The role of certain mortality factors in the underperformance of *Aristaea* (*Parectopa*) *thalassias* (Meyrick) as a biological control agent of *Leptospermum laevigatum* F. Muel.

Dissertation submitted in fulfilment of the requirements for the Degree of Master of Science, University of Cape Town

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Abstract

Leptospermum laevigatum is indigenous to the coasts of south and central New South Wales in Australia. It has become invasive in South Africa since its introduction in the 1800s, competing with the native fynbos across the coastal regions of the Western Province and into the Eastern Cape Province. Due to its aggressive spread, it became a significant concern and is considered a highly invasive species requiring a concerted management plan in South Africa. Biological control of *L. laevigatum* has been implemented since the 1980s with limited success to date. *Aristaea (Parectopa) thalassias* is one of two biological control agents introduced against *L. laevigatum* and is present across the plant's distribution along the coastal regions of the Western Cape Province and into the Eastern Cape Province in South Africa.

In order to understand the limited success of *A. thalassias*, this study considers the biology of the immature stages, larval mortality due to parasitoids, predation and overcrowding, and seasonal influences on development and survival. The findings showed that *A. thalassias* is present throughout the year, completes its life cycle in 58 days (as measured in spring), and thus has the potential to complete several overlapping generations throughout the year. Levels of overall mortality of larvae, pre-pupae and pupae averaged over a 20-month period indicated seasonal variability, with highest mortality in autumn and winter. Mortality attributed to parasitism was highest in summer (maximum 25% in one year). Furthermore, predation and overcrowding (maximum 15%) were also low, and thus these mortality factors are unlikely to account for the low efficacy of the agent. Despite this, mortality through other unknown factors was at times found to be high, especially for first instar larvae in mined leaves (maximum 58% in one year). The

peak oviposition period was not aligned with the peak in production of new leaves, suggesting a possible phenological mismatch between *L. laevigatum* and its biological control agent *A. thalassias* which could curb population expansion. Whilst mortality resulting from unknown factors (*i.e*., not investigated in the scope of this study) might be playing an important role in the performance of *A. thalassias*, specific factors investigated during this study could not explain why the moth is not more prolific.

Determining why any particular biological control agent is ineffective is often not a straightforward exercise because it may be the result of a complex of interacting factors, which are not easily discernible. The findings of this study show that this is probably true for *A. thalassias* in South Africa and much more work is needed to resolve the reasons for its underperformance as a biological control agent.

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Chapter 1

General Introduction

Invasive weeds: A global problem

Globally, ecosystems are susceptible to alien plant invasions, threatening human health, the environment, and the economy (Pimentel *et al.* 2001; Early *et al.* 2016). The occurrence of problematic, invasive species originates from intentional and unintentional introductions of plants that are moved between geographic regions and continents without their natural enemies (Pyšek *et al.* 2011; Faulkner *et al*. 2017). In South Africa, for example, commercial forestry, e.g*., Pinus* species (Hoffmann *et al*. 2011), dune stabilisation, e.g., *Acacia* species (Dennill *et al*. 1999), the ornamental plant industry, e.g., *Sesbania punicea* (Cav.) Benth. (Fabales: Fabaceae) (Hoffmann and Moran 1991), the aquarium trade, e.g., *Eichhornia crassipes* (C.Mart) Solms (Commelinales: Pontederiaceae) (Cilliers 1991), and the need for shade and fodder plants, e.g., *Prosopis laevigata* (Humb. & Bonpl. ex Willd.) M.C.Johnst (Fabales: Fabaceae) (Zimmermann 1991), have all played important roles in driving deliberate introductions. For the most part however, plant introductions via trade routes (Westphal *et al.* 2008; Hulme 2009), are accidental, e.g., crofton weed (*Eupatorium adenophorum* Spreng (Asterales: Asteraceae) in China (Yan *et al*. 2001), *Hypericum perforatum* L. (Malpighiales: Hypericaceae) in South Africa (Gordon and Kluge 1991), and *Chromolaena odorata* (L.) King and Robinson (Asterales: Asteraceae) in many parts of the world (Day and McFadyen 2012; Zachariades *et al*. 1999; 2009).

The naturalisation of invasive species occurs in three stages: arrival, establishment and population spread (Sakai *et al.* 2001; Holmes *et al*. 2009; Faulkner *et al*. 2017). When their populations increase, alien invasive species can cause major changes to

ecosystems in several ways, including, but not limited to reduction in water quality and quantity; threating indigenous species; stimulating and intensifying fires; disrupting ecosystem functions and agricultural production (Mooney and Cleland 2001; Dukes and Mooney 2004; Morales and Traveset 2009).

Invasive weeds in South Africa

South Africa has a problem with alien plant invasions from various taxonomic groups (Richardson and van Wilgen 2004; Holmes *et al.* 2005; Moran *et al.* 2005; van Wilgen *et al.* 2020; Faulkner *et al*. 2020), these include shrubs, trees, climbers and grasses that have invaded both terrestrial and freshwater ecosystems (Holmes *et al*. 2005; Henderson 2007; Richardson and Rejmanek 2011). Of all the provinces in South Africa, the Western Cape is considered the most extensively vulnerable to plant invasions, particularly the species-rich Fynbos Biome (Richardson and van Wilgen 2004; Latimer *et al.* 2004; Holmes *et al.* 2005), one of the world's biodiversity hotspots (Myers *et al.* 2000; Forest *et al.* 2007).

Management of alien invasive plant species

Alien invasive plant species are managed mainly by means of three techniques: mechanical, chemical, and biological control methods (Fowler *et al.* 2000; Kelton and Price 2009). Mechanical control is comprised of the physical removal or severe damaging of the invasive plants through uprooting, chain dragging and bulldozing (DiTomaso 2000). Chemical control involves the application of registered herbicides on alien invasive plants. Application of the chemicals can be done by means of helicopter aerial spraying, ground applicators and backpack sprayers. The dosage and timing of applications are critical factors that determine the overall efficacy of the techniques when applying herbicides (DiTomaso 2000; Ruffner and Barnes 2012;

Hunter 2019). While both chemical and mechanical control methods are used for management of alien invasive plant species, they are costly and unsustainable, given the scale on which they have to be applied when implementing control measures (van Wilgen *et al*. 2001; Howle *et al*. 2010; Williams *et al*. 2010; Sheley *et al*. 2011).

The third method used to control alien invasive plant species is biological control, which is the subject of this study. Biological control relies on the premise that alien invasive plants become problematic because, when moved between regions and continents, they are removed from the specialist natural enemies that normally damage them and curb their growth and reproduction in their natural habitat (Keane and Crawley 2002; Colautti *et al*. 2004). Biological control entails the introduction of specialist natural enemies, or control agents, (i.e., mostly herbivorous insect, mite, and pathogen species) into the regions where their host plants have been introduced and have become a problem (Eilenberg *et al*. 2001; Hajek and Eilenberg 2018).

Biological control is usually applied as a stand-alone control method but can be enhanced when integrated with other methods (Moran *et al.* 2005; Kaur *et al.* 2014; Barrett 2017). It involves several stages, which include: starting with finding a suitable agent for a target weed; importing the agent from the country of origin; and running tests in quarantine to assess host specificity. This stage is followed by an official application to release, a process which varies slightly from one country to the next, and then finally, mass-rearing (where appropriate) and release of the agent, with follow-up monitoring of establishment and evaluation of the success of the agent (Littlefield and Sobhian 2000; McClay and Balciunas 2005). There are different ways of undertaking the host-specificity assessment. These may be used in any combinations, including a series of no-choice and choice laboratory experiments to test for host selection behaviour, and includes feeding, oviposition and development

studies on selected target and non-target species (Heard *et al*. 2010). The degree of host specificity of the agent is investigated to prevent or minimise the risk of direct and indirect non-target plant effects (Hight *et al*. 2003).

The herbivorous insects that have been used as agents include different feeding guilds, incorporating chewers, suckers, borers, gallers and miners, which cause damage to different plant parts, including leaves, stems, roots, flower buds, flowers, and seeds (Stiling and Cornelissen 2005). Although it is quite common to use multiple agents, often targeting different plant parts to achieve the desired impact (Harris 1991; Hoffmann and Moran 1998), there are other cases, where the use of one effective agent as opposed to many is sufficient for successful control to be achieved (e.g., Denoth *et al.* 2002). If successful, the damage inflicted by the biological control agents causes a decline in population densities and rates of spread of the problem plants (Paynter 2005; Clewley *et al.* 2012; Jones *et al.* 2018).

History of biological control programmes

The history of biological control against alien invasive plant species dates back to 1902 in Hawaii where biological control was implemented against *Lantana camara* L. (Lamiales: Verbenaceae) (Zachariades *et al.* 2017). Ever since, biological control has been extended to several other weed species and has not only shown to be efficacious but also self-sustainable as a management method for invasive species globally (Moran *et al*. 2013; Zachariades *et al*. 2017; Hinz *et al*. 2019; 2020). In South Africa, biological control dates back to 1913 when large densities of the drooping prickly pear, *Opuntia monacantha* Haw (Caryophyllales: Cactaceae) were significantly reduced by means of the cochineal insect *Dactylopius ceylonicus* (Green) (Hemiptera: Dactylopiidae) released as a biological control agent on the invasive cactus species

(Paterson *et al.* 2011; van Wilgen *et al.* 2013; Moran *et al*. 2013; Zachariades *et al*. 2017). To date, there are more than 554 invasive weed species worldwide against which biological control by means of invertebrates or fungi has been implemented with varying degrees of success (Winston *et al*. 2014; Hinz *et al*. 2020). In South Africa, 325 biological control agents have been introduced into quarantine for evaluation against 87 plant species, of which 71 have been rejected, 73 shelved, 181 have been released (Klein 2011; Zachariades 2018 unpublished data). The success rate of these agents on their targeted plants varies, with some alien invasive plants now considered under complete or substantial control (Zimmermann *et al.* 2004; van Wilgen *et al*. 2013). For example, the control of *Opuntia stricta* Haw (Caryophyllales: Cactaceae) using *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) and *Dactylopius opuntiae* (Cockerell) (Hemiptera: Dactylopiidae) is one of the biggest success stories of biological control programmes in Australia, which reduced the plant biomass by 90% since its introduction, resulting in no need for further interventions (Julien and Griffiths 1998).

Constraints to biological control agents

Although biological control is widely considered a potentially effective method for the management of invasive plant species, the success of any programme may ultimately be influenced by various factors such as the level of abundance of a biological control agent on its host plant (McFadyen 1998; Urban *et al*. 2011). Ideally, an agent will only be effective if it is damaging on its host and if it becomes abundant enough to cause and sustain high levels of damage (Randall *et al*. 2017; Sutton *et al.* 2018). However, this is not always the case; some agents are never sufficiently damaging, usually because they never reach high levels of abundance in the field (Wang *et al*. 2011; Pitcairn 2018) or because the targeted plant species can tolerate or compensate for

the levels of damage inflicted by the agents (Raghu *et al*. 2006). The abundance of agents is influenced by both biotic (e.g., predation, parasitism, competition) (Swope and Satterthwaite 2012; Goldson *et al*. 2014) and abiotic (e.g., climate extremes) factors (Milan *et al*. 2006; Zalucki *et al.* 2007; Magagula 2011).

The case of Leptospermum laevigatum *in South Africa*

Leptospermum laevigatum (Gaertn.) F.Muell.,(Myrtales: Myrtaceae), commonly known as Australian myrtle or Victorian tea tree, is one of the most significant invasive species to threaten coastal dunes in the fynbos biome in South Africa (Burrell 1981; Lam 2002; Gordon 2011). It is a tree that can grow to 8 m in height with a canopy of up to 5 m in diameter. Mature branches and the lower parts of the main stem bear characteristic papery and flaky bark (Burrell 1981; Lam 2002; Gordon 2011). Individuals can live for up to 150 years, producing flowers and fruit capsules in winter, spring, and summer, depending on their location (Burrell 1981; Ellis and Allen 2013). Each fruit capsule (8 mm wide) contains 80 - 130 tiny seeds (Gordon 2011). It is a serotinous species, retaining its seed for up to three years without disturbance, but capsules on felled or dying, and fire-damaged branches release seeds within hours.

Leptospermum laevigatum is indigenous to the coastal dunes of New South Wales, eastern Victoria, northern Tasmania, and Bass Strait Islands in Australia (Burrell 1981; Lam 2002; Gordon 2011; Dorchin and Adair 2011). Besides South Africa, *L. laevigatum* is now naturalised in other countries such as the United States of America and New Zealand (Lam 2002). It is known to commonly grow in sandy soil containing free calcium carbonate, but also occurs in disturbed areas and along roadsides in Australia (Bennett 1994; Lam 2002).

In South Africa, *L. laevigatum* was introduced in the 1800s to stabilise dunes in the Western Cape Province; however, it became a significant concern as it spread aggressively across the coastal regions of the Western Cape Province and into the Eastern Cape Province (Evans 2001; Dorchin and Adair 2011; Gordon 2011). In South Africa, invasive species are categorised according to their level of invasiveness (NEMBA 2014). Due to the level at which it is threatening native ecosystems, especially in the Cape Floristic Region (Henderson 2001), *L. laevigatum* is listed as a category 1b invader, meaning that it must be removed wherever it occurs (NEMBA 2014).

Current control methods rely on mechanical, chemical, and biological control. However, mechanical and chemical control of Australian myrtle are labour-intensive and expensive (Dorchin and Adair 2011; Gordon 2011). Therefore, biological control remains a primary goal for providing a long-term, reliable, and affordable method to supress the growth of *L. laevigatum* (Gordon 1999).

The leaf-mining moth, *Aristaea (Paractopa) thalassias* (Meyrick) (Lepidoptera: Gracillariidae) (Plate 1) which is native to Australia, was introduced into South Africa in 1993 as a biological control agent for *L. laevigatum*. This was preceded by a gall-forming midge, *Dasineura strobila* Dorchin (Diptera: Cecidomyiidae) introduced in the mid-1980s (Gordon 1999; 2011). Both agents are established on *L. laevigatum* but they are not achieving significant impact on the invasiveness of the weed (Gordon 2011).

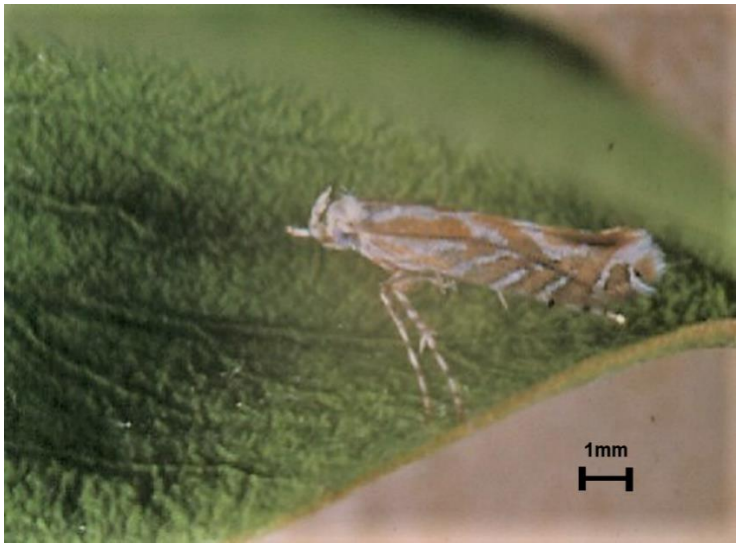


Plate 1.1. *Aristaea thalassias* adult on a *Leptospermum laevigatum* leaf. (Photo: A.J. Gordon).

Although it is now well established and widespread in South Africa, the damage that *A. thalassias* causes, particularly on adult trees, is typically superficial and patchy (Gordon 2011). As a first step towards resolving the reasons for the apparent failure of *A. thalassias* as a biological control agent, the present study was undertaken to identify possible curbs on the moth's ability to reach higher population levels than are currently achieved. This study investigated the biology of *A. thalassias*, including development times for each life stage under natural conditions and overwintering of the immature stages (Chapter 2). The second part of this study identified and quantified some of the causes of mortality in the immature stages of *A. thalassias,* specifically the role of parasitoids, predation and overcrowding on mortality rates seasonally (Chapter 3). Finally, the need for more in-depth follow-up studies to identify why *A. thalassias* does not achieve higher levels of abundance in South Africa are discussed, along with the aspects that should be prioritised going forward (Chapter 4).

Chapter 2

The developmental biology of *Aristaea thalassias,* a biological control agent of *Leptospermum laevigatum*

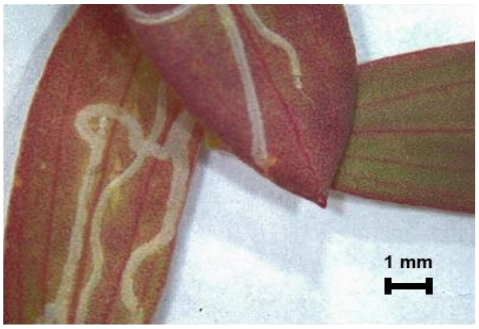
Introduction

Little is known about the life history of *Aristaea thalassias* in either its native or introduced ranges. The female moths lay their eggs on the adaxial and abaxial surfaces of newly formed leaves at the tips of stems (Gordon 1999; 2011). The early-instar larvae feed on parenchyma while mining within the leaf, forming distinctive threadlike serpentine mines (Plate 2.1a) which become broader as the larva develops (Gordon 1999; 2011). Eventually all the parenchyma is consumed, at which stage the hollow leaves become air-filled and puffy (Plate 2.1b) before falling from the plant (Gordon 1999; 2011). Prior to leaf drop, each final instar larva moves from the puffy leaf and settles on the surface of an adjacent, young healthy leaf. The pre-pupa uses silk to roll the leaf into a tube which forms a chamber in which it pupates (Plate 2.1c).

Although it has been reported that the moth takes about 64 days to complete its life cycle in summer and 94 days in autumn (Gordon 1999), nothing further has been reported about the phenology of *A. thalassias* in South Africa. Some of the important aspects where there is insufficient and inadequate knowledge and information with regards to the phenology of *A. thalassias* include: the developmental duration of each of the life stages; the number of larval instars; which stages overwinter and if there is a dormant phase in the life cycle; and whether the timing of the insect's life cycle is synchronised with that of its host.

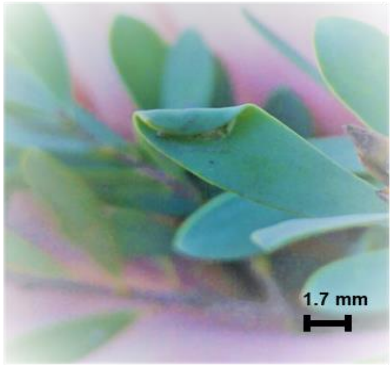
Understanding the phenology of the insect is a fundamental requirement for any study of the mortality factors that are constraining its population densities. To gain a better

understanding of the developmental biology of *A. thalassias* on *L. laevigatum* in South Africa, field and laboratory studies were undertaken. The methods and findings are reported here.



2.1a

2.1b



2.1c

Plate 2.1. Typical damage caused by different immature stages of *A. thalassias*. Plate 2.1a; the serpentine mine typical of the early instar larvae, Plate 2.1b; an internal view of the air-filled chamber in a “puffy” *L. laevigatum* leaf that develops at the end of larval development and Plate 2.1c; a *L. laevigatum* leaf rolled by an *A. thalassias* pre-pupa to form a pupation chamber (Photos: Dr Candice Lyons).

Methods

Development of A. thalassias

During the month of June 2018, 50 similarly-sized (≈15 cm high) *L. laevigatum* saplings which were clear of any signs of *A. thalassias* were collected from a site near Wemmershoek (33°52'S,19°02'E) in the Franschoek area. The saplings were dug up with as much soil as possible surrounding their roots and were transplanted, on site, singly into plastic plant-pots (20 cm diameter). The pots were filled with the same sandy soil from which the saplings were obtained in the field and no fertiliser or different type of soil was added. The saplings were transported to the ARC campus and given an initial spray treatment with an organic insecticide (Efekto Oleum) to ensure that they were free from any *A. thalassias.* They were then maintained in a shade house (Alnet 40% shade cloth) and watered two times a week.

Three months later, 25 successfully transplanted saplings were selected for the development trial. At the onset of September (in both 2016 and 2017), when adult moths are known to be present in the field (Gordon 1999), the potted seedlings were labelled (1 to 25) and dated on the outer surface of the individual pots using a permanent marker and transported to Walshacres Farm (34°42'S, 19°44'E) near Stanford, a site where *A. thalassias* was known to be abundant (Gordon 1999). The potted saplings were left in the field over a period of three days to allow egg laying by the adult female moths.

Leaves of individual plants were inspected for the presence of viable eggs daily during the trial. To track the duration of development from egg to adult, each egg was assigned an identity by marking the leaf on which it was laid using coloured woollen thread tied around the petiole. On those plants where eggs were present, multiple

eggs (up to ten) were sometimes found on individual leaves. Each leaf bearing a single egg was marked using different coloured woollen threads to distinguish a specific egg on a particular plant (e.g., leaf one of plant one marked with a red woollen thread, leaf two on plant one with blue woollen thread, leaf three on plant one with pink thread, etc). The colour of the markers was not linked to time of oviposition but was used to identify and track the development of each egg on each labelled plant. The labelled plants were returned to an outdoor nursery under ambient temperature conditions and were monitored every 24 hours to record the length of each life stage (egg, larva in mined leaf, larva in puffy leaf, and pre-pupae and pupal stages in rolled leaves). The daily temperature measurements for the duration of the trial were obtained from Agricultural Research Council- Institute of Soil, Climate and Water in Stellenbosch.

The different life stages of *A. thalassias* were categorized as follows: (E) the presence of spherical, transparent to white eggs on leaf surfaces; (M) first-instar larvae in mines; (L) 2nd and 3rd instar larvae within puffy leaves; (P) the pre-pupal stage within rolled leaves; and (R) pupae within rolled leaves. The 24-hour monitoring took place between September and December, after which the immature stages of the cohort of *A. thalassias* had all completed their development.

The determination of larval instars

The number of larval instars was determined by dissecting 30 mined and 30 puffy leaves that were haphazardly collected monthly for a period of 20 months from Walshacres Farm (between July 2015 and February 2017). The leaves were placed in plastic freezer bags (300 x 200 mm), to prevent desiccation, and transported in an air-conditioned vehicle to the laboratory for processing. The leaves were dissected to remove the larvae. From these dissections, 445 larvae were removed and stored in

plastic Eppendorf tubes (1.5 ml) in 70% ethanol and were later mounted on microscope slides, using Canada Balsam (according to methods described in Thakur 2016) or DPX mounting solution (in the absence of Canada Balsam), in order to measure the width of head capsules and the length of larvae, and thereby separate the different larval instars (Ghafoor 2011). A Leica microscope, with digital camera (EZ4W) and eyepiece graticule, was used to photograph the specimens (see plate 2.2) and to record head capsule measurements in micrometres (*µm*) at forty times magnification.

Plate 2.2: 3rd instar larva of *A. thalassias* mounted in DPX mounting solution.

The monthly samples that were collected to measure larval instars, and their frequency over different months, were grouped according to different austral seasons. Samples collected between September and November were grouped as “spring”, those collected between December and February as “summer”, those collected between March and May as “autumn”, and those collected June to August, as “winter”.

Statistical Analysis

To determine the developmental period of each life stage, the mean number of days spent in each stage was calculated. The data was tested for normality and homogeneity of variance to assess whether they met the conditions of Analysis of Variance (ANOVA) using the Shapiro-Wilks tests. ANOVA was performed using the GLM procedure of SAS statistical software.

The average daily temperatures (minimum and maximum) and the duration of each life stage were calculated using Microsoft Excel.

To determine the number of larval instars, frequency histograms of the complete set of head capsule widths were plotted using descriptive statistical analysis (Ott and Longnecker 2001; Panzavolta 2007; Calvo and Molina 2008; Castañeda-Vildózola *et al*. 2016). The statistical methods used assumed that head capsule measures were normally distributed for each instar as described by Sokal and Rohlf (1995) and Hunt and Chapman (2001) and that each peak of the resulting multimodal curve represented a different instar (Hunt and Chapman 2001). Generally, the number of class intervals selected to determine larval instars from head capsule measurements is determined by the number of measurements in the data set, with smaller intervals appropriate for large data sets and large intervals for small data sets (Ott and Longnecker 2001). In this study, visualization of distinct instars was optimum when a class interval of 13.5 *µm* was used. This interval corresponds with those used in other similar studies (e.g., Panzavolta 2007; Calvo and Molina 2008). Based on Dyar's rule (Dyar 1890) and Gaines and Campbell (1935), head width growth pattern follows a perfect geometrical progression which can be obtained by a straight line if the logarithms of the head measurements are plotted against the number of instars. Linear

regression of natural log was done for the measurements made during this study to ensure the results conformed to Dyar's rule (Dyar 1890).

All analyses were performed using XLSTAT software (version 7.5.2, Add-in soft, New York, USA) or using STATISTICA 10.0 (Stat soft, Tulsa, OK, USA) and SAS statistical software 9.4 (SAS institute Inc., Cary, NC, USA).

Results

Development duration of immature stages of A. thalassias

The development durations of each life stage of *A. thalassias* are shown in Table. 2.1. Larvae within puffy leaves accounted for the longest phase of the life cycle, which made up almost half of the average development time of 58 days from egg to adult (Table. 2.1). The development of eggs and first instar larvae constituted the shortest part of the life cycle, at 8 and 9 days respectively.

Table 2. 1. Mean (± SE) development time (days) for each of the sub-adult life stages of *A. thalassias,* and mean (±SE) minimum, maximum and daily temperature conditions when measurements were made between September and December.

Life stages	**Development time**	**T_{min}(°C)**	**T_{max} (°C)**	**Daily Temperature (°C)**
Egg	8 ± 0.6	9.7 ± 0.1	21.9 ± 0.1	15.8 ± 0.1
Larvae in mines	9 ± 0.6	10.1 ± 0.1	23.5 ± 0.1	16.8 ± 0.1
Larvae in puffy leaves	26 ± 1.5	9.1 ± 0.5	22.6 ± 0.1	15.8 ± 0.1
Pre-pupal and pupal stages in rolled leaves	15 ± 1.2	12.4 ± 0.5	26.6 ± 0.1	19.5 ± 0.1

Number of larval instars

The head capsule widths ranged from 60 - 546 *µm*. Frequency distribution analysis of the head capsule widths of *A. thalassias* produced three peaks at 141 ± 2.2, 254 ± 3.3 and 428 ± 3.2 *µm* indicating the existence of three larval instars (Fig. 2.1). The regression analysis showed a highly significant linear relationship (R^2= 0.94; p < 0.001) between head capsule widths and larval instars (Fig. 2. 2).

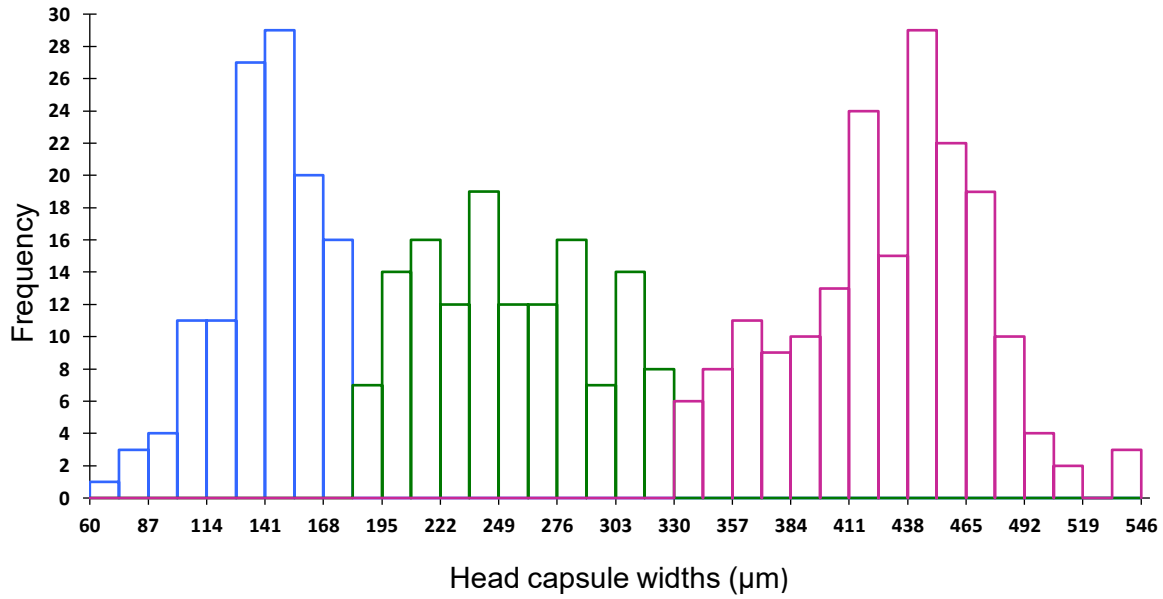


Figure 2. 1. Frequency distribution of head capsule widths (*µm*) of *A. thalassias*. The bars illustrate the instar distributions; blue coloured bars representing instar 1; green, instar 2 and pink, instar 3. The data presented are the measurements recorded over a three-year period (2015, 2016 and 2017).

The three larval instars were found throughout the year except that there were no first instars found in February (Fig. 2.3). There were two prominent peaks for the frequency of first instars in January and November ($F_{(10,111)}$ = 4.17, p < 0.001). While there were

significant differences in the numbers of second instars recorded for different months ($F_{(11,123)}$ = 2.20, p < 0.001), the numbers of third instars observed in the different months were not significantly different ($F_{(11,175)}$ = 1.77, p = 0.06) (Fig. 2.3).

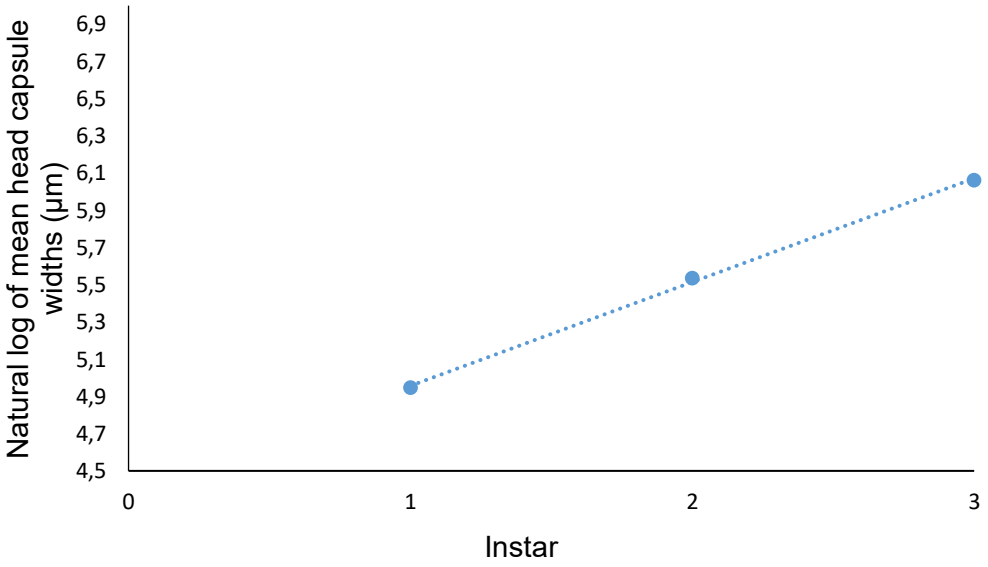


Figure 2. 2. Linear regression of natural log (ln) of mean larval head capsule widths against instars.

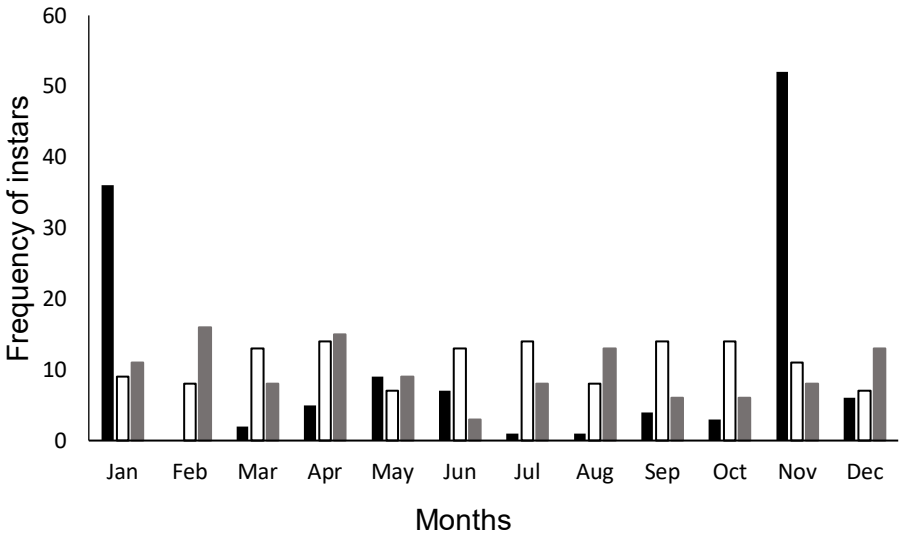


Figure 2. 3. Frequency distribution of head capsule widths (*μm)* of *A. thalassias*. The bars illustrate the instar distributions; black shaded bars representing instar 1; unshaded bars, instar 2 and grey shaded bars, instar 3. The data presented are for cumulative measures made on larvae (n = 445) collected over a three-year period between 2015 and 2017.

Discussion

Gordon (1999) reported on the biology of *A. thalassias* and recorded that the development from egg to adult is 94 and 64 days in autumn and summer, respectively. This study recorded that development in spring is 58 days. This pattern indicates, not unexpectedly, that development rates are predominantly determined by temperature regimes at different times of the year (e.g., Powell and Logan 2005). The more rapid development of the immature stages of *A. thalassias* in spring was probably influenced by the availability of new leaves which are rich in nutrients at that time of the year (e.g., De Bruyn *et al.* 2002; Jones and Despland 2006). Indeed, environmental conditions are seemingly most suitable for *A. thalassias* in spring, a trend that is well known in herbivorous insects (e.g., Wermelinger and Seifert 1998). Although winter

development times were not measured, it is probable that they would be longer compared to other seasons due to lower temperatures (Schmolz and Lamprecht 2000) and, possibly, inferior host plant quality during winter months (e.g., Zilahi-Balogh *et al*. 2003; Tullett *et al.* 2004).

These observed development periods would allow *A. thalassias* to be multivoltine with five to six generations a year as suggested by Gordon (1999) and reported for other micro-lepidopterans (e.g., Jackson and Sweeney 1995), an attribute commonly considered beneficial when selecting candidate agents for biocontrol programmes (Boughton *et al*. 2012).

This study also showed that out of the 58 days development period from egg to adult, *A. thalassias* spends most time in the larval stages (35 days in mined and puffy leaves) (Table 2.1). The longer development duration recorded in these larval stages is common among insect species as a result of the increased time needed to assimilate nutrients, and grow (Weed and Casagrande 2010; Montezano *et al*. 2014; Folgarait *et al.* 2018).

The findings in this study showed that the first-instar larvae create the serpentine mines while the second and third instars feed on parenchyma tissues until the leaf is hollow and puffy and has lost its ability to photosynthesize leading to leaf abscission (Fig. 2.1). In contrast, Gordon (1999) reported the possibility of more than three larval instars by observing ecdysis and that feeding on parenchyma tissues only begins after a third moult, suggesting that the insect goes through three larval instars within a serpentine mine. Inconsistency in the determination of number of instars is not uncommon and is due to various factors including methodological problems (e.g., counting the number of moults; imprecise mathematical models) as well as host plant

quality and quantity and environmental factors (e.g., photoperiod, humidity, temperature) (Jackson and Sweeney 1995; Bentancourt *et al*. 2003; Jones and Despland 2006; Esperk *et al*. 2007; Kingsolver 2007; Barraclough 2014). The methodologies used in this study differ from those described in Gordon (1999), which could explain the reason for the differences in the determination of the number larval instars. Further observations of larval moults could clarify the reasons for the differences in numbers of instars recorded in the two studies and provide more clarity on the phenology of *A. thalassias*.

This study has demonstrated that *A. thalassias* has the potential to sustain high population densities, if not constrained by unfavourable extraneous conditions (e.g., natural enemies, competition – investigated in the next chapter). The observations on its developmental biology provide no indications that environmental factors or host plant unsuitability are inhibiting population increases of *A. thalassias* and hence accounting for its limitations as a biological control agent of *L. laevigatum* in South Africa. The presence of larvae throughout the year, even with proportionately lower numbers of early instars in colder months, shows that *A. thalassias* does not have a dormant overwintering phase as suggested by Gordon (1999); rather, development slows down, but continues, during colder months (e.g., Jackson and Sweeney 1995). However, information on the degree to which seasons influence the development of *A. thalassias* needs to be investigated further, especially seasonal influences on adult activity, oviposition patterns and fecundity, which all, ultimately influence its efficacy as a biocontrol agent.

Chapter 3

Factors influencing immature-stage mortality, and abundance, of *Aristaea thalassias*

Introduction

The alien invasive weed biological control programme in South Africa has registered a number of notable successes (Zhao *et al*. 2015; van Wilgen *et al.* 2020). The objective of the release of biological control agents is to reduce the abundance, rate of spread and invasiveness of the target weed species. As such, the different biological control agents have registered varying levels of success in controlling weed infestations due to the limiting factors encountered in the field (Hoffmann and Moran 1998). Some biological control agents, though showing potential during testing, are not always as successful when released, and therefore, understanding why they fail is often challenging (Schulz *et al.* 2019). Several factors have been identified and shown in various studies as the reason for the inefficacy. One of these is failure of the agents to establish and thus buildup populations large enough to cause significant damage to the host plant species, perhaps as a consequence of natural enemy-induced mortality (Paynter *et al*. 2010; Boughton *et al*. 2012; Schulz *et al.* 2019). The ability of a host plant to tolerate the damage inflicted by the agent also has an influence on the effectiveness of the agent (Garren and Strauss 2009; Heimpel and Mills 2017; Schulz *et al.* 2019).

The use of biological control against *L. laevigatum* has been conducted for decades with limited success. *Aristaea thalassias* is one of two introduced, but poorly performing biological control agents of the plant (Gordon 2011). In the field, leaf miners have demonstrated varying levels of success; some either have a negligible impact

(e.g., *Cremastobombycia lantanella* Busck (Lepidoptera: Gracillariidae), Baars and Neser 1999), whilst others substantially control their targeted host (e.g., *Phytomyza vitalbae* Kaltenbach (Diptera: Agromyzidae), Gourlay *et al.* 1999). Host plant dynamics (plant quality and age), parasitism, predation and competition are amongst factors that commonly affect leaf miners in the field (Eber 2004; Flores 2007; Zalucki *et al*. 2007; Sinclair and Hughes 2010; Kirichenko *et al.* 2019).

Parasitism is often the most significant factor regulating insect herbivore populations (Connor and Tarvener 1997). The way parasitoids and predators act on insect populations varies with each species (Swope and Satterthwaite 2012). Parasitoids and predators that regulate populations of their host need to respond in a density-dependent manner, where the mortality they cause increases proportionately with increasing populations of their host (Hassell 1985; Denno *et al*. 2003).

In the case of biological control agents, species are introduced to a region outside of their native range for use in biological control programmes, and freed from their specialist natural enemies, where they have the potential to thrive (Paynter *et al.* 2010; Boughton *et al.* 2012). However, they may suffer elevated levels of mortality by generalist parasitoids or predators that they may encounter in their introduced range (Paynter *et al*. 2010; Byrne *et al*. 2011; van Nouhuys *et al*. 2012; Herron-Sweet *et al*. 2015).

The impact of natural enemies on biological control agents also varies. Some agents remain effective regardless of natural enemies (e.g., Urban *et al*. 2011); while in other instances, some species are negatively impacted by local predators or parasites (Baars and Neser 1999). The impact by natural enemies is like that of competition

among and within species, with some insects impacted by competition to a greater extent than others.

Competition is usually a response to overcrowding and it is often correlated with a variety of factors, including availability of resources. At high population densities, insect species may compete for resources (Sugiura *et al.* 2007), sometimes leading to cannibalism (Okano and Okuda 2018). The impact of competition for food resources is mostly noticeable in the immature stages of endophagous insects, particularly leaf miners that feed and complete their development on one leaf (Stiling *et al*. 1984; Sugiura *et al*. 2007; Thiery *et al.* 2014) compared to ectophagous insects that can move from one leaf to another when crowded, and when forage becomes scarce. These various impacts will in turn lead to increases in mortality (Thiery *et al.* 2014).

In biological control programmes in general, little has been reported on the impacts of competition on the relative success of agents (Crowe and Bourchier 2006; Urban *et al.* 2011; Nzama *et al.* 2014). However, it has been demonstrated that either interspecific or intraspecific competition between and within biological control agents may result in the exclusion of the other and render it an unsuccessful biological control agent (e.g., Hoffmann *et al*. 1993; Boivin and Brodeur 2006).

Although *A. thalassias* has become well-established and widespread on *L. laevigatum* in South Africa, it has a limited impact on the growth or survival of its host (Klein 2011). Currently, there is insufficient knowledge of the limiting factors that may contribute to the low impact of *A. thalassias* but Gordon (2011) suggested that local natural enemies of the immature stages of the moth may be keeping its populations at low levels. Field studies were therefore undertaken to determine the validity of this assumption. The study investigated the role of specific causes of mortality (parasitism, predation, and

overcrowding) in the different immature life stages of *A. thalassias* and whether it is prone to elevated levels of mortality as previously suggested. Furthermore, the aims included determining which life stages are most prone to mortality; how mortality varies seasonally; and how oviposition activity of *A. thalassias* varies seasonally. The methods and findings are reported here.

Methods

Study area

The study was conducted at Walshacres Farm (34°42'S, 19°44'E) near Stanford. The farm, which is currently used for cattle grazing and wildflower harvesting, is invaded by several weed species, in particular *L. laevigatum* and *Acacia saligna* (Labill.) H.L.Wendl. (Fabaceae). The site was chosen on the basis that there was a dense infestation of *L. laevigatum* with both large and small trees on which the biological control agent, *A. thalassias* was common and widespread.

Impact of parasitism, and predation on mortality

Several factors potentially contributing towards mortality and abundance of *A. thalassias* were investigated over a period of 20 months and included field as well as laboratory observations. The processing of samples was undertaken in the laboratory at the Agricultural Research Council, Plant Health and Protection, Vredenburg Campus, Stellenbosch).

Between July 2015 and March 2017, samples were collected from the field site on a weekly basis. Thirty branch tips, each approximately 20 cm long, bearing mined, puffy, and rolled leaves were removed from both small trees (<1 m height) and large trees (>2 m height). The samples from both small and large trees were placed in groups in

separate plastic bags, labelled with the tree size, date of collection, the name of the site and the person collecting as well as the type of leaf for each life stage contained. The leaves were placed in plastic freezer bags (300 x 200 mm) to prevent desiccation and transported in an air-conditioned vehicle to the laboratory for processing. Where possible, leaves were processed immediately on arrival in the laboratory; those that could not be processed immediately were stored in the refrigerator and processed the following day (i.e., within 24 hrs).

Thirty leaves containing early (first) instar larvae (mined leaves), second and third instar larvae (puffy leaves), pre-pupae and pupae (rolled leaves) were removed from the branches of both small and large plants, respectively; placed in separate petri dishes with labels recording tree size, date of collection, the name of the site. The leaves were inspected for the presence and absence of larvae, pre-pupae, and pupae. The numbers of live and dead individuals were recorded, and mortality was attributed to one of the following causes: predation, parasitism, or 'unknown' factors (i.e., no specific cause of mortality could be ascertained). Parasitism of *A. thalassias* larvae, pre-pupae and pupae was confirmed upon inspection of the various stages, and when parasitoid larvae, pupae or exuviae were seen together with the dead *A. thalassias* individuals within the mined, puffy, or rolled leaves. Where possible, the parasitoid larvae and pupae found were reared in the laboratory by placing them in Eppendorf tubes (1.5 ml). The vials were sealed with white tissue paper to allow a flow of air and kept under ambient conditions until emergence of any adult parasitoids. Each of the sample tubes included a label detailing: date of collection and date of emergence; the life stage of the moth within which the parasitoid was found (larvae, pre-pupal or pupal stage); site name and the name of the collector. The parasitoids which were reared

successfully were subsequently sent for identification by Dr S. van Noort of the Iziko Museum, Cape Town, South Africa.

Predation as a possible cause of mortality was recorded concurrently with the parasitism study. Predation of larvae in mined leaves was recorded if there were perforations in the surface of mines. On the puffy and rolled leaves, predation was recorded when leaves were torn or obviously damaged. In all cases larvae, pre-pupae or pupae were missing from the leaf. Leaves containing all immature stages of *A. thalassias* were inspected for the presence of predatory mites and, in particular, physogastric females of *Pyemotes ventricosus* (Newport) (Prostigmata: Pyemotidae), along with dead hosts (larvae, pre-pupae and pupae). These mites have been observed within galls of *D. strobila* on *L. laevigatum* (Gordon 2011; Impson and Lyons 2021) and other gall midges (Hoschele and Tanigoshi 1993).

Impact of larval crowding on mortality

During the investigation into mortality factors described above (i.e., between July 2015 and March 2017), and when the leaves were being inspected for the presence or absence of parasitoids and predators; observations were made to record incidences of overcrowding. This was determined when two or more larvae were observed within a single mine or multiple mines were present in one leaf, and where one or all larvae were dead without any signs of parasitism or predation. Puffy and rolled leaves were also examined for the presence of multiple larvae and pupae. The date of collection, plant size (large or small), the total number of larvae in one leaf, and the number of live and dead larvae within one leaf was also recorded.

Parasitism on mortality of A. thalassias *eggs*

To confirm observations made during previous sampling regarding parasitism of eggs, a total of 500 leaves were collected in November 2018 from Walshacres Farm. The leaves all bore one or more eggs and were placed in plastic bags labelled with the date of collection, name of the site and collector, and transported to the laboratory for processing. The samples that could not be processed on the day of collection were stored in the refrigerator to prevent desiccation and were processed the following day (i.e., within 24 hrs). Each leaf was placed in a petri dish and eggs (n = 651) were inspected and dissected under the microscope for the presence and signs of parasitism. To determine whether eggs were parasitized, a method used by Cônsoli *et al.* (2000) was adopted which entailed examination of eggs for discolouration (i.e., parasitised eggs turn black) and for signs of parasitoid larvae or pupae being discernible within the eggs.

Sleeve trials to determine which life stages of A. thalassias *are being targeted by parasitoids*

As a follow on to the first part of this study and to determine which parasitoids were targeting *A. thalassias* and what life stage was most impacted by parasitoids, a separate sleeve trial was carried out in the field at Walshacres Farm. The study was conducted for a period of twelve months (from March 2017 to March 2018). Every two weeks, 10 fine mesh bags (300 x 200 mm in size) were secured to a tree by sleeving five different branches that had puffy leaves and another five that had rolled leaves. On each visit, different large trees were used for sleeving, and prior to sleeving the branches and leaves were inspected to ensure exclusion of any other insects. The sleeves were secured onto the branches at one end and tied closed using woollen

thread. This method ensured that there was no interference of predators or parasitoids from the outside. All sleeves were left *in situ* for two weeks after which the branch, together with the sleeve, was removed, placed in a plastic bag packed loosely, so as not to crush any insects that may have been present inside the bags and transported back to the laboratory for inspection. This procedure also enabled determination of the seasonality of parasitoids. Any adult *A. thalassias* or parasitoids that had emerged in the sleeves as well the month and date of collection were recorded. Parasitoids were retained for identification and placed in Eppendorf tubes (1.5 ml), labelled with the date of collection and emergence, the life stage where it was collected, site name and the name of the collector. The parasitoids which were reared successfully were subsequently sent for identification by Dr S. van Noort of the Iziko Museum, Cape Town, South Africa.

All the weekly and fortnightly samples of larvae, pre-pupae and pupae that were collected to measure levels of mortality, and data collected to measure the seasonal impact of percentage parasitism on mortality, were grouped according to different austral seasons. Samples collected between September and November were grouped as “spring”, those collected between December and February as “summer”, those collected between March and May as “autumn”, and those collected June to August, as “winter”.

Measurement of plant parameters

Because it had been reported that *A. thalassias* causes more damage on saplings and small trees (<1 m height) than on larger (>2 m height), older trees (Gordon 2011), an investigation into the leaf phenology of the plant was undertaken. Host plant growth parameters were measured by haphazardly selecting and marking three branches (20

cm long) using a ribbon on each of five large and five small trees (i.e., n = 15 per tree size category). The numbers of new and old leaves on the marked branches were recorded once a week for a year (from January to December 2016). New leaves occur only on the terminal extremities of the branches and are characterised by being soft in texture, covered with fine hairs and easily crushed. Older leaves occur anywhere along the branches and are robust and with limited numbers, or a complete absence, of visible hairs.

Assessment of A. thalassias *seasonal oviposition activity*

To assess the oviposition activity (e.g., Micieli and Campos 2003) of *A. thalassias* throughout the year, three branch tips of approximately the same size (15 cm) were randomly collected from 15 large and 15 small trees every two weeks for a year (March 2017 to March 2018). The number of eggs laid on leaves on each of these branch sections was recorded. Only branch tips were inspected because eggs are only found on newly formed leaves which only develop on the terminal extremities of the branches (Gordon 1999).

Statistical analysis

To determine if there were seasonal differences in mortality of each life stage, mean numbers of dead and live larvae, pre-pupae and pupae at each sampling date were calculated using Microsoft Excel. The data were tested for normality and homogeneity of variance to assess whether they met the conditions of ANOVA using the Shapiro-Wilks test. For cases where data did not meet the conditions for ANOVA, non-parametric tests were performed.

To assess whether the mean values between mortality for each life stage (larvae in mines, larvae in puffy leaves and pre-pupae and pupae in rolled leaves) and season

were significantly different, a t - independence test was performed. To determine if the impact of parasitism on mortality differed across different months, a non-parametric Kruskal-Wallis ANOVA was performed, with incidence of parasitism as the dependent variable and month as the independent variable. A histogram demonstrating proportions of larval mortality in the mining stage over different months was generated using XLSTATS. Incidences of parasitism contributing to seasonal mortality of larvae, pre-pupal and pupal stages of *A. thalassias* were calculated using the following formula:

$$\frac{Total\ \ number\ of\ incidences\ of\ parasitism/month}{Total\ number\ of\ larvae/prepupae/pupae\ found} \times 100$$

To determine if levels of parasitism on each life stage of *A. thalassias* (larvae in puffy leaves and pre-pupae and pupae in rolled leaves) differed across seasons, a non-parametric Kruskal-Wallis ANOVA was performed, with parasitoid emergence as the dependent variable and season and each life stage (larvae, pre-pupal and pupal stages) as independent variables. Percentage parasitism of each life stage of *A. thalassias* within sleeves was calculated using the following formula:

$$\frac{Total\ \ number\ of\ parasoids\ emerged/month}{Total\ number\ of\ larvae/prepupae/pupae\ found} \times 100$$

To determine if there were any significant differences in the number of dead larvae in mined leaves that could not be attributed to any specific cause between months, an ANOVA was performed using the Generalised Linear Model procedure of SAS statistical software. Percentage mortality of *A. thalassias* larvae in mined leaves of *L. laevigatum* attributed to unknown factors was calculated using the following formula:

$$\frac{Total\ \ number\ of\ dead\ larvae/month}{Total\ number\ of\ larvae\ in\ mined\ leaves} \times 100$$

To determine the growth parameters *of L. laevigatum*, the mean number of new *L. laevigatum* leaves per branch was calculated for each month (January to December). To determine if there were any significant differences in the number of new *L. laevigatum* leaves per branch (i.e., growth) between months, an ANOVA was performed using the Generalised Linear Model procedure of SAS statistical software.

To obtain an understanding of oviposition activity of *A. thalassias*, each month, the numbers of eggs laid per branch on new leaves of *L. laevigatum* were counted and means were calculated for each month (January to December). To determine if there were any significant differences in the number of eggs laid on new *L. laevigatum* leaves between months, an ANOVA was performed using the Generalised Linear Model procedure of SAS statistical software.

All analyses were performed using Excel or using XLSTAT software (version 7.5.2, Add-in soft, New York, USA) or using STATISTICA 10.0 (Stat soft, Tulsa, OK, USA) and SAS statistical software 9.4 (SAS institute Inc., Cary, NC, USA).

Results

The impact of season on overall mortality of different life stages showed significant differences across all life stages with significantly more dead larvae than live larvae in mined leaves in autumn (t_{1560} = -5.30, p < 0.001) and in winter (t_{1558} = -13.9, p < 0.001) whereas there was no significant difference between the numbers of live and dead larvae in mines in either spring (t_{2570} = 2.00, p = 0.82) or summer (t_{2266} = 0.32, p = 0.75) (Fig. 3.1a). The number of dead and live larvae followed a different trend in puffy leaves. The number of dead larvae in puffy leaves was significantly lower than live larvae in all seasons (autumn: t_{1618} = 7.58, p < 0.001; spring: t_{2626} = 21.18, p < 0.001; summer: t_{2286} =19.80, p < 0.001; winter: t_{1558} = 7.02, p < 0.001) (Fig. 3.1b). In the case

of pre-pupae in rolled leaves, the number of dead pre-pupae was significantly higher than live pre-pupae in all seasons (autumn: t_{1556} = -7.33, p < 0.001; spring: t_{2640} = -3.95, p < 0.001; summer: t_{2214} = -10.18, p < 0.001; winter: t_{1558}= -3.25, p < 0.001) (Fig. 3.1c). In the case of pupae in rolled leaves, the number of dead pupae was significantly higher than live pupae in autumn (t_{1556} = -8. 25, p < 0.05) compared to spring where the number of live pupae were significantly higher that dead pupae (t_{2640}= 6.81, p < 0.05). There were no significant differences between the number of dead and live pupae in either summer (t_{2214} = -0.85, p = 0.4) or winter (t_{1558} = -0.47, p = 0.63) (Fig. 3.1d).

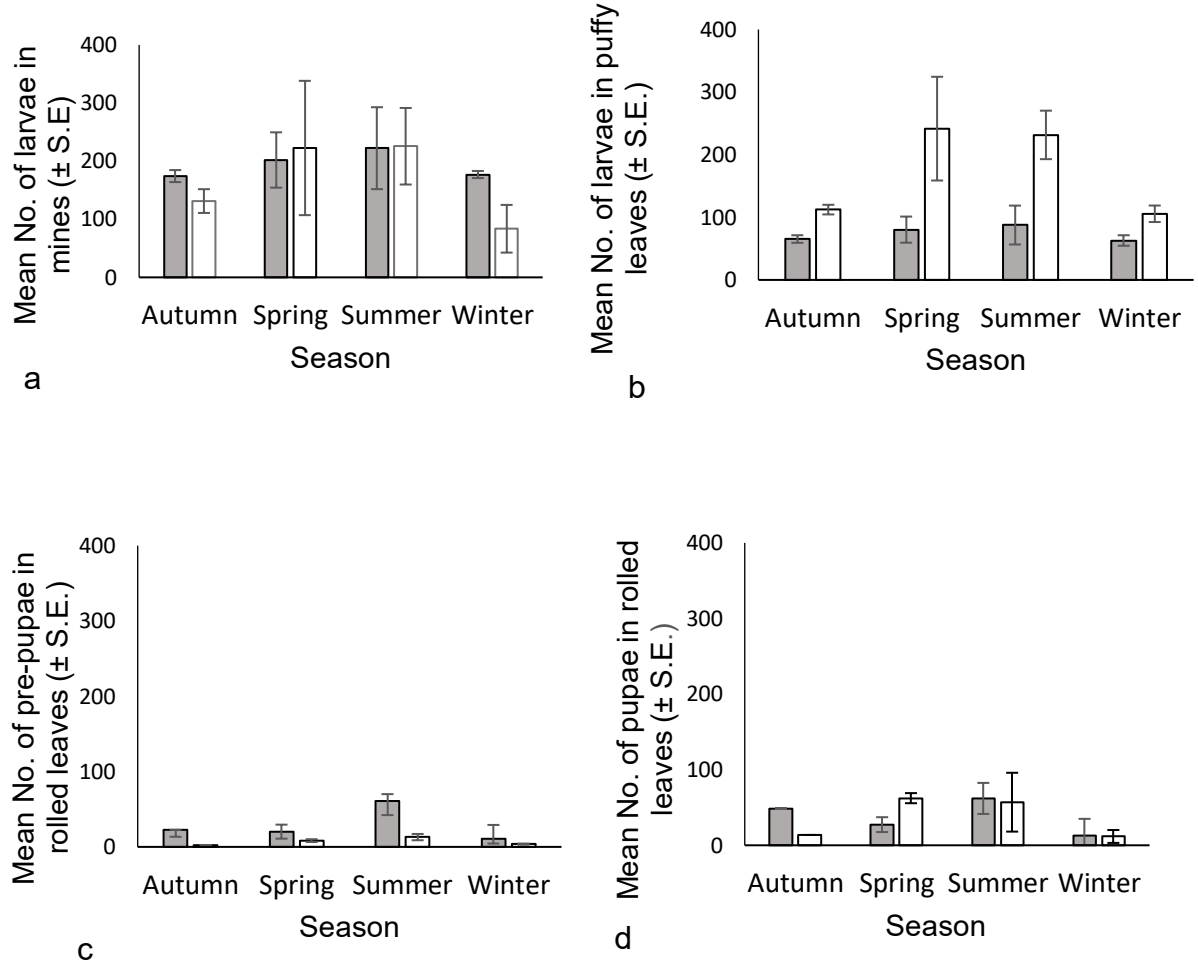


Figure 3. 1. Mean (± S.E.) numbers of dead (shaded bars) and live larvae (unshaded bars) in mines (a); dead and live larvae in puffy leaves (b); dead and live pre-pupae in rolled leaves (c); and live and dead pupae in rolled leaves (d) of *A. thalassias* across seasons. The data presented are for cumulative means measured over three years (2015, 2016 and 2017).

Parasitism, larval crowding, and predation

The inspection and dissection of eggs, in addition to the inspection of early instar larvae in mines, revealed no signs of parasitism of these life stages. However, percentage parasitism varied significantly with month for larvae in puffy leaves and for pre-pupae and pupae in rolled leaves (χ^2 = 199.,20, df = 11, p < 0.001) with levels

being highest during the warmer months and reaching a maximum of 25% in February 2016 (Fig. 3.2).

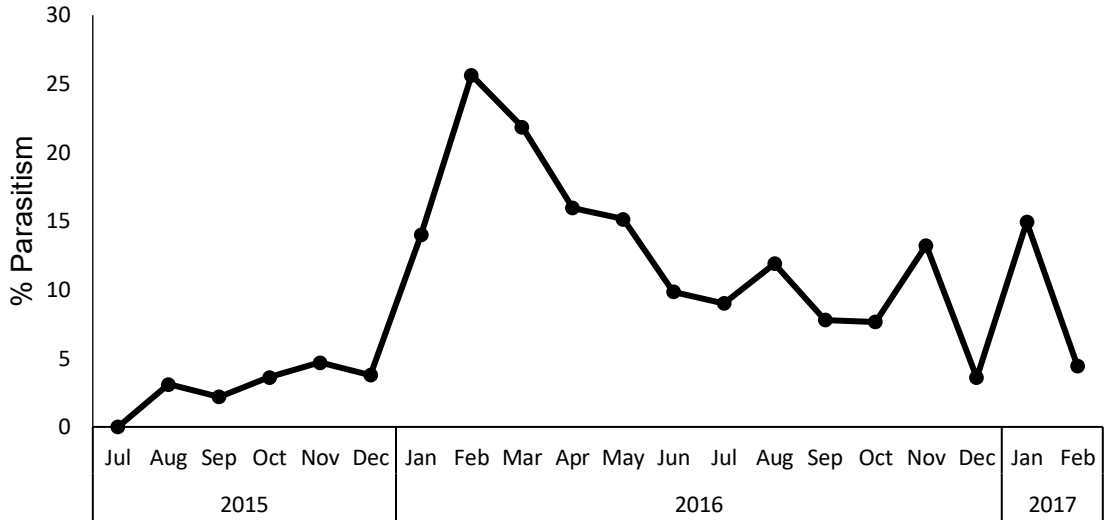


Figure 3. 2. Incidence of parasitism (%) on *A. thalassias* larvae in puffy leaves and pre-pupae and pupae in rolled leaves combined from July 2015 to February 2017.

In conjunction with this, the results of the sleeve experiment showed that levels of parasitoid emergence from larvae in puffy leaves were not significantly different to those from pre-pupae and pupae in rolled leaves and that both life stages were equally impacted by parasitoids ($\chi^2 = 2.88$, df = 1, $p = 0.894$) (Table 3.1). When the impact of season was taken into consideration in the sleeving experiment, the results showed that levels of parasitism varied significantly with season ($\chi^2 = 28.90$, df = 3, $p < 0.001$) for larvae in puffy leaves and for pre-pupae and pupae in rolled leaves (Table 3.1). Even though parasitoids were present in other seasons, they were encountered more regularly during summer months (January and February) in the larvae in puffy leaves. In the case of the pre-pupal and pupal stages in rolled leaves, parasitoid activity was

less distinct with peaks in parasitism recorded in summer (January and February), autumn (April and May) and winter (July) (Table 3.1).

Table 3. 1. Emergence of parasitoids (%) from sleeved samples of larval (puffy leaves) stages and pre-pupal and pupal (rolled leaves) stages of *A. thalassias* on *L. laevigatum* over different months in 2017. Total numbers of parasitoids that emerged each month are shown in parentheses.

Season	Month	Larvae in puffy leaves	Pre-pupae and pupae in rolled leaves
Summer	Jan	25 (15)	15 (4)
	Feb	13 (6)	19 (5)
Autumn	Mar	5 (4)	0 (0)
	Apr	0 (0)	8 (1)
	May	0 (0)	10 (1)
Winter	Jun	4 (1)	11 (1)
	Jul	4 (1)	20 (1)
	Aug	5 (1)	7 (1)
Spring	Sep	0 (0)	0 (0)
	Oct	5 (2)	0 (0)
	Nov	3 (2)	0 (0)
Grand total		64(41)	90 (14)

All of the 45 identified specimens of parasitoids attacking the immature stages of *A. thalassias*, were found to be native, with 42 specimens belonging to the Eulophidae and three to the Pteromalidae. From these families two genera, *Cirrospilus* sp. and *Pteromalus* sp. were recorded from larvae in puffy leaves and pre-pupae and pupal stages in rolled leaves while *Minotetrastichus* sp. was only recorded from larvae in the puffy leaves (Table 3.2).

Table 3. 2. Native parasitoid species attacking different immature (larvae in puffy leaves and pre-pupae and pupae in rolled leaves) stages of *A. thalassias,* a biocontrol agent for *L. laevigatum* in South Africa.

Life stage	**Order**	**Family**	**Subfamily**	**Genus**
Larvae, pre-pupae and pupal stages	Hymenoptera	Eulophidae	Eulophinae	*Cirrospilus* sp.
Larvae in puffy leaves	Hymenoptera	Eulophidae	Tetrastichinae	*Minotetrastichus* sp.
Pre-pupae and pupae in rolled leaves	Hymenoptera	Pteromalidae	Pteromalinae	*Pteromalus* sp.

There were no predatory mites found during examination of larvae, pre-pupae or pupae within puffy and rolled leaves. However, on occasion, small holes or openings were observed at the end of mines, and in such cases, the larvae were missing (45 out of a total 12437 leaves inspected) or dead (1), such observations were believed to be associated with predation since there were no parasitoid exuviae found within the mines.

Mortality of larvae in mines, which could not be attributed to a specific cause, varied throughout the years but was highest in winter (June to August) compared to other months ($F_{11, 64} = 3.61$, $p < 0.001$) (Fig. 3.3).

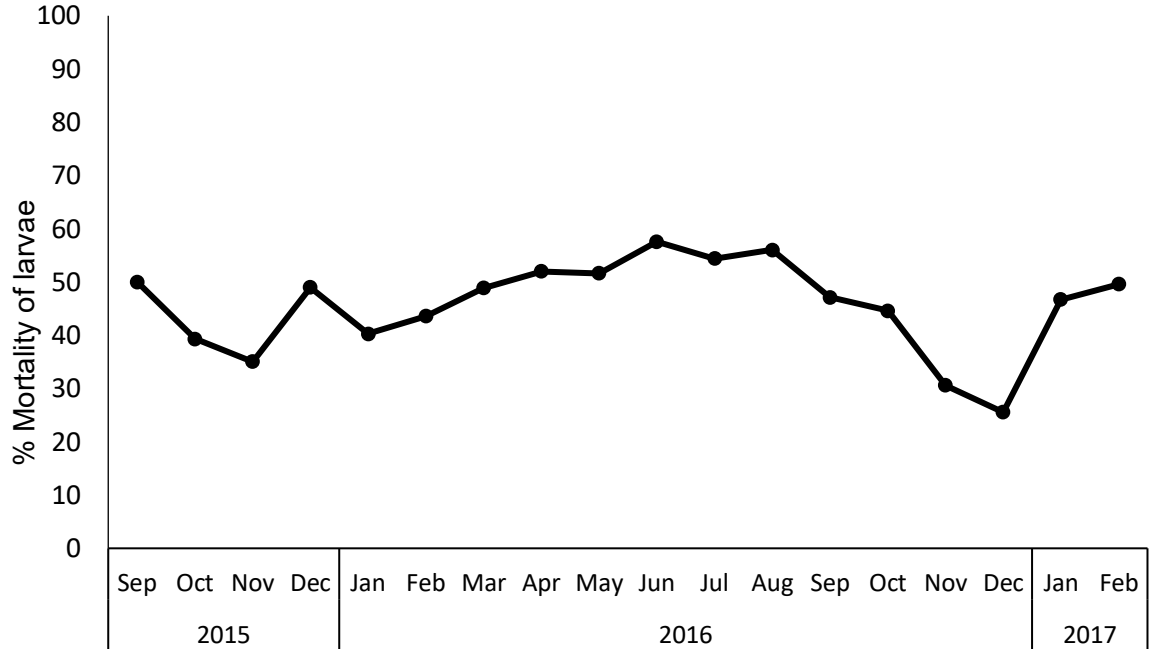


Figure 3. 3. Mortality (%) (solid black line) of *A. thalassias* larvae in mined leaves of *L. laevigatum* attributed to unknown factors from September 2015 to February 2017.

The incidence of two (or more) larvae occurring within a single leaf was only ever recorded in mined leaves, and no more than one larva (if any) ever survived. Mined leaves with only one larva were more frequent than mines with a total of 2, 3, 4 and 5 larvae throughout all seasons (Fig. 3.4 a-d). During the investigation into the impact of overcrowding, larval mortality in mined leaves differed across seasons with winter having the highest mortality (62%) followed by autumn (44%). However, this data only reflects mortality where individual larvae occurred within mines and is therefore independent of any influence of competition on survival (Fig. 3.4 a-d).

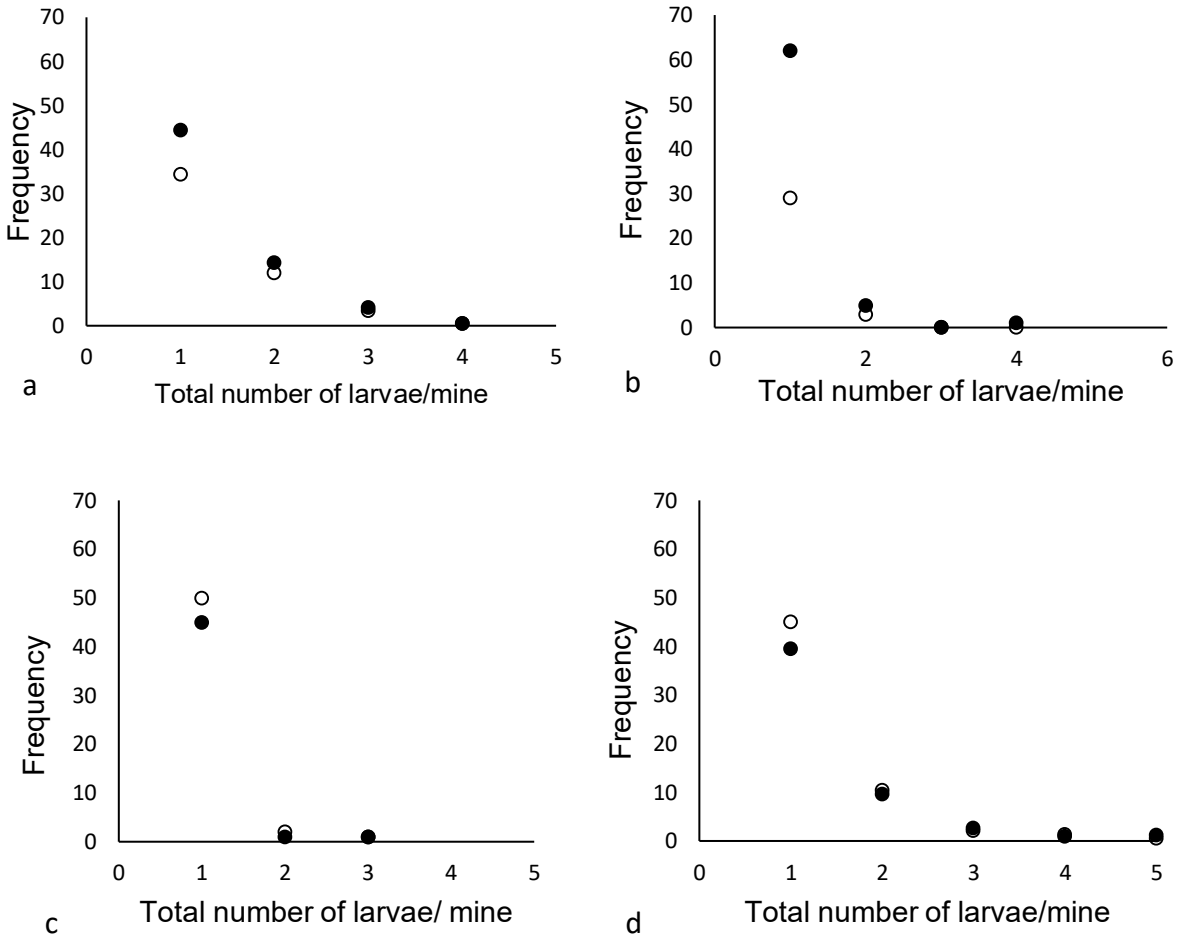


Figure 3. 4. Frequency (%) of live (unshaded dots) and dead (shaded dots) larvae of *A. thalassias* in mines with different number of larvae per leaf across seasons; (a) autumn, (b) winter, (c) spring, and (d) summer.

Plant phenology

The number of new leaves produced varied significantly across seasons ($\chi^2 = 232.36$, df = 11, $p < 0.001$) with most new leaves produced per branch in spring (Fig. 3.5). The results indicate that there was a significant interaction between the number of leaves produced per branch monthly and the tree size (large and small trees) with small trees producing more new leaves per branch than large trees in October and December ($F_{11, 418} = 8.53, p < 0.001$) (Fig. 3.5).

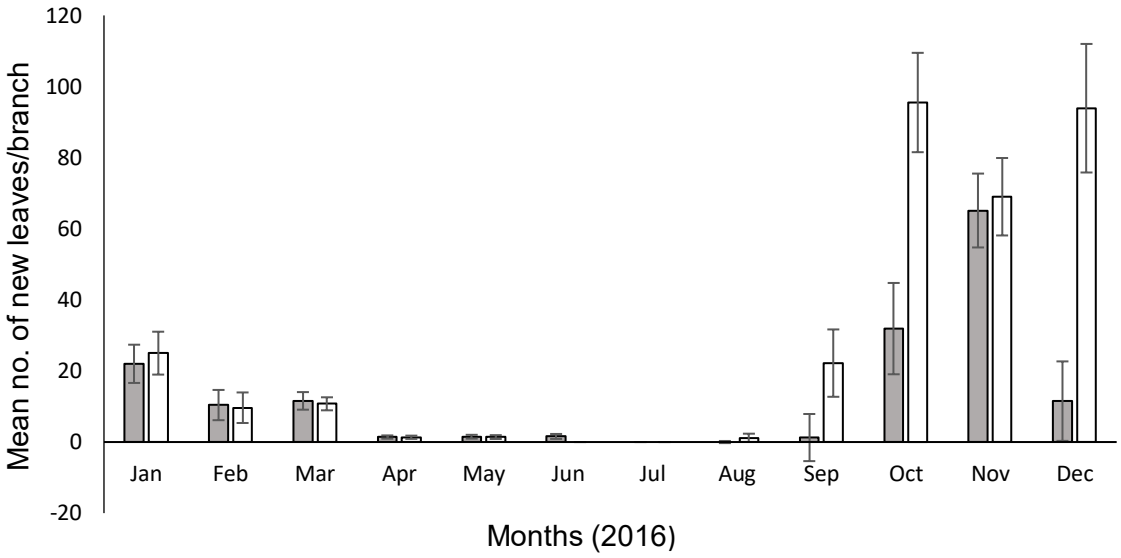


Figure 3. 5. The mean (± S.E.) number of new leaves per branch on large (> 2 m height) (shaded bars) and small (< 1 m height) (unshaded bars) *L. laevigatum* trees during different months in 2016.

A. thalassias *seasonal oviposition activity*

There was a significant variation in the number of eggs laid by *A. thalassias* over different months (χ^2= 141.53, df = 12, p < 0.001) with most eggs laid in summer (January to Feb) and autumn (March) (Fig. 3.6). The results showed that there was a significant interaction between the number of eggs laid monthly and tree size, with more eggs per branch being present on large rather than small trees in January and March ($F_{11, 636}$ = 8.53, p < 0.001) (Fig. 3.6).

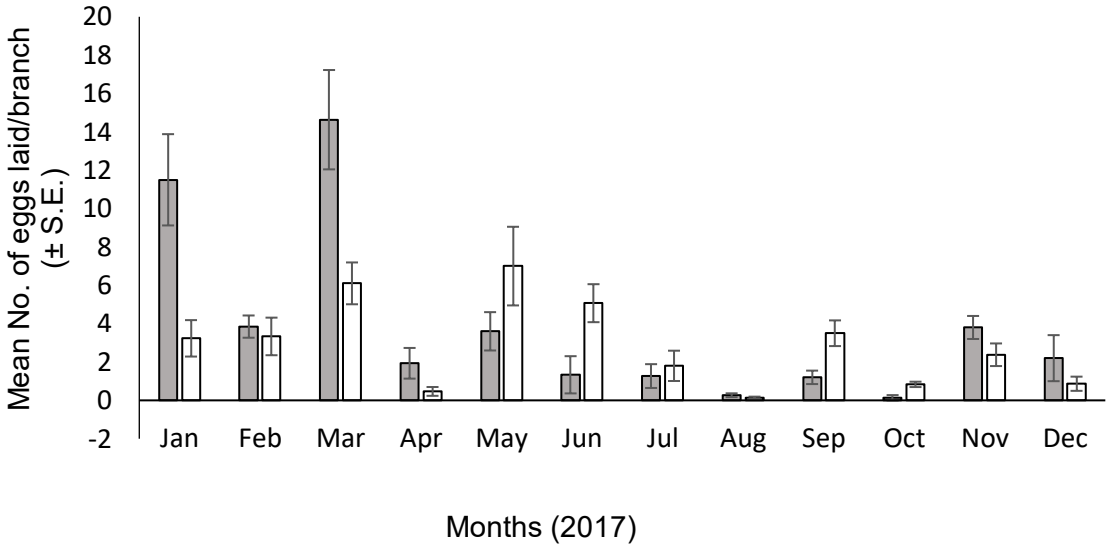


Figure 3. 6. The mean (± S.E.) number of eggs laid per branch by *A. thalassias* on the new leaves of large (> 2 m height) (shaded bars) and small (< 1 m height) (unshaded bars) *L. laevigatum* trees during different months in 2017.

Discussion

The establishment of biological control agents in the field can be impacted by natural enemies, with leaf miners considered to be more susceptible to generalist parasitoids and predators (Paynter *et al*. 2010; Byrne *et al*. 2011). For example, leaf miners in the family Agromyzidae in South Africa are often attacked by a wide range of parasitoids from hymenopteran families such as Eulophidae, Pteromalidae and Braconidae (Nzama *et al.* 2014). Parasitism was generally low in this study (25% maximum), and only recorded on larvae in puffy leaves and pre-pupae and pupae (Table 3.1). Interestingly similar parasitoid families were recorded by Nzama *et al.* (2014) in their study on the leaf mining fly, *Calycomyza eupatorivora* Spencer (Diptera: Agromyzidae), which likewise had limited impact on the overall mortality of this fly. Whilst parasitism was only noticeable in these specific life stages, it is possible that *A. thalassias* could have been targeted by endoparasitoids at an earlier life stage (i.e.,

first instar) but only suffered mortality at the end of the development, as has been observed with parasitoids in other circumstances (e.g., Harvey *et al*. 2016). Additionally, when final instar larvae leave the puffy leaf to roll another leaf to pupate, they may become more susceptible to predation. Similar to other studies (e.g., Hawkins *et al.* 1997), pre-pupae have been reported to be more susceptible to predation when they leave their host plant or the safety of the leaf to move around before pupation.

According to Cornell & Hawkins (1993) insect biological control agents that are parasitized in their native range are likely to be parasitized in the introduced range. However, this does not necessarily mean parasitism in the introduced range will have an impact on the efficacy of agents (Hill *et al*. 2001; Nzama *et al.* 2014). The degree to which parasitoids have an impact on the agent varies for different species; some have no impact whereas in other cases the impact is significant (Paynter *et al*. 2010). Despite elevated levels of parasitism, *A. thalassias* was observed to be damaging on its host in its native range in Australia, which formed the basis for its selection as a biological control agent in South Africa (Gordon 1999). In keeping with observations in Australia, the results of this study have shown no indication that parasitoids are having an effect on the abundance of *A. thalassias* in the introduced range either. Moreover, some studies on predator-prey interactions have demonstrated how the regulation of some insect species by parasitoids and predators is density dependent (Gould *et al.* 1990; Roland 1994; Bonsall and Hassell 1995). Although, in the case of *A. thalassias*, the aspect of density dependent mortality was not investigated, it would need further observations in order to understand its influence on mortality of the agent.

In addition to the generally low levels of mortality resulting from parasitism, the results demonstrated that predation of the immature stages of *A. thalassias* is also low, and thus unlikely to be a contributing factor to the limited success of this agent.

Despite the overall low mortality attributed to factors that were investigated, the study demonstrated that, with the exception of the egg stage, the immature stages of *A. thalassias* are affected by varying levels of mortality in the field which occur throughout the year (Fig. 3.1a-d). Mortality is often associated with various factors other than parasitism and predation (e.g., larval crowding, host plant quality and quantity, and environmental factors) and in some cases with unknown factors (Eber 2004; Zalucki *et al*. 2002; Sugiura *et al*. 2007; Thiéry *et al.* 2014; Doak and Wagner 2015). In the case of *A. thalassias*, it is the larvae in mines and pupae in rolled leaves that were most prone to mortality factors (Fig. 3.1a and d) compared to the larvae in puffy leaves. Mortality was at its lowest in spring compared to other seasons (Fig. 3.1b). This low mortality in spring (Fig. 3.1b) could be a consequence of its coincidence with the time of the season when there is an abundance of new growing tender shoots which are more suitable for larval development and survival. Similar studies by Scheirs *et al*. (2002) and De Bruyn *et al*. (2002) have associated the availability of new leaves with increased nutritional compounds and in turn greater performance and survival of a grass leaf miner *Chromatomyia milii* (Kaltenbach) (Diptera: Agromyzidae:) on *Holcus lanatus* L. (Cyperales: Poaceae). Moreover, the slower recovery of both predator and parasitoid populations after winter, especially in a cold climate as experienced at the study site could have had an influence in the low mortality in spring (e.g., Foccoli 2002).

Highest levels of overall mortality were recorded for the first instar larvae in mined leaves, particularly during winter months compared to other months (Fig. 3.3). Given the limited impacts of parasitism and predation on this life stage, the high mortality recorded could not be attributed to any specific cause. Additionally, although there were incidences of larval crowding within mines, periods of larval crowding did not correspond with increasing mortality as was previously reported by Gordon (2011). In the current study, larval mortality was higher in mined leaves with single larvae compared to mines with multiple larvae (Fig. 3.4). Furthermore, there were far fewer leaves encountered with multiple larvae in comparison to leaves with individual larvae, indicating that larval crowding is unlikely to be playing an important role in mortality and the performance of the moth.

Although impacts of climatic factors on seasonal patterns of mortality were not investigated, there was a significant difference in number of live and dead larvae in mined leaves between the seasons, with the proportion of live larvae highest in spring and summer (Fig. 3.4) suggesting that climatic factors could be impacting mortality to some extent. *Aristea thalassias* is generally known for its sensitivity to leaf quality (Gordon 1999), preferring young nutritious and less tough leaves. During winter months, nutritional quality of the foliage reportedly declines, secondary compounds accumulate, and toughness increases making the leaves less suitable for feeding, development, and survival as is the case with other plant species (e.g., van Asch and Vissern 2007; Despland 2018; Fuentealba *et al*. 2017). The unavailability of new leaves in winter may have disrupted the development and survival of the early instar larvae.

The presence of viable eggs in the field throughout the year showed that the moth is active during all seasons but more pronounced in the warmer summer months

(January to March) (Fig. 3.6). Although many leaf miners are known to oviposit on new growth of their host plants (e.g., Eber 2004; Ayabe and Shibata 2008), and although *A. thalassias* has been reported to do the same (Gordon 2011), the present study found that peak abundance of eggs occurred at the end of the growing season of *L. leavigatum*. This coincided with the time when new leaves were becoming scarce and possibly the time where there were incidences of overcrowding/ more than one larva observed within a mine in summer (Figure 3.4).

The results of this study showed that there is a possible mismatch in the phenological synchrony of *A. thalassias* and its host plant which may affect both the timing of eggs being laid on suitable young leaves, as well the subsequent survival and development of neonate larvae. This type of phenological asynchrony is not uncommon among herbivorous insects (e.g., Visser and Holleman 2001; Russell and Louder 2004; Jones and Despland 2006; van Asch and Visser 2007; Singer and Parmesan 2010). A further indication that phenology of the host plant can be problematic was the failure of initial releases of *A. thalassias* at a site near Hermanus (34°24'S, 19°19'E) where there were limited numbers of new leaves available because it was the end of the growing season (Gordon 1999).

Timing of oviposition by insects is associated with availability and abundance of food and oviposition resources (Kouki 1993; Awmac and Leather 2002; Hoffman and Rao 2010; Sezen *et al*. 2021). Mismatches between the phenologies of host plants and their herbivores may occur because of climate differences between the native and introduced ranges of the two (e.g., Huang and Hao 2018; Renner and Zohner 2018). Although there is no background information on the phenology and life history of *A. thalassias* in its native range, it is known that it was collected in New South Wales and released in coastal regions of the Western and Eastern Cape in South Africa (Gordon

1999). These regions were found to be climatically similar when compared by Dennill and Gordon (1991) who investigated the efficacy of *Trichilogaster acaciaelongifoliae* Froggatt (Hymenoptera: Pteromalidae), a biological control agent of *Acacia longifolia* (Andrews) Willdenow (Fabales: Fabaceae). It was thus assumed that the phenologies of *L. laevigatum* and *A. thalassias* would also be well synchronized. However, the observations from the present study indicate that this was not the case.

On the other hand, annual variability in climate, especially drought periods in hot dry regions such as South Africa, may influence survival and development of herbivorous insects, both directly and indirectly by changes induced through water stress on their host plants (Jamieson *et al*. 2012; Lawal *et al*. 2019). The drought that occurred in the Western Cape during the time of this study (Otto *et al.* 2018) may have negatively impacted the host plants (e.g., by reducing the quality and quantity of new foliage, as well as the timing of such growth). The response of insect herbivores to physiological changes in drought-stressed plants varies with feeding guild (Huberty and Denno 2004; Gely *et al*. 2020) with leaf miners usually being detrimentally affected by reductions in water and nutrient levels in their host plants (De Bruyn *et al*. 2002; Salgado and Saastamoinen 2019; Gely *et al*. 2020).

While findings from this study have given insights into some of the mortality factors influencing *A. thalassias* on *L. laevigatum* in South Africa, the evidence gathered on mortality of the immature stages did not identify parasitism, predation or overcrowding as being key factors to explain the low field efficacy of the agent. A notable omission in this study was adult mortality which might be curbing population expansion and keeping *A. thalassias* at low levels. Of special significance would be the possibility that one or more stages of the life cycle of *A. thalassias* are being influenced by density

dependent mortality which is well known to be pivotal in regulating species' populations (Umbanhowar and Hastings 2002).

More extensive studies across the distribution of *A. thalassias* in the introduced range are needed to further investigate the possible role of predation and parasitoids in regulating the populations of the moth, similar to studies on the winter moth, *Operophtera brumata* (L.) (Lepidoptera: Geometridae), a model species in studies of insect population dynamics (East 1974). Furthermore, investigations into additional 'unknown' mortality factors, which were out of the scope of this study would be beneficial. It is also recommended that fecundity patterns should be determined in order to understand the reproduction of *A. thalassias* and the influence this may have on its field abundance. Finally, the ability of *L. laevigatum* to compensate for insect damage should be part of these studies.

Chapter 4

Discussion and recommendations

Management of weeds using biological control has been applied for decades with varying levels of success (Schwarzländer *et al*. 2018; Hill *et al*. 2020). Some weed management successes have been achieved using biological control as a stand-alone control method (Zimmermann *et al*. 2004) or using an integrated approach where biological control is used with other different control measures simultaneously in an integrated management approach (Moran *et al.* 2005; Clewley *et al.* 2012; Suckling 2013). In other instances, biological control agents have negligible impact or fail completely to control their target plant (Zachariades *et al.* 2017). The failures of biological control agents have been attributed to a variety of factors which affect the performance and behavior of biological control agents in the field, including climate incompatibility, host plant dynamics, natural enemies, and competition (Paynter *et al.* 2005; Coetzee *et al.* 2011). For instance, competition leads to resource depletion which in turn affects the size, fecundity, and fitness of the insect (Dai *et al*. 2019) and consequently, the performance and survival of the insect agent. Climatic factors (i.e., extremely cold temperature) have also been reported to limit the survival, proliferation and establishment of agents in the field (e.g., Cowie *et al.* 2016).

The preliminary studies that were conducted to evaluate the host specificity of *A. thalassias* had proven the moth to be a suitable and potentially effective agent for *L. laevigatum* as it could feed and damage the leaves of its host plant (Gordon 1999). However, despite this reported compatibility of the plant and its introduced agent, *A.*

thalassias is still having a negligible impact on its host plant *L. laevigatum* (Gordon 2011; Klein 2011).

To understand the reasons for the low efficacy of *A. thalassias*, this study investigated several aspects of its biology on *L. laevigatum* in South Africa. The phenology, mortality, and oviposition activity of the moth were investigated under natural conditions to assess the adaptation of *A. thalassias* to its host plant *L. laevigatum*. The development time (Chapter 2) and year-round egg abundance in the field (Chapter 3) support the suggestion that *A. thalassias* is likely to be producing multiple generations throughout year as previously reported by Gordon (1999). Although multivotinism was not investigated in detail in this study, it is one of the attributes often sought after when selecting candidate agents for biocontrol programmes (Boughton *et al*. 2012), with the idea that more generations per year, allow for more rapid population build up. The investigation into the developmental biology of the immature stages of *A. thalassias* also provided useful information in determining which life stages were present in the mined, puffy, and rolled leaves and, in turn, which might be most vulnerable to mortality.

In keeping with Wang *et al.* (2011), findings from the current study show that the success of an agent deployed for the biological control of a target weed is not always predictable. There are other factors that were not measured in this study such as oviposition rates, fecundity, egg and adult mortality, thermal physiology and development over all seasons which may provide further insights into the performance of *A. thalassias*. For example, the number of eggs an insect can produce is an important trait that indicates its potential to generate field populations that are sufficient to cause significant impact on target weed populations (Picciau *et al*. 2017; Seehausen *et al.* 2019). This is critical for all agents, but mostly in instances where a weed is well-

established, and the insect agent would need to reach extremely elevated levels to cause sufficient damage to suppress their host plant (Bourchier *et al.* 2019).

Generally, development rates of insects are predominantly determined by temperature regimes at different times of the year (Chidawanyika and Terblanche 2011). Their fitness, feeding habits, metabolism, dispersal, reproduction, behavior, development, and survival are influenced by environmental conditions (Griffith 2018). As such, the efficacy of a biocontrol agent against a target weed may be limited by climate (Byrne *et al.* 2004; Coetzee *et al.* 2007; Robertson and Zachariades 2008; Skandalis *et al*. 2011; Griffith 2018). While this study has demonstrated the ability of A*. thalassias* to complete its life cycle on *L. laevigatum* under ambient conditions in spring, development of the agent during other seasons was not investigated here. There are other factors that need further investigation which could impact on the agent, including management of the plant, in terms of large clearing operations, and the ability/inability of the agent to find new populations on which to thrive.

In Chapter 3, the potential causes of mortality of the immature stages, and oviposition activity of *A. thalassias* in the field, were investigated. The highest mortality faced by *A. thalassias* is at the first instar stage, (within the serpentine mines), which may explain the low impact the agent is having on the host plant, since many early instar larvae are not able to develop and thus cause sufficient feeding damage for reductions in photosynthesis or leaf abscission. Similar observations were made by Nzama *et al*. (2014) who showed that mortality of the early developmental stages of a leaf-mining fly *C. eupatorivora*, a biological control agent of *C. odorata* in South Africa limited the levels of damage inflicted by the fly in the field. Also, the mortality recorded in pre-pupal and pupal stages (in rolled leaves) could further be contributing to reduced populations of the moth (e.g., Bonsignore *et al*. 2019). The second and third instar

larvae within puffy leaves, the stage that exerts the greatest impact on *L. laevigatum* by causing leaf abscission, suffered less overall mortality than that recorded for early instar larvae within mined leaves. This distinction also supports the interpretation that high mortality of the first instar larvae could be the driving force in the poor success of this agent.

Long term studies on population dynamics of insects have demonstrated that density dependent factors such as parasitism and predation play a key role in regulating insect populations (Roland 1994; Bonsall and Hassell 1995). However, evidence of the influence of density dependence and relationships with natural enemies on weed biological control agents is lacking. Some studies have either demonstrated parasitoids having a minimal impact on successful agents, even under high levels of parasitism (e.g., Kula *et al.* 2010; Boughton *et al.* 2012; Wheeler *et al*. 2017), or in cases where parasitism is low and agent mortality is high, parasitism is assumed to have no direct effect on biological control (Nzama *et al*. 2014). On the other hand, however, impacts of parasitism on biocontrol agents has also been reported as being substantial, where unsuccessful agents are heavily parasitized (e.g., Paynter *et al.* 2010). Although this study showed that parasitism was low on *A. thalassias*, like other studies (e.g., Nzama *et al*. 2014), there was insufficient evidence to suggest that parasitoids are regulating the overall levels of abundance of *A. thalassias* in South Africa. Long term studies are needed in order to understand if density dependent regulators, including predation and parasitism, determine the population density of *A. thalassias* in the field. Furthermore, studies to investigate the additional 'unknown' factors, which were having more of an influence on mortality, than any of the investigated factors were, are recommended.

Due to sensitivity of leaf miners to leaf quality and age, their impact is often observed on actively growing plants and seedlings rather than old and crowded trees (Baars and Neser 1999). Gordon (2011) made similar predictions that mining of new leaves by *A. thalassias* could be more prevalent on seedlings and small trees than large old trees of *L. laevigatum*. In this study, *A. thalassias* was prevalent on both large and small trees, with large trees having highest levels of oviposition activity. The agent prefers feeding on new growth of leaves, however, the large trees generally have very few of these compared to the small trees and consequently there are generally fewer mines observed in large trees than small trees (Gordon 2011). For this reason, it would therefore seem the agent is more likely to have a significant impact on small trees than large trees. The nature of trees retaining mature leaves for one or more years and largely only producing new flush of growth during the growing season is common among perennial trees (Pérez-Llorca and Munné-Bosch 2021). Therefore, the scarcity of new growth of leaves on mature perennial trees could be one of the reasons leaf miners generally perform poorly as biological control agents against weedy perennials (Zalucki *et al.* 2007; Sinclair and Hughes 2010). If this is the case, biological control using *A. thalassias* could be used in conjunction with other methods such as mechanical control where the growth of new seedlings after clearing or fire would be curtailed by biological control and the overall impact of the weed reduced. Furthermore, investigations into the impacts of leaf toughness on larval mortality would make a valuable contribution into understanding the low efficacy of the agent, *A. thalassias,* and are therefore recommended.

For a biological control programme to be successful, it is ideal that the phenologies of both the host plant and its herbivorous natural enemies be in synchrony (Posledovich *et al.* 2018). However, when there is a malalignment, due to climatic factors for

example, it could negatively affect the performance of the natural enemy and render the biological control efforts ineffective (Fuentealba *et al.* 2017; Renner *et al.* 2018; Despland 2018). It is likely that the drought that occurred in Western Cape region (Otto *et al.* 2018) during the time that this study was carried out could have compounded mismatches between the phenologies of *L. laevigatum* and *A. thalassias,* thus inducing mortality of the agent. When drought occurs, plants experience several changes such as disruption in water flow, nutrient deficiency and loss of chemical defense compounds (Fahad *et al.* 2017; Sharma *et al.* 2020). Drought-stressed plants are therefore sometimes more vulnerable to herbivory (Gely *et al*. 2020). However, the negative impacts of drought on plants may render them unsuitable for some types of herbivores (Connor *et al.* 1994; Yarnes and Boecklen 2006; Mutamiswa *et al.* 2019; Gely *et al*. 2020). Since *A. thalassias* is known for its preference for young leaves, desiccation of *L. laevigatum* leaves as a result of drought may have restricted the performance of the agent in the field (as reported in Chapter 3).

On the other hand, mismatches between host plant and insect agent in the introduced range are often associated with climate differences between the two regions (Byrne *et al.* 2002). When a mismatch is evident, it becomes necessary to determine the climates of the native and introduced ranges along with the tolerances of the insect to climate extremes (May and Coetzee 2013). Although climate differences between the native (New South Wales in Australia) and introduced range (Eastern and Western Cape in South Africa) for *A. thalassias* were not investigated in this study, Dennill and Gordon (1991) investigating *T. acaciaelongifoliae,* found these regions to be climatically similar. This needs to be validated for *A. thalassias* with respect to differences in its performance between its native and introduced ranges as it may have different eco-physiological requirements compared to *T. acaciaelongifoliae*.

Leptospermum laevigatum is a competitor to the native fynbos vegetation and considered a highly invasive species that needs a successful management programme in South Africa. The post release assessment of *A. thalassias* as a biological control agent of *L. laevigatum* has provided insights on constraints to the current biological control programme but further investigation is needed to determine why *A. thalassias* is not performing more effectively and how its performance as a biological control agent might be enhanced.

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