MIGRATION AND DISPERSAL OF THE WESTERN LEOPARD TOAD
Amietophrynus pantherinus
IN A FRAGMENTED AGRICULTURAL LANDSCAPE

By

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Abstract

Habitat alteration, primarily associated with human expansion and consumption, has been identified as the leading threat to biodiversity worldwide. The net result of an ever increasing human population is the loss of available habitat to species, affecting individual survival, together with the fragmentation of habitat across a landscape, resulting in an increased chance of a genetic bottlenecks and localized extinction. Although many organisms are experiencing the deleterious effects of these processes, amphibians appear to be suffering more than other vertebrate groups. One species that has experienced significant impact through habitat alteration and urbanization is the Western Leopard Toad (Bufonidae: *Amietophrynus pantherinus*). In the south-western portion of the Western Cape Province, South Africa, the population is large and genetically diverse with numerous potential breeding sites. However, extinctions in the disjunt eastern area, where there are a low number of known breeding sites (currently 7 identified), have raised concern over the conservation of this management unit. In this study radio-telemetry and population genetic data were used to investigate fine scale, short term migration patterns in Western Leopard Toads to specifically assess the role of agricultural land-use and habitat fragmentation on long-term dispersal dynamics. Within urban areas previous studies indicated that toads use gardens as foraging areas and terrestrial refuges during non-breeding periods of the year. Urban areas also have short distances between breeding sites allowing toads to successfully disperse, there by forming a stable metapopulation. In rural areas however, the habitat is dominated by agricultural land-use practices with greater distances between breeding sites (up to 26 km). Results from individual radio-tracking data revealed a preference for dense shrubs and bushes as post-breeding terrestrial refuges together with mowed fields for migration movement. Mark-recapture data showed an average stop over time in the breeding pond of 9 days and suggests that male Western Leopard Toads remain around the breeding pond, while females begin return migration after breeding. Across the seven known breeding sites microsatellite analysis revealed three distinct genetic clusters, which maintain a degree of ongoing connectivity, up to 26 km, despite the human altered landscape. Knowledge of habitat use together with the long term effects of habitat alteration and fragmentation are essential for effective conservation management, not only for Western Leopard Toads, but for a range of small vertebrates sharing similarly transformed habitats. This study provides base-line data for further studies into Western Leopard Toad migration and dispersal, and makes a number of recommendations towards the conservation planning for a variety of species which share the same ecosystem.

Chapter 1: General Introduction

Habitat alteration, which includes urban (McKinney 2002) and agricultural development (McLaughlin & Mineau 1995), harvesting of natural resources (Fearnside 2005), introduction of invasive species (Mooney & Cleland 2001), and climate change (Parmesan & Yohe 2003), have been identified as the leading threats to biodiversity worldwide (Pimm *et al.* 1995; Wilcove *et al.* 1998; Rahbek & Colwell 2011). Ultimately, habitat alteration stems from increasing human population size and the associated per capita consumption (Pletscher & Schwartz 2000). Although it is difficult to assess the direct relationship between spatial habitat loss and species extinctions, the traditional rule of thumb when applying a species-area relationship suggests that when 90% of a habitat area is lost, roughly 50% of the species will go extinct (Rahbek & Colwell 2011). However, a more accurate reflection of species loss in relation to habitat loss is applying a true endemics-area relationship (see Fig 1.1) (Kinzig & Harte 2000; Rahbek & Colwell 2011; also see He & Hubbell 2011).

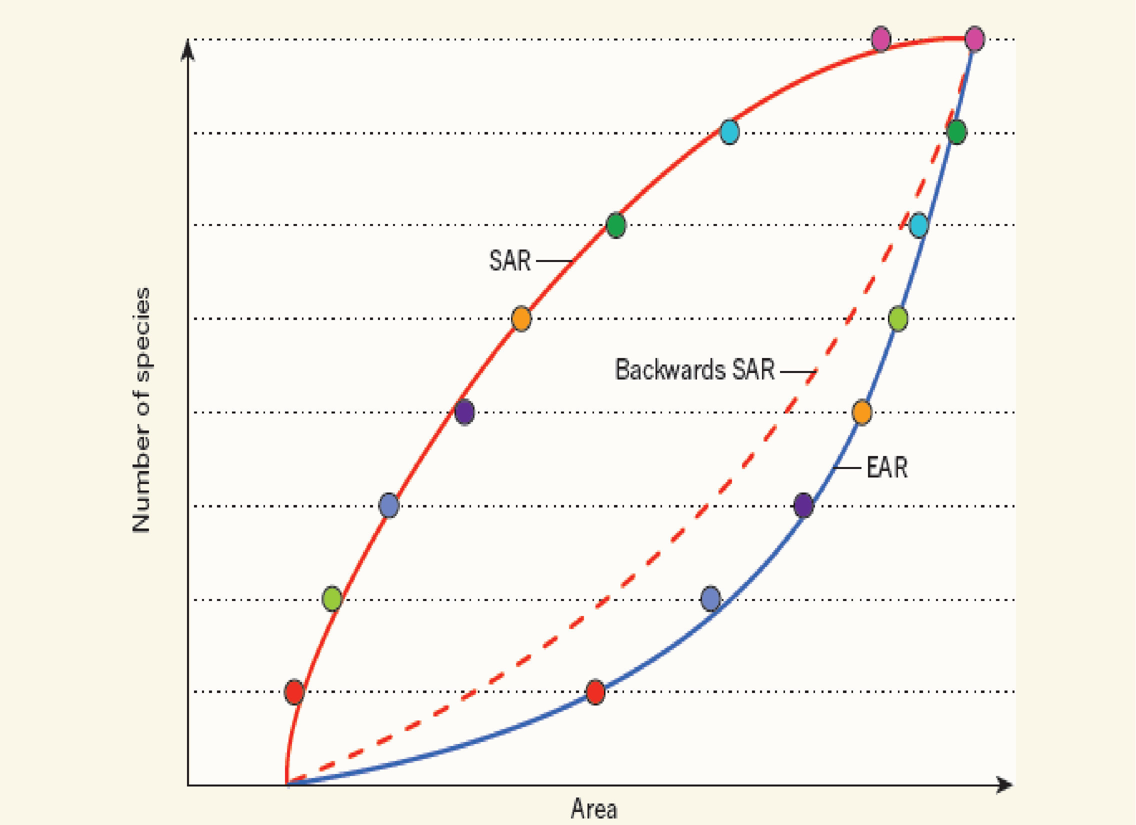


Fig 1.1 Estimating species extinctions due to habitat loss**.** This hypothetical example shows the contrast between use of the backwards species–area relationship (SAR), traditionally used to predict extinctions, and the true endemics–area relationship (EAR) that correctly estimates extinctions with increasing area lost (Reproduced from Rahbek & Colwell 2011)

Humans have transformed 40-50% of all ice-free land surfaces (Chapin III *et al*. 2000) and it has been estimated that the current extinction rates of species worldwide are 100-1000 times greater than pre-human rates, and regardless of methodology, most scientists agree there is an extinction crisis (Pimm *et al.* 1995). Such is the extent of human impact on extinction rate that our current period has been described as the 6th mass extinction in the planet's history, also referred to as the Anthropocene (Wilson 1992; Pimm *et al*. 1995; Leakey & Lewin 1996; Wake & Vredenburg 2008; Zalasiewicz *et al*. 2011).

Although human activities are impacting species in all classes, amphibians appear to be affecting more than other vertebrate groups (Stuart *et al.* 2004; Beebee & Giffiths 2005). Amphibian populations are vulnerable to a multitude of factors including over-exploitation, pollution, introduced species, climate change, endocrine-disrupting pollutants, UV-radiation, and diseases like chytridiomycosis (Blaustein *et al.* 1994; Houlahan *et al.* 2000; Collins & Storfer 2003); however, habitat loss and fragmentation is still the number one threat to amphibians (Cushman 2006).

Concerns over amphibian population declines were highlighted at the First World Congress of Herpetology, in 1989, and the need to accurately assess the state of amphibian populations worldwide became apparent. It has been found that 43.2% (2468) of amphibian species are experiencing some sort of population decline, with 32.5% (1856) of amphibian species listed as globally threatened (Vulnerable, Endangered, or Critically Endangered) on the IUCN Red Data List. The level of threat to amphibians is most likely underestimated since 22.5% (1294) of species are data deficient, and it is very likely that a significant portion of these data deficient species is also globally threatened (Stuart *et al.* 2004). Although amphibians, in a global context, are under heavy extinction pressure, not all are experiencing the same level of threat.

Declines caused by habitat loss are important in most amphibian families, yet certain other factors pose extra supplementary threats for decline to more specific amphibian families (Stuart *et al.* 2004). The variation between amphibian families in the number and proportion of rapidly declining species can be attributed to geographic distribution and the human activities associated with the area, such as overexploitation for human consumption of the family Ranidae, particularly in Asia (Stuart *et al.* 2004). Some families are endemic to certain regions of the world, e.g. Heleophrynidae to South Africa (Frost *et al.* 2006), and these families tend to suffer most where enigmatic declines, where populations are declining even without habitat change and biodiversity should be protected, take place. This has been shown to have a major impact on the family Bufonidae (Stuart *et al*. 2004). Furthermore, different habitat types and different geographical areas have different proportion of amphibian species (See Table 1). Within sub-Saharan Africa, there are several regions of high anuran diversity, a few of which correspond to previously recognized biodiversity hotspots, including the Upper Guinean Forests, the Cameroon Volcanic Line, and Eastern Arc Mountains (Brooks *et al*. 2002; Stuart *et al.* 2004). When these regions of high anuran diversity go under distress, the proportion of amphibian species and families in decline increases. Different habitat types experience different levels of threat, i.e. deforestation in the Amazon, and the species that occur in habitats with high level of threat are at a greater risk of extinction.

Table 1 Table, redrawn from Stuart *et al*. 2004, depicting habitat preference of rapidly declining and enigmatic decline species amphibians with percentages in relation to all amphibian species. Percentages are independent of each other, as some species are included in more than one category.

Habitat preferences	Total number of species (%)	Number of rapidly declining species (%)	Number of enigmatic-decline species (%)
Forest	4699 (81.8)	365 (82.6)	187 (90.3)***↑
Savanna	487 (8.5)	7 (1.6)***↓	0 (0.0)***↓
Shrubland	814 (14.2)	47 (10.6)*↓	14 (6.8)***↓
Grassland	953 (16.6)	81 (18.3)	39 (18.8)
Flowing water	2650 (46.1)	277 (62.7)***↑	164 (79.2)***↑
Marshes/swamps	760 (13.2)	43 (9.7)*↓	14 (6.8)**↓
Still water bodies	2030 (35.3)	107 (24.2)***↓	28 (13.5)***↓
Artificial terrestrial habitat	1304 (22.7)	40 (9.0)***↓	22 (10.6)***↓
Tropical lowland habitats	3392 (59.1)	212 (48.0)**↓	79 (38.2)***↓
Tropical montane habitats	2714 (47.3)	251 (56.8)***↑	155 (74.9)***↑

*p<0.05

**P<0.01

***P<0.001

↑Significantly higher than average; ↓significantly lower than average

Habitat loss/fragmentation and the current extinction crisis

Human altered habitats, from farmlands to cities, are generally not considered suitable for wildlife; such habitats have, in turn, seen dramatic losses in biodiversity. Industrial agriculture is the main cause of habitat loss while urbanization is the major hindrance to species recovery (Noss *et al.* 1995). In the United States, half of the total land area is devoted to agriculture, with massive single crop industrial agriculture the leading cause of habitat destruction (Noss *et al.* 1995). As a result, nearly 90 percent of recent wetland losses in the United States are due to agricultural practices (Noss & Peters 1995). Those species which rely heavily on US wetlands for survival are now on the brink of extinction. Of the 77 species endangered as a result of wetland loss in the United States, the largest agricultural states of Florida and California, not surprisingly, have the greatest number of species endangered as a result of agricultural practices (Czech *et al.* 2000).

Habitat loss and alteration also occurs naturally through time by means of droughts, floods, fires, etc. and whether natural or anthropogenic, these processes play a critical role in local population viability and turnover, and are therefore key components in landscape population dynamics. Habitat loss directly reduces the resources available to the population within that habitat. The reduction of resources, in turn, decreases the carrying capacity of the area (Lande 1987; Hanski & Ovaskainen 2000). Localized populations may then decrease as the competition for limited resources increases, leading to mortality or emigration. The reduction in population size increases the risk of a stochastic extinction event (Foley 1994). In general there are four types of uncertainty to which a population may be subject: demographic stochasticity, environmental stochasticity, natural disasters, and genetic stochasticity (Shaffer 1981). Since a population will eventually become exposed to one or more of these factors, which become more important as population size decreases, a population must maintain a certain population size, known as the minimum viable population, in order to remain extant. A minimum viable population is the smallest an isolated population can be while still maintaining a 99% chance of survival over a period of 1000 years, despite effects from demographic, environmental, and genetic stochasticity, and natural catastrophes (Shaffer 1981). If habitat loss reduced the amount of resources available to the population, where the carrying capacity is lower than the minimum viable population, the population faces extinction from stochastic events.

On top of reduced resources, if habitat becomes fragmented during the habitat loss process, the loss of connectivity between habitat areas may reduce the chances for a species to obtain certain remaining resources in the area (see Fig 1.2). Blomquist & Hunter (2009) showed that Northern leopard frogs (*Lithobates pipiens*) utilize different areas in the habitat, based on certain microhabitat conditions (e.g. leaf litter moisture), during different seasons of the year. If the connectivity between *L. pipiens* summer breeding sites and overwintering habitat is lost, then the ability for animals to migrate to and from breeding areas would be reduced (if not completely eliminated), eventually causing the population to go extinct. Additionally, if the lost habitat contained all of one critical resource in the area (for example: a breeding site (Sutherland 1996)), the population would inevitably become extinct.

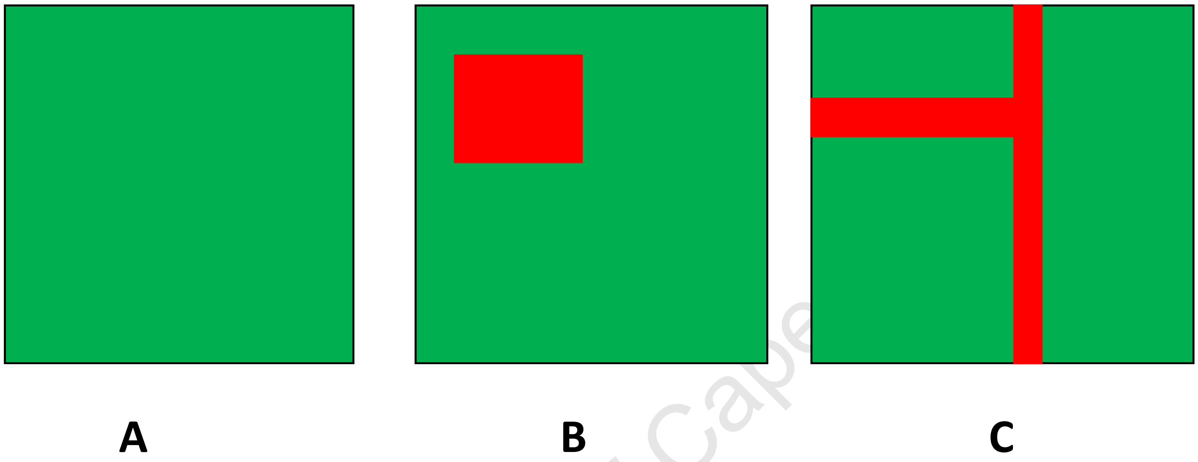


Fig 1.2 Shows an area of suitable habitat (green) that experiences habitat loss (red). In area B habitat loss is experienced without fragmentation, and area C habitat loss is experienced with fragmentation

Metapopulation dynamics and the disruptive role of land habitat loss

Habitat fragmentation separates populations at a landscape level which can interact with each other, provided suitable habitat for movement is present. These interacting populations are known as a metapopulation (Levins 1969), and even in an ecologically pristine environment, metapopulations are a naturally occurring system. Metapopulation ecology runs on the assumption that across any landscape, suitable habitat for any species occurs as a network of idealized habitat patches, varying in area, degree of isolations, and suitability (see Fig 1.3) (Hanski 1998). In classical metapopulation theory, each sub-population exists in relative independence of the others and eventually goes extinct due to stochastic events; the smaller the population, the more prone it is to extinction (Hanski 1991; Smith & Green 2005).

Each sub-population within the metapopulation has a finite life-span, and relies on immigration from another to re-colonize the habitat which has been left open by the extinct sub-population. This re-colonization may also occur before the total extinction of a sub-population, known as the ‘rescue effect’

(Brown & Kodric-Brown 1977; Funk *et al.* 2005). This ability to rescue (or simply re-colonize) demonstrates the necessity for population connectivity as no single sub-population can guarantee the long-term survival of a species (Levins 1969). Metapopulations rely on dispersal between sub-populations in order to maintain healthy populations, genetic diversity, or in the event of local extinction, some degree of recolonisation; this requires stable source/sink dynamics. (Semlitsch 2008).

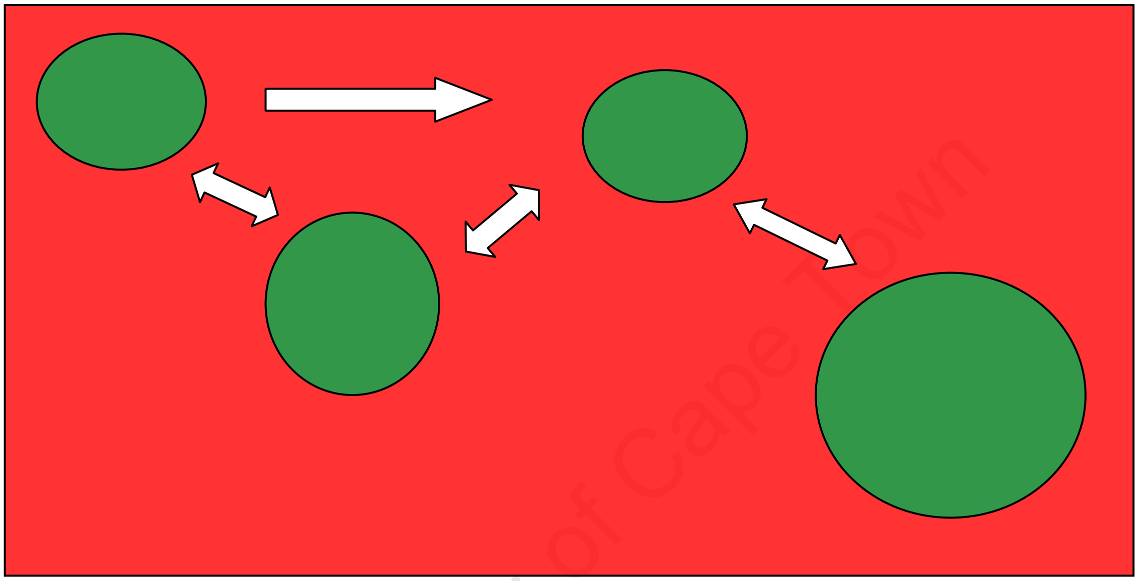


Fig 1.3 Metapopulation scenario where sub-populations residing within suitable habitat patches (green), are connected via dispersal within a landscape consisting of unsuitable habitat for living, but still allows dispersal between sub-populations. The bidirectional arrows indicate scenarios where dispersal take place in both directions and the unidirectional arrow indicates a scenario where dispersal can take place one way, and a barrier to dispersal in the reciprocal direction exists.

Since metapopulations are naturally occurring systems across a landscape, and not a product of fragmentation, which rely heavily on the movement of individuals to maintain viability, the ability for individuals to move between populations is just as important on a landscape level as habitat fragmentation is at a localized population level (Levins 1969; Trenham & Schaffer 2005; Semlistch 2008). If a localized population experiences changes in the habitat that makes it unsuitable for living, such as the removal of a food source, the patch becomes unsuitable and therefore unavailable to emigrating animals. This outcome of localized habitat change affecting landscape level metapopulation dynamics has been observed in a wide range of terrestrial taxa (for reviews see: Hanski 1991; Harrison 1991; Andren 1994).

Within a metapopulation geographically distant sub-populations can be genetically linked across a landscape via the stepping stone process through sub-populations of closer geographic distance. When an animal disperses from its natal population to another sub-population, eventually, either the individual will emigrate from the new sub-population or its offspring with emigrate from their natal population to a new population, connecting the two geographically distant sub-populations (Hanski 1991). If a centrally located population (B) becomes extinct due to an unfavourable change in habitat, outer populations (A & C) which were once linked become isolated from each other if the species in question cannot disperse over the new geographical distance created (between A & C). This interrupts the metapopulation dynamic, putting the species at risk across the landscape (see Fig 1.4).

A ←→ B ←→ C   A → ~~B~~ ← C   A -------------- C

Fig 1.4 Simplified metapopulation scenario in which populations A and C are connected through the stepping stone process via population B until the habitat for population B becomes unsuitable for living causing an extinction event of population B. Populations A and C are now isolated from each other

Habitat change can also affect population connectivity indirectly by creating barriers to movement between habitat patches (Bennett 1990). Even if a landscape naturally contains patches of suitable habitat, the rest of the area, although deemed ecologically unsuitable, may still be suitable for movement between the occupied habitat patches. A change in habitat, resulting in a barrier to movement between patches, would not directly hinder the viability of a population, but would eliminate the rescue effect or potential for re-colonization in the event of a stochastic extinction event (Forman & Alexander 1998).

These types of population separations occur naturally (e.g. changes in drainage dynamics that influence the size and flow of rivers). If the separation became permanent the two populations would eventually over time evolve into two genetically different species (Endler 1980; Stebbins 1966). On the other hand, human-driven habitat change can occur over much shorter time spans (in terms of landscape and biological processes) and usually at large scales. When combining such large scale habitat changes, over short periods of time, a species cannot adapt to the new conditions quickly enough (if at all), and if this habitat loss reduces a resource (or resources) below the threshold of the minimum viable population, the population will go extinct. How much habitat has to be destroyed before this threshold is reached depends upon how much of a habitat generalist the species is (Hamer & McDonnell 2008). Generalist species with higher rates of colonization can tolerate the greatest degree of habitat loss, while habitat specialists are more susceptible to extinction (Hamer & McDonnell 2008).

Amphibians and metapopulation dynamics

Many, if not most, amphibian species naturally occur in metapopulations (Alford and Richards 1999); furthermore, pond breeding amphibians often fit a classical metapopulation model (Marsh & Trenham 2001). The persistence of amphibian populations depends largely on processes that occur on at least two spatial scales: those of local pond dynamics and those at the level of landscapes (Semlitsch 2008). Further, recent research has provided evidence on the relationships between certain amphibian species' decline and specific attributes of habitat loss and fragmentation (Sinsch 1992; Todd et al 2009). For example, species with large dispersal abilities are more vulnerable to dispersing mortality (e.g. desiccation and predation: Rothermel & Semlitsch 2002), whereas species with limited dispersal abilities over longer time periods, become isolated by fragmentation and ultimately become extinct (Semlitsch *et al.* 1996; Skelly *et al.* 1999; Cushman 2006).

At a localized level, the biphasic life-history of pond-breeding amphibian populations requires both aquatic and terrestrial habitat. Traditionally, efforts to conserve amphibians have been directed towards aquatic habitats because of their fundamental role in breeding behaviour and larval development, but this approach often fails to address the importance of terrestrial habitats in local amphibian ecology (Sztatecsny & Schabetsberger 2005). Post-metamorphic amphibians live, forage, and overwinter in terrestrial habitats adjacent to aquatic reproduction sites (Semlitsch 2008). Consequently, amphibians require suitable terrestrial habitat, within a particular proximity to their breeding areas, for growth and survival during the non-breeding portions of the year (Guerry & Hunter 2002; Todd *et al.* 2009). Furthermore, it has been shown that post-metamorphic survival, which is largely based on suitable terrestrial refuges, has to exceed a certain level in order to maintain local population viability. Trenham et al. (2000) reported that juvenile survival of *Ambystoma californiense* to first reproductive year must average 18% in order to maintain a healthy population. In their case study population, the survival rate of juveniles was 5%, leading them to conclude that the population was a sink, and would inevitably go extinct.

Pond-breeding amphibians also require appropriate contiguous habitats that link their terrestrial activity centres to aquatic reproduction sites (Semlitsch *et al*. 1996; Skelly *et al*. 1999; Rothermel & Semlitsch 2002; Becker *et al.* 2007). Loss of local connectivity between terrestrial and aquatic environments can negatively affect amphibians and has been shown to lead to population declines in biphasic amphibians (Harper *et al.* 2008). Even in unfragmented habitats, amphibian populations experience relatively frequent localized extinction events and turnover (Trenham *et al.* 2003), making dispersal critical for recolonisation of local populations and maintenance of regional metapopulations (Semlitsch *et al.* 1996).

At a landscape level, connectivity amongst amphibian populations is predominantly influenced by juvenile dispersal (Sinsch 1992; Guerry & Hunter 2002; Cushman 2006). For example, Preisser *et al.* (2001) have shown that adult wood frogs (*Rana sylvatica*) and adult *Ambystoma sp.* moved within a relatively short distance from the breeding ponds (100+ m), while the juveniles moved up to ten times farther (1000+m) away from the breeding pond (Preisser *et al.* 2001). Habitat loss and fragmentation can significantly reduce a juvenile's ability to disperse across landscapes (Cushman 2006).

It has also been shown that post-metamorphic juvenile amphibians are selective for the habitat type in their dispersal corridors (Funk *et al.* 2005). In a number of forest dwelling amphibian species, including wood frogs (*Rana sylvatica*) (De Maynadier & Hunter 1999), spotted salamander (*Ambystoma maculatum*), small-mouthed salamander (*Ambystoma texanum*), and the American toad (*Anaxyrus americanus*) (Rothermel & Semlitsch 2002), juveniles show a preference for wooded areas with closed canopy as their dispersal corridor. Additionally, juveniles moving within the forested areas were found to move farther than those found moving in clear cut habitats (Rothermel & Semlitsch 2002), suggesting that continuous forested habitats are essential for the connectivity of metapopulations for species in boreal forests. Furthermore, both adult and juvenile densities can decline sharply over a gradient of habitat from mature forest to recently clear-cut habitat (De Maynadier & Hunter 1999), indicating that not only may juveniles seek out preferential habitat for dispersal, but that those that do not find suitable habitat can be prevented from moving far enough to reach another population. The loss of these mature forest corridors connecting populations would reduce the suitable habitat for juvenile dispersal of these species, potentially leading to isolation and possible extinction of populations (De Maynadier & Hunter 1999). Since juvenile dispersal has been shown to play a pivotal role in amphibian population viability, the effects of habitat fragmentation on juvenile dispersal can be viewed as one of the key issues in the conservation of pond breeding amphibians (Cushman 2006).However, patterns of individual juvenile dispersal remain a difficult area of study, as their small body size and behavioural characteristics make them difficult to sample (Semlistch 2008).

In order to understand the effects of habitat loss and fragmentation on a species, it is important to assess site-specific impacts as well as multiple interacting factors across a landscape (Cushman 2006). Most current research on amphibian population viability focuses on external factors affecting movement at both a local population and landscape level, in order to understand the many facets of and challenges for amphibian management and conservation (Semlitsch 2008). Habitat area, at a local level, and isolation, at a landscape level, are key factors in long term population survival (Cushman 2006).

Amphibian migration and dispersal

Amphibian population viability is largely dependent on the dispersal success of individuals at both a local and landscape level (Sinsch 1992; Rothermel 2004; Cushman 2006; Semlitsch 2008).

Local population persistence appears to be highly dependent on the ability of individuals to complete the annual migration (Nakazawa & Yamamura 2006), in the case of pond breeding amphibians, to spawning ponds and wetlands from terrestrial habitat and back (Rittenhouse & Semlitsch 2007; Semlitsch 2008). The distance and direction of these migrations may differ considerably among sites depending on the quality of terrestrial habitats. Additionally, the availability of suitable refuges versus local predation pressure (Jehle & Arntzen 2000; Kovar *et al.* 2009), together with sufficient continuous habitat for movement are also critical to the completion of the migration process.

At a landscape level dispersal is vital for population persistence. Dispersal, as applied to the study of amphibian ecology, is the unidirectional movement of an individual away from its natal area to another breeding site not belonging to the local population (Semlitsch 2008). Dispersal is related to several ecological processes such as intraspecific competition (competition for resources amongst a single species), interspecific competition (competition between different species), and avoidance of inbreeding (Hamilton & May 1977; Bengtsson 1978; Clobert *et al.* 2001; Bowler & Benton 2005). For pond breeding amphibians, dispersal is largely performed by juveniles, although it has been shown that breeding adults do make up a small portion of dispersing animals (Gamble *et al.* 2007). A number of studies on toad species, including the common spadefoot (*Pelobates fuscus*), the natterjack toad (*Epidalea calamita*), and the common toad (*Bufo bufo*), (Reading *et al.* 1991; Sinsch 1992; Hels 2002; Smith & Green 2006) all indicate strong philopatry within the Bufonidae. Philopatry describes the behaviour of individuals who remain within their natal population (Greenwood 1980). For example, assuming movement was possible, Hels (2002) reports that only ~1% of common spadefoot individuals collected were found to be in more than one pond, out of five, over the course of a four year study period. Since the adult common spadefoots remain steadfast to a site, the juveniles are expected to move further than adults. Nevertheless, Smith and Green (2006) have shown that dispersal in the Fowler's Toad (*Bufo fowleri*) is neither sex biased nor age biased, showing that even though juveniles may play the biggest role in dispersal, preserving the features that allow adult dispersal cannot be ruled out as a conservation priority.

Since juvenile pond breeding amphibians generally metamorphose at a small size their locomotory abilities are limited in comparison to adults and travel shorter distances (often >1km), have lower rates of travel, and have lower stamina than large individuals (Goater *et al.* 1993, Beck and Congdon 2000). Dispersal to distant populations take place during several discrete events which will allow the juveniles time to reach a larger body size more conducive to longer terrestrial movements (see Fig 1.5). There is no evidence that juvenile amphibians have any form of orienteering ability towards new habitat sites and movement appears to be more random than that of adults (Sincsch 1997). Dispersal will typically cease once an animal reaches reproductive maturity and after first reproduction at a new pool (Semlitsch 2008).

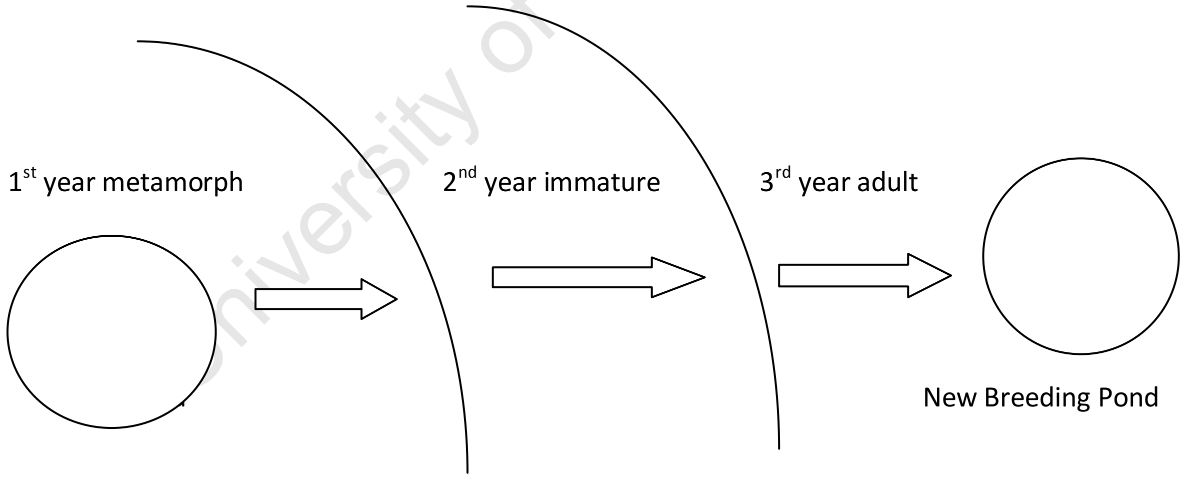


Fig 1.5 Model of a juvenile based dispersal scenario redrawn from Semlitsch 2008. The scenario suggests several distinct stages of dispersal, assuming that an individual will continue movement until reaching adult stage. Arched lines represent point where the individual will find a terrestrial refuge, before continuing to move towards a new breeding site.

Given that habitat quality for any one species is such an important factor in the success of these movement behaviours, a direct assessment of habitat quality would be ideal. That is, however, difficult. Fortunately, getting a finer view of the migration and dispersal processes can give a fairly good idea of the quality of habitat, as good habitat quality would promote successful dispersal and migration. In this way, populations can be assessed at a local and landscape level in order to develop conservation strategies for amphibian species which are already in danger of extinction (Cushman 2006).

The Western Leopard Toad

A species for which fine-scale migration and dispersal data is urgently needed is the endangered Western Leopard Toad (WLT), *Amietophrynus pantherinus* Smith 1828 (IUCN *et al*. 2011). The WLT is a large Bufonid, up to 140mm snout-vent length (females), endemic to the south-western tip of Africa (de Villiers 2004). The species distribution lies within the Cape Fynbos biome of the winter rain fall region of South Africa. Populations which used to occur between the areas of Pringle Bay and Kleinmond (see Fig 1.6) appear to have become extinct in the last 20 years (Measey & Tolley 2011). The species is found around bodies of water such as large wetlands, vleis, dams, and ephemeral ponds (de Villiers 2004) and whilst historically associated with fynbos habitat, the species is often found taking up residence in suburban gardens and on farm land; this is because the modern-day distribution of WLTs is not restricted to pristine natural habitats, but determined rather by the annual availability of appropriate aquatic habitats for breeding (du Preez & Carruthers 2009).

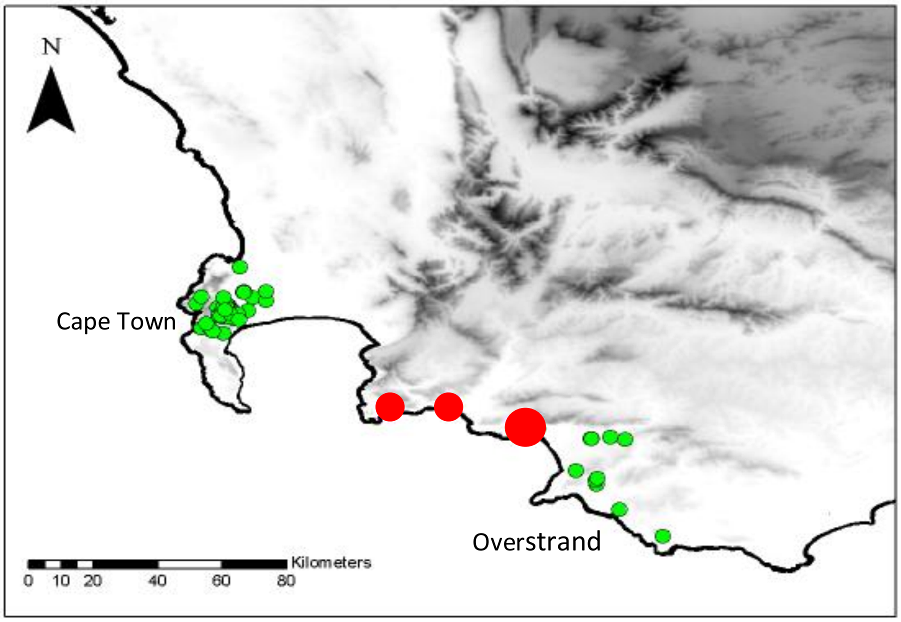


Fig 1.6 Range map of the Western Leopard Toad (*Amietophrynus pantherinus*), based on currently identified breeding sites (green), as well as extinct breeding sites (red) (Data received from G. J. Measey)

Western leopard toads can be identified by a clear dorsal pattern of chocolate coloured 'blotches' on a bright yellow background, with a continuous yellow line running down the entire back. Dorsal patterns are thought to be unique to each individual. The top of the head is a reddish coloration and the underside is cream. Two large and distinct reddish coloured parotid glands sit just behind the eyes (de Villiers 2004; du Preez & Carruthers 2009). The Western leopard toad is commonly confused with Ranger's toad, *Amietophrynus rangeri*, and the Eastern Leopard Toad, *Amietophrynus pardalis*. Some key distinguishing characteristics that differentiate *A. rangeri* from the Western Leopard Toad are the distinct symmetrical dorsal markings and the dark patches behind the eye that are not fused together (du Preez & Carruthers 2009). *A. pardalis* is difficult to distinguish morphologically from *A. pantherinus* however they do not naturally occur in sympatry (Eick *et al*. 2001). Like most of its bufonid relatives, the Western Leopard Toad is predominantly terrestrial, spending most of the year away from water, where it feeds primarily on invertebrate species, with one notable prey item being snails (de Villiers 2004).

Breeding behaviour coincides with the onset of the winter rainfall season, in late July through August (Cherry 1992). The WLT is an explosive breeder, gathering en mass at breeding sites including dams and small ponds where breeding activity taking place across a period of 4-5 nights. During this time, males attract mates, emitting a call described as a slow snore (Cherry 1992). Surprisingly, they do not attempt to displace other males that have engaged females in amplexus, which is rare amongst explosive breeders (Cherry 1992). Eggs are laid within shallow marshy areas, usually to avoid predators such as fish, in strings consisting of around 25,000 eggs. Tadpoles feed on algae, and after about 10-12 weeks metamorphosis takes place (du Preez & Carruthers 2009).

The Western Leopard Toad's current distribution comprises two disjunct 'populations' separated by 100 km, one population located in the Greater Cape Town Area (West of False Bay) and the other located in the Overstrand region (East of False Bay), estimated to have been separated for approximately five thousand years, the result of a period of regional drying during the Holocene (Measey & Tolley 2011). Both populations occupy habitats which are heavily influenced by human presence in very different ways.

The Greater Cape Town metropole is highly influenced by anthropogenic habitat change, primarily urbanised and suburban settings. Previous studies on WLTs in this area found that the species has adjusted to the new habitat setting and may even prefer it (Pers. Comm. Dr. G.J. Measey). Radio-tracked toads regularly favour paved roads as migration corridors as opposed to the green corridor surrounding their wetland habitats (Pers. Comm. Dr. G.J. Measey; also seen in other anurans Seabrook & Dettmann 1996; Brown *et al*. 2006). Also, individual toads were found utilizing suburban gardens as their terrestrial refuge during the non-migratory portion of the year (Pers. Comm. Dr. G. J. Measey). Suburban gardens provide a variety of essential resources for toads including loose soil for burrowing, vegetation, food, and standing water (ex: ornamental ponds). The ability of the species to adjust to a highly urbanised environment, not usually associated with providing suitable habitat for wildlife, suggests that this species has a certain level of tolerance to habitat alteration.

In contrast to the suburban sprawl of the greater Cape Town metropole the Overstrand area is heavily influenced by agricultural land-use practices and small town development (Driver *et al.* 2005). The area is characterised by a mosaic of farm land, grazing pasture, natural fynbos habitat and tree stands (both native and alien), with scattered wetlands both man-made and natural. Western Leopard Toads appear to be more prevalent in the highly urbanised Cape Town area; where there are currently 40+ confirmed breeding sites; this is in contrast to their Overstrand area where only 10 breeding sites have been identified, three of which (between Kleinmond and Pringle Bay) have gone extinct in the last 20 years due to human housing development (Measey & Tolley 2011). Across a similarly sized geographic area, the Overstrand 'population' is also genetically less diverse than the Greater Cape Town area 'population' (Measey & Tolley 2011). The disparity in success between the two areas leads to questions centred on why the species seems to be declining in the rural/agriculture landscape, and, whether their decline is linked to disruptions to processes of migration and dispersal in the highly fragmented agricultural setting of the Overstrand.

Project Aims

This study focuses on the influence of habitat fragmentation on patterns of migration and dispersal in the Western Leopard Toad. Migration and dispersal are investigated across an agricultural mosaic in the Overstrand region of the Western Cape, South Africa using a number of techniques that allow inferences at different temporal and spatial scales. These include radio telemetry and mark-recapture together with the use of microsatellite DNA markers analysed using a population genetic approach. The main aims of the study are:

1. To investigate fine scale, short term migration patterns in WLTs using adult movement data collected during the breeding season.

Because movement towards water resources during the breeding season is critical to the persistence of local populations, specific conservation actions, policies, and management need to be informed by a number of aspects of migration. To paint a more complete picture of the Western Leopard Toad migration process: Questions of interest include; how long do animals utilize wetland and aquatic habitats during the breeding season? How far do animals move away from their breeding pools at the end of the breeding season? What habitat types do animals use as their terrestrial refuge during the non-migratory period of the year?

2. To quantify the degree of population genetic structuring and connectivity among WLT sampling sites across an area of the Overstrand region.

   To assess the role of agricultural land use and habitat fragmentation on long-term dispersal dynamics in Western Leopard Toads, data from DNA markers can provide insight into the degree to which the species is structured into genetic ’pools’, and how these are linked via dispersing individuals. Questions of interest include how many genetically unique ‘pools’ occur in the Overstrand? How genetically diverse is each individual ‘pool’? Is there any evidence of recent connectivity i.e. gene flow, between these ‘pools’? To what degree do agricultural landscapes act as barriers to gene flow and thereby influence the distribution of WLT genetic variation in the area? What is the geographical distance (in km) for dispersal? Does WLT exhibit a Sex-biased dispersal pattern?

Ultimately it is the goal of the study to provide stakeholders (Government, NGO’s, landowners) with a set of recommendations, based on empirical data for the conservation of the species across the Overstrand region, as well as to add a further body of knowledge to the disciplines involved in amphibian conservation.

Chapter 2: Methods

Study Site

This study took place in the Overstrand region of the Western Cape, South Africa (see Fig 2.1). The Overstrand region is an area of low elevation bordered by the Cape Fold Mountain range to the North and the Atlantic Ocean to the South, and represents the eastern most distribution of the Western Leopard Toad. Across vast areas the landscape has been highly transformed by human activity, in particular agricultural land-use development and urbanisation. The majority of the area comprises privately owned land with an array of vegetation cover, from pristine coastal fynbos habitat, to grassy fields, with some areas dominated by invasive plant species. The study of landscape level dispersal in Western Leopard Toads included seven known breeding sites in the Overstrand area (Pers. Comm. GJ Measey & A de Villiers), Stanford Town Dam (34°26'36.06"S; 19°27'16.09"E), Vaalvlei (34°26'30.95"S; 19°30'30.98"E), Grootbos (34°32'24.37"S; 19°24'49.14"E), Flower Valley (34°32'54.10"S; 19°28'17.40"E), Owl's Nest (34°34'13.30"S; 19°28'3.80"E), Klein Paradise (34°39'9.25"S; 19°31'59.87"E), and Buffeljarsvlei (34°44'13.51"S; 19°39'11.06"E). Six of these breeding areas are privately built farm dams and one a natural wetland vlei (Buffeljarsvlei) within the Agulhas National Park (see Fig 2.1). Approximately 50km separates the eastern most breeding site and the westernmost field site within the Overstrand area.

Figure 2.1 Google Earth image of the Overstrand area with the seven identified breeding sites

Data for localized migration behaviour were collected on privately owned land at Klein Paradise Country House (see Fig 2.1). The Klein Paradise breeding site was chosen after a preliminary survey at two sites; activity levels over a 1km area surrounding the dams were surveyed at both the Klein Paradise and Owl's Nest breeding sites. These two sites were chosen for the surveys, based on activity levels recorded in July 2009 (Pers. Comm. GJ Measey), as the sites most likely to yield the greatest number of animals for mark-recapture data collection. The breeding sites at both locations represent man-made permanent farm dams stocked with fish (mainly trout) for recreational purposes. Explosive breeders are often associated with ephemeral ponds during the breeding season, since the very short nature of the breeding period requires a body of water for a limited period of time. Furthermore, the absence of fish in these temporary ponds reduces predation on pre-metamorphic juveniles. Klein Paradise was also chosen due to the absence of grazing livestock (sheep) which were present at Owl's Nest. Livestock could influence WLT habitat selection, favouring more covered habitat to avoid trampling.

Figure 2.2 Klein Paradise breeding site, with a 1km radius (2km diameter indicated by the red line) used for vegetation survey

Klein Paradise was also the preferred site based on the variety of vegetation types present, where eight different habitat types were identified within the 1km radius survey area (see Fig 2.2): indigenous fynbos veld (25%), mowed grass fields (25%), tall un-mowed grass (5%), open fields (20%), wetland with tall reeds (2%), brush piles (man-made piles of braches and wooded material) (2%), man-made habitats (docks, patios, guest houses, etc) (1%), closed canopy trees (20%; both natural and alien). Habitat percentages were estimated using a printed image of Fig 2.2 over graph paper with 1cm x 1cm dimension boxes before the initial field surveys. Habitat types in the image were indentified during the initial survey of the field sites. All habitat types occur within 0.25km of the breeding dam. Fynbos veld only occurred in the area north of the breeding dam site, with the rest of the property comprising a heterogeneous mix of both indigenous and exotic plant species. Of the two breeding sites surveyed only Klein Paradise had natural veld within the survey radius, providing an opportunity to investigate not only WLT migration behaviour in a rural landscape setting, but also whether exhibited behaviours differ between natural areas vs. disturbed areas.

A. Assessing migration behaviour of the Western Leopard Toad at a local scale

i) How long do they stay?

Sampling was conducted at night time, starting just after dark, when the toads are most active. Sampling was conducted of the whole pond by row boat, a meter off the reed line, in a clockwise rotation. Sampling sessions were conducted for 2 hours, in order to eliminate a biased in capture numbers between evenings. A single toe clip was collected for each newly captured animal using sterilised surgical scissors; the tip to the first articulation on the inside toe on the left foot (UCT Ethics 2010/V17/SD-R) was collected for both DNA sampling and identification of previously capture individuals. Each toe clip was stored in 96% ethanol for later genetic work; additional data was also collected for each individual including sex (for estimations of sex-ratios), GPS location, recaptures, SVL (mm), and mass (g). Between each toe clipping session the surgical scissors were disinfected using ~ 1% sodium hypochlorite solution so as to prevent the spread of chytrid fungus amongst individuals (Johnson *et al.* 2003). Samples were then stored at -20°C. All unused tissue has been accessioned into the DNA biobank at the South African National Biodiversity Institute for future genetic studies on *A. pantherinus*.

Photographs were also taken of the dorsal patterns for each individual for each capture event. Recaptures were identified by the toe clipping on the inside left foot. Recapture photographs were then cross referenced with original capture photographs to identify an individual's recapture history at the breeding site during the breeding season. Stop-over duration, the time between when the animal arrives and leaves the breeding pond, was calculated using the Cormack-Jolly-Seber (CJS) models (Cormack 1964; Jolly 1965; Seber 1965), in the program SODA (Schaub *et al.* 2001). The CJS model calculates total stop over time by combining two separate probabilities. First a recruitment analysis, essentially a survival analysis run backwards, was performed by calculating probability estimates of the animal's presence before the time of capture, at each individual capture event, and the probability of capture (Pradel 1996). Next the program calculates probability estimates of the animal's presence after the capture event by calculating survival, under the assumption that survival is equal to presence at the stop over site, and the probability of capture during each sampling evening (Lebreton *et al*. 1992). Both factors are assumed to be time dependent in the CJS model. The total stop over is then the sum of the recruitment analysis and the survival analysis (Schaub *et al.* 2001). The model was run with 500 bootstraps. The process of bootstrapping creates a distribution of capture histories by randomly sampling capture histories from the given data set. The more times this is done the greater the precision of the calculation of the mean (Schaub *et al*. 2001). The output from SODA provides a stopover for each sampling event (n=15), and a mean value was calculated for the entire breeding period.

ii) Radio-tracking

Radio-telemetry was used to investigate the fine-scale movement and migration behaviour of Western Leopard Toad in terms of what habitat they move through and total distance migrated from the breeding dam to their non-breeding terrestrial refuge. The Western Leopard Toad is highly suitable for a radio-telemetry study because its size makes the attachment of radio-transmitters easier compared to smaller amphibian species (Kusano *et al.* 1995). Captured animals kept in a bucket with moistened vegetation to prevent desiccation. Individuals were tagged (UCT Ethics 2010/V17/SD-R) with Holohil Systems Ltd. BD2-HX (weight: 1.2g) transmitters as per a previous radio-tracking study on the species (Feldman & Measey in prep). Transmitters were tied around the animal's waist, like a belt, using fishing line encased in catheter tubing, in order to prevent any chafing of the animal's skin. The belts were tied to a snug fit in order to make sure they could not be pulled off over the pelvic region. In order to ensure the tags were not tied too tightly, and thereby potentially injuring the animal or restricting movement, the first attempt at tying would be purposely too loose, and then adjusted accordingly. Animals were then released after a period of 30 minutes, in order to ensure there were no lingering effects from capture stress.

Both males and females were tagged in this study, however, only females which had deposited eggs were tagged. Gravid females which lay eggs lose a lot of body mass and volume (20-44% Bull 2006; Bionda *et al.* 2011), and attaching the tags prior to the eggs being deposited may result in the animal slipping off the tag once the body size decreases. Furthermore, tags tied around the waist may have interfered with the female's ability to deposit eggs. Females were distinguished from males, by the absence of nuptial pads which males have present on the inside of their forearms.

(iii) Migration distances

Like many amphibians WLT movements usually occur at night (Cherry 1992) to prevent desiccation (Semlitsch 2008), while using shelter sites during the daytime (Schwarzkopf & Alford 1996). Each animal was tracked to its day-time resting point and its position was recorded with a Garmin® Colorado 300 Global Positioning System (GPS), along with details on the vegetation category the animal was found in. Transmitters were removed at the end of the study period, when male calling had ceased and no animals were captured at the breeding pond.

GPS waypoints were uploaded into ArcGIS Explorer and each individual's GPS points were plotted. From this, the total distance travelled as well as the net displacement, the linear distance from the first recorded GPS point to the last, were calculated from the point of release, and averaged. In a recent review of 14 studies on amphibian species [7 on caudates, 7 on anurans (3 on bufonids)] by Rittenhouse & Semlitsch (2007) the authors suggest that amphibians do not generally make use of habitat within 30m of the breeding pools as terrestrial refugia, but instead utilize this habitat for cover during the breeding period, then migrate away from the breeding pond in search of a non-breeding period terrestrial refuge. Therefore, average distances and displacements were also recalculated after the exclusion of individuals that remained within 30m of the breeding pond after radio tracking had ceased.

(iv) Habitat use

Many studies of habitat use by wild animals make use of radio-tracking data to determine whether a species uses habitats available to it at random, to rank habitats in order of relative use (Conner *et al.* 2003; Miaud & Sanuy 2005), to compare use by different groups of animals (e.g. males and females Muths & Guyer 2003; Bartelt *et al.* 2004), to relate habitat use to environmental and ecological variables (e.g. temperature and food abundance Anderson *et al.* 1999; Seebacher & Alford 2002), or to examine the effects of habitat on movement and home range size (Aebischer *et al.* 1993). All available radio-tracking techniques however contain at least one shortcoming affecting the validity of the analysis, often resulting with type I or type II statistical errors (Alldredge & Ratti 1986; White & Garrott 1990; Alldredge & Ratti 1992; Aebischer *et al*. 1993).

A commonly used method compares frequencies of use and proportional availability of habitats using a χ^2 test (Aebischer *et al*. 1993). Determination of which habitat types a species uses is generally made by comparing habitat availability to the percentage of use for each habitat type (Neu *et al*. 1974; Byers *et al*. 1984). Tests using estimates of available habitat (e.g. from sampling), opposed to measured (e.g. from a map),should compare use and availability with the χ^2 test for homogeneity rather than a goodness-of-fit test (Marcum & Loftsgaarden 1980); however, these tests are more likely to indicate selection when there is none (Thomas &Taylor 1990), leading to a type I statistical error.

Several problems emerge when grouping animals together to describe the 'average' animals' behaviour in terms of habitat selection. Insufficient sampling and small sample sizes can lead to two separate forms of non-independence (Aebischer 1993). First, the positions of sequentially collected radio locations from tagged animals can be serially correlated (Swihart & Slade 1985). Animals exhibit individual variation in behaviour, as well variation based on different groups within the species e.g. sex and age class. The non-independence of proportions of habitat type, as a result of vegetative heterogeneity, is likely to result in an apparent preference for a habitat type due to individuals avoiding another habitat type; so the interpretation of absolute preference of habitat types is difficult (Neu *et al.* 1974, Byers *et al.* 1984). As a general rule, available habitat is usually defined from the total study area with some sort of arbitrary boundary selection for the study area (Johnson 1980, Porter & Church 1987).

An alternative method to account for these shortcomings is to group animals together, looking at each animal individually, and then assessing the proportion of habitat used within the individual's trajectory (Aebischer *et al*. 1993). However, one problem with this method is when animals make the bulk of their long distance movement in a single night but this can be accounted for by including data for as many individuals as possible from the sampled area.

To investigate habitat selection at the Klein Paradise breeding site, Gabriel's (1978) index of selectivity (W) was used to determine the relationship between the availability of different habitat types and the proportion of radio tracking points within each habitat type. Expressed as the log form, W yields values that range between $-\infty$ (negative selection) and $+\infty$ (positive selection), with values of 0 indicating an absence of any selection. The formula used to calculate the selectivity index is: $W = p1q2/p2q1$, where $p1$ is the percentage of radio track points occupying a particular habitat type; $p2$ is the percentage of area covered by that particular habitat type; $q1 = (100 - p1)$, and $q2 = (100 - p2)$ (Gabriel 1978).

Selection is defined as the process of choosing resources, in this case habitat, while preference is the likelihood of a resource being chosen if offered on an equal basis with others (Johnson 1980). It is important the time of sampling be taken into account when interpreting the data, as different times within the day will represent different behaviours for the species. Since each point was taken during the day time, and toad movements take place during the night, the point represents the most likely habitats individuals selected as their temporary refuge during the migration process. Using the proportion of radio-tracked points in each habitat type compared to the total amount of radio tracked points, each point is given equal weight. Comparing percent usage by individuals to the proportion of habitat will skew the data, by giving a heavier weight towards animals which were tracked for fewer days (i.e. an animal tracked for only one day, will show 100% usage of the habitat it was found in).

B. Landscape level dispersal and the population genetic structure of Western Leopard Toads in the Overstrand region

i. Microsatellite markers and the study of dispersal in natural populations

Given the geographical scale over which dispersal processes can occur, and that on average only a small percentage of individuals successfully disperse among natural populations, it is often impossible to determine the true pattern of dispersal behaviours by following individual animals alone. Consequently, the chances that any one individual being followed will in fact disperse are very low, and in amphibians because dispersal is generally undertaken by post-metamorphic juveniles, alternative methods to determine dispersal patterns are needed.

With the advancement of techniques from the field of molecular biology, the genetic characterization and study of natural population trends, at both localized and landscape levels, is possible. Population processes studied using molecular approaches include distinguishing current restricted gene flow from past gene flow, and investigating the direction and relative timing of events (such as range expansions), relatedness and parentage, dispersal and migration, inbreeding, and effective population size (N_e) (Avise 1994; Luikart & England 1999; Sannucks 2000)

In this study microsatellite markers were used to study patterns of dispersal among WLT populations. Microsatellite loci are composed of short repeated nucleotide sequences (e.g., [CA]n) generally consisting of two, three or four nucleotides and motifs can be repeated up to 100 times (Queller et al 1993). Microsatellites, compared with allozymes, offer the advantage that, in principle, several thousand potentially polymorphic markers are available, and have mutation rates that range from 10^{-6} to 10^{-2} per generation (Schlotterer 2000). These frequent mutations are predominantly a result of strand slippage during DNA replication, where repeats are either added or deleted but also arise as a result of unequal crossing over at meiosis (Blouin *et al.* 1996). Microsatellites generally occur in non-coding regions of the genome, where random mutations can accumulate more rapidly than in coding regions. Microsatellites are also co-dominantly inherited. The speed at which these microsatellites mutate, combined with the co-dominant trait, make them powerful tools in identifying patterns of allelic divergence between closely related populations (Selkoe & Toonen 2006).

Population connectivity via the processes of dispersal and gene flow is an important aspect in maintaining a healthy, genetically diverse, metapopulation. Population connectivity also helps understand the source-sink dynamics and extinction-recolonization frequencies which affect the maintenance of within-species genetic diversity (Whitlock & Barton 1997). These recolonization processes balance the effect of stochastic events which over time may eventually cause an extinction event at one or more breeding sites. Since microsatellites mutate at such a rapid rate, populations become differentiated over time due to genetic drift. As populations become different, individuals can be identified with respect to their source gene pools. The measure of between population connectivity can be obtained from identifying individuals that do not fit the genetic profile of the population they were captured in. These individuals could either be immigrants or their offspring (Jehle & Arntzen 2002), indicating immigration. Since many amphibians naturally occur in metapopulations and have a relatively short generation turn over time, they are ideal organisms for studying local and regional population structures. Microsatellites appear to be particularly useful for establishing population structure (Jehle & Arntzen 2002).

ii. Laboratory Methods: DNA extraction and microsatellite genotyping

Tissue samples for microsatellite analysis were collected from six breeding sites from previous sampling (collected by G. J. Measey), and were further supplemented by toe clippings taken for mark-recapture purposes at Klein Paradise, as well as toe clippings from the other breeding sites during the breeding period. The collection of toe-clipped samples was carried out with ethics approval from the University of Cape Town (UCT Ethics 2010/V17/SD-R). A standard salt extraction protocol (following Bruford et al 1992) was used to extract high quality, total genomic DNA from the tissue samples.

Ten species-specific microsatellite primers were recently developed for the Western Leopard Toad (Feldheim *et al.* in prep). Primer pairs were initially considered successful if at least one individual displayed positive amplification in PCR. For each primer pair, PCR conditions were then optimised and tested for allelic polymorphisms.

For each polymorphic locus the final PCR reaction volume of 10µl contained: 1X buffer, 0.6pmol/ µl each forward and reverse primers, 0.16µl dNTPs (10µM each) (Promega), 1 unit SuperTherm® Taq (Applied Biosystems) and DNA template (30ng/ µl) 1.0µl. Magnesium chloride ($MgCl_2$) (25mM) volumes and annealing temperatures vary according to the locus, and reagents across a range of conditions were tested in order to produce the best quality PCR product (See Appendix I). PCRs were run at an initial denaturing stage of 94°C for 5:00 minutes, then followed by 40 cycles of 94°C for 0:30, annealing temperature 0:30, 72°C for 0:45, 72° for 5:00. The PCR product was then stored at 4°C until viewed on a 2% agrose gel under UV-light stained with GoldView®.

Once ideal PCR conditions were identified, eight individuals were initially used to assess polymorphism of each locus. Loci were considered polymorphic if at least one individual contained a different allele than other individuals tested. Microsatellite genotyping was carried out at the University of Stellenbosch's Central Analytical Facility. Allele sizes were identified using the program PeakScanner® (Applied Biosystems, US) and sized using 50-300bp size standard (Applied Biosystems). Samples with ambiguous peaks were given an allele size of '0' for that particular locus. Seven loci were used to genotype 113 individuals from the 5 breeding sites. Details of the loci are reported in Appendix I**.**

Data Analysis:

(i) Landscape level population genetic structure across the Overstrand region of the Western Cape, South Africa

To identify the degree to which the sampled locations of WLTs represented unique and differentiated gene pools, the number of individual local 'populations' (*K*) was estimated and graphical models were viewed in the program STRUCTURE v2.3 (Pritchard *et al.* 2000). Using multi-locus genotype data to infer population structure, STRUCTURE implements a Bayesian clustering method to reconstruct ancestry and assign individuals to 'population' gene pools (Pritchard et al 2000). The clustering model assumes that there are *K* number of populations present in the data set, each of which is characterized by a defining set of allele frequencies at each locus. For any given data set the program calculates the probability of *K,* as defined by the user, and is then used to explore the level at which a set of samples may be structured into gene pools across, as in this case, landscapes. The number of genetic clusters present in the dataset, *K*, is inferred based on the estimated probability of *K*, where ln Pr(X|*K*). Individuals in the sample are assigned to single 'populations', or jointly to two or more populations if their genotypes indicate that they are the result of extensive admixture (Pritchard et al 2000).

In this study population structure was explored using *K* = 1-5, for each of the sampled breeding sites, with a burn-in period of 100,000 and run for 100,000 iterations. Pritchard *et al.* (2000) warns that the final value of *K* as estimated from ln Pr(X|*K*) must be carefully interpreted with respect to its biological value, and therefore graphical models (Triangular and Bar Plot) must also be viewed.

Genetic diversity of each unique local population and interpopulation connectivity throughout the region was calculated in the program GenAlEx (Peakall & Smouse 2006). GenAlEx operates under user-defined populations, in this case the STRUCTURE output, and estimates the relationship between them based on allele frequencies. Genetic variation (Fst) was calculated in GenAlEx using a pairwise AMOVA test (Excoffier et al 1992) among populations. An Fst value of over 0.2 generally indicates significant genetic differentiation between populations (Avise 1994). An alternative calculation of genetic distance (Rst) was also calculated. Rst is the equivalent of Fst for loci undergoing stepwise mutation, and may therefore be more appropriate for microsatellite data although this expectation has not always been realised in empirical studies (Slatkin 1995).

Population genetic analyses, like the estimation of Fst, have traditionally been based on the comparison of allele frequencies between population samples. With the development of highly variable genetic markers, like microsatellites, enough information can be collected on an individual organism by where the scale of analysis can be shifted from populations to individuals (for review see Estoup & Angers 1998). This shift in analytical scale allows for genetic analysis to provide data on population ecology quantitatively similar to that obtained with traditional field techniques (e.g. Woods *et al.* 1999). This has prompted the pursuit of genetic methods to study and individual's population of origin (Paetkau *et al.* 1995; Rannala & Mountain 1997; Waser & Strobeck 1998; Cornuet *et al.* 1999; Pritchard *et al.* 2000; Eldridge *et al.* 2001; Guinand *et al.* 2002; Maudet *et al.* 2002; Wilson & Rannala 2003).

Paetkau et al. (1995) demonstrated that genetic analysis can assign individuals to their population origin by using the probability of drawing that individual's microsatellite genotype profile from the allelic distribution observed across a series of study populations. A population assignment test assigns an individual, with a known genotype and known capture location, to a breeding site based on the allele frequency at the breeding site (Peakall & Smouse 2006). Unlike Fst values which measure genetic differentiation between different populations based on allele frequencies, the population assignment test is based on the individual genotypes sampled, and can provide an indication of recent immigration events. The program calculates each individual's expected genotypic frequency from each loci and the log transformed to give a log-likelihood value. An individual is assigned to the population with highest (least negative) likelihood value; the further from the diagonal line points fall the more likely that the sampled individual belongs to that breeding site. If the individual is incorrectly assigned to a breeding site (e.g. sampled in site A but genetically assigned to site B) then it can be inferred that the individual or its parent immigrated from another area (Peakall & Smouse 2006). A pair-wise population assignment test was conducted for each population.

(ii) Sex-Biased Dispersal

Depending on a species' life history strategy, the cost/benefit difference of dispersal is expected to affect sexes differently (Perrin & Mazalov 2000; Smith & Green 2006). This may lead to a sex-biased dispersal pattern, where individuals of the migrating sex disperse into a non-natal area where they breed, while the philopatric sex stays nears their natal site (Palo *et al*. 2004). Greenwood (1980) hypothesises that sex biased dispersal revolves around resource partitioning and if dispersal is solely to avoid inbreeding, there should be no sex-bias seen in dispersal behaviour (Greenwood 1980; Johnson & Gaines 1990). Female-biased dispersal is to be expected when there is some kind of resource partitioning by males where as male-biased dispersal is to be expected when male distribution is determined female distribution (Greenwood 1980).

Greenwood's hypothesis has been supported in regards to amphibians, yet not as readily and uniformly as seen with birds and mammals. In the North American bullfrog *Rana catesbeiana* genetic evidence revealed female-biased dispersal (Austin *et al.* 2003) supporting Greenwood's hypothesis, however, mark-recapture data has previously indicated that male *R. catesbeiana* move larger distances than females (Raney 1940). In general the Bufonide mating system is polygynous (Wells 1977). Western Leopard Toads, unlike most explosive breeders, do not demonstrate male 'scramble competition' (Cherry 1992). Sexual selection is highly likely to be via female choice (Sullivan 1983) where females judge males and are unlikely to breed more than once a year and as there is no resource partitioning by males in polygynous mating systems, one would expect male biased dispersal (Smith & Green 2006). However, Smith & Green (2006) reported an absence of sex biased dispersal in *Bufo fowleri* which is known to be polygynous. The contradictory evidence seen in anurans suggests either Greenwood's hypothesis is wrong, or there are elements of anuran life history that invalidate its application to anurans (Smith & Green 2006).

To determine if adult dispersal in Overstrand WLTs is characterised by a sex bias, sex specific assignment tests were carried out in the program GenAlEx 6 (Peakall & Smouse 2006). For each individual, GenAlEx calculates a log likelihood assignment test value (similar to the population assignment) test. An Assignment Index correction (*AIc*) for each individual is calculated as: Individual (log likelihood – mean log likelihood of the population) (Peakall & Smouse 2006). AIc values will average zero for each population, while negative values will characterize individuals with a higher probability of being immigrants. The genetic signal of sex- biased dispersal is indicated when there is a difference in the frequency distribution of AIc values among males and females (Favre *et al*. 1997; Mossman & Waser 1999). A Kolmogorov-Smirnov Test (Massey Jr 1951) was used to tested using Statistica 10 (StatSoft, Inc), for differences in frequency distribution implemented in GenAlEx.

(iii) Isolation by distance

Having explored the level at which sampled individuals are genetically structured across the sampling locations, testing that structure against a well established theoretical model can provide greater insight into the dispersal patterns of species. In this study, population structure was tested against Wright's isolation by distance model (IBD, Wright 1943). Dispersal distance is one of the primary factors influencing the spatial portioning of genetic variation, particularly in species with low dispersal capabilities such as amphibians. Wright's theory proposes that as geographic distance increases so does the genetic difference between populations, creating a genetic distance gradient that co-varies with geographic distance. One disadvantage this poses is the program STRUCTURE is not well suited to a data set that is characterised by a dispersal scenario that fits the IBD model. This is because individuals which fall into the middle of the gradient may have a 'mixed membership' from multiple groups, making it difficult to accurately determine distinct populations; as a result, a strong positive relationship brings into question the output of the STRUCTURE analysis (Pritchard 2000).

The Isolation by Distance Web Service (Jensen *et al.* 2005) was used to calculate whether isolation by distance mechanism is occurring. A Mantel Test, with 30,000 bootstraps, for matrix correlation was run using a measure of genetic distance (pair-wise Fst) versus geographic distance (calculated from ArcGIS Explorer) (Mantel 1967; Smouse & Long 1992). If genetic and geographic distances are not significantly correlated, then other factors must be involved in shaping the distribution of genetic variation across the sampling locations and therefore the identification of this could play an important role in conservation planning and management as applied to WLTs.

Chapter 3: Results

A. Assessing migration behaviour of the Western Leopard Toad at a local scale

Individual toads were captured and marked at their breeding pond at Klein Paradise Farm over 15 consecutive nights (16 days; July 24-Aug 8 2010) when the males began their chorus and ended after two consecutive nights of no captures, which coincided with the stopping of chorus behaviour (Pers Obs). In total 206 individual animals were captured, with an operation sex ratio of 14.8:1 (males n=193, females n=13). Two distinct periods of immigration (July 24th-July 28th & July 30th-August 7th) into the breeding pond characterized the data collection period, with peaks on July 24th & August 1st (see Fig 3.1). The first period of immigration had fewer total new individuals (previously unmarked, n=87) than the second period (n=119). The first period averaged 10.75 recaptured individuals (standard error: ±1.66) per sample evening and the second period averaging 15.3 (standard error: ±0.77) recaptured individuals per sample evening. The Cormack-Jolly-Seber model suggests an average stop over time of nine nights, five nights minimum and 12 days maximum, at the breeding pond, over the course of 15 nights. Stop over time was not calculated between sexes because none of the 13 females were recaptured during any of the following sampling evenings.

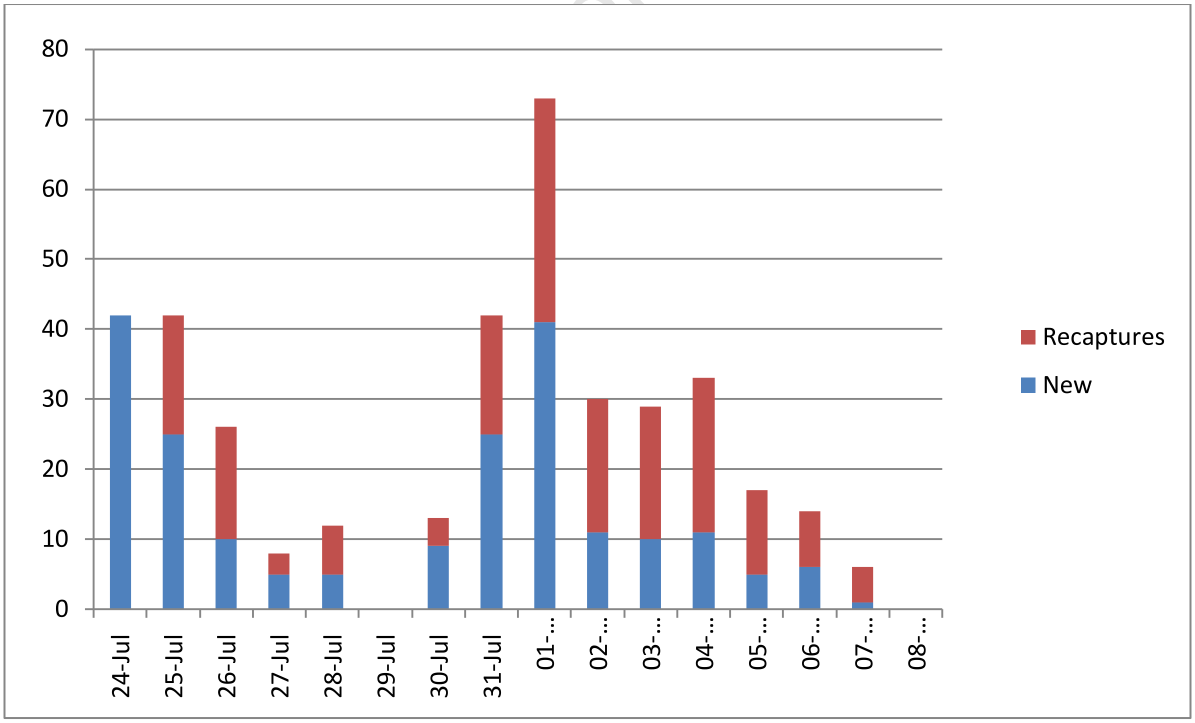


Fig 3.1 Total numbers of individuals captured during each evening sample session at the Klein Paradise breeding pond, with new captures represented by blue and previously marked individuals represented by red.

A total of 16 captured individuals (13 males, three females) were then radio-tracked during the study period. Seven of these individuals were caught during the initial mark-recapture evening sessions and tracked for the full 16 days of the study period. A further six individuals were captured after the first evening capture session and tracked for the remaining days of the study period. Three individuals were captured during the initial capture session and radio tracking ceased early due to two detached radio-transmitters and one due to predation (the half eaten carcass was found with the radio-transmitter attached). After the migration period was deemed to be over, i.e. once calling at the breeding pond had ceased and two consecutive nights of zero captures occurred, the radio transmitters of the remaining animals (those that had not been stationary for five consecutive days) were removed from the individual toads. Three animals did not have radio-transmitters removed because it was not possible to locate them using the radio-antennae.

Table 2: Radio-tracking data, distance travelled, distance from pond, and number of days tracked for all individuals tagged.

Represents those animals that did not travel farther than 30m from the breeding pond and were assumed not to have begun their return migration, with Mean being the recalculated mean excluding these animals

ID (Sex)	Distance travelled (m)	Distance from pond (m)
480 (M)	520	70
461 (F)	1570	1140
617.2 (M)	40	350
401 (M)	22	22
380 (F)	390	370
218 (M)	200	190
939 (M)	660	420
900 (F)	390	160
878 (M)	110	10
839 (M)	340	80
759 (M)	810	620
659 (M)	150	30
699 (M)	470	370
Mean	436	295
Mean*	575	377

The mean distance (n=13 individuals) animals travelled was 436m (standard error: ±103.82) with an average displacement (linear distance from start point to end point) of 295m (standard error: ±51.62) from their point of release (i.e. the breeding pond) (see Table 2). In order to eliminate the biased from the animals with lost transmitters or the one deceased by predation, these individuals were excluded from all calculations. Of the 13 animals, three were consistently found within 30m of the breeding pond. These animals were removed from the sample set and the mean recalculated. The remaining 10 animals travelled a mean distance of 575m (standard error: ±122.99) with a displacement of 377m (standard error: ±100.47) from their point of release (see Fig 3.2). Mean displacement of females (n=3) was 515m (standard error: ±297.9) and 199m (standard error: ±75.21) for males (n=13).

Individual migration away from the pond took place in several steps. Nine individuals made an initial movement out of the breeding pond within the 30m buffer zone adjacent to the pond; since radio-tracking was done during the day it is unknown if these individuals returned to the pond in the evening, as there were no recaptures with radio-transmitters attaches. All individuals remained in within the 30m buffer zone between 1-2 days before making a second, longer distance, movement into terrestrial habitat where two individuals remained for the remainder of the study. Eight individuals made a third movement after a period of 2-7 days remaining idle. In all cases this third movement was shorter than the second.

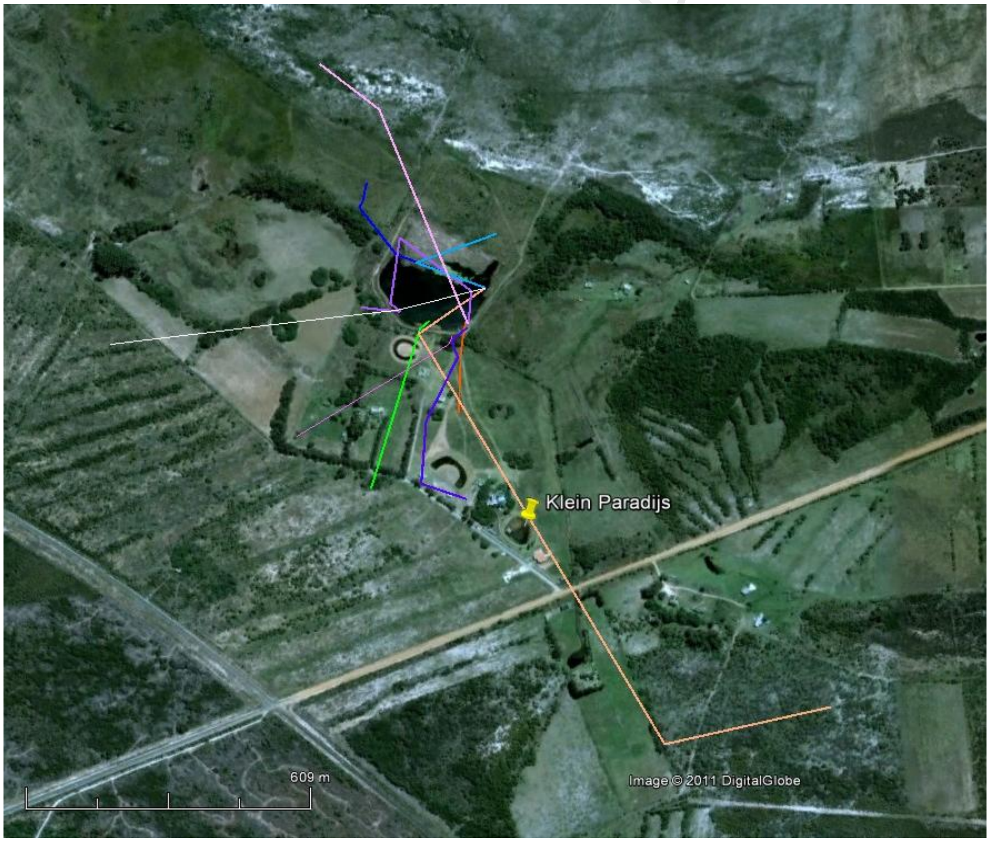
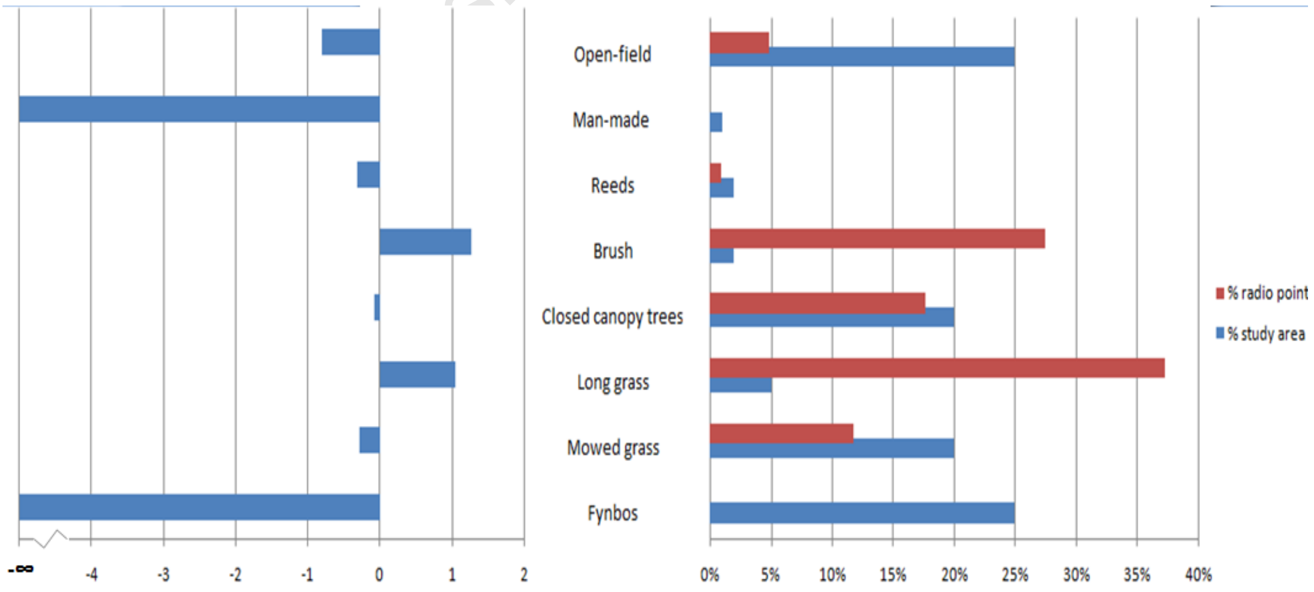


Figure 3.2: Google Earth imagine of radio-tracked paths of migrating animals away from the breeding pond at Klein Paradise in 2010

Because the presence or absence of preferred habitat can hinder migration to and from the breeding pond, the habitat which individuals moved to get to their terrestrial refuge was also assessed. Figure 3.3 shows the proportions of WLT resting points for migrating animals (those which travelled beyond 30m from the breeding pond) found in the different habitat types plotted against the proportion of available habitat, as well as the selectivity indices for each habitat type.

In total 102 radio-tracked points were taken over the course of the radio tracking period. Tall grass recorded the most radio-tracked locations with 38.4%, followed by, brush 20.1%, closed-canopy trees 13.8%, wetland reeds 13.2%, mowed grass 7.6%, man-made 3.8%, open field 3.1%, and fynbos 0%. Out of the 10 animals tracked to what was assumed to be final resting places, four were found in tall grass, two were found in mowed grass and two in brush, and one individual being found in closed-canopy trees and one in open field.

Strong positive selection was reported for long grass (5% of the study site, ~38% radio points) and brush (2% of the study site, ~20% radio points). Negative selectivity was revealed for most of the habitat types present at the site, A Selectivity index of $-\infty$ results from zero radio-points being recorded, regardless of the proportion the habitat type makes up within the study area. This was shown for both man-made and fynbos habitats, and interpretation of this must be taken with care. Fynbos habitat however made up 25% of the initial area surveyed at Klein Paradise, whereas man-made habitat made up only 1%, suggesting that strong negative selection for the fynbos habitat type is likely.



(a) **(b)**

Fig 3.3 Habitat selection by Western Leopard Toads at Klein Paradise. (a) The selectivity indices of WLT for each habitat type. (b) The proportional distribution of resting points during individual WLT migrations, compared to the proportional availability of each habitat type. Negative selectivity values indicate rejection or avoidance and positive values show preferences for a particular substratum (Gabriel 1978).

B. The population genetic structure of Western Leopard Toads in the Overstrand region

Microsatellite markers

A final set of 10 species-specific microsatellite primer pairs were tested and optimised for successful PCR amplification in this study. Optimal conditions were developed for eight of the 10 primer pairs (See Appendix I). Of the eight primers, seven were found to be polymorphic and used in the study. Allelic frequencies for all loci genotyped in this study are reported in Appendix II. A total of 106 unique alleles were identified across the seven loci in 113 individuals. Sample sizes for the individual study sites were Vaalvlei n=10, Stanford n=9, Owl's Nest n=40, Klein Paradys n=41, and Buffeljagsvlei n=13. Klein Paradise and Buffeljagsvlei had individuals genotyped from the 2010 breeding season, as only the Klein Paradise breeding site had breeding activity occurring during that breeding season, and one individual was found crossing the road within proximity of Buffeljagsvlei in 2010. All the other breeding sites had samples collected from previous breeding seasons (2009).

Differences in the number of animals genotyped per locus were generally due to repeatedly failed PCR amplifications. The number of alleles per locus ranged from four (WLT_76) to 20 (WLT_76) with a mean of 15 alleles per locus. Significant departures from Hardy–Weinberg were observed at one locus for the Klein Paradise (WLT_40) and Buffeljagsvlei (WLT_1) breeding sites, and two loci at the Owl's Nest (WLT_40 &WLT_44) breeding site (Appendix III), possibly due to homozygosity in rare alleles for single individuals (Morin *et al.* 2009). Allele frequencies per locus per population are reported in Appendix IV. No consistent patterns in allele frequencies were observed across the loci or populations, therefore all seven loci were used in the remaining analyses. The mean observed and expected heterozygosities across loci were Hexp=0.80 (StDev 0.11) and Hobs=0.79 (StDev 0.08), respectively.

Population structure

Using the Bayesian approach of the program STRUCTURE, three genetically distinct clusters were inferred across the WLT breeding sites sampled in the Overstrand region of the Western Cape (see fig 3.4). Using exhaustive sampling of the data set, the probability of data (L(K)) for the admixture and correlated frequencies model was calculated (Pritchard 2000). The highest L(K) average for each value of K (ranging from 1 to 4), was observed for K = 3.

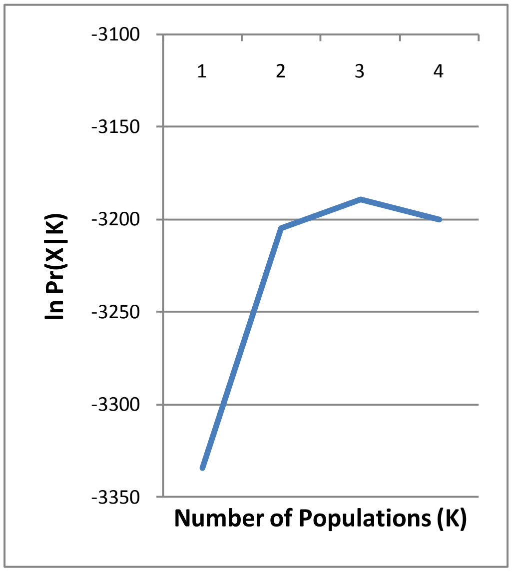


Figure 3.4: Estimated probability of the number of populations in the study area

The graphical outputs (See Appendix V & VI) suggest that Stanford-Vaalvlei & Owl's Nest breeding sites form one genetic cluster, with Klein Paradys and Buffeljagsvlei representing their own genetic cluster. In Population 1 (Stanford, Vaalvlei, & Owls Nest), loci WLT_44 and WLT_40 showed a significant deviation from Hardy-Weinberg equilibrium and in population 2 (Klein Paradise) locus WLT_1 showed a significant deviation from Hardy-Weinberg equilibrium. The fact that deviations are apparent at certain loci and not all of them indicates that selection, opposed to other factors contributing to deviations from Hardy-Weinberg equilibrium (i.e. non-random mating), may be occurring in the population on those particular loci.

Population genetic differentiation

Overall genetic differentiation for the entire region was Fst = 0.069 (P < 0.001). Differentiation between pairs of populations were all quite similar (see Table 3) ranging between Fst = 0.068 and Fst = 0.071. All pair-wise estimates differed significantly from zero (p < 0.001). Rst calculation produced only one significant difference between population 1 (Stan-Vaal-Owls) and population 2 (Klein Paradise) (See Appendix VII).

Table 3 Pair-wise AMOVA: Fst values between the 3 genetically distinct populations in the Overstrand area

	Stan-Vaal-Owls n=59	Klein Paradise n=41	Buffeljagsvlei n=13
Stan-Vaal-Owls		0.001	0.001
Klein Paradise	0.069		0.001
Buffeljagsvlei	0.068	0.071	

*Fst values below the diagonal, p-values above the diagonal

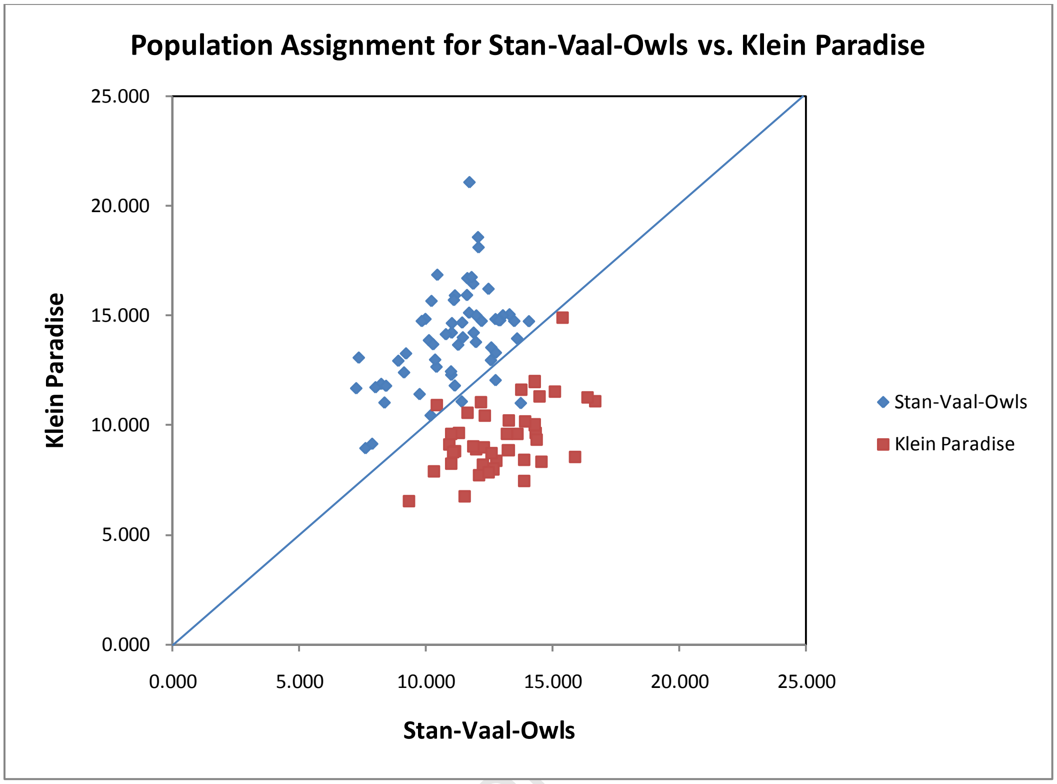
In total Population 1 (Stan-Vaal-Owls) 91 alleles of which 31 were private, Population 2 (Klein Paradise) had 65 alleles of which 10 are private, and Population 3 (Buffeljagsvlei) had 47 alleles of which 2 are private. Of the three genetic clusters, the Stan-Vaal-Owls population was the most genetically diverse across all measures of diversity (see Table 4). Population one averaged a higher number of effective alleles (Ne) which enables meaningful comparisons of allelic diversity, across loci with diverse allele frequency distributions. The measure of the number of effective alleles provides an estimate of the number of equally frequent alleles in an ideal population with homozygosity equivalent to the actual population (Peakall & Smouse 2006). Population 1 also had a higher expected herterozygosity than the other two populations, indicating this population is the most genetically diverse.

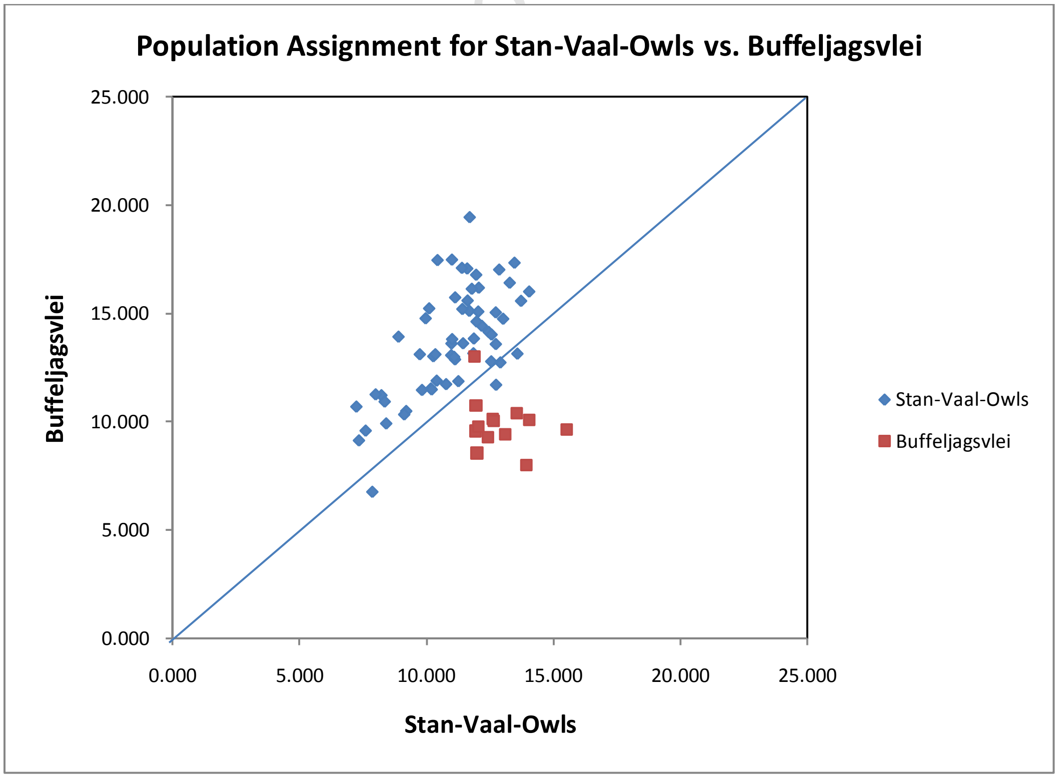
Table 4: Alleic patterns across populations

Population	**Stan-Vaal-Owls**	**Klein Paradise**	**Buffeljagsvlei**
N	59	41	13
Na	13.286	9.286	6.714
Na Freq. >= 5%	6.286	5.000	5.429
Ne	6.878	4.704	4.804
No. Private Alleles	4.429	1.429	0.286
He	0.820	0.778	0.772

N – sample size, Na- Mean no. alleles, Na Freq. >=%5 – Mean no. of alleles with a frequency greater or equal to 5%, Ne- Mean no. of effective alleles, No. Private Alleles, He – Mean Expected Heterozygosity

Results from the population assignment test revealed that ~91% of the animals could be assigned to the population they were sampled in. Pair-wise population assignment test revealed three individuals with genetic signatures from the Stan-Vaal-Owls found in Klein Paradise and four in Buffeljagsvlei. One individual with a genetic signature from Klein Paradise population and one individual from the Buffeljagsvlei population was found in the Stan-Vaal-Owls population, and one individual with a genetic signature from Klein Paradise was found in Buffeljagsvlei (see Fig. 3.5).





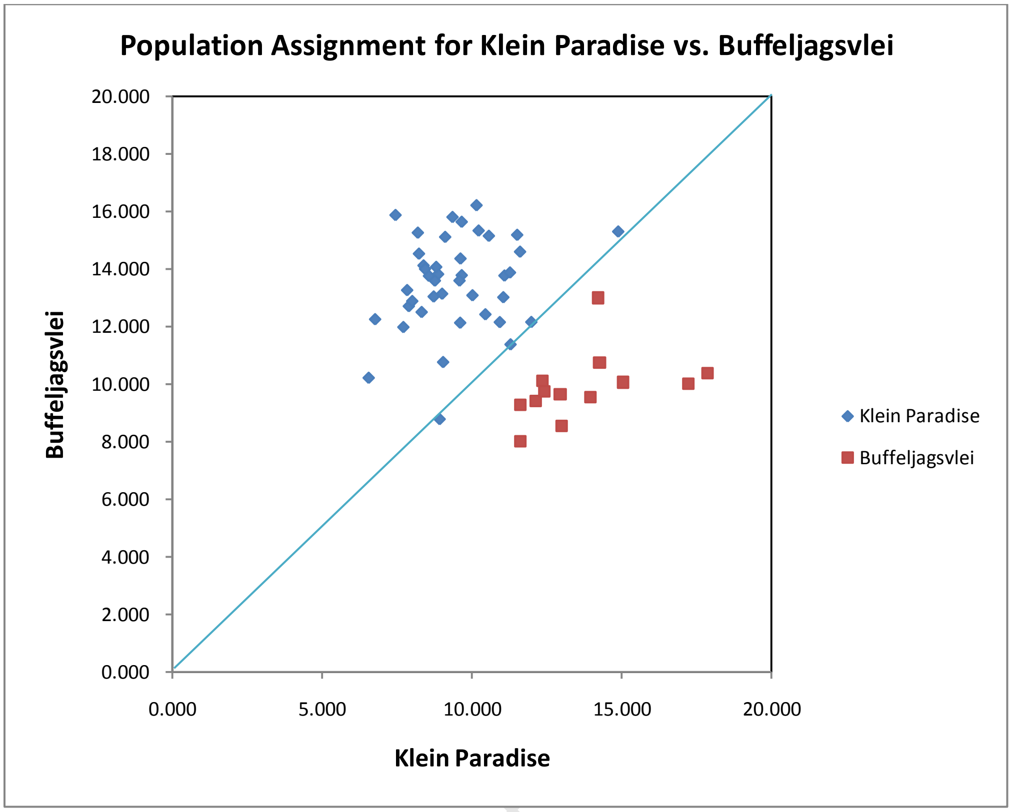


Fig 3.5: Pair-wise population assignment tests indicating recent migrations between three identified genetic clusters (Stan-Vaal-Owls, Klein Paradise, and Buffeljagsvlei) in the Overstrand region.

Sex-Biased Dispersal

Of the total number of animals genotyped, 102 were of known gender; the remaining samples were either sampled as tadpoles or the gender was unclear. Due to the strict requirements of the sex-biased dispersal analysis package implemented in Genalex, individuals with gaps (missing data from one or more loci) in their genotype profiles could not be included in the analysis, and therefore the final analysis was based on only 81 animals (62 males and 19 females). Although females did have a slightly lower mean assignment index (-0.456) than males (-0.263), there was no significant difference ($p>0.10$) between the two sexes.

Isolation by distance

The correlation between genetic distance (as measured by Fst) and geographical distance was evaluated using a Mantel test with 30,000 permutations. The Mantel Test did not support a pattern of isolation by distance (Z = 3.45, p= 0.46), indicating that there is no correlation between genetic and geographical distances in WLTs in the Overstrand study area.

Chapter 4: Discussion

Many species of amphibians appear vulnerable to both the loss and fragmentation of nonbreeding habitat (Cushman 2006). The extent, pattern and quality of terrestrial habitat in landscape mosaics have shown to play significant roles in localised and landscape level movement patterns in pond breeding amphibians (Cushman 2006; Semlistch 2008). The Western Leopard Toad has had much of its distribution experience anthropogenic habitat change, and as a result has seen it listed as endangered of extinction. Investigation into the effects of agricultural habitat change on the Western Leopard Toad was carried out a both local and landscape levels, as movement towards water resources during the breeding season is critical to the persistence of local populations and connectivity of local populations across a landscape is key in species survival across its distribution.

Analysis of radio-tracking and mark-recapture data over a 16 day period provided valuable insight into short term migration patterns of the Western Leopard Toad, and fine scale habitat use in an agricultural influenced landscape. Microsatellite analysis revealed metapopulation structure with interconnected populations, in a highly fragmented landscape. Knowledge of habitat uses of a species is essential for effective conservation management, to ensure the persistence of a species through future generations.

Arrival and stop-over: length and duration of Western Leopard Toad migration

Individual population survival is key in maintaining a species' continued existence across a landscape. In pond-breeding amphibians an individual population revolves around its breeding pond (or a group of closely situation ponds) (Marsh *et al.* 1999; Semlitsch 2008). The short term fitness of any individual population will rely on the successful, regular reproduction. For pond-breeding amphibians, this success requires the migration from terrestrial refuges inhabited during the non-breeding portion of the year, to the breeding pond (Sinsch 1990). The terrestrial habitat surrounding the breeding pond is important in facilitating breeding migration and the survival of post-metamorphic animals during the non-breeding season; and is therefore important to the long term survival of population.

In this study Western Leopard Toads showed typical explosive breeding behaviour (Cherry 1992), where an increased activity of arrival to the breeding pond began on the first evening of the study period, followed by a sharp decrease in arrival activity in the consecutive evenings, for a period of 7 days. Following the first period of arrival activity there was a second phase of arrivals to the breeding pond which was also followed by a sharp decline in arrival over the consecutive nights, a period of 8 days. Capture numbers began rising a few days before the August 1st peak suggesting that animals may have arrived before the July 24th peak, when the study began. This may have skewed the calculation of stop-over as animals arriving before the study period, July 24th, would have had their capture histories started on July 24th, or later.

Asynchronous patterns of arrival in amphibians have been reported to result in a male-biased operational sex-ratio (the ratio of actively mating males and females) and increased competitive interactions in the breeding season (Lode *et al*. 2005). Cherry (1992) reported operational sex-ratios for Western Leopard Toads, over the course of three breeding seasons, fluctuating between 2.4:1 and 6.9:1 at Sun Valley, in the Cape Peninsula. At Klein Paradise in 2010 the operational sex-ratio was 14.8:1 (M=193; F=13), which is high, even in Bufonids where sex-ratios are typically male biased (See table 3 in Zug & Zug 1979; Gittins 1983; Loman & Madsen 2010). Although the number of females captured could have been the result of human error in search and capture methods, it is unlikely that this error would explain such a large disparity as observed during the 2010 breeding season, compared to that seen in Cherry (1992). A possible explanation for the disproportionate number of males to females could be the differential responses to environmental cues that stimulate the migration to the breeding site. Alternatively, unaccounted for females were present at the breeding pond prior to the study period or arrived after the study period finished.

Environmental and meteorological factors have long been assumed to be important as attendance triggers for anurans (Blankenhorn 1972; Obert 1975; Alderton 1985; Woolbright 1985; Henzi *et al* 1995). It has also been proposed that operational sex ratios are interdependent on the presence of the other sex. Males have been known to restrict their calling activity to occur only when numerous females are present and that females preferentially attend large choruses, increasing the chance for reproductive success in males and allowing for selection in females (Fellers 1979; Bradbury 1981; Godwin & Roble 1983; Krebs & Davies 1984; Green 1990). This argument however would require that a mechanism by which numbers of opposite sex can be assessed (Fellers 1979; Green 1990). For females, this could logically be associated with the intensity of male calling. Males, however, would have to have a way of predicting female attendance, because chorusing begins before the arrival of females (Henzi *et al.* 1995). Little is known about which abiotic factors act as environmental triggers for pond-breeding amphibians; however, onset and duration of breeding activity of several anuran species have been associated with the rainy season (Aichinger 1987; Gascon 1991; Donelly & Guyer 1994; Vaira 2005).

The presence of two peak activities in arrivals suggests there were two triggers during the breeding season. Environment cues such as amount of rain fall (Bragg 1945), rate of rainfall (Bragg & Smith 1942), temperature (Mayhew 1962; Zweifel 1968) and lunar cycle (FitzGerald & Bider 1974) have all been shown to trigger amphibian breeding. The number of recaptured individuals compared to the number of new individuals during the second peak suggests that these environmental cues may have caused some individuals to return to the breeding pond in an attempt to increase their chances of reproductive success. Although sequential polyandry has only been reported in a few anuran species, its widespread taxonomic and geographic distribution suggests it may be common and may prove beneficial in terms of reproductive fitness (Byrne & Roberts 2011). Roberts & Byrne (2011) found that offspring of Grey Foam Nest Tree frog, *Chiromantis xerampelina,* from polyandrous matings had both significantly higher mean survival and reduced variance in offspring survival. Further studies into WLT polygamy could provide opportunities for research significantly contributing to the understanding anuran evolution and even the development of sexual-selection and life-history theory (Byrne & Roberts 2011).

Migration takes place in two parts. In respect to amphibians the first portion of migration is the movement from terrestrial refuge to the aquatic breeding habitat. The second portion is the returning from the aquatic breeding habitat to the terrestrial habitat which the animal will reside for the non-breeding portion of the year. The period of duration at the breeding site and departure time from the breeding site based on recapture data showed a distinct difference in the behaviour of males and females. No females were recaptured during any sample evening. The most likely explanation for this is that females can only reproduce once in any breeding season; after eggs are deposited the female will begin the return portion of migration. Assuming this to be the case, and not a product of sampling error, the stop-over calculation from recapture data would more apply to that of males rather than females. Stop-over calculation suggests that animals remained in the breeding pond for an average of nine nights, five nights minimum and 12 days maximum. There are two possible explanations to describe the males behaviour in time spent at the breeding pond. Either the males behave like females and leave after breeding has taken place or the more likely scenario, based on the number of recaptures during the second arrival peak during the study, that males remain after breeding events and continue to stay in order to reproduce on more than one occasion in a breeding season.

The process of migration and reproduction are heavily taxing on energy requirements. Theoretically this would suggest that larger animals would be advantaged to complete the migration and successful reproduction. With respects to males, this would enable them to remain in the breeding pond longer increasing their chance of one or more successful reproductions, if polygamy is characteristic of WLT breeding. If polygamy is not characteristic of WLT reproduction, then larger males which can outcompete smaller males and would be expected to leave the breeding pond earlier. Further investigation into size demographics of males in relation to stop-over would help indicate to the nature male WLT breeding behaviour.

Migration distance and core habitat area use by Western Leopard Toads

The post-breeding migration to terrestrial refuges of mature Western Leopard Toads at Klein Paradise was comparable to that of other migration studies on amphibians. In a review by Semlitsch & Bodie (2003) a range of average migration distances of 142-289m from pond edge was reported over a broad range of phylogenetic lineages. The higher than mean distance (377m, se: ±100.47) observed in WLT in this study, compared to those presented by Semlitsch & Bodie (2003), may be attributed to the fact that large toads are much more mobile, especially being relatively large even in comparison to other anurans. In respect to other similarly large Bufonids, Bartelt *et al.* (2004) has reported a maximum post-breeding migration distance of 2440m in *Anaxyrus* (*Bufo*) *boreas* and Leblois *et al.* (2000) has reported a maximum nightly movement of 1300m in *Rhinella* (*Bufo*) *marinus*, a same maximum distance recorded during the study, by one radio-tracked WLT individual occurring over one night.

Distance moved by individuals helps to understand the total extent of the core habitat used by a species and not just the breeding location, which has been the traditional focus of amphibian conservation efforts (Sztatecsny & Schabetsberger 2005). Using data from 11 species, Rittenhouse & Semlitsch (2007) estimate that, on average, a core habitat extending 93m from the breeding pond supports 50% of the population, 664m from the breeding pond supports 95% of the population, and 852m from the breeding pond supports 99% of the population. Species with greater mobility, such as large toads, averaged 703m of core habitat extending from the breeding pond to support 95% of the population. The low mean, compared to Rittenhouse & Semlistch (2007) reporting for large toads, of the average post-breeding migration distance, in relation to core habitat area, seen in WLT at Klein Paradise could be attributed to the low sample size, particularly that of female numbers. In some amphibian species females have been shown to move greater distances than males (Semlitsch 2008), in theory because they are larger and therefore more mobile. This was observed in 2010 for WLT as the distance travelled by females was 515m (se: ±297.9) and 199m (se: ±75.21) for males. A sample size more representative of the population, of males and females, would give a better indication of core habitat for WLT around Klein Paradise.

A further consideration in regards to migration distance and core habitat for WLT around Klein Paradise could be a limitation on the data due to time frame of study. Radio-tracking was stopped after a 16 days (when captures at the breeding pond ceased) and even though breeding activity was assumed to have ceased, male toads may have remain close to the pond within auditory range, which was indicated during the sampling session through a high number of recaptures. A longer time frame of radio-tracking data would give a better indication of post breeding migration distances and core habitat around the breeding pond.

Habitat use: choice or no choice?

The availability of suitable habitat for migration can be an important factor in determining success during migration (Patrick et al. 2008). Breeding sites lacking connectivity to suitable terrestrial habitat may be population sinks due to high mortality of juveniles during emigration (Rothermel 2004). The post breeding migration movements of the Western Leopard Toads saw a preference of long un-mowed grass and thick brush piles as day time refuges in between movement events. Habitat selection comes with varying degrees of trade-offs, for example resistance of movement greater in dense vegetation, however risk of predation would be lowered (Rittenhouse *et al.* 2009; Blomquist & Hunter Jr. 2009).

First, WLT may have used the path of least resistance for long distance travels, assuming that a dense understory makes movement harder for migrating animals (Patrick *et al.* 2006), and therefore have chosen these open canopy habitats for long distance movements. This behaviour has been observed in other anurans, such as *Lithobathes clamitans* (Birchfield & Deters 2005) utilizing short grasses on a golf course allowing for fast movement. In one radio-tracked WLT individual, its day time terrestrial refuge was found in a burrow next to a dense bush, in an open-field after a long distance movement event. Use of such vegetated refuges may be a common strategy to avoid predation among anurans that use open-canopy habitats. For example, the Western Toad (*Bufo boreas*) used open-canopy habitats only when adequate ground cover was available (Bartelt *et al.* 2004)

Cover is an important variable in the risk perception of anurans (Blomquist & Hunter Jr. 2009), and WLT may have resorted to day time refuges in more dense areas (long grass and brush piles) because of reduced predation risk from diurnal predators (such as birds). WLT may have chosen to make night time movements more in open-canopy habitats because they lack dense understory that provides cover from ground predators, such as mammals and reptiles which are mainly active at night, and then returning to dense vegetation during the day.

Canopy cover and understory density also may be important during migration for thermo- and hydro-regulation. Amphibian migrations are often timed to occur with rain events or optimal temperatures (Todd & Winne 2006), and long distance movements through open canopy occurring at night time would prevent direct exposure to the sun. This preference for vegetation with dense cover was further supported by WLT using the wetland reeds, within the 30m 'buffer zone' from the breeding pond, as terrestrial refuge prior to the second phase of migration. Wetland reeds provide a dense understory for cover, the necessary moisture for hydro-regulation, as well as an abundance of insect life that would support nutritional needs. Since physical components of vegetation such as density, cover, etc have been shown to be important factors in amphibian migration, vegetation structure is likely to be more important than habitat/vegetation type. This aspect was not taken into consideration in this and investigation on WLT movements in relation to vegetation structure could provide value insight into migration of this species. However, the data complied did provide certain valuable aspects in habitat use during WLT migration.

Interestingly, in this study natural veld was negatively selected for (i.e avoided), with none of the radio tracked individuals utilizing this habitat despite being one of the most readily available habitats in terms of percentage of area covered (25%). Results from the selectively index, as with migration distances and core habitats, need to be interpreted with caution. The limitations on the data by time the time frame of study could give a misrepresentation into habitat selectivity. However, as discussed with respect to vegetative density and movement, natural veld would not be used as a thoroughfare. Negative selection for natural veld may also be a consequence of the physical positioning of the natural veld area being uphill from the breeding pond at the study site, as active vertical migration in adult amphibians is rare (Sztatecsny & Schabetsberger 2005). In order to fully determine if WLT actively chose other habitats over natural veld, a study with a field site containing lower elevation natural veld would be needed.

The results obtained from radio-tracking at Klein Paradise show that WLT are utilizing disturbed habitats for movement and terrestrial refuge, and they are not reliant on natural fynbos habitat. Bufonids often utilize naturally disturbed habitats, where disrupted soil is easier to burrow in and in turn stimulates the movement of invertebrate prey upon which they feed (Sullivan 1995). Amphibian species which utilize disturbed habitats are habitat generalists (Kolozsvary & Swihart 1999), which appear to be better able to survive in urban and suburban landscapes (Hamer & McDonnell 2008). This would appear to be the case with WLT, as it has shown the ability to utilize both urban and rural habitat changes, and in terms of conservation, habitat generalists pose a much simpler challenge than specialists (McKinney 2002). Despite showing a tolerance to both rural and urban habitat change, WLT populations have been documented to go extinct in their Eastern distribution and the species is label as endangered primarily due habitat change.

The selection for disturbed habitats combined with the avoidance of fynbos habitat may also reflect on the management approaches used in areas of fynbos. All ecosystems are naturally subject to disturbances, and fynbos ecosystems rely on natural occurring fires (with a fire return interval of 4-45 years; Van Wilgen *et al*. 1992), and WLT avoidance of natural veld may suggest that the fynbos system in the area does not have enough natural disturbance. Human influenced fire suppression is common, and in order to re-establish WLT to fynbos habitat, fire management plans may be necessary to restore the fynbos habitat to a more natural system.

Population genetic connectivity across the Overstrand Western Leopard Toad metapopulation

The survival of a species relies on the interpopulation dynamics across a broad scale (Hanski 1998). The connection of individual populations ensures healthy genetic variability and recovery from stochastic population degeneration. The ability for individual animals to move across landscapes is vital, and as with fine scale migration movement, habitat can play an influential role in facilitating the dispersal process.

In this study three genetically distinct Western Leopard Toad clusters were identified across the landscape level spatial scale, from five previously identified breeding sites included in genetic analysis. One genetic cluster contained three breeding sites, Stanford, Vaalvlei, & Owl's Nest (Stan-Vaal-Owls), spanned a range of 13.75km. This geographical range also includes the two other unanalyzed breeding sites. Semlitsch (2008) reports that the use of multiple ponds in a small area has been described in annual breeding migration of pond breeding amphibians, suggesting a cluster of ponds can constitute a single breeding unit that is genetically distinct. The results observed in the Stan-Vaal-Owls population, would therefore suggest that the Klein Paradise breeding site would form part of the Stan-Vaal-Owls population given that the geographical distance between Klein Paradise and Owl's Nest breeding sites is shorter (11.5km) than the maximum distance between breeding sites in the Stan-Vaal-Owls population.

Since this is not the case something else other than geographical distance must be driving the separation of the three genetic clusters in Overstrand.

The result from the population assignment test shows interchange between all three genetically distinct clusters with distance not appearing to be an absolute hindrance for dispersal for WLTs in the Overstrand area. Gene flow exists between the Stan-Vaal-Owls population and Klein Paradise. More interesting is the similar level of gene flow between Buffeljagsvlei and both Klein Paradise and Stan-Vaal-Owls (with Klein Paradise having a slightly higher level), despite Buffeljagsvlei being twice as far from the Stan-Vaal-Owls population as Klein Paradise. The similarity seen between interpopulation gene flow, despite geographical distance, can probably be attributed to the fact that the sample sizes from each of the far ranging breeding sites, Buffeljagsvlei, Stanford, & Vaalvlei, were small in comparison to that of Klein Paradise and Owls Nest, and therefore the results of an accurate measure of gene flow must be interpreted with caution (Pritchard *et al.* 2000). The evidence, however, from gene flow calculations together with recent migrations (from the assignment tests) suggests that dispersing individuals move directly from Buffeljagsvlei to the Stan-Vaal-Owls population. Nonetheless geographical distance cannot be completely ruled out as a part player, in spite of negative isolation by distance tests, given that the results observed may have been influenced to some degree by sample size and that the geographical distance between the Cape Town area and the Overstrand region has produced two completely disjunct populations (Measey & Tolley 2011). Although results should be interpreted with caution, recent movement between all the breeding sites has occurred, a distance of 26km (Stan-Vaal-Owls to Buffeljagsvlei), meaning WLT is capable of long distance dispersal and that the Overstrand landscape does facilitate some degree interpopulation connectivity.

Evidence from empirical studies has largely shown that a species' mobility is matrix-dependent, at both a localized level and landscape level affecting distribution, density, and genetic structuring (Fahrig *et al.* 1995; Carr & Fahrig 2001; Joly *et al.* 2001; Vos *et al.* 2001; Rothermel & Semlitsch 2002; Lampert *et al.* 2003; Stevens *et al.* 2004; Funk *et al.* 2005; Spear *et al.* 2005). The general pattern shows that a species' mobility is enhanced by the presence of preferred habitat types (Stevens *et al.* 2006).The results from radio-tracking at Klein Paradise show that the WLT utilizes disturbed habitats for movement. Since the Overstrand area is largely influenced and disturbed by agricultural practices, it would seem that there is enough appropriate habitat to allow for movement throughout the region.

If geographical distance and habitat availability are not the main drivers shaping population genetic divergence among Overstrand WLT populations then some other factors within the landscape must be shaping the metapopulation structure. The Overstrand area is composed of lowland hills and this may present a challenge to unhindered movement by WLT. If WLT is showing avoidance towards elevational inclines this may in fact play a major role not just in the local migrations, but the landscape level movements as well. The presence of hills across the Overstrand landscape between breeding sites would force toads to move around the hills instead of over, despite being physically able to do as seen in the Cape Town area (Pers. Comm. G J Measey). This added distance of going around as opposed to over may in fact create a larger than necessary distance deterring WLT individuals from dispersing to alternative breeding sites (provided appropriate habitat is available), and actually funnel them in one direction, which in this case, may be the already existing breeding populations available.

Alternatively, the genetic differentiation seen amongst WLT populations in the Overstrand could be attributed to a declining number of toads present at each population. Populations of most species which have declined in spatial distribution and number may also be subject to declines in levels of genetic diversity at a localised population which would in turn create a larger genetic difference between population units within the metapopulation (Scribner 2001). Since genetic differentiation is being seen, even in the presence of connectivity, between WLT populations in Overstrand, it is likely that WLT numbers in each population are already low enough to start creating more genetically unique clusters, as fewer individuals would mean fewer dispersing individuals. Although declining toad numbers at each population seems to be supported in terms of habitat change, small numbers at the breeding ponds could be a construct of the time in which breeding sites were developed. Since most breeding sites in the Overstrand area are developed through agricultural development, they would be relatively new in term of availability for the toads in the area. This could be a factor for toad numbers, as not enough time may have passed since colonization for large numbers of toads to be present.

The management of WLT: facilitating the processes of localized migration and dispersal

Ideally, potentially detrimental land use activities (such as lawn mowing) should be refrained from anywhere the Western Leopard Toad exists, however, it is important that certain levels of habitat disturbances take place as WLT seems to favour this. At a localized level it is important that an appropriate core habitat size is available by the persistence of a localized population, but also contains all the habitat requirements, such as cover, movement corridors, etc. Although a general picture of habitat selection for post breeding adult Western Leopard Toads was observed at Klein Paradise, studies into microhabitat (see Bartelt 2004) conditions and vegetation structure should be done in order to paint a better picture of true selection. Even so, the results from this study show clearly that toads need

(i) areas of dense reeds near and around their breeding ponds in order to adequately protect themselves from predators, (ii) open habitat which allow for long range movements, and (iii) dense brush for their terrestrial refuge.

Although, small-scale active habitat management may be appropriate for species of concern in some situations, it would be unwise to extrapolate to large-scale habitat management, such as clear cutting for long range movement, for such species. For local level management, the priority is ensuring that migration from terrestrial refuges to breeding sites is possible. Since the WLT has shown to use disturbed, open-habitat, for long range movements, the creation of cleared paths through dense vegetation from the breeding ponds could channel toads to ideal terrestrial refuges. This would mimic natural disturbances such as large animals creating game trails from terrestrial habitat to water sources. Furthermore, channelling toads towards ideal terrestrial refuges would also direct toads away from unfavourable areas that are likely to lead to mortality (i.e. roads).

At a landscape level the most immediate concern for the Western Leopard Toad, as with most amphibians, is the development of more suitable wetlands to be used as breeding sites (Semlitsch & Bodie 1998). Given that all populations within a metapopulation system have a finite life span, a stochastic event causing a current loss of one population could be drastic to the metapopulation dynamics. Therefore the development of further breeding areas, to establish new populations, would be crucial for maintaining a functioning and diverse metapopulation. Currently the decreasing number of breeding sites in the Overstrand region can be attributed to two things. First, historical breeding sites have been built over or built around by anthropogenic structures, either completely obliterating the site or producing run-offs which change the composition of the water in the site, making it inhabitable for the aquatic stage of the amphibian life cycle. Second the presence of alien vegetation in the area has increased the uptake of ground water which would normally be present for ephemeral pond systems. Although alien vegetation clearing may transform the area to allow ephemeral pond systems to return, this may not be sufficient in order to preserve the WLT in the area. Since privately used dams appear to be sufficient in choice for WLT, and most private farms have some sort of private dam, then one would expect that are many available bodies of water for breeding sites. Since only seven active breeding sites have been documented in the Overstrand area, either these unused water bodies are unsuitable for WLT breeding or WLT have strong site fidelity. In order to assess whether the unused dams are unsuitable, further studies on what characteristics determine suitable breeding sites, and using these guidelines as recommendations for landowners. Assuming that these unused dams are suitable for WLT breeding, then high site fidelity could be an hindrance for dispersal and colonisation. Finding a way to encourage those few dispersing individuals to find and utilize these sources is key. In this, establishing a rescue and recolonisation dynamics to maintain a regional population must be assessed within the constraints of the entire landscape. Although breeding habitat may be of "primary importance to long term population survival" (Harrison & Fahrig 1995), other habitats may be critical if individuals are to survive to reproductive maturity.

General Conclusions

Anthropogenic habitat change has been identified as the leading cause of global biodiversity loss (Pimm *et al*. 1995). Habitat change directly impacts the localized area in which the change occurs as well as a broader scale, fragmenting habitat across a landscape and isolating populations. The current extinction crisis has led for the increased need for species specific investigations on the impact of habitat change, in order to develop appropriate conservation strategies. Amphibians are an ideal taxonomic group for this as they are of global concern due to reported declines (Wake & Morowitz 1991; Blaustein *et al.* 1994) and extinctions (Pounds & Crump 1994). Their natural history further makes them ideal candidates as they are affected by both aquatic and terrestrial dynamics and because they may constitute the greatest biomass among vertebrates in some ecosystems (Burton & Likens 1975).

The results from this study have shown that pristine habitats are not required for the survival of the Western Leopard Toad, which is positive news given much of their remaining distribution has been changed by anthropogenic activity. Nevertheless despite their resilience to anthropogenic habitat change, the Western Leopard Toad's overall distribution has decreased, suggesting that further breeding ponds are needed to maintain the species across its remaining geographical distribution as well as promoting recolonisation into historical distribution. Given that WLT do use man-made dams as breeding sites, one would expect that with the increasing number of dams in the agricultural landscape, potential breeding sites would also increase. This has shown not to be the case as no new breeding sites in the Overstrand area have been identified. In fact, with only one previously identified breeding site out of seven having breeding activity in the 2010 breeding season, the trend may be gearing toward fewer suitable breeding areas and potential extinction within WLT's Eastern distribution. Despite the constraints of a low sample size, the results from Klein Paradise in 2010 have given a preview into WLT's patterns in terrestrial habitat use. Further investigation into fine scale terrestrial habitat requirements as well as aquatic habitat requirements could provide essential into developing the existing agricultural dams into suitable areas for maintain WLT subpopulation.

The results from genetic analysis across the Overstand landscape show that geographical distance for dispersal in WLT is far, making colonisation of newly created breeding areas possible. The creation of new suitable habitat and restoration of former suitable habitat is importance to the survival of this species, as well as a host of other amphibian species which inhabit the area. As the number of suitable breeding sites along with areas of suitable terrestrial habitat increases, the dispersal abilities of WLT could eventually lead to colonisation of new subpopulations creating a healthier and more diverse metapoplation over the landscape.

This study further emphasizes the value of developing new methods and advanced statistical models, such as molecular techniques (Quellar 1993; Selkoe & Toonen 2006), to compliment more traditional methods such as mark-recapture and radio-tracking (Aebischer *et al.* 1993; Semlitsch 1998). Through analysis similar to those presented in this study, an understanding of the biological consequences of habitat change can be understood at a localized and landscape level. Hopefully, this study will stimulate further efforts into research of how habitat change affects a species at both local and landscape level spatial scales, with priorities on conservation implication and the development of management techniques which involve habitat restoration.

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Appendix

Appendix I: Details of each of the 7 polymorphic loci used in analysis

Primer	Primer Sequence	Primer Label	Fragment Size	PCR Annealing Temperature	PCR $MgCl_2$ Concentration
WLT_1	F: TGA ATT CCG CTA TTT TTC GTT R: ATC CGG TGA GTA GCC ATG AA	HEX	173 base pairs	55-57°C	1.5-1.75mM
WLT_25	F: CAG CGT GTC CGT ATG ATA GGT R: GCC CTC CCA TAT CTA TCT GTCT	FAM	168 base pairs	55-58°C	0.75-1.25mM
WLT_36	F: CCT GAA TGG GAT TAG TCA ACT G R: GAA CTC GGA CAA CCC CTT TA	HEX	252 base pairs	53-55°C	1.0-1.5mM
WLT_40	F: TTG TGT GCT CTG TGC TCT CC R: AGG ACC GCA CGT TAC AGT TT	FAM	173 base pairs	46-48°C	1.25-1.5mM
WLT_44	F: ACC CAT TTT TCG CAG TCA AG R: TGG AGG AGG ATA ACC TGG AA	HEX	239 base pairs	55-56°C	1.0-1.25mM
WLT_76	F: CCC CTT ATC ATA TAT ACA GCA CCA R: GGA GCT TAA CTG CCT GGA TG	FAM	186 base pairs	60-61°C	1.5-2.0mM
WLT_88	F: AGT GCA AAA CAC CCC AAG AC R: GGG CTC CTT TAC CCC AAT TA	FAM	214 base pairs	54°C	1.0-1.25mM

Appendix II: Details of each locus with respect to each breeding site

Pop	Locus	N	Na	Ne	I	Ho	He	F
Buffeljagsvlei	**WLT_1**	13	8.000	5.541	1.875	1.000	0.820	-0.220
	WLT_36	13	9.000	6.036	1.981	0.923	0.834	-0.106
	WLT_40	13	10.000	7.860	2.162	0.769	0.873	0.119
	WLT_88	13	8.000	5.633	1.862	0.923	0.822	-0.122
	WLT_25	13	6.000	4.122	1.573	0.692	0.757	0.086
	WLT_44	13	9.000	6.377	2.005	0.846	0.843	-0.004
	WLT_76	10	4.000	3.509	1.305	0.800	0.715	-0.119
Klein Paradise	**WLT_1**	41	14.000	8.260	2.346	0.805	0.879	0.084
	WLT_36	41	15.000	8.180	2.332	0.878	0.878	0.000
	WLT_40	40	14.000	8.579	2.361	0.900	0.883	-0.019
	WLT_88	41	13.000	6.320	2.060	0.805	0.842	0.044
	WLT_25	41	12.000	7.373	2.151	0.780	0.864	0.097
	WLT_44	41	18.000	6.975	2.354	0.805	0.857	0.060
	WLT_76	41	4.000	2.988	1.218	0.585	0.665	0.120
Owl's Nest	**WLT_1**	40	13.000	6.612	2.177	0.800	0.849	0.057
	WLT_36	40	15.000	8.163	2.337	0.725	0.878	0.174
	WLT_40	38	14.000	9.531	2.392	0.658	0.895	0.265
	WLT_88	39	12.000	5.200	1.902	0.897	0.808	-0.111
	WLT_25	24	12.000	8.794	2.287	0.958	0.886	-0.081
	WLT_44	37	16.000	6.071	2.221	0.676	0.835	0.191
	WLT_76	32	4.000	2.616	1.133	0.656	0.618	-0.062
Stanford	**WLT_1**	9	8.000	3.767	1.658	0.556	0.735	0.244
	WLT_36	9	7.000	3.306	1.532	0.667	0.698	0.044
	WLT_40	9	8.000	6.231	1.937	0.778	0.840	0.074
	WLT_88	9	6.000	3.115	1.407	0.889	0.679	-0.309
	WLT_25	9	6.000	4.263	1.613	0.778	0.765	-0.016
	WLT_44	9	6.000	3.240	1.455	0.778	0.691	-0.125
	WLT_76	9	4.000	3.522	1.311	1.000	0.716	-0.397
Vaalvlei	**WLT_1**	10	9.000	5.405	1.942	0.800	0.815	0.018
	WLT_36	10	8.000	5.263	1.834	0.900	0.810	-0.111
	WLT_40	10	7.000	5.000	1.752	0.700	0.800	0.125
	WLT_88	10	7.000	4.255	1.635	0.700	0.765	0.085
	WLT_25	7	6.000	4.455	1.631	0.571	0.776	0.263
	WLT_44	10	5.000	2.410	1.158	0.800	0.585	-0.368
	WLT_76	9	4.000	3.176	1.228	0.667	0.685	0.027

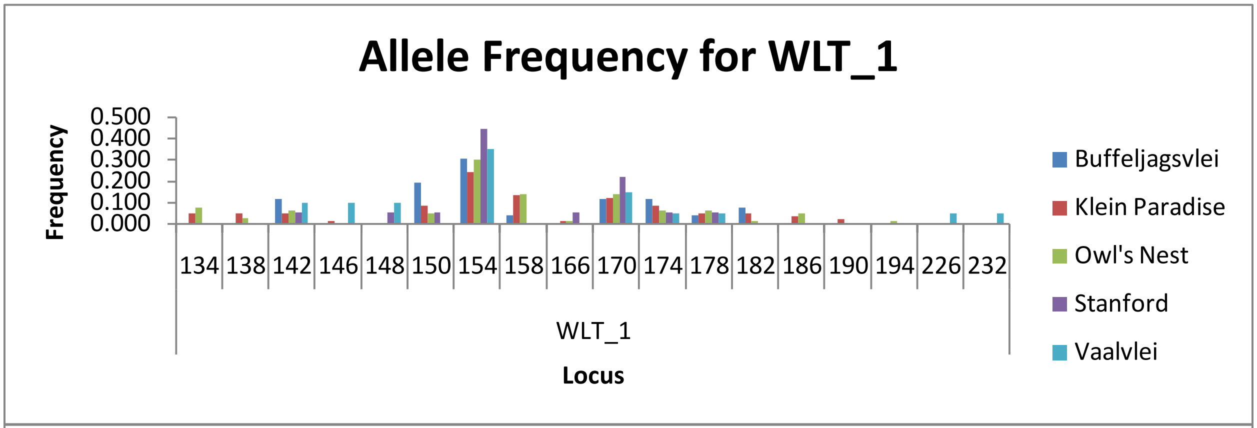
N- Sample sizes, Na- No. alleles, Ne - No. effective alleles, Ho- Observed Heterozygosity He-Expected Heterozygosity, and F-Fixation Index

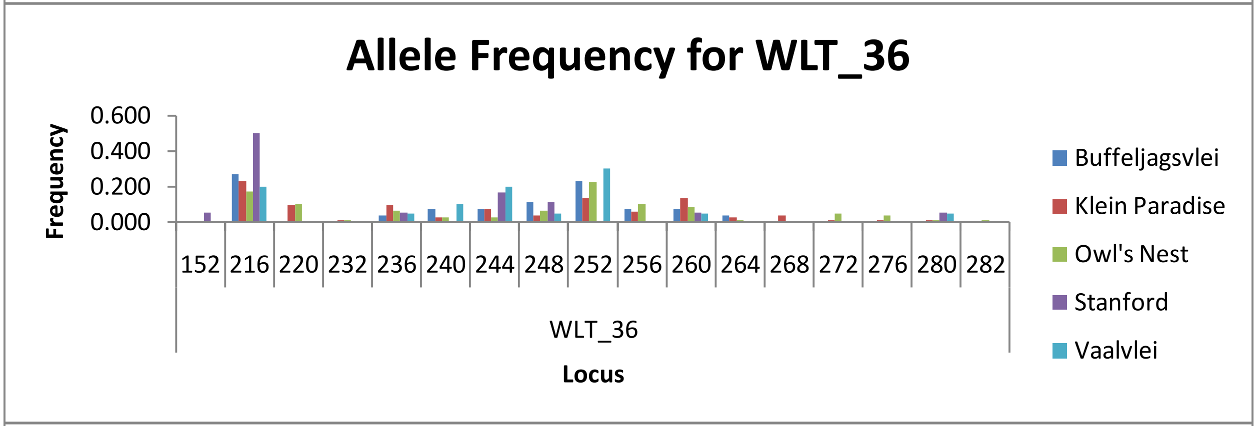
Appendix III: Calculations of Hardy-Weinberg Equilibrium per locus at each samples breeding site (n=5)

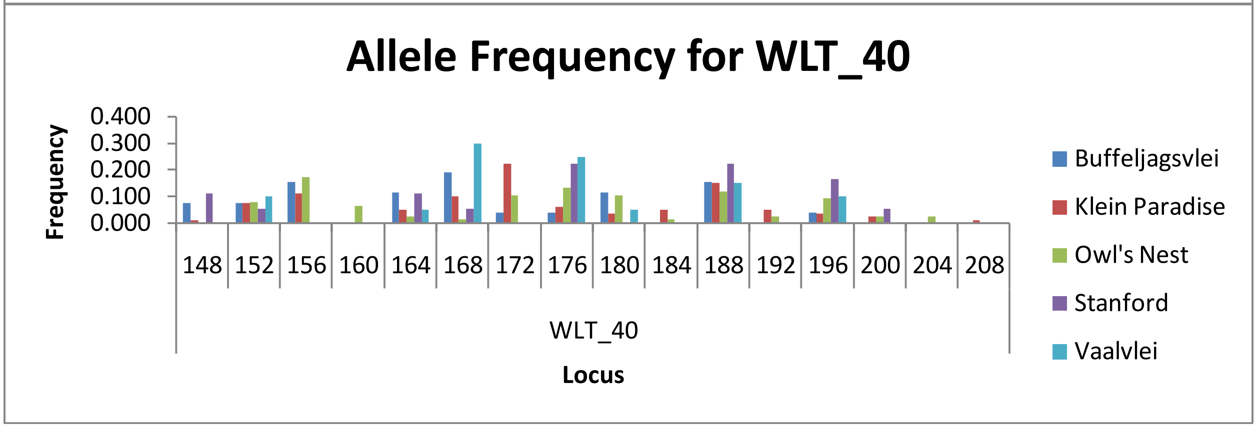
Pop	Locus	DF	ChiSq	Prob	Signif
Buffeljagsvlei	WLT_1	28	24.989	0.628	ns
	WLT_36	36	49.347	0.068	ns
	WLT_40	45	70.417	0.009	**
	WLT_88	28	27.482	0.492	ns
	WLT_25	15	12.307	0.656	ns
	WLT_44	36	24.917	0.918	ns
	WLT_76	6	3.035	0.804	ns
Klein Paradise	WLT_1	91	116.576	0.037	*
	WLT_36	105	107.681	0.409	ns
	WLT_40	91	79.858	0.792	ns
	WLT_88	78	72.453	0.656	ns
	WLT_25	66	43.825	0.984	ns
	WLT_44	153	148.817	0.580	ns
	W_76	6	8.269	0.219	ns
Owls Nest	WLT_1	78	95.260	0.089	ns
	WLT_36	105	103.264	0.530	ns
	WLT_40	91	174.735	0.000	***
	WLT_88	66	66.209	0.470	ns
	WLT_25	66	47.224	0.961	ns
	WLT_44	120	224.744	0.000	***
	WLT_76	6	6.200	0.401	ns
Stanford	WLT_1	28	29.250	0.400	ns
	WLT_36	21	23.444	0.321	ns
	WLT_40	28	21.250	0.815	ns
	WLT_88	15	13.444	0.568	ns
	WLT_25	15	14.592	0.481	ns
	WLT_44	15	8.111	0.919	ns
	WLT_76	6	9.000	0.174	ns
Vaalvlei	WLT_1	36	36.451	0.448	ns
	WLT_36	28	29.028	0.411	ns
	WLT_40	21	29.778	0.097	ns
	WLT_88	21	11.773	0.946	ns
	WLT_25	15	13.564	0.559	ns
	WLT_44	10	4.444	0.925	ns
	WLT_76	6	4.413	0.621	ns

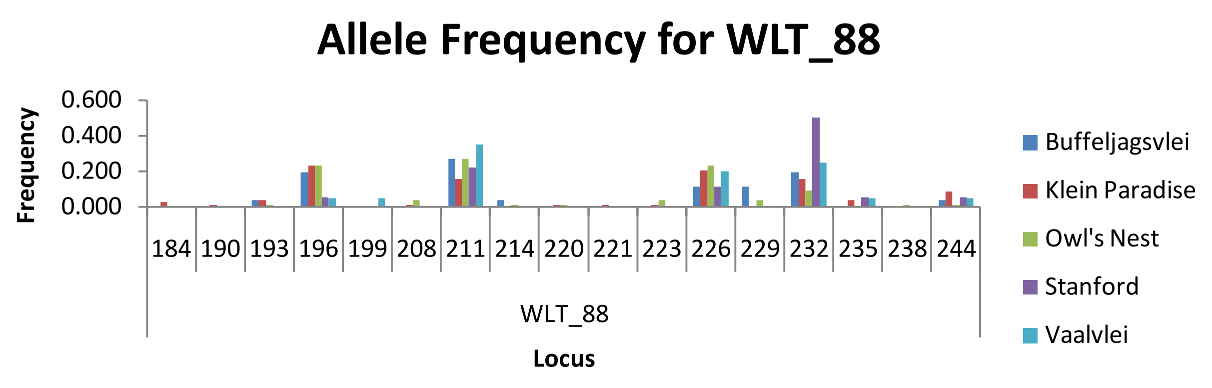
Key: ns=not significant, * P<0.05, ** P<0.01, *** P<0.001

Appendix IV: Number of Alleles & Sizes and Allele frequency per locus (n=7), broken down for each sampled breeding site (n=5)





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220
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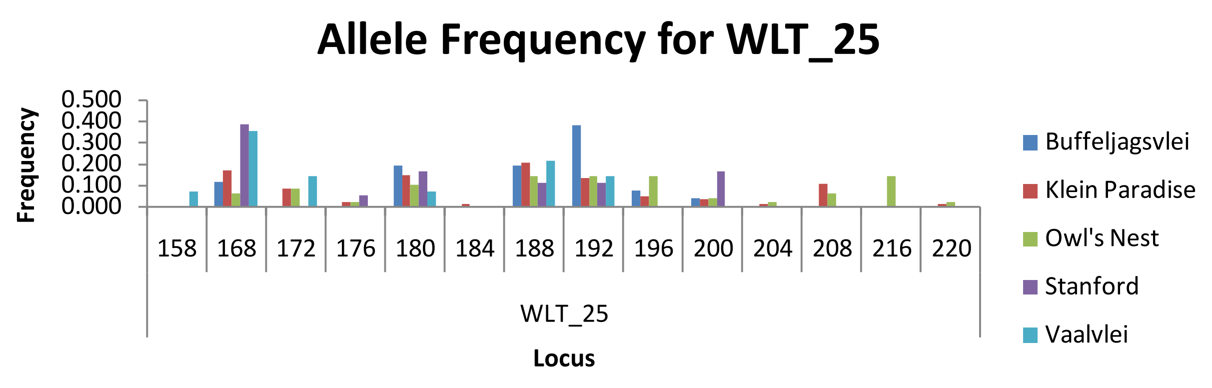
235
238
244
WLT_88
Locus
Buffeljagsvlei
Klein Paradise
Owl's Nest
Stanford
Vaalvlei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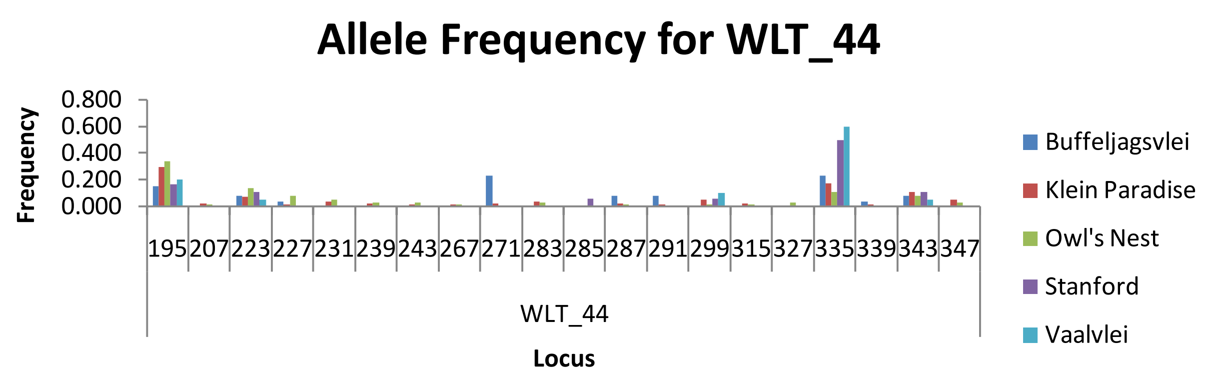
Locus
Buffeljagsvlei
Klein Paradise
Owl's Nest
Stanford
Vaalvlei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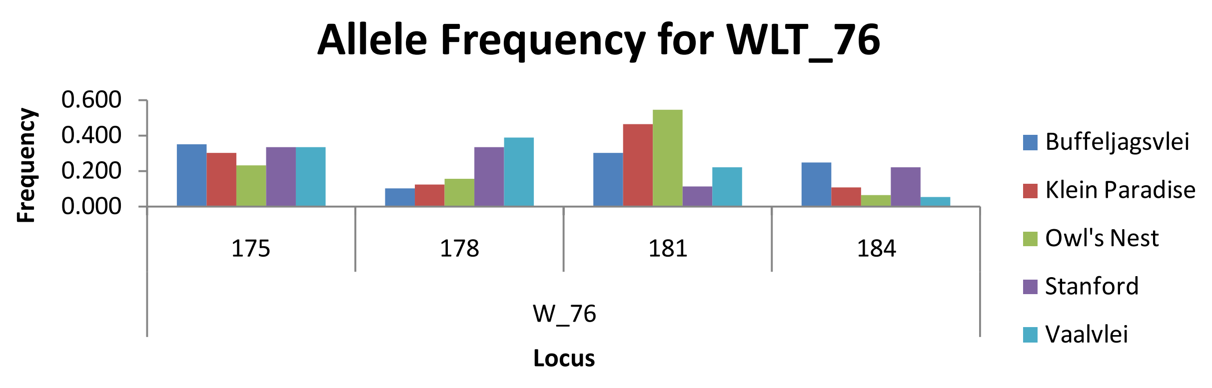
Buffeljagsvlei
Klein Paradise
Owl's Nest
Stanford
Vaalvlei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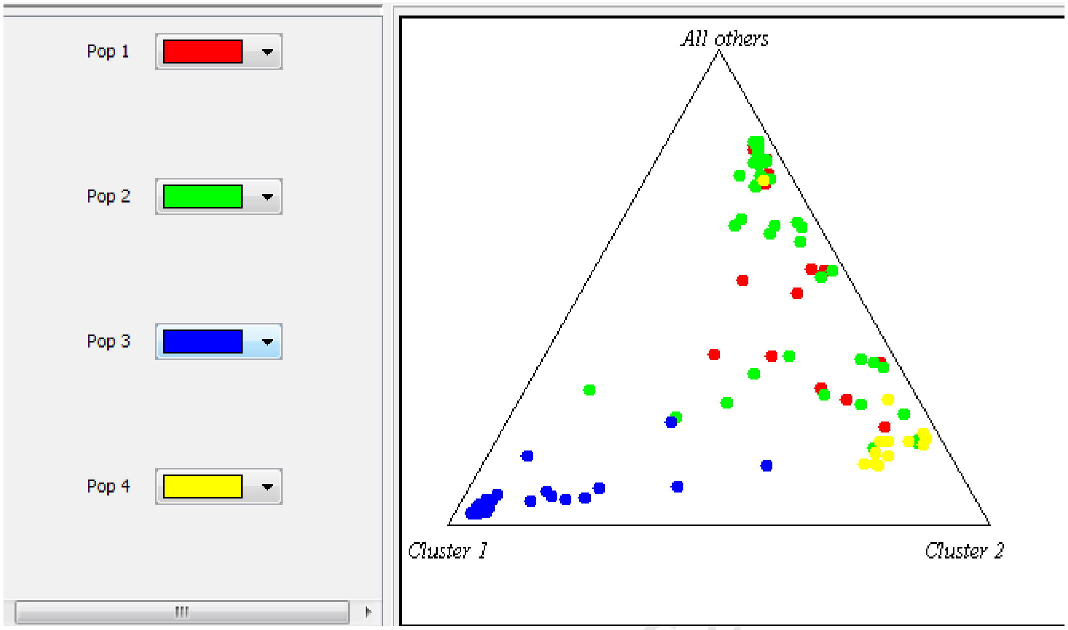
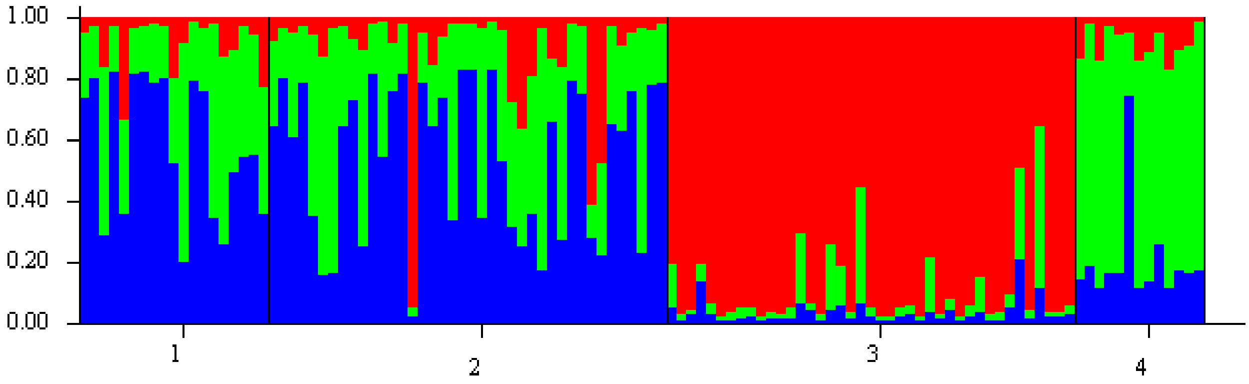
W_76
Locus
Buffeljagsvlei
Klein Paradise
Owl's Nest
Stanford
Vaalvlei

Appendix V: Triangle plot (STRUCTURE v2.3) of the population structure of the four breeding areas in Overstrand. Pop 1 = Stanford-Vaalvlei breeding sites, Pop 2 = Owl's Nest, Pop 3 = Klein Paradys, Pop 4 = Buffeljagsvlei, showing three distinct populations, with Pop 1 & 2 representing one genetic cluster, and populations 3 & 4 each representing their own genetic cluster



Appendix VI: Bar plot (STRUCTURE v2.2) of the population structure of the four breeding areas in Overstrand. Pop 1 = Stanford-Vaalvlei breeding sites, Pop 2 = Owl's Nest, Pop 3 = Klein Paradys, Pop 4 = Buffeljagsvlei showing three distinct populations, with Pop 1 & 2 representing one genetic cluster, and populations 3 & 4 each representing their own genetic cluster



Appendix VII: Pair-wise AMOVA: Rst values for between the 3 genetically distinct populations in the Overstrand area

Stan-Vaal-Owls	Klein Paradise	Buffeljagsvlei	
	0.001	0.094	Stan-Vaal-Owls
0.091		0.220	Klein Paradise
0.035	0.010		Buffeljagsvlei

*Rst values below the diagonal, p-values above the diagonal