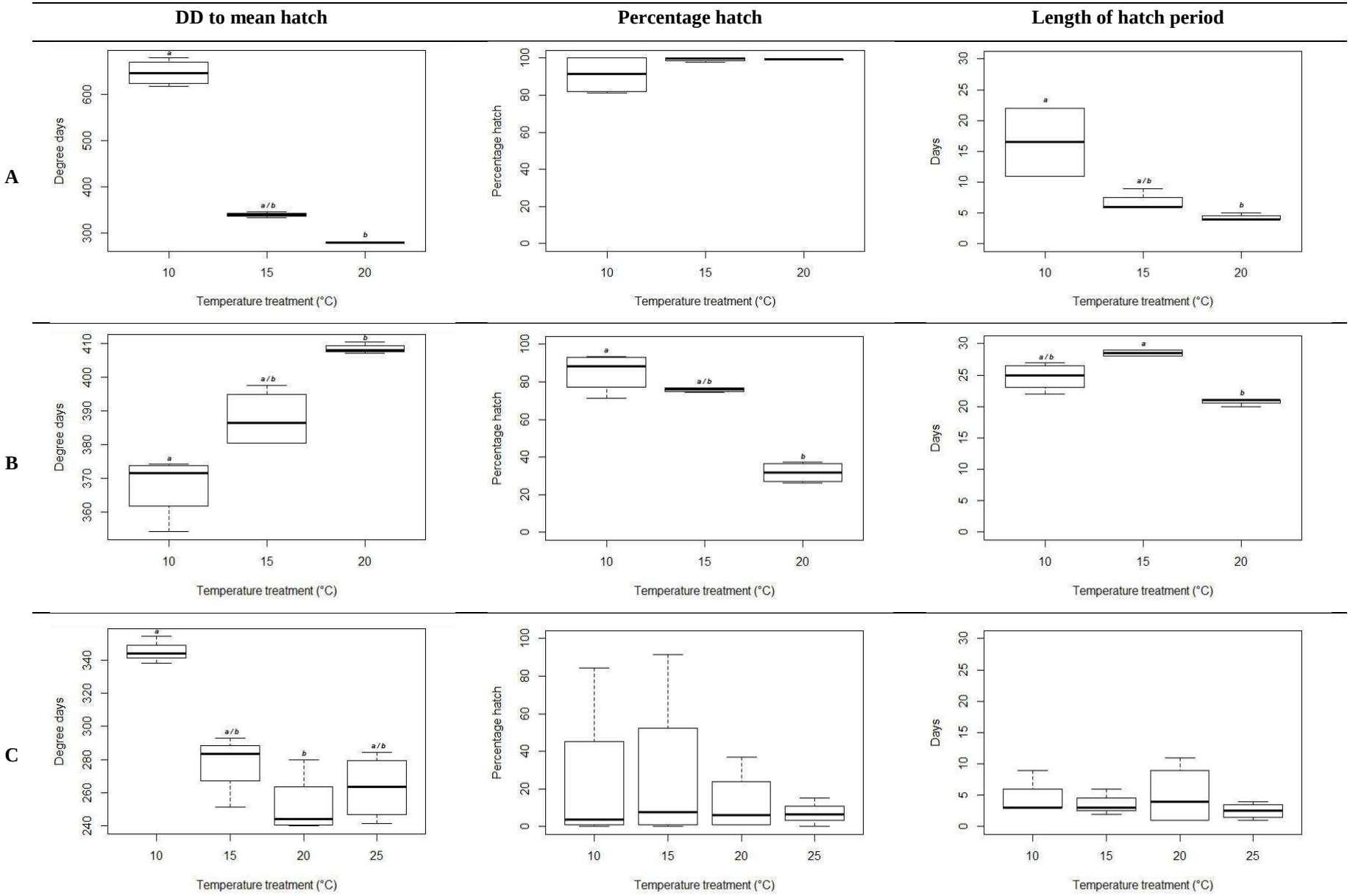


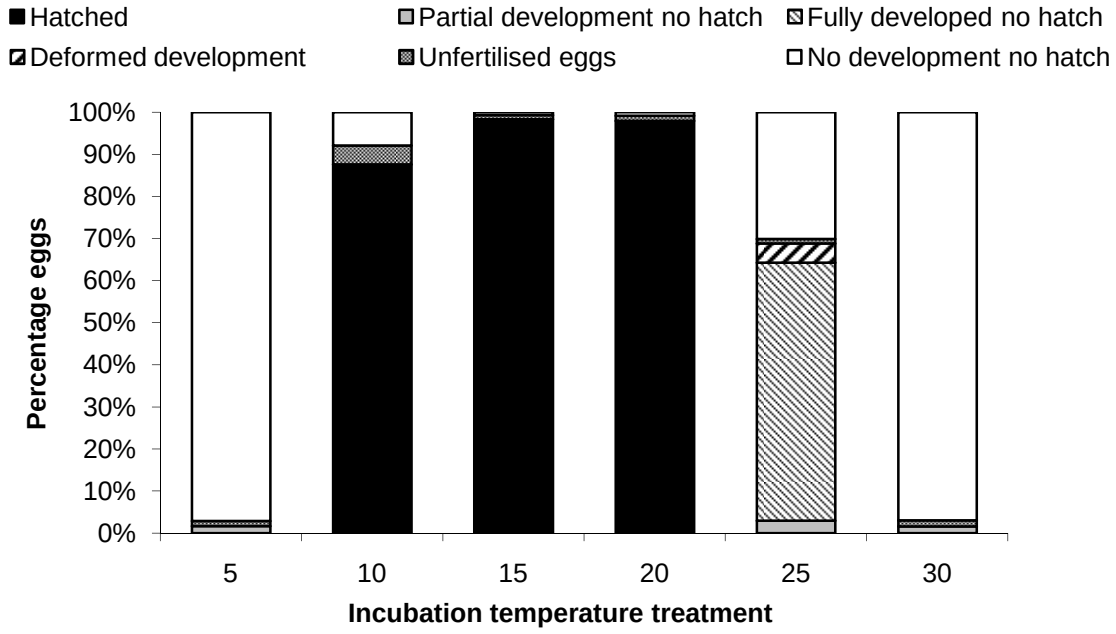
Fig. 5.7. Box plots summarising egg hatch parameter data for A) *Lestagella penicillata*, B) *Aphanicercella scutata* and C) *Chimarra ambulans* at different incubation temperatures. Data were analysed with a Kruskal Wallis test followed by a Nemenyi test (*a* and *b* indicate significantly different groups $p < 0.01$, $n = 4$ for each group while *a / b* denotes a group that is not significantly different from a or b). Note different scales on the y-axes for Degree Days (DD) to hatch plots

The percentage hatch of eggs[39] was significantly affected ($p < 0.05$, Kruskal Wallis test) by incubation temperature only for *A. scutata* (Table 5.4) which exhibited high percentage hatches (75-95%) at 10 and 15°C with a considerably lower % hatch (approximately 32%) recorded at 20°C (Fig. 5.7). Percentage hatch in this species thus appeared to roughly decrease with increasing temperature. No multiple comparisons tests for percentage hatch were performed for *L. penicillata* or *C. ambulans*, both of which showed no significant differences in the Kruskal Wallis test (Table 5.4). *A. scutata* revealed a highly significant difference ($p < 0.01$, Nemenyi test) in the percentage hatch between the 20 and 10°C temperature treatments, however with no significant differences being observed for 10 vs. 15°C or 20 vs. 15°C (Fig. 5.7).

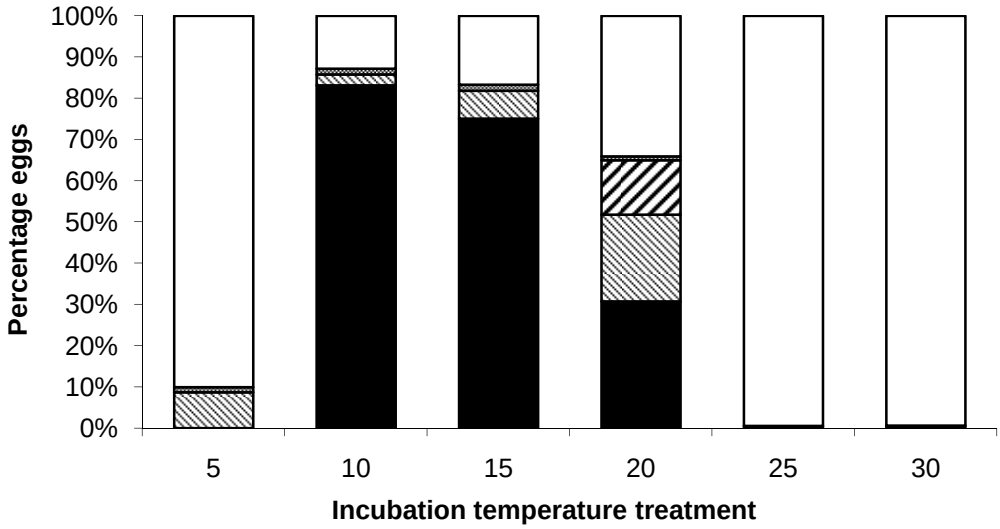
Additionally, the length of the hatch period (time from the appearance of the first hatchling to the last hatchling) was shown to be significantly affected ($p < 0.05$, Kruskal Wallis test) only by experimental incubation temperature treatment for *L. penicillata* and *A. scutata* (Table 5.4). In the case of *L. penicillata* the hatch period appeared to be less variable and more synchronous (shorter) with increasing temperatures, while for *A. scutata* the greatest variability in the length of the hatch period (ranging from 22 to 27 days) was observed at 10°C (Fig. 5.7). The most synchronous hatch in *A. scutata* was observed at 20°C (average length of 21 days), followed by 10°C (average length of 25 days) with the longest and least variable hatch period observed at 15°C (average length of approximately 28 days) (Fig. 5.7). The length of the hatch period for *C. ambulans*, while generally short and synchronous, was highly variable and showed no trends in relation to incubation temperature treatment (Fig. 5.7). Multiple comparisons revealed a significantly ($p < 0.01$, Nemenyi test) longer length of hatch for *L. penicillata* at the 10°C treatment when compared to the 20°C treatment, with no significant differences observed between treatments at 20 vs. 15°C and 10 vs. 15°C (Fig. 5.7). *A. scutata* showed a highly significant ($p < 0.01$, Nemenyi test) difference in the length of the hatch period between treatments at 15 and 20°C, however with no significant differences observed among treatments at 20 vs. 10°C and 10 vs. 15°C (Fig. 5.7).

The proportion of unfertilised eggs in each species among temperature treatments was consistent. The highest proportion of unfertilised eggs were observed in *C. ambulans* (ranging from approximately 1 to 8% among treatments), while low to negligible proportions (0-4%) were observed for *L. penicillata* and *A. scutata* among treatments (Fig. 5.8).

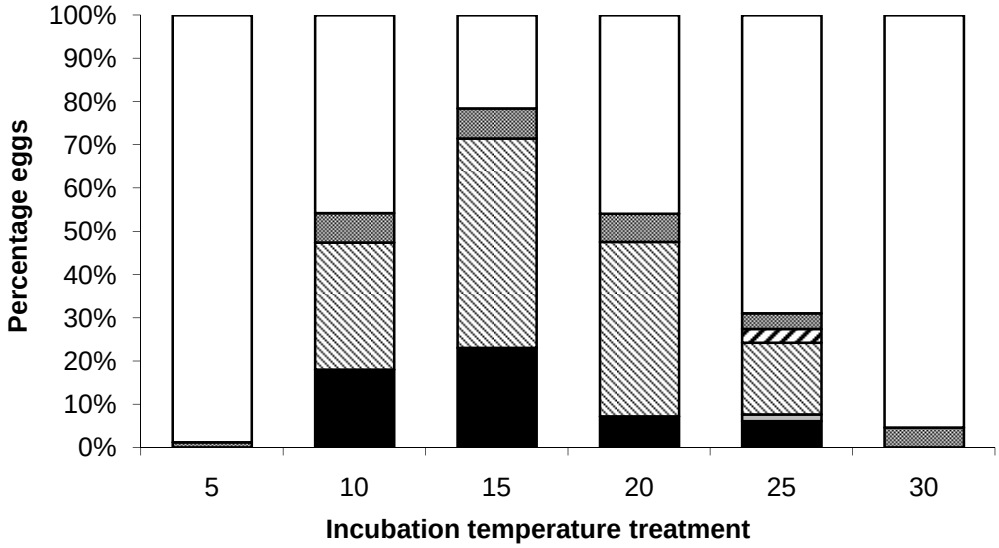
[39]The figures of percentage hatch presented here for each species are calculated as the proportion of the total eggs, excluding unfertilised eggs, which successfully hatched.



A) Lestagella penicillata



B) Aphanicercella scutata



C) *Chimarra ambulans*

Fig. 5.8. The percentage of the eggs of three species of South African aquatic insect categorized according to their final developmental outcome at several incubation temperatures (°C).

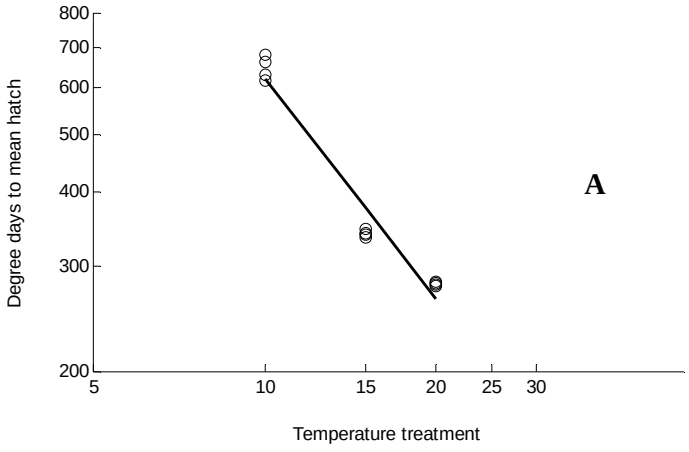
For *C. ambulans* less than half of the eggs that showed signs of full development at each temperature (10, 15, 20 and 25°C) actually hatched (Fig. 5.8). In *L. penicillata* a high percentage hatch failure was observed only in the 25°C treatment, where almost 65% of the total eggs showed signs of full development (to stage S13) but then failed to hatch (Figs. 5.4, 5.8). Additionally, a small proportion of eggs (~5%), of both *L. penicillata* and *C. ambulans* at 25°C also showed signs of deformed development (Fig. 5.8). In these instances the resulting embryos were distorted, disproportionate or considerably smaller than those observed in the treatments at which successful hatches were observed. *A. scutata* showed signs of fully developed yet unhatched eggs as well as deformed embryos at 20°C (Fig. 5.8).

The proportion of fully developed but unhatched eggs showed an increasing trend with increasing temperature from 10 to 20°C (Fig. 5.8). However, *A. scutata* was also the only species for which fully developed but unhatched eggs occurred in the 5°C treatment (Figs. 5.5, 5.8). Instances of partial egg development and no hatch were low for each species, among all the treatments and appeared to occur only at extreme temperatures (5, 25 and 30°C) (Figs. 5.4-5.6, Fig. 5.8).

5.3.4 Thermal reaction norms: degree day requirement to mean hatch

Power equations best explained the relationship between DD requirement to mean hatch and temperature, and in all species highly significant ($p < 0.01$) fits were observed (Fig. 5.9). Linear fits of the log-transformed data revealed negative regression gradients (referred to as reaction norms) of -1.23 and -0.46 for *L. penicillata* and *C. ambulans* respectively (Fig. 5.9A, C), while *A. scutata* (Fig. 5.9B) exhibited a positive regression slope of 0.15 (Fig. 5.9).

Following the method of Pritchard *et al.* (1996), the data relating DD requirement to hatch for *C. ambulans* at the 25°C treatment were excluded from the analyses of thermal reaction norms. This is because all replicates at this temperature showed an increase in the DD requirement to hatch when compared to replicates at 10, 15 and 20°C.



Power equation:

$y = 10592x^{-1.23256}$

($R^2 = 0.96$, $p < 0.01$)

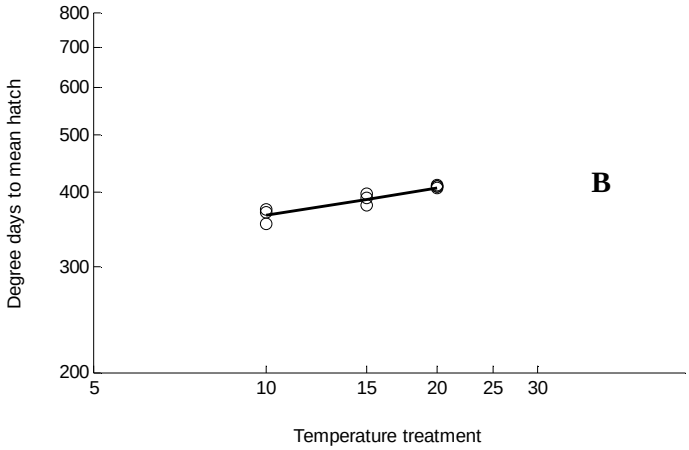
Log transformed equation:

$y = -1.23256x + 9.267817$

($R^2 = 0.96$, $p < 0.01$)

Reaction norm:

($b = -1.23256$)



Power equation:

$y = 259.44x^{0.150361}$

($R^2 = 0.86$, $p < 0.01$)

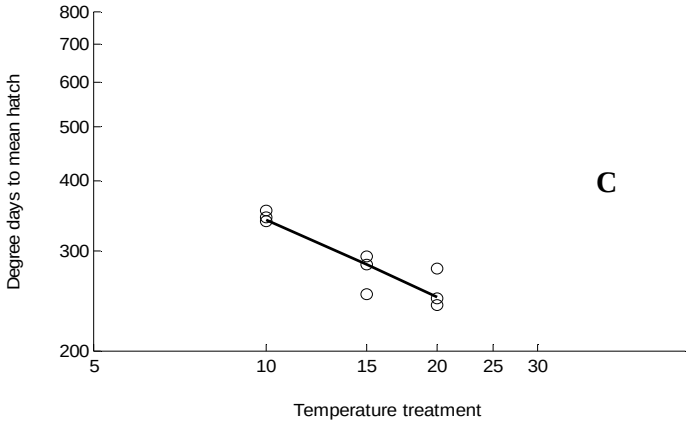
Log transformed equation:

$y = 0.150361x + 5.558521$

($R^2 = 0.86$, $p < 0.01$)

Reaction norm:

($b = 0.150361$)



Power equation:

$y = 978.97x^{-0.45776}$

($R^2 = 0.84$, $p < 0.01$)

Log transformed equation:

$y = -0.45776x + 6.886505$

($R^2 = 0.84$, $p < 0.01$)

Reaction norm:

($b = -0.45776$)

Fig. 5.9. Thermal reaction norms (Log-transformed relationship between Degree Days requirement to mean hatch) at different temperature treatments (°C) calculated for three species of South African aquatic insect A) *Lestagella penicillata*, B) *Aphanicercella scutata* and C) *Chimarra ambulans*. Untransformed power equations defining the same relationship are given for each species.

5.3.5 First-instar hatchlings

Newly hatched larvae of *C. ambulans* were on average the largest of the three species, followed by *A. scutata* and *L. penicillata* (Table 5.5, Fig. 5.10). While only a small number of first-instar hatchlings (n = 12) of each species were measured, recorded values of the coefficient of variation (C.V.) for each of the body part measurements were generally low, indicating a low percentage of variation among these individuals and thus a reasonable reflection of size measurements. Mean values (± std. dev.) and C.V. for specific measurements, as well as body length, of first-instar hatchlings of the three species are indicated in Table 5.5.

Table 5.5. Size of first-instar hatchlings (n = 12) of the mayfly *Lestagella penicillata*, the stonefly *Aphanicercella scutata*, and the caddisfly *Chimarra ambulans.* Mean values (mm) are presented in bold face with standard deviation in parentheses and C.V. (coefficient of variation) values as percentages.

Species	Body length (BL)	Interocular distance (IOD)	Head capsule width (HCW)	Head capsule length (HCL)
Lestagella penicillata	**0.36** (± 0.045) 12.4%	**0.08** (± 0.001) 1.8%	-	-
Aphanicercella scutata	**0.56** (± 0.052) 9.3%	-	**0.14** (± 0.001) 1.0%	-
Chimarra ambulans	**1.11** (± 0.067) 6.0%	-	-	**0.18** (± 0.005) 2.7%

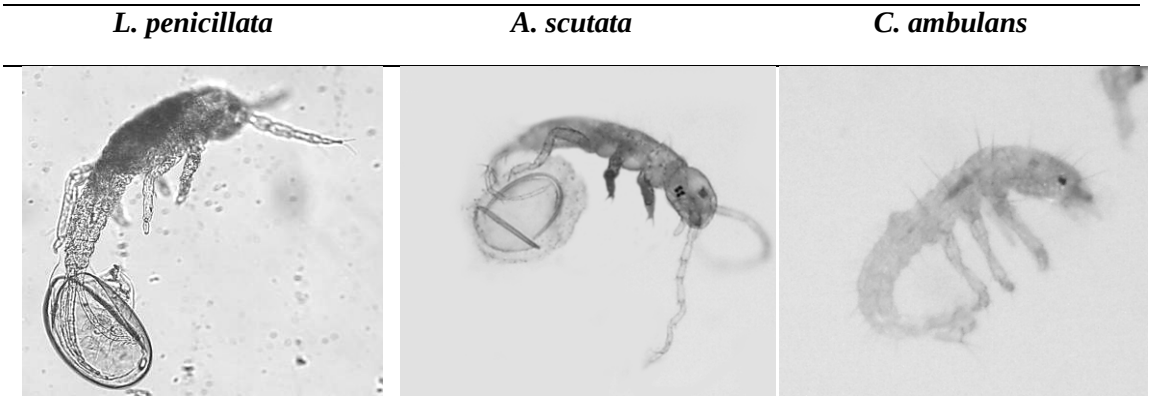


Fig. 5.10. Newly hatched first-instar larvae of *Lestagella penicillata*, *Aphanicercella scutata* and *Chimarra ambulans.*

Further analysis also revealed the existence of a clear positive trend between average egg volume and the average body length of first-instar hatchlings when comparing each of the species (Fig. 5.11). Measurements of the specific hardened body parts of hatchlings of each species also confirmed the presence of first-instar hatchlings in samples collected monthly for life-history analyses (see Chapter 4). First-instar hatchlings of *L. penicillata* had five antennal segments (including the scape and pedicel), and four cercal segments. Hatchlings of *A. scutata* however, had nine antennal segments (including the

scape and pedicel) and four cercal segments. Antennal counts were not made for *C. ambulans* as the number of larval instars (which can be determined by antennal segment counts) was established in Chapter 4.

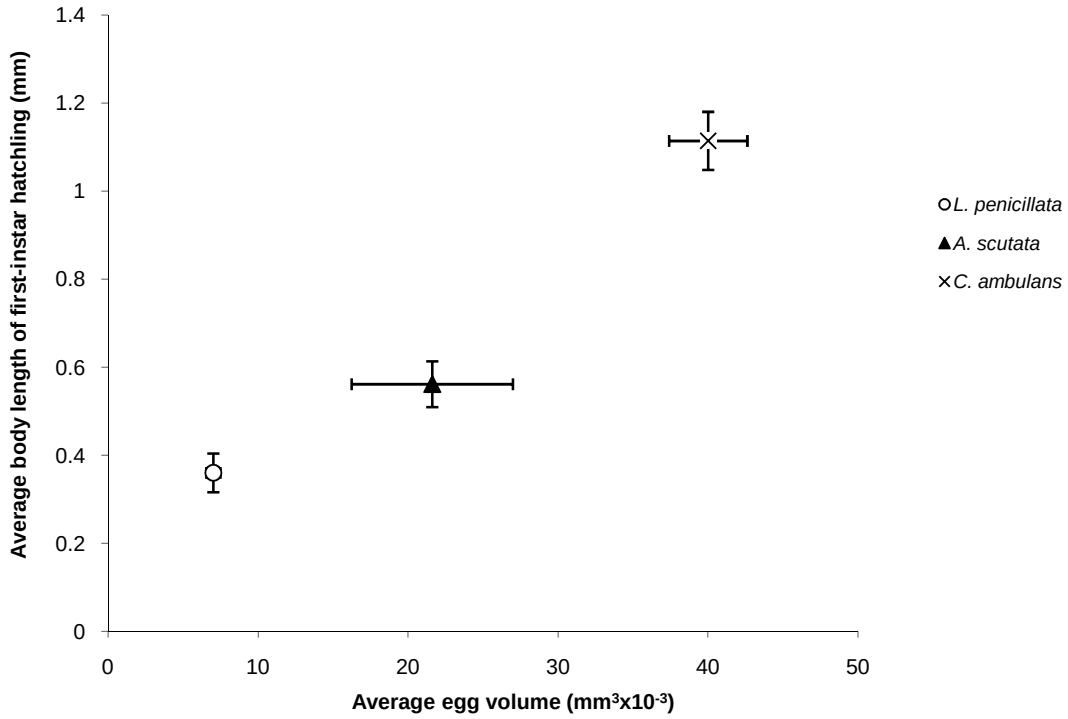


Fig. 5.11. Comparison of average body length of first-instar hatchlings (n = 12) and average egg size (n = 100) for the mayfly *Lestagella penicillata* (crosses), the stonefly *Aphanicercella scutata* (triangles) and the caddisfly *Chimarra ambulans* (open circles). Error bars indicate standard deviation.

5.4 Discussion

The timing of emergence and oviposition as well as the duration of egg development, the hatching period and overall hatching success are crucial factors in the life-histories of aquatic insects. Late emergence and oviposition may result in eggs being exposed to suboptimal or potentially lethal conditions for egg development (thermal extremes, floods), leading to delayed hatching or hatching failure. This is highlighted by work conducted by Encalada & Peckarsky (2006) who showed that high stream discharge early in the emergence season covered suitable potential oviposition sites and thereby strongly delayed the onset of oviposition in Baetis bicaudatus (a species which exhibits specialised oviposition behaviour or non-random selection of sites for oviposition)- a process which the authors in turn suggested could have strongly affected recruitment. Similarly, premature or delayed hatching may result in high mortality of the new generation of early-instar nymphs if they too are exposed to suboptimal or potentially lethal conditions for larval development (predation, thermal extremes, floods, lack of food) (see for example Peckarsky *et al.* 2000, 2001). Such adverse impacts affect the

recruitment and thus the numbers of survivors that will successfully mate and oviposit in the following season – thereby ultimately reducing the potential success of a species (Giberson & Rosenberg 1992).

The collection of egg development data is thus essential for the interpretation of life-history data, specifically with regard to the identification and separation of growth cohorts – which in turn affects estimations of growth rates, mortality rates and also production (Humpesch 1980a, 1980b). Where such data are collected through laboratory experiments in which certain environmental variables like temperature can be manipulated, they also provide valuable information regarding optimal temperatures for development as well as both lethal and sublethal thermal limits.

Egg hatching success and egg development period are directly related to water temperature for most aquatic insects (Brittain 1990, Lillehammer *et al.* 1989, Suter & Bishop 1990, Pritchard *et al.* 1996). Additionally the relationship between water temperature and both oviposition period and egg development, in turn, determines the timing of hatching and when young larvae will be present in the habitat (Knispel *et al.* 2006). Flow however can result in physical damage or displacement of eggs without attachment apparatus. Low flows coupled with high temperatures as well as extreme high flows coupled with low temperatures, in conjunction with the timing of the shifts between these states (regime shifts), thus all act as seasonal time constraints affecting the timing of life-history traits (especially oviposition and hatching). Changes in photoperiod can often act as early indications for these regime shifts (Chapter 2) and can be used by aquatic insects as cues for life-history traits. Species have evolved specific adaptations (egg morphology: attachment apparatus/threads; nymphal morphology: dorso-ventral flattening; pupation) to either cope with these environmental constraints enabling certain niche environments to be occupied. Alternatively, they have evolved life-histories that avoid unfavourable conditions all together - e.g. through emergence or through the egg stage (where conditions are unfavourable to the nymph or adult).

Aquatic insects that occur In high altitude Northern Hemisphere Alpine streams that drain glaciers have adapted to withstand high flows during the warmer more favourable parts of the year as a result of elevated spring temperatures resulting in snow melt (Brown *et al.* 2006, Milner *et al.* 2009). In non-glacial catchments elsewhere in the Northern Hemisphere, harsh winter conditions can result in streams and rivers in exposed/non wooded areas (see Hrachowitz *et al.* 2010 and Imholt *et al.* 2010) freezing over - causing either the larvae or the eggs of aquatic insects to enter into a period of diapause (Danks 2007). Unlike these examples from the Northern Hemisphere, in parts of the Southern Hemisphere like the Western Cape of South Africa, high flows prevail in winter when rainfall is highest and temperatures are coldest. Conversely summer is dominated by high temperatures and low flows. These Mediterranean-type climatic conditions represent a unique array of seasonal time constraints for aquatic insects. While milder winters without regular freezing conditions enable aquatic insects to continue growing, the insects must be able to withstand high flows. When warmer conditions begin to prevail

they must be able to withstand low flows (reduced oxygenation) and high temperatures (sometimes exceeding lethal limits). While the rivers studied in this thesis all experience these general seasonal constraints, they differ somewhat in the timing and also their capacity to buffer environmental extremes (e.g. through groundwater/shading) as evidenced by differences in the timing and magnitude of regime shifts (Chapter 2). These differences in turn affect the timing of life-history traits, most noticeably those associated with oviposition, emergence and hatching (see Chapter 4). The information presented here can thus be used to elucidate these life-history differences.

5.4.1 Insights into oviposition site choice from egg morphology

Hatching success, larval growth, recruitment and fitness of the offspring are all affected by the suitability of the site selected for oviposition by adult female insects (Rausher 1979, Resetarits 1996, Hoffmann & Resh 2003). As such, strong selection pressure should exist for the ability to choose specific sites for oviposition based on the best conditions for larval development (Rausher 1979, Resetarits 1996). Inherently though, the characteristics of the eggs themselves represent adaptations to the specific conditions present in the sites available to the female for oviposition (Resetarits 1996). Observations of the morphology of eggs of aquatic insects thus provide useful information regarding the evolution of life-history traits, environmental conditions suitable for embryonic development and even the seasonal timing of life-history events (e.g. environmental conditions at times for oviposition and hatching).

The external morphological characteristics of the eggs of all three species show evidence of distinctive ecological specialisation and adaptation to local environmental conditions. Additionally, field observations suggested that the oviposition site selection by females of all three species were non-random and discriminatory, ensuring the oviposition of eggs into suitable instream conditions for the development of both the embryo and the nymphs. Females that oviposit eggs in conditions that are close enough to optimal conditions such that their offspring survive would be at a selective advantage. However, further quantitative studies on oviposition site selection, such as those conducted by (Resetarits 1996, Hoffman & Resh 2003), would be needed to confirm these notions.

Lestagella penicillata

The relatively small eggs of *L. penicillata* showed no external signs of a protective membrane or attachment apparatus/threads, but Scanning Electron Micrographs (SEM) would be required to confirm these observations (see Fig. 5.3). The average length of the eggs (length = ~173µm and volume = ~7mm^{3}x10^3) is within the range reported by Brittain (1982) for the large majority of mayflies (between 150-200µm) (Table 5.2). *L. penicillata* utilised small pools, located upstream and gently fed by subsurface flows, for oviposition during a narrow time period in early summer, when moderate temperatures and low flow conditions prevailed (see Appendix 5A). Similar oviposition flight

behaviour to that of *L. penicillata* has been reported for most mayflies including the Ephemeridae, *Heptageniidae* and *Leptophlebiidae* (Brittain 1982), while Suter & Bishop (1990) have reported similar oviposition site selection flights in four species of south Australian mayflies (*Baetis soror*, *Nousia fuscula*, *N. inconspicua* and *Atalophlebia australis*).

The morphological characteristics of the eggs, *viz.* absence of attachment apparatus, thus appear to be well suited to the environmental conditions of the favoured oviposition sites, as sites experiencing faster flow conditions could result in a) the eggs being carried considerably further downstream to potentially unsuitable sites for development (e.g. sites exhibiting higher water temperatures) and b) the eggs being physically damaged as a result of more turbulent flows. The small size and lack of attachment apparatus of eggs of *L. penicillata* are characteristics allowing eggs of this species to be naturally washed, via a gentle current, into either the rhithron or into deeper interstitial spaces within the substrate. This could be advantageous, as these areas would provide more protected "nursery" areas for egg development and are less susceptible to drying out over the warmer summer months.

Aphanicercella scutata

Eggs of *A. scutata*, were slightly larger (length = ~195µm and volume = ~22mm^{3}x10^3) (see Table 5.2) than those of *L. penicillata,* and fell within the range of volumes reported for other species of Plecoptera *viz.* 2.0-38.5mm^{3}x10^3 (Gillooly & Dodson 2001). The appearance of the gelatinous substance on the eggs of *A. scutata*, as described, is consistent with the findings of Brittain (1973) and many others (e.g. Jewett 1960, Khoo 1964, Hynes 1976, Rościszewska 1991, Merritt & Cummins 1996) for other stonefly species. These authors suggest that this substance is in fact a gelatinous membrane which swells upon contact with water (through its absorptive properties), forming a sticky coating, which not only serves to anchor the eggs to the substrate but also provides protection to the eggs from physical injury and desiccation. Kishimoto & Ando (1985) reported similar expansion and eventual disappearance of the gelatinous membrane coating the eggs of the stonefly *Kamimuria tibialis*.

The presence of a gelatinous membrane on the eggs of *A. scutata* enables this species to oviposit eggs at a broad range of flow conditions within a suitable range of temperatures. Oviposition was observed during August, when high flows and low water temperatures still prevailed in all streams. Females freely oviposited extruding egg masses into the slightly slower flowing sections of the stream nearer the banks (see Appendix 5A).

Chimarra ambulans

The caddisfly *C. ambulans* was found to have the largest eggs of the three species, with an average size (length = ~275µm and volume = ~40mm^{3}x10^{-3}) (see Table 5.2) well within a range (100-400µm in length) reported for several other species of Trichoptera in Merritt & Cummins (1996). Oviposition occurred over a long period extending from mid-spring to late-autumn. The glue-like substance

covering eggs of this species (Fig. 5.3) is excreted by cement glands of the female during oviposition (Dodson 1935) and fixes the eggs to the substrate. Miyakawa (1973) reported the presence of a similar substance on the eggs of another trichopteran species, *Stenopsyche griseipennis*. The adhesive characteristic of this glue-like substance, similar to that of *A. scutata* eggs, allows the eggs of *C. ambulans* to be oviposited at sites incurring a wide range of flow conditions that persist from spring through to summer (both spates and baseflow conditions as well as cold and warm temperatures). This said, based on preliminary observations,[40] the eggs of this species do not appear to be desiccation resistant, unlike observations made for eggs of many stoneflies (see Khoo 1964, Hynes 1970). Females of *C. ambulans*, while clinging to the substrate surface, will enter the water and continue to crawl to the submerged underside of large partially exposed stones in riffles to oviposit eggs. Such stones were repeatedly observed as sites for oviposition by females. Eggs are thus oviposited in sites optimal for both egg development and larval growth, as a) the underside of partially exposed stones in gentle current during spring and summer are unlikely to dry out, b) these positions are not easily accessed predators and c) as flows increase during autumn, the underside of stones are not as exposed as the upper surfaces, presenting a better location for the development and growth of these filter feeders. Hoffman & Resh (2003) also point out that a) such stones (moderate – large cobbles) are stable and not easily displaced in high flows (barring floods), b) eggs are not easily dislodged as velocity and shear stress are reduced on the underside of stones, c) substrate and interstitial spaces surrounding the underside of stones provide refugia for newly hatched larvae in high flows, d) siltation and algae growth, both detrimental to egg development, are reduced on the underside of such stones, and e) stones in riffles offer higher oxygen concentrations to developing eggs and larvae.

5.4.2 Embryogenesis: the finer details of egg development limits

The more basal winged insects (Odonata, Ephemeroptera and Plecoptera) share similar embryology. As a result of anatrepsis (direction of motion of the embryo as it passes in an arc over the posterior pole) and the elongation of the germ band, they share a developmental stage in which the embryo is S-shaped (Kishimoto & Ando 1985, Tojo & Machida 1997, Panfilio 2008) (see Figs. 5.4, 5.5). This type of blastokinesis (specific movements of anatrepsis and katatrepsis) is thus considered an ancestral trait amongst the winged insects (Tojo & Machida 1997). The Trichoptera however, being a more derived order, show quite a different sequence of embryological development. The ancestral trait of an S-shaped embryo is not visible (anatrepsis and katatrepsis do not occur) (Fig. 5.6), embryonic growth is non-blastokinetic and instead certain aspects of the embryology are shared with the Lepidoptera (Miyakawa 1973, Anderson & Lawson-Kerr 1977, Panfilio 2008).

[40]Although this was not reported, two egg masses of this species were intentionally removed from water filled petri dishes and left to dry out over the period of 48 h. They were subsequently replaced in water and monitored for signs of development. Eggs appeared misshapen and no further signs of development were observed.

The visual comparison of the progression of developmental stages in the eggs of each of the species studied, using photographic methods, has allowed for a finer distinction of the upper and lower thermal limits for embryonic development as well as successful hatching (as opposed to just recording time to hatching). Additionally, the use of defined stages of embryonic development has allowed for estimates to be made of rate development.

Lestagella penicillata

The developmental range for *L. penicillata* appears to have a lower limit of between 5 and 10°C and an upper limit of between 20 and 25°C, with successful hatching occurring only at temperatures of 10, 15 and 20°C. These limits are based on the fact that no signs of development beyond stage S2 were observed for eggs of *L. penicillata* at temperatures of 5° C and 30° C, while at 25°C eggs showed signs of development to the penultimate embryonic stage S12, but failed to hatch (Fig. 5.4).

There is generally considerable variation in the optimal temperature range recorded for mayfly hatching and therefore there is no clearly defined temperature range within which the eggs of most mayfly species exhibit high percentage hatches (Brittain 1990). Clearly both phylogenetic and environmental determinants influence temperature-related developmental rates. The results presented here are however similar to ranges of roughly 10-25/30°C reported for successful hatching in several species of Australian mayflies (see Suter & Bishop 1990, Brittain & Campbell 1991, Brittain 1995, Parnrong & Campbell 2001) and 3-21/25°C for the majority of the Ephemeroptera from temperate areas in Europe and North America (Brittain 1982 see also Elliott & Humpesch 1980, Humpesch 1980a, 1980b, Brittain 1990). The reported temperature range for successful hatching in European and North American species is slightly lower than that reported for Australian species. While for the most part the egg development characteristics of Epehemroptera between Australia and the Northern Hemisphere are similar, the slight differences in the range of temperatures for successful hatching have been attributed to generally warmer water temperatures, milder winters and a greater degree of hydrological variability in Australian rivers compared to those from Europe and the North America (Brittain 1991).

Aphanicercella scutata

The developmental temperature range for *A. scutata* appears to have a lower limit a few degrees above 5°C and an upper limit of between 20 and 25°C (data indicated an upper limit closer to 20°C), with successful hatching occurring only at temperature treatments of 10, 15 and 20°C. No development beyond stage S1 was observed in eggs at temperature treatments of 25 and 30°C (Fig. 5.5). At 5°C, however, eggs showed signs of development to the final embryonic stage S13 but failed to hatch.

The temperature range presented here for embryonic development as well as successful hatching in eggs of *A. scutata* coincides well with the range of successful hatch temperatures reported for the majority of plecopteran species of between 10 and 15°C (Brittain 1990). Unlike the majority of

Ephemeroptera in which hatching generally tails off at around 5°C, however, most species of Plecoptera also exhibit high percentage hatches at temperatures of between 5 and 10°C with some species having successful hatching others at temperatures as low as 2°C (Brittain 1990- see also Harper 1973, Elliott 1988, Lillehammer *et al.* 1989,).

Chimarra ambulans

At temperature treatments of 5 and 30°C, eggs of *C. ambulans* showed no signs of development beyond stage S2, while eggs successfully hatched at all other temperatures (10, 15, 20 and 25°C) (Fig. 5.6). As such the temperature range for egg development appears to have a lower limit of between 5 and 10°C and an upper limit between 25 and 30°C.

Studies that relate the egg development of Trichoptera to different water temperature treatments are rare. Of the few detailed studies available (Wagner 1986, Hildrew & Wagner 1992, Jackson & Sweeney 1995, Enders & Wagner 1996) none deal with *C. ambulans.* Reported ranges in these studies have yielded variable results for thermal tolerances: 6-18°C for *Plectrocnemia conspersa* (Leptoceridae) (Hildrew & Wagner 1992), 10-20°C for *Apatania fimbriata* (Limnephilidae) (Enders & Wagner 1996) and 2-14°C for *Chaetopteryx villosa* (Limnephilidae) (Wagner 1986). Only one study found (Jackson & Sweeney 1995) provided information (though only for egg development at a single constant temperature) for a closely related yet unidentified species from the genus *Wormaldia,* which falls within the family Philopotamidae - the study revealed a successful hatch at 20°C. As such it is very difficult to make any generalisations with regards to thermal ranges for development or hatching in the Trichoptera.

5.4.3 Defining optimal and lethal thresholds for egg development using egg hatch parameters

Egg hatch parameters are useful in providing information regarding the thermal threshold for egg development, the thermal optimum for egg development as well as the timing and synchrony of hatching, all of which constitute crucial information to studies of life-histories.

Thermal thresholds for egg development can be inferred from egg percentage hatch data, in conjunction with information on the embryonic developmental stage and in this case with categorical information on the developmental outcome of the eggs (e.g. percent of eggs exhibiting deformed embryos). Very low percentage hatches (~0-5%) or marked decreases in percentage hatch can indicate an approach toward a thermal threshold for development. Similarly, the retardation of growth beyond a certain embryonic stage, and or the presence of deformed embryos can also indicate an approach toward, or a surpassing of, a thermal threshold. Pritchard *et al.* (1996) define the upper thermal limit or maximum as “that temperature above which the number of DD (species with negative or zero slope thermal reaction norms) or days (species with positive reaction norms) required to complete development begins a

consistent increase." As such, thermal reaction norms can also be used to estimate or infer the thermal limits for development. However, caution should be taken when inferring thermal optima using only reaction norms, especially if only a small range of experimental temperatures were assessed.

Thermal optima can be also be inferred from percentage hatch data, where the highest percentage hatch or consistently high hatches would indicate a thermal optimum. So too, the DD requirement to hatch can give an indication of thermal optima, where the shortest time requirement leading to a moderately high percentage hatch (>50%) would indicate a thermal optimum.

Synchrony can be inferred from the length of hatch, such that a total hatch period occurring over a short period of time (say 2-5 days) would be synchronous whereas hatches taking as long as 20-30 days to complete, could be considered asynchronous. This is because in such a scenario (depending on the growth rate of the species) hatchlings from day two could have already undergone a moult to the second instar by the time the total hatch is complete on day 30.

Lestagella penicillata

The DD requirement to mean hatch for eggs of *L. penicillata* decreased with increasing temperature following a power law (Figs. 5.7, 5.9). Hatching at the 10°C treatment took significantly longer (646.8 DD ~64 days) than hatching at the 15 and 20°C treatments (340.3 and 279.8 DD respectively ~22 and 14 days) (Table 5.3). Similar ranges of temperatures for hatching and corresponding DD requirement values have been obtained for four closely related North American species of Ephemerellidae, by Sweeney & Vannote (1981). Average values for DD to first hatching in *Ephemerella funeralis* are especially close at 59-82, 22-36, 18-21 and 17-24 days at temperatures of 10, 15, 20, and 25°C.

Using the DD requirement data alone, one could infer an optimum thermal range between 15 and 20°C[41] for *L. penicillata*. However when considering the percentage hatch data in conjunction with the DD requirement to mean hatch data, it is observed that in all treatments where a successful hatch was observed, a high percentage hatch (between 85 and 99%) was also recorded – with no significant differences among treatments. The most variable hatch (82-100 %) was observed at 10°C while hatches between 97 and 100% were observed in all replicates at temperatures treatments of 15 and 20°C (Fig. 5.7). As such, this could suggest a thermal optimum existing instead between a wider range of temperatures (10-20°C).

The presence of almost fully developed embryos as well as deformed embryos at the temperature treatment of 25°C (Fig. 5.8) in conjunction with a 0% hatch (Fig. 5.7), indicates a surpassing of the thermal maximum or threshold for development at 25°C. The thermal threshold for development in this

[41]Note that since there was no hatching at 25°C, there is no information for hatching success for temperatures between 20 and 25°C. Therefore, strictly speaking, the upper limit for the optimum range could be anything up to (but not including) 25°C.

species would then be estimated to be between 20 and 25°C, most likely just below 25°C, as fully developed embryos were still present in the 25°C treatment.

The length of the hatch period in *L. penicillata* was significantly shorter (4-5 days) and more synchronous at the 20°C temperature treatment when compared to the 10°C treatment (11-22 days) (Fig. 5.7). Similar findings of asynchronous growth at lower temperatures have been reported in studies of stoneflies and mayflies from the Northern Hemisphere (Elliott 1972, Harper 1973, Humpesch 1980a, 1980b). The longer DD requirement to hatch and longer asynchronous hatch period observed for this species at lower temperatures provides good evidence to account for the discrepancies in the appearance of first-instar nymphs observed in the life-history data (Chapter 4) amongst the sites. First-instars occurred approximately 30 days later at sites exhibiting lower temperatures over summer (Rooi-Els Kloof and Eerste rivers) while the period of the peak emergence period of adults was approximately 30 days longer compared to sites exhibiting higher average temperatures and more variable thermal regimes (Molenaars and Wit rivers) (see Chapter 2).

Aphanicercella scutata

The DD requirement to mean hatch for eggs of *A. scutata*, unlike the requirement for other species, showed a positive relationship with temperature, requiring fewer accumulated DD at lower temperatures (Fig. 5.7, 5.9) and greater DD at higher temperatures. Positive reaction norms have most commonly been observed amongst species of Plecoptera from North America and Europe (see Pritchard *et al.* 1996, Lillehammer *et al.* 1989) but a single species of Ephemeroptera (*Rithrogena loyolea*), restricted to cold mountain streams in Europe, has also been found to exhibit a positive reaction norm.

In general the DD requirement to mean hatch for *A. scutata* was very similar to that observed in three closely related species of *Nemoura* reported in Gillooly & Dodson (2001), *viz.*: *Nemoura cinerea* (Brittain & Lillehammer 1987), *Protonemura meyeri* (Strange 1985) and *Protonemura praecox* (Elliott 1988). The range of values (days to mean hatch) reported for these aforementioned species in order were: 30 days, 29 days and 34 days at 10°C, 22 days, 18 days and 24 days at 15°C and 17 days, 15 days and 19 days at 20°C.

Based on the DD requirement to mean hatch, the thermal optimum for *A. scutata* would appear to occur between 10 and 15°C, perhaps even closer to 10°C. Percentage hatch data showed no differences however, between the relatively high hatches at 10 and 15°C (85.2 and 75.8% respectively) but did show a marked decrease in the percentage hatch between the treatments at 10 and 20°C, with the percentage hatch at 20°C declining almost 2-3 fold to only ~31.7% (Fig. 5.7 and Table 5.4). The marked decrease in hatching at 20°C for *A. scutata* was initially thought to perhaps be a consequence of laboratory conditions (e.g. low oxygen concentrations at higher temperatures). Britain (1990) in a review of plecopteran development, however, claimed that hatching success at an optimal ranges of 10-

15°C was invariably high, but rapidly decreases at temperatures over 20°C. This suggests that the decreased hatch at 20°C of *A. scutata* represents natural low hatch percentages rather than artefacts of laboratory conditions. Percentage hatch data thus indicate a thermal optimum for egg development between 10 and 15°C and a thermal threshold existing between 20 and 25°C. The thermal threshold is likely closer to 20°C as deformed embryos were already visible in the 20°C treatment, while no signs of development were noted in the 25°C treatment (Fig. 5.8).

Hatching in *A. scutata* appears relatively asynchronous with a long hatching period (>20 days) observed in all temperature treatments for which a successful hatch was recorded. Hatching period was shortest at 20°C (20-21 days) followed by 10°C (22-26 days) and 20°C (28-29 days) although the length of hatch was only significantly different between treatments at 15 and 20°C (Fig. 5.7 and Table 5.4) These observations suggest that the hatching period of *A. scutata* is shortened at suboptimal conditions, while under more optimal conditions an asynchronous hatch occurs. Alternatively the apparently quickened hatch at 20°C could be an artefact of the fact that substantially fewer eggs actually hatched at this temperature. Nevertheless hatching period was substantially longer and more asynchronous at all temperature treatments for *A. scutata* in comparison to *L. penicillata* and *C. ambulans*. This relatively asynchronous hatch could explain the longer emergence period observed in *A. scutata* (May-August ~3-4 months) when compared to that observed for *L. penicillata* (October-November ~2-3 months) (see Chapter 4). Additionally the DD requirement (36 days at 10°C – this temperature roughly coinciding with winter temperatures over the period of emergence and oviposition) in conjunction with the length of the hatch period (22-28 days) suitably account for the presence of first-instar nymphs of this species occurring for approximately four consecutive months after the first signs of adult emergence (see Chapter 4).

Chimarra ambulans

Eggs of *C. ambulans* exhibited the widest thermal range for development (lower limit between 5 and 10°C and upper limit between 25 and 30°C) relative to the other species studied. Eggs of *C. ambulans* also showed the fastest development times and lowest thermal requirement at all incubation treatments, followed by *L. penicillata* and *A. scutata*. Development time or DD requirement to mean hatch decreased at increasing temperature treatments of 10, 15 and 20°C following a power law (Figs. 5.7, 5.9). At the temperature treatment of 25°C however, the DD requirement was higher than that observed at 20°C suggesting that a temperature of 25°C is above the thermal optimum for hatching in this species. Additionally, the presence of deformed embryos at temperatures of 25°C indicates thermal stress at this temperature. The DD requirement (252.1 DD) to mean hatch at 20°C for *C. ambulans* equates to roughly 12 days (Table). This DD requirement is almost identical to the values of 240 DD at 20°C ~12 days obtained by Jackson & Sweeney (1995) for egg development of a closely related, though unidentified, tropical species of *Wormaldia* (Philopotamidae).

Percentage hatch in this species was the most variable with the general trend being one of decreasing percentage hatch with increasing temperature – though this trend was not shown to be significant, presumably as a result of the high level of variability (Fig. 5.7 and Table 5.4). Experiments would however need to be repeated to determine the origin of the high variability in percentage hatch observed, which could either be a true reflection of natural levels or be an artefact of the fact that mating and oviposition occurred in an artificial laboratory setup. At temperature treatments of 10, 15, 20 and 25°C a high percentage of eggs showed full development but failed to hatch (Fig. 5.8). It remains uncertain, though, whether this too is a true reflection of eggs in nature or whether this is a result of laboratory conditions affecting egg development[42]. It is possible that fungal infections and low oxygenation levels at higher temperature treatments (20 and 25°C) may have been the cause. Nevertheless the decrease in percentage hatch at higher temperatures (averages as low as 12.4% at 20°C and 7% at 25°C) suggests that the thermal limit is close to being reached at 25°C.

It is difficult to estimate a thermal optimum for this species based on values of percentage hatches alone, as hatches were variable at all temperature treatments. However, highest hatches were recorded for treatments at 15°C followed by 10°C with a marked decrease observed at 20°C, suggesting the potential for a thermal optimum between 10 and 20°C for this species. However, eggs from the same species collected from a different location (Window Stream, Kirstenbosch) and incubated with two replicates at 20°C, exhibited two high percentage hatches of 79.3 and 80.9% respectively, suggesting that the thermal optimum may in fact be greater than 20°C. This observation relates well to the potential thermal optimum derived from "DD to mean hatch" data.

The length of the hatching period, while exhibiting variability at all incubation treatments, was still relatively short compared to that of the other species, at an average of around 3.5 days for all treatments. No significant differences were observed among treatments. This species therefore exhibits a synchronous hatch. However, as female oviposition appears to occur continuously over a long period of up to 8 months (perhaps extending throughout the year in certain rivers) (see Chapter 4), this could result in the appearance of seemingly asynchronous hatch in the field data, which is in fact caused by the presence of overlapping newly hatched generations.

5.4.4 Environmental cues for oviposition, emergence and development

The timing of oviposition and length of the egg development period in *L. penicillata* suggest that this species may utilise the timing of the positive late-spring (November) thermal regime shift as a cue for

[42] Although this was not reported, egg hatching experiments for this species were in fact repeated two further times in an attempt to ascertain and minimise the degree of variability in the percentage hatch data. The first repeat experiment utilised a flow through system to constantly circulate water over the eggs (in an attempt to increase dissolved oxygen concentrations), while the second utilised the same flow through system but with an added diluted dose of aquarium anti-fungicide (in attempt to prevent any fungal infection). Surprisingly in both repeat experiments (each having four replicate egg masses per temperature treatment) no hatching occurred at any temperature treatment.

oviposition (see Chapters 2, 4). This regime shift is timed with the abating of winter and spring high flows to summer baseflow conditions (see Chapter 2) – presenting an optimal time period for the oviposition of these eggs which a) do not have attachment apparatus, b) develop optimally at moderate temperatures and c) need to develop before the onset of peak summer temperatures which might adversely impact hatch success. For *A. scutata*, more rapid and synchronised development of nymphs appears to be cued with the first negative thermal regime shift of the year in autumn (coinciding with the March equinox) – indicating the onset of low temperatures and higher flows (see Chapters 2, 4). Following this negative thermal regime shift, *A. scutata* appear to emerge throughout winter (perhaps to avoid high flows), with oviposition subsequently appearing to be synchronised with the first positive thermal regime shift in early spring. This regime shift coincides with the September equinox and indicates the onset of warmer conditions and the abating winter spates to moderate flows (see Chapters 2, 4). This presents an optimal time for the development of the eggs of this species as they a) are fixed in place and protected from turbulent/high flows by a gelatinous sticky coating and b) develop optimally with higher hatch success at low temperatures. Similar to *L. penicillata*, emergence of the over wintering population/pupae of *C. ambulans* appears to be synchronised with the late spring positive thermal regime shift (November) (see Chapters 2, 4). Emergence as well as oviposition appears to occur continuously throughout summer but stops abruptly in conjunction with first negative thermal regime shift in autumn. This regime shift coincides with the March equinox perhaps acting as a cue to indicate high flows and low temperatures (see Chapter 2). The summer period presents the optimal conditions for development of eggs of this species which develop rapidly and optimally at a wider range of temperatures than the other species even at peak summer temperatures.

5.4.5 Thermal reaction norms and evolutionary origins

Lestagella penicillata

The slope of the thermal reaction norm for *L. penicillata* was found to be steeply negative (b = -1.23) (Fig. 5.9). This value, while falling within a broad range reported for many other ephemeropteran species (b = -1.75 to 0.0 with the median around -0.75), is more negative than the majority of species (Pritchard *et al.* 1996). Eggs of almost all the temperate zone mayflies in the study by Pritchard *et al.* (1996) exhibited warm adaptation and negative slopes, though with values slightly lower than those observed for Odonata (b = -1.75 to -0.75 with the median around -1.25) which are known to be warm adapted (see Pritchard *et al.* 1996). Increasingly negative slopes indicate that a greater number of DD are required at lower temperatures to complete development, and *vice versa*. As such, species exhibiting negative slopes are considered to be warm-adapted species. The explanation for this finding given by Pritchard *et al.* (1996) is that the close phylogenetic relationship between mayflies and Odonata might imply that they shared a similar (tropical, warm water) environment during their origin and evolution. More specifically, this may hold true for the Teloganodidae which are thought to have had their origin

in more tropical waters of Gondwanaland, at a time when the global climatic conditions were generally cooler and moister than at present (Endrödy-Younga 1988, Barber-James et al 2008). However, as the southern African climate became warmer and more arid during the Mesosoic, these invertebrates along with other Gondwanan fauna would have incurred subsequent isolation in montane refugia (Endrödy-Younga 1988). Compared to the slope of the thermal reaction norm of two closely related species in the Ephemerellidae from North America (see Sweeney & Vannote 1981), *Ephemerella funeralis* and *E. subvaria* (*b* = -0.39 and -0.64 respectively), *L. penicillata* shows a markedly steeper and more negative slope. It is possible that the more variable climate in the Southern Hemisphere, coupled with higher average water temperatures over summer and milder winters, have resulted in a greater degree of adaptation to warm waters than its Northern Hemisphere counterparts.

Aphanicercella scutata

The thermal reaction norm for the stonefly *A. scutata* showed a weakly positive slope (*b* = 0.15) (Fig. 5.9). In contrast to the negative slope recorded for *L. penicillata*, a positive slope indicates that fewer DD are required at lower temperatures for egg development. Pritchard *et al.* (1996) reported that positive slopes were commonly recorded for the Plecoptera, more so than in any other insect order, suggesting frequent cold adaptation and cold water origins. The values of slopes (*b*) reported by Pritchard *et al.* (1996) for Plecoptera range from -0.75 to 1.0 with the median between -0.25 and 0.25. Power law equation constants *a* and *b*, as well as the slope for the thermal reaction norm reported for *A. scutata* in this study compare well to those reported for four closely related Southern Hemisphere species of Notonemouridae from Australia in the genus *Austrocercella* (See Brittain 1991, Pritchard *et al.* 1996). Values for *A. scutata* are most similar to those recorded for *Austrocercella illiesi* (power law equation constants *a* = 227, *b* = 0.124), a species widespread in the Australian Alps, with an autumn emergence period, and an egg development period during the winter months of June/July/August (Brittain 1991), coinciding with that of *A. scutata*.

Studies by Brittain (1991) and Pritchard *et al.* (1996) both highlight the point that the Southern Hemisphere Notonemouridae (including *Austrocercella*) and its sister taxon from the Northern Hemisphere, the Nemouridae, share similar ranges of reaction norms. Brittain (1991) found that both regression constants from equation (2) (section 4.2.3 this chapter) for *Austrocercella* were more similar to those found for colder stenothermal species of Nemouridae (Lillehammer *et al.* 1989) rather than cold-cool eurythermal species in the Nemouridae. As all species of *Austrocercella* are restricted to higher elevation mountain streams in the Australian Alps, they would experience cold environments during egg development. This ties in well with the fact that *Aphanicercella*, including *A. scutata*, is considered to belong to a relictual stenothermal Gondwanan family (the Notonemouridae) currently restricted to higher elevation/mountainous regions of the South Western Cape (Day 2005, Stevens 2009). It should be noted, though, that the lack of steepness of the slope in *A. scutata* could suggest

adaptation to variable thermal regimes that are characteristic of temperate streams and lakes (Pritchard *et al.* 1996). Findings in this study for *A. scutata* nevertheless lend further support to the conclusions of Brittain (1991) that the Notonemouridae have a cold-adapted life cycle.

Chimarra ambulans

While Pritchard *et al.* (1996) did not include trichopterans in their study, the moderately negative slope (b = -0.45) calculated for *C. ambulans* (Fig. 5.9) is similar to values they observed for several species of Diptera and Ephemeroptera as well as some warm-adapted plecopterans.

It is generally accepted that the basal group of the Trichoptera (Spicipalpia, comprising Glossosomatidae, Rhyacophilidae, Hydroptilidae, Hydrobiosidae, all of which make closed unpermeable silk cocoons) originated in cool well oxygenated lotic waters (Ross 1956, Wiggins & Wichard 1989, Frania & Wiggins 1997, Wiggins 2004). The Spicipalpia are thus believed to represent the primitive conditions and origins of the entire order Trichoptera (Wiggins 2004). Several advantageous evolutionary adaptations (e.g. increased respiratory efficiency through the use of permeable silk and open cocoons as well as tubular retreats) however, are thought to have enabled Trichoptera to invade lotic habitats with decreasing current and oxygen availability and higher summer water temperatures, with some even invading lentic waters. In turn this is thought to have lead to the radiation of more derived extant groups, such as the Integripalpia and the Annulipalpia (to which the family Philopotamidae belong), which exhibit the aforementioned adaptations (Wiggins, 2004). The Philopotamidae have their origins in the middle to late Triassic period, in what would then have been a tropical belt extending across modern day North America and Western Europe (de Moor & Ivanov 2008). The warm water origins of the family are reflected in the most speciose and widespread genus *Chimarra*. This genus is well represented in the tropical and sub-tropical areas of Asia and Africa and as a whole has been described as being warm-adapted (Blahnik 1998).

The negative slope obtained for *C. ambulans* in this study also suggest adaptation to and perhaps origins in warm waters, this in agreement with the assertions of Blahnik (1998). The negative slope, being substantially weaker than the slope for *L. penicillata* however, and the fact that the species shows a higher percentage hatch at slightly lower temperatures of 10-15°C, could represent an adaptation over time to more variable thermal conditions that include warmer waters. A higher percentage hatch at moderate to low water temperatures compared to high water temperatures could also reflect site specific adaptation to the water temperatures experienced by this species at the Elandspad River site.

5.4.6 First-instars, egg size and clues to life-history evolution

The characteristics of the eggs represent adaptations to specific conditions present in the sites available to the female for oviposition (Resetarits 1996). It has been proposed and shown that female body size is directly related to egg size and also the number of eggs produced in aquatic insects (Smith & Fretwell

1974, Sweeney 1978, Brittain 1982, Berrigan 1991). It has also been shown in this study and in others that the size of first-instar hatchlings is positively related to egg size (Lillehammer *et al.* 1989, Corkum *et al.* 1997). Additionally it has been shown for several ectothermic animals, including fish and insects, that females developing and ovipositing at lower temperatures produce larger eggs (Fleming & Gross 1990, Avelar 1993, Sheader 1996). Phenotypic plasticity has been proposed to account for much of the variation in egg size at lower temperatures, yet the mechanisms underlying this widespread phenotypic plasticity are not yet understood (Azevedo *et al.* 1996, Crill *et al.* 1996, Blanckenhorn 2000, Fox & Czesak 2000, Fischer *et al.* 2003). Of the several explanations proposed, one is that selection favours the production of larger offspring at lower temperatures to shorten development time and thereby shorten generation time (Green 1966, Perrin 1988, Fischer *et al.* 2003). This suggestion will be considered in the following paragraphs, in light of the three species investigated in this study.

The size of first-instar nymphs is positively related to egg size (Fig. 5.11). Eggs and first-instar nymphs of *L. penicillata* were the smallest, followed by those of *A. scutata* and *C. ambulans* (Table 5.2 and Fig. 5.11). Based on life-history data presented in Chapter 4, *L. penicillata* exhibits the longest larval development period (11-13 months) and is univoltine. *A. scutata* was also found to be univoltine, exhibiting an extended development period (8-14 months) as well as a longer emergence period, compared to *L. penicillata.* In contrast, *C. ambulans* appeared to exhibit variable voltinism linked with the shortest development time[43] (estimated between 2-5 months). The total development time for two closely related species, *Chimarra mosleyi* and an unidentified species from the genus *Wormaldia*, were shown to have total development times (at 20°C) of as little as 1.5 and 4 months respectively allowing for a bivoltine life cycle (see Jackson & Sweeney 1995 and Cudney & Wallace 1980 respectively).

Larger eggs produced by *A. scutata* and *C. ambulans* (both more derived species than *L. penicillata*) are associated with quicker larval development (only marginally so in the case of *A. scutata*) and perhaps greater flexibility with regard to life-histories under more variable climatic conditions than *L. penicillata*. This is especially true in the case of the multivoltine species *C. ambulans*, which shows a great affinity for warm waters, while for *A. scutata* this might be less applicable as this species appears to already be existing at the thermal limit for its development. The more basal species *L. penicillata* produces smaller eggs, potentially as a result of its warm water origin and thus has a longer development and life cycle/generation time, with perhaps a less flexible life-history. However, because of the lengthy life cycle it is subject to a wide range of seasonal temperature fluctuations, including summer temperatures to which it is adapted.

In effect these data suggest that egg size and corresponding first-instar nymph size are inversely related to generation time. Additionally Green (1966) and Perrin (1988) proposed that selection favours the

[43]The life-history for *C. ambulans* was shown to be complicated and still needs to be thoroughly resolved – this would require rearing experiments in which the total development time for individual larvae under different temperature treatments can be monitored.

production of larger offspring at lower temperatures to shorten development time and thereby shorten generation time. These observations appear to explain the trends observed in the relationship between eggs size and first-instar size for the three orders in this study. However, phylogenetic constraints might equally explain generation length, with longer life cycles being associated with a greater number of instars. This is discussed further in Chapter 7.

CHAPTER 6

The role of temperature in larval growth rates, survival and adult emergence of *Lestagella penicillata*

Summary

Knowledge of aquatic individuals' thermal tolerance limits, as well as an understanding of the sublethal effects of temperature on other phenological traits (e.g. emergence, growth, egg development and hatching, and fecundity), is of critical importance in determining not only an individual's life cycle but also the potential response of ecosystems to altered thermal regimes. Additionally, where such information can be coupled with fundamental life-history data in conjunction with long-term temperature data the ability to further model and forecast ecosystem responses (based on predicted future climate change scenarios) then becomes possible. Collecting such data from the field can be complicated and difficult to achieve and is thus better suited to being collected through rearing/mortality experiments conducted under controlled laboratory conditions. This chapter reports on the results of laboratory rearing experiments that were conducted on two genetically distinct lineages of the larger putative species complex of *L. penicillata*, collected from the Window Stream and Molenaars River sites. The major aim was to assess whether differences existed between the lethal and sublethal effects of temperature on these individuals specifically in terms of nymphal growth rates, upper Lethal Temperature (LT_{50}) mortality, and timing of emergence. Importantly, suspected differences in growth rates among *L. penicillata* populations (Molenaars River and Window Stream) were experimentally evaluated in this chapter before the genetic analyses had been completed (Chapter 3). Using a novel, thermostatically controlled, flow-through system design, the sublethal effects of temperature on growth rates, body size, and the timing of emergence in conjunction with thermal tolerance limits (using a static LT_{50} experimental procedure) were assessed for a range of water temperature treatments within a CE room. The results of the experiment confirmed estimates of thermal optima for growth (obtained in Chapter 4) and allowed for life-history trait differences between two genetically distinct lineages to be evaluated and contrasted under common environmental conditions, providing insight into the degree to which genetic differences vs. phenotypic plasticity affect these traits. In particular, individuals from both Window Stream and the Molenaars River showed optimal growth rates converging at between 16-18°C in accordance with GLM estimates for optimal growth of this species (Chapter 4), possibly suggesting that this is a common thermal optimum range for growth in the genus. Individuals from the Molenaars River, however, showed better growth than those from the Window Stream individuals at all temperature treatments, likely owing to genetic differences in conjunction with adaptation to a warmer thermal regime that is experienced in the Molenaars River. This conclusion was further supported by the observation that individuals from the Molenaars River showed higher upper LT_{50} limits than those from Window Stream. The delayed timing of emergence of *L. penicillata* collected from both sites in laboratory experiments when compared to natural populations appeared to be an adaptive plastic response in the life-history trait of this species. Further differences in the timing of emergence were observed between individuals collected from the two sites; these too are also thought to be as a result of the different natural thermal regimes experienced at the two sites to which the species have become

adapted. Notably, body size and the number of moults appeared to be regulated by the inter-moult duration, which generally decreased with increasing temperature. Differences under the same temperature treatments were again observed between individuals from the Molenaars River and Window Stream, providing further evidence of genetic differentiation between the two lineages.

6.1 Introduction

Temperature plays a fundamental role in the ecology of aquatic insects, with altered thermal regimes influencing the timing of emergence and hatching, growth and metabolic rates, fecundity, and ultimately determining the survival and distribution of different species (Ward & Stanford 1979, Brittain 1982, Ward & Stanford 1982, Perry *et al.* 1987, Brittain & Campbell 1991, Atkinson 1995, McKie *et al.* 2004). Certain species have more flexible life-histories (e.g. bi- tri- or multi-voltine) and are thus able to respond to different temperature regimes by adjusting their growth rates and emergence times accordingly (Nebeker 1971a, 1971b, Hynes 1976, Vannote & Sweeney 1980, Perry *et al.* 1987, Brittain & Campbell 1991, McKie *et al.* 2004). Increases in water temperature have been shown to accelerate larval development and to cause an earlier onset of emergence in some species as well as reduced size at maturity (Brittain 1976a, Sweeney 1978, Ward & Stanford 1979, Perry *et al*. 1987, Lillehammer *et al.* 1989, Rader & Ward 1989, Panov & McQueen 1998, McKie *et al.* 2004, Harper & Peckarsky 2006). While accelerated growth and earlier emergence may not pose major negative effects for the survival and fitness of more adaptable eurythermic species, increased temperatures resulting in suboptimal conditions for growth for less adaptable species (e.g. stenothermic univoltine species) could reduce fitness and overall reproductive success of these species by altering life-history traits such as timing of emergence, size at emergence, fecundity and egg hatching success (Sweeney 1978, Brittain & Saltveit 1989, Harper & Peckarsky 2006). Indeed any aquatic species, not only those that are stenothermic in origin, that exists at a thermal optimum where size and fecundity are maximised would be vulnerable to such changes in life-history traits as a result of thermal regime alterations (Harper & Peckarsky 2006).

The causes of thermal alteration to aquatic riverine habitats are numerous and include amongst others: river regulation and modifications of flow regime through the construction of dams and associated hypolimnetic (cold) or epilimnetic (warm) releases (Webb *et al.* 2003, Arthington *et al*. 2010, Olden & Naiman, 2010), inter-basin transfers, riparian vegetation condition or deforestation in turn affecting the amount of incoming solar radiation (Rutherford *et al.* 1997, Caissie 2006), upwelling and subsurface water or groundwater interactions (Sear *et al.* 1999, Story *et al.* 2003) and of course thermal changes related to global climate change (Hogg *et al.* 1995, Durance & Ormerod 2007, Woodward *et al.* 2010). As is evident from the above, many of the drivers of thermal alteration are anthropogenic in nature (Dallas & Ketley 2011) and as such the increasing demand for freshwater and its associated goods and services needs to be coupled with the need to ensure and maintain freshwater ecosystem integrity (Arthington *et al.* 2010, Olden & Naiman 2010). This major challenge for both freshwater scientists and managers has recently emerged as one of the most important resource issues facing the planet (Olden & Naiman 2010). In this regard it is essential that scientists provide managers with the research that can enable them to make informed decisions and develop suitable management guidelines.

Knowledge of aquatic individuals' thermal tolerance limits, as well as an understanding of the sublethal effects of temperature on other phenological traits (e.g. emergence, growth, egg development and hatching, and fecundity), is therefore of critical importance in determining the potential response of ecosystems to altered thermal regimes (Dallas & Ketley 2011). Additionally, where such information can be coupled with fundamental life-history data in conjunction with long-term temperature data, modelling and forecasting ecosystem responses based on predicted future climate change scenarios is made possible (Panov & McQueen 1998).

Two main experimental methods, dynamic (non-lethal) and static (lethal), exist for determining thermal tolerance limits (Lutterschmidt & Hutchinson 1997, Terblanche *et al.* 2007, Dallas & Ketley 2011, Dallas & Rivers-Moore 2012). In the dynamic method individuals are subjected to temperature increases or decreases at a constant rate until they exhibit a behavioural stress response or physiological failure (e.g. increased swimming, followed by loss of attachment to substrate and or immobility) at which point the temperature or critical thermal endpoint (CTE) is recorded and noted either as a critical thermal maxima (CTmax) or minima (CTmin) (Terblanche *et al.* 2007, Dallas & Ketley 2011). The overall CTE for a taxon is determined through calculating the arithmetic mean of end points for a sample of several individuals of the same species (Dallas & Ketley 2011). In the static method individuals are either subjected to a range of constant temperatures for the same duration of time or they are subjected to a single constant temperature for varying durations (Terblanche *et al.* 2007). The Lethal Temperature (LT_{50}) is then calculated as the temperature at which 50% of the individuals in a sample survive in the particular time frame (Dallas & Ketley 2011). This static lethal method is a robust method that has been used for many years and was initially designed for calculating median lethal concentrations (LC_{50}) values in eco-toxicology studies and toxicity bioassays (Hamilton *et al.* 1977), but is also applicable for studies where median lethal temperature limits are the focus hence the use of the term LT_{50} instead of LC_{50}. Generally the static method uses varied temperatures over a constant duration (normally anywhere from four to 10 days) and commonly involves 20 to 30 individuals per temperature treatment. There can be upwards of four treatments (Dallas & Ketley 2011), though a minimum of at least six individuals at each temperature treatment is required to provide statistically meaningful results (Stephan 1977). Dynamic methods on the other hand are relatively quick (1-2 h) to perform and require fewer individual (approximately 30 organisms in total) to obtain results. For these reasons the CTM method has gained popularity and much interest over recent years compared to LT_{50} methods. Both however remain suitable and useful experimental methods for determining thermal limits of aquatic individuals.

For determining sublethal effects of temperature on phenological traits such as emergence cues and growth rates, experiments involving the regular monitoring of live insects held at different temperature treatments in simulated stream environments have proven to be effective and have provided valuable information (e.g. Brittain 1976a, Lillehammer *et al.* 1989, Pritchard & Zloty 1994, Elliott 2009).

However such experimental setups can be far more challenging and costly to construct. This is in contrast to experimental setups used for determining sublethal temperature effects on egg growth, which are easier to assemble and maintain, as they do not necessarily require a simulated flow environment, filtration or food provision (see Chapter 5).

Using a new design for constructing simple, cost effective, self-contained and thermally controlled simulated stream flow-through systems, the primary aims of this study were a) to determine the sublethal effects of temperature on growth rate, timing of emergence and also size at emergence in two representative populations of a putative species complex of the mayfly *L. penicillata* (Teloganodidae) (see Chapter 3) and b) to determine the upper thermal limits of these populations using the flow-through setup concurrently as a long-term (25-day) static LT_{50} experiment.

More specifically, this information was intended to better interpret the field-collected phenology and life-history data collected for this putative species complex (Chapters 3, 4) by linking field data with laboratory experiments on growth. Additionally this data can be used to gain insight into thermal tolerance limits by comparison with egg development experiments (Chapter 5) and by comparing growth trends, phenological and morphological traits in two genetically distinct lineages of the putative species complex reared under the same environmental conditions (Chapter 3).

6.2 Methods

6.2.1 Field sampling

Samples of *L. penicillata* were collected from two sites previously utilised in this study, namely the Molenaars River and Window Stream sites (Fig. 4.2, Chapter 4). Based on life-history information previously obtained for this species, sites were sampled in late September/early October 2011 following the onset of sexual dimorphism, but prior to the emergence period (Chapter 4). This was done a) in order to be able to distinguish the sex of collected specimens, thereby preventing confounding results that might occur from sexually dimorphic growth and b) to as far as possible ensure information regarding the timing of emergence was captured during the course of the laboratory experiments, whilst at the same time mitigating the effects of mortality owing to potential flaws in the experimental setup (i.e. slightly later-instar individuals would be more likely to survive and emerge than early-instar individuals).

Riffle areas at these sites were sampled by gently picking up, shaking and brushing the surfaces of submerged cobbles at the opening of a standard square frame kick net (30cm x 30cm x 60cm), which was fitted with an 80µm mesh. Invertebrate samples were returned to the laboratory where they were aerated and allowed to acclimate for 72 h in a CE room set to ± 11°C with full spectrum flourescent lighting, which provided a light/dark photoperiod of 12:12 h (light from 07:00 to 19:00). The CE room was set at approximately 11°C in order to match ambient stream temperatures calculated for the

Molenaars River for the month of September (see Chapter 2, Table App2B.2 in Appendix 2B). Small alga covered pebbles, collected separately during sampling at the Window Stream site, were placed in the containers housing the invertebrates as a source of food prior to acclimation. Hourly water temperature records were collected for both sites by means of HOBO® TidbiT® v2 loggers (Onset Computer Corporation 2008) and were available for the time period from November 2008 to May 2009, i.e. prior to the commencement of the experiment.

6.2.2 Laboratory methods

Following the acclimation to the ambient stream temperature (control temperature) specimens/individuals were moved into the laboratory where they were sorted, identified and sexed using a dissecting microscope. Suitable test individuals of female *L. penicillata* were subsequently aged (by counting antennal segments) and photographed using a Leica DM750 compound microscope fitted with a digital camera. During this process all individuals were kept in aerated containers and allowed to acclimate slowly to room temperature (~24°C) over a period of three to four hours at a rate of roughly 3-4°C/h. Approximately one hour was required to age and photograph 30 test individuals. With four intended experimental temperature treatments (10°C, 15°C, 20°C, 25°C) [44] each populated with 15 individuals from the two sites, ageing and photographing took roughly four hours in total. Individuals that were to be placed in the control temperature chambers were aged and photographed first after which they were immediately moved back into the CE room and placed in the experimental temperature chambers. The same process was repeated for the experimental temperatures in order of increasing temperature. Individuals were placed in the experimental temperature chambers as their environmental temperature reached the intended experimental temperature. However, individuals intended for the 25°C treatment were placed in their experimental chambers once they had acclimated to room temperature at approximately 24°C. While this was not an entirely precise or accurate acclimation process it served the intended purpose of preventing the individuals from experiencing thermal shock when being placed initially in the experimental temperature chambers (Nebeker & Lemke 1968).

Experimental temperature chamber design

Experimental chambers were designed to be cost effective, contained, thermally controlled, re-circulating flow-through systems with built in filtration, and able to hold up to 15 test individuals in separate housings (Fig. 6.1). The design consisted of a reservoir (rectangular plastic crate filled with 26 litres of conditioned tap water) which could be heated using standard thermostatically-controlled aquarium heaters in the case of treatments from 15-25°C. A single aquarium powerhead pump with attached filtration canister and filtration medium was used to circulate water, at an adjustable rate,

[44] While it was the intention to have four temperature treatments (10, 15, 20, & 25°C) with two replicate chambers at each temperature, this was not achieved owing to difficulties with calibration of heaters used in the experiment. Final temperatures used in the experiment were 10.65, 10.70, 15.76, 16.83, 19.76, 21.75, 25.61 and 26.96°C (see Results and Discussion).

through standard aquarium tubing around the perimeter of the reservoir. Standard garden irrigation connectors were used to connect tubing to the each of the individual housings and to the submerged pump itself. Individual housings consisted of polytop glass vials fitted with a piece of 80µm mesh under the plastic lid (to prevent escape of the individual). The lid of each vial had a portion cut out to allow for an overflow effect and a small hole to allow for direct connection to the tip of a standard automatic pipette end which itself was inserted into the opening of a T connector. In order to ensure that neither a) water temperature in the housings was impacted by air temperature (i.e. if the housing is elevated mostly above the water of the reservoir) nor b) ineffective overflow (i.e. if the housing is submerged), plastic S-hooks were used to elevate the tubing and the attached organism housings such that the lids of the housings were about 2 cm above the level of water in the reservoir.

Fig. 6.1. Design of temperature chambers and organism housings used in growth and LT_{50} experiments. Diameter is denoted by the symbol Ø.

The flow-through system provided a stable average flow rate of approximately 1.875litres/h to the housing of each test individual. The subsequent overflow allowed for the removal of nutrient build up from each housing, circulating it back into the reservoir for filtration, re-heating and re-circulation. The reservoirs were topped up with fresh water every two weeks. Three replicate measurements of dissolved oxygen concentration and saturation, recorded in each temperature chamber setup prior to the commencement of the experiment, revealed high average levels of oxygen saturation (>95%) and concentration (>8mg/l) at all temperature treatments, suggesting that suitable aeration was provided by the experimental setup. Conditioned tap water used to fill the temperature treatment chambers had an average pH of 7.6 and average electrical conductivity of approximately 295µS/cm.

Temperature treatments

Two temperature chambers, identical to that depicted in Fig. 6.1, were set up at each of four intended temperature treatments from 10 to 25°C at roughly 5°C intervals (thus two intended replicates at each of 10, 15, 20, 25°C). However even after calibration, heaters did not appear to maintain the same temperatures in each replicate resulting rather in a setup consisting of two controls at ~11°C and a single temperature chamber replicate at each of several temperatures: ~16°C, ~17°C, ~20°C, ~22°C, ~26°C and ~27°C (see Results section). Each replicate contained between seven and eight individuals from *both* the Molenaars River and Window Stream sites such that a total of 15 individuals were tested in each chamber.

All temperature chambers were placed in the same CE room used for initial acclimation purposes and set at ± 11°C. This temperature was used as the control temperature, while higher temperatures needed for all other treatments were obtained using aquarium heaters. As these heaters did not maintain a very constant temperature, HOBO® TidbiT® v2 water temperature loggers (Onset Computer Corporation 2008) were placed in each temperature chamber to record water temperatures every 15 minutes for one month of the experiment duration for reference purposes.

In each of the vials housing test individuals, two small alga covered pebbles (roughly equally sized) were placed as a food source with the intention of keeping food as a non-limiting factor for growth. Based on the results of a pilot study to determine survival/growth in relation to the frequency of three food replacement treatments (weekly, bi-weekly, monthly), pebbles were replaced with fresh pebbles collected from Window Stream site every two weeks.

Growth experiments

Results obtained in pilot studies in conjunction with information collected for newly hatched first-instar individuals (Chapter 5), suggested that a simple sequence of post-embryonic antennal growth exists for *L. penicillata*. In this sequence the meriston (first generative annulus of the flagellum) divides into two annuli which do not undergo further division. In first-instar nymphs, two non-dividing annuli (known as

singletons) are present in addition to the meriston, bringing the total number of annuli to three. In this manner the antenna is lengthened from near its base by one additional annulus per moult (e.g. from instar one to instar two antennal segments/annuli increase from three to four). Similar simplistic antennal growth sequences have been found for other Ephemeroptera, and for some Polyneoptera (Isoptera- Fuller 1920, Blattaria, Zygentoma and other Ephemeroptera- Qadri 1938). In other Polyneoptera there are more complicated antennal growth patterns (e.g. Plecoptera - Khoo 1964, Brittain 1973, Orthoptera - Burnett 1951, Mantophasmatodea - Hockman *et al.* 2009).

Based on the simple antennal growth sequence observed, antennal counts as well as measurements of interocular distance (IOD), body length (BL), head capsule width (HCW) and thorax width (TW) were used to determine the initial age (instar number) and size of individuals prior to being placed in experimental treatment chambers. As far as possible the youngest and smallest individuals collected from each site were used in the experiment. Upon commencement of the experiment, individuals were monitored daily for deaths and moults until all individuals had either died or emerged. Where deaths occurred, the dead insect as well as the housing was removed from the temperature chamber. Dead individuals were preserved in 70% ethanol for reference. Where moults occurred, the exuvia itself was viewed under a compound microscope to obtain an accurate count of the number of antennal segments (instar stage) of the individual prior to moulting - these data were also used to confirm the antennal growth sequence through to emergence. The organism that had moulted was immediately photographed live using a calibrated dissecting Leica EZ4 microscope fitted with a digital camera in order to obtain post-moult size measurements. In order to standardize the way in which images of the individuals were taken, the individual was first carefully transferred (using a pipette) onto a microscope slide with a few drops of water from the its housing. A cover slip was then placed over the individual to be photographed in order to gently flatten the insect and prevent distortion of the digital image to be captured. The digital image was subsequently analysed in Leica imaging software (Leica Application Suite - Image Analysis) in order to obtain the various size measures (IOD, BL, HCW and TW) of the newly moulted insect. Successive measurements recorded in this manner after each moult provided a measure of growth for each individual in the experiment. After being photographed the individual was then returned to its housing in the temperature chamber. This method provided the quickest, most effective and least intrusive means to record growth measurements from live individuals on a regular basis. Only two to five minutes were required for a single individual to be measured in this manner (the time recorded from being removed from the temperature chamber to being photographed and subsequently replaced).

Morphometric relationships between size measurements (IOD, HCW, TW and BL) were calculated for individuals from each study site as well as at each temperature treatment. Similarly, summary tables of size, instar, as well as antennal counts were produced for black wingpad individuals that developed

during the experiment (under laboratory conditions) in comparison to black wingpad individuals collected from the wild from each study site.

LT_{50} experiments

The setup used for the growth experiments was simultaneously used to determine long-term survival and LT_{50} values. The constant temperatures used for the growth experiment were selected in order to provide insight into growth at the upper and lower thermal limits experienced by *L. penicillata* under natural conditions at the selected study sites. As such, a range of mortalities from 0 to 100% were expected from the lower to upper thermal limits respectively, thereby providing suitable data for LT_{50} analyses. Temperature chambers and individual housings were checked for survival every 24 h for the duration of the growth experiment, although for the purposes of the LT_{50} experiments, survival data for only the first 25 days (600 h) were used. After being gently prodded or squirted with water from a pipette, individuals that did not respond with any signs of movement were considered dead and were subsequently removed from the experiment and placed in 70% ethanol for reference. The same setup was used simultaneously for both the growth experiment and the LT_{50} experiments, and it should be noted that individuals were provided with a source of food for the duration of the LT_{50} experiment (see temperature treatments section for details). Individuals that emerged to the subimago stage during the course of the 600 h LT_{50} experiments, though few in number, were *not* recorded as mortalities in the experiments and were instead recorded as surviving individuals for the purposes of mortality calculation. LT_{50} values were specifically noted at the 168 h duration, as no emergences were recorded at any treatment during this time period thus providing comparable LT_{50} results between individuals from the Molenaars River and Window Stream sites at each temperature treatment without any potential effects of emergence.

6.2.3 Statistical analyses

For the LT_{50} experiments, estimates of upper LT_{50} values were obtained using the program SPEARMAN (USEPA TSK Programme Version 1.5) which utilises the Trimmed Spearman-Karber statistical method (see Hamilton *et al.* 1977). No lower LT_{50} values were measured in this experiment. Upper LT_{50} estimates along with upper and lower 95% confidence limits were calculated at 24 h intervals for a 600 h (25-day) total duration. These individual estimates were plotted against temperature treatment and analysed using linear regression. Where mortality was not greater than 50% in any of the temperature treatments at a given duration, LT_{50} values could not be estimated using SPEARMAN. Similarly confidence intervals are not shown for data points where they could not be reliably calculated using SPEARMAN (where the Trim >50%) (see Hamilton *et al.* 1977 for details). All morphometric relationships were analysed using linear regression and plotted in R (R Core Team 2012). Growth measurements were also plotted using R (R Core Team 2012).

6.3 Results

6.3.1 Temperature treatments

Temperature records collected from loggers placed within each of the temperature chambers revealed that the heaters did not provide the same temperatures in each of the intended replicates, barring that of the control at 10°C (Table 6.1) where heaters were not used. Variation in some cases was fairly high and also differed among replicates (Table 6.1). For this reason temperature chambers, excepting those at 10°C were *not* considered true replicates and instead were used to provide a range of exposure temperatures from cold (± 10°C) to hot (± 27°C). Data obtained from each temperature chamber were therefore considered separately for both the LT_{50} and the growth experiments and this inadvertently provided a greater range of experimental temperatures than was originally intended. This additional information was used to further inform both experiments.

Table 6.1. Summary of water temperature data collected from experimental temperature chambers used in the growth and LT_{50} experiments. The total number of *L. penicillata* (n) from the Window Stream and the Molenaars River study sites exposed at each temperature treatment are indicated. ^{CON} denotes control temperatures.

	Experimental temperature chambers							
	10#1^{CON}	**10#2^{CON}**	**15#1**	**15#2**	**20#1**	**20#2**	**25#1**	**25#2**
Mean (°C)	10.68	10.70	15.76	16.83	19.76	21.75	25.61	26.96
Maximum (°C)	11.32	11.47	16.25	19.03	20.46	22.71	25.87	28.19
Minimum (°C)	10.39	10.39	15.03	15.58	17.77	18.87	23.49	24.34
Std. deviation.	0.096	0.116	0.180	0.313	0.400	0.557	0.226	0.251
Window (n)	7	8	7	8	8	7	7	8
Molenaars (n)	8	7	8	7	8	7	7	8

6.3.2 LT_{50} experiments

The LT_{50} values for *L. penicillata* from the Window Stream and the Molenaars River sites, calculated at 24 h intervals for the duration of the 600 h experiment, are shown in Fig. 6.2 and Fig. 6.3 respectively. Lethal temperatures calculated for individuals from both sites were generally stable up to the 216-240 h duration after which they declined with increasing duration as mortality rates increased, especially at the higher temperature treatments. Individuals of *L. penicillata* from from the Molenaars River exhibited upper LT_{50} temperatures that were higher than those from the Window Stream after both the 168 h (23.20°C vs. 26.45°C) and 600 h duration (20.13°C vs. 23.24°C) (Figs. 6.2, 6.3). Regression analyses of LT_{50} values against exposure duration for *L. penicillata*, calculated separately for both study sites, were statistically significant ($p < 0.01$). These regression analyses revealed that individuals from the Molenaars Rivers had a steeper gradient describing the relationship between LT_{50} values and exposure duration compared to those from the Window Stream ($y = -0.00859x + 27.69$, $R^2 = 0.93$, and $y = -0.00635x + 24.01$, $R^2 = 0.92$, respectively).

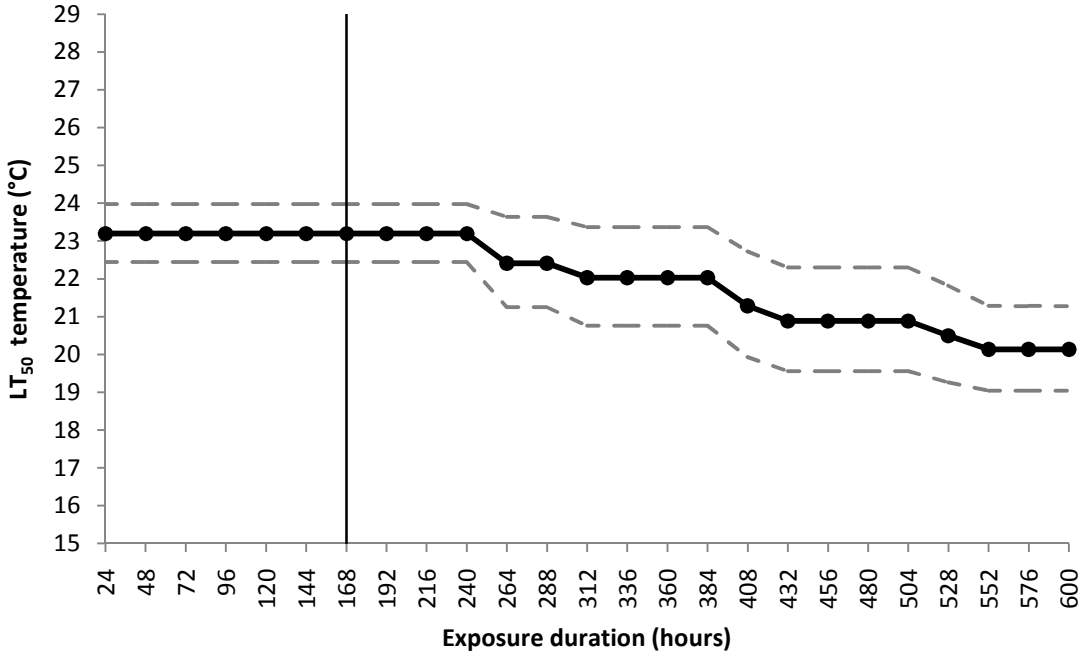


Fig. 6.2. LT_{50} values calculated using the trimmed Spearmann-Karber method every 24 h for a total duration of 600 h for *L. penicillata* collected from Window Stream. Dashed grey lines indicate upper and lower 95% confidence intervals. The solid vertical line denotes a duration of 168 h before which no emergence of adults was recorded in any temperature treatments.

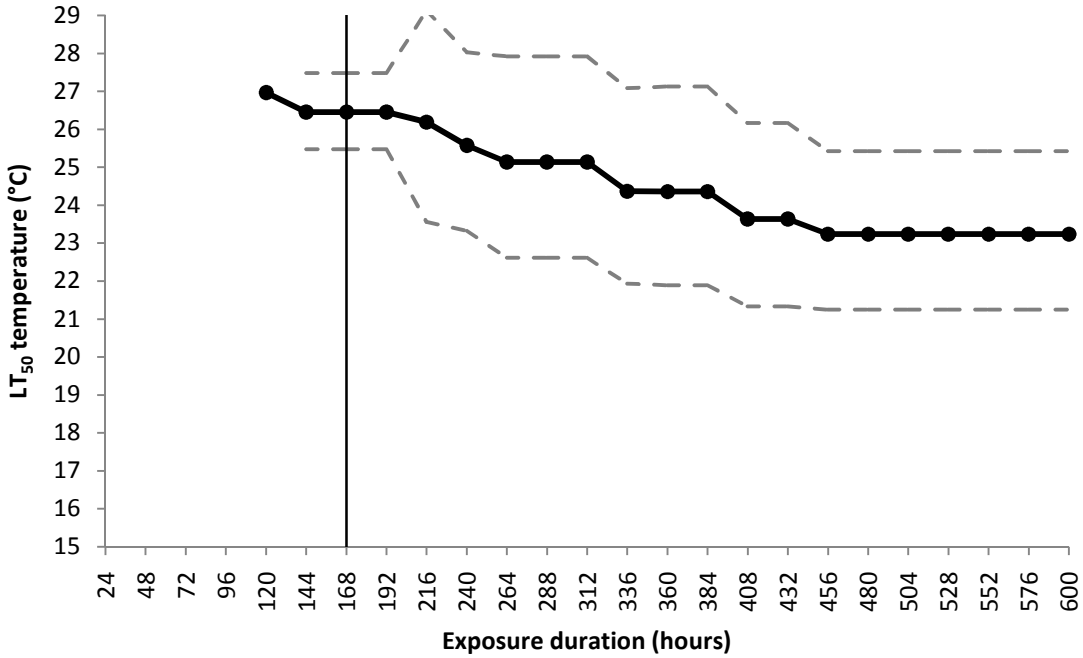


Fig. 6.3. LT_{50} values calculated using the trimmed Spearmann-Karber method every 24 h for a total duration of 600 h for *L. penicillata* collected from the Molenaars River. Dashed grey lines indicate upper and lower 95% confidence intervals. The solid vertical line denotes a duration of 168 h before which no emergence of adults was recorded in any temperature treatments.

The proportion of mortality after 168 h at each temperature treatment for *L. penicillata* from the Window Stream and the Molenaars River sites are shown in Figs. 6.4 and 6.5 respectively. *L.*

penicillata from Window Stream experienced 100% mortality at both the 25.61°C and the 26.96°C treatment after 168 h whereas mortality proportions of only 28.6% and 62.5% were recorded for individuals from the Molenaars River at the same temperature treatments after the same duration exposure. A maximum of ca. 68% mortality occurred in the Molenaars River population at 27.5 °C.

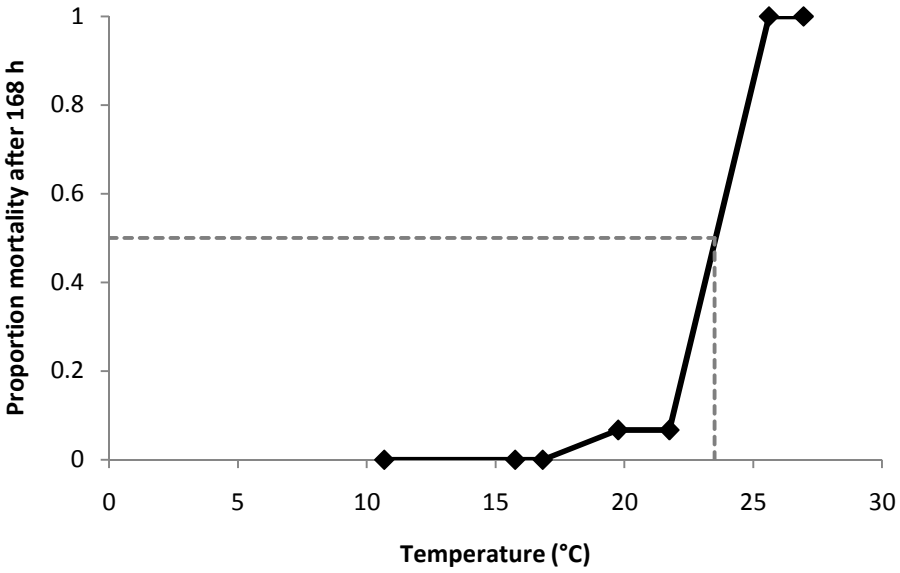


Fig.6.4. The proportion mortality at each temperature treatment of *L. penicillata* collected from Window Stream calculated after 168 h exposure. The dashed grey line indicates 50% mortality.

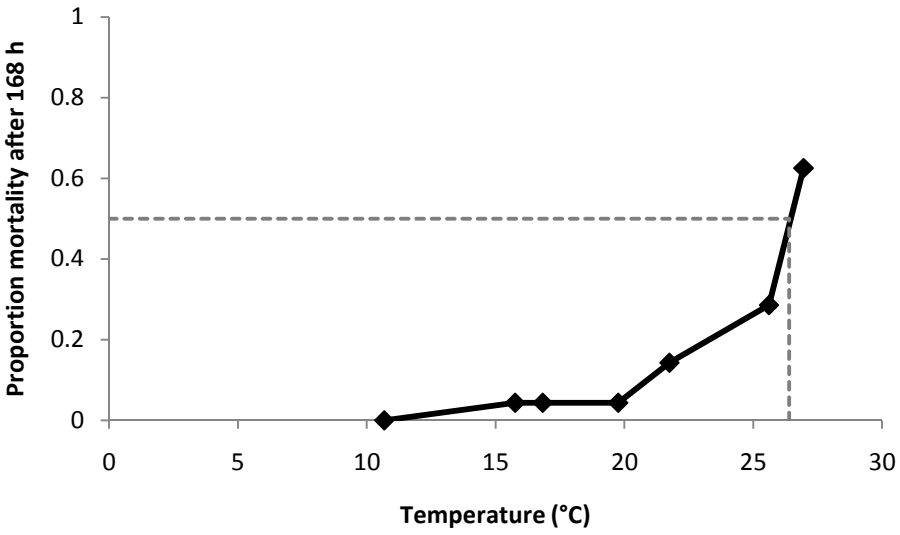


Fig. 6.5. The proportion mortality at each temperature treatment of *L. penicillata* collected from the Molenaars River calculated after 168 h exposure. The dashed grey line indicates 50% mortality.

6.3.3 Antennal growth

The number of antennal segments was used as an indicator of instar number (and relative age) of each individual of *L. penicillata.* The antennal segment count, as a surrogate for age, was used to stage specimens at the start of the experiment as well as at the penultimate moult – the black wingpad stage -

prior to emergence and also at death. These data are summarised and compared for each temperature treatment in Tables 6.2 and 6.3 respectively. In the experiments a greater proportion of individuals from the Molenaars River reached black wingpad stage and subsequently emerged than was observed for individuals from the Window Stream (Tables 6.2, 6.3). Additionally the average age or instar at which these emergences occurred at all temperature treatments for individuals from the Molenaars River was generally instar 14 (Table 6.3). This was two moults less than the average instar at emergence for individuals collected from Window Stream (instar 18 at control temperatures) (instar 17 at the 15.76°C and 16.83°C treatments) (Table 6.2). No moults to black wingpad stage were observed in specimens collected from Window Stream at temperature treatments of 19.76°C and higher (Table 6.2), while individuals collected from the Molenaars River were found to emerge at all temperature treatments (Table 6.3).

Table 6.2. Summary of age and black wingpad (BW)/emergence information of individuals of *L. penicillata* collected from Window Stream used in growth experiments. ^{CON} denotes control temperature. The two replicates at the control have been pooled and averaged.

	Temperature treatment (°C)						
	10.68^{CON}	**15.76**	**16.83**	**19.76**	**21.75**	**25.61**	**26.96**
Number of BW individuals	4	5	7	0	0	0	0
Total number of mortalities	11	2	1	8	7	7	8
Average instar at BW	18	17	17	-	-	-	-
Average instar at start of experiment	12	12	13	13	13	13	13
Average instar at death	17	17	17	14	13	13	13

Table 6.3. Summary of age and black wingpad (BW)/emergence information of individuals of *L. penicillata* collected from the Molenaars River used in growth experiments. ^{CON} denotes control temperature. The two replicates at the control have been pooled and averaged.

	Temperature treatment (°C)						
	10.68^{CON}	**15.76**	**16.83**	**19.76**	**21.75**	**25.61**	**26.96**
Number of BW individuals	10	6	7	7	2	1	2
Total number of mortalities	5	2	0	1	5	6	6
Average instar at BW	15	15	15	15	14	15	15
Average instar at start of experiment	13	13	13	14	13	14	14
Average instar at death	15	15	15	15	14	15	14

6.3.4 Growth experiments

Plots summarising size (increase in IOD) over time, of individuals of *L. penicillata* from both study sites at each temperature treatment are provided in Appendix 6A. The same data were collated to produce plots of growth (% change in IOD per day) against instar number at each temperature treatment for all individuals from the Window Stream and the Molenaars River sites respectively (Figs. 6.6, 6.7). Individuals from both the Window Stream and the Molenaars River exhibited higher growth rates at

higher temperatures, with fastest growth at 15.76°C and 16.82°C. Growth rates at the 19.76°C treatment were almost as low as growth in the controls (10.7°C) (Figs. 6.6, 6.7). Growth rates of *L. penicillata* from Window Stream in the 15.76°C and 16.82°C treatments initially increased for instars 12-14 after which growth rates declined to values only slightly higher (at instar 17) than what they were at instar 12. The trends in growth rate of *L. penicillata* from Window Stream at the 15.76 and 16.82°C treatments were in contrast to growth rates for individuals in a) the control where such a pronounced initial rise was not visible and values stayed more or less stable and b) the 19.76°C treatment where growth rates exhibited a slight decline. Similar trends were observed but were not as clear in the growth rates of individuals from the Molenaars River. At the warmest temperature treatments of 21.75, 25.61 and 29.69°C, insufficient data were available to produce growth curves for *L. penicillata* from either study site, owing to a combination of mortality and emergence.

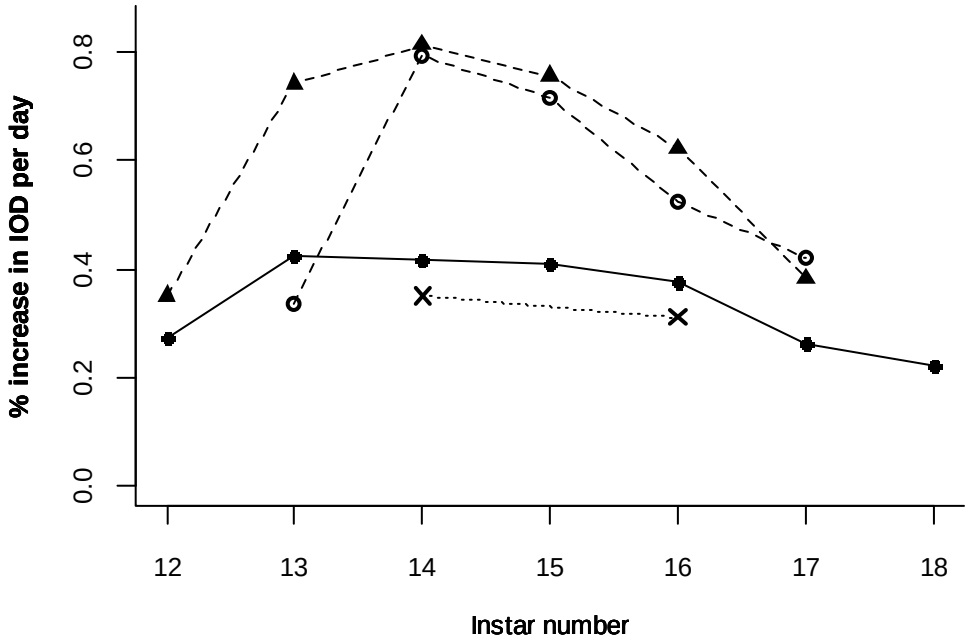


Fig. 6.6. Growth of *L. penicillata* (percentage increase in interocular distance -IOD- per day) collected from the Window Stream shown against instar number at different experimental temperature treatments. Solid line with closed circles = 10.68°C (both control replicates merged), dashed line with open circles = 15.76°C treatment, dashed line with closed triangles = 16.83°C treatment, dotted line with crosses = 19.76°C treatment.

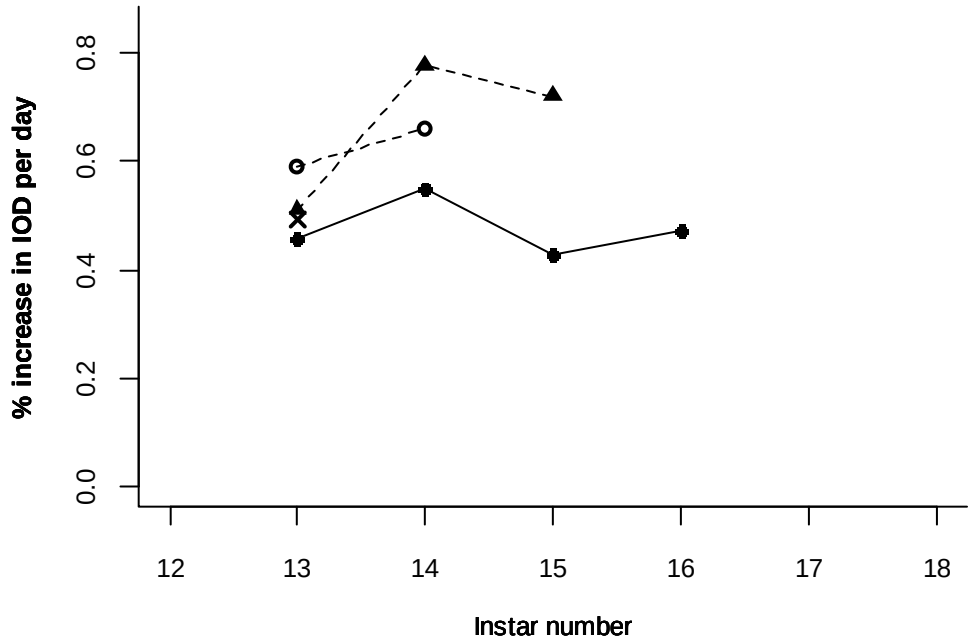


Fig. 6.7. Growth of *L. penicillata* (percentage increase in interocular distance -IOD- per day) collected from the Molenaars River shown against instar number at different experimental temperature treatments. Solid line with closed circles = 10.68°C (both control replicates merged), dashed line with open circles = 15.76°C treatment, dashed line with closed triangles = 16.83°C treatment, cross = 19.76°C treatment.

The average growth against temperature treatment for *L. penicillata* from both study sites is shown in Fig. 6.8. The graph comprises the average growth rate of all the individuals from each site in each of the temperature treatments, irrespective of instar number. *L. penicillata* from the two sites showed almost identical trends in growth, with optimum growth occurring in the 16.83°C treatment, after which growth declined in the 19.76°C treatment to levels similar to those observed in the control (Fig. 6.8.). The average proportion of growth per day in all temperature treatments was however consistently higher for *L. penicillata* collected from the Molenaars River when compared to *L. penicillata* from the Window Stream, which also revealed greater variability (Fig. 6.8.).

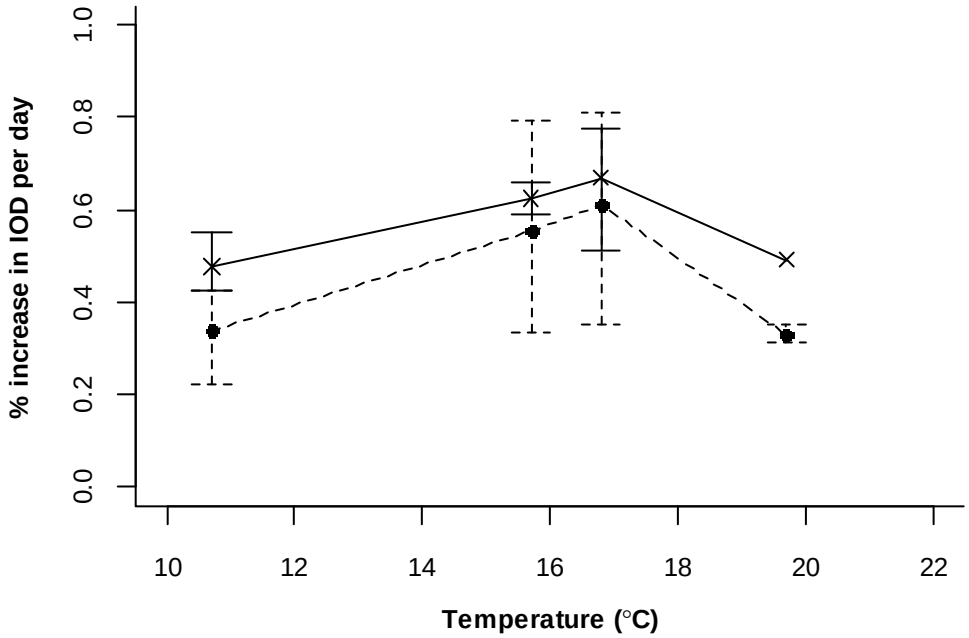


Fig. 6.8. Average growth of *L. penicillata* (percentage increase in interocular distance -IOD- per day) from collected from the Window Stream and the Molenaars River sites at different experimental temperature treatments. Dashed line with closed circles denotes Window Stream, solid line with crosses denotes Molenaars River. Bars indicate range of growth values.

6.3.5 Inter-moult duration

The average time (number of days) between moults was found to decrease by a factor of almost 2.5 at the highest temperature treatments compared to the control temperature for *L. penicillata* collected from both the Window Stream and the Molenaars River, with individuals from the latter exhibiting longer inter-moult duration at the control than the former (Fig. 6.9). Trend analysis revealed that power curves best explained the relationship between temperature and the average time between moults for *L. penicillata* from both sites (Fig.6.9). For *L. penicillata* from both sites the average time between moults decreased with increasing temperature, however a steeper gradient was observed in insects from the Molenaars site. The equation of the power curve fitted to the data from the Molenaars River was $y = 895.78x^{-1.37}$, $R^2 = 0.96$ while the equation of the curve fitted to the data from the Window Stream was $y = 239.14x^{-0.93}$, $R^2 = 0.93$ (see Fig. 6.9). On average moults at the control occurred every 30 days compared to every 15 days at 19.76°C and every 12 days at 25.61°C.

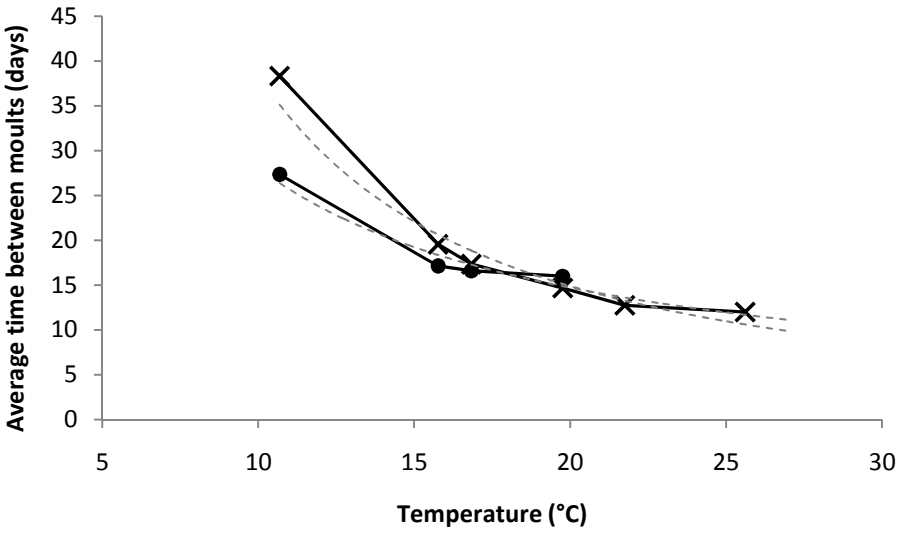


Fig. 6.9. Average time taken between moults of *L. penicillata* collected from the Window Stream and the Molenaars River sites at different experimental temperature treatments. Solid line with closed circles denotes Window Stream, solid line with crosses denotes Molenaars River. Dashed grey lines represent power curves fitted to data from both sites (Molenaars River: $y = 895.78x^{-1.37}$, $R^2 = 0.96$; Window Stream: $y = 239.14x^{-0.93}$, $R^2 = 0.93$).

6.3.6 Morphometric relationships

Linear trends revealing isometric growth were observed in morphometric relationships of all body size measurements recorded from individuals used in the growth experiments from both the Window Stream and the Molenaars River (Figs. 6.10 and 6.11 respectively). Summaries of the regression analyses however indicated that the gradients of these linear relationships differed slightly for *L. penicillata* collected from the two study sites (Table 6.4). It should be noted that these plots represent pseudo-replicated points as the same individuals (roughly 60 individuals per site) were measured each time they moulted.

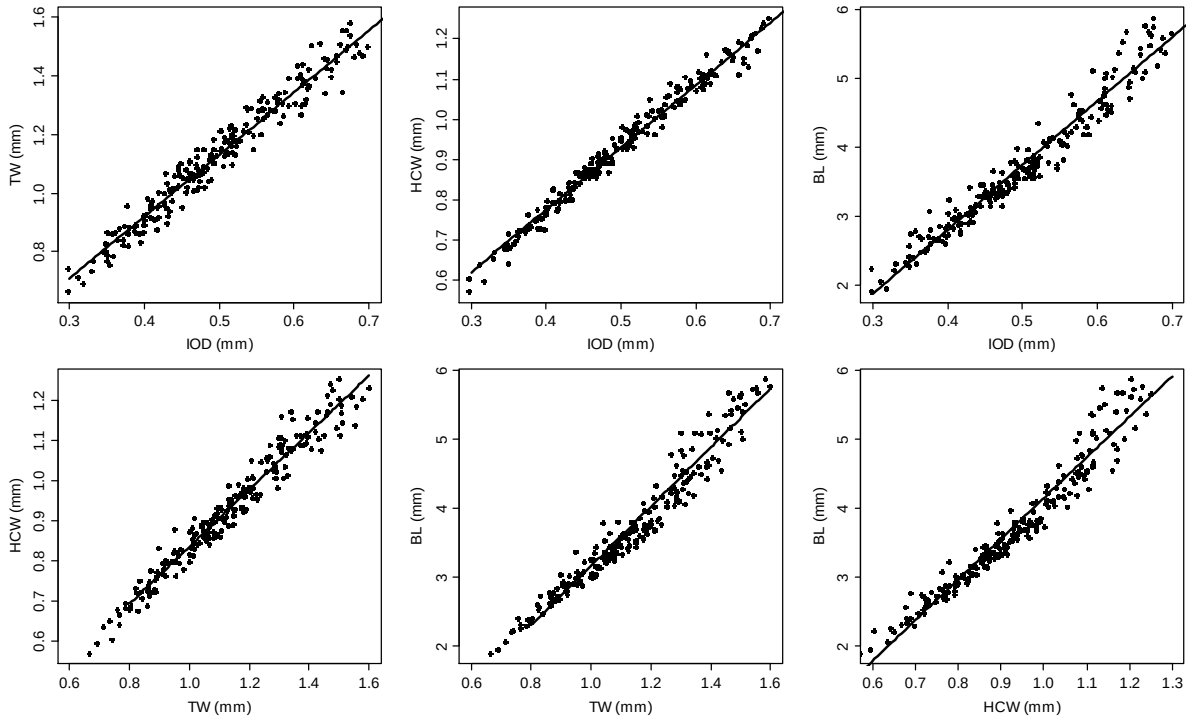


Fig. 6.10. Morphometric relationships between body part measurements (BL- body length, HCW- head capsule width, IOD- inter-ocular distance, TW- thorax width) of *L. penicillata* collected from the Window Stream used in growth experiments. Data points represent measurements recorded from the same individuals (initial n = 60) after each successive moult. Solid line represents regression line.

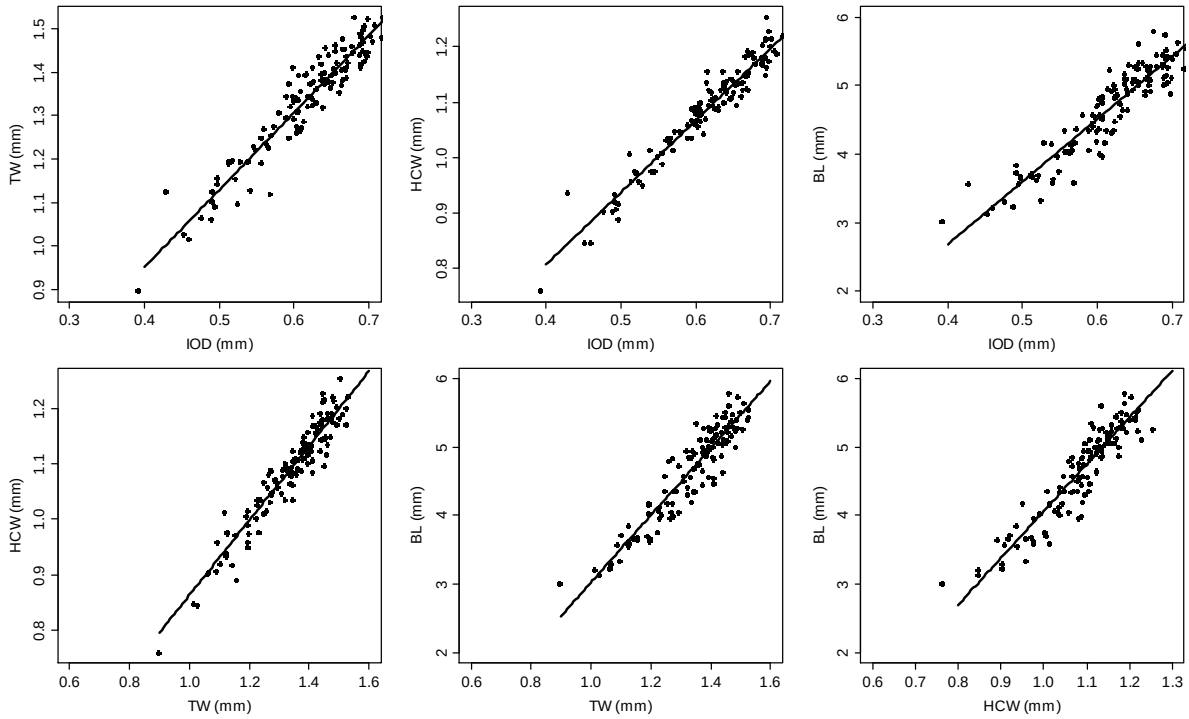


Fig. 6.11. Morphometric relationships between body part measurements (BL- body length, HCW- head capsule width, IOD- inter-ocular distance, TW- thorax width) of *L. penicillata* collected from the Molenaars River used in growth experiments. Data points represent measurements recorded from the same individuals (initial n = 60) after each successive moult. Solid line represents regression line.

Table 6.4. Regression analysis summary of morphometric relationships between body part measurements (BL- body length, HCW- head capsule width, IOD- inter-ocular distance, TW- thorax width) of *L. penicillata* collected from the Window Stream and the Molenaars River sites used in growth experiments. Standard deviation values are indicated in brackets.

	Gradient (*m*)	**Intercept (*y*)**	**R^2**	**Significance**
Window Stream				
TW vs. IOD	2.113 (0.028)	0.076 (0.014)	0.955	$p < 0.001$
HCW vs. IOD	1.555 (0.014)	0.152 (0.007)	0.980	$p < 0.001$
BL vs. IOD	9.292 (0.129)	-0.911 (0.066)	0.951	$p < 0.001$
HCW vs. TW	0.710 (0.009)	0.125 (0.011)	0.957	$p < 0.001$
BL vs. TW	4.296 (0.060)	-1.129 (0.070)	0.950	$p < 0.001$
BL vs. HCW	5.889 (0.089)	-1.738 (0.085)	0.942	$p < 0.001$
Molenaars River				
TW vs. IOD	1.786 (0.048)	0.236 (0.030)	0.884	$p < 0.001$
HCW vs. IOD	1.299 (0.026)	0.287 (0.017)	0.931	$p < 0.001$
BL vs. IOD	9.197 (0.297)	-1.001 (0.188)	0.841	$p < 0.001$
HCW vs. TW	0.673 (0.017)	0.190 (0.023)	0.900	$p < 0.001$
BL vs. TW	4.905 (0.146)	-1.883 (0.199)	0.863	$p < 0.001$
BL vs. HCW	6.843 (0.218)	-2.778 (0.241)	0.845	$p < 0.001$

Comparisons of the body size measurements of field-caught black wingpad individuals with those of black wingpad individuals obtained in the growth experiments is summarised Table 6.5. Body size measurements of naturally collected black wingpad individuals of *L. penicillata* from the Window Stream were on average marginally smaller (but with two to three fewer moults) than black wingpad individuals from growth experiments. In comparison, naturally collected black wingpad individuals from the Molenaars River were slightly larger, but with the same number of moults as those obtained from growth experiments. At the Window Stream, emergence of *L. penicillata* was observed to occur during the summer months of December through to January, with a peak in December. At the Molenaars River emergence was recorded from October through to November, with a peak observed in late October/early November (see Chapter 5).

Table 6.5. Comparison of body part measurements (BL- body length, HCW- head capsule width, IOD- interocular distance, TW- thorax width) and average instar of black wingpad specimens of *L. penicillata* obtained from laboratory growth experiments and also from natural populations from the Window Stream and the Molenaars River sites. Standard deviation values are indicated in brackets.

Site	N	Treatment	IOD (mm)	HCW (mm)	TW (mm)	BL (mm)	Ave. instar
Experiments							
Window	4	10.68°C	0.680 (0.048)	1.207 (0.068)	1.467 (0.124)	5.409 (0.412)	18
Window	5	15.76°C	0.657 (0.027)	1.158 (0.054)	1.470 (0.125)	5.457 (0.418)	17
Window	7	16.83°C	0.662 (0.018)	1.152 (0.032)	1.461 (0.081)	5.416 (0.269)	17
Molenaars	10	10.68°C	0.679 (0.027)	1.169 (0.038)	1.449 (0.064)	5.218 (0.357)	15
Molenaars	6	15.76°C	0.660 (0.027)	1.148 (0.023)	1.430 (0.059)	5.079 (0.215)	15
Molenaars	7	16.83°C	0.644 (0.011)	1.121 (0.020)	1.389 (0.038)	4.983 (0.253)	15
Molenaars	7	19.76°C	0.684 (0.019)	1.177 (0.042)	1.448 (0.043)	5.271 (0.239)	15
Molenaars	2	21.75°C	0.679 (0.019)	1.182 (0.047)	1.416 (0.047)	5.316 (0.284)	14
Molenaars	1	25.61°C	0.688	1.169	1.410	5.124	15
Molenaars	2	26.69°C	0.684 (0.033)	1.149 (0.055)	1.431 (0.112)	5.487 (0.206)	15
Natural							
Window	10	Natural	0.656 (0.026)	1.184 (0.065)	1.381 (0.068)	5.480 (0.375)	15
Molenaars	10	Natural	0.694 (0.054)	1.247 (0.077)	1.540 (0.154)	6.110 (0.506)	15

6.3.7 Comparison of natural stream temperatures at each site

Water temperature records collected at each study site prior to the experiment revealed that the Molenaars River was on average almost 2°C warmer than the Window Stream over the late-spring month of November and almost 2.5°C warmer over the summer months of December- February, with a noticeably higher standard deviation over this period (Fig. 6.12). Water temperatures appeared to converge somewhat at both sites over the autumn months of March-May, along with generally lower standard deviation values (Fig. 6.12). Water temperature data available from both sites are summarised in (Table 6.6).

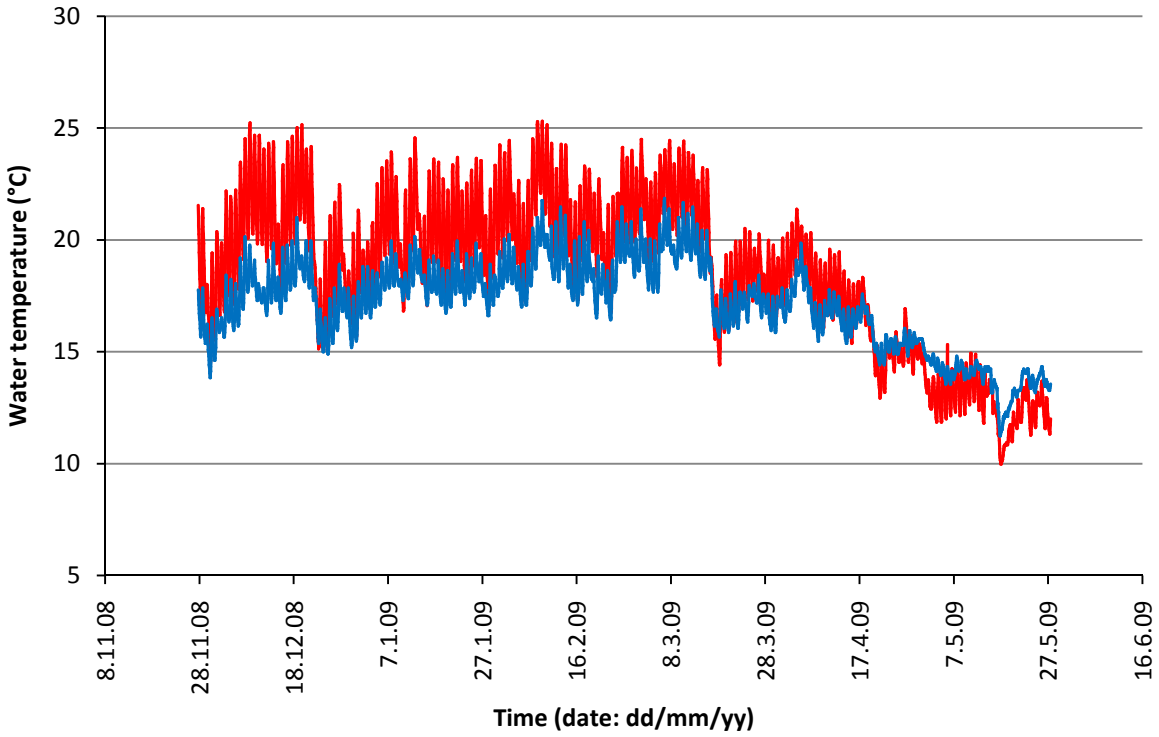


Fig. 6.12. Comparison of hourly water temperature data collected from the Window Stream (blue) and the Molenaars River (red) study sites from November 2008 to May 2009 prior to the commencement of the growth experiments.

Table 6.6. Comparison of seasonal average, maximum and minimum water temperature calculated from hourly water temperature data collected from the Window Stream and the Molenaars River study sites from November 2008 to May 2009 prior to the commencement of the growth experiments.

Site	Season	Month(s)/year	Ave. water temp. (std.dev.)	Max. water temp.	Min water temp.
Molenaars	late spring	Nov (2008)	17.88 (1.782)	21.53	14.43
Window	late spring	Nov (2008)	15.85 (0.975)	17.86	13.85
Molenaars	summer	Dec (2008)-Feb (2009)	20.46 (2.074)	25.31	15.06
Window	summer	Dec (2008)-Feb (2009)	18.08 (1.184)	21.76	14.61
Molenaars	autumn	Mar-May (2009)	16.66 (3.448)	24.48	9.98
Window	autumn	Mar-May(2009)	16.23 (2.152)	21.86	11.24

6.4 Discussion

Thermal tolerance limits and the sublethal effects of temperature on phenological traits such as growth, and emergence cues, gleaned from laboratory studies, provide a valuable component to life-history studies based on serial field samples. Such data not only allow for a more accurate interpretation and confirmation of field-collected life-history data but also provides information which is important for the generation of management guidelines for protection of aquatic ecosystems in the face of on-going alteration of thermal regimes. In this study the phenological traits of two genetically distinct lineages of the *L. penicillata* species complex collected from sites experiencing differing natural thermal regimes (Window Stream colder/less variable - Molenaars River warmer/more variable) were differentially

expressed when reared under laboratory conditions at a range of constant water temperatures, while photoperiod and food source were kept constant.

6.4.1 Antennal growth

This is the first study to detail the post-embryonic antennal growth as well as the total number of nymphal instars of a South African mayfly. The simple antennal growth sequence observed, in which the meriston of the first annulus divides into two to produce an additional segment each moult, was confirmed to continue until emergence. It provides a simple and useful means of accurately identifying the instar (a surrogate for age) of *L. penicillata* in ecological studies.

The total number of nymphal instars in both populations of female *L. penicillata* was found to be variable in the laboratory, both within and among temperature treatments, ranging from 13 to 16 in the Molenaars River population and from 16 to 18 in the Window Stream population. In contrast, the natural populations from both sites in the field were found to have consistently undergone 15 instars (i.e. the same number of antennal segments) by the time they reached maturity (black wingpad stage). While certain aquatic insect taxa have a fixed number of instars (e.g. most Trichoptera have five larval instars, one pupal instar and one adult instar), other insect orders (particularly basal groups such as Ephemeroptera - Brittain 1976a, and Plecoptera - Elliott 2009) can have a variable number. The total number of nymphal instars in *L. penicillata* is towards the lower end of the range that has been reported for other ephemeropteran families of between 10 and 50 (Brittain 1982).

6.4.2 Upper thermal tolerance limits

The 168 h (7-day) and 600 h (25-day) duration LT_{50} values determined in this study provide an indication of thermal tolerance limits over time frames that are better suited to gauge the gradual ecological-physiological effects of thermal alteration expected from deforestation, hypolimnetic and epilimnetic dam release strategies and even scenarios of global climate change (e.g. more variable climatic conditions for certain months of the year). Additionally, they offer a useful contrast to the more common and shorter term (4-day) LT_{50}'s or CTM experiments. LT_{50} values obtained at the 168 h (7-day) and 600 h (25-day) duration differed for *L. penicillata* collected from the two sites, with those from Window Stream showing thermal limits that were lower by almost 3.2°C (at both durations) compared to those from the Molenaars River. The values reported here for the 168 h duration (23.20°C Window Stream vs. 26.45°C Molenaars River) are substantially lower than those previously reported by Dallas & Ketley (2011) for this species (also collected from Window Stream) using a 96 h LT_{50} (29.5°C), an extrapolated 168 h LT_{50} (27.8°C) and CTM experiments (CTmax of 33.2°C). While the results from the 600 h duration show even lower thermal tolerance limits for *L. penicillata* from both sites (20.13°C Window Stream vs. 23.24°C Molenaars River) when compared to those of the 168 h duration, the results should be interpreted with caution owing to the effects of emergence on mortality calculations as

well as wider confidence intervals. Nevertheless these results suggest that this species may be more thermally sensitive than previously thought and somewhat closer to limits reported for other Gondwanan relictual fauna (e.g. Notonemouridae genus *Aphanicerca*) (see Dallas & Ketley 2011 and Dallas & Rivers-Moore 2012).

The above mentioned discrepancies in the LT_{50}'s of this study and that of Dallas & Ketley (2011) could however be as a result of a) a true reflection of thermal limits at longer duration exposures compared to short term exposures, b) different instar individuals collected at different times of the year being used in the two experiments, where thermal limits might potentially vary with instar (i.e. ontogenetically)[45] (see Lutz 1968, Heiman & Knight 1975, Bowler & Terblanche 2008), c) slightly different experimental methods (thermostatically controlled flow through system vs. aerated containers kept in different CE rooms for each temperature treatment) or d) the effects of different acclimation methods used (Terblanche *et al.* 2005, 2007, Jumbam *et al.* 2008). Recent experimental work (Dallas & Ross-Gillespie 2013, unpublished data) has shown that the thermal tolerance limits (168 h or 7-day LT_{50}) calculated for *L. penicillata,* collected from Window Stream in August 2013, were 22.15°C. This value is far closer to the 168 h LT_{50} of 23.2°C reported in this study and could suggest that *L. penicillata* have a higher thermal tolerance during early-instar stages compared to later-instar stages. Early-instar individuals would experience warmer water temperatures over the summer months of December/January through to March/April compared to later-instar individuals which experience lower water temperatures in early spring (September/October) following winter rains and high flows. The potential for ontogenetic shifts in thermal tolerance limits, while highly likely, would however need to be confirmed through further experimentation.

The findings of Chapter 3, suggested that there exists a putative species complex of *L. penicillata* with individuals from the Molenaars River and Window Stream sites representing two distinct lineages of this species complex. The results presented here indicate that differences do indeed exist between thermal tolerance limits of these two genetically divergent lineages of a larger species complex. Additionally, the LT_{50} results (both the 168 h and 600 h duration) are also in close accord to the findings of experiments conducted on the thermal limits for egg development in this species (*L. penicillata* collected from Window Stream) (Chapter 5) which showed an upper thermal limit for egg development of between 20 and 25°C - thus providing two independent lines of evidence for defining upper tolerance limits for this species. Overall these findings of thermal limits, both the LT_{50} results and the egg development results of Chapter 5, also coincide with reference water temperature data collected from the two sites (Fig 6.12 and Table 6.6) (see also Chapter 2). These data revealed that Molenaars River was on average approximately 2°C warmer than Window Stream with greater standard deviation as well as maximum temperatures experienced over the hottest parts of the year (December-February).

[45] *L. penicillata* used in the study by Dallas & Ketley (2011) were collected in early autumn (the months of March/April), where as in this study *L. penicillata* were collected in October.

In effect this suggests that warmer thermal regimes with greater variability can result in individuals evolving higher thermal tolerance limits and a greater capacity to respond to altered thermal regimes - obviously where this is within the limits of other aspects of their developmental biology (e.g. egg development limits, dissolved oxygen limits for respiration). The differences in the variability of thermal regimes at the two sites relates to the genetic divergence observed between the two populations at these sites - likely interspecific genetic differences.

6.4.3 Effects of temperature on growth and thermal optima for growth

Growth rate in relation to instar showed an initial increase at instars 12-13 in the control and 15.76°C treatments and at instars 13-14 in the 16.83°C temperature treatments, followed by a period of sustained higher growth which gradually declined at later instars prior to emergence (Figs. 6.6, 6.7). This initial increase in growth rate could be owing to individuals adjusting to water temperatures warmer than ambient stream temperatures from which they were initially collected. In contrast, the declining growth at later instars and larger body sizes is a trend commonly observed in many ectothermic organisms including aquatic insects (McDiffett 1970, Knight *et al.* 1976, Brittain 1983, Atkinson 1994, Angilletta *et al.* 2004). Such a decline could also be as result of greater amounts of energy potentially being directed to reproductive development (e.g. gonadal growth) and developmental reorganisation as opposed to growth (e.g. body size) at higher temperatures in late-instar aquatic insects especially those just prior to their penultimate moult or black wingpad stage (Knight *et al.* 1976, Angilletta *et al.* 2004).

When reared under the same environmental conditions in the laboratory, *L. penicillata* collected from the Molenaars River consistently exhibited higher growth rates at all temperature treatments when compared to *L. penicillata* collected from the Window Stream (Fig. 6.8). However, the thermal optimum for growth for individuals from both sites converged at around 15-18°C, with a noticeable decline in growth at temperatures exceeding 18°C. The apparent capacity of the population of *L. penicillata* from the Molenaars River to grow at faster rates than the population from the Window Stream at temperatures outside the thermal optimum is suggestive of adaptation to differing natural thermal regimes (Fig. 6.12 and Table 6.6). The Molenaars incurs a more variable thermal regime and as such, growth of individuals from this river could be expected to be less affected at a greater range of temperatures within the thermal tolerance limits (Fig. 6.12). Ultimately this site specific adaptation to differing thermal regimes and environmental conditions at the respective locations (Fig. 6.12 and Table 6.6) could result in genetic divergence between these populations. Alternatively, speciation and accumulation of general genetic divergence could include local thermal adaptation however local thermal adaptation might also be expected to occur in the absence of speciation. In either case, genetic differentiation has been shown in Chapter 3 and might also explain the slight differences observed in morphometric relationships (more specifically the regression gradients) for the two populations of *L. penicillata* (Figs. 6.10, 6.11 and Table 6.4).

Overall trends in growth rate and thermal optima for growth, calculated from laboratory experiments, coincided closely with those modelled from monthly field samples which comprised life-history data for populations of this species complex occurring in several localities (Chapter 4).

6.4.4 Effects of temperature on emergence

In *L. penicillata* from the Window Stream, the slowest growth rates observed in the control resulted in delayed emergence (two to three months), longer survival (up to six months longer than normal), additional moults (an average of three extra moults) being added to the life-history, as well as a slightly larger body size at emergence compared to individuals in warmer treatments and those found naturally (Table 6.5). At warmer temperature treatments (15.76°C and 16.83°C) inter-moult duration decreased by a factor of 1.6 (from 27 days at the control to 17 days at 15.76°C) (Fig. 6.9), resulting in the earlier onset of emergence compared to the control. Emergence timing at these warmer treatments coincided more closely with those that emerged naturally but were still delayed by up to one month. Individuals that emerged at these treatments were found to have added an additional two moults on average to their natural life cycle even though they emerged at sizes comparable to individuals that emerged under natural conditions. Such a finding supports the "compensation scenario" (Esperk *et al*. 2007) whereby additional moults and a greater number of instars is incurred under adverse conditions (i.e. conditions that are suboptimal for growth but where temperatures are not yet approaching the upper tolerance limits) where a species-specific threshold size has to be reached before emergence can take place (Nijhout 1975, 1994). The population from the Window Stream did not show accelerated emergence at temperature treatments warmer than the natural thermal regime at the time of emergence (19.76°C, 21.75°C, 25.61°C and 26.96°C). This might be because these temperature treatments approached upper LT_{50} tolerance limits for this population (168 h - 23.20°C and 600 h - 20.13°C) and as such only mortalities were recorded.

Individuals of *L. penicillata* from the Molenaars River at the control were found to emerge almost two months later than those under natural conditions, yet at the same average age/instar as and comparable body size to individuals that emerged under natural conditions (Table 6.5). This can be explained by the fact that average inter-moult duration was twice as long at the control (± 38days) compared to the 15.76°C treatment and this was observed to be even longer than Window Stream individuals at the control (Fig. 6.9). At warmer treatments (15.76°C and 16.83°C), body size measurements and instar at the time of emergence were comparable to individuals measured under natural conditions though emergence was still delayed by approximately one and half months. At both the 19.76°C, 21.75°C treatments emergence occurred approximately one month later than natural, while at the 25.61°C and 26.96°C treatments the timing of emergence of experimentally reared individuals appeared to coincide with the timing of emergence for individuals collected naturally. Similar findings of both advancement and retardation of emergence, in conjunction with changes in inter-moult, as a result of altered water

temperatures have been observed for other ephemeropterans: *Leptophlebia vespertina* (Brittain 1976a), *Leptophlebia cupida* (Clifford *et al*. 1979), six species of the genus *Ephemerella* (Sweeney & Vannote 1981).

The observed retardation and delayed emergence of *L. penicillata* in growth experiments compared to those under natural conditions thus appears to be an adaptive plastic response in the life-history (Atkinson 1994, Angiletta *et al.* 2004, Robinson & Buser 2007). This could be directly related to natural thermal regimes (Fig. 6.12) experienced at the respective sites to which the two species of *L. penicillata* have become adapted. However, the very different response in terms of inter-moult duration exhibited by *L. penicillata* from the Window Stream compared to *L. penicillata* from the Molenaars River at the control temperature provides further evidence for genetic differentiation between these populations. This plastic response of emergence observed for populations of *L. penicillata* could have allowed this species complex to become more temporally and spatially resilient to altered thermal regimes (Harper & Peckarsky 2006), in turn leading to its current widespread distribution throughout the Western Cape of South Africa. Based on its greater tolerance in the upper thermal zone, it is predicted that the *Lestagella* species at the Molenaars River might be expected to have a wider distribution within the Western Cape Province.

6.4.5 Water temperature effects on body size at emergence

The Window Stream population exhibited slowest growth rates, increased inter-moult duration and additional moults being recorded at the control temperature, which resulted in greatly delayed emergence (up to five additional months) and slightly larger body size at emergence compared to their counterparts in nature. These findings were in accordance with the temperature-size rule or thermal plasticity of body size (Atkinson, 1994, 1995, Atkinson *et al*. 2003, Angiletta *et al.* 2004).

However the population from the Molenaars River at the control also revealed slower growth, but had a comparatively higher inter-moult duration which resulted in no additional moults being added and a marginally smaller body size at emergence compared to their counterparts in nature. While Clifford *et al.* (1979) reported that inter-moult periods for *Leptophlebia cupida* became progressively longer as nymphs became older, in this case nymphs from the Molenaars River at the control temperature were not older than those from the Window Stream yet exhibited longer moult durations. Thus the different growth response at the low control temperatures between these populations, under the same laboratory conditions, is attributed to genetic differences. Additionally the findings that a) the experimental population from the Molenaars River was slightly smaller in body size at the time of emergence at the colder control temperature when compared to their natural counterparts and b) that no major differences were observed between experimental individuals that emerged at each temperature treatment, are intriguing as these are not in accordance with the temperature size rule (Atkinson, 1994, 1995, Atkinson *et al*. 2003, Angiletta *et al.* 2004). This may also be attributable to the natural variation of body size

measurements observed at each instar (Table 6.5) or possibly to a small degree of intraspecific genetic divergence between individuals used in the experiments. However, another perhaps more plausible explanation for this might be a nutritional factor (Brittain 1976b, Brittain 1982, Cabanita & Atkinson 2006). While both laboratory populations were provided with excess food, this food was collected from the Window Stream a shaded stream with different environmental conditions (potentially lower algal production) compared to the Molenaars River (an open site with higher algal production). As such, the population from the Molenaars River was provided with a food source collected from the Window Stream to which the population naturally occurring in this stream could have been pre-adapted to but not the population from the Molenaars River - potentially resulting in slightly reduced growth. This said one cannot discount other factors not measured in this study (e.g. effect of variable thermal regimes as opposed to constant thermal regimes on growth, effects of varying food availability under natural conditions to growth) that may have resulted in such findings, especially since growth rates of ephemerellid mayflies from both open and closed sites in a study by Hawkins (1986) were shown to be similar.

6.4.6 Experimental setup and limitations

The experimental flow-through system designed and used in this study proved to be efficient, reliable and cost effective for rearing *L. penicillata.* The same setup could be used to rear many different species of aquatic organisms with simple modifications made to the housings and adjustments of the flow rates of the pumps. Basic pilot studies would also be beneficial as they can be used to determine the correct food source/rate of food change.

Water temperatures collected during the course of the growth experiments using HOBO® TidbiT® v2 water temperature loggers (Onset Computer Corporation 2008) revealed that heaters used in the experimental setup were unreliable even after being calibrated against a thermometer. It is therefore suggested that all future calibration procedures be undertaken using similar loggers (logging at 15 minute intervals) over a period of at least two days. Alternatively, with a larger budget, higher quality heaters could be used or the system could be modified for use in precision water baths. Despite the fact that heaters were unable to provide replicate temperatures at the intended interval of 10°C, 15°C, 20°C and 25°C, the final temperature range obtained in the static temperature setup provided a suitable experimental method for determining growth rates and also thermal tolerance limits using LT_{50} procedures.

Future studies of this species should aim to determine growth rate and secondary productivity for the entire life cycle. Smaller individuals, perhaps even first or second instar, can be used to commence the growth experiment and sex can be determined retrospectively to compare growth rates in male and female individuals. Information of this sort will help to determine whether different thermal optima for growth exist at different stages of development or at successive instars. For instance several studies

(Brittain 1976a, Sweeney & Vannote 1978, Clifford *et al.* 1979) have shown that mayflies exhibit ontogenetic shifts in growth rates. LT_{50} experiments could thus also be repeated over a long term but with individuals of different instar stages/relative ages to determine if indeed upper thermal tolerance limits too also change at different stages of the life cycle. Additionally growth rates at all instars can be compared to determine whether a) earlier-instar individuals might exhibit higher growth rates in comparison with later-instar individuals and b) whether a period of synchronisation is achieved through rapid growth, that exists in later-instar individuals and 60-90 days prior to the penultimate moult of many species (see Newbold *et al.* 1994), under controlled constant temperature environments (i.e. in the absence of environmental cues such as photoperiod, increasing variation in water temp, increasing/decreasing availability of food).

CHAPTER 7

General discussion and synthesis

A central theme of ecology is to understand and explain the spatial and temporal differences in species assemblages in different environments at various scales (Levin 1992). This entails identifying and investigating the environmental triggers and drivers of life cycle phenology. Species responses incorporate a number of inter-related traits combined into complex adaptations that are ultimately expressed as life-history phenology (see Verberk *et al.* 2008). Aquatic insects inhabit variable and dynamic systems and are subject to a wide range of physical fluctuations ranging from floods to droughts and also temperature changes (Resh *et al.* 1988, Lytle 2002, Arthington *et al.* 2006). Because of the dynamic habitat they occupy and the numerous selective pressures they face (e.g. food availability, predation, varying thermal regimes, environmental disturbances such as floods), aquatic insects in lotic systems provide an ideal framework to understand how spatial and temporal differences in the adaptations of aquatic organisms, specifically life-history patterns, evolve in response to the environment. Streams in Mediterranean-type ecosystems, as found in the south-western parts of South Africa, are well suited to such investigations as they are generally considered to be governed by predictable seasonal cycles (Gasith & Resh 1999, Dallas 2013) yet are also "predictably unpredictable" (Davies *et al.* 1995) (i.e. high flows are predictable every winter, yet the magnitude and number of floods within a season are unpredictable). In this thesis six perennial streams in the Western Cape of South Africa were selected to provide a gradient of thermal and hydrological regimes (Chapter 2) against which to contrast the life-history traits of three genera of aquatic insects (*Lestagella*: Ephemeroptera, *Aphanicercella*: Plecoptera and *Chimarra:* Trichoptera).

Life-history patterns of aquatic insects elsewhere have revealed great variability across different environments, especially with respect to aspects such as voltinism, larval development time, timing of hatching and emergence synchronisation (Clifford 1982, Brittain 1990, Merritt & Cummins 1996). Variability in life-history patterns both within and among populations of different (and even the same) species occurs as a result of the interaction of genetics and the environment, which together drive trait adaptations (Scheiner 1993, Lytle 2008, Pfennig *et al.* 2010). Ideally an organism would be able to adjust its life-history pattern or behaviour to either cope with or avoid the environmental change or disturbance. Phenotypic plasticity facilitates this process. The local adaptation of metapopulations of a species is a first step to coping with local environmental conditions. This adaptation can result in some degree of genetic variation between the metapopulations giving rise to a number of genotypes. Certain traits can be plastic for a given genotype though and the degree to which these plastic trait responses effect classical adaptation lies in the process by which they effect fitness and are exposed to selection (Whitman & Agrawal 2009, Robinson 2013). Subsequent phenotypic plasticity of the genotypes therefore likely reflects a finer scale process by which organisms cope with/avoid environmental changes. Concurrently though, the adaptive process can also be constrained by the phylogenetic history of the organism (Resh *et al.* 1994, Lytle 2008). For example some basal taxa are evolutionary conservative, with all clade members being restricted to certain life cycle durations, number of moults and also development trajectories and this in turn can define the environments they can inhabit as well

as their distribution ranges (see McKie *et al.* 2004, Poff *et al.* 2006b). Studies which are able to incorporate molecular analyses in conjunction with life-history information are therefore able to provide greater insight into the role that phenotypic plasticity and genetics play in governing the variability in life-history patterns sometimes observed at the population level (Avise 1994). For this reason molecular analyses were used to compare the genetic divergence of target taxa amongst sites used in this study (Chapter 3) in order to better interpret detailed life-history information (Chapter 4).

A common shortfall of life-history studies that use fairly course sampling regimes and/or inappropriate sampling equipment, however, is a lack of information regarding the fate of eggs and first-instar larvae (Hynes 1970, Suter & Bishop 1980, Butler 1984, Elliott 2009). Such information is crucial to correctly interpreting life-history patterns and the timing of specific traits, e.g. oviposition and hatching (Knispel *et al.* 2006). In this study while a fine mesh net (80µm) was used to ensure the collection of first-instar hatchlings, gaps were nevertheless observed in the field-collected life-history data. Detailed egg-development experiments for each of the three target taxa in relation to water temperature were therefore conducted (Chapter 5) to better elucidate field-collected life-history data, as well as to provide useful information regarding the thermal tolerances of egg development.

Nevertheless, life-history information gathered *in situ* for populations in different environments, even when accompanied by molecular analyses, may in many cases be insufficient to determine how trait values respond to specific environmental conditions (e.g. temperature) via phenotypic plasticity. This is because of any number of additional site specific conditions that are not held constant among sites that can influence life-history responses (e.g. food availability, predation) (Sweeney & Vannote 1986, Robinson *et al.* 1992). In these circumstances a combination of field data and controlled-environment experiments conducted in the laboratory or reciprocal transplant experiments provides a basis for teasing apart the extent to which certain life-history traits are plastic vs. genetically controlled and which factors are responsible for driving these specific adaptive or non-adaptive traits (e.g. Postma *et al.* 1995a, 1995b, Miller & Hendricks 1996, Zhang & Malmqvist 1996, Shama & Robinson 2009). Such an approach, using a controlled-environment laboratory experiment, was used here to investigate a number of traits including growth rate, timing of emergence, size at maturity and inter-moult duration, in individuals of one of the target taxa, *Lestagella*, collected from two different study sites (Chapter 6).

Overall, understanding how phenotypic plasticity or trait heritability can affect an individual organism's or a population's ability to cope with different environmental conditions through the expression of variable life-history patterns is important for effective conservation, setting appropriate guidelines for environmental flows and the Ecological Reserve, informing policy and also predicting impacts of global climate change and the ability of species to adapt to such changes.

7.1 The evolution of life-histories

Major driving forces behind such adaptations are temporal and spatial changes in a habitat which in turn affect its suitability for feeding, survival, reproduction and the overall success of a species. Over time, the environment changes and the degree, duration and predictability of these changes are key factors influencing key adaptations (Verberk *et al.* 2008). Adaptations can be morphological, behavioural or physiological in nature and collectively they influence an organism's life-history pattern (Lytle 2008, Robinson 2013). Owing to the dynamic nature of lotic ecosystems, different suites of abiotic variables (e.g. substrate, channel morphology, depth), environmental conditions (e.g. water quality, temperature, flow) in conjunction with varying biotic factors (e.g. predation, competition) provide a multitude of habitats for aquatic insects in which to complete their life cycles. The influence of these variables on life cycle timing and duration are often interrelated and complex but have a pronounced effect. Aquatic insects exhibit a range of life cycles that vary in duration from less than three weeks (e.g. some Baetidae, Chironomidae, Culicidae) (Merritt & Cummins 1996, Reynolds & Benke 2005) to several years (e.g. Ameletidae, Elmidae, Perlidae, Polymitarcidae, and some genera within the Odonata and Megaloptera) (Pritchard & Zloty 1994, Sweeney *et al.* 1995, Merritt & Cummins 1996). Syntopic species do not necessarily all display a single life-history pattern and similarly those with the same life-history pattern are not always limited to the same habitat (Verberk *et al.* 2008). As the study of life-histories incorporates an organism's entire development from egg to senescence, for aquatic insects, this includes the close inspection of a number of life-history traits such as: life cycle length or total development time, growth rates, size at maturity, timing of emergence, adult life-span and dispersal ability, fecundity, egg development time-requirement, quiescence and diapause, timing of hatching and hatching success and mortality. Adaptations leading to changes in these, often interrelated, traits incur cost-benefit tradeoffs with respect to overall fitness (Stearns 1976, Southwood 1988, Stearns 1989, Rowe & Ludwig 1991, Nylin & Gotthard 1998). Natural selection pressures then ensure that adaptations with the lowest cost-benefit tradeoffs prevail in a given landscape.

As such, aquatic insects have evolved a number of life-history patterns and adaptations allowing them to maximise fitness, utilise favourable periods for growth and timing for emergence, evade unfavourable conditions (e.g. droughts, spates and lethal temperature thresholds), minimise competitive effects and essentially survive comfortably within the constraints of the abiotic and biotic environment (Merritt & Cummins 1996). For example when organisms are faced with "seasonal time constraints" (Rowe & Ludwig 1991, Johansson & Rowe 1999) for development (such as winter freeze periods or summer droughts) and growth rates are maximised, trade-offs between age and size at maturity can be expected. Insects may develop at a faster rate and emerge earlier in order to avoid unfavourable conditions, but at the cost of emerging at a smaller size thereby reducing fitness (Nylin & Gotthard 1998). However when differences in growth rate have adaptive value that is optimised, such trade-offs

are not necessarily expected and some insects that develop at faster rates and emerge earlier can still do so at larger body sizes (Abrams *et al.* 1996, Nylin & Gotthard 1998, Shama & Robinson 2006).

The degree to which growth rate can be optimised in aquatic insects occurring in different environmental conditions is particularly important when considering the evolution of life-histories, their flexibility and specifically traits such as development time, emergence synchronisation and voltinism.

Clifford (1982) showed that 60% of Northern Hemisphere mayflies exhibited a univoltine life cycle, followed by 30% exhibiting multivoltinism (the majority of which were baetids) and only 4% semivoltinism and 3% showing variable life cycles (e.g. switching from bivoltinism to univoltinism - again recorded mainly in Baetidae). He noted extensive flexibility in many but not all mayfly species in different parts of their distribution range. In stark contrast to Ephemeroptera which show a propensity for shorter life cycles, Plecoptera exhibit longer, largely univoltine and semivoltine life cycles with virtually no occurrence of multivoltinism (Brittain 1990), with an exception being *Nemurella pictetii* (see Lieske & Zwick 2008). The Trichoptera on the other hand have also exhibited a mixture of mainly univoltinism as well as semivoltinism, and multivoltinism, with large degrees of flexibility recorded from species in different parts of their range (see Cudney & Wallace 1980, Elliott 1981, Resh *et al.* 1984, Bowles & Allen 1992, Reiso & Brittain 2000)

Life-history duration of both Ephemeroptera and Plecoptera increases with latitude, with a general tendency for semivoltinism in arctic and alpine areas which represent the limit of the distribution range of Ephemeroptera but the optimum distribution range for Plecoptera (Clifford 1982, Brittain 1990). Univoltinism in both orders is most common in cool temperate areas while multivoltinism and continuous asynchronous development (Ephemeroptera only) occur in the warm temperate and tropical areas (Brittain 1990). Because of these life-history differences (primarily with regard to voltinism), Ephemeroptera and Trichoptera are at an advantage over Plecoptera in warmer environments, but not in cold environments which are better suited for Plecoptera (Brittain 1990). Interestingly, while no clear relationship has been observed between body size and voltinism with respect to the phylogeny of extant Ephemeroptera species, in many cases small body sizes are associated with multivoltinism and almost all larger body sizes of Ephemeroptera and Plecoptera species with semivoltinism (although there are some large Ephemeroptera that do not exhibit semivoltinism) (Clifford 1982). For Ephemeroptera of intermediate body size no, clear trends are evident (Clifford 1982)- this perhaps owing to the fact that the size-at-maturity rule can become decoupled or show exceptions when growth rate is adaptive (Abrams *et al.* 1996). Although not definitive, the number of instars or moults exhibited during larval development does however appear to correlate broadly with patterns of voltinism, with fewer moults generally evident in multivoltine species and a greater number of moults in semivoltine species.

Most ephemeropteran families exhibit 12-19 instars, however estimates as high as 35-45 instars have been made for *Stenonema canadiense,* a univoltine species of Heptageniidae (Ide 1935) and up to

approximately 34 instars for another univoltine species *Leptophlebia cupida* (Clifford *et al.* 1979). However, *Leptophlebia vespertina* also a univoltine species, revealed 17-19 instars (Brittain 1976a). In contrast, the oviviparous multivoltine species *Callibaetis floridanus* is estimated to have 9-11 instars (Trost & Berner 1963) while another multivoltine species, *Cloeon* sp. has between 10-14 instars (Gupta *et al.* 1993). Degrange (1959), however, reported between 20-29 instars for the multivoltine baetid species *Cloeon simile*. In Plecoptera the average number of instars is reported to range from 12-33 (Elliott 2009) but this is based on a limited number of studies. Elliott (2009) provided a comprehensive study of the number of larval instars in a number of European species and reported that for herbivorous species of Plecoptera the average number of instars ranges from 10-17, while for smaller carnivorous species this value ranges from 12-16 and in larger carnivorous species it reaches 12-23. The semivoltine species *Dinocras cephalotes* takes three years to develop to maturity, progressing through 15-19 instars (Elliott 2009) although 33 instars have also been reported (Schoenemund 1925) for this species. This is close to the values reported for two other semivoltine species that can take up to three years to develop *viz. Perla bipuncta* and *Perla abdominalis* which exhibit 18-23 instars and up to 22 instars respectively (Samal 1923, Hynes 1970, Elliott 2009). In contrast the large majority of Trichoptera have only 5 larval instars followed by a pupal stage (Cudney & Wallace 1980, Elliott 1981, Resh *et al.* 1984, Bowles & Allen 1992, Reiso & Brittain 2000). In general the basal insecta have a greater number of instars than the groups exhibiting a pupal stage and this is largely a phylogenetic pattern rather than an ecological pattern.

As the trend between the number of instars and voltinism is not exact, it is important that additional confounding factors such as egg development duration (along with delayed hatching), inter-moult duration, and sexual dimorphism are considered when interpreting voltinism in relation to the number of instars alone. This is because a short inter-moult duration for example would make it possible for a multivoltine species to exhibit a relatively large number of instars. Equally so, a delayed or extended hatch period of eggs would allow species to exhibit multiple or overlapping cohorts (appearing multivoltine) even if larvae exhibited a relatively large number of instars.

Studies on temperature/development relationships in aquatic insects, particularly those focussed on the egg stage, therefore provide a particularly useful component for investigations of the life-history patterns, evolutionary origins and distribution ranges adopted by different taxa (Clifford 1982, Pritchard & Mutch 1985, Pritchard *et al.* 1996). This is because the egg stage is considered to be more independent of the external environment compared to the larval or adult stages, thereby retaining the more primitive characteristics present in the ancestral lineage (Brittain 1990).

This said, a pertinent question arises as to how much of the observed differences in voltinism, life-history pattern and distribution range is as a result of phylogenetic constraint versus true phenotypic plasticity?

In a study by Poff *et al.* (2006b) examining trait responses in relation to phylogeny for aquatic invertebrate taxa occurring across a range of environments, it was shown that life-history traits (*viz.* voltinism, development, synchronisation of emergence, adult life span, adult ability to exit, adult ability to survive desiccation) are less labile and therefore more phylogenetically constrained than other ecological and or behavioural traits (e.g. thermal preference – for stenothermy, or eurythermy - and habitat preference) with the exception of voltinism - which was comparatively quite labile when a large spectrum of lotic insects were considered. However when only EPT taxa were considered voltinism was found to be less labile owing to some taxonomic groups showing less variation in voltinism among genera.

They suggest that this finding might relate to the fact that convergent evolution would play a role in shaping traits (e.g. behavioural and ecological) that are more labile and responsive to local selection therefore a species' phylogenetic history would not necessarily correlate with the presence of a specific trait. Furthermore they propose that it makes logical sense if one considers that taxonomic constraints in life-history traits can relate to reproductive success (e.g. emergence synchrony) and the continuation of the species, whereas diversification of ecological and behavioural traits relate to the optimisation of spatially and temporally variable local resources and the habitat.

Phylogenetic constraint may however play a significant role in shaping the life-history patterns (particularly development time) that are exhibited by relictual Gondwanan aquatic insect taxa that are over-represented in Mountain streams of the Western Cape (Picker & Samways 1996, Day 2005).

7.1.1 The role of phenotypic plasticity

Identifying the causal mechanisms for adaptation of species traits is often very difficult, but is normally attributed to a complex interaction of genetic and environmental effects (Via & Lande 1985, Scheiner 1993, Lytle 2008, Pfennig *et al.* 2010). While for a long time genetic mutation was considered to be the ultimate basis for evolutionary theory, phenotypic plasticity has now become widely recognised as being adaptive (i.e. acted upon by natural selection) in many circumstances (West-Eberhard 1989, 2003, Pfennig *et al.* 2010). Phenotypic plasticity, through processes such as genetic accommodation and genetic assimilation, is now considered (along with environmental variation, genetic mutation and natural selection acting on gene frequencies) to be an equally important means of driving speciation, diversity, micro- and even macro evolution (West-Eberhard 1989, 2003, Price *et al.* 2003, Schlichting 2004, Lytle 2008, Whitman & Agrawal 2009, Pfennig *et al.* 2010).

Phenotypic plasticity can be described as the capacity of a single genotype to display variable phenotypes that are induced by exposure to different environmental conditions (Pigliucci 2001, Fordyce 2006, Whitman & Agrawal 2009). Such phenotypically plastic changes can be either adaptive (permanent changes sometimes beneficial as a result of past selection - e.g. life-history shifts, diapause)

or non-adaptive (reversible – e.g. nutrition effects on body size, manipulation of hosts by parasites or pathogens) and are often brought about by environmental stimuli (e.g. temperature or oxygen levels) or specific cues (e.g. photoperiod or chemicals released from predators) which can also be harmful (e.g. toxins) (West-Eberhard 2003, Whitman & Agrawal 2009). Responses can be either active anticipatory plasticity (i.e. where an organism uses abiotic cues of seasonal change - such as photoperiod or drying - to avoid imminent environmental change) or passive responsive plasticity (i.e. where an organism experiences the environmental change and then reacts) (Whitman & Agrawal 2009).

Shama & Robinson (2006) using a controlled field experiment showed that populations of the alpine caddisfly *Allogamus uncatus* from permanent and temporary streams in Switzerland exhibited sex-specific life-history plasticity in response to photoperiod (treatments were current vs. late- where a longer photoperiod was used to impose a time constraint by mimicking late summer) and hydroperiod (treatments were constant vs. drying). In the late photoperiod but constant hydroperiod treatment both sexes from temporary as well as permanent streams exhibited a shortened developmental period. Additionally the growth rates of both sexes from both stream types were affected by both hydroperiod and photoperiod cues. Generally growth rates increased with a combination of a late photoperiod and a drying hydroperiod, but sexually dimorphic growth was evident in that females had higher growth rates than males. Males and females from the permanent stream showed a decline in growth rate and mass at emergence under the current photoperiod but drying hydroperiod treatment. In all cases sexually dimorphic growth meant that males emerged earlier and smaller than females but interestingly only female mass at emergence (from both stream types) differed among both time constraint treatments, while male mass at emergence from both stream types were found to stay constant. In a subsequent study using the same species Shama & Robinson (2009) showed that populations can exhibit markedly different phenotypically plastic responses even at a microgeographic scale. Phenotypically plastic responses in size at metamorphosis for *Baetis bicaudatus* have also been suggested to have been the result of predator pressure, namely fish and carnivorous Plecoptera (Peckarsky *et al.* 2001, 2002, 2005). In their study, smaller size at metamorphosis in males and females was observed in streams containing fish versus those without fish and further decreased as densities of predatory Plecoptera increased. Furthermore Deere *et al.* (2006), showed that thermal tolerance limits (both upper and lower and super cooling point) in five oribatid mite species varied in a phenotypically plastic manner in relation to both acclimation temperature (see also Chown *et al.* 2009) and the predictability of the environment (marine vs. terrestrial) they inhabit. For one species *Halozetes begicae* the upper lethal tolerance limit differed by up to 4.16°C depending on acclimation temperature, while the super cooling point for *Halozetes marionensis* differed by up to 15.3°C.

Some of the most dramatic phenotypically plastic responses are those related to shifts in voltinism with some of the most striking examples coming from aquatic insects. A case in point is a study conducted by Sand & Brittain (2009) in which the voltinism of a single species *Baetis rhodani* was observed to

shift from univoltine to semivoltine at increasing altitudes along the same river system in the mountains of central southern Norway. The authors suggested that a combination of differences in water temperature and possibly other factors such as food resources were responsible for the observed shift.

7.2 So what do the taxa in this study reveal?

Field data collected for *L. penicillata, Aphanicercella* spp. and *C. ambulans* revealed subtle differences in life-history traits observed for the same species in the different study rivers in terms of size at maturity, growth rates, and the timing of hatching and emergence (Chapter 4).

At all study rivers *L. penicillata* exhibited a univoltine, slow seasonal cycle (Hynes 1970) with a single cohort or generation evident within a year. The larval development period typically extended from hatching during the early-summer (early December to early January) through to the emergence period which occurred over the late-spring to early-summer months of October to December. The timing of the emergence period appeared to coincide with the onset of warming water temperatures and abating flows, such that oviposition and egg development were prevented from occurring during a combination of highest water temperatures and lowest flow conditions during the mid-summer (late January to February) as well as during periods of higher flows occurring in autumn (March/April). However the timing of hatching was estimated to have occurred up to one month earlier in rivers with warmer overall water temperatures. Lower growth rates and smaller size at maturity were observed in rivers with colder overall water temperatures - this in disagreement with the temperature-size rule for ectotherms (Atkinson 1994, Angilletta *et al.* 2004); however several species of Ephemeroptera in particular have been cited as exceptions to this rule Atkinson (1995) presumably as a result of growth rate being adaptively controlled (Abrams *et al.* 1996). Additionally the onset of emergence was delayed and extended by up to one month in rivers with colder overall water temperatures.

For *Aphanicercella* spp. the life-history was similarly found to be that of a univoltine, slow seasonal cycle (Hynes 1970) in all rivers with a single cohort or generation evident within a year. The larval development period contrasted with that of *L. penicillata* and generally extended from hatching in the late winter months of August/September over summer to a more extended emergence period spanning from early to late winter (May/June-September). The timing of the emergence period coincided closely with the onset of high flows/floods and cold air and water temperatures, suggesting a) a possible flood avoidance mechanism or b) timed emergence for optimal conditions specifically for adult survival. Differences in life-history traits among the study rivers were likewise subtle, and included: hatching (different estimated hatch dates for the different species of *Aphanicercella* and variable hatch dates for the same species in different rivers); growth rates (most pronounced differences were between species, followed by water temperature with fastest growth and larger size at maturity occurring in rivers with colder water temperatures). Growth appeared slower over the warmer summer months and faster over

the colder winter months - however as data from cohorts from two different years spanned these periods, this pattern could not be confirmed.

In contrast to the above two taxa *C. ambulans s*howed a non-seasonal or asynchronous cycle (Hynes 1970), with multiple, potentially overlapping generations/cohorts occurring within the period of a year. Overlapping cohorts are typical of many Trichoptera (Elliott 1968, Ulfstrand 1968) and in this case for *C. ambulans* the cohorts were difficult to differentiate in spring and summer when growth appeared to be rapid. The voltinism or number of generations produced in a year by this species was suspected to vary amongst the study rivers in accordance to site groupings based on analyses of hydrological and thermal regimes. Trivoltinism was suspected in the study river with the warmest water temperatures (Wit), to bivoltinism in rivers with slightly cooler water temperatures (Molenaars, Elandspad and Wolwekloof) changing to univoltinism in the rivers with the coldest water temperatures (Eerste and Rooi-Els Kloof). Generally *C. ambulans* exhibited continuous emergence as well as continuous recruitment of first-instar larvae throughout the year, except for a hiatus in the coldest months. As these cold winter months also correspond to periods of high flow, this hiatus could be attributed to a combination of a) avoidance of high flows and cold temperatures (likely in the form of a pupal stage or an overwintering generation (V. Ross-Gillespie, pers. obs., 2010) and b) an artefact of sampling in spate conditions. The observed flexibility in voltinism of *C. ambulans* has been noted for other species of *Chimarra* in studies from the Northern Hemisphere (see Williams & Hynes 1973, Cudney & Wallace 1980, Parker & Voshell 1982, 1983, Benke *et al.* 1984, Bowles & Allen 1992).

Egg development experiments were conducted in the laboratory for each taxon in relation to water temperature in order to more accurately interpret the field-collected life-history, particularly with regard to the timing of hatching in the field. The delayed hatching of *L. penicillata* in the field in rivers experiencing lower water temperatures could to some degree (barring potential genetic differences) be explained by laboratory observations of slowest egg development being observed at low temperatures (64 days at 10° compared to 14 days at 20°C). Additionally in these laboratory experiments the duration of the hatch period was longest and less synchronous at low temperatures (11-22 days at 10°C compared to 4-5 days at 20°C) which in turn might have explained the longer emergence period of adults in the field in these streams. Overall, successful hatching was observed to occur at temperatures between 10 and 20°C, with a lower limit between 5 and 10°C and an upper limit between 20 and 25°C with the resultant strongly negative reaction norm for egg development indicating an adaptation to warm water (Pritchard *et al.* 1996). This finding was in agreement with the more tropical Gondwanan origins suspected for the family Teloganodidae (Barber-James *et al.* 2008). However, it should be noted that the upper thermal limit for egg development in *L. penicillata* of 20-25°C is not that high relative to some eurythermic species (e.g. *Baetis rhodani* exhibit >50% hatching success at temperatures as high as 25-27°C- Elliott & Humpesch 1980) and that in fact while this family may have had origins in a more tropical area than that in which it now exists, the global climate at the time of Gondwanaland is

suspected to have been substantially cooler. Additionally the current distribution of *L. penicillata* is limited to higher lying cooler Gondwanan refugia (Rivers-Moore *et al.* 2013b) - which suggests a propensity for cold water.

Similarly, the extended emergence period clearly observed in *A. scutata* in the field when compared to *L. penicillata* could be explained by observations of laboratory experiments on eggs of the species. In these experiments *A. scutata* generally had a longer hatch period (>20 days) at all temperature treatments compared to *L. penicillata* (<10 days in 15 and 20°C treatments and up to ~20 days in the 10°C treatment). This longer hatch period which would in effect translate to the nymphal cohort of *A scutata* being more spread out in terms of size as the year progressed leading to an extended emergence period. Additionally the number of days to median hatch was shorter at warmer temperatures (36 days at 10°C compared to 20 days at 20°C) but unlike the other taxa the corresponding DD requirements were higher at warmer temperatures (367.8 DD at 10°C, 387.6 DD at 15°C and 408.5 DD at 20°C). In this species successful hatching occurred at temperatures also ranging from 10 to 20°C, but with a lower limit just slightly higher than 5°C and an upper limit between 20 and 25°C. Interestingly, the highest hatching success was observed at 10 and 15°C, with a sharp decline at 20°C indicating the sensitivity of this species to warmer water temperatures. This was supported further by the resultant reaction norm for egg development which was found to be positive, indicating an adaption to cold environments for this taxon. This finding is interesting considering the general uncertainty regarding the origins of the Notonemouridae (Stuckenberg 1962, Stevens & Picker 1995, Zwick 2000, Stevens 2009) and the Plecoptera as an order (Zwick 2000, Fochetti & de Figueroa 2008). The thermal responses of the eggs of *A. scutata* supports the notion of a cold water or stenothermic origin.

In *C. ambulans* indications of multivoltinism and shorter life cycles at warmer water temperatures were supported by egg experiments which revealed that of all the taxa studied *C. ambulans* had the shortest development time (from 34 days at 10°C to 11 days at 25°C), lowest thermal requirement (from 345.4 DD at 10°C to 252.1 DD at 20°C and 263.3 DD at 25°C), shortest hatch duration (3.5 days on average for all treatments) and widest range of temperatures for successful hatch (10-25°C: with the lower limit between 5 and 10°C and upper limit between 25 and 30°C). Hatch success, however, was generally low and variable, with lowest levels recorded at 25°C - suggesting an approach of tolerance limits at the temperature. The weakly negative reaction norm for egg development calculated for this species suggested adaptation over time to more variable thermal conditions but with a propensity for warmer water. This is in agreement with the Pangaean origins of the Trichoptera from the Middle and Late Triassic period (~230mybp) (de Moor & Ivanov 2008). This period is thought to have been characterised by a generally warm and uniform climatic conditions with summer temperatures >30°C during the end-Triassic (Dickins 1993, McElwain *et al.* 1999) facilitating rapid dispersion of insect groups across the supercontinent. The Philopotamidae appear to have their origins in what then would

have been a tropical belt across modern day North America and Western Europe, according to interpretations of late Triassic fossil deposits of this taxon (de Moor & Ivanov 2008).

Using a novel GLM approach, it was established that for all studied taxa temperature had the most pronounced effect on growth rate, and therefore development time, followed by flow and finally other physicochemical variables. Multivariate analyses (Appendix 4A) provided additional support for these findings in showing that temperature was the variable that explained the most variation in the stage compositional data, followed by physicochemical variables and then flow variables (see Appendix 4A for further details). Using the GLM approach, the thermal optimum for growth for *L. penicillata* was estimated to occur between 13°C and 20°C, compared to colder temperatures of less than 16°C for *Aphanicercella spp*. In contrast, *C. ambulans* exhibited substantial tolerance to a range of water temperatures, with optimal growth occurring at temperatures above 15°C (even to as high as 21.5°C) but a marked decline in growth at temperatures below 15°C. Collectively these data, representing the sublethal effects of temperature on growth in sensitive indicator taxa, provide particularly useful information for setting thermal guidelines for the Ecological Reserve (see later). Additionally they correspond well with thermal tolerance limits for egg development (Chapter 5). An additional variable in the GLM called "River" accounted for the combined effects on growth by other factors not explicitly considered in the model (e.g. food and genetic divergence) but associated with each separate study river. This variable revealed some noticeable effects on growth especially for *L. penicillata* and *Aphanicercella* spp. but not to the same degree in *C. ambulans* and thus warrants further investigation.

At the time of sampling and field data analysis, the taxonomy indicated that a single species of *Lestagella* and *Chimarra* occurred across the six rivers (different species of *Aphanicercella* in the study sites were taxonomically confirmed), leading to the assumption that these differences in life-history traits must have arisen from a) drastically different conditions of food quantity/quality at each site and/or physico-chemical characteristics of the rivers - both thought to be unlikely considering the similarity of local site conditions and available physico-chemical data presented (Chapter 2) or b) phenotypically plastic life-history responses. Subsequent genetic analyses (Chapter 3) however, surprisingly revealed a cryptic species complex within *L. penicillata* comprising two major clades and five distinct species with high genetic divergence occurring between some of the study sites. Divergence ranged from 0% (between individuals collected from the same site) to a maximum of 28.7% (between individuals from two distinct clades - Window Stream and the Wit River). These findings suggest that a taxonomic revision of the genus is required. For *Aphanicercella*, molecular analyses confirmed the species status of the four morphologically identified species and so provided support for a genetic basis of life-history responses - as was expected. In contrast, genetic analyses suggested a single species of *Chimarra, viz. C. ambulans* occurred across the six study sites with relatively low intraspecific divergence amongst populations averaging 1.83%. Samples from Window Stream (Table Mountain), however, showed high genetic divergence (13.9%) from the clade containing the specimens

from the six study sites, suggesting the presence of a previously unidentified species there. Similar to *L. penicillata* though, the genetic results suggested that the taxonomy of *Chimarra* should be revisited along with morphological comparisons to confirm the findings presented here. Importantly, the indication of single species of *C. ambulans* occurring at the six study sites provided support for the notion that the different life-history responses observed across the study sites could be as a result of phenotypic plasticity - in response to differences in thermal regime.

Genetic analyses also yielded the unexpected yet important finding that specimens collected from Window Stream for each of the target taxa all showed congruent high genetic divergence with respect to their populations collected from the six study sites. This is in support of a growing body of research that posits Table Mountain as being geographically isolated from the nearest Cape Fold Mountain range across the divide of the Cape Flats (Stuckenberg 1962, Picker & Samways 1996, Sharratt *et al.* 2000, Daniels *et al.* 2013). Examples of taxa which reveal isolation on Table Mountain amongst others are species of freshwater *Mesamphisopus* isopods (Gouws *et al.* 2010, McDonald & Daniels 2012), as well as species of *Peripatopsis* velvet worms (Daniels *et al.* 2009, 2013). The Cape Peninsula Mountains are characterised by fragmented mountain blocks, supporting remnant Afromontane forest patches that are separated by low lying valleys, steep gorges and sheltered ravines (Cowling *et al.* 2009). Table Mountain is one such mountain block that is separated from the rest of the interior Cape Fold Mountains (Hottentots Holland and Jonkershoek) by a long (50km) sandy stretch known as the Cape Flats. For extensive periods during the Miocene/Pliocene this stretch became inundated with seawater from marine transgressions (up to 200m) leading to the extinction of low lying flora and fauna (Daniels *et al.* 2013). Subsequently, as a result of major climatic ameliorations during the Miocene/Pliocene and Pleistocene, this area was subject to periods of shifts between xeric and mesic conditions which ultimately lead to progressive aridification and contractions of forest patches to higher altitudes (Mucina & Rutherford 2006, Cowling *et al.* 2009). Lower lying areas such as the Cape Flats, that were unlikely to have supported forest areas, became exposed dry corridors thereby acting as dispersal barriers for certain mountain invertebrate taxa (e.g. velvet worms) (Daniels *et al.* 2001, Wishart & Hughes 2003, Gouws *et al.* 2004, McDonald & Daniels 2012). The high-lying forest patches in contrast would have provided a multitude of habitats and microclimates in effect acting as altitudinal buffers and refugia for organisms in response to climate changes (Daniels *et al.* 2013). In this manner the high-lying mountains such as Table Mountain have facilitated the persistence and survival of several ancient Gondwanan fauna that would have evolved under cooler climate conditions. Additionally however, during periods of marine transgressions, other taxa besides stenothermic Gondwandan taxa that became isolated on Table Mountain would as a result have undergone speciation and local adaptation to cooler forested conditions. Ultimately a combination of these two scenarios has lead to Table Mountain and other high lying areas of the Cape Fold Mountains exhibiting high levels of endemicity both in terrestrial and freshwater habitats (Picker & Samways 1996). Generally none of the Gondwanan stream fauna have managed to disperse elsewhere in South Africa or occupy other types of water bodies -

hence they are considered to be conservative (Assoc. Prof. Mike Picker, pers. comm., University of Cape Town, 2013).

While molecular analyses helped to elucidate a genetic basis for life-history responses in *Aphanicercella* and potentially the *L. penicillata* species, phenotypic plasticity and some degree of genetic differentiation appeared to be governing the varied life-history responses of *C. ambulans*. Controlled-environment experiments (Chapter 6) allowed for further investigation of the role that phenotypic plasticity versus genetics played in regard to the subtle life-history differences observed in two lineages of *L. penicillata* from two different sites. In particular, observations were made in relation to changes in water temperature.

The two sites selected were the Molenaars River situated on the on the interior Cape Fold Mountains and Window Stream situated on the slopes of Table Mountain on the Cape Peninsula. These two sites are approximately 80km apart and are separated by the Cape Flats and the Cape Fold Mountains. They incur substantially different natural thermal regimes as evidenced by maximum and minimum water temperatures recorded over the summer months December-February (Molenaars River max. = 25.31°C, min. = 15.06°C; Window Stream max. = 21.76°C, min. = 14.61°C) and autumn months March -May (Molenaars River max. = 24.48°C, min. = 9.98°C; Window Stream max. = 21.86°C, min. = 11.24°C). Window Stream is a first order headwater mountain stream but is thermally stable and buffered (December-February average water temperature and standard deviation = 18.08°C ± 1.184), likely as a result of shading from forest canopy cover and groundwater inputs from the Table Mountain. It is not inhabited by any predatory fish. The Molenaars River in contrast is a wider second order foothill river draining a much larger catchment of mountain fynbos. It is an open channel river and is more thermally variable (December-February average water temperature and standard deviation = 20.46°C ± 2.074). It is also currently inhabited by several species of predatory fish (Rainbow Trout, Smallmouth Bass, Sharptooth Catfish) while historically it was inhabited by the endemic and now endangered indigenous Whitefish, along with Cape Kurper and Galaxias (Jeremy Shelton, pers. comm., University of Cape Town, 2013)

Phenotypic plasticity can be investigated by subjecting individuals of a species to different temperature treatments under controlled laboratory conditions where other factors are held constant (e.g. flow and photoperiod), as was done for individuals (females only) of two putative species of *Lestagella* collected from the Window Stream and Molenaars River (Chapter 6). Since this experiment a) could not be conducted on larvae reared from eggs, as attempts were unsuccessful, and b) used a relatively short acclimation period of only 72 h, it was subject to environmental (acclimation differences) and first generation maternal effects (see Postma *et al.* 1995a, 1995b, Kavanaugh 1998). On the other hand, it was free from artificially induced environmental effects that might influence laboratory reared organisms (Bernardo 1996). Nevertheless, results indicated that both species exhibited lower growth

rates at lowest temperature treatments (~0.35%[46] at ~11°C for Window Stream and ~0.48% at ~11°C for Molenaars River) and highest temperature treatments (~0.30% at ~20°C for Window Stream and ~0.50% at ~20°C for Molenaars River), i.e. in their sub-optimal ranges. Both species also increased the inter-moult duration at colder temperatures (27 days at ~11°C compared to 16 days at ~20°C for Window Stream and 38 days at ~11°C compared to 15 days at ~20°C for Molenaars River), illustrating a generalised phenotypically plastic response to temperature. Growth rates in both species appeared to converge to an optimum growth rate of about 0.6% at the ~17°C treatment in accordance with optimum growth rates determined for this genus from the GLMs (Chapter 4).

By comparing the growth rates of the two species under controlled conditions (at identical temperatures on an identical food source and at the same instar), the effects of genetic control could also be explored. Individuals from the Molenaars River exhibited the highest growth rates at all temperatures, as well as the greatest increase in inter-moult duration at cold temperatures, but the total number of moults (i.e. age at maturity) did not change between laboratory and natural populations (black wingpad nymphs at all treatments along with those measured from a natural population were at instar 15). Individuals from Window Stream on the other hand, while having slightly lower growth rates and not increasing inter-moult duration at colder temperatures to the same extent, revealed an interesting growth response of adding extra moults at colder temperature treatments (up to 3 additional moults) compared to the natural populations (black wingpad nymphs measured from a natural population were at instar 15 compared to non-black wingpad laboratory individuals which were at instar 18). These extra moults substantially extended the life cycle of the laboratory population from Window Stream (up to 6 months longer than individuals in natural populations), allowing individuals to attain larger size at maturity (average IOD of 0.680mm for laboratory black wingpad individuals vs. average IOD of 0.656mm for natural population black wingpad individuals). While flexibility in the number of moults has been recorded for species of Ephemeroptera (Degrange 1959), the response exhibited here by female *L. penicillata* from Window Stream of adding extra moults suggests a genetically controlled adaptation to prolong the life cycle, perhaps even enabling semivoltinism, under a) colder conditions or b) no pressure from potential environmental constraints (i.e. timing of oviposition in order to avoid hot summer temperatures and onset of winter flows). Such an adaptation would enable females of this species to obtain a bigger size at maturity and thus greater fecundity and overall fitness (Vannote & Sweeney 1980, Ward & Stanford 1982, Honěk 1993). Interestingly this adaptive response appears to have been lost in individuals from the Molenaars River which have presumably adapted to warmer water conditions in genetic isolation from those in Window Stream.

A further noteworthy comparison made between individuals of the two putative species was that of thermal tolerance limits and temperature induced mortality. Using a long-term static lethal temperature experiment (LT_{50}), individuals from the Molenaars River showed a higher upper lethal thermal

[46]Percentage increase in interocular distance (IOD) per day

tolerance limit (168 h LT_{50} of 26.45°C) compared to those from Window Stream (168 h LT_{50} of 23.20°C). These differences are assumed to be of genetic origin, caused by local adaptation to site conditions.

Collectively these observations provide an interesting platform for hypothesising the evolution of life-history patterns of these two species. Based on the temperature data presented in the thesis, although these are somewhat limited, the Molenaars River appears to be a warmer river than the Window Stream, and thermally less stable. For this reason the Molenaars River is more likely to reach the thermal limits for egg and larval development of *L. penicillata* especially over the warm summer months (December to February), whilst also likely incurring greater levels of hydrological disturbance (floods)(as it is a second order foothill river in a large catchment compared to a first order mountain stream). Furthermore one could assume that historically, this would have been the case as well - although historical daily flow records are not available for Window Stream. As a result, individuals of *L. penicillata* in the Molenaars River might have adapted to exhibit less flexibility in terms of length and timing of their life cycle - owing to the early onset of adverse conditions, seasonal environmental constraints, particularly increased temperatures, high flows, or even predation - in essence they have to be quick to emerge and oviposit during a period of gentle flows and cool but warming water temperatures (the eggs do not have attachment apparatus - Chapter 5). In contrast, *L. penicillata* in Window Stream could have adapted to cooler water temperatures, less variable thermal regimes, and importantly an absence of predatory fish. There are therefore fewer environmental constraints to inhibit the life-history flexibility of the individuals of this stream. As such, they retain the potential to respond with an increase in the inter-moult duration, as well as in the number of moults at colder temperatures, and they are better suited to take advantage of these conditions. This flexibility was evident in the much extended life cycle exhibited by individuals from Window Stream in the laboratory setup, which in turn allowed them to reach larger sizes and therefore potentially gain a fitness advantage in terms of greater fecundity. It is likely that phylogenetic constraints (e.g. a minimum number of required larval instars before reaching sexual maturity (perhaps 15) prevent both of these species from drastically shortening their life cycles under warmer more favourable conditions, through either a) shortening inter-moult duration or b) reducing the number of moults. Neither species were able to shorten inter-moult duration much below 15 days - Window Stream individuals revealed a lowest inter-moult duration of 15 days and Molenaars River individuals 12 days. Perhaps the multivoltine Baetidae have adapted greater flexibility of inter-moult duration, such that they are able to substantially reduce the duration under warm conditions thereby allowing a relatively large number of moults in some cases (e.g. 20-29 moults reported by Degrange 1959) to be completed in a short period of time?

Adaptation to local site conditions of warmer water temperature and possibly greater food resources (Ewart-Smith 2012) has resulted in *L. penicillata* from Molenaars showing higher thermal tolerance and a higher growth rate compared to *L .penicillata* from Window Stream (which experiences colder

temperatures and possibly lower productivity like other mountain streams in the Western Cape -see King 1981, 1982, King *et al.* 1988) - and ultimately the two species exhibit different genetics. This genetic basis, along with some degree of plasticity in growth rate, is what appears to be controlling the life-history differences (*viz.* emergence, development time) in these two putative species.

Similar to *L. penicillata*, *Aphanicercella* showed subtle differences in life-history responses for different species in different rivers. Like *L. penicillata*, *Aphanicercella* also represents a basal insect order, exhibiting similarly poor dispersal abilities, and with a large number of moults (see Brittain 1990). One might therefore expect that similar evolutionary trends could be observed as discussed above for *L. penicillata*. However, without conducting a reciprocal transplant or controlled-environment experiments for species in this genus the effects of phenotypic plasticity vs. genetic divergence cannot be explored further here.

In contrast to these two aforementioned taxa, individuals of *C. ambulans* from the six study sites were shown to be genetically similar (intraspecific divergence of 1.83%) and assumed to be the same species at each of the study sites - geographical populations thus appear to have predominantly phenotypically plastic life-history responses. The substantial differences found with respect to voltinism (and presumably growth rates), and the extent of these differences raises the question as to why this species, in contrast to the others, is able to exhibit this degree of phenotypic plasticity.

C. ambulans being within a more recently diverged group than the other two target taxa (de Moor & Ivanov 2008) is suspected to have had vastly different evolutionary origins thus leading to the expression of a markedly different life-history pattern. Firstly, this taxon exhibits far fewer larval instars, in effect meaning that phylogenetic constraint on development time is not as pronounced compared to species with longer conservative life-histories. However, despite a less conservative life-history, the complex interaction of factors that affect life cycles (nutrition, photoperiod) cannot be ignored as they clearly play important roles in regulating life cycle length and timing (Khoo 1964, Lock & Williams 1981, Johansson *et al.* 2001, Shama & Robinson 2006, Danks 2007, Boggs 2009). Secondly, *C. ambulans* goes through a pupal stage, which enables avoidance of unfavourable conditions – and allows for a synchronising of emergence at more favourable conditions. Thirdly this taxon exhibits a wider range of thermal tolerance for successful egg development (10-25°C for *C. ambulans* compared to 10-20°C for the other taxa) and shows fast and synchronous egg development in favourable conditions (allowing for the generation of several cohorts or multivoltinism). It further has relatively large eggs compared to the other taxa (Chapter 5) thereby potentially conferring a relatively greater degree of fitness on larvae - in turn leading to increased survival of juveniles. It also specifically selects optimal oviposition sites (V. Ross-Gillespie, pers., obs. 2010 and see Resetarits 1996) and protects eggs against high flows by cementing them to the oviposition substrate. Collectively these factors contribute to the great flexibility in life-history data observed for *C. ambulans* and result in

almost continuous adult emergence and recruitment throughout the year with flexible voltinism, especially in rivers with warmer temperatures.

It is my assertion that, in contrast to more flexible life-histories expressed in more recent lineages (e.g. Trichoptera, Lepidoptera, Diptera), conservative life-histories expressed in more basal taxa that are phylogenetically constrained because of past evolutionary histories (e.g. Ephemeroptera and Plecoptera) requiring a large number of moults and continuous larval development leading to a longer life cycle with no quiescent (pupal) stage, will potentially be less able to exhibit phenotypic plasticity. This has profound implications when one considers the changes to riverscapes predicted from global climate change in conjunction with ongoing anthropogenic activities, particularly with regards to temperature and precipitation. Determining the degree to which aquatic biota will be able to respond to such changes is of primary concern.

7.3 The impact of climate change on life-histories and adaptive management mitigation

Riverscapes already face a suite of existing stressors (e.g. hydrological alteration, abstraction, impacts from surrounding land use) and global climate change is expected to amplify these stressors, potentially resulting in further cascading effects (Rivers-Moore *et al*. 2012). In a developing and water-scarce country like South Africa, where unfortunately data are also limited, the implications of global climate change on freshwater ecosystems are considerable.

Changes in water temperature are likely to have a substantial impact with expected cascade effects taking place at multiple levels of ecological organisation from the individual/population to the community and ultimately the ecosystem (Geyer *et al*. 2011). With the growing appreciation of the necessity of incorporating thermal regimes into environmental flow assessments and to inform policy (see Olden & Naiman 2010 and Rivers-Moore *et al.* 2013a), there is understandably a need to collect relevant and fundamental data, conduct suitable experiments, use the collected data to glean a spatial and temporal perspective on the ecosystem and to model responses of the system under climate change scenarios (see Heller & Zavaleta 2009). It is crucial, however, that these activities are implemented in a framework of adaptive management and conservation (see for example Kingsford *et al.* 2011). This can be achieved by incorporating later steps (successes and failures) in the management process for the future, as this will inform and allow for the re-assessment of earlier steps in an ongoing iterative learning process (Lee 1999, Tompkins & Adger 2004). As natural and social systems evolve and even co-evolve over time, this management approach is similarly one that does not return to a prior state but instead anticipates projected or actual changes in order to mitigate impacts and take advantage of certain conditions (Tompkins & Adger 2004). In this manner, adaptive management can increase the present-day resilience of the system, in turn enabling greater flexibility in response to long-term threats of climate change (Tompkins & Adger 2004).

The data presented in this thesis have direct importance for establishing thermal guidelines for the Ecological Reserve in South Africa and provide an important first step in the adaptive management process by providing fundamental baseline data. Analyses of flow and temperature data (Chapter 2) (although the latter are limited) give valuable initial insights into the spatial and temporal variability of these ecosystems and allow for classification criteria to be identified based on specific ecologically relevant environmental variables. In turn, such approaches can be used to develop a classification for reference rivers across the country and enable riverscapes to be grouped into larger management units - i.e. at the regional scale. Genetic analyses (Chapter 3) provide a basis for understanding differences in biotic responses that might be observed in relation to environmental change for taxonomic groups or even species that have wide distribution ranges. Life-history information (Chapter 4) in conjunction with egg development experiments (Chapter 5) provide fundamental information that is relevant for all future ecological investigations of riverscapes and or the taxa - though more data of this kind are needed for South African aquatic invertebrate fauna if informed adaptive management decisions are to be made. The data presented in this thesis showed that differences in hydrological and thermal regimes of South African rivers do indeed induce a plastic response in the life-history traits of the representative aquatic insect species inhabiting them – particularly in those insect taxa (e.g. *Chimarra*) exhibiting more flexible life- histories and less phylogenetic constraint (shorter life cycles of fewer instars). This in turn suggests that generalisations in terms of thermal guidelines made at a broad national-scale for the Ecological Reserve might be inappropriate and would at the very least need to be conceived at a regional or even local scale. Furthermore these plastic life-history trait responses should, where possible be evaluated through a combination of laboratory experiments (common environment experiments for rearing, molecular analyses, egg development experiments – such as those presented in Chapters 3, 5 and 6) and field work (Chapter 4) in order to distinguish potential underlying genetic drivers of trait differences vs. true phenotypically plastic responses. While smaller levels of plasticity would be evident within a species, larger differences in life-cycle patterns and adaptation to thermal differences might be expected when considering interspecific comparisons. This is particularly important as cryptic species complexes, which can often be overlooked by taxonomy, act to confound life-history trait and thermal tolerance studies. Such data would provide much needed information regarding the sub-lethal effects of temperature on aquatic taxa (i.e. growth rate, emergence timing and hatching success of eggs). For instance, the upper and lower thermal tolerance limits for egg development derived for the representative species (Chapter 5), in conjunction with LT_{50} values (Chapter 6) as well as the optimum temperature ranges for growth (obtained from GLM's and rearing experiments – Chapters 4 and 6) have provided fundamental information necessary in the first step of forming thermal guidelines for the Ecological Reserve. This is evidenced by the fact that if water temperatures of Ecological Reserve flows in summer (December-February) should exceeded an upper limit of 25°C (e.g. surface release flows from dams upstream) they could be severely detrimental to the successful egg development of conservative species particularly *L. penicillata*, *A. scutata* (likely the entire genus *Aphanicercella*) and

to a degree also the more flexible species *C. ambulans*. Increased hatching failure at these temperatures could result in a dramatic drop in population sizes, and if sustained for several years in sequence could lead to the eventual loss of the species from the river. Similarly, long term LT_{50} experiments for *L. penicillata* showed that if summer water temperatures should exceed an upper limit of 20-23°C for 600h 50% mortality of nymphs can be expected in places. Life-history data revealed important times of the year for species where cues (emergence/hatching) are closely tied to thermal and hydrological regimes. These regimes are likely to be altered by climate change and so might result in the life-cycle being put out of sync with its normal annual pattern. Similarly, voltinism indicated which species are conservative and which are flexible and therefore likely to cope with changes made to the thermal and hydrological regimes. The GLM approach using this life-history data provided a range of temperature for optimal growth for each species and provided insight as to the effect of additional environmental variables on growth. Using this data, it is evident that should Ecological Reserve flows over summer be below 13°C (e.g. bottom release flows from dams upstream), growth rates in warm adapted species such as *L. penicillata* and *C. ambulans* can be expected to decline markedly. In *C. ambulans* the decline in growth rate could be coupled with a change in voltinism (decrease in the number of generations produced in the year) while for *L. penicillata* evidence from Chapter 6 would suggest it could shift to a semivoltine life cycle. For *Aphanicercella* spp. on the other hand which are cold adapted, colder summer water temperatures would likely result in increased growth rates without a noticeable change in voltinism.

This thesis presents a template of what data are necessary for incorporating thermal guidelines for aquatic insects into environmental flows/Ecological Reserve and how they can be collected and interpreted. In particular, the GLM modelling approach adopted in this thesis could be useful for assessing the range of thermal optima for a specific taxon at the regional scale (e.g. Western Cape). These life-history data and GLM approaches, when used in conjunction with thermal limits for egg development (Chapter 5) and lethal upper thermal tolerance limits (Chapter 6) (using LT_{50} and CTM methodologies), provide a solid foundation for establishing thermal guidelines for the Ecological Reserve at the regional scale for these and other taxa. Furthermore, the data presented in this thesis have been used to provide one of the first approaches to modelling the effects of climate change on aquatic insects in South African rivers.

An example of how such data can be practically applied in order to better inform adaptive management processes is highlighted in a paper co-authored by myself along with Dr. Nick Rivers-Moore and Dr. Helen Dallas, recently published (Rivers-Moore *et al.* 2013b). Data presented in this thesis (thermal limits for egg development of *L. penicillata* in conjunction with life-history information for the same species) were used in this co-authored paper to examine the potential impacts of temperature changes on this species under several climate change scenario analyses. Methods involved linking biotic responses (hatching success) to thermal triggers and the transgression/exceedance of a chronic stress

temperature threshold. The chronic stress temperature threshold was calculated based on an averaging statistic (Maximum Weekly/7-day moving Average Temperature - MWAT) using agglomerative techniques (duration curves and cumulative DD) for 31 river sites in the Western Cape. The threshold MWAT for a species is calculated using an optimal temperature (OT) or range of preferred temperatures from laboratory derived growth curves (Brungs & Jones 1977) (or the GLM approach used for field data presented in this thesis) and an incipient lethal upper temperature (ILUT) calculated from LT_{50} and CTM experiments (see equation 7.1).

$$MWAT = OT + \frac{(ILUT - OT)}{3} \qquad (7.1)$$

Hourly water temperatures collected from the 31 sites were used to assess the frequency and duration (days) of MWAT threshold transgressions/exceedances both under current climatic conditions and under scenarios of global climate change. This is important because the rationale behind using the MWAT as a temperature limit is based on data showing that moderate fluctuations in temperature can be tolerated by organisms as long as the ILUT is not exceeded for long periods (Brungs & Jones 1977). As such, at sites where the MWAT was exceeded (either under current climatic conditions or global climate change scenarios) for more than 30 days, breeding failure was considered to occur (this assumption was based on egg development experiments conducted in Chapter 5 of this thesis that showed that prolonged exposure to temperatures outside of the range for successful egg development resulted in hatch failure).

Results from the analyses using MWAT thresholds indicated that under a conservative climate chage prediction, based on recent studies (Lester *et al.* 2011, Turak *et al.* 2011, Viers & Rheinheimer 2011) of a 2°C increase in water temperature, the thermally suitable habitat modelled for *L. penicillata* (which is already limited under current climatic conditions) was predicted to contract further by approximately 30% as a result of breeding failure in thermally marginal habitats that would be affected by this degree of temperature increase.

The findings in Rivers-Moore *et al.* (2013b) in conjunction with those presented in this thesis suggest that global climate change is likely to have greatest impacts on conservative and phylogenetically constrained univoltine species (such as *L. penicillata* and *Aphanicercella spp*), especially where such species are stenothermic and inhabit thermally marginal habitats (e.g. Gondwanaland relictual fauna). In contrast, less conservative eurythermic and multivoltine species, such as *C. ambulans* exhibit greater flexibility and are likely to be favoured by such temperature changes. For these species, warmer conditions (still within lethal limits) would be favourable for egg development and also result in increased growth rates and the production of a greater number of generations per year. This could have important management implications in the case of a eurythermic and multivoltine pest species (e.g.

Simulium chutteri), where an increased number of generations per year owing to warmer conditions, under different flow scenarios, would equate to an increased number of outbreaks of this species (see Rivers-Moore *et al.* 2013b for further details).

Overall these findings illustrate the usefulness of data such as those presented in this thesis in understanding and predicting, through modelling techniques, how different species will respond to changing environmental conditions. They also highlight the need for further research in order to gain a fuller picture of the impacts of climate change on our river ecosystems and their inhabitants.

7.4 Proposals for addressing current research lacunae

Information regarding water temperature is vital in the interpretation of life-history studies and individual species responses, not only in the face of ongoing anthropogenic impacts on river systems, but also climate change research. Therefore it is recommended that appropriate systems be installed to measure and record long-term water temperature trends at all existing DWA gauging stations across the country. Various technologies exist that could simplify this process: for example data loggers could be installed at existing gauging weirs that use cellular or satellite uplink systems to record and automatically upload data (real time) to cloud servers (e.g. the HOBO-U30-GSM Cellular Data Logger: www.onsetcomp.com/products/data-loggers/u30-gsm). This would avert the need and associated costs of employing/hiring personnel to manually collect the data.

This study highlights the value of collecting life-history of aquatic invertebrates, especially within a country in which data of the sort are so limited, and linking these to thermal and hydrological regimes of rivers. While only three target species were analysed in the study presented here, similar studies conducted on additional taxa from more rivers across the country (perhaps even including responses of species in temporary rivers) would allow for a much needed reference databaseto be created (see for e.g. Merritt and Cummins 1996). Such data on their own would be highly valuable to the fields of ecology and taxonomy, but if they are analysed in relation to variables such as flow and water temperatures over a range of habitats and environments, the value of the studies would increase exponentially. Future studies could contrast the life-histories of various functional feeding groups, endangered or threatened species, invasive species, or even pest species requiring careful management within altered or natural habitats (e.g. mosquitoes, midges, and black flies). It is the opinion of the author that life-history studies, which are relatively inexpensive to conduct and do not require advanced facilities or technology, should be prioritised in the field of freshwater research in South Africa. Perhaps such studies could even be incorporated into existing river monitoring programmes carried out by the government, such as the River Health Programme (www.dwa.gov.za/iwqs/rhp/index.html), where it could allow for additional job creation and skills development.

Where possible, life-history studies should be conducted in conjunction with egg development experiments and subsequent rearing experiments. A methodological problem area for such studies would be the collection of fertilised eggs. Light trapping techniques, emergence traps or sweep netting for gravid females might be considered. Additionally though, artificial fertilisation could be attempted as a means to obtain fertilised eggs. While these studies are generally more labour intensive, require an effective laboratory setup (such as a flow through setup, suitable lighting, accurate temperature control) and more often than not require initial pilot studies, they often provide the most accurate data.

Specifically, some key areas for potential future research might include:

1. Assessing the life-histories of target aquatic taxa in altered versus natural environments – where the predictability of one or another environmental variable has increased or decreased significantly (e.g. channelised compared to natural streams, above and below impoundments)

2. Assessing the life-histories of the same species occurring in temporary rivers vs. perennial rivers using similar assessment techniques. This would provide a far greater range of predictability values against which to compare life-history patterns. Deeper substrate sampling methods could perhaps be employed to obtain samples that could yield information on taxa that seek refugia during unfavourable conditions.

3. Given that few rivers/streams have water temperature data spanning more than two years, one could model water temperatures from historical air temperature records and assess them using predictability indices to provide an estimate of the predictability range of water temperatures in Southern African rivers.

4. A logical next step, after having obtained reliable life-history information, would be to assess the secondary production or cohort production interval of target species in relation to environmental variables (e.g. thermal regime, groundwater vs. surface runoff dominated streams), using a planned sampling regime and quantitative sampling methods.

5. Conducting surveys on aquatic macroinvertebrate fauna in rivers across South Africa using rapid DNA Barcoding techniques. Such an approach could a) provide an indication of species diversity in different regions across the country b) allow for a reference library of genetic sequence data to be established and c) highlight additional cryptic species complexes and thus areas for taxonomic investigation.

Research in these areas is likely to yield a wealth of valuable information that will contribute greatly to management operations and guidelines, policy formation and effective conservation. Additionally it will

provide a solid foundation for a diverse range of future research topics in a country where data of the sort are desperately lacking. Given the habitat diversity, range of climatic conditions, as well as the thermal and hydrological variability exhibited by rivers across the country, the potential outcome of such proposed research is very exciting.

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Appendices

APPENDIX 2A

Variables derived from temperature and flow metrics

Table App2A.1. Hydrologic parameters and their respective ecosystem influence as assessed by Indicators of Hydrologic Alteration (IHA) software Version 7 developed by Richter *et al.* (1996). Table from The Nature Conservancy (2006).

IHA Parameter Group	Hydrologic Parameters	Ecosystem Influence
1. Magnitude of monthly water conditions	Mean or median value for each calendar month Mean annual flow Annual C.V. of variation of flow Colwell's Predictability Index Constancy/predictability _____ *Subtotal 12 parameters*	- Habitat availability for aquatic organisms - Soil moisture availability for plants - Availability of water for terrestrial animals - Availability of food/cover for fur-bearing mammals - Reliability of water supplies for terrestrial animals - Access by predators to nesting sites - Influences water temperature, oxygen levels, photosynthesis in water column
2. Magnitude and duration of annual extreme water conditions	Annual minima, 1-day mean Annual minima, 3-day means Annual minima, 7-day means Annual minima, 30-day means Annual minima, 90-day means Annual maxima, 1-day mean Annual maxima, 3-day means Annual maxima, 7-day means Annual maxima, 30-day means Annual maxima, 90-day means Number of zero-flow days Baseflow index: 7-day minimum flow/mean flow for year _____ *Subtotal 12 parameters*	- Balance of competitive, ruderal, and stress- tolerant organisms - Creation of sites for plant colonization - Structuring of aquatic ecosystems by abiotic vs. biotic factors - Structuring of river channel morphology and physical habitat conditions - Soil moisture stress in plants - Dehydration in animals - Anaerobic stress in plants - Volume of nutrient exchanges between rivers and floodplains - Duration of stressful conditions such as low oxygen and concentrated chemicals - in aquatic environments - Distribution of plant communities in lakes, ponds, floodplains - Duration of high flows for waste disposal, aeration of spawning beds in channel sediments
3. Timing of annual extreme water conditions	Julian date of each annual 1-day maximum Julian date of each annual 1-day minimum _____ *Subtotal 2 parameters*	- Compatibility with life cycles of organisms - Predictability/avoidability of stress for organisms - Access to special habitats during reproduction or to avoid predation - Spawning cues for migratory fish - Evolution of life-history strategies, behavioural mechanisms
4. Frequency and duration of high and low pulses	Number of low pulses within each water year Mean or median duration of low pulses (days) Number of high pulses within each water year Mean or median duration of high pulses (days) Flood Free Season Percentage Floods in 60 day period _____ *Subtotal 4 parameters*	- Frequency and magnitude of soil moisture stress for plants - Frequency and duration of anaerobic stress for plants - Availability of floodplain habitats for aquatic organisms - Nutrient and organic matter exchanges between river and floodplain - Soil mineral availability - Access for waterbirds to feeding, resting, reproduction sites - Influences bedload transport, channel sediment textures, and duration of substrate disturbance (high pulses)
5. Rate and frequency of water condition changes	Rise rates: Mean or median of all positive differences between consecutive daily values Fall rates: Mean or median of all negative differences between consecutive daily values Number of hydrologic reversals _____ *Subtotal 3 parameters* *Grand Total* *33 parameters* _____	- Drought stress on plants (falling levels) - Entrapment of organisms on islands, floodplains (rising levels) - Desiccation stress on low-mobility stream edge (varial zone) organisms

Table App2A.2. Hydrologic parameters showing negligible multicollinearity (<0.8) used in the multivariate analyses that guided site selection. The respective IHA parameter group from which the variable was calculated is indicated.

IHA parameters with multicollinearity less than 0.8	IHA Parameter Group
Mean annual flow	1
Annual C.V. flow	1
Flow predictability	1
Constancy / predictability	1
7 Day min	2
Baseflow index	2
Date of maximum flow	3
Low pulse count	4

Table App2A.3. Variables derived from daily water temperatures data that were calculated using metrics developed by Rivers-Moore *et al.* (2010).Table from Rivers-Moore *et al.*(2010). See also (Rivers-Moore *et al.* 2008a, 2008b)

Annual descriptive statistics		Mean annual temperature SD of mean annual temperature Annual coefficient of variability Predictability (Colwell 1974) Annual range (mean) SD of annual range Annual co. eff. var. of range Summer range Winter range
Group 1	Monthly **magnitudes** (measure of central tendency) Oct - Sept Coefficient of monthly means gives an expression of environmental contingency Constancy = degree to which monthly means vary from month to month Contingency = extent to which flows vary within a month	Mean spring temperature (1 Sep - 30 Nov) Mean summer temperature (1 Dec - 28 Feb) Mean autumn temperature (1 Mar - 31 May) Mean winter temperature (1June - 31 Aug)
Group 2	Magnitude and Duration of annual extreme water temperature conditions 1-day minimum (Based on moving averages of different durations)	3-day minimum 7-day minimum 30-day minimum 90-day minimum (winter min) 1-day maximum 3-day maximum 7-day maximum 30-day maximum 90-day maximum (summer max) Degree days (annual) Degree days (monthly) Degree days (seasonal) Mean daily maximum Mean daily minimum Maximum diel range
Group 3	Timing - Julian date of maximum and minimum metrics (thermal triggers)	Date of minimum (Or date of 7 coldest days) Date of maximum (Or date of 7 warmest days)
Group 4	Frequency and duration (successive days of event above or below a threshold) (successive days exceeding MWAT) Min. temp threshold duration	Min. temp threshold count Max. temp threshold count Max. temp threshold duration Min. temp threshold Max. temp threshold Duration between two temperatures (an upper and lower as determined either by the temperature data or biological cues)
Group 5	Rate and frequency of a change in conditions (i.e. the abruptness and number of intra annual cycles of environmental variation)	Rate of change in daily range with downstream distance Rate of change in max. temp threshold exceedance with downstream distance

Table App2A.4. Thermal parameters showing negligible multicollinearity (<0.8) used in the multivariate analyses that guided site selection. The respective thermal parameter group from which the variable was calculated is indicated.

Thermal parameters with multicollinearity less than 0.8	Thermal Parameter Group
Annual C.V. of variation of temperature	1
Thermal predictability	1
Mean daily minimum	2
July monthly mean	2
November monthly mean	2
3 Day moving average of minimum temperature	2
Minimum temperature threshold exceedance count	4
Maximum temperature threshold exceedance count	4

APPENDIX 2B

Temperature and flow metric outputs for the six study rivers

Daily flow data for the six study rivers was analysed using Indicators of Hydrological Alteration (IHA) Version 7 software. This software calculated ecologically relevant parameters derived from daily flow using metrics developed by Richter *et al.* (1996). For each river, barring the Elandspad River, 20 years of flow data were analysed for the period from 1988-2008. In the Elandspad only 19 years of data were available. The output generated from IHA is presented in Table 2B.1.

Table App2B.1. Flow metrics calculated for six rivers within the Western Cape, South Africa. Data analysed were collected by the department of water affairs for the period from 1988 – 2008. For the Elandspad River only data from 1989-2008 were available.

Flow variables	Eerste River	Elandspad River	Molenaars River	Rooi-Els Kloof River	Wit River	Wolwekloof River
Mean annual flow	0.79	2.81	5.12	0.25	4.14	3.16
Annual C. V.	2.16	3.79	2.66	1.55	2.57	3.00
Flow predictability	0.41	0.54	0.51	0.56	0.39	0.32
Constancy/predictability	0.45	0.5	0.46	0.66	0.33	0.64
% of floods in 60d period	0.33	0.37	0.36	0.44	0.29	0.30
Flood-free season	29	36	31	47	9	0
1-day minimum	0.001	0.238	0.390	0.059	0.106	0.001
3-day minimum	0.004	0.238	0.398	0.061	0.108	0.005
7-day minimum	0.006	0.240	0.405	0.062	0.119	0.007
30-day minimum	0.009	0.256	0.454	0.065	0.135	0.051
90-day minimum	0.023	0.277	0.532	0.074	0.192	0.097
1-day maximum	14.890	45.570	96.690	4.287	90.470	18.410
3-day maximum	8.008	33.180	62.290	2.691	64.490	7.451
7-day maximum	5.291	19.880	40.490	1.895	37.010	4.773
30-day maximum	2.913	9.551	18.840	0.981	18.200	2.171
90-day maximum	2.103	6.616	12.690	0.553	11.570	1.287
Baseflow index	0.007	0.086	0.075	0.279	0.026	0.017
Date of minimum	71	84	72	120	61	112
Date of maximum	190	181	188	192	191	211
Low pulse count	12	4	4	4	5	4
Low pulse duration	3	17	17	7	13	7
High pulse count	16	13	12	8	16	5
High pulse duration	2	4	4	4	4	5
Number of reversals	134	75	90	78	86	54

Temperature metrics were developed by Rivers-Moore *et al.* (2010) and applied to daily water temperature data (computed from hourly water temperature data) collected for six rivers in the Western Cape, South Africa for the period from February 2009 to April 2010 (Table 2B.2). Annual metric values were calculated for the period from 01 March 2009 to 28 February 2010.

Table App2B.2. Temperature metrics calculated for six rivers within the Western Cape, South Africa. Metrics were calculated for the period from 01 March 2009 – 28 February 2010.

Temperature variables	Eerste River	Elandspad River	Molenaars River	Rooi-Els Kloof River	Wit River	Wolwekloof River
Max. threshold	18	18	18	18	18	18
Min. threshold	12	12	12	12	12	12
Annual Mean (°C)	15.13	15.60	15.66	14.98	15.71	15.81
Annual std. dev. (°C)	3.66	4.34	4.40	3.42	4.91	4.19
Annual C. V.	24.20	27.83	28.09	22.82	31.27	26.53
Pred. Colwell (P)	0.59	0.60	0.61	0.64	0.56	0.59
Constancy/predictability	0.46	0.35	0.36	0.42	0.31	0.36
Min mean	13.70	14.10	14.30	14.29	13.94	14.08
Max mean	16.91	17.55	17.21	15.77	17.75	18.09
Range in mean	1.10	1.14	0.98	0.50	1.31	1.35
Jan	19.93	21.66	21.89	19.11	22.88	22.12
Feb	19.36	20.76	20.89	18.84	21.40	20.92
Mar	18.78	20.55	19.97	18.33	21.51	19.76
Apr	16.69	16.78	16.83	16.53	17.13	16.42
May	12.58	12.42	12.39	13.06	12.20	12.69
June	11.35	10.79	10.81	10.36	10.46	11.94
Jul	10.98	10.13	10.14	10.14	9.80	11.10
Aug	10.76	10.21	10.14	10.44	9.83	10.84
Sep	11.25	11.18	11.21	12.01	10.67	11.41
Oct	13.85	14.24	14.17	14.67	13.69	13.75
Nov	14.79	15.63	15.61	14.93	15.28	15.24
Dec	17.37	19.09	19.00	17.34	19.25	19.04
Min 1 day Mov. Ave.	6.94	7.67	7.59	6.69	7.12	5.95
Min 3 day Mov. Ave.	7.42	7.90	7.84	6.88	7.40	7.14
Min 7 day Mov. Ave.	8.71	8.54	8.47	7.82	7.95	7.71
Min 30 day Mov. Ave.	9.45	9.09	9.02	9.18	8.66	8.56
Min 90 day Mov. Ave.	9.85	9.52	9.44	9.64	8.96	8.87
Max 1 day Mov. Ave.	25.79	27.19	27.04	21.60	29.17	27.06
Max 3 day Mov. Ave.	25.19	26.18	26.13	21.37	28.21	26.30
Max 7 day Mov. Ave.	24.32	25.64	25.40	21.13	27.78	25.84
Max 30 day Mov. Ave.	23.04	25.06	24.49	20.39	27.10	24.95
Max 90 day Mov. Ave.	22.24	24.05	23.60	19.95	25.39	23.84
Degree days	5396.1	5549.5	5554.9	5337.0	5567.9	5530.7
Degree days max.	6041.2	6253.5	6105.3	5631.7	6278.5	6365.1
Range in max temp.	2.64	2.47	2.11	2.06	3.47	3.71
Min. threshold count	150	152	153	130	171	165
Max. threshold count	151	157	152	113	149	151
Min threshold duration	79	101	102	76	113	109
Max threshold duration	100	101	100	61	99	103
Julian Day min	163	163	163	163	162	160
Julian Day max	325	324	325	364	326	322

APPENDIX 2C

Modelled water temperature data for the Wolwekloof River

Water temperature data in the Wolwekloof River was not available for the period 5 May to 19 October 2009, owing to a water temperature logger located within the riffle habitat at the site being washed away by winter spates. Additionally, a replacement logger malfunctioned, producing unreliable temperature readings from February 2009 to April 2010. Water temperatures for this period were however recorded from another properly functioning logger within a pool habitat at the same site. Regression analyses were performed on water temperatures readings recorded from these two habitats (riffle and pool) for a period of overlap before the riffle logger malfunctioned (Fig. App2C.1).

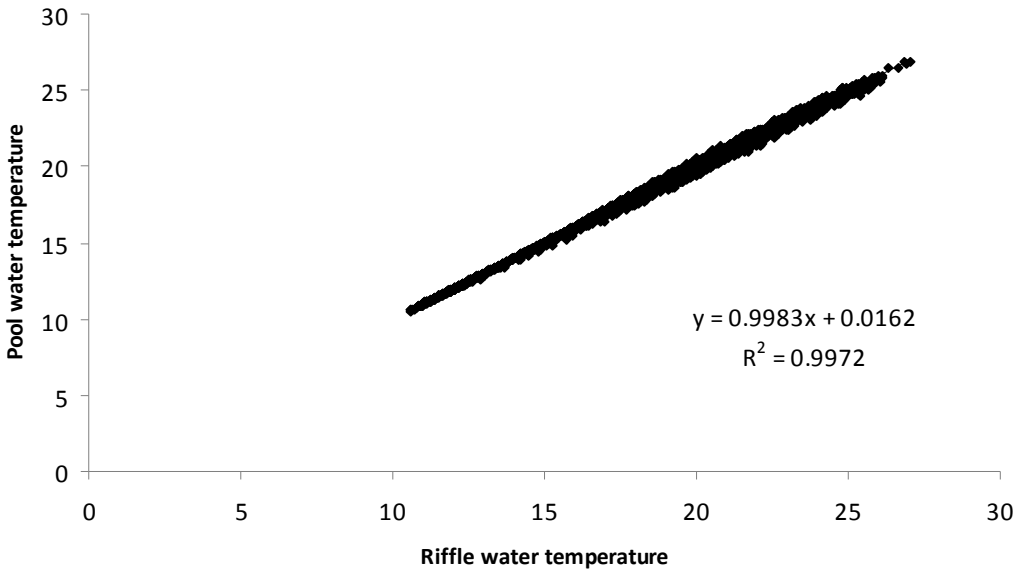


Fig App2C.1. Regression of hourly water temperature data collected from pool and riffle habitats in the Wolwekloof River from 19 Oct 2009 to 27 January 2010.

Water temperatures recorded from the two habitats were found to be almost identical (R^2 = 0.99, p < 0.001). As such water temperatures from the pool habitat were used to infer water temperatures missing for the period from February 2010 to April 2010.

Mean, minimum and maximum daily water temperatures in the Wolwekloof were estimated from hourly (for some months 2-hourly) air temperatures and relative humidity values (collected for the Berg River site – same catchment as the Wolwekloof River approximately 5 kms apart),for the period where data were missing, using models developed by Rivers-Moore *et al.* (2010) (Table App2C.1). Hourly and 2-hourly air temperatures were converted to daily means before applying the model.

Table App2C.1. Models developed by Rivers-Moore *et al.* (2010)

All data (complex) – Unadjusted:	
Mean	- F(4, 9876) = 8062 $WT = -2.33 + 0.70(AT) + 0.08(RH) + 0.93(SO) + 0.001(Alt)$ **(Model1)**
Min	- F(3, 9877) = 8062 $WT = -4.33 + 0.71(AT) + 0.09(RH) + 0.87(SO)$
Max	- F(4, 9876) = 8062 $WT = 0.70(AT) + 0.07(RH) + 1.01(SO) + 0.001(Alt)$

Where WT is water temperature, AT is Air temperature, RH is relative humidity, SO is stream order and Alt is site altitude.

Values for these variables were obtained from site description data displayed in Tables 2.1, 2.2 and 2.3 of Chapter 2. The results of Model 1 yielded an over estimation of winter temperatures and slight underestimation of summer temperatures. The model was therefore adjusted to better fit mean water temperatures over winter by using complete data sets of water and air temperatures obtained for the Wit and Elandspad rivers. Constants were adjusted until modelled water temperatures best reflected the observed mean water temperatures for these two rivers (Fig. App2C.2). The constants that were adjusted in the model for the winter period are shown in bold face in Table 2C.2.

Table App2C.2. Adjusted models based on those developed by Rivers-Moore *et al.* (2010). Values in bold face indicate adjusted values.

All data (complex) winter – Adjusted	
Mean	- F(4, 9876) = 8062 $WT = \mathbf{-6.00} + 0.70(AT) + 0.08(RH) + 0.93(SO) + 0.001(Alt)$ **(Model 2)**
Min	- F(3, 9877) = 8062 $WT = \mathbf{-6.70} + 0.71(AT) + 0.09(RH) + 0.87(SO)$
Max	- F(4, 9876) = 8062 $WT = \mathbf{0.58}\ (AT) + \mathbf{0.038}(RH) + 1.01(SO) + 0.001(Alt)$

Where WT is water temperature, AT is Air temperature, RH is relative humidity, SO is stream order and Alt is site altitude.

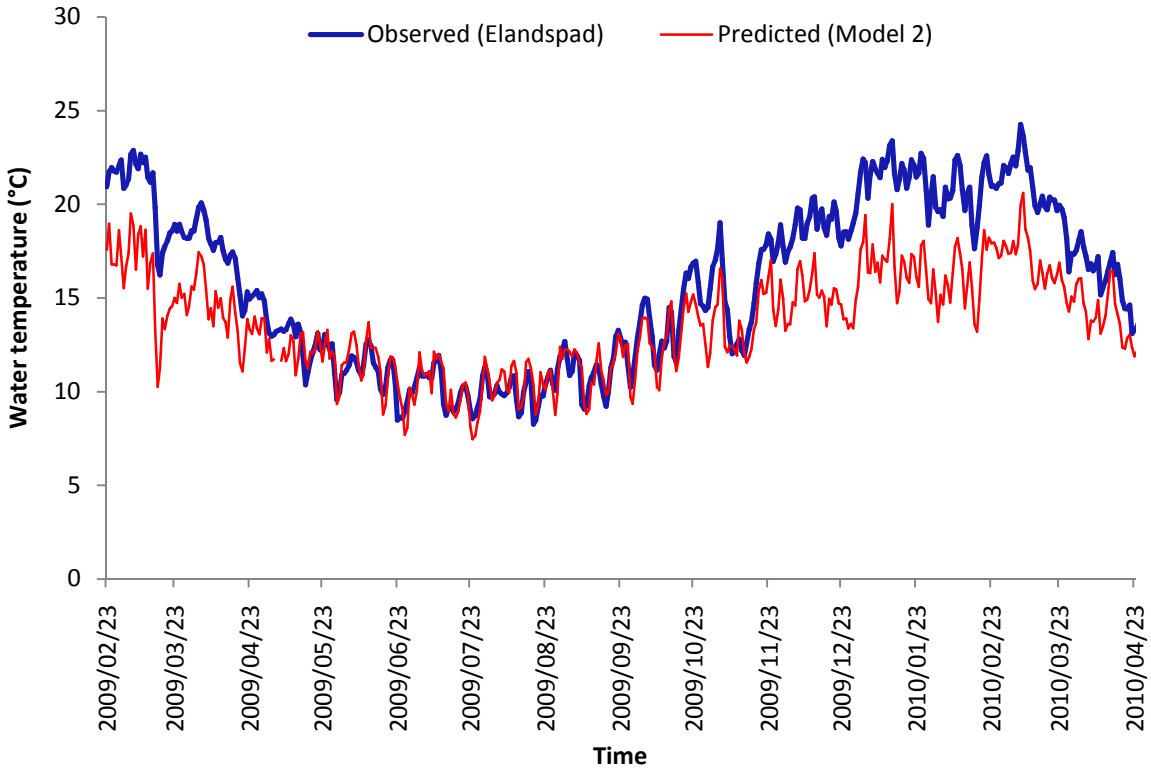


Fig App2C.2. Modelled water temperatures using a winter-adjusted model in relation to observed water temperatures collected for the Elandspad River for the period February 2009 to April 2010.

The model using adjusted constants was then reapplied to the Wolwekloof air temperatures (Model 2) (Fig. App2C.3). This adjusted model showed a better estimation of winter temperatures. This was repeated for the models that were used to estimate mean minimum temperatures and mean maximum temperatures. As winter was the period that was being modelled in the Wolwekloof the model was adjusted to provide a better fit only for the winter period. The model formula (Model 2) with adjusted constants is shown in Fig. App2C.3.

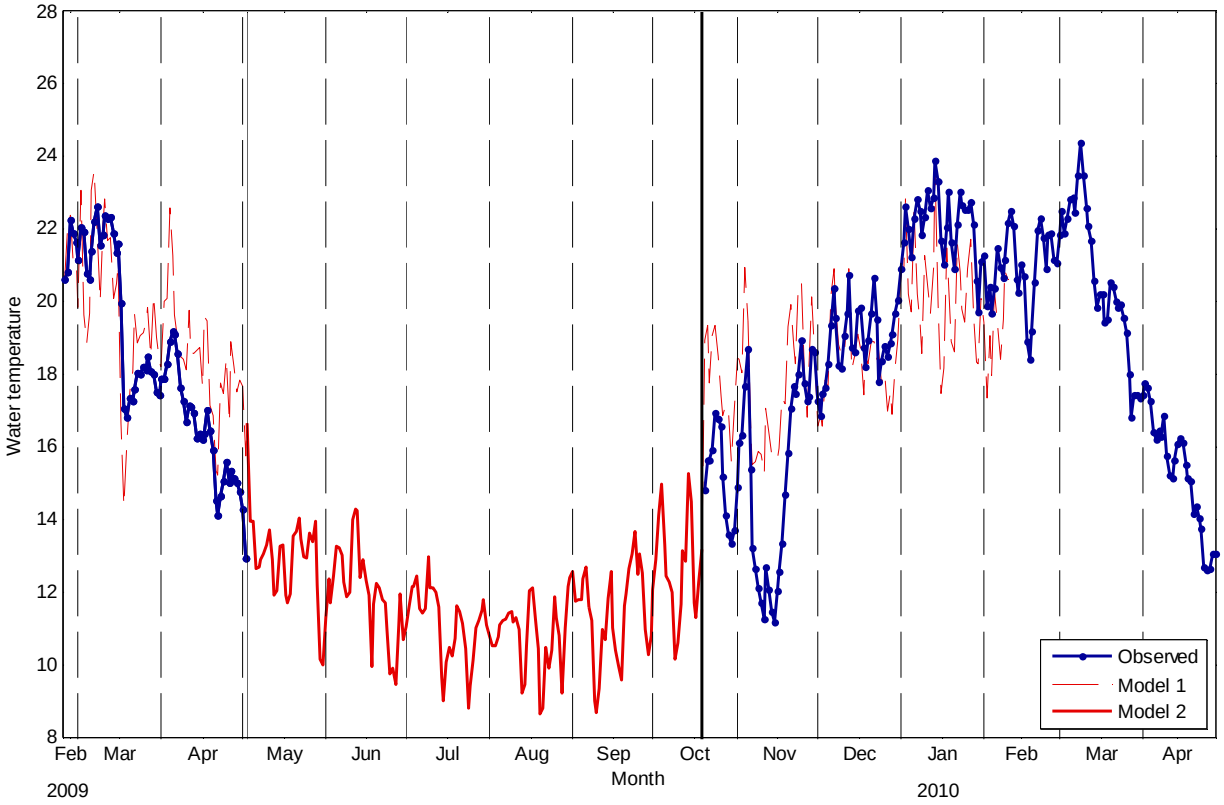


Fig App2C.3. Water temperatures modelled using a winter-adjusted model (Model 2) in relation to observed water temperatures collected for Wolwekloof River for the period February 2009 to April 2010. Model 1 represents an unadjusted model.

APPENDIX 2D

Outputs from hydrological and thermal regime shift analyses

The Sequential T-test Analysis of Regime Shifts (STARS) software, v3.2 (see Rodionov 2004), was used to analyse daily flow and water temperature data for the six study rivers.

Table App2D.1. Hydrological regime shift outputs for mean daily flow data collected from each of the six study rivers for the period from February 2009 to April 2010.

River	Date of Regime shift	Magnitude of Regime shift (RSI of Daily Range)	Description of regime	Duration of regime	Mean Daily flow (m^3/sec)
Eerste	09/02/01	0	Summer baseflow	103	0.03
Eerste	09/05/15	1.12	Mid-autumn spate	5	4.11
Eerste	09/05/20	-1.14	Decline after mid-autumn spate	9	0.28
Eerste	09/05/29	0.32	Late autumn spate	6	2.52
Eerste	09/06/04	0.49	Early winter 1 spate	3	9.86
Eerste	09/06/07	-1.93	Decline after early winter 1 spate	16	2.10
Eerste	09/06/23	0.68	Early winter 2 spate	4	5.87
Eerste	09/06/27	-1.37	Decline after early winter 2 spate	15	0.99
Eerste	09/07/12	0.47	Mid-winter spate	4	5.66
Eerste	09/07/16	-0.76	Decline after mid-winter spate	33	1.24
Eerste	09/08/18	0.13	Late winter spate	5	3.37
Eerste	09/08/23	-0.32	Decline after late winter spate	77	1.08
Eerste	09/11/08	1.38	Late spring spate	5	5.61
Eerste	09/11/13	-1.16	Decline after late spring spate to baseflows	167	0.14
Elandspad	09/02/01	0	Summer Baseflow	123	1.19
Elandspad	09/06/04	0.76	Early winter 1 spate	3	33.41
Elandspad	09/06/07	-1.57	Decline after early winter 1 spate	16	7.13
Elandspad	09/06/23	0.51	Early winter 2 spate	3	31.82
Elandspad	09/06/26	-2.17	Decline after early winter 2 spate	135	3.39
Elandspad	09/11/08	0.80	Late spring spate	6	17.14
Elandspad	09/11/14	-0.90	Decline after late spring spate to baseflows	166	0.50
Molenaars	09/02/01	0	Summer Baseflow	123	2.29
Molenaars	09/06/04	0.52	Early winter 1 spate	4	52.62
Molenaars	09/06/08	-0.90	Decline after early winter 1 spate	15	14.26
Molenaars	09/06/23	0.39	Early winter 2 spate	3	64.34
Molenaars	09/06/26	-1.45	Decline after early winter 2 spate	135	6.68
Molenaars	09/11/08	0.64	Late spring spate	6	36.62
Molenaars	09/11/14	-0.71	Decline after late spring spate to baseflows	166	1.02
Rooi-Els Kloof	09/02/01	0	Summer Baseflow	142	0.18
Rooi-Els Kloof	09/06/23	4.08	Early winter 1 spate	3	4.03
Rooi-Els Kloof	09/06/26	-3.97	Decline after early winter 1 spate	136	0.38
Rooi-Els Kloof	09/11/09	0.79	Late spring spate	7	1.32
Rooi-Els Kloof	09/11/16	-0.42	Decline after late spring spate to baseflows	164	0.13
Wit	09/02/01	0	Summer Baseflow	103	0.24
Wit	09/05/15	0.34	Mid-autumn spate	5	32.26
Wit	09/05/20	-0.29	Decline after mid-autumn spate	9	3.97
Wit	09/05/29	2.20	Late autumn spate	10	37.36
Wit	09/06/08	-0.75	Decline after late autumn spate	34	13.83
Wit	09/07/12	0.06	Mid-winter spate	7	27.81
Wit	09/07/19	-0.45	Decline after mid-winter spate	23	12.30
Wit	09/08/11	0.93	Late winter spate	13	28.72
Wit	09/08/24	-0.96	Decline after late winter spate	18	19.17
Wit	09/09/11	-1.03	Late winter decline after sustained high winter flows	60	2.32
Wit	09/11/10	0.13	Late spring spate	8	13.84
Wit	09/11/18	-0.36	Decline after late spring spate to baseflows	162	0.27
Wolwekloof	09/02/01	0	Summer Baseflow	103	0.12
Wolwekloof	09/05/15	0.86	Mid-autumn spate	4	7.82
Wolwekloof	09/05/19	-1.09	Decline after mid-autumn spate	35	2.23
Wolwekloof	09/06/23	0.45	Early winter 2 spate	3	9.28
Wolwekloof	09/06/26	-1.94	Decline after early winter 2 spate	16	0.02
Wolwekloof	09/07/12	0.02	Mid-winter spate	5	4.62
Wolwekloof	09/07/17	-0.38	Decline after mid-winter spate	114	0.49
Wolwekloof	09/11/08	1.61	Late spring spate	2	12.21
Wolwekloof	09/11/15	-0.34	Decline after late spring spate to baseflows	165	0.01

Daily flow data were analysed using the Ordinary Least Squares (OLS) parameter estimator in conjunction with the prewhitening function. A default sub sample setting size was used and the significance level was set to 0.05. A cut off length of 5 was used and the Huber parameter was set to 1 (see Rodionov & Overland 2005 for details).

Table App2D.2. Thermal regime shift outputs for mean daily range in water temperature data collected from each of the six study rivers for the period from February 2009 to April 2010.

River	Date of Regime shift	Magnitude of Regime shift (RSI of Daily Range)	Description of Regime	Duration of regime shift (Days)	Mean daily range in water temperature (°C)
Eerste	09/03/20	-0.05	Start of early autumn	179	2.15
Eerste	09/09/16	0.28	Start of early spring	27	3.64
Eerste	09/10/15	0.12	Start of mid-spring	20	5.02
Eerste	09/11/04	-1.81	Spring cold front	9	1.83
Eerste	09/11/16	2.05	Start of late spring/summer	99	5.43
Eerste	10/02/20	-0.18	Start of early autumn	55	3.62
Eerste	10/04/16	-0.22	Start of mid-autumn/winter	7	2.21
Elandspad	09/04/15	-1.06	Start of early autumn	28	2.53
Elandspad	09/05/13	-0.36	Start of mid-autumn/winter	128	1.49
Elandspad	09/09/18	0.08	Start of early spring	28	2.74
Elandspad	09/10/16	0.39	Start of mid-spring	20	4.42
Elandspad	09/11/05	-1.57	Spring cold front	10	1.89
Elandspad	09/11/15	2.04	Start of late spring/summer	112	5.59
Elandspad	10/03/06	-0.33	Start of early autumn	38	4.33
Elandspad	10/04/14	-0.13	Start of mid-autumn/winter	11	3.12
Molenaars	09/03/23	-0.10	Start of early autumn	23	1.03
Molenaars	09/04/15	-0.25	Start of mid-autumn/winter	134	1.52
Molenaars	09/08/27	0.15	Start of early spring	32	2.25
Molenaars	09/09/28	0.22	Start of mid-spring	30	3.49
Molenaars	09/10/28	0.31	Hot spell	8	5.08
Molenaars	09/11/05	-2.28	Spring cold front	9	1.92
Molenaars	09/11/14	2.07	Start of late spring/summer	115	4.85
Molenaars	10/03/09	-0.12	Start of early autumn	37	3.56
Molenaars	10/04/15	-0.27	Start of mid-autumn/winter	11	2.55
Rooi-Els Kloof	09/04/22	-0.37	Start of mid-autumn/winter	118	1.12
Rooi-Els Kloof	09/08/18	0.18	Start of early spring	87	1.62
Rooi-Els Kloof	09/11/13	0.39	Start of late spring/summer	114	2.03
Rooi-Els Kloof	10/03/07	-0.26	Start of mid-autumn/winter	50	1.30
Wit	09/04/03	-0.03	Start of early autumn	13	4.59
Wit	09/04/16	-0.44	Start of mid-autumn/winter	164	1.87
Wit	09/09/27	0.06	Start of mid-spring	40	3.99
Wit	09/11/06	-0.39	Spring cold front	8	1.71
Wit	09/11/14	1.11	Start of late spring/summer	28	4.41
Wit	09/12/12	0.78	Start of early summer	87	7.06
Wit	10/03/09	-0.06	Start of early autumn	41	4.82
Wit	10/04/19	-0.52	Start of mid-autumn/winter	6	2.85
Wolwekloof	09/04/16	-0.51	Start of mid-autumn/winter	187	1.07
Wolwekloof	09/10/19	0.75	Start of mid-spring/artefact	17	3.70
Wolwekloof	09/11/06	-1.48	Start of spring cold front mid-	9	1.14
Wolwekloof	09/11/15	1.81	Start of late spring/summer	129	4.41
Wolwekloof	10/03/24	-0.02	Start of early autumn	24	3.26
Wolwekloof	10/04/17	-0.05	Start of mid-autumn/winter	7	2.06

Daily water temperature data (mean daily range in water temperature) were analysed using the Ordinary Least Squares (OLS) parameter estimator in conjunction with the prewhitening function. A significance level of 0.01 was set – such that only regime shifts significant at this level of confidence were detected and displayed. A cut off length of 10 was used and the Huber parameter was set to 1 (see Rodionov & Overland 2005 for further details).

APPENDIX 3A

Descriptions of the setal patterns of mature and early instar nymphs of species of the stonefly genus *Aphanicercella.*

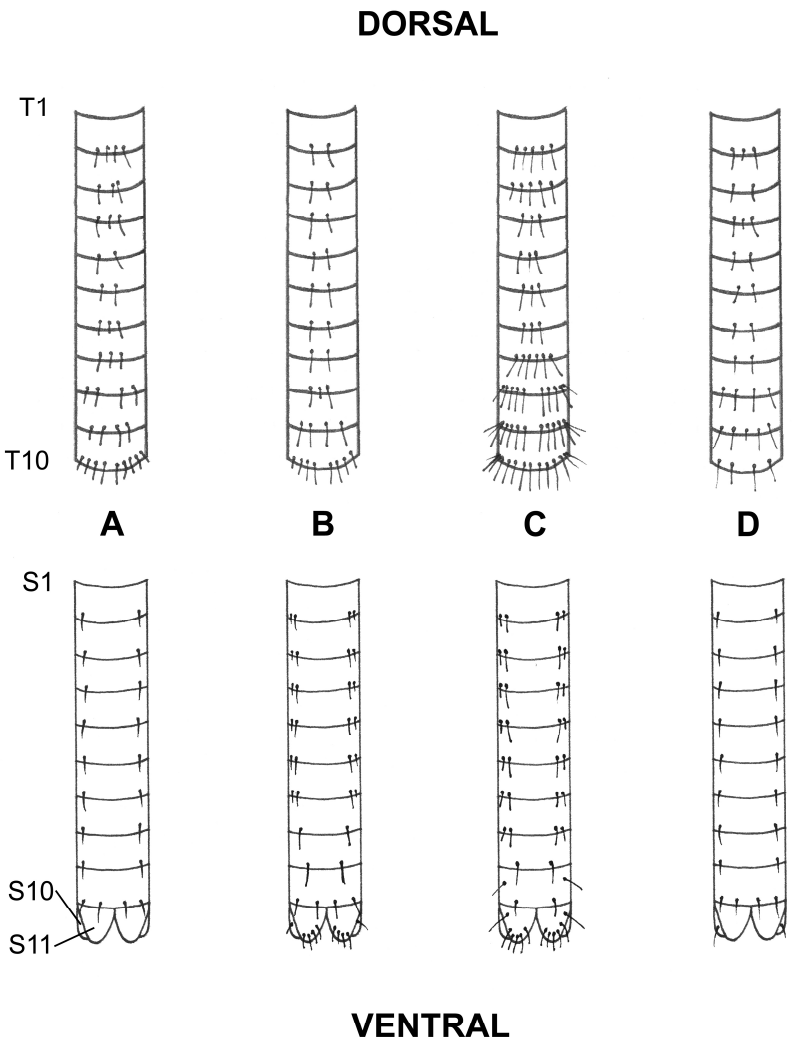


Fig. App3A.1. Abdominal setal patterns of mature nymphs of four species of *Aphanicercella*. **A**, *Aphanicercella clavata*; **B**, *Aphanicercella barnardi*; **C**, *Aphanicercella flabellata*; **D**, *Aphanicercella scutata*. Setal patterns were used to distinguish nymphs collected and analysed for the study of life-histories (Chapter 4). Abbreviations: S = sternite; T = tergite.

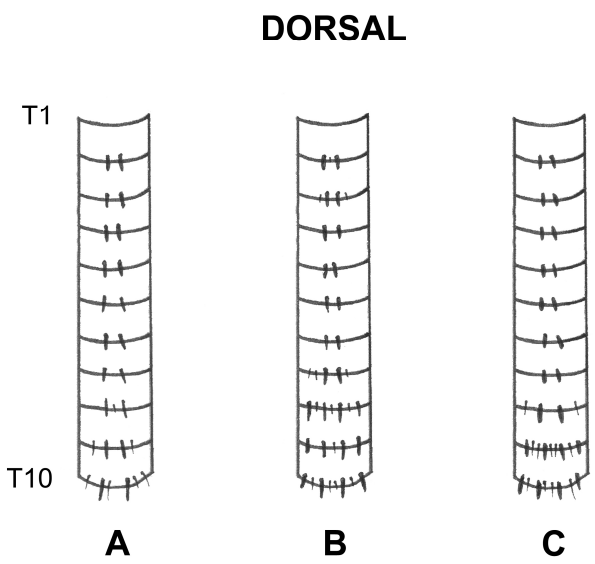


Fig. App3A.2. Abdominal setal patterns of immature nymphs (approximately instars 1-5) of three species of *Aphanicercella*. **A**, *Aphanicercella barnardi*; **B**, *Aphanicercella flabellata*; **C**, *Aphanicercella scutata* at 40X magnification. Setal patterns were used to distinguish immature nymphs collected and analysed for the study of life-histories (Chapter 4). Abbreviations: T = tergite.

Table App3A.1. Average number of setae per abdominal segment for four species of *Aphanicercella* collected in this study - compared to data from Stevens (2009). Range in brackets.

Dorsal abdominal hairs

	This study				**From Stevens (2009)**	
Abdominal segment	*A. barnardi* *n = 11*	*A. clavata* *n = 1*	*A. flabellata* *n = 10*	*A. scutata* *n = 19*	*A. clavata sp.* *n = 19*	*A. scutata* *n = 2*
1	1.9 (1-2)	4	3.1 (1-5)	2.15 (2-3)	2.5(2-5)	1.0(0-2)
2	2	3	3.0 (1-5)	2.15 (2-3)	2.4(2-4)	2
3	2	3	2.7 (2-3)	2.2 (2-3)	2.3(2-4)	2
4	2	2	2.6 (1-4)	2.0 (1-3)	2.1(1-4)	2
5	2	2	2.4 (1-3)	2.0 (1-3)	2.0(1-3)	1.5(1-2)
6	2.0 (1-3)	3	3.0 (2-4)	2.0 (1-2)	2	2
7	2.1 (2-3)	3	5.6 (2-8)	2	2.0(1-3)	2
8	2.5 (2-3)	4	8.1 (7-10)	4.0 (3-5)	3.1(2-6)	3.5(3-4)
9	4.5 (2-5)	4	9.4 (5-12)	5.8 (4-9)	5.1(4-9)	6.5(6-7)
10	7.0 (5-10)	9	9.9 (8-14)	7.0 (4-11)	5.7(3-9	9.0(6-12)

Ventral abdominal hairs

	This study				**From Stevens (2009)**	
Abdominal segment	*A. barnardi* *n = 4*	*A. clavata* *n = 1*	*A. flabellata* *n = 1*	*A. scutata* *n = 1*	*A. clavata sp.* *n = 19*	*A. scutata* *n = 2*
1	4	2	4	2	0	0
2	4	2	4	2	2.0(1-4)	2.0(0-4)
3	4	2	4	2	1.9(0-4)	3.0(2-4)
4	4	2	4	2	1.9(0-4)	2
5	4	2	4	2	1.8(0-2)	3.0(2-4)
6	3.5 (2-4)	2	4	2	1.9(1-3)	3.0(2-4)
7	2	2	4	2	1.9(1-2)	2
8	2	2	2	2	2.2(1-4)	2.5(2-3)
9	4	4	4	4	3.8(1-6)	6
10	9.8 (9-10)	0	12	2	0.6(0-4)	2

APPENDIX 3B

Images of all specimens collected for genetic analyses from the three genera (*Lestagella, Aphanicercella* and *Chimarra*) assessed in this study.

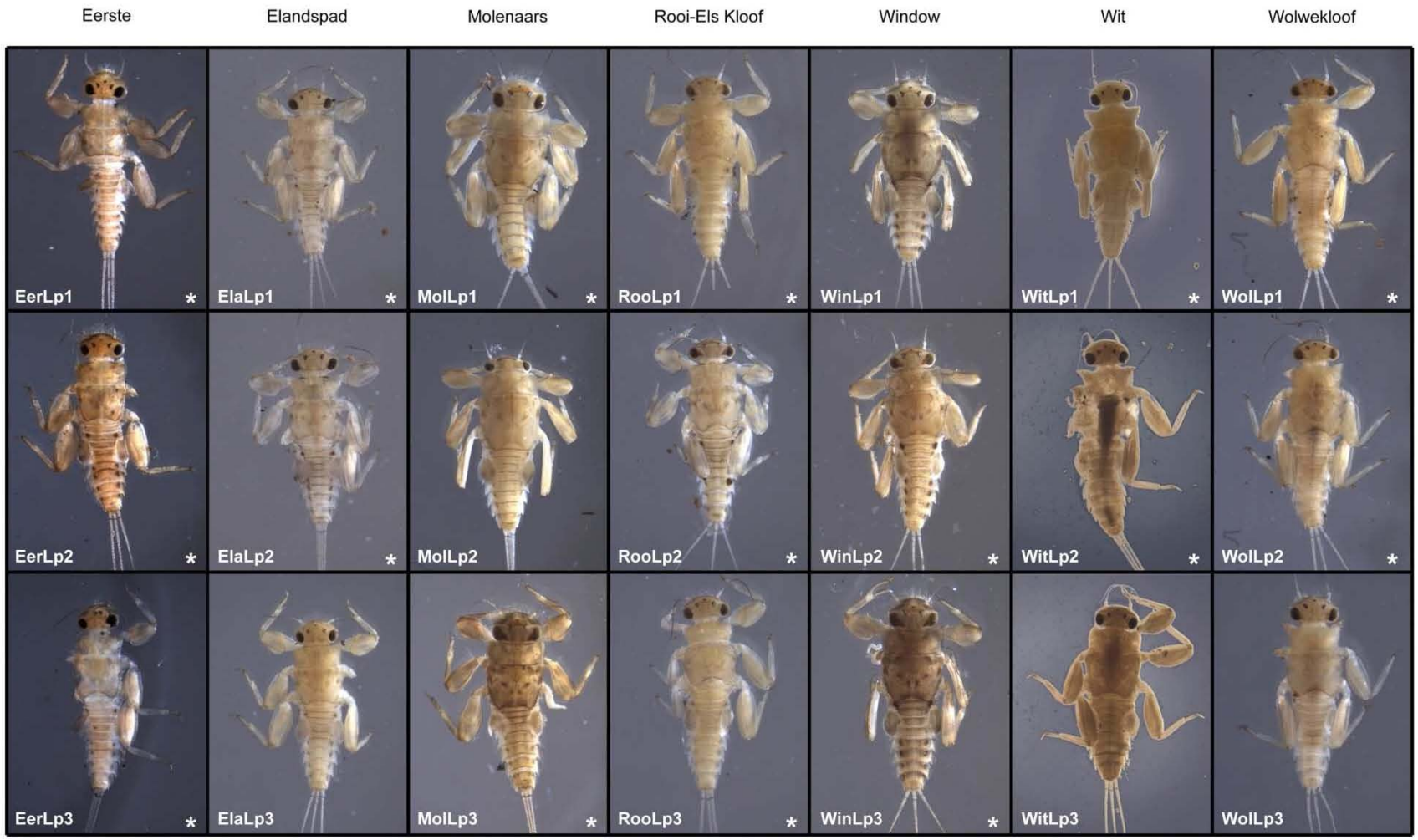


Fig. App3B.1. Images of specimens of *Lestagella penicillata* (nymphs) collected for genetic analysis from seven localities in the Western Cape, South Africa. Specimen sample codes are indicated in the bottom left corner of each image. * Denotes specimens from which DNA was successfully sequenced for use in phylogenetic analyses. Images were taken using a Leica EZ 4 dissecting microscope equipped with a digital camera.

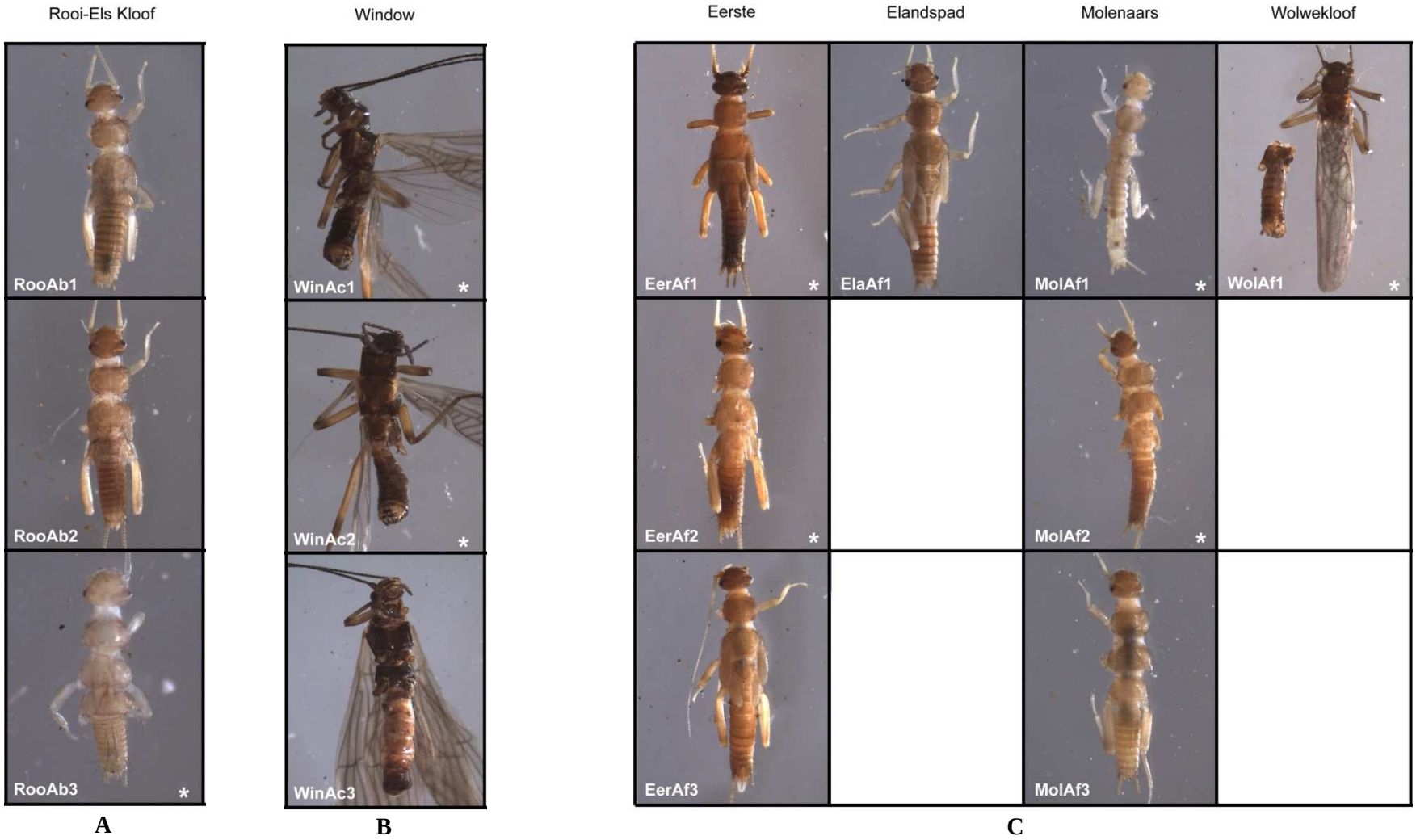


Fig. App3B.2. Images of specimens of *Aphanicercella* collected for genetic analysis from several localities in the Western Cape, South Africa. Specimen sample codes are indicated in the bottom left corner of each image. **A**, *Aphanicercella barnardi* (nymphs); **B**, *Aphanicerella clavata* (adults); **C**, *Aphanicercella flabellata* (nymphs and a single adult). * Denotes specimens from which DNA was successfully sequenced for use in phylogenetic analyses. Images were taken using a Leica EZ 4 dissecting microscope equipped with a digital camera.

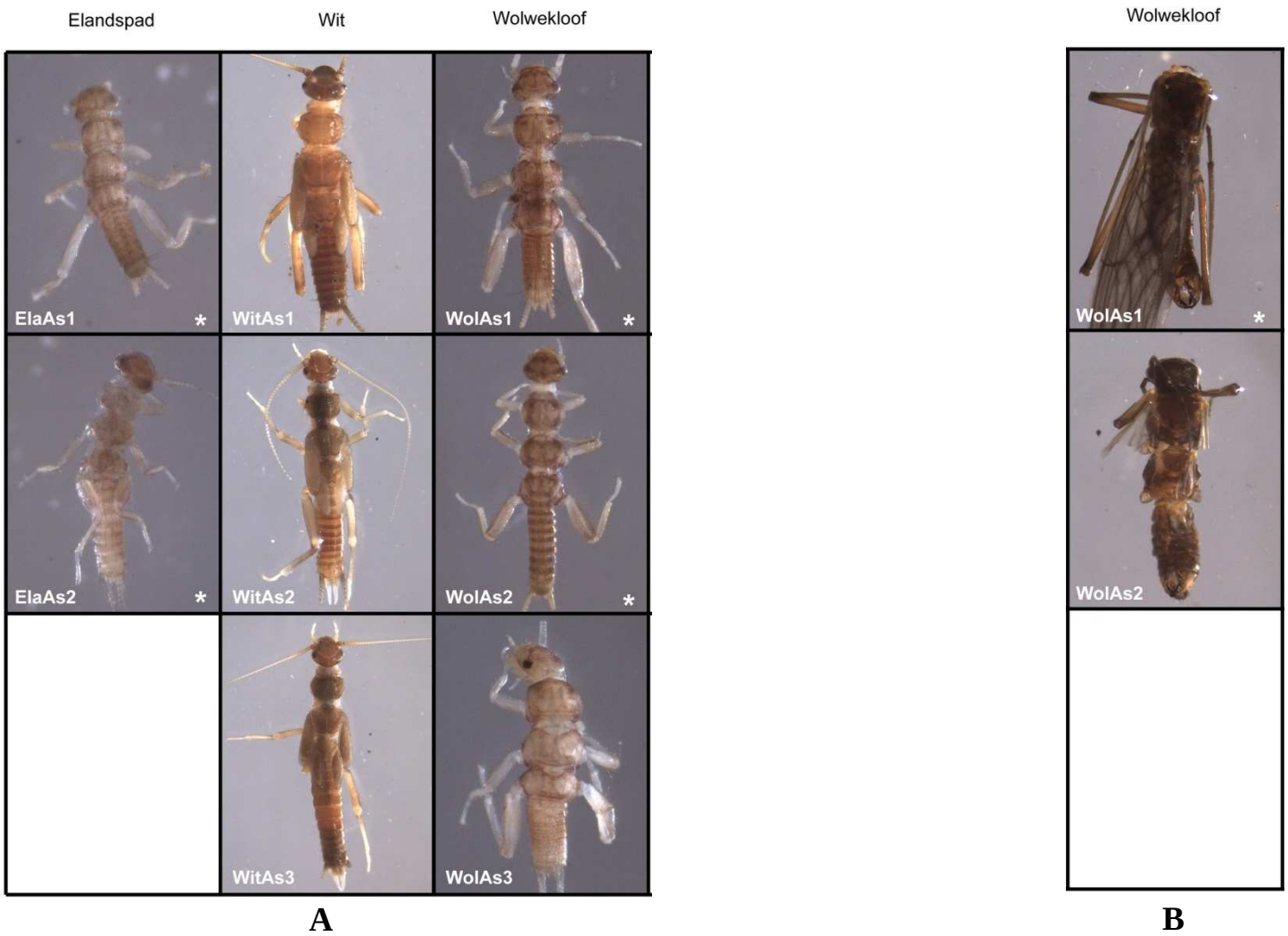


Fig. App3B.3. Images of specimens of *Aphanicercella* collected for genetic analysis from several localities in the Western Cape, South Africa. Specimen sample codes are indicated in the bottom left corner of each image. **A**, *Aphanicercella scutata* (nymphs); **B**, *Aphanicerella scutata* (adults). * Denotes specimens from which DNA was successfully sequenced for use in phylogenetic analyses. Images were taken using a Leica EZ 4 dissecting microscope equipped with a digital camera.

Fig. App3B.4. Images of specimens of *Chimarra ambulans* (larvae) collected for genetic analysis from seven localities in the Western Cape, South Africa. Specimen sample codes are indicated in the bottom left corner of each image. * Denotes specimens from which DNA was successfully sequenced for use in phylogenetic analyses. Images were taken using a Leica EZ 4 dissecting microscope equipped with a digital camera.

Fig. App3B.5. Images of specimens of *Chimarra ambulans* (adults) collected for genetic analysis from a single locality (Window stream) in the Western Cape, South Africa. Specimen sample codes are indicated in the bottom left corner of each image. * Denotes specimens from which DNA was successfully sequenced for use in phylogenetic analyses. Images were taken using a Leica EZ 4 dissecting microscope equipped with a digital camera.

APPENDIX 3C

Accession number of the CO1 DNA barcode region sequences for species used as outgroups in the phylogenetic trees constructed for each genus assessed in this study.

Sequences were obtained from GenBank using a BLASTn search.

Ephemeroptera:

Eurylophella temporalis, family Ephemerellidae

GenBank Accession number: JQ662039.1

Plecoptera:

Aphanicerca capensis, family Notonemouridae

GenBank Accession number: AF429300.1

Trichoptera:

Dolophilodes distinctus, family Philopotamidae

GenBank Accession number: ADR69530.1

APPENDIX 3D

Results of the maximum likelihood fit analysis of nucleotide substitution models for each genus assessed in this study.

Table App3D.1. Maximum likelihood fits of 24 different nucleotide substitution models for specimens of *Lestagella penicillata*. NOTE- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) are also presented (Nei and Kumar 2000). Non-uniformity of evolutionary rates among sites may be modelled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (R) are shown for each model, as well. They are followed by nucleotide frequencies (f) and rates of base substitutions (r) for each nucleotide pair. Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 640 positions in the final dataset. Dark grey shading denotes the model used for constructing the ML phylogram while light grey shading denotes the model used for NJ and ME phylogram construction.

Model	# Par.	BIC	AICc	lnL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G	A=> T	A=> C	A=> G	T=> A	T=> C	T=> G	C=> A	C=> T	C=> G	G=> A	G=> T	G=> C
TN93+I	37	4825.27	4555.63	-2240.68	0.64	n/a	6.51	0.22	0.32	0.25	0.21	0.02	0.02	0.3	0.01	0.11	0.01	0.01	0.14	0.01	0.32	0.02	0.02
T92+I	34	4825.79	4577.99	-2254.89	0.63	n/a	4.21	0.27	0.27	0.23	0.23	0.03	0.02	0.18	0.03	0.18	0.02	0.03	0.22	0.02	0.22	0.03	0.02
TN93+G+I	38	4834.58	4557.65	-2240.69	0.64	200.00	6.52	0.22	0.32	0.25	0.21	0.02	0.02	0.3	0.01	0.11	0.01	0.01	0.14	0.01	0.32	0.02	0.02
K2+I	33	4834.64	4594.12	-2263.96	0.63	n/a	4.03	0.25	0.25	0.25	0.25	0.02	0.02	0.2	0.02	0.2	0.02	0.02	0.2	0.02	0.2	0.02	0.02
T92+G+I	35	4835.12	4580.04	-2254.9	0.00	0.20	4.54	0.27	0.27	0.23	0.23	0.02	0.02	0.19	0.02	0.19	0.02	0.02	0.22	0.02	0.22	0.02	0.02
HKY+I	36	4836.6	4574.24	-2250.99	0.62	n/a	3.67	0.22	0.32	0.25	0.21	0.04	0.03	0.16	0.02	0.19	0.02	0.02	0.25	0.02	0.17	0.04	0.03
K2+G+I	34	4842.17	4594.37	-2263.07	0.60	2.96	4.30	0.25	0.25	0.25	0.25	0.02	0.02	0.2	0.02	0.2	0.02	0.02	0.2	0.02	0.2	0.02	0.02
HKY+G+I	37	4842.49	4572.84	-2249.29	0.57	1.82	4.18	0.22	0.32	0.25	0.21	0.03	0.02	0.17	0.02	0.2	0.02	0.02	0.26	0.02	0.18	0.03	0.02
GTR+I	40	4848.61	4557.12	-2238.41	0.64	n/a	7.08	0.22	0.32	0.25	0.21	0	0.03	0.31	0	0.1	0.02	0.02	0.14	0	0.33	0.03	0
T92+G	34	4854.61	4606.81	-2269.29	n/a	0.52	2.99	0.27	0.27	0.23	0.23	0.03	0.03	0.17	0.03	0.17	0.03	0.03	0.21	0.03	0.21	0.03	0.03
GTR+G+I	41	4857.91	4559.15	-2238.41	0.64	200.00	7.06	0.22	0.32	0.25	0.21	0	0.03	0.31	0	0.1	0.02	0.02	0.14	0	0.33	0.03	0
HKY+G	36	4861.24	4598.88	-2263.32	n/a	0.53	2.95	0.22	0.32	0.25	0.21	0.04	0.03	0.15	0.03	0.18	0.03	0.03	0.24	0.03	0.16	0.04	0.03
K2+G	33	4862.74	4622.22	-2278.01	n/a	0.53	2.94	0.25	0.25	0.25	0.25	0.03	0.03	0.19	0.03	0.19	0.03	0.03	0.19	0.03	0.19	0.03	0.03
TN93+G	37	4868.1	4598.45	-2262.1	n/a	0.53	2.95	0.22	0.32	0.25	0.21	0.04	0.03	0.18	0.03	0.16	0.03	0.03	0.21	0.03	0.19	0.04	0.03
GTR+G	40	4891.66	4600.17	-2259.94	n/a	0.54	2.92	0.22	0.32	0.25	0.21	0.05	0.05	0.18	0.04	0.16	0.02	0.04	0.22	0.02	0.19	0.03	0.02
T92	33	4980.45	4739.94	-2336.87	n/a	n/a	2.31	0.27	0.27	0.23	0.23	0.04	0.03	0.16	0.04	0.16	0.03	0.04	0.19	0.03	0.19	0.04	0.03
HKY	35	4982.73	4727.65	-2328.71	n/a	n/a	2.32	0.22	0.32	0.25	0.21	0.05	0.04	0.14	0.03	0.17	0.03	0.03	0.23	0.03	0.15	0.05	0.04
K2	32	4985.25	4752.01	-2343.91	n/a	n/a	2.30	0.25	0.25	0.25	0.25	0.04	0.04	0.17	0.04	0.17	0.04	0.04	0.17	0.04	0.17	0.04	0.04
TN93	36	4991.68	4729.31	-2328.53	n/a	n/a	2.32	0.22	0.32	0.25	0.21	0.05	0.04	0.15	0.03	0.16	0.03	0.03	0.22	0.03	0.16	0.05	0.04
GTR	39	5009.48	4725.28	-2323.5	n/a	n/a	2.32	0.22	0.32	0.25	0.21	0.07	0.05	0.15	0.05	0.16	0.02	0.04	0.22	0.02	0.16	0.03	0.03
JC+I	32	5019.62	4786.39	-2361.1	0.59	n/a	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
JC+G	32	5025.07	4791.84	-2363.82	n/a	0.32	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
JC+G+I	33	5034.37	4793.85	-2363.82	0.00	0.32	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
JC	31	5134.76	4908.81	-2423.31	n/a	n/a	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

Table App3D.2. Maximum likelihood fits of 24 different nucleotide substitution models for specimens of *Aphanicercella*. NOTE- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) are also presented (Nei and Kumar 2000). Non-uniformity of evolutionary rates among sites may be modelled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (R) are shown for each model, as well. They are followed by nucleotide frequencies (f) and rates of base substitutions (r) for each nucleotide pair. Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 629 positions in the final dataset. Dark grey shading denotes the model used for constructing the NJ, ME and ML phylogram

Model	# Par.	BIC	AICc	lnL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G	A=> T	A=> C	A=> G	T=> A	T=> C	T=> G	C=> A	C=> T	C=> G	G=> A	G=> T	G=> C
T92+G	28.00	3507.70	3309.56	-1626.69	n/a	0.16	2.69	0.31	0.31	0.19	0.19	0.04	0.02	0.14	0.04	0.14	0.02	0.04	0.23	0.02	0.23	0.04	0.02
T92+G+I	29.00	3516.25	3311.04	-1626.42	0.39	0.37	2.72	0.31	0.31	0.19	0.19	0.04	0.02	0.14	0.04	0.14	0.02	0.04	0.23	0.02	0.23	0.04	0.02
HKY+G	30.00	3518.41	3306.13	-1622.96	n/a	0.17	2.50	0.27	0.35	0.19	0.18	0.05	0.03	0.13	0.04	0.14	0.03	0.04	0.26	0.03	0.20	0.05	0.03
TN93+G	31.00	3525.32	3305.97	-1621.87	n/a	0.19	2.42	0.27	0.35	0.19	0.18	0.05	0.03	0.10	0.04	0.17	0.03	0.04	0.31	0.03	0.14	0.05	0.03
HKY+G+I	31.00	3526.87	3307.52	-1622.65	0.40	0.43	2.55	0.27	0.35	0.19	0.18	0.05	0.03	0.13	0.04	0.14	0.02	0.04	0.26	0.02	0.20	0.05	0.03
TN93+I	31.00	3527.53	3308.18	-1622.97	0.68	n/a	2.28	0.27	0.35	0.19	0.18	0.05	0.03	0.10	0.04	0.16	0.03	0.04	0.30	0.03	0.15	0.05	0.03
GTR+G	34.00	3531.05	3290.49	-1611.11	n/a	0.25	2.22	0.27	0.35	0.19	0.18	0.10	0.04	0.09	0.07	0.17	0.00	0.05	0.32	0.00	0.13	0.01	0.00
GTR+I	34.00	3532.65	3292.10	-1611.91	0.65	n/a	2.16	0.27	0.35	0.19	0.18	0.10	0.04	0.09	0.07	0.17	0.00	0.06	0.32	0.00	0.13	0.01	0.01
HKY+I	30.00	3533.01	3320.73	-1630.26	0.54	n/a	2.01	0.27	0.35	0.19	0.18	0.06	0.03	0.13	0.04	0.13	0.03	0.04	0.24	0.03	0.18	0.06	0.03
TN93+G+I	32.00	3533.69	3307.26	-1621.51	0.42	0.50	2.49	0.27	0.35	0.19	0.18	0.05	0.03	0.10	0.04	0.17	0.03	0.04	0.31	0.03	0.15	0.05	0.03
GTR+G+I	35.00	3539.74	3292.12	-1610.91	0.39	0.63	2.25	0.27	0.35	0.19	0.18	0.10	0.04	0.09	0.07	0.17	0.00	0.05	0.32	0.00	0.13	0.01	0.00
K2+G	27.00	3558.42	3367.35	-1656.59	n/a	0.18	2.41	0.25	0.25	0.25	0.25	0.04	0.04	0.18	0.04	0.18	0.04	0.04	0.18	0.04	0.18	0.04	0.04
K2+G+I	28.00	3566.82	3368.68	-1656.25	0.42	0.46	2.45	0.25	0.25	0.25	0.25	0.04	0.04	0.18	0.04	0.18	0.04	0.04	0.18	0.04	0.18	0.04	0.04
T92	27.00	3569.94	3378.87	-1662.35	n/a	n/a	1.75	0.31	0.31	0.19	0.19	0.05	0.03	0.12	0.05	0.12	0.03	0.05	0.20	0.03	0.20	0.05	0.03
GTR	33.00	3574.32	3340.83	-1637.29	n/a	n/a	1.75	0.27	0.35	0.19	0.18	0.11	0.04	0.09	0.09	0.16	0.01	0.06	0.29	0.01	0.13	0.01	0.01
HKY	29.00	3576.50	3371.29	-1656.54	n/a	n/a	1.75	0.27	0.35	0.19	0.18	0.06	0.03	0.12	0.05	0.12	0.03	0.05	0.23	0.03	0.18	0.06	0.03
T92+I	28.00	3579.02	3380.87	-1662.34	0.00	n/a	1.75	0.31	0.31	0.19	0.19	0.05	0.03	0.12	0.05	0.12	0.03	0.05	0.20	0.03	0.20	0.05	0.03
TN93	30.00	3579.26	3366.98	-1653.38	n/a	n/a	1.77	0.27	0.35	0.19	0.18	0.06	0.03	0.09	0.05	0.15	0.03	0.05	0.28	0.03	0.13	0.06	0.03
K2+I	27.00	3600.16	3409.09	-1677.46	0.28	n/a	1.81	0.25	0.25	0.25	0.25	0.04	0.04	0.16	0.04	0.16	0.04	0.04	0.16	0.04	0.16	0.04	0.04
JC+G	26.00	3614.13	3430.12	-1688.98	n/a	0.20	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
K2	26.00	3614.50	3430.50	-1689.17	n/a	n/a	1.75	0.25	0.25	0.25	0.25	0.05	0.05	0.16	0.05	0.16	0.05	0.05	0.16	0.05	0.16	0.05	0.05
JC+G+I	27.00	3623.06	3431.99	-1688.91	0.44	0.61	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
JC	25.00	3664.26	3487.32	-1718.59	n/a	n/a	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
JC+I	26.00	3664.32	3480.32	-1714.08	0.12	n/a	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

Table App3D.3. Maximum likelihood fits of 24 different nucleotide substitution models for specimens of *Chimarra ambulans*. NOTE- Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) are also presented (Nei and Kumar 2000). Non-uniformity of evolutionary rates among sites may be modelled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (R) are shown for each model, as well. They are followed by nucleotide frequencies (f) and rates of base substitutions (r) for each nucleotide pair. Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 525 positions in the final dataset. Dark grey shading denotes the model used for constructing the ML phylogram while light grey shading denotes the model used for NJ and ME phylogram construction.

Model	# Par.	BIC	AICc	lnL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G	A=> T	A=> C	A=> G	T=> A	T=> C	T=> G	C=> A	C=> T	C=> G	G=> A	G=> T	G=> C
HKY+G	30.00	3147.94	2941.12	-1440.43	n/a	0.13	7.81	0.28	0.36	0.22	0.14	0.02	0.01	0.12	0.02	0.19	0.01	0.02	0.32	0.01	0.25	0.02	0.01
T92+G	28.00	3152.66	2959.62	-1451.70	n/a	0.13	8.16	0.32	0.32	0.18	0.18	0.02	0.01	0.16	0.02	0.16	0.01	0.02	0.29	0.01	0.29	0.02	0.01
T92+I	28.00	3153.85	2960.80	-1452.29	0.63	n/a	8.45	0.32	0.32	0.18	0.18	0.02	0.01	0.16	0.02	0.16	0.01	0.02	0.29	0.01	0.29	0.02	0.01
TN93+G	31.00	3156.15	2942.44	-1440.09	n/a	0.14	7.81	0.28	0.36	0.22	0.14	0.02	0.01	0.10	0.02	0.21	0.01	0.02	0.36	0.01	0.21	0.02	0.01
HKY+G+I	31.00	3156.84	2943.14	-1440.43	0.00	0.13	7.81	0.28	0.36	0.22	0.14	0.02	0.01	0.12	0.02	0.19	0.01	0.02	0.32	0.01	0.25	0.02	0.01
TN93+I	31.00	3159.75	2946.05	-1441.89	0.63	n/a	8.34	0.28	0.36	0.22	0.14	0.02	0.01	0.15	0.01	0.17	0.01	0.01	0.29	0.01	0.29	0.02	0.01
T92+G+I	29.00	3161.56	2961.63	-1451.70	0.00	0.13	8.16	0.32	0.32	0.18	0.18	0.02	0.01	0.16	0.02	0.16	0.01	0.02	0.29	0.01	0.29	0.02	0.01
TN93+G+I	32.00	3165.05	2944.46	-1440.09	0.00	0.14	7.81	0.28	0.36	0.22	0.14	0.02	0.01	0.10	0.02	0.21	0.01	0.02	0.36	0.01	0.21	0.02	0.01
GTR+G	34.00	3178.22	2943.86	-1437.77	n/a	0.14	7.46	0.28	0.36	0.22	0.14	0.01	0.03	0.10	0.01	0.22	0.01	0.04	0.37	0.00	0.20	0.02	0.00
GTR+I	34.00	3183.03	2948.67	-1440.17	0.63	n/a	8.23	0.28	0.36	0.22	0.14	0.01	0.02	0.14	0.01	0.17	0.01	0.03	0.29	0.00	0.29	0.02	0.00
GTR+G+I	35.00	3187.12	2945.88	-1437.77	0.00	0.14	7.46	0.28	0.36	0.22	0.14	0.01	0.03	0.10	0.01	0.22	0.01	0.04	0.37	0.00	0.20	0.02	0.00
K2+G	27.00	3210.44	3024.28	-1485.04	n/a	0.14	6.80	0.25	0.25	0.25	0.25	0.02	0.02	0.22	0.02	0.22	0.02	0.02	0.22	0.02	0.22	0.02	0.02
HKY	29.00	3211.64	3011.70	-1476.73	n/a	n/a	2.31	0.28	0.36	0.22	0.14	0.05	0.03	0.10	0.04	0.15	0.02	0.04	0.26	0.02	0.20	0.05	0.03
T92	27.00	3217.79	3031.63	-1488.71	n/a	n/a	2.30	0.32	0.32	0.18	0.18	0.05	0.03	0.13	0.05	0.13	0.03	0.05	0.23	0.03	0.23	0.05	0.03
TN93	30.00	3218.10	3011.28	-1475.51	n/a	n/a	2.33	0.28	0.36	0.22	0.14	0.05	0.03	0.08	0.04	0.17	0.02	0.04	0.29	0.02	0.16	0.05	0.03
K2+G+I	28.00	3219.34	3026.29	-1485.04	0.00	0.14	6.80	0.25	0.25	0.25	0.25	0.02	0.02	0.22	0.02	0.22	0.02	0.02	0.22	0.02	0.22	0.02	0.02
HKY+I	30.00	3220.54	3013.72	-1476.73	0.00	n/a	2.31	0.28	0.36	0.22	0.14	0.05	0.03	0.10	0.04	0.15	0.02	0.04	0.26	0.02	0.20	0.05	0.03
K2+I	27.00	3233.36	3047.20	-1496.50	0.48	n/a	3.15	0.25	0.25	0.25	0.25	0.03	0.03	0.19	0.03	0.19	0.03	0.03	0.19	0.03	0.19	0.03	0.03
GTR	33.00	3235.73	3008.25	-1470.97	n/a	n/a	2.33	0.28	0.36	0.22	0.14	0.06	0.05	0.08	0.05	0.18	0.01	0.07	0.29	0.00	0.16	0.04	0.00
K2	26.00	3268.86	3089.58	-1518.70	n/a	n/a	2.26	0.25	0.25	0.25	0.25	0.04	0.04	0.17	0.04	0.17	0.04	0.04	0.17	0.04	0.17	0.04	0.04
JC+G	26.00	3315.48	3136.21	-1542.01	n/a	0.27	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
JC+G+I	27.00	3324.06	3137.90	-1541.84	0.34	0.54	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
JC+I	26.00	3340.95	3161.68	-1554.74	0.20	n/a	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
JC	25.00	3344.62	3172.24	-1561.03	n/a	n/a	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08

Abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.

APPENDIX 4A

Multivariate analysis of life-history data submitted as part of a PRIMER+PERMANOVA statistical training course (Included for the interested reader).

AIM: to interrogate life-history data in relation to environmental and physicochemcial variables using multivariate techniques

METHODS

For statistical analyses, hourly water temperature data were converted to daily and then monthly mean, minimum, maximum, range and standard deviation values. Similarly daily flow records were converted to monthly mean, range and standard deviation values. Owing to the fact that a) no single species of *Aphanicercella* occurred consistently at each of the study sites and b) in some cases low numbers of one or two co-occurred, the three species of *Aphanicercella* collected from the different study sites were pooled for the purposes of this study to provide more information at the genus level for this taxa and its occurrence in each river.

Biological data collected in this study consisted of species stage compositional data i.e.relative numbers of individuals of each species in predetermined size class bins each month for the six study rivers. Size classes have been pre-defined to represent the approximate size range of each of the specific instars present in each species (e.g. five size classes for the Trichopteran *C. ambulans*, each size class represents a single larval instar). For detailed information on how size classes/numbers of instars were determined for each species see Dallas *et al.* (2012).

This stage compositional data essentially represents the life history data for each species across the six rivers and consists of a matrix with river/sampling month representing the samples and the specific size classes for each of the three taxa representing the variables. The cells are populated with the relative numbers of individuals (or relative abundances) present in each of the size classes of the three taxa collected each month from the study rivers.

Spreadsheet checks

Biological data were checked in PRIMER for the presence of samples containing all zero's, as these samples represented an artefact of sampling – the result of sampling rivers shortly after a spate or high flow event. Only one such sample (the sample from the Eerste River in the month of June) was detected and subsequently removed as it represented an outlier in the biological data. The biological data were then transformed using a fourth root transformation technique, commonly applied to species assemblage data, so as to down-weight the abundance of dominant species and minimise the bias these dominant species cause in analyses of similarity between samples. In this case the

transformation served to down-weight the abundance of individuals in specific dominant size classes of the three taxa. Fourth root transformation was applicable as abundances in size classes for a given sampling month ranged from 0 or 1 to up to 400 plus. Using the Bray-Curtis distance measure, a resemblance matrix of the biological data was then formed to show similarities of samples based on species stage compositional data. This measure is particularly appropriate for this type of data as it ignores negative matches and similarities are therefore not based on the absence of particular size classes in a species from a river in a given sample.

For the environmental data, similar checks to those conducted for the biological data were performed in Primer to detect all zero samples or missing values. Relationships between pairs of variables were visualised using draftsman's plots and variables showing high degrees of multi co linearity with other variables ($r > 0.8$) were noted. Additionally those variables with skewed distributions were identified and adjusted accordingly using appropriate transformations. The variable "turbidity" was the only variable that required an individual log transformation as a result of the presence of a few extremely high values relative to other values resulting in right skewness. After the transformation of this particular variable, all environmental data were normalised to minimise the effects of the different scales used to measure individual variables. A resemblance matrix of the environmental data was formed using the Euclidean distance measure. Additionally a Principal Component Analysis (PCA) was used to determine which environmental variables account for the greatest variation in the environmental data when visualised with a season overlay. This in turn was used to help inform the selection of variables for analyses such as BEST/BIOENV as well as DISTLM.

Main multi-dimensional scaling plots (MDS)

Variation and similarity in the stage compositional data among the rivers and sampling times were visually analysed using a combination of Multi-dimensional Scaling plots, cluster analyses and associated SIMPROF tests.

Cyclicity test

As samples were collected monthly over the period of a single year, it was assumed that entire life-history cycles would be observed in univoltine, bivoltine and multivoltine species. This inherently introduces a degree of cyclicity in the data and implies that samples will be closely related to/dependent on time. In order to investigate this assumption, a model matrix for cyclicity was constructed based on seasons, where numeric values 0, 0.25, 0.5, 0.75 were assigned to each season respectively. Using the RELATE function (set at 9999 permutations, and using the spearman rank correlation method), the resultant resemblance matrix from this model matrix was tested against the resemblance matrix of the stage compositional data.

ANOSIM

To determine whether the combined stage compositional data for all of the species differed as a result of the effects from the *a priori* defined groups "time" (season effect) and " river" (site effect),a two way crossed ANOSIM with replicates was employed. Permutations (set to 9999) were used to determine significance levels of the r-statistics reported for main effects and pair-wise tests. In this case ANOSIM tested the null hypothesis of 'no time effect' on stage compositional data allowing for the fact that there may be differences between rivers and similarly that there is 'no river effect' allowing for the fact that there may be time effects.

2STAGE MDS

In addition to the tests for cyclicity using a model matrix, second stage MDS plots were constructed to visually compare differences in the stage compositional data of the species through time and across the study rivers. Second stage MDS plots are particularly useful for analysing time series and data captured using a repeated measures design (Clarke & Gorley 2001). In this case the second stage MDS plot split a single similarity matrix in which sample groups were defined using two factors (an outer factor "river" and an inner factor "time") and proceeded to analyse similarities between these groupings. Second stage MDS plots were constructed using stage compositional data from each species separately, as well as with data from all species combined. This in turn allowed for a direct visual comparison of life history data of each species across the rivers.

PERMANOVA

While ANOSIM can provide information regarding the effects of "river" and "time" on stage compositional data (using ranked similarities), it cannot however provide information on the effect of an interaction of these two factors or partition variability accordingly among factors (Anderson *et al.* 2008), and thus PERMANOVA was used.

PERMANOVA was conducted using the combined species stage compositional data, because results for all species together would be similar to the results of a single species – especially since in this case a single species (*L. penicillata*) was more dominant across all rivers in each of the sampling months. A two way crossed fixed effects design was employed, with "river" and "season" employed as factors and having 6 and 4 levels respectively. River was selected as a factor to determine whether stage compositional data of the selected genera were affected by differential thermal and hydrological regimes in these rivers. Season was selected as a factor to determine whether stage compositional data of the selected genera showed similar differences within each of the seasons across the rivers. While it may seem more logical to have made "river" a random effect in this design, it is important to note that the rivers in this study were not randomly selected to represent a sample of possible river systems

(with associated thermal and hydrological regimes) but instead they were deliberately pre-selected to represent a wide range of thermal and hydrological regimes (where analysis of 20 years of historical flow data was used as a proxy for selecting sites that would exhibit an equally wide range of temperature variability). A main effects PERMANOVA was conducted (sum of squares type III partial, permutation method under a reduced model with 9999 maximum permutations) and a subsequent PERMANOVA was run to analyse PAIR-WISE tests of the interaction term "river x season", for pairs of the factor "river."

PERM DISP

To test the null hypothesis, in this case that there is no difference in the multivariate dispersion among the groups of the stage compositional data, in terms of their within group dispersions, the PERM DISP test was utilised in conjunction with PERMANOVA. PERM DISP in this case was employed to help determine if the differences (if detected)in PERMANOVA between the stage compositional data among rivers and different seasons could be as a result of differences in the mean stage compositional data of these groups, or as a result of variability of the stage compositional data within these groups, or a mixture of both. PERM DISP was thus performed on the combined species stage compositional data using both the group factors "river" and "season" respectively.

DistLM – comparison to BEST

In order to determine which environmental variables or groups of environmental variables best accounted for the variation observed in the stage compositional data, a combination of DIST LM (with associated dbRDA analysis) and BIOENV functions were used and compared. For DIST LM, environmental variables were related to the stage compositional data (selection criterion: R^2, selection procedure: all specified) using grouped variables. An indicator was used to group the environmental variables into suites of similar ecologically meaningful variables (i.e. temperature related variables, flow related variables and physicochemical related variables). This allowed for variables showing an appreciable degree of co linearity (e.g. mean monthly temperature, mean minimum monthly temperature and number of monthly temperature regime shifts) to be grouped together as opposed to being removed altogether from the analysis. This was deemed an important step, as these variables while showing a degree of co linearity, in fact have quite different effects on the ecology of aquatic insects and *should* therefore be included in analyses to provide additional information regarding the larger effect of temperature in its entirety. This approach would be similar to conducting a PCA on all of the environmental variables and then relating the scores of the first two axes to the stage compositional data. However the PCA does not retain all the information related to each individual variable and therefore the above mentioned approach was employed.

While the BEST/BIO ENV function included in PRIMER essentially allows for a similar approach to relating environmental data to biological data, and also specifies combinations of variables that best explain the variance in the biological data (using rank order correlations of dissimilarities and inter point distances from environmental variables), it cannot apportion variation to these variables individually or collectively (Anderson *et al.* 2008). DIST LM on the other hand fits a linear model of predictor variables (or groups of predictor variables), in response to the biological data cloud or stage compositional data in this case, and also partitions the variability that is explained by each environmental variable (Anderson *et al.* 2008). Multiple permutations based on the resemblance matrices and selection of variables then allow for p-values to be generated for each variable/pair of variables to determine whether these variables in fact explain a statistically significant proportion of variation among the stage compositional data (Anderson *et al.* 2008). This makes this approach more useful in teasing out which environmental variables have a greater influence on, and statistically significant relationship with, the stage compositional data.

RESULTS

Figure 1 shows the 2-dimensional MDS ordination of the samples of stage compositional data collected monthly from six rivers, based on √√- transformed abundances and a Bray-Curtis similarity matrix. Superimposed on the MDS plot are symbols denoting the seasons of the year to which the monthly samples correspond, as well as the clustering of samples at the 40% similarity level based on a cluster analysis (Fig. 3). Distinct clusters of samples are visible which bear a fairly strong relationship to the month of sampling or, on a coarser scale, the four seasons of the year (2D Stress = 0.15). Overlap between these clusters is evident, and the samples reveal an overall circular form, indicative of an underlying cyclic response. Additionally, the cluster analysis reveals a similar grouping of samples into seasons, however certain clusters of outliers associated with each season are visible. Dispersion appears greatest in samples collected from spring and summer and in particular samples collected from the Wit River.

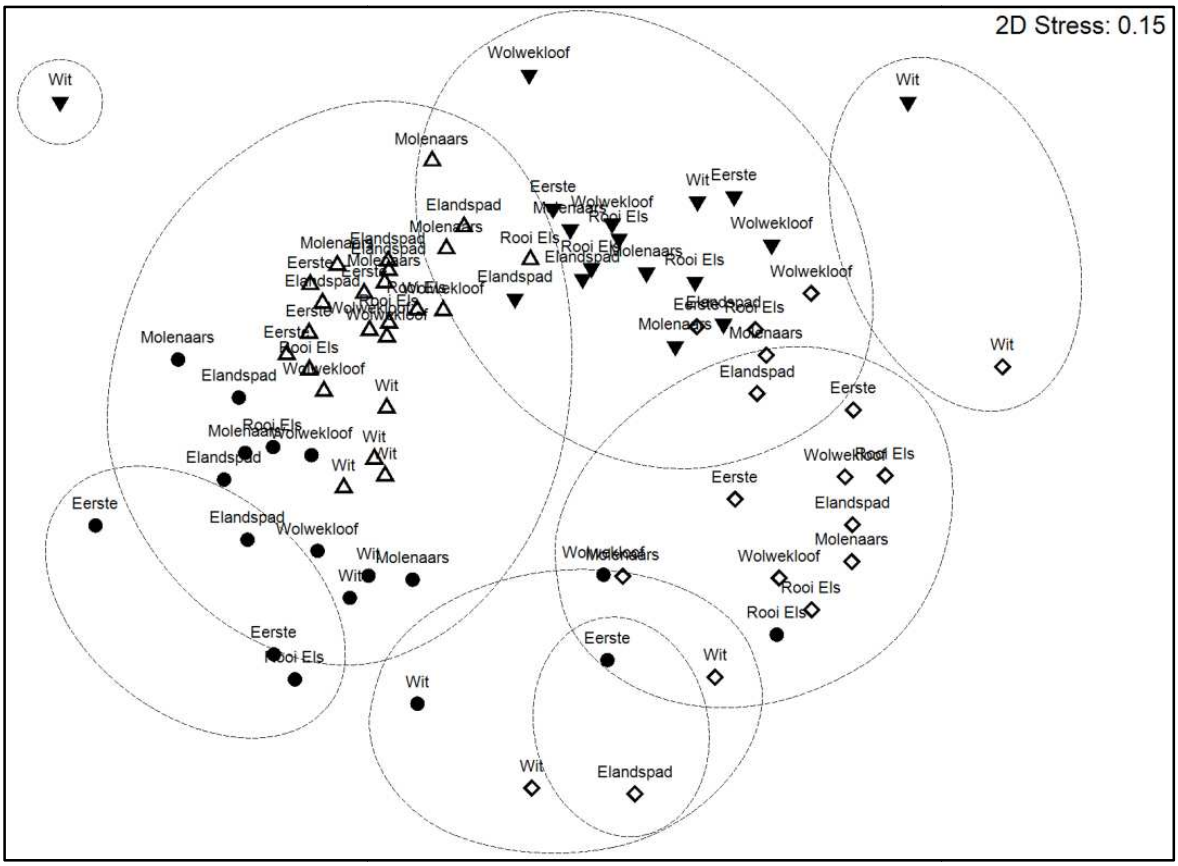


Fig. 1. A non metric multi-dimensional scaling plot of stage compositional data for three aquatic insect genera collected at monthly intervals from April2009-April2010 from six rivers in the Western Cape. Symbols indicate groupings of samples into four seasons (Open triangles = autumn; Solid triangles = winter; Open diamonds = spring; Closed circles = summer). Dashed line represents 40% similarity level of samples.

The stage compositional data when matched to a model matrix based on seasonal cyclicity, using the RELATE test, showed a highly significant rank correlation (ρ = 0.583, p = 0.001, T = 9999 simulations), indicating the presence of an underlying seasonal effect acting on each of the studied genera acrossall the rivers (Fig. 2).

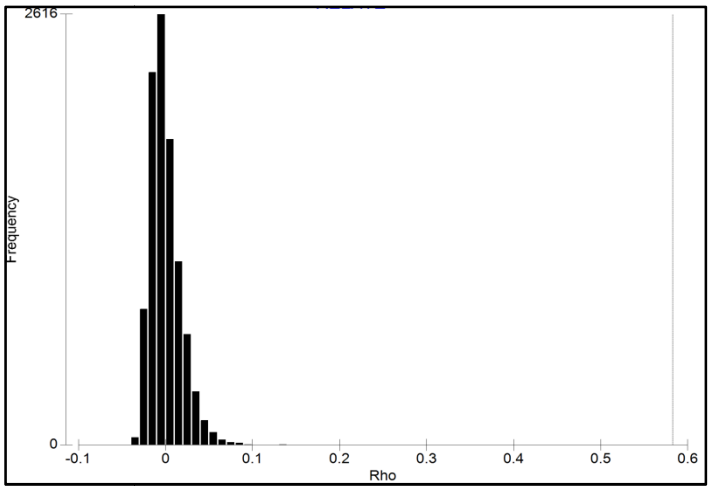


Fig. 2. Simulated distribution of the test statistic *R* under the null hypothesis of "no cyclicity effect" for a RELATE test matching a cyclicity matrix to stage compositional data. Dashed line representsan observed *R* value of 0.583.

A cluster analysis of the √√- transformed stage compositional data from all rivers, using the Bray-Curtis similarity measure and associated SIMPROF test (Fig. 3), revealed a similar seasonal grouping of samples. Two significant major clusters (as well as three samples from the Wit River) separated out at a similarity of approximately 23%. These two clusters generally grouped samples collected during summer and autumn together and those collected during winter and spring together. Significant subdivisions in these two major clusters tended to group the samples from each of the seasons individually – in most cases these samples were grouped with a high level of similarity (~60%). Some degree of overlap between samples collected from subsequent seasons and a few outlying samples (the majority of these being from the Wit River) are visible in the dendrogram.

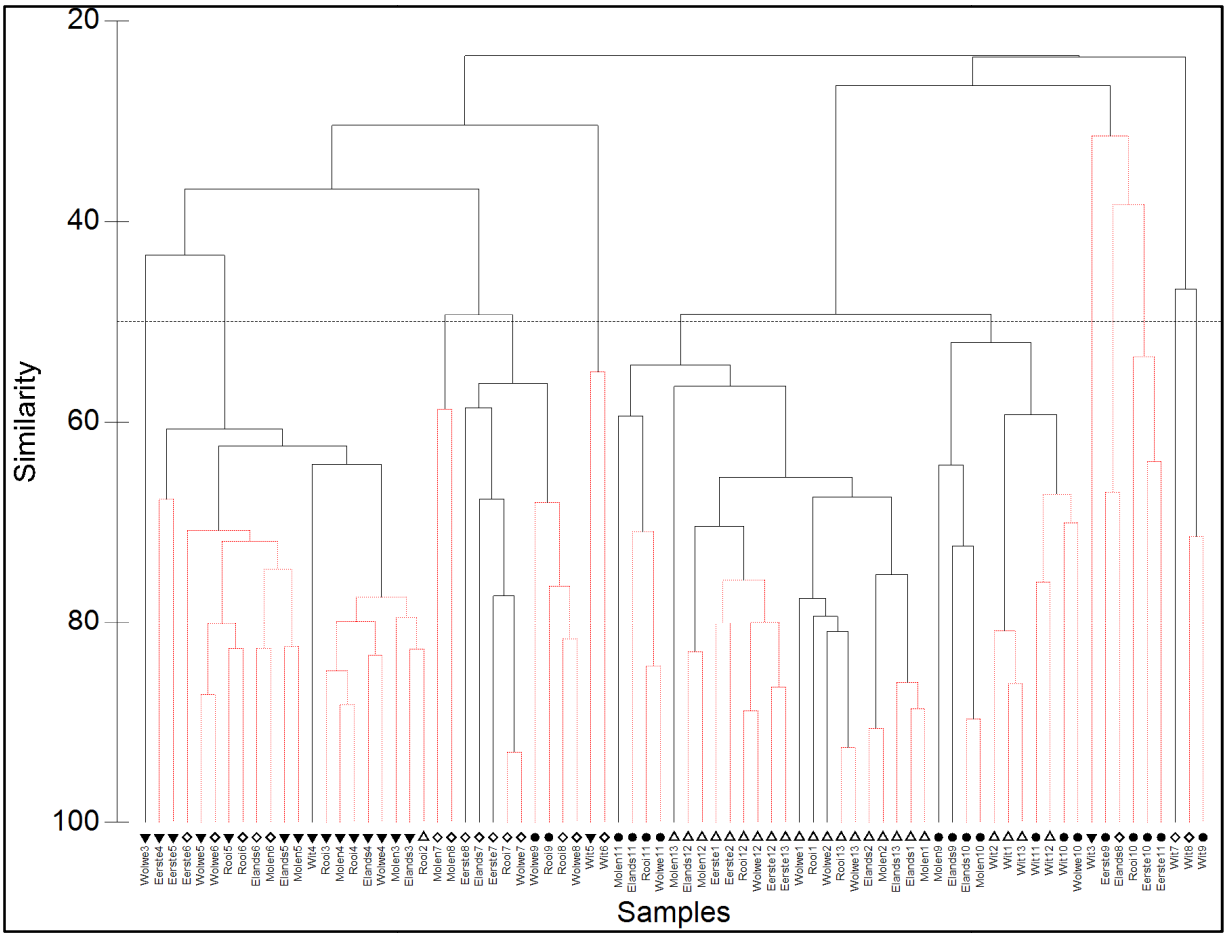


Fig. 3. Dendrogram using group-average linking on Bray-Curtis sample similarities from √√-transformed stage compositional data for three aquatic insect genera collected at monthly intervals from April 2009–April 2010 from six rivers in the Western Cape. Symbols indicate groupings of samples into four seasons (Open triangles = autumn; Solid triangles = winter; Open diamonds = spring; Closed circles = summer). Dashed horizontal line indicates 50% similarity slice

Two-way crossed ANOSIM tests revealed significant effects (p = 0.01, T = 9999 simulations) of the *a priori* defined grouping factors "River" and "Season" on the stage compositional data (Fig. 4a, b). The global R statistics calculated for the "River" and "Season" grouping factors were R = 0.375 and R = 0.763 respectively, suggesting a stronger effect of season across all rivers, as opposed to a stronger effect of river across all seasons. Nevertheless both grouping factors were found to be significant. Pairwise tests revealed highly significant differences between all pairs of seasons when tested across the rivers (p = 0.01 and R > 0.7), with the lowest global R statistic calculated between the seasons winter and spring (R = 0.526, p = 0.01). In contrast, pair wise tests of rivers across all seasons revealed the greatest differences between the Wit River and each of the other rivers (p < 0.05 in all cases) as well as between the Eerste River and the Elandspad River (R = 0.569, p = 0.04).

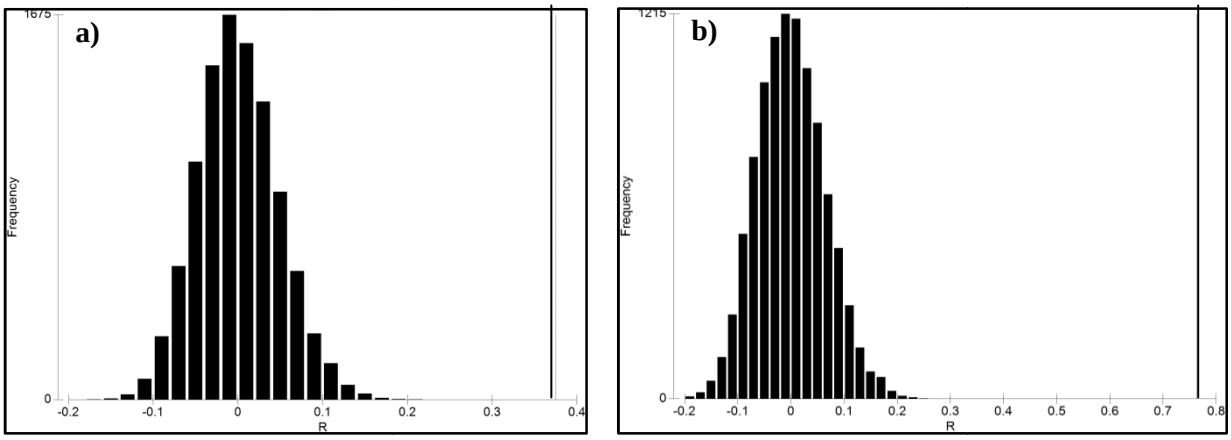


Fig. 4. Two-way crossed ANOSIM results indicating the simulated distribution of the test statistic *R* under the null hypothesis of a) "no river effect" and b) "no season effect" for combined stage compositional data. Solid lines represent observed values for *R* (*R* = 0.375 and *R* = 0.763 respectively).

The difference in combined stage compositional data through time compared across each of the six study rivers is shown in Fig. 5(a correlation of sites $\rho > 0.8$ has been superimposed). Similarly Figs. 6a, b, c reveal differences in the stage compositional data through time compared across rivers, in relation to each genus individually (a: Lestagella, b: Chimarra, c: Aphanicercella). Figure 5 reveals a high correlation in stage compositional data through time between a cluster containing the Eerste, Wolwekloof and Rooi-Els rivers, and an additional cluster incorporating the Elandspad and Molenaars rivers. The Wit River is shown to be most dissimilar to both of these clusters. While in general the individual genera revealed similar patterns to the combined stage compositional data, subtle differences in the clustering of rivers was observed in each plot for each genus, suggesting a non-uniform response of the species across the rivers.

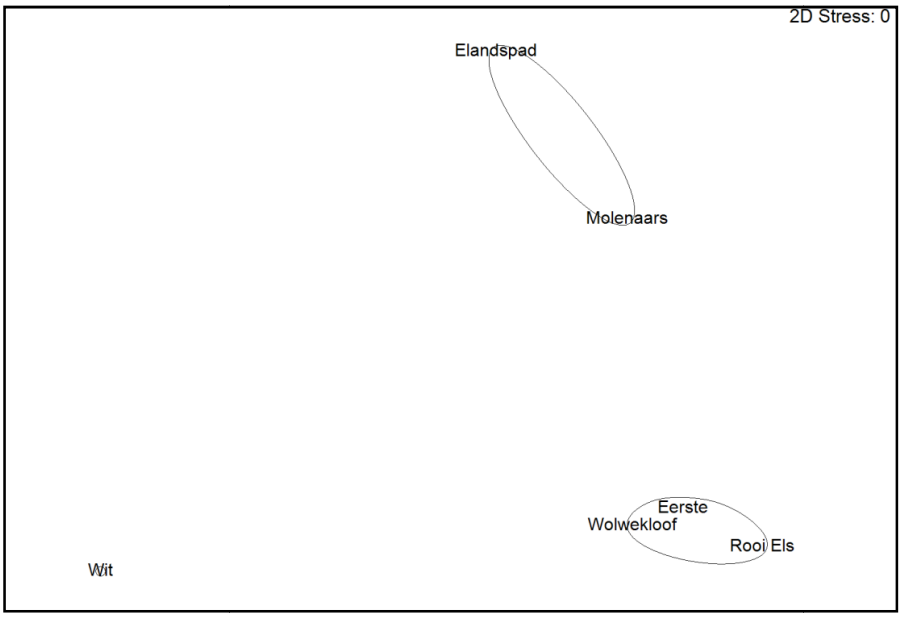


Fig. 5. Second stage MDS plot of combined stage compositional data using "river" as outer factor and "time" (sampling month) as inner factor. Groupings are shown for the ($\rho > 0.8$) correlation level.

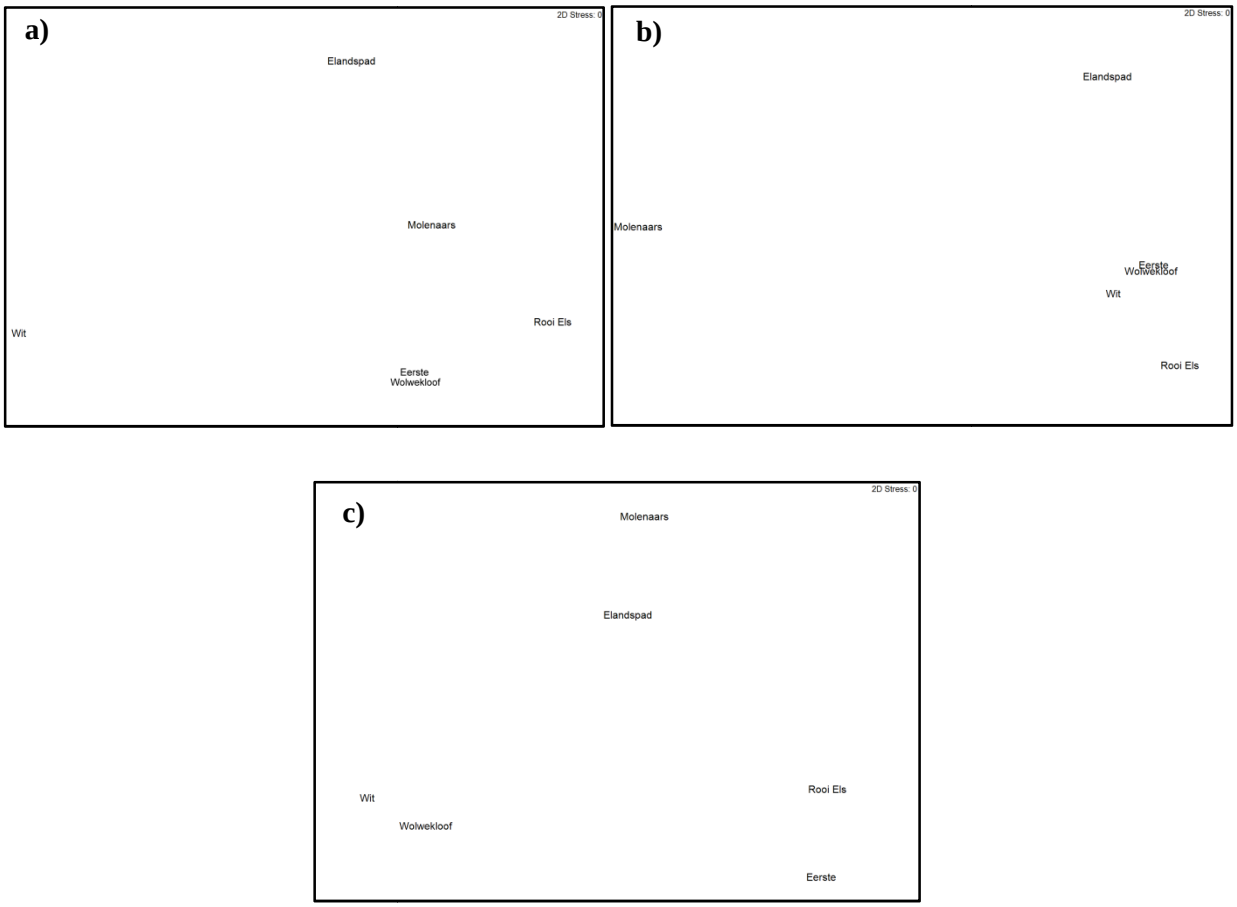


Fig. 6. Second stage MDS plots for stage compositional data relating to each genus of aquatic insect studied a) *Lestagella penicillata*, b) *Aphanicercella* spp. and c) *Chimarra ambulans* using "river" and "season" as inner and outer factors respectively.

PERMANOVA revealed significant differences to occur among stage compositional data for each of the study rivers based on season and river effects ($p < 0.001$; Table 1). Additionally, the interaction of river+season was significant. Further *a posteri,* pair-wise tests (data not shown) revealed that stage compositional data for the genera differed in all rivers between the seasons autumn and spring and autumn and summer as well as between winter and summer. In the Molenaars, Wolwekloof and Rooi-Els rivers, stage compositional data differed between the seasons winter and spring, while no differences between these two seasons were detected in the remaining rivers. However in all the rivers except the Rooi-Els and Wit, stage compositional data was shown to differ between spring and summer. In general, stage compositional data collected in the autumn and summer seasons differed the most across rivers.

Table 1. Results of a main test fixed effects two-way crossed PERMANOVA testing for differences in stage compositional data of three genera of aquatic insect across rivers and seasons. Samples were collected monthly from six rivers in the Western Cape from April 2009-April 2010.Each term was tested using 9999 permutations. Estimates of multivariate variation are given for each factor.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
River	5	18219	3643.7	4.0826	0.0001	9911
Season	3	85261	28420	31.843	0.0001	9935
River x Season	15	27064	1804.3	2.0216	0.0001	9844
Residual	53	47303	892.51			
Total	76	1.783E5				

A PERMDISP test (deviations from centroid) for the homogeneity of variances between the stage compositional data relating to the grouping factor "river" revealed no significant results ($F_{5,71}$=0.753, p = 0.71) using permutations (T = 9999 simulations) to generate significance values. However an additional PERMDISP test for the homogeneity of variances between the stage compositional data relating to the grouping factor "season" (Table 2) revealed significant results ($F_{3,73}$=5.837, p = 0.004), with pairwise comparisons revealing which seasons showed highest differences in variance, namely autumn, when compared to spring and summer.

Table 2. PERMDISP test results for homogeneity of variances in combined stage compositional datafor three aquatic insect genera for pair-wise comparisons of levels of the factor season. Tests used 9999 permutations.

Groups	t	P(perm)
(autumn, winter)	1.307	0.267
(autumn, spring)	3.799	0.001
(autumn, summer)	4.872	0.0001
(winter, spring)	1.265	0.320
(winter, summer)	1.888	0.133
(spring, summer)	0.765	0.487

Results of the distance-based linear modelling variation partitioning showed that each of the three groups of environmental variables (T = Thermal variables, F = Flow variables, P = Physicochemical variables), when assessed separately without accounting for variation explained by other variables (marginal tests), were significantly related (p < 0.05) to the stage compositional data and explained reasonable amounts of the total variation (34%, 11% and 29%) (Table 3). Sequential tests, using the "all specified" selection procedure, and the "R^2 selection" criterion, showed that of the grouped environmental variables, the thermal variables explained the most variation, followed by physicochemical variables and then flow variables (Table 3). The cumulative proportion of variance in the stage compositional data explained by a combination of these groups of variables was approximately 60% with the fixed linear model revealing an appreciable R^2 value of 0.6.

Table 3. Table of results from distance-based liner model fitting three groups of environmental variables to combined stage compositional data for three genera of aquatic insect collected monthly from six rivers in the Western Cape from April 2009-April 2010. Marginal and sequential test results using the all specified selection procedure are shown. An overall model fit of R^2 = 0.6 was obtained.

Marginal tests

Group	SS (trace)	Pseudo-F	P	Prop.	res.df	regr.df
T(Thermal)	60480	7.289	0.001	0.3392	71	6
F(Flow)	19383	1.732	0.011	0.10871	71	6
P(Physicochem)	51121	5.7077	0.001	0.28671	71	6

Sequential tests

Group	R^2	SS(trace)	Pseudo-F	P	Prop.	Cumul.	res.df	regr.df
+T(Thermal)	0.3392	60480	7.289	0.001	0.3392	0.3392	71	6
+F(Flow)	0.41219	13015	1.6392	0.041	7.2994E-2	0.41219	66	11
+P(Physicochem)	0.59602	32777	5.5515	0.001	0.18383	0.59602	61	16

When compared to the best results obtained from a BIOENV test, it is observed that similarly the combination of variables best accounting for variation in the stage compositional data for two variables are mean monthly minimum temperature and mean monthly maximum temperature (ρ = 0.385). An overall best rank correlation (ρ = 0.399) was calculated from a combination of five variables (mean monthly minimum temperature, mean monthly maximum temperature, mean channel width, number of temperature regime shifts and mean electrical conductivity).

The distance-based redundancy analysis (Fig. 7) revealed that monthly mean minimum and maximum temperature variables were most strongly correlated with the first dbRDA axis (multiple partial correlations = -0.604 and -0.501 respectively), while mean turbidity and mean electrical conductivity were most strongly correlated with the second dbRDA axis (multiple partial correlations = 0.555 and 0.463 respectively). The first two axes of the dbRDA cumulatively accounted for 78.5% of the variation in the fitted DISTLM model and 46.8% of the total variation in the stage compositional data.

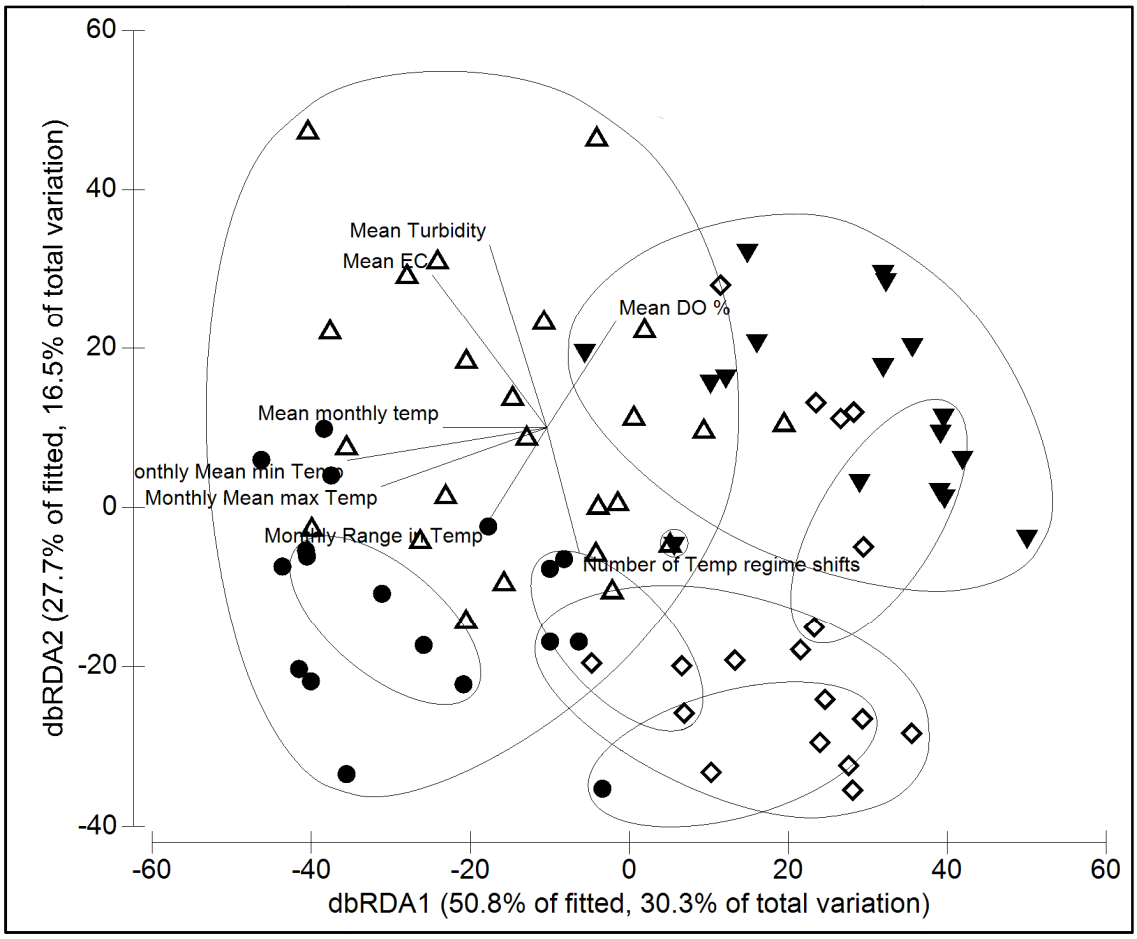


Fig. 7. Constrained ordination plot of the first two axes resulting from a distance based redundancy analysis (dbRDA) of combined stage compositional data for three aquatic insect genera collected monthly from six rivers in the Western Cape to explanatory environmental variables. Only rank correlations (ρ > 0.3) of environmental variables are shown by vectors. Symbols indicate groupings of samples into four seasons (Open triangles = autumn; Solid triangles = winter; Open diamonds = spring; Closed circles = summer). Groupings of samples relate to a 40% similarity level.

DISCUSSION

Stage compositional data of the study taxa were found to show differences across the six study rivers, when analysed in conjunction with one another as well as when they were analysed individually. The same species of aquatic insect has been shown to exhibit different life history responses in six rivers, each with similar physical characteristics and located over a relatively small geographical range (~100km), but incurring substantially different thermal and hydrological regimes.

These differences co-inside with the similarities of sites based purely on environmental variables (see Chapter 2 of this thesis). This suggests that the aquatic insect taxa studied here exhibit site specific life-history responses across the rivers examined. As suspected, seasonality was found to be a major underlying factor driving the structure of the combined stage compositional data/life history-responses of the study taxa across the rivers. Additionally the effect of river was shown to be significant,

suggesting that the sites selected for this study exhibit a range in thermal and hydrological regime sufficient to induce life history responses in the biota inhabiting these rivers.

The seasons summer and autumn when compared across the rivers were shown to be the most variable in relation to the stage compositional data. For summer, this could be explained as a result of the high degree of variability in temperature recorded over the summer months for each river – this having a noticeable effect on the date of emergence of adults and also hatching of eggs for the mayfly *L. penicillata*. The Wit River for instance, which experienced higher mean temperatures over late spring and summer (up to 5°C warmer) compared to the Rooi-Els Kloof River, exhibited an earlier peak emergence date of adults (up to 1 month earlier) of this mayfly and also earlier hatching of eggs (also 1 month earlier). This would obviously have caused a great degree of variability on the stage compositional data collected from these rivers over these months, as both larger and smaller size classes of individuals would have been recorded at different times. In autumn a major contributing factor would have been the differential adult emergence dates of the stonefly species from the genus *Aphanicercella* recorded across the rivers, which also would have had a similar effect on both the relative abundance (fewer individuals in emergence months, as adults leave the stream) and distribution of size class data (very large individuals dominate until emergence, after which slower growing smaller size classes become dominant) recorded in these months. The relative abundances of the *C. ambulans* would also have greatly influenced the stage compositional data for the months of autumn and summer. In autumn, as the temperatures drop and high flows dominate, this genus radically drops off in numbers; the timing of this sudden drop off in numbers is dependent on a combination of average temperatures and the first high flows, which differs across rivers. Over the summer months, rivers which experience a greater degree of warming and experience this warming earlier in the season (e.g. Wit and Wolwekloof rivers), have a sudden boom in numbers of this genus occurring. This is in contrast to colder rivers (Rooi-Els Kloof and Eerste rivers) in which this boom in numbers occurs later in the season. These combined effects from each genus are very likely to have resulted in the seasons of summer and autumn exhibiting a greater degree of variability in the stage compositional data, compared to more stable months

It is likely that the second stage MDS plots for each individual taxa reveal genera specific preferences for certain environmental variables unique to certain rivers (or groups of rivers), as such the unique clusters of rivers in these plots should be examined further and related to the environmental data.

Additionally it should be noted that the amount of total variation in the stage compositional data left unexplained (~50%) using the model fitted with the DIST LM function is as a result of additional environmental variables that were not measured or considered in this specific study (e.g. algal biomass, photoperiod etc).

Of the environmental variables measured in this study, temperature was found to have the greatest effect on life history/stage compositional data across the six study rivers, followed by physicochemical properties and then flow. It is interesting to note that just several measures of temperature, flow and physicochemical properties alone accounted for over half the variation observed in the life-history/stage compositional data for three separate genera. This indeed reveals that these species are influenced by a combination of environmental factors (not only flow and temperature) that need to be identified, measured/collected at each location and tested using the multivariate techniques used here. Quite surprising was the fact that flow variables accounted for the least variation in the DISTLM model, suggesting that flow might indeed have less of an impact on life history responses of genera occurring in perennial rivers, in comparison to temperature and water chemistry properties. It is possible that temperature - having more of a direct effect on the physiology (growth rates, metabolic rates etc, indirectly through promoting algal production and food growth) of aquatic insects - outweighs the effect of flow which might have more of an indirect effect on physiology and more of a direct control on mortality as well as relative abundances. For this reason a similar study could be undertaken to investigate the life-history responses of the same species in both temporary and perennial rivers.

ACKNOWLEDGEMENTS

The author would like to thank Prof. John Field, Dr. Dawit Yemane and Dr. Lara Atkinson for their assistance with statistical analyses.

APPENDIX 4B

Transformation of data for GLM's

Investigation of the field data showed slightly different behaviour of the insects in the six study rivers. In particular, simple regressions for *Lestagella* and *Aphanicercella* indicated that the average growth rates differed amongst the rivers (see Figs. 4.3, 4.5 in Chapter 4). The rivers were chosen to represent a range of thermal and hydrological conditions, and the natural question that follows is if (and to what extent) the differing growth rates can be attributed to temperature, flow or physicochemical variables.

A general linear model (GLM) is one tool for undertaking such an investigation, and to estimate what effect temperature, flow, etc have on growth. A GLM was therefore set up, using categorical data[47] (input variables temperature, flow etc were binned into appropriate[48] bins). A problem quickly arose – because of the cyclic nature of temperature, there were no data to estimate the effect of high temperatures in the winter months and correspondingly there were no data to estimate the effect of low temperatures in the summer months. Consequently in the "month" category, there was no one month which spanned all the temperature bins that could be used as a reference level to which to compare the other months. But because of the time trends in the data (as evidenced by the individuals insects growth with time), the "month" category had to be included in the GLM. A method was therefore devised to de-trend the data (i.e. remove the time-effect), and involved the following steps:

1. Fit a growth curve to river-aggregated data
2. Use the estimated growth curve to transform the data so that no obvious trends with time remain
3. Check that the transformed data are normally distributed
4. Use the transformed data in the GLM

Given the monthly variation in insect sizes, finding a suitable growth curve was not entirely straightforward. Six growth curves were explored: linear, quadratic, exponential, von Bertalanffy, logistic and Gompertz. In each case, the relevant growth curve was fit to the data, and the resulting estimated parameters were used to first linearise the data, and finally transform the data, as explained in Table App4B.1 and Table App4B.2 below.

Note that in the following discussions, '*T*' and '*L*' relate to the three study species in the following manner:

[47] The use of continuous data makes the assumption that the response variable has a linear relationship with the predictor variables. Since this has not been established in this study, it was considered better to use categorical data.
[48] Bins were chosen so that the spread of the data points in the bins was as equal as possible.

Table App4B.1. Definitions of the measure of time (*T*) and size (*L*) used for each of the three taxa in the descriptions that follow

Species	***T***	***L***
Lestagella	Days since hatch	Inter-ocular distance (IOD)
Aphanicercella	Days since hatch	Head capsule width (HCW)
Chimarra	Instar number	Head capsule length (HCL)

The equations below describe how the data can be linearised.

Table App4B.2. Equations and transformed variables for each of the six transformations applied.

Growth Model	**Model**	**Linearised form**	**Transformed variable**
Linear	$L = mT + c$	$L = mT + c$	$L' = (L - c)/T = m$
Quadratic	$L = (mT + c)^2$	$\sqrt{L} = mT + c$	$L' = (\sqrt{L} - c)/T = m$
Exponential	$L = ae^{bT}$	$\ln L = \ln a + bT$	$L' = (\ln L - \ln a)/T = b$
von Bertalanffy	$L = L_\infty\left(1 - e^{-\kappa(T - t_0)}\right)$	$\ln(1 - L/L_\infty) = -\kappa T + \kappa t_0$	$L' = (\ln(1 - L/L_\infty) - \kappa t_0)/T = -\kappa$
Logistic	$L = a/(1 + e^{-b(T - c)})$	$\ln(a/L - 1) = -bT + bc$	$L' = (\ln(a/L - 1) - bc)/T = -b$
Gompertz	$L = ae^{be^{cT}}$	$\ln(-\ln(L/a)) = \ln b - cT$	$L' = (\ln(-\ln(L/a)) - \ln b)/T = -c$

So for example, if growth can be adequately modelled with a quadratic function, then a plot of $\sqrt{L}$ against *T* should yield a straight line, and a plot of $L' = (\sqrt{L} - c)/T$ against *T* should yield a random scatter of points around the line $L' = m$. Similarly, if growth can be modelled with the von Bertalanffy model, then a plot of $\ln(1 - L/L_\infty)$ against *T* should yield a straight line, and a plot of $L' = (\ln(1 - L/L_\infty) - \kappa t_0)/T$ should yield a random scatter of points around the line $L' = \kappa$.

Of primary importance is the fact that the transformed data L' in each case is a measure of the gradient of the growth model (e.g. *m* for the linear model, κ for the von Bertalanffy model), and thus gives a measure of growth rates of the individuals. Therefore when L' is used as the response variable in the GLM, one is able to explore the effects of environmental variables on growth directly, whereas the original data (untransformed measures of IOD, HCW, HCL) give absolute sizes and not estimates of growth, as they don't incorporate a change in size over time.

Days since hatch

Note that 'days since hatch' (*T*) for *Lestagella* and *Aphanicercella* were derived from the estimated hatch date given for each river (see Figs 4.7 and 4.8 in Chapter 4). In any given month and for any given river, an average age was calculated for the individuals collected by calculating the number of

days that had passed between the estimated hatch date for that river and the sampling date for that month.

Instar number

Since the field data collected for *Chimarra* showed several cohorts present simultaneously in the rivers each month, it was not straight forward to track the cohorts though consecutive months. Consequently backward regression could not be used to estimate a hatch date. However, a frequency histogram of the head capsule lengths (of all the individuals measured from all rivers) revealed five clear instar groups (Fig. App4B.1 below), and this could be used to obtain size limits for each of the five instars. Given this information, each individual could be assigned an instar number based on its head capsule length, and the instar number was used as the time variable (*T*) in the growth model.

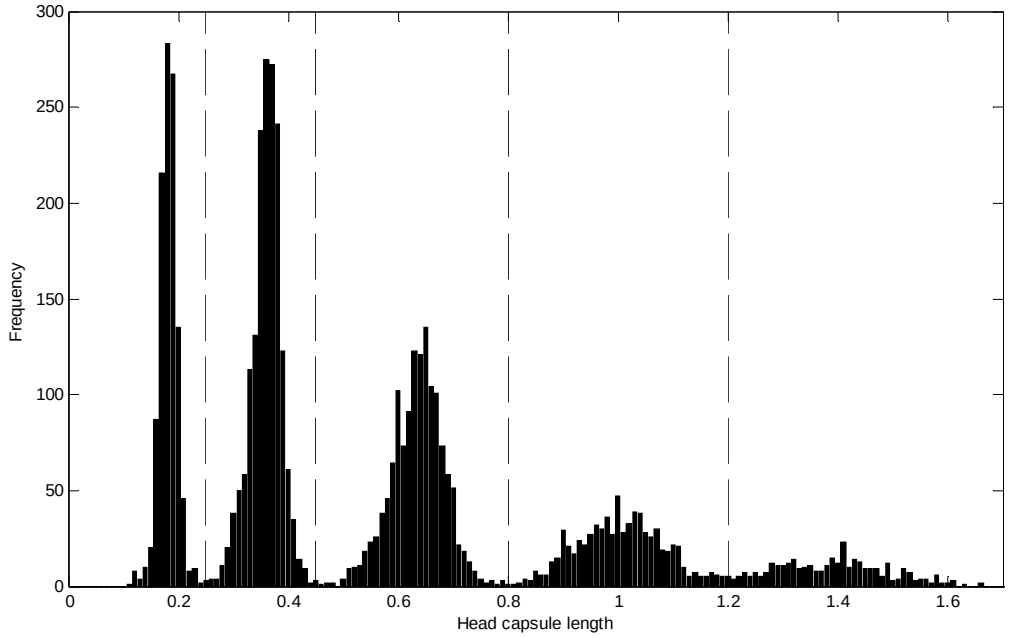


Fig. App4B.1. Frequency histogram of the head capsule lengths of the *Chimarra ambulans* individuals collected from all rivers, clearly showing grouping into five larval instars. The vertical dashed lines indicate the chosen size limits for the five instars.

Figures App4B.2 to App4B.4 illustrate the transformation process and final transformed data for each of the six growth models. A final growth model was chosen for each of the three species based on the fit of the model (r^2 value) as well as a qualitative assessment of the normality of the transformed data. The simplest model that performed well on both accounts was chosen for each of three species, as follows: the linear growth model for *Lestagella* (r^2 = 0.75), the exponential growth model for *Aphanicercella* (r^2 = 0.69), and quadratic growth model for *Chimarra* (r^2 = 0.98).

Although the r^2 values are not extraordinarily good, this is not entirely unexpected given the large data set and the natural variation in sizes found each month in the collected data. Since the aim of this exercise was not primarily to fit a growth curve, but rather to de-trend the data, and since the transformed data no longer showed clear trends with time and were reasonably normally distributed, the results were considered satisfactory.

Three potential sources of error are introduced in the transformation process described in this appendix:

(1) The time factor in the growth model is in all cases based on an estimated age for the individuals. In the case of *Lestagella* and *Aphanicercella*, the underlying assumption is that all the individuals collected each month are of the same age, namely the number of days since the estimated hatch date, i.e. an average age for that river. Under this assumption, the variation in size observed in each river then becomes a natural variation in growth, when it could in fact be a result of slightly differing ages of the individuals. But since the individual aging of all the samples is not possible, the average age seems the next best measure. For the *Chimarra* species, fitting growth models using instar number yielded very good results with high r^2 values. Given the clarity of the instar division illustrated in Fig. App4B.1, it seems that the assigning of instar number is fairly reliable.

(2) While the de-trending of the data appears to be reasonable, it is obviously not perfect, and some degree of error may be introduced if not all of the time-dependence is removed.

(3) Estimated hatch date was calculated using the second generation visible in cohort analyses for both *Aphanicercella* and *Lestagella* (Figs. 4.3, 4.5 in Chapter 4) as individuals in the first generation showed much greater variance in sizes making regression analyses less reliable. In so doing the hatch date obtained for the second generation was the same hatch date applied to the first generation - this assumes that the hatching occurs at roughly the same time each year in both of these species.

The above points should be kept in consideration when interpreting the GLM results, although general trends should still be reliable.

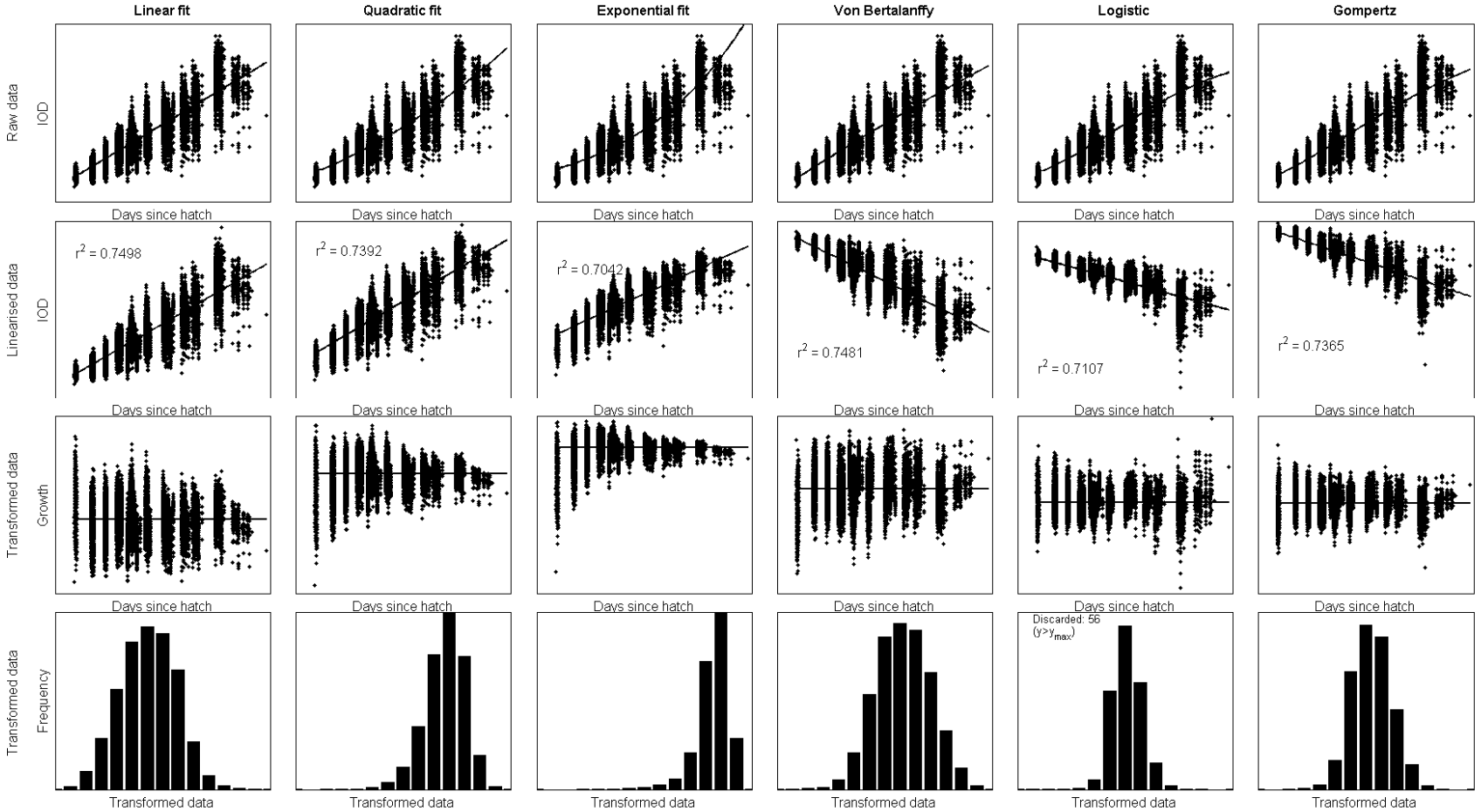


Fig. App4B.2. Illustration of the transformation of the *Lestagella penicillata* IOD to obtain a measurement of growth. The six columns correspond to the six growth models that were applied. The first row shows the original data with the fit to the growth models indicated with solid lines. The second row shows the IOD data after they were linearised using the growth model parameters (see Table App4B.2). Note that the better the linear fit in the second row, the better the overall fit to the growth model in question. The third row shows the final transformed data. A good transformation is one where there is no obvious trend in the transformed data with time. Finally, the fourth row shows the frequency histograms of the transformed data to check for normality. The linear model was chosen as the final growth model to transform the data.

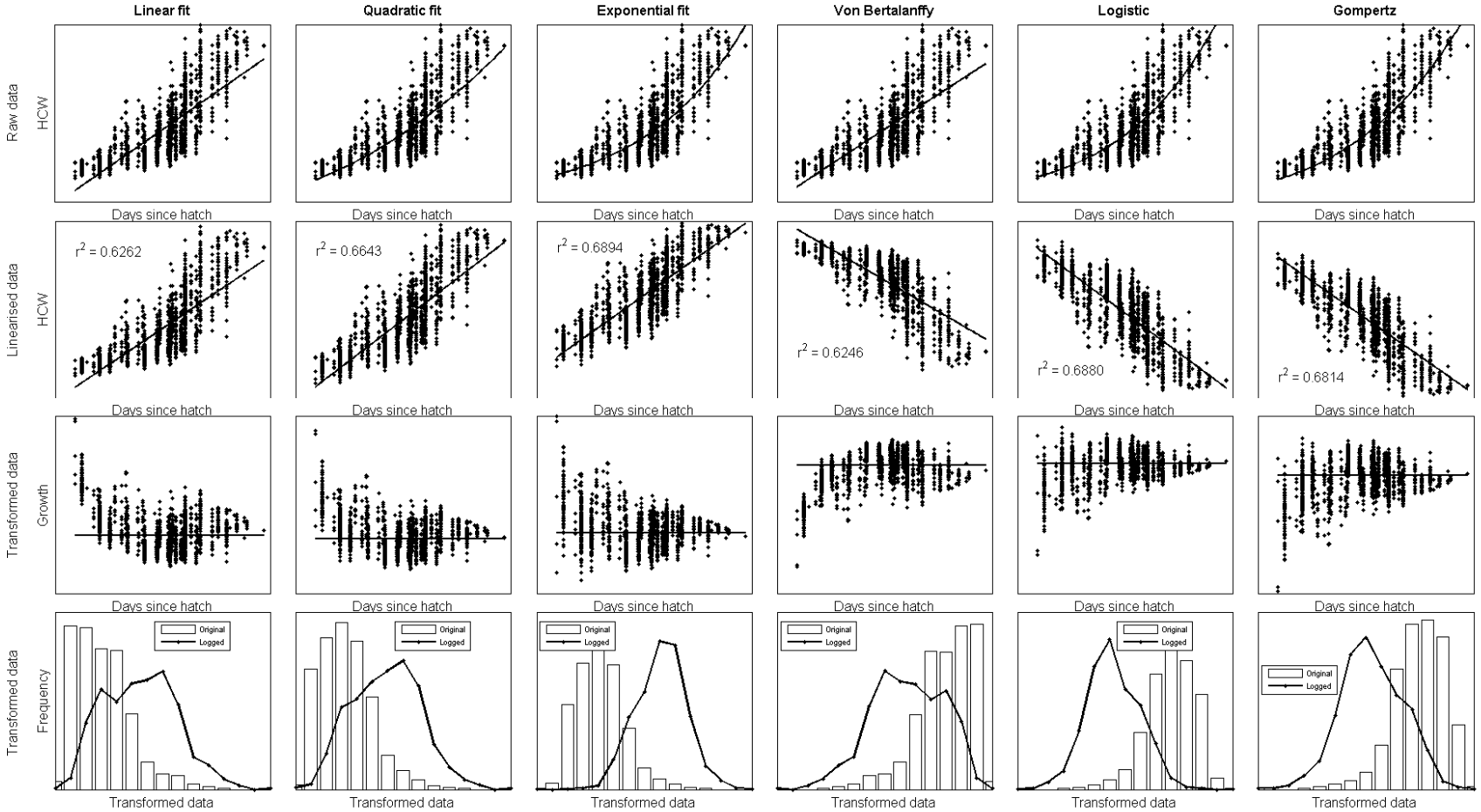


Fig. App4B.3. Illustration of the transformation of *Aphanicercella barnardi*, *Aphanicercella, flabellata* and *Aphanicercella scutata* HCW to obtain a measurement of growth. The six columns correspond to the six growth models that were applied. The first row shows the original data with the fit to the growth models indicated with solid lines. The second row shows the HCW data after they were linearised using the growth model parameters (see Table App4B.2). Note that the better the linear fit in the second row, the better the overall fit to the growth model in question. The third row shows the final transformed data. A good transformation is one where there is no obvious trend in the transformed data with time. Finally, the fourth row shows the frequency histograms of the transformed data to check for normality. Since the data appeared to be log-normally distributed, the final step of the transformation was to log the data. The distributions of the final transformed data are indicated by the solid lines. The exponential model was chosen as the final growth model to transform the data.

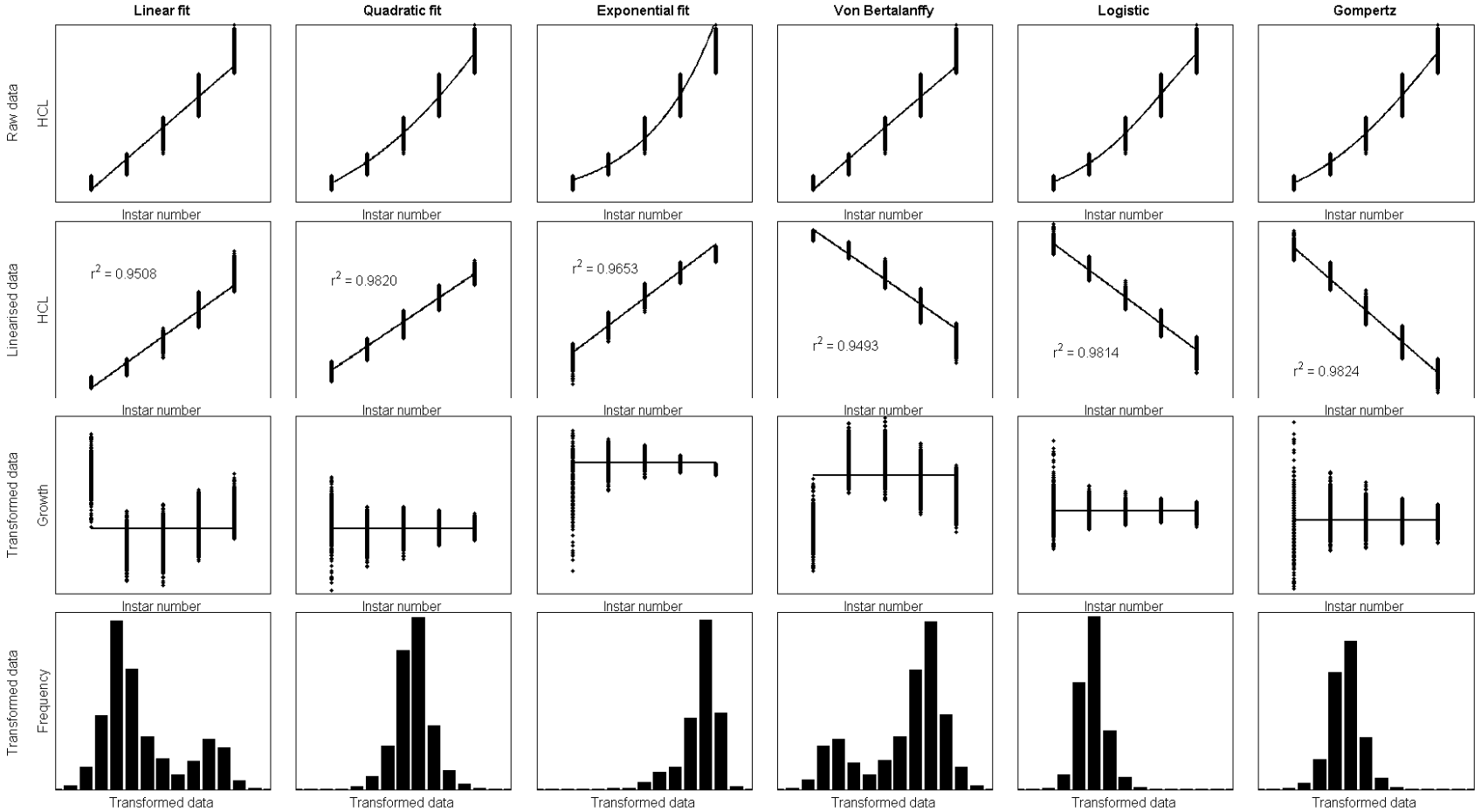


Fig. App4B.4. Illustration of the transformation of the *Chimarra ambulans* HCL to obtain a measurement of growth. The six columns correspond to the six growth models that were applied. The first row shows the original data with the fit to the growth models indicated with solid lines. The second row shows the HCL data after they were linearised using the growth model parameters (see Table App4B.2). Note that the better the linear fit in the second row, the better the overall fit to the growth model in question. The third row shows the final transformed data. A good transformation is one where there is no obvious trend in the transformed data with time. Finally, the fourth row shows the frequency histograms of the transformed data to check for normality. The quadratic model was chosen as the final growth model to transform the data.

APPENDIX 4C

Validation of GLM assumptions

Typical graphical outputs of the "plot" command in R (R Core Team 2012) are shown for the optimal Generalised Linear Models used for each taxon. These plots are used to assess model validation and adherence to statistical assumptions by examining the following:

1) residuals (standardised) against predicted or fitted values (upper left plot of Figs. App4C.1, App4C.3 and App4C.5) and a scale- location plot where the square root of the absolute standard deviance residuals (lower left plot of Figs. App4C.1, App4C.3 and App4C.5) is plotted against fitted values. Both plots are used to check for homogeneity. If no clear pattern/structure or trend can be observed in the data (i.e. scatter markedly increasing as the fitted values get higher) then homogeneity can be assumed.

2) a histogram or normal Quantile-Quantile plot (Q-Q plot) of the residuals (standardised or standard deviance) to verify normality (upper right plot of Figs. App4C.1, App4C.3 and App4C.5). If the majority of the points are in a straight line then normality can be assumed - though some degree of deviation can be acceptable at the ends of the line as these deviations in most cases are caused by several outliers.

3) potential and influential observations by plotting leverage against residuals (lower right plot of Figs. App4C.1, App4C.3 and App4C.5). Leverage indicates whether an observation has an extreme value of the explanatory variable. Where a point has a large influence (high effect on the parameter estimates of the model) it can be decided to remove that point. The Cook distance statistic aids this process by being superimposed as contour lines. A Cook distance larger than 1 is considered the threshold upon which action should be taken.

4) potential autocorrelation in the residuals (i.e assessing the similarity between observations as a function of the time lag between them - in this case months) obtained from each river (Figs App4C.2, App4C.2 and App4C.6). If any of the lines representing lags 1-10 clearly surpass the 0.05 significance threshold and show a high ACF value, then similarity is present between the observations from these time intervals and an autocorrelation function would need to be incorporated into the GLM to account for this.

Lestagella penicillata GLM

For *L. penicillata* a minor increase in the spread was observed in the residuals vs. fitted plot as predicted values increased but this increase in spread was not clearly pronounced and appeared to be as a result of only a few outliers. As no clear trend, pattern or structure was evident in the residuals vs. fitted or scale-location plots, homogeniety was assumed. The normal Q-Q plot revealed that residuals

were normally distributed, while the residuals vs. leverage plot showed that Cook distance values were very low, with no observations approaching the threshold for concern. Assumptions for the GLM were therefore considered to be met. Similarly ACF plots revealed no sign of autocorrelation of observations among the lag periods.

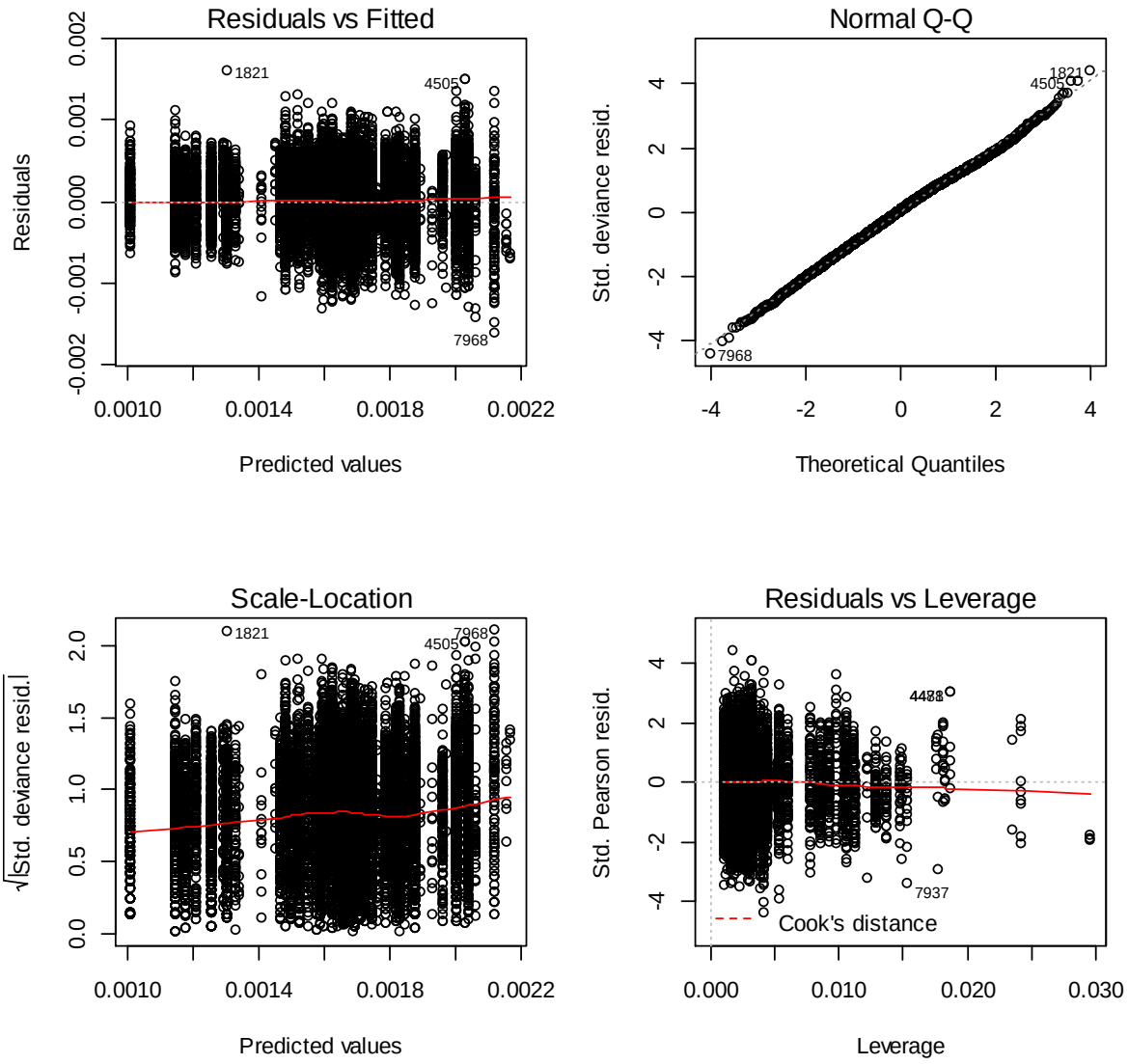


Fig App4C.1. Plots used to validate assumptions of the GLM for *Lestagella penicillata*.

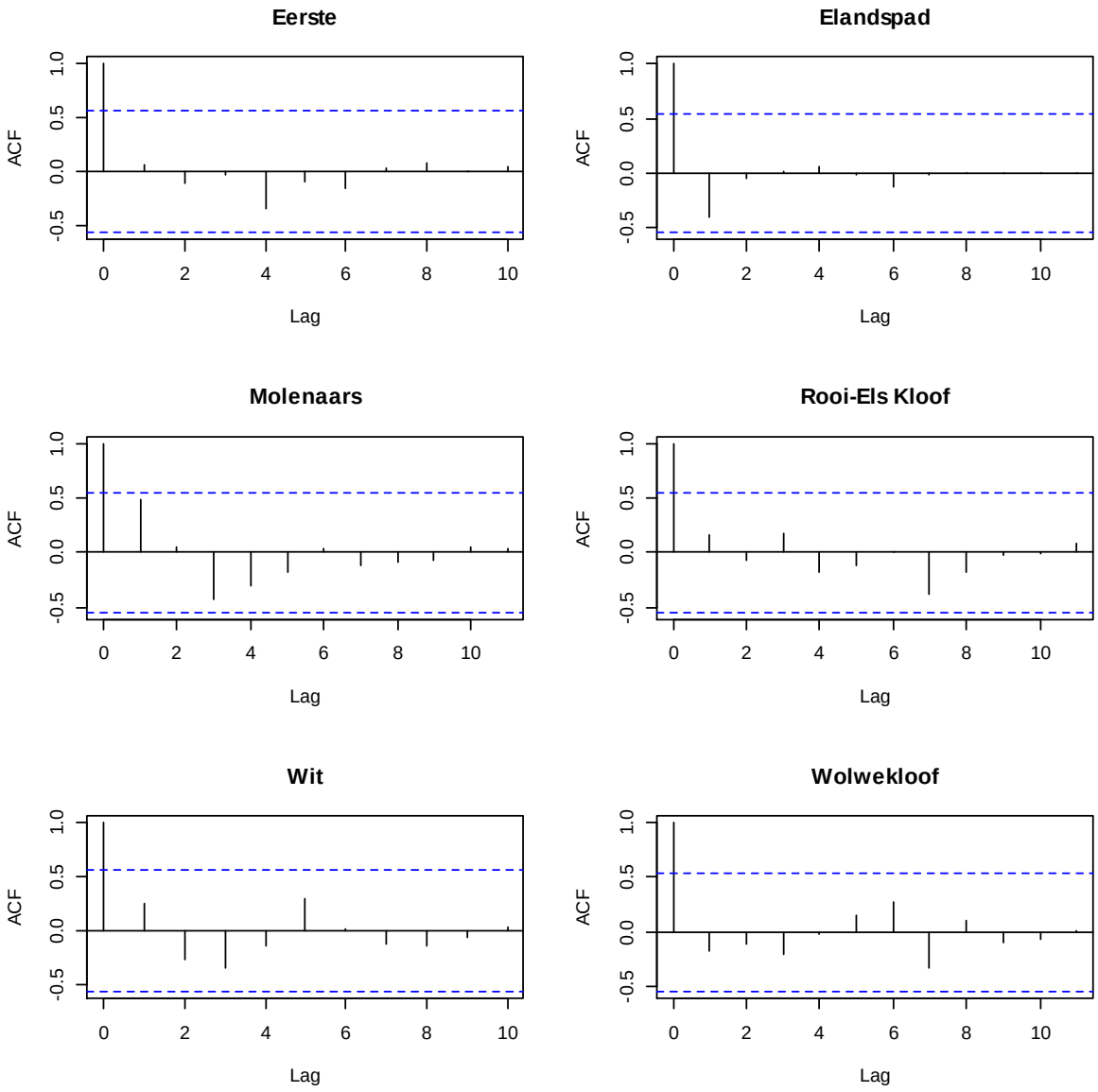


Fig App4C.2. Plots used to assess effects of autocorrelation in residuals of data *Lestagella penicillata*. A mean residual value was calculated for each month. Dotted horizontal line indicates autocorrelation above a significance threshold of 0.05. The horizontal axis shows "Lag" period in number of months.

Aphanicercella spp. *GLM*

For *Aphanicercella* spp. no clear patterns were observed in the residuals shown in the residuals vs fitted plot and the scale-location plot and homogeneity of variance was therefore assumed. While the normal Q-Q plot showed some deviation of standard deviance residuals from the theoretical quantiles at lower values, the majority of the values showed normality. Deviation was considered acceptable and was partially expected as far fewer small individuals compared to medium and larger sized individuals were collected that could therefor inform this model. The low Cook distance values in the residuals vs. leverage plots again revealed no pattern or observations near the the threshold for concern. GLM assumptions were therefore considered to be met. Similar to *L. penicillata* the ACF plots showed no signs of autocorrelation.

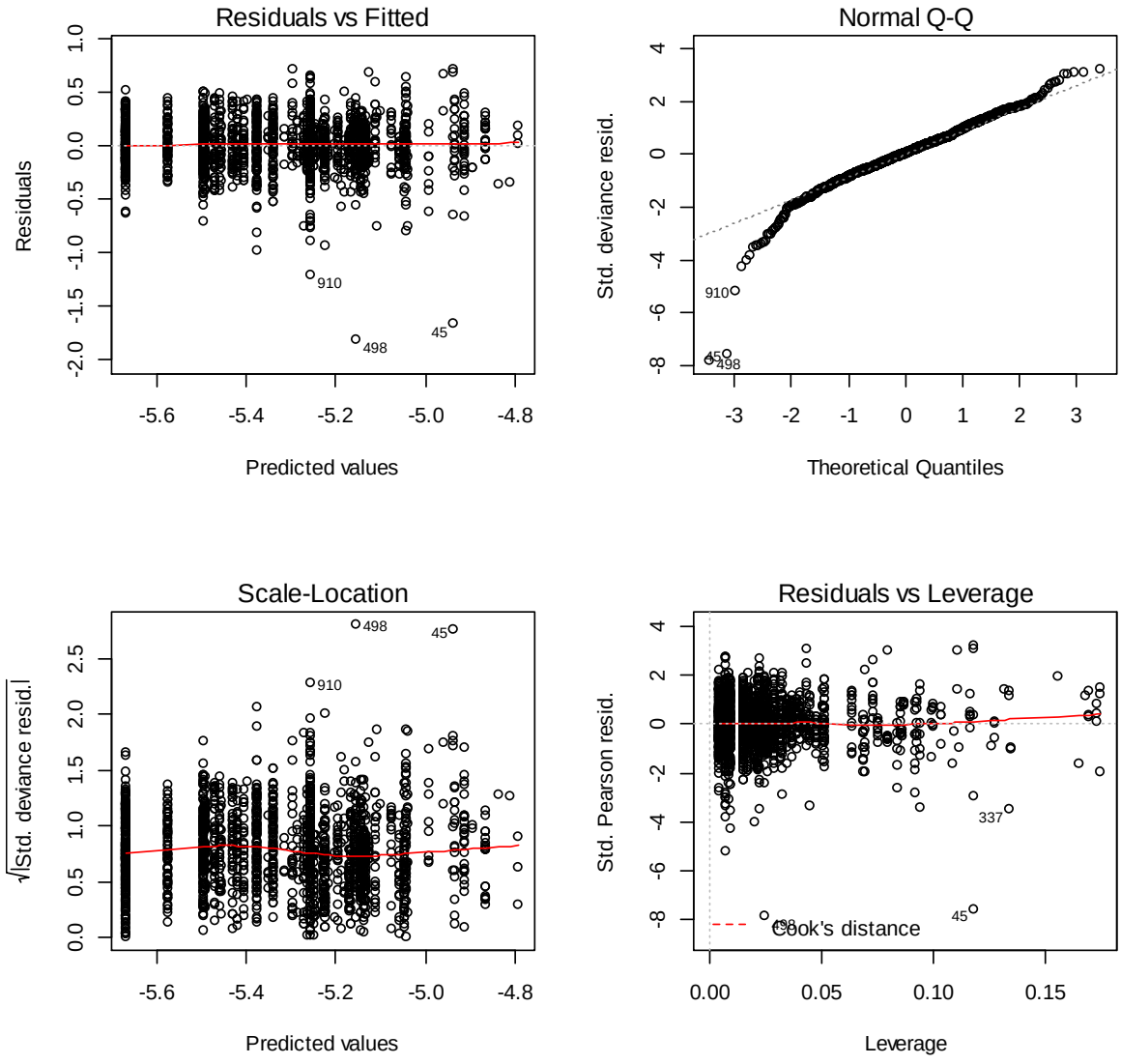


Fig App4C.3. Plots used to validate assumptions of the GLM for *Aphanicercella spp*.

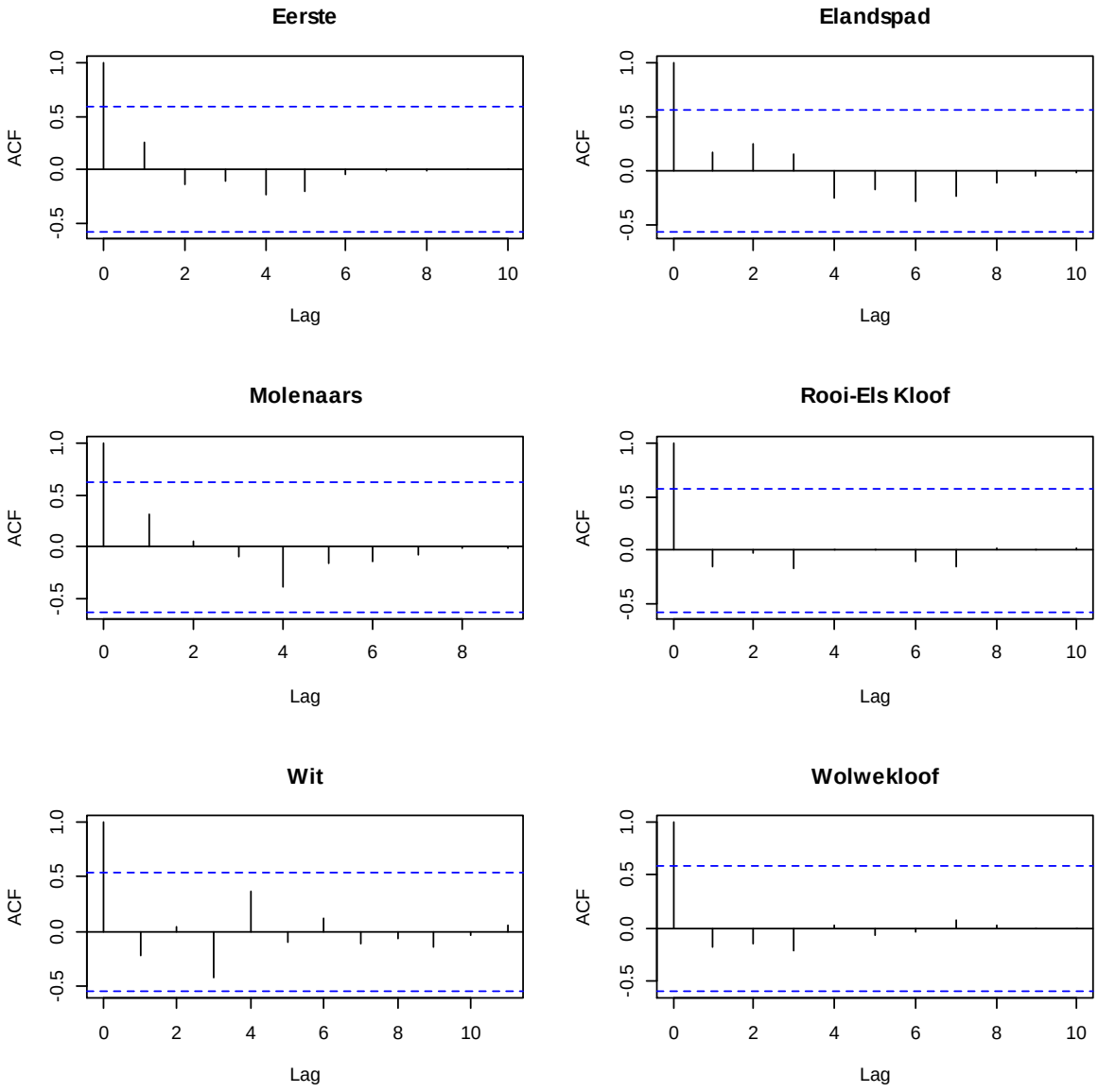


Fig App4C.4. Plots used to assess effects of autocorrelation in residuals of data for *Aphanicercella spp*. A mean residual value was calculated for each month. Dotted horizontal line indicates autocorrelation above a significance threshold of 0.05. The horizontal axis shows "Lag" period in number of months.

Chimarra ambulans GLM

For *C. ambulans* plots of residuals vs fitted values in conjucntion with scale location plots exhibited no clear patterns or structure and therefore homogeneity of variances was assumed. The normal Q-Q plots showed more deviation than was observed for Lestagella and Aphanicercella and revealed a symmetric distribution of residuals but with heavier tails than would be expected from a normal distribution. While the majority of values were normal, the deviation in the residuals suggests that the model struggled to accurately predict values at both ends of the possible range of values. This was not entirely unexpected as individual cohorts could not be tracked in *C. ambulans* and as such an estimate of growth was more difficult to obtain for this taxon from field data alone (laboratory experiments would have be needed to confirm growth rates but such experiments were out of the scope of this thesis). The growth estimate produced by the model was therefore essentially a measure of how much growth was put on from one instar to the next, rather than how fast individuals within a single cohort were growing. Low Cook distance values were observed in the residuals vs. leverage plots and no observations were near the the threshold for concern. Apart from potentially problematic normality the assumptions of the GLM were considered to be met. It is acknowledged that the model for *C. ambulans* has shortcomings, but given the time constraints it was the best model fit for the data available. Apart from only weakly significant autocorrelation (not considered to be of major concern) of residuals at time lag 1 in the Rooi-Els Kloof river, no autocorrelation was otherwise observed in the residuals for the data used in the model.

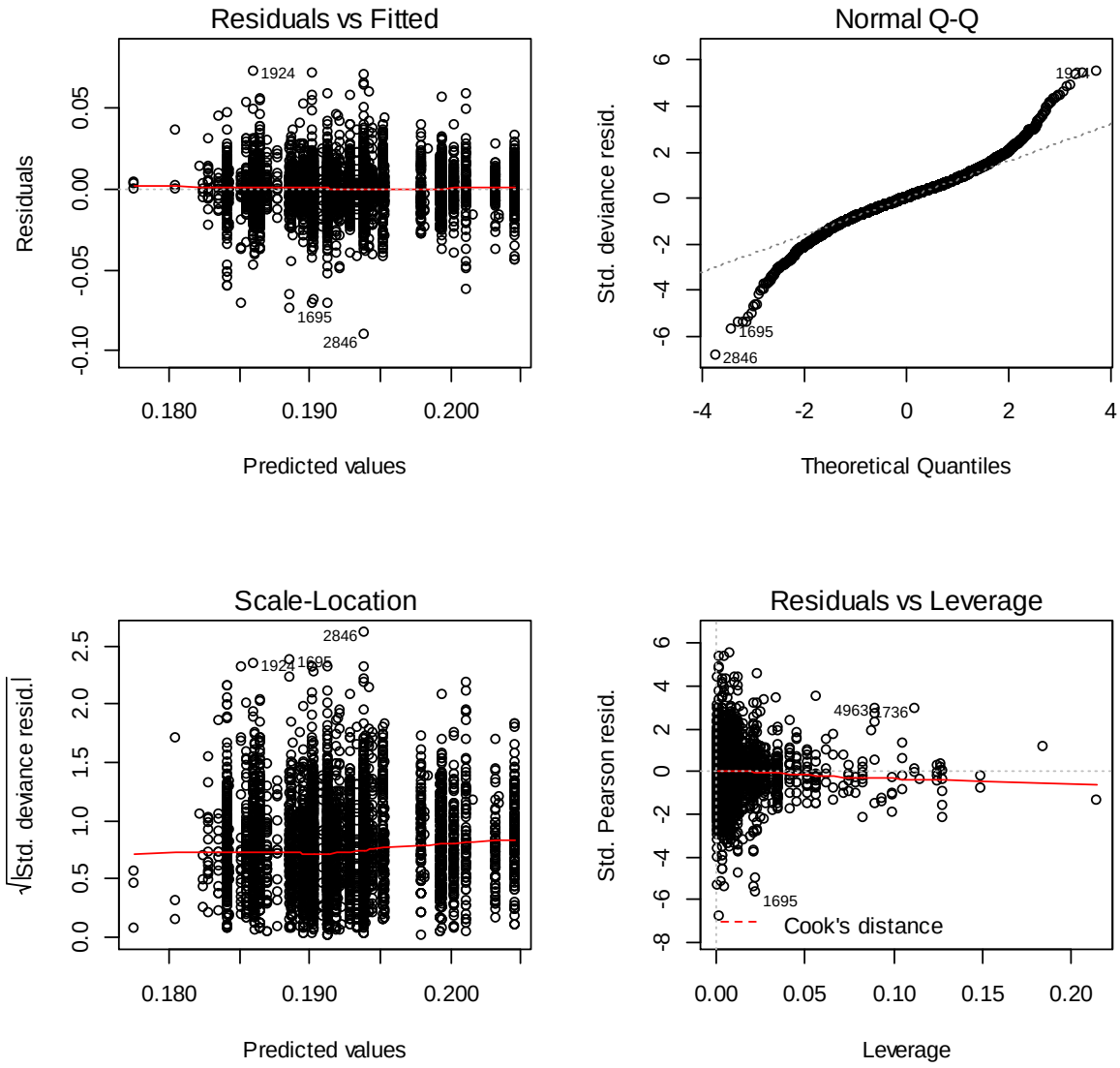


Fig App4C.5. Plots used to validate assumptions of the GLM for *Chimarra ambulans*.

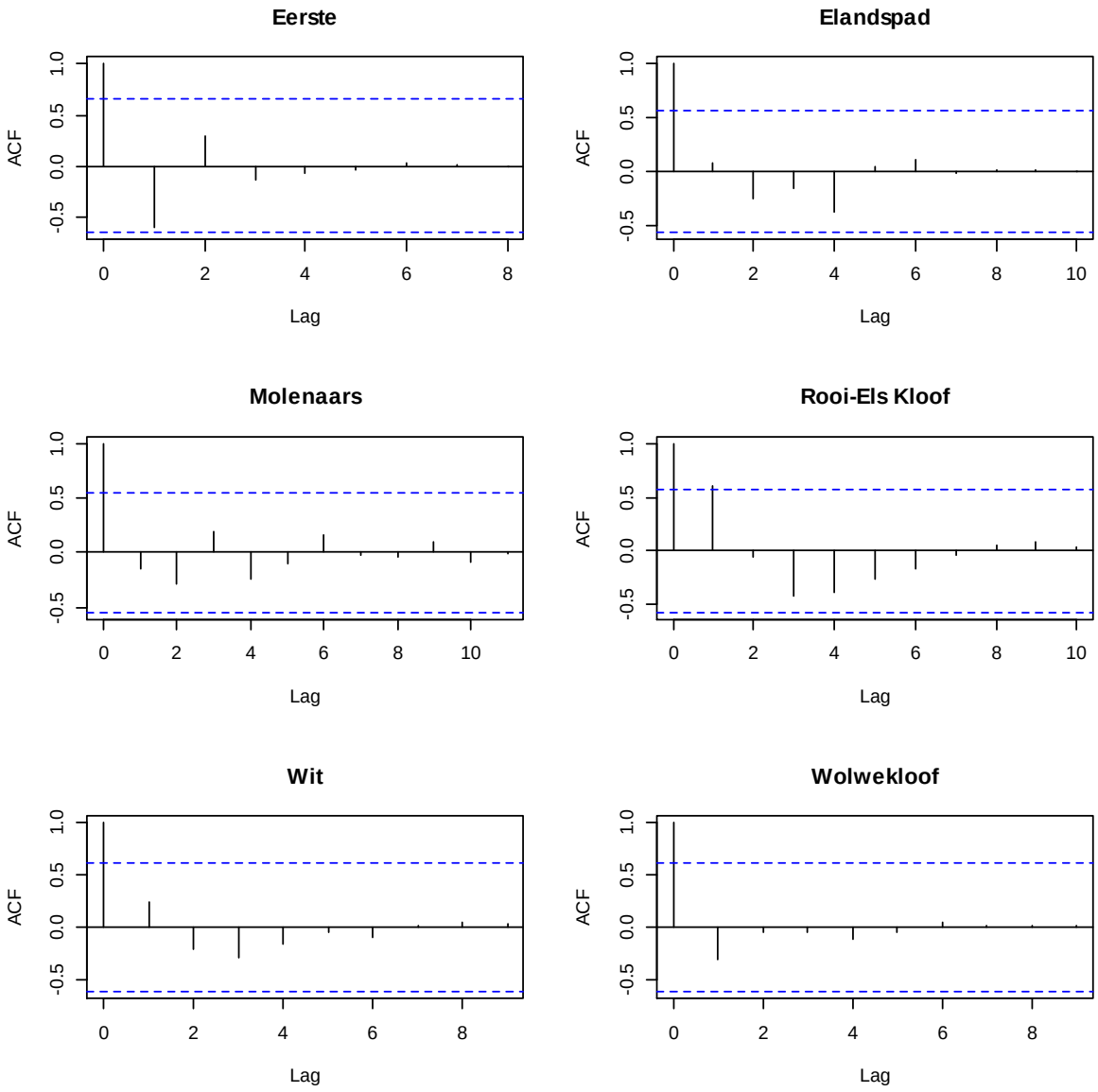


Fig App4C.6. Plots used to assess effects of autocorrelation in residuals of data for *Chimarra ambulans*. A mean residual value was calculated for each month. Dotted horizontal line indicates autocorrelation above a significance threshold of 0.05. The horizontal axis shows "Lag" period in number of months.

APPENDIX 4D

Sexual dimorphism in *Lestagella penicillata*

Differential growth of the eyes in nymphs of *L. penicillata* was first observed from the month of August 2009 and was evident until nymphs emerged in October/November 2009. Nymphs were observed to start forming crescent shaped eyes, which eventually became turbinate. Male mayflies commonly develop turbinate eyes, which in turn allows for excellent vision and an advantage for sexual reproduction.

This sexually dimorphic growth in the eyes of male and female *L. penicillata* nymphs, proved to be a problem when recording measurements of interocular distance. This was because the exact position from where the measurement was taken became subjective. The position where standardised measurements of interocular distance were recorded in females and small nymphs (not exhibiting sexually dimorphic growth) is indicated by (Position A Fig. App4D.1).

Had measurements of males been taken between the inner edges of the middle of the eyes, these measurements would most definitely have been biased (Position B Fig. App4D.1). Measurements of males were therefore recorded between eyes at the bottom of the crescent (Position C Fig. App4D.1). However measurements, even when they were taken from this position, were not comparable to measurements taken in previous months or to those recorded for females, using the standard measurement technique. For this reason measurements recorded from males were dealt with separately in the simple regression analysis, as they introduced extra variance and a potential bias. Note however that the total number of identified males was roughly 10% of the entire dataset, thus making it unlikely that a small bias in the IOD measurement will have a substantial impact on the analyses. As such the full dataset was used in the GLM analysis.

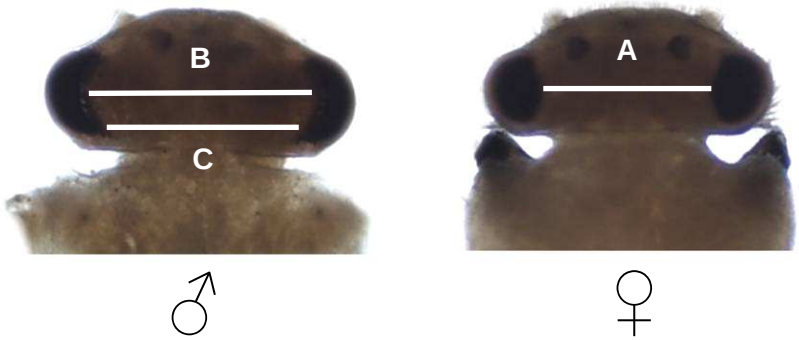


Fig. App4D.1. Head capsules of male and female nymphs of *Lestagella penicillata* showing the sexually dimorphic growth of the eyes. The horizontal line located at position A in the female shows the standard interocular distance measurement. Position B shows the biased measurements in the males, while position C indicates where actual measurements were recorded in males.

APPENDIX 4E

Complete GLM estimate outputs

Lestagella penicillata

Call:

glm(formula = TransIOD ~ relevel(as.factor(River), as.character(ref_level[1])) + relevel(as.factor(Temp.mean), as.character(ref_level[2])) + relevel(as.factor(Temp.std.monthly), as.character(ref_level[3])) + relevel(as.factor(Flow.mean), as.character(ref_level[4])) + relevel(as.factor(DO), as.character(ref_level[5])) + relevel(as.factor(EC), as.character(ref_level[6])) + relevel(as.factor(pH), as.character(ref_level[7])) +

relevel(as.factor(Turbidity), as.character(ref_level[8])) + relevel(as.factor(Channel.width), as.character(ref_level[9])), family = gaussian(link = "identity"), data = GLM1)

Deviance Residuals:

Min	1Q	Median	3Q	Max
-1.603e-03	-2.443e-04	1.242e-05	2.580e-04	1.600e-03

Coefficients:	**Estimate**	**Std. Error**	**t value**	**Pr (>|t|)**	**Signif.**
(Intercept)	1.73E-03	4.12E-05	41.918	<2E-16	***
relevel(as.factor(River),as.character(ref_level[1]))1	-3.04E-04	4.62E-05	-6.577	4.94E-11	***
relevel(as.factor(River),as.character(ref_level[1]))2	-2.01E-04	5.86E-05	-3.428	0.000609	***
relevel(as.factor(River),as.character(ref_level[1]))3	-1.06E-04	6.15E-05	-1.722	0.08507	.
relevel(as.factor(River),as.character(ref_level[1]))5	-1.77E-04	5.25E-05	-3.38E+00	0.00074	***
relevel(as.factor(River),as.character(ref_level[1]))6	-1.61E-04	3.89E-05	-4.14E+00	3.54E-05	***
relevel(as.factor(Temp.mean),as.character(ref_level[2]))1	-2.82E-04	3.33E-05	-8.48E+00	<2E-16	***
relevel(as.factor(Temp.mean),as.character(ref_level[2]))2	-2.39E-04	4.13E-05	-5.78E+00	7.45E-09	***
relevel(as.factor(Temp.mean),as.character(ref_level[2]))3	-1.03E-05	4.11E-05	-2.50E-01	0.80271	
relevel(as.factor(Temp.mean),as.character(ref_level[2]))4	2.55E-05	2.44E-05	1.05E+00	0.295232	
relevel(as.factor(Temp.mean),as.character(ref_level[2]))5	3.02E-05	3.23E-05	9.35E-01	0.349702	
relevel(as.factor(Temp.mean),as.character(ref_level[2]))6	5.02E-05	1.97E-05	2.55E+00	0.010826	*
relevel(as.factor(Temp.mean),as.character(ref_level[2]))8	-6.26E-05	2.13E-05	-2.94E+00	0.003248	**
relevel(as.factor(Temp.mean),as.character(ref_level[2]))9	-2.56E-04	2.58E-05	-9.91E+00	<2E-16	***
relevel(as.factor(Temp.std.monthly),as.character(ref_level[3]))2	-6.48E-06	1.80E-05	-3.60E-01	0.718534	
relevel(as.factor(Temp.std.monthly),as.character(ref_level[3]))3	1.38E-04	1.73E-05	7.97E+00	1.65E-15	***
relevel(as.factor(Temp.std.monthly),as.character(ref_level[3]))4	1.84E-04	2.70E-05	6.84E+00	8.43E-12	***
relevel(as.factor(Temp.std.monthly),as.character(ref_level[3]))5	1.54E-05	2.45E-05	6.29E-01	0.529384	
relevel(as.factor(Flow.mean),as.character(ref_level[4]))2	-3.31E-05	2.51E-05	-1.31E+00	0.188715	
relevel(as.factor(Flow.mean),as.character(ref_level[4]))3	-3.18E-05	2.73E-05	-1.17E+00	0.243361	

relevel(as.factor(Flow.mean),as.character(ref_level[4]))4	1.55E-05	3.07E-05	5.04E-01	0.614122	
relevel(as.factor(Flow.mean),as.character(ref_level[4]))5	-6.71E-05	3.75E-05	-1.79E+00	0.073552	.
relevel(as.factor(Flow.mean),as.character(ref_level[4]))6	2.19E-04	5.13E-05	4.27E+00	2.01E-05	***
relevel(as.factor(Flow.mean),as.character(ref_level[4]))7	9.61E-05	5.77E-05	1.66E+00	0.096221	.
relevel(as.factor(DO),as.character(ref_level[5]))2	-1.48E-04	2.19E-05	-6.75E+00	1.55E-11	***
relevel(as.factor(DO),as.character(ref_level[5]))3	-8.41E-05	2.27E-05	-3.71E+00	0.000207	***
relevel(as.factor(DO),as.character(ref_level[5]))4	-8.54E-05	2.12E-05	-4.03E+00	5.50E-05	***
relevel(as.factor(DO),as.character(ref_level[5]))5	-7.78E-05	1.94E-05	-4.01E+00	6.04E-05	***
relevel(as.factor(EC),as.character(ref_level[6]))1	2.58E-04	2.49E-05	1.03E+01	<2E-16	***
relevel(as.factor(EC),as.character(ref_level[6]))2	4.04E-05	2.38E-05	1.70E+00	0.089856	.
relevel(as.factor(EC),as.character(ref_level[6]))3	6.41E-06	2.71E-05	2.37E-01	0.812999	
relevel(as.factor(EC),as.character(ref_level[6]))4	6.95E-05	2.91E-05	2.38E+00	0.017154	*
relevel(as.factor(pH),as.character(ref_level[7]))1	1.06E-04	2.66E-05	4.00E+00	6.48E-05	***
relevel(as.factor(pH),as.character(ref_level[7]))2	2.34E-04	2.79E-05	8.37E+00	<2E-16	***
relevel(as.factor(pH),as.character(ref_level[7]))3	2.28E-05	2.03E-05	1.12E+00	0.260928	
relevel(as.factor(pH),as.character(ref_level[7]))5	-4.58E-05	2.73E-05	-1.67E+00	0.0942	.
relevel(as.factor(Turbidity),as.character(ref_level[8]))1	-1.89E-04	2.00E-05	-9.45E+00	<2E-16	***
relevel(as.factor(Turbidity),as.character(ref_level[8]))2	1.30E-05	2.08E-05	6.23E-01	0.533302	
relevel(as.factor(Turbidity),as.character(ref_level[8]))3	8.75E-05	1.98E-05	4.43E+00	9.48E-06	***
relevel(as.factor(Turbidity),as.character(ref_level[8]))5	-2.16E-05	2.05E-05	-1.06E+00	0.291522	
relevel(as.factor(Channel.width),as.character(ref_level[9]))1	-9.82E-05	2.35E-05	-4.18E+00	2.90E-05	***
relevel(as.factor(Channel.width),as.character(ref_level[9]))3	-1.72E-04	2.98E-05	-5.77E+00	8.27E-09	***
relevel(as.factor(Channel.width),as.character(ref_level[9]))4	1.29E-04	3.85E-05	3.36E+00	0.000778	***
relevel(as.factor(Channel.width),as.character(ref_level[9]))5	1.01E-04	4.50E-05	2.24E+00	0.025439	*

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 1.327508e-07)

Null deviance: 0.0029764 on 16287 degrees of freedom

Residual deviance: 0.0021564 on 16244 degrees of freedom

AIC: -211648

Number of Fisher Scoring iterations: 2

Aphanicercella spp.

Call:

glm(formula = TransHCW ~ relevel(as.factor(River), "5") + relevel(as.factor(Temp.mean), "9") + relevel(as.factor(Temp.std.monthly), "4") + relevel(as.factor(Flow.mean), "1") + relevel(as.factor(DO), "1") + relevel(as.factor(EC), "4") + relevel(as.factor(pH), "3") + relevel(as.factor(Turbidity), "2") + relevel(as.factor(Channel.width), "2"), family = gaussian(link = "identity"), data = GLM2)

Deviance Residuals:

Min	1Q	Median	3Q	Max
-1.8179	-0.1325	0.0039	0.1389	0.7081

Coefficients:	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**	**Signif.**
(Intercept)	-5.475529	0.055685	-98.33	<2E-16	***
relevel(as.factor(River),"5")1	0.364635	0.09264	3.936	8.62E-05	***
relevel(as.factor(River),"5")2	0.312462	0.055318	5.649	1.90E-08	***
relevel(as.factor(River),"5")3	0.220943	0.061734	3.579	0.000355	***
relevel(as.factor(River),"5")4	0.350318	0.065883	5.317	1.20E-07	***
relevel(as.factor(River),"5")6	0.148419	0.089345	1.661	0.096864	.
relevel(as.factor(Temp.mean),"9")1	0.227641	0.106169	2.144	0.032167	*
relevel(as.factor(Temp.mean),"9")2	0.130051	0.107912	1.205	0.228315	
relevel(as.factor(Temp.mean),"9")3	0.28146	0.082111	3.428	0.000623	***
relevel(as.factor(Temp.mean),"9")4	0.188145	0.089277	2.107	0.035229	*
relevel(as.factor(Temp.mean),"9")5	0.071528	0.092398	0.774	0.438965	
relevel(as.factor(Temp.mean),"9")6	0.140133	0.077185	1.816	0.069619	.
relevel(as.factor(Temp.mean),"9")7	0.139727	0.068152	2.05	0.040499	*
relevel(as.factor(Temp.mean),"9")8	-0.018774	0.077097	-0.244	0.807644	
relevel(as.factor(Temp.std.monthly),"4")1	-0.06128	0.04874	-1.257	0.208826	
relevel(as.factor(Temp.std.monthly),"4")2	-0.093484	0.055873	-1.673	0.094483	.
relevel(as.factor(Temp.std.monthly),"4")3	-0.083099	0.056667	-1.466	0.142718	
relevel(as.factor(Temp.std.monthly),"4")5	-0.074404	0.059365	-1.253	0.210257	
relevel(as.factor(Flow.mean),"1")2	-0.025869	0.061962	-0.417	0.676373	
relevel(as.factor(Flow.mean),"1")3	-0.037324	0.065804	-0.567	0.570654	
relevel(as.factor(Flow.mean),"1")4	-0.189553	0.05387	-3.519	0.000445	***
relevel(as.factor(Flow.mean),"1")5	0.020984	0.07438	0.282	0.77789	
relevel(as.factor(Flow.mean),"1")6	-0.051408	0.069474	-0.74	0.459425	
relevel(as.factor(Flow.mean),"1")7	0.046402	0.106506	0.436	0.663129	
relevel(as.factor(Flow.mean),"1")8	0.111707	0.119936	0.931	0.351788	
relevel(as.factor(DO),"1")2	0.012682	0.067545	0.188	0.85109	
relevel(as.factor(DO),"1")3	0.05569	0.055638	1.001	0.316999	
relevel(as.factor(DO),"1")4	-0.00892	0.058906	-0.151	0.879662	

relevel(as.factor(DO),"1")5	-0.108828	0.057791	-1.883	0.059855	.
relevel(as.factor(EC),"4")1	0.006612	0.06872	0.096	0.923362	
relevel(as.factor(EC),"4")2	-0.167508	0.083788	-1.999	0.045751	*
relevel(as.factor(EC),"4")3	0.008852	0.068827	0.129	0.897678	
relevel(as.factor(EC),"4")5	-0.030207	0.046862	-0.645	0.519283	
relevel(as.factor(pH),"3")1	0.020416	0.047997	0.425	0.670625	
relevel(as.factor(pH),"3")2	0.001343	0.056116	0.024	0.980912	
relevel(as.factor(pH),"3")4	-0.055565	0.043149	-1.288	0.198012	
relevel(as.factor(pH),"3")5	-0.105745	0.06882	-1.537	0.124592	
relevel(as.factor(Turbidity),"2")1	-0.005275	0.052377	-0.101	0.919785	
relevel(as.factor(Turbidity),"2")3	-0.009317	0.040464	-0.23	0.817916	
relevel(as.factor(Turbidity),"2")4	-0.107504	0.0534	-2.013	0.044259	*
relevel(as.factor(Turbidity),"2")5	-0.076227	0.044035	-1.731	0.083631	.
relevel(as.factor(Channel.width),"2")1	0.071557	0.039357	1.818	0.069218	.
relevel(as.factor(Channel.width),"2")3	0.061152	0.070975	0.862	0.38903	
relevel(as.factor(Channel.width),"2")4	0.196399	0.090782	2.163	0.030651	*

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 0.05483391)

Null deviance: 163.922 on 1706 degrees of freedom

Residual deviance: 91.134 on 1662 degrees of freedom

AIC: -65.531

Number of Fisher Scoring iterations: 2

Chimarra ambulans

Call:

glm(formula = TransHCL ~ relevel(as.factor(River), "2") + relevel(as.factor(Temp.mean), "3") + relevel(as.factor(Temp.std.monthly), "4") + relevel(as.factor(Flow.mean), "4") + relevel(as.factor(DO), "4") + relevel(as.factor(EC), "3") + relevel(as.factor(pH), "2") + relevel(as.factor(Turbidity), "5") + relevel(as.factor(Channel.width), "4"), family = gaussian(link = "identity"), data = GLM3)

Deviance Residuals:

Min	1Q	Median	3Q	Max
-0.089459	-0.007030	0.000137	0.007137	0.072057

Coefficients:	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**	**Signif**
(Intercept)	1.88E-01	3.79E-03	49.491	<2e-16	***
relevel(as.factor(River),"2")1	1.23E-02	6.24E-03	1.977	4.81E-02	*
relevel(as.factor(River),"2")3	7.39E-04	2.40E-03	0.309	7.58E-01	
relevel(as.factor(River),"2")4	8.47E-03	4.93E-03	1.718	0.085774	.
relevel(as.factor(River),"2")5	1.28E-02	5.22E-03	2.452	1.42E-02	*
relevel(as.factor(River),"2")6	1.07E-02	6.52E-03	1.641	0.100859	
relevel(as.factor(Temp.mean),"3")1	-1.26E-02	6.10E-03	-2.07	0.038528	*
relevel(as.factor(Temp.mean),"3")2	1.64E-03	6.53E-03	0.251	0.802179	
relevel(as.factor(Temp.mean),"3")4	-3.40E-03	6.31E-03	-0.539	0.589909	
relevel(as.factor(Temp.mean),"3")5	-2.72E-03	5.36E-03	-0.508	0.611801	
relevel(as.factor(Temp.mean),"3")6	-4.56E-03	6.50E-03	-0.701	0.483226	
relevel(as.factor(Temp.mean),"3")7	-5.22E-03	5.89E-03	-0.885	0.376256	
relevel(as.factor(Temp.mean),"3")8	-2.84E-03	5.86E-03	-0.484	0.628058	
relevel(as.factor(Temp.std.monthly),"4")1	-2.93E-03	2.49E-03	-1.178	0.238913	
relevel(as.factor(Temp.std.monthly),"4")2	-3.27E-03	2.81E-03	-1.165	0.244214	
relevel(as.factor(Temp.std.monthly),"4")3	6.03E-04	2.28E-03	0.265	0.791352	
relevel(as.factor(Temp.std.monthly),"4")5	-1.53E-02	3.86E-03	-3.977	7.06E-05	***
relevel(as.factor(Flow.mean),"4")1	-5.09E-03	4.44E-03	-1.145	0.252422	
relevel(as.factor(Flow.mean),"4")2	-8.41E-03	4.21E-03	-2	0.045537	*
relevel(as.factor(Flow.mean),"4")3	1.40E-03	2.78E-03	0.502	0.615533	
relevel(as.factor(Flow.mean),"4")5	-1.96E-03	3.60E-03	-0.544	0.586175	
relevel(as.factor(Flow.mean),"4")6	1.08E-02	5.48E-03	1.968	0.0491	*
relevel(as.factor(Flow.mean),"4")7	-5.59E-04	2.61E-03	-0.214	0.830462	
relevel(as.factor(Flow.mean),"4")8	4.79E-03	4.32E-03	1.109	0.267592	
relevel(as.factor(DO),"4")1	1.10E-03	2.85E-03	0.387	0.698532	
relevel(as.factor(DO),"4")2	3.21E-03	3.46E-03	0.929	0.352916	
relevel(as.factor(DO),"4")3	3.40E-03	2.62E-03	1.296	0.194904	
relevel(as.factor(DO),"4")5	-8.59E-05	3.29E-03	-0.026	0.979183	

relevel(as.factor(EC),"3")1	7.44E-03	2.85E-03	2.617	0.008899	**
relevel(as.factor(EC),"3")2	1.30E-02	3.16E-03	4.124	3.79E-05	***
relevel(as.factor(EC),"3")4	1.02E-02	3.40E-03	2.988	0.002824	**
relevel(as.factor(EC),"3")5	1.09E-02	2.99E-03	3.631	0.000285	***
relevel(as.factor(pH),"2")1	-2.03E-03	2.04E-03	-0.993	0.320801	
relevel(as.factor(pH),"2")3	1.94E-03	1.90E-03	1.019	0.308033	
relevel(as.factor(pH),"2")4	1.43E-03	2.19E-03	0.65	0.515946	
relevel(as.factor(pH),"2")5	3.31E-03	2.83E-03	1.17	0.24207	
relevel(as.factor(Turbidity),"5")1	-1.87E-03	1.97E-03	-0.948	0.342986	
relevel(as.factor(Turbidity),"5")2	-7.17E-03	2.23E-03	-3.217	0.001302	**
relevel(as.factor(Turbidity),"5")3	-4.55E-03	1.68E-03	-2.707	0.006815	**
relevel(as.factor(Turbidity),"5")4	-5.34E-03	5.10E-03	-1.048	0.29469	
relevel(as.factor(Channel.width),"4")1	2.89E-03	6.50E-03	0.444	0.657168	
relevel(as.factor(Channel.width),"4")2	-3.29E-03	5.06E-03	-0.65	0.516016	
relevel(as.factor(Channel.width),"4")3	-1.69E-03	5.11E-03	-0.331	0.74036	
relevel(as.factor(Channel.width),"4")5	3.51E-03	3.77E-03	0.93	0.352387	

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 0.0001734407)

Null deviance: 1.01796 on 5228 degrees of freedom

Residual deviance: 0.89929 on 5185 degrees of freedom

AIC: -30396

Number of Fisher Scoring iterations: 2

APPENDIX 5A

Collection of eggs from *Lestagella penicillata* (Ephemeroptera: Teloganodidae), *Aphanicercella scutata* (Plecoptera: Notonemouridae) and *Chimarra ambulans* (Trichoptera: Philopotamidae).

Life-history data for the three species, previously collected from six rivers in the Western Cape, South Africa, from 2009-2010 was used to determine the appropriate time of year to collect adults of each of the species (Chapter 4 this thesis). Sites were selected for the collection of adults based on the following criteria a) confirmed presence of the species in question b) abundances of the species in question c) travelling distance and d) ease of access to the sampling site.

Lestagella penicillata

Adult females and naturally oviposited eggs of *Lestagella penicillata* were collected from Window Stream, Table Mountain (see Fig. 5.1 in Chapter 5). On the 6th January 2011 a small pool measuring 1.20m x 1.05m x 0.18m and indirectly connected to the main channel) was positively identified as an oviposition site owing to the presence of dead female imagos (~25) floating on the surface of the water. Two plastic containers (230mm x150mm x 80mm) were submerged in this pool to collect oviposited eggs. Oviposition flights were observed at this pool shortly after sunset (between 18:30-19:15pm). The conditions at the time of these flights were warm, slightly humid and still. Swarms of females were observed flying upstream (at a height of approximately 1m) to this particular pool after which they circled the pool once or twice and then started hovering over the water surface of the pool. No mating flights or male adults were observed in these swarms suggesting that the females had already mated and were ready to oviposit. From a hovering position about 30 cm above the water, females made several short descent flights, making contact with the surface of the water on each occasion, presumably ovipositing eggs freely into the water. This observed oviposition behaviour is common also in the vast majority of mayflies Brittain (1982). Several adult females adhered to the water surface and died. Others which successfully completed several descent flights flew to surrounding riparian vegetation. The following morning the plastic containers were retrieved and returned to the laboratory where successfully collected eggs (+3000) were sorted under dissecting microscope.

Aphanicercella scutata

The emergence period (adult flight period) for this species was shown to roughly extend from May to August. Adult stoneflies are generally abundant during these winter periods of the year in cooler fast flowing rivers or mountain streams and they can easily be collected, using an aspirator, from the underside of stones, logs and debris on the stream banks. Sweep nets can also be used to collect adults from riparian vegetation. Three trips were therefore made to the Wit River in June, July and August 2011, in an attempt to collect gravid females and fertilised eggs.

During the trips made in June and July 2011, black wingpad nymphs were collected from the surfaces of stones-in-current and adults were collected using an aspirator from the underside of exposed stones in the stream bed. The black wingpad nymphs were placed in the holding tank to obtain sub imagos and imagos while the adults were sexed and inspected for eggs. On both occasions none of the collected females showed signs of protruding egg masses and upon dissection it was observed that eggs in the ovarian ducts were still immature.

During a subsequent trip on 29 August 2011, adults were again collected from the underside of exposed stones in the stream bed however in addition to these adults, females were observed flying to and from as well as clinging to the underside of riparian vegetation about 1.5 meters above the waters surface. Approximately 35-40 females were collected gently by means of an aspirator into a small glass vial containing some river water. Upon closer inspection, the adult females were found to be carrying egg masses protruding from the tip of their upwardly-bent abdomens. The small circular-shaped egg masses were white in colour and held together with a gelatinous substance. On contact with the surface of the water in the vials, the egg masses came apart explosively, successfully dispersing the eggs in the process, after which the individual eggs (+11000) sank to the bottom of the vial.

Chimarra ambulans

The emergence period for *C. ambulans* extends roughly from late spring (October/November) through to late autumn/early winter (May/June) (see Chapter 4). Adults appeared to be present in abundance in most of the study rivers over this period.

Multiple trips were made to collect adults of this species during the period from December 2011 to June 2012. Initially adults were collected from Window Stream, located on the slopes of Table Mountain, Western Cape, as logistically this was the closest stream to collect from on a regular basis (see Fig. 5.1 in Chapter 5). The collected insects were first sexed (this was done roughly according to body size, females having noticeably larger abdomens) after which a few females were dissected to look for the presence of mature eggs in the oviducts. In some females upon making the initial incision into the abdomen the eggs freely flowed out whilst in others, no eggs were located, and then in others only very small undeveloped eggs were observed in connected filaments within the oviducts. Adults were found to mate freely and continuously in captivity (both in small glass vials and also in larger plastic containers) with single females mating with more than one males. In some instances the adults remained in copula for more than 30 minutes whilst in other cases mating was as short as a minute, with disturbances such as nudging and shoving from other competing males being common. With this type of mating behaviour being exhibited, it was very difficult to know which of the females had successfully mated (i.e. which had incurred successful fertilisation of eggs), especially since males of several species of Trichoptera, perhaps also *C. ambulans*, employ a wide array of strategies relating to sexual competition (e.g.scraping sperm deposited from a previous male out of the female genital tract

using a modified penis, before mating). Initially several adults (~20) were left overnight in the laboratory in 2 litre plastic ice cream tubs half filled with water, aerated by means of an airstone, with one or two large river stones (the stones were sterilised in boiling water to prevent confusion from hatching of any other possible eggs/egg masses already on the stone). The containers were then covered with fine mesh to prevent adults escaping. Visual inspections in the morning revealed several dead adults floating on the water surface, and others drowned at the bottom of the container. The stones were inspected and in two cases several orange coloured egg masses l layer thick and in an oval shape were found deposited on the submerged portion of the stones.

This revealed that females were able to successfully mate and also oviposit eggs within an artificial laboratory setup. Following this finding, flies were collected from both the Window Stream and Elandspad River (see Fig. 5.1 in Chapter 5) and separated into small oviposition chambers. Approximately 15-20 adults (a combination of both sexes) were placed into each chamber. The clear plastic circular chambers each had a petri dish filled with water as well as several half submerged sand blasted perspex plates placed into them along with a small piece of grape and a piece of folded filter paper to provide a dry surface for resting. All chambers were covered with a fine mesh. The half-submerged sand-blasted perspex plates placed in the filled petri dishes were hoped to provide suitable surfaces for oviposition for female adults. Chambers were kept overnight in a large cooler box along with some ice, while a glass lid was placed over the cooler box to enable a natural light cycle but maintain a cool temperature inside.

Upon inspection the following morning, several egg masses had been oviposited on the submerged pieces of perspex in the containers. These pieces of perspex (3 pieces each with an egg mass) were retrieved and placed in separate petri dishes filled with approximately 50ml of fresh filtered (60µm) and aerated tap water as in the case of *A. scutata*.

APPENDIX 5B

Artificial fertilisation experiments using eggs of *Lestagella penicillata*

Crucial to any experiment regarding the development of eggs in aquatic insects, is the actual collection of the eggs themselves. Three methods exist for the collection of eggs 1) Fertilised eggs can be collected from catching mated, gravid females ready to oviposit, through the use of hand nets or light trap in the wild, 2) Fertilised eggs masses can be obtained by collecting substrata onto which the eggs have been deposited (in cases where eggs have attachment threads) or 3) Unfertilised eggs can be collected from mature emerging females that have been either caught in the wild or reared in the laboratory.

In this study initial attempts to capture mated female adults of *L. penicillata* in the wild during October/November 2010 were unsuccessful. Mature or black wingpad nymphs were therefore collected from algae covered stones in-current from a nearby stream, Window Stream (see Fig. 5.1 in Chapter 5) and returned to the laboratory. The nymphs and some of the stones they were collected from were placed in a holding tank, fitted with a vertical net and ¾ filled with chilled (10°C) aerated reservoir water. The tank was kept in a constant temperature room set at 10°C with a 12:12 hour light cycle. Partially submerged netting was placed on the insides of the holding tank in order to allow emerging nymphs to climb out of the water and upwards in to the vertical net. Nymphs were left to emerge as sub-imagos and then finally as mature imagos. Mature male and female imagos were collected for the purposes of a) obtaining unfertilized eggs and sperm and b) attempting artificial fertilization of the eggs in controlled laboratory conditions at several temperatures. Two separate artificial fertilisation experiments were conducted. In the first, aerated tap water from a reservoir (bubbled off for more than 24 hours) was used as the fertilisation medium, while in the second experiment a standard insect saline solution (Hoar & Hickman 1975) was used as the fertilization media.

Artificial fertilisation experiment 1

Eggs were extracted from eight female imagos. In this procedure the insect was decapitated and a small incision was made in the posterior tip of the abdomen. Eggs were then gently forced out of the female by compressing the abdomen. Extracted eggs were immediately placed in two to three drops of aerated tap water. The testes from seven male imagos were carefully removed and placed immediately into aerated tap water. Sperm was squeezed from the testes (Fig. App5B.1) within the drops of water containing the eggs and then gently mixed. Eggs and sperm were left for 30 minutes before the mixture of sperm and eggs were placed in 50ml of reservoir water (room temperature) within glass petri dishes (2cm deep, 4.5cm diameter).

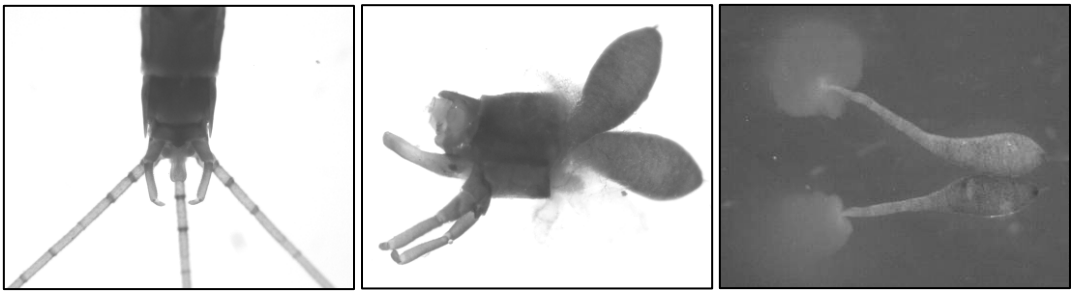


Fig. App5B.1 Photographs showing the sequence of removing the external genitalia from male *Lestagella penicillata* and obtaining sperm from the testes for mixing with unfertilised eggs in artificial fertilisation experiments.

Following this, approximately 100-200 eggs were pipetted into 12 separate petri dishes (two replicate dishes at each of six different experimental temperatures) and the dishes were filled with 50ml of reservoir water. Dishes were then transferred into controlled temperature environments set at 5, 10, 15, 20, 25 and 30°C. Similar studies in the literature have shown that light and photoperiod does not affect the development of eggs (Britain 1982). For this reason lighting was not controlled in these initial artificial fertilisation experiments. For the dishes at 20, 25, and 30°C constant temperatures were obtained by floating the dishes in water in aquaria fitted with thermostatically controlled heaters calibrated prior to use. The experiment commenced on the 5th November 2010 after which eggs were monitored daily for signs of development. Digital photographs of the eggs were also taken at each experimental temperature at five-day intervals starting from the first day of the experiment in order to track the rate of development of the eggs. Dishes were topped up with aerated tap water every second day and carefully cleaned when the first signs of algal and fungal build up was observed.

Artificial fertilisation experiment 2

Following the same protocol as outlined in the artificial fertilisation experiment 1, eggs and sperm were collected from four female imagos and seven male imagos. In this experiment the eggs and sperm were gently mixed in drops of insect saline which was used as the fertilization medium. After the mixture of sperm and eggs had been left for 30 minutes eggs were separately placed in petri dishes containing sterilized, aerated river water which was used as the incubation medium in this experiment. The experiment commenced on the 12th December 2010 and was monitored according to the same protocol outlined in the artificial fertilisation experiment 1. After 40 days no signs of development were observed in either of the artificial fertilisation experiments. The experiments were thus terminated and it was concluded that further artificial fertilisation experiments would be necessary to determine whether this method is in fact possible with this species of mayfly to obtain fertilised eggs.

APPENDIX 5C

Egg hatch parameter data: *Lestagella penicillata*

Table App5C.1 Egg development in *L. penicillata* from Window Stream, South Africa.

Temp. (°C)	No. eggs	Date oviposited	Unfertilised eggs	Incubation period (days)		Hatch duration (days)	Degree days to mean hatch	Hatch success (%)
				First hatch	Mean hatch			
5	173	07/01/2011	1	-	-	-	-	-
5	140	07/01/2011	0	-	-	-	-	-
5	191	07/01/2011	3	-	-	-	-	-
5	89	07/01/2011	3	-	-	-	-	-
10	55	07/01/2011	2	52	63	22	630.1	100
10	52	07/01/2011	1	59	62	11	617	100
10	51	07/01/2011	3	63	68	11	678.4	81.3
10	43	07/01/2011	3	52	66	22	661.8	82.5
15	76	07/01/2011	2	19	23	9	346.4	100
15	132	07/01/2011	1	19	23	6	341	99.2
15	117	07/01/2011	1	19	22	6	334.4	100
15	84	07/01/2011	0	19	23	6	339.5	97.6
20	130	07/01/2011	3	13	14	4	282	99.2
20	115	07/01/2011	3	13	14	4	280.4	99.1
20	203	07/01/2011	0	13	14	5	279.4	99.0
20	133	07/01/2011	1	13	14	4	277.4	99.2
25	172	07/01/2011	1	-	-	-	-	-
25	147	07/01/2011	2	-	-	-	-	-
25	89	07/01/2011	0	-	-	-	-	-
25	52	07/01/2011	2	-	-	-	-	-
30	80	07/01/2011	4	-	-	-	-	-
30	90	07/01/2011	2	-	-	-	-	-
30	150	07/01/2011	5	-	-	-	-	-
30	43	07/01/2011	0	-	-	-	-	-

APPENDIX 5D

Egg hatch parameter data: *Aphanicercella scutata*

Table App5D.1 Egg development in *A. scutata* from the Wit River, South Africa.

Temp. (°C)	No. eggs	Date oviposited	Unfertilised eggs	Incubation period (days)		Hatch duration (days)	Degree days to mean hatch	Hatch success (%)
				First hatch	Mean hatch			
5	189	29/08/2011	3	-	-	-	-	-
5	390	29/08/2011	6	-	-	-	-	-
5	440	29/08/2011	4	-	-	-	-	-
5	307	29/08/2011	3	-	-	-	-	-
10	405	29/08/2011	5	31	35	22	354	93.5
10	590	29/08/2011	9	31	37	24	369.4	83.7
10	635	29/08/2011	12	31	37	27	374.2	92.6
10	628	29/08/2011	6	31	37	26	373.4	71.1
15	320	29/08/2011	3	18	25	29	380.4	74.5
15	432	29/08/2011	6	18	25	28	380.4	76.8
15	671	29/08/2011	6	17	26	29	392.3	76.6
15	374	29/08/2011	11	18	27	28	397.5	75.5
20	544	29/08/2011	4	15	20	20	408.2	37.2
20	691	29/08/2011	8	15	20	21	407.2	26.2
20	543	29/08/2011	5	15	21	21	410.4	27.5
20	409	29/08/2011	4	15	20	21	408	36.1
25	521	29/08/2011	2	-	-	-	-	-
25	296	29/08/2011	1	-	-	-	-	-
25	408	29/08/2011	4	-	-	-	-	-
25	501	29/08/2011	3	-	-	-	-	-
30	442	29/08/2011	4	-	-	-	-	-
30	534	29/08/2011	4	-	-	-	-	-
30	422	29/08/2011	3	-	-	-	-	-
30	394	29/08/2011	1	-	-	-	-	-

APPENDIX 5E

Egg hatch parameter data: *Chimarra ambulans*

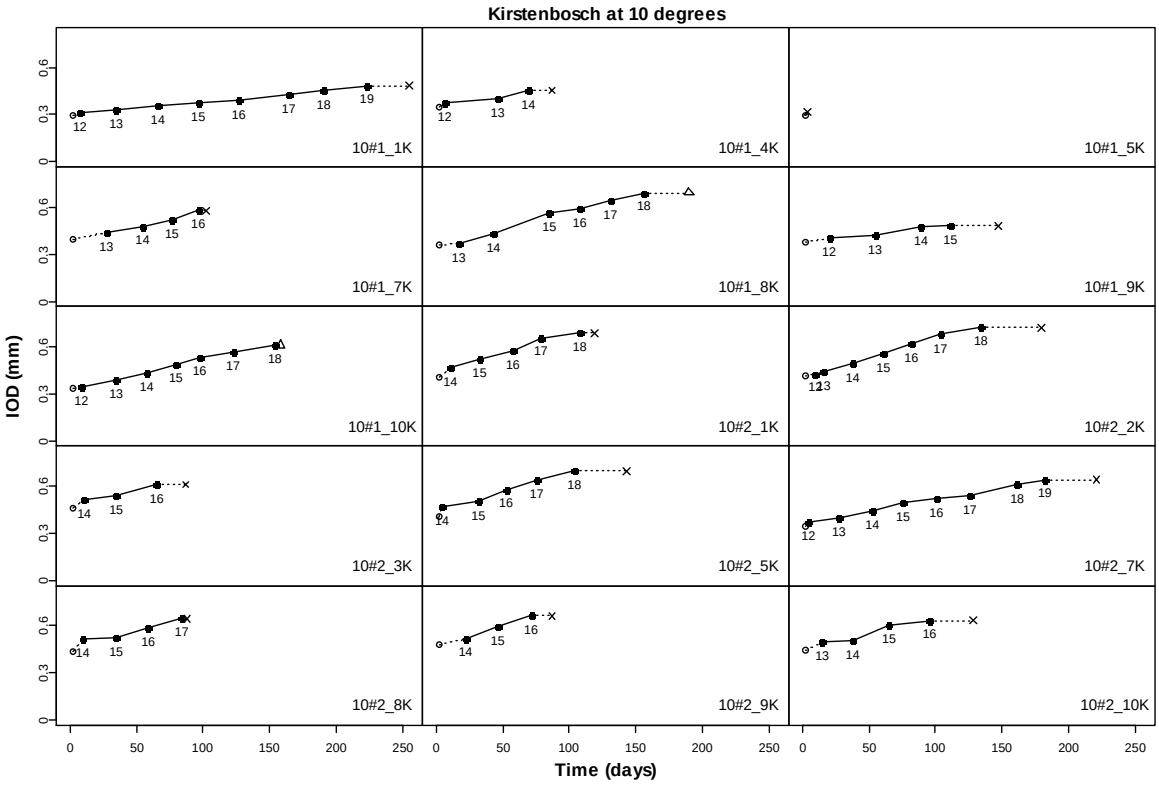
Table App5E.1 Egg development in *C. ambulans* from Elandspad River, South Africa.

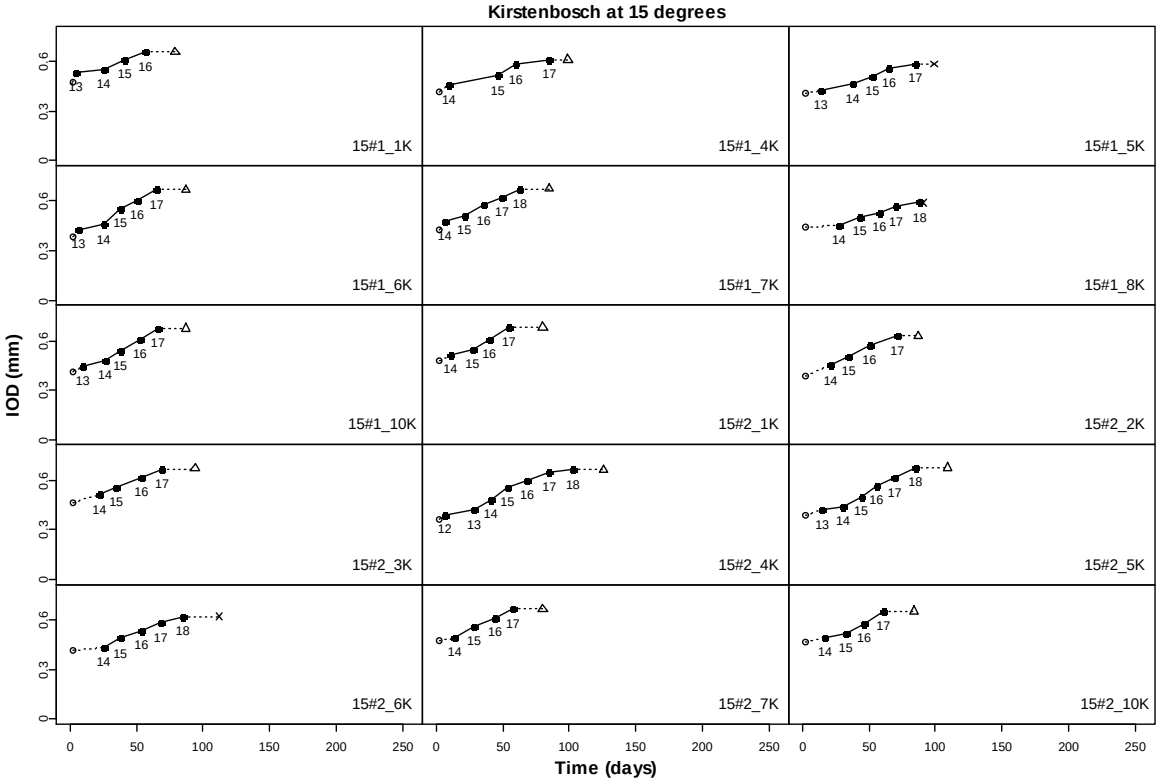
Temp. (°C)	No. eggs	Date oviposited	Unfertilised eggs	Incubation period (days)		Hatch duration (days)	Degree days to mean hatch	Hatch success (%)
				First hatch	Mean hatch			
5	282	16/03/2012	2	-	-	-	-	-
5	208	22/02/2012	0	-	-	-	-	-
5	313	24/03/2012	8	-	-	-	-	-
5	258	29/03/2012	3	-	-	-	-	-
10	254	16/03/2012	43	-	-	-	-	0
10	329	21/03/2012	15	34	34	3	344	1.6
10	164	22/03/2012	2	33	34	3	338	6.2
10	184	24/03/2012	3	33	35	9	354.1	84.5
15	201	22/02/2012	25	17	19	6	283.7	91.5
15	241	22/02/2012	11	16	17	3	251.3	1.7
15	108	21/03/2012	11	19	20	2	293	13.4
15	221	24/03/2012	86	-	-	-	-	0
20	210	16/03/2012	37	10	12	10	241	11
20	111	22/03/2012	5	12	12	1	240	0.9
20	165	21/03/2012	78	11	12	7	247.4	36.8
20	257	24/03/2012	18	14	14	1	280	0.8
25	145	24/03/2012	18	10	11	4	284.3	6.3
25	560	28/03/2012	29	11	11	1	275	0.2
25	350	28/03/2012	0	9	10	2	241.5	15.1
25	419	28/03/2012	6	9	10	3	252.5	6.8
30	201	28/03/2012	22	-	-	-	-	-
30	152	29/03/2012	3	-	-	-	-	-
30	147	29/03/2012	11	-	-	-	-	-
30	321	29/03/2012	2	-	-	-	-	-

APPENDIX 6A

Size increase over time of individuals of *L. penicillata* used in growth experiments from each site and at each temperature treatment

Crosses denote death of the individual, while open triangles represent emergence. Open circles represent initial size measurements not used in the calculation of growth rates. Dotted lines denote periods not used in the calculation of growth rates.




Kirstenbosch at 15 degrees
IOD (mm)
Time (days)
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15#1_5K
15#1_6K
15#1_7K
15#1_8K
15#1_10K
15#2_1K
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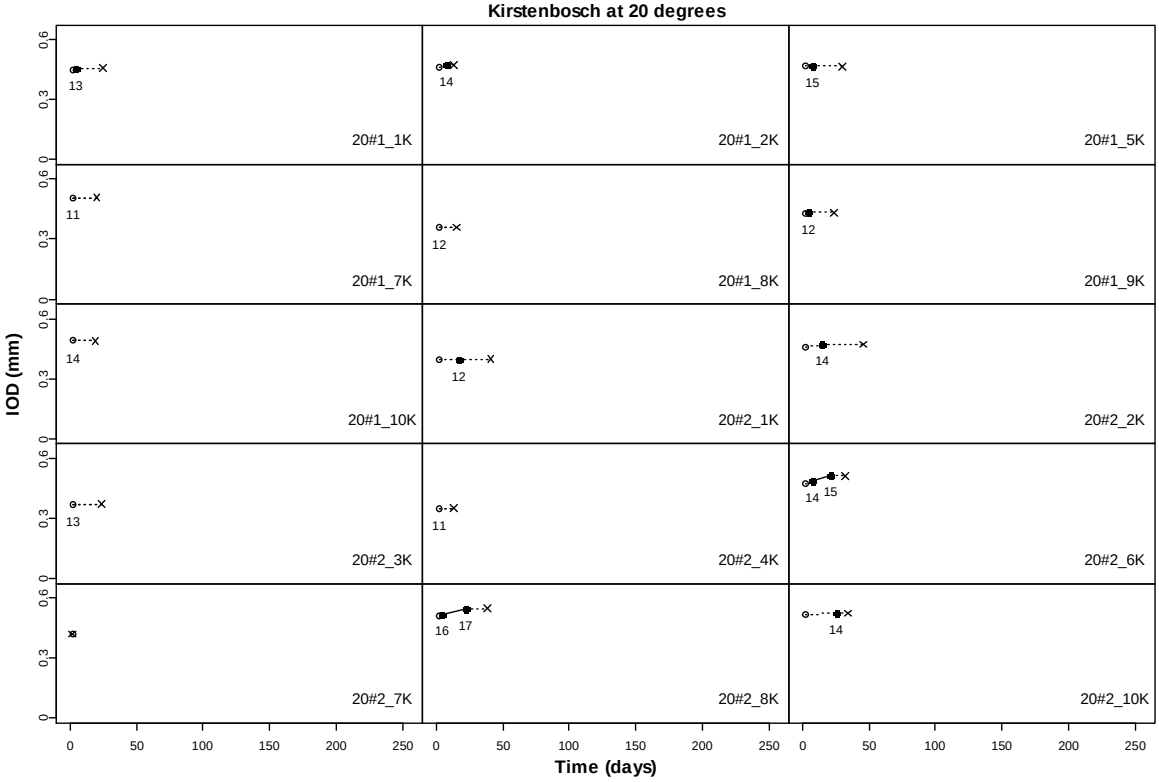
Kirstenbosch at 20 degrees
IOD (mm)
Time (days)
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20#1_5K
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20#1_8K
20#1_9K
20#1_10K
20#2_1K
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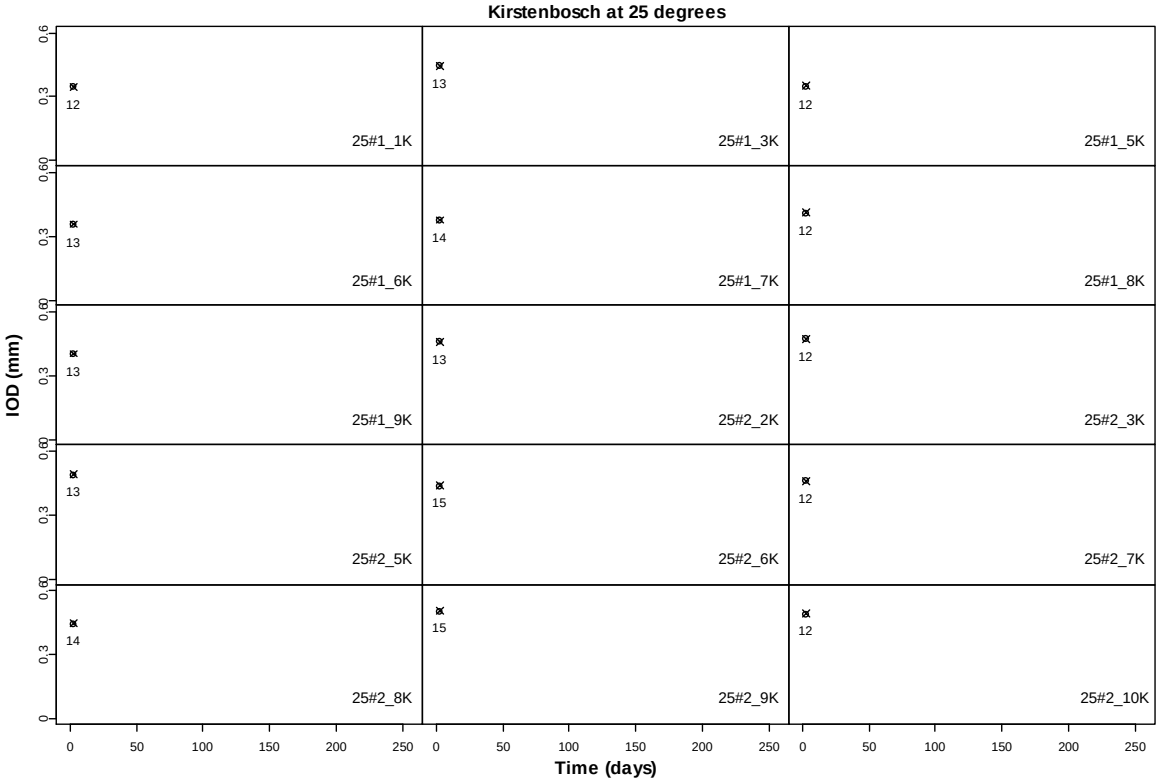
Kirstenbosch at 25 degrees
IOD (mm)
Time (days)
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25#1_3K
25#1_5K
25#1_6K
25#1_7K
25#1_8K
25#1_9K
25#2_2K
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25#2_7K
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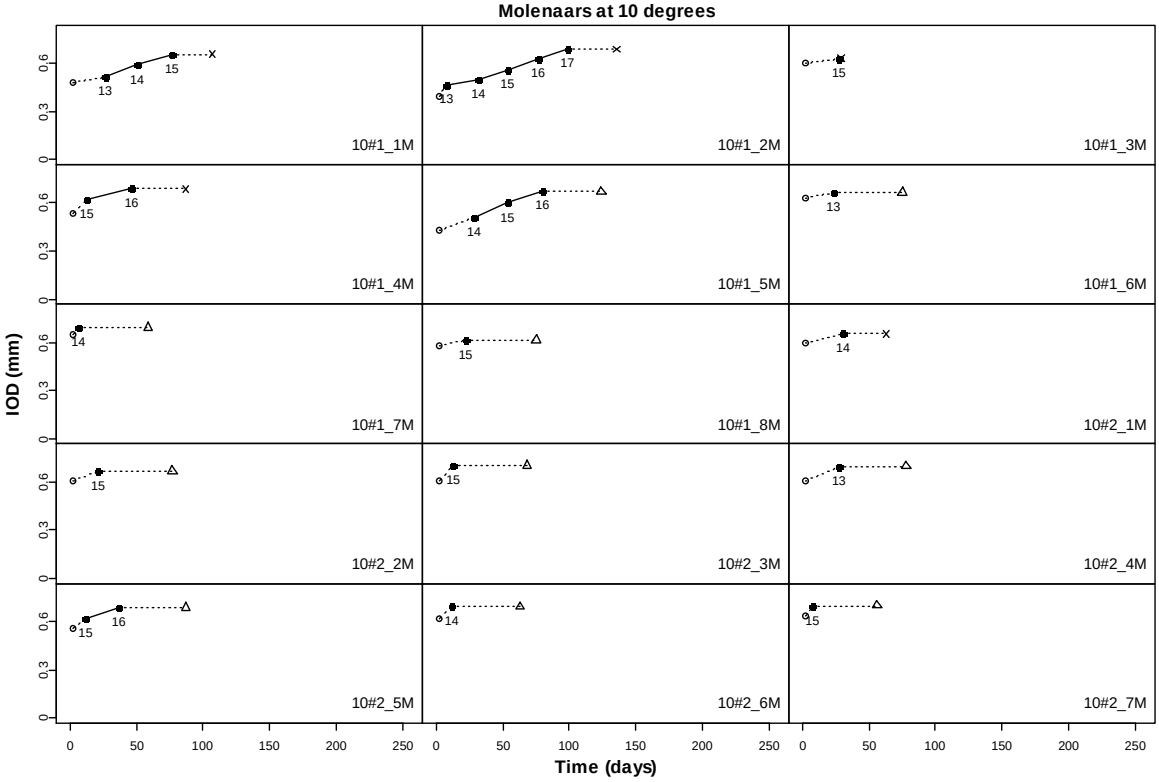
Molenaars at 10 degrees
IOD (mm)
Time (days)
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10#1_3M
10#1_4M
10#1_5M
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10#1_7M
10#1_8M
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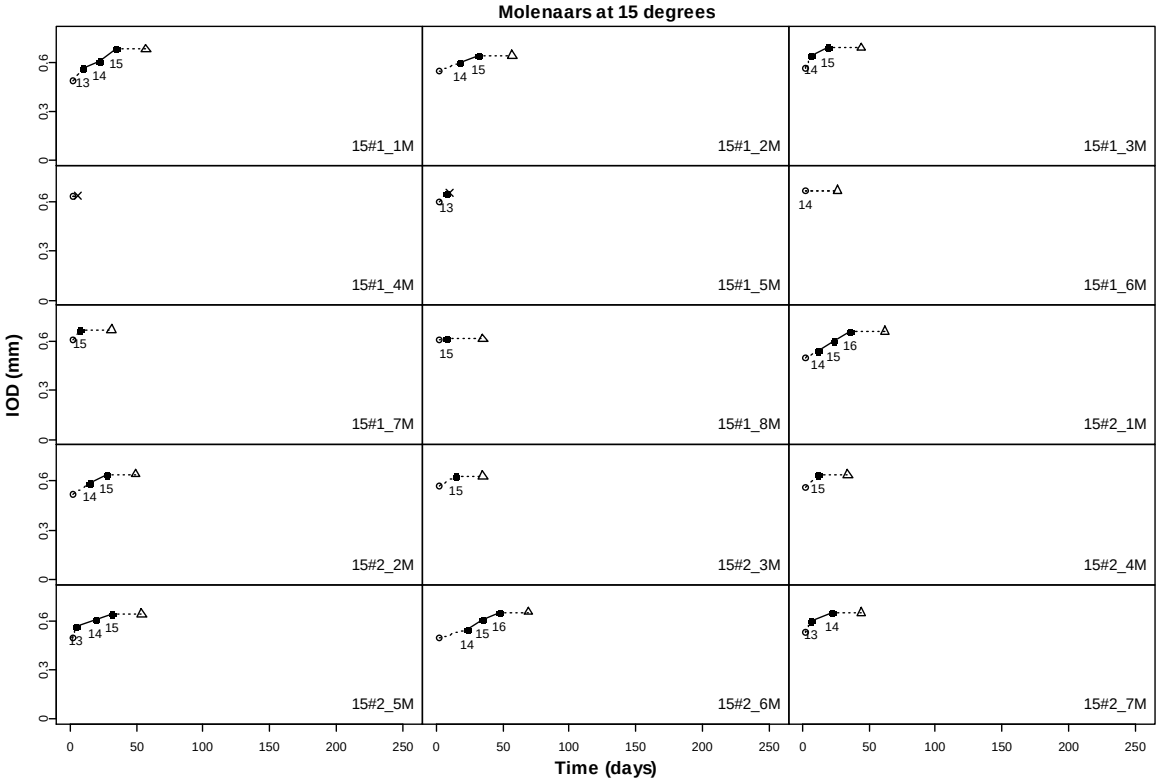
Molenaars at 15 degrees
IOD (mm)
Time (days)
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15#1_2M
15#1_3M
15#1_4M
15#1_5M
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15#1_7M
15#1_8M
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15#2_4M
15#2_5M
15#2_6M
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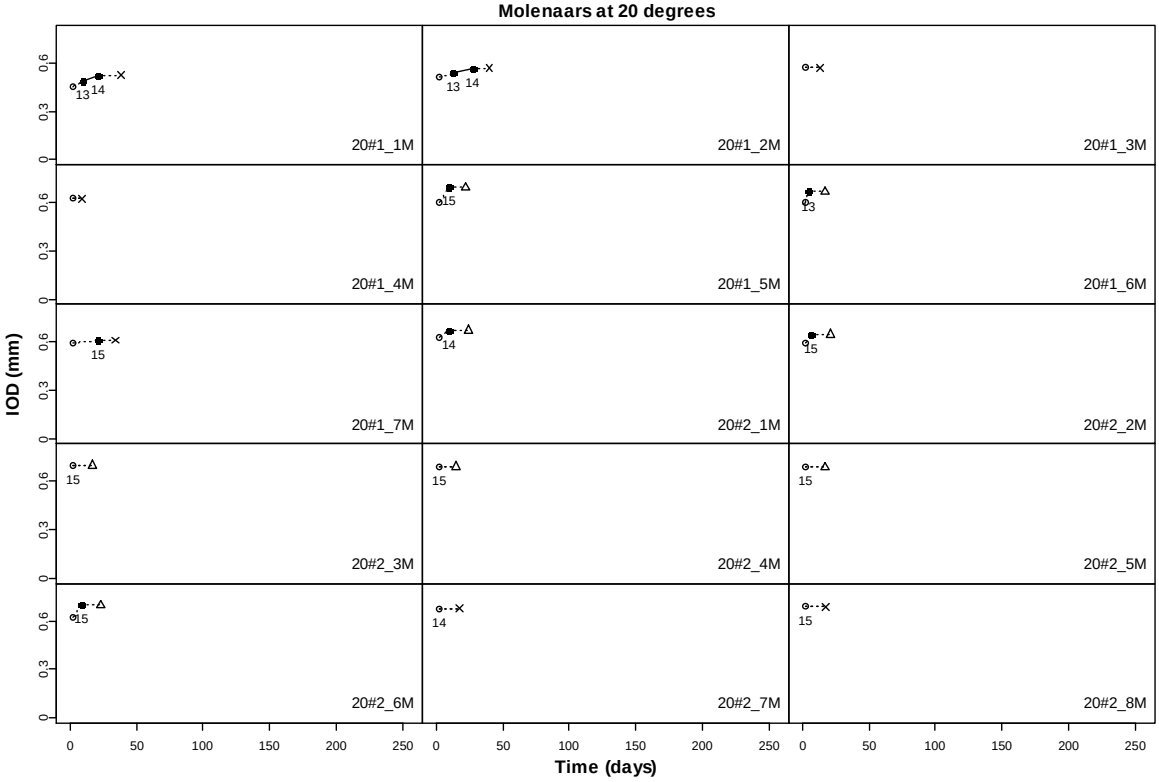
Molenaars at 20 degrees
IOD (mm)
Time (days)
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20#1_2M
20#1_3M
20#1_4M
20#1_5M
20#1_6M
20#1_7M
20#2_1M
20#2_2M
20#2_3M
20#2_4M
20#2_5M
20#2_6M
20#2_7M
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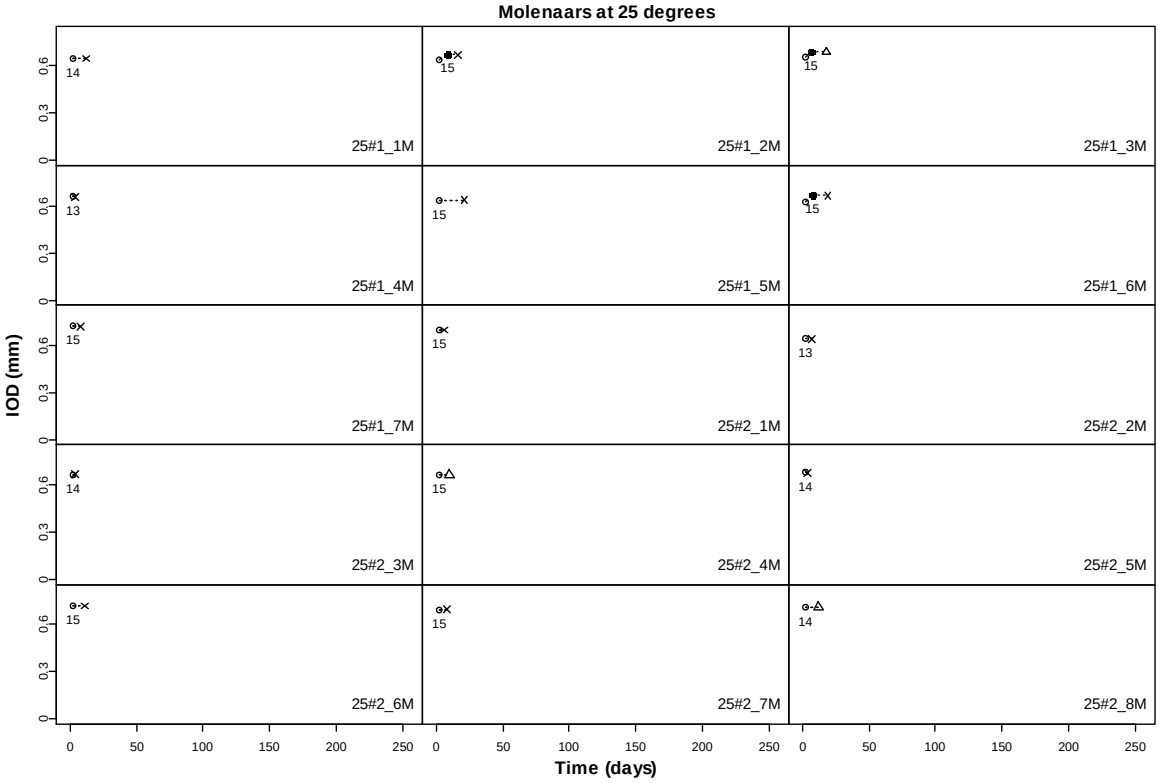
Molenaars at 25 degrees
IOD (mm)
Time (days)
25#1_1M
25#1_2M
25#1_3M
25#1_4M
25#1_5M
25#1_6M
25#1_7M
25#2_1M
25#2_2M
25#2_3M
25#2_4M
25#2_5M
25#2_6M
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25#2_8M