The thermal physiology of *Stenopelmus rufinasus* and *Neohydronomus affinis* (Coleoptera: Curculionidae), biological control agents for the invasive alien aquatic weeds *Azolla filiculoides* and *Pistia stratiotes* respectively

THESIS

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ABSTRACT

Water lettuce, *Pistia stratiotes* L. (Araceae), and red water fern, *Azolla filiculoides* Lam. (Azollaceae), are floating aquatic macrophytes that have become problematic invaders in numerous South African waterbodies. Two weevils, *Neohydronomus affinis* Hustache 1926 (Coleoptera: Curculionidae) and *Stenopelmus rufinasus* Gyllenhal 1936 (Coleoptera: Curculionidae), are successful biological control agents of these two species, respectively, in South Africa. However, nothing is known about the thermal physiology of these two species Therefore, the aim of this study was to investigate the thermal physiologies of these two species to explain their establishment, distribution and impact in the field.

Laboratory based thermal physiology trials showed that both weevils were widely tolerant of cold and warm temperatures. The CT_{min} of *N. affinis* was determined to be 5.5 ± 0.312°C and the CT_{max} was 44 ± 0.697°C, while the CT_{min} of *S. rufinasus* was 5.4 ± 0.333°C and the CT_{max} was 44.5 ± 0.168°C. In addition, the lower lethal temperatures were -9.8 ± 0.053°C and -7.2 ± 0.19°C, and the upper lethal temperatures were 42.8 ± 0.053°C and 41.9 ± 0.19°C respectively. These results suggest that both species should not be limited by cold winter temperatures, as previously thought. This is evident in the field, where *S. rufinasus* has established widely on *A. filiculoides*, despite local cold climates in some areas of the plant's distribution. Even though *N. affinis* has a similar thermal range, and should therefore theoretically reflect a similar distribution to *S. rufinasus* throughout South Africa, its distribution is limited by the range of its host, which is restricted to the warmer regions of the country, as is its biocontrol agent.

Using the reduced major axis regression method, the development for *N. affinis* was described using the formula $y=12.976x+435.24$, while the development of *S. rufinasus* was described by

y=13.6x+222.45. These results showed that *S. rufinasus* develops much faster, in fact almost twice as quickly, than *N. affinis*. Using these formulae and temperature data obtained from the South African Weather Service, *N. affinis* was predicted to complete between 4 and 9 generations per year in South Africa, while *S. rufinasus* was predicted to complete between 5 and 14 generations per year around the country.

This study showed that although the native range of these two species is warm temperate to tropical, they possess sufficient thermal plasticity to not only establish, but also damage their respective host plants in far cooler climates. Thus, in South Africa *N. affinis* and *S. rufinasus* are limited by the distribution of their target weeds and not climate.

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CHAPTER ONE

INTRODUCTION

1.1 Problem statement

This thesis focuses on insects that have been used as agents to control or suppress two invasive aquatic weeds, through biological control. Invasive aquatic weeds are plants that are deliberately or unintentionally introduced into water bodies outside of their native range, which result in considerable economic, social and environmental damage (Hill 2003). The biological control programme against aquatic weeds in South Africa has been highly successful with the complete control of four of the five main species (Hill and Coetzee 2017). However, one of the criticisms levelled at biological control is that it is not 100% effective, in other words the agents do not always establish, and if they do, they do not always reduce weed populations to an acceptable level. One of the main reasons given for the lack of effectiveness is climate incompatibility (Byrne *et al.* 2004). This thesis investigates the thermal physiology of two tropical insects that have been used against two aquatic weeds in subtropical to cool temperate climates in South Africa to further understand the role of climate, and in particular temperature, in success or failure of weed biological control programmes.

1.2 Biological control of weeds in South Africa

Biological control of weeds involves the introduction of host-specific insects and pathogens in order to reduce populations of the targeted species to below an economic or ecologically defined threshold (McFadyen 1998). According to Neser and Annecke (1973), the first deliberate release of a biological agent in South Africa was in 1913 with the introduction of the South African cochineal insect, *Dactylopius ceylonicus* (Green) (Hemiptera: Dactolopiidae) for drooping prickly pear, *Opuntia monacantha* Haw. (Cactaceae). The cochineal attained

complete control of the cactus, which shortly thereafter was no longer considered a problem (Moran and Zimmermann 1991; Moran *et al.* 2013). Since then, a number of weed biological control programmes have been initiated, thus South Africa has had a long history of using biological control as a means to reduce populations of invasive alien weeds, mostly those that impact the environment and water supply, and less so agricultural weeds (Moran *et al.* 2013). Zachariades *et al.* (2017), in a recent review, note that in South Africa, biological control contributes significantly to the control of 34 of the 59 invasive alien plant species on which biological control agents have been established. Fourteen of these target species are considered to be under complete control, with no need for any other control intervention (Klein 2011). Three of the most successful biological control programmes have been against Australian *Acacia* species, cacti and, importantly for this thesis, floating macrophytes (Klein 2011).

1.3 Floating aquatic weeds worldwide, and specifically in South Africa

There are three main problematic floating aquatic plant species native to South America that have been introduced in many parts of the world, both in tropical and subtropical regions, which have had significant socio-economic and ecological impacts (Mitchell *et al.* 1990). These species are *Eichhornia crassipes* (Mart.) Solms-Laub. (Pontederiaceae) (water hyacinth); *Pistia stratiotes* L. (Araceae) (water lettuce) and *Salvinia molesta* D.S. Mitch. (Salviniaceae) (salvinia) (Coetzee and Hill 2009). In South Africa, an additional two species make up the notorious "big bad five", *Myriophyllum aquaticum* (Vell. Conc.) Verd. (parrot's feather), which is not free-floating, and *Azolla filiculoides* Lam. (Azollaceae) (red water fern), while *Salvinia minima* Baker (Salviniaceae) and *Azolla cristata* Kaulf. (Azollaceae) (Mexican azolla) are in the early stages of establishment (Coetzee *et al.* 2011).

There are two drivers of invasion of these macrophytes in South Africa following their establishment: the lack of co-evolved natural enemies in their adventive range (McFadyen 1998); and disturbance; the presence of nitrate- and phosphate-enriched waters, associated with urban, agricultural and industrial pollution that promotes plant growth (Coetzee and Hill 2012). Whilst the latter driver is difficult to address without a major intervention from government, the former can be remedied through the introduction of biological control agents. The biological control programme against aquatic weeds in South Africa has been highly successful (see Coetzee *et al.* 2009; Hill and Coetzee 2017, and references therein), but there is still the perception that it is not quite as successful as it has been in more tropical parts of the world (Hill and Olckers 2001; Julien 2001). Whilst this variable success has been ascribed to climate, in particular temperature, it has seldom been investigated. However, an exception was the study by Coetzee *et al.* (2007) who investigated the thermal physiology of the water hyacinth biological control agent, *Eccritotarsus catarinensis* (Carvalho) (Hemiptera: Miridae) and predicted its establishment and efficacy on the weed throughout South Africa, suggesting that this insect would be less effective in the cooler, high elevation sites characterized by cold winters. Field surveys have shown that *E. catarinesis* has established far wider than anticipated (Coetzee *et al.* 2011) and suggested that microclimates on host plants could be important. Thus, I investigated the thermal limits of two species of weevil, *Neohydronomus affinis* Hustache 1926 (Coleoptera: Curculionidae) and *Stenopelmus rufinasus* Gyllenhal 1936 (Coleoptera: Curculionidae) that have been used in South Africa for the control of *P. stratiotes* and *A. filiculoides* respectively, to determine if their performance and ultimate impact on the weed throughout South Africa could be linked to the climate in which the weed occurs.

1.4 Thermal physiology of insects

Insect physiology is the branch of biology dealing with the functions and activities of living insects, including all physical and chemical processes (Klowden 2013). Physiological studies are broadly defined to be helpful to ecologists, but generally assess the interactions between organisms and their environment (Schmidt-Nielsen 1972), in this case temperature. The science of physiology details how organisms work, and how these functions differ during the individuals' lifetimes, and over long evolutionary periods – through the changes in the environment in which the organism lives (Schmidt-Nielsen 1972). Thermal physiology may be a significant factor underlying the ecological and evolutionary success of organisms (Gilchrist 1995; Pörtner and Farrell 2002), as the geographic distributions of many species are limited by climate (Gaston 2003). Thus insect thermal physiology is the study of the response in insects to temperature change (Clarke 1967).

Seastedt (2014) identified temperature, nutrients, moisture and fire as abiotic factors that all affect the fitness of both species involved in biological control initiatives: the target weed species and the biological control agent itself, however, my study only focuses on temperature as the main abiotic factor that indirectly influences the control success in the biocontrol programmes. According to Somero (2010), it is important to identify physiological factors that are involved in predicting where a species will occur and how will it perform in certain climates. While the majority of studies investigating the relationship between insects and their thermal environment have been largely related to agriculture and forestry (Trudgill *et al.* 2005), experimental tests of the physiological responses of individual organisms to different types of environmental changes (i.e. temperature and rainfall) can be used to predict future biological control performance (Pörtner and Farrell 2002; Hoffmann and Sgrò 2011; Huey *et al.* 2012). Although thermal physiology is not always assessed in biological control programmes, as it is

time consuming, it is important in predicting the possible success rate of the insects released onto the target weed, and does not require extensive funding to complete (McEvoy and Coombs 1999; Coetzee *et al.* 2007). Pre-release thermal physiology studies have been considered to be important to choose a well-adapted agent before release (May and Coetzee 2013), although there is no evidence to suggest that any agent has not been released in the world due to thermal incompatibility. Therefore, these studies are now considered to be more important in post-release evaluations, to explain the performance of an agent in the field (McClay and Hughes 1995). Indeed, Byrne *et al.* (2004) state that some 44% of weed biological control agents fail to establish because of climatic incompatibility of the agent, usually an insect, to its new area of introduction.

1.4.1. *Responses to extreme temperatures*

Insects have a variety of thermal responses to extremes of temperature, and two of these values are easily measured. The first is the upper and lower critical temperature (CT_{max} or CT_{min}), these are the temperature extremes at which the insect immediately loses locomotory function. Here, individuals are exposed to temperatures changing at a given rate and the temperature at which they are knocked down or show spasms is recorded (Roberts *et al.* 1991; Klok and Chown 1997; Gilbert and Huey 2001). Beyond these temperatures, the insect cannot respond to any further change in temperature in the same direction, and therefore becomes vulnerable to predation or further temperature excess. This type of thermal limit assessment is a dynamic measure of thermal tolerance. The second, a static measure, is upper and lower lethal temperatures (LT_{50}s) which defines extreme temperature limits from which organisms cannot recover after a prolonged exposure, and are referred to as mortality assays. These thermal limits can be determined in a few days of experimentation, by exposing groups of the insects to given extreme temperatures for a fixed period, following which recovery (% survival in the group) is

assessed at 'room' temperature after several hours or days (Krebs and Loeschcke 1995), and give some indication of the thermal tolerance of the insect.

Deciding on which method to use in determining an insect's response to extreme temperatures should be based on the rationale for the study. Stress levels in mortality assays (i.e. lethal temperatures) are often severe in comparison to knockdown assays (i.e. critical thermal limits), suggesting that these experiments should be limited to more sedentary stages of insects that are not able to respond behaviourally, such as egg and pupal stages, or galling/mining species. The results of knockdown assays would be more relevant to ecological studies, such as those investigating the survival and fertility of mobile insects in the field, because the stress levels would be less severe, allowing a behavioural response (Sørensen *et al.* 2001).

1.4.2. *Temperature-dependent development*

In predicting the distribution and success of a biological control agent, or understanding reasons for the lack of establishment and impact, insect development (degree–day) models, using temperature and time to predict the number of generations that an insect can complete at a given locality are useful (Coetzee *et al.* 2007). Insect development time is strongly dependent on environmental temperature (T_e); as T_e increases, rate of development increases up to an optimum temperature (T_o), above which development rapidly decreases and ceases. Conversely, as T_e decreases, developmental rates slow down, until the lower developmental threshold temperature (T_b) is reached, beyond which development ceases. Generally, the rate of insect development is linear between T_b and T_o, which allows the calculation of two thermal constants – the number of degree days, DD, required to complete development at the base temperature, T_b. Determination of these constants allows for comparison of developmental rates between species at temperatures within their developmental range, and comparisons of

temperature thresholds and optima, providing an understanding into the adaptation and ecological strategies of species in relation to their thermal environment (Trudgill *et al.* 2005).

Determining the degree day requirements of biological control agents has been conducted in a handful studies to improve understanding of the agents field distribution and subsequent level of control. For example, McClay and Hughes (2007) concluded that some of the variation in establishment of the stem-mining weevil, *Mecinus janthinus* Germar (Coleoptera: Curculionidae), a European species that has been established in North America as a biological control agent against the invasive European weeds *Linaria vulgaris* P. Mill. and *Linaria dalmatica* (L.) P. Mill. (Scrophulariaceae), may be due to a lack of sufficient time for *M. janthinus* to develop to the adult (overwintering) stage in less favourable climates. Similarly, Coetzee *et al.* (2007) attributed the lack of control by the mirid, *E. catarinensis*, on water hyacinth, to cold winter temperatures in the high lying interior of South Africa, and the delphacid, *Megamelus scutellaris* Berg (Hemiptera: Delphacidae) was also predicted to fare no better as a control agent of water hyacinth in similar cold high lying areas of South Africa (May and Coetzee 2013).

Thus, studies such as these, and those investigating responses to extreme temperatures, provide insight into why biological control agents thrive in some areas, but fail to establish in others, based on their thermal requirements. This provides the rationale for this thesis, which aims to understand the thermal requirements of two biological control agents released against two aquatic weeds in South Africa, as a means to explain their success.

1.5 The study system

1.5.1 *Azolla filiculoides*

Description and distribution

Azolla filiculoides is a floating fern that is indigenous to South and western North America (Lumpkin and Plucknett 1980), but has been introduced into Europe, southern Africa, China, Japan, southern Australia and Scandinavia (West 1953; Birkenbeil 1974; Bernhardt 1991; Kohler 1995; Ferreira *et al.* 1998; Janes 1998a,b; Hussner and Lösch 2005; Rune and Jørgensen 1997). The first description of *A. filiculoides* was from 1783, when the genus *Azolla* was botanically established by Lamarck (Kannaiyan and Kummar 2006). The origin of the generic name *Azolla* is thought to be derived from a conjugation of two Greek words meaning 'to dry' and 'to kill', inferring that the fern is killed by drought (Moore 1969; Lumpkin and Plucknett 1980). Species within the genus *Azolla* has no generally accepted taxonomic framework, primarily as a result of the plant's diminutive structure, morphological and phenotypic plasticity (Stergianou and Fowler 1990; Saunders and Fowler 1992) resulting in numerous synonyms. Currently, there are seven extant species and the genus is divided into three sections (Saunders and Fowler 1992; 1993): section *Azolla*, which includes *A. filiculoides* Lam., *A. rubra* R. Br. (often regarded as a variety of *A. filiculoide*s), *A. caroliniana* Willd., *A. microphyll*a auct. Non Kaulf, and *A. mexicana* Presl; section Rhizosperma, which includes *A. pinnata* R. Br. var. *africana* (Desv.) R.M.K. Saunders and K. Fowler, stat. Et comb. Nov., *A. pinnata* R. Br. var. *asiatica* R.M.K. Saunders and K. Folwer, subsp. nov., and *A. pinnata* R. Br. var. *pinnata*; and section Tetrasporocarpia, which includes *A. nilotica* Decne. ex Mett. Taxonomic problems have primarily centred around the five closely related species in section *Azolla*, with the consequence that research is focussing on cytological studies to resolve these (Stergianou and Fowler 1990; Evrard and van Hove 2004; Madeira *et al.* 2013). A more recent revision of the genus *Azolla* using molecular techniques has combined *A. microphylla* and *A. mexicana* into a

single species, and a new species, *Azolla cristata* Kaulf. which had previously been misidentified as *A. pinnata* var. *africana* is also invasive around the world (Madeira *et al.*2013; 2016).

Azolla filiculoides is a small aquatic heterosporous fern, rarely larger than 25 mm (O'Keeffe 1986) (Fig. 1.1). The genus is unique in that it grows in association with a heterocystous cyanobacterium (blue-green alga), *Anabaena azollae* Strasburger (Nostocales: Nostocaceae), which is located in cavities in the dorsal leaf-lobes (Ashton and Walmsley 1984). This symbiotic association is the only one known between a pteridophyte and a cyanobacterium (Ashton and Walmsley 1984). The *Azolla* macrophyte consists of a main rhizome, which branches into secondary rhizomes. These all bear alternately arranged small leaves. Ventrally, unbranched adventitious roots hang down into the water from nodes. Nutrients are absorbed directly from the water by the roots. In very shallow water, however, the roots may touch the soil thus deriving nutrients from it (Wagner 1997). Rao (1936) reported that in *A. pinnata*, once the roots attain a length of 40-50 mm they drop off. Root hairs found along the length of the root provide accommodation for a large number of protozoa, algae and soil particles (Rao 1936).

As with many other plant species, *A. filliculoides* exhibits an increased growth rate with increased photoperiod (Shi and Hall 1988). The interactive effect of light intensity, photoperiod, temperature, pH and nutrient availability have all been shown to have an effect on the species' sporulation (Janes 1998a). Janes (1998b) conducted laboratory experiments to demonstrate that a maximum germination rate was reached at 20ºC; while a constant temperature of 5ºC resulted in the cessation of germination. Although *A. filiculoides* grows best at 15-20ºC (Tung and Watanabe 1983; Watanabe and Berju 1983), Janes (1998a) described *A.*

filiculoides as a frost tolerant species as plants were able to survive ice for a week although some parts of the plants above the ice were killed.

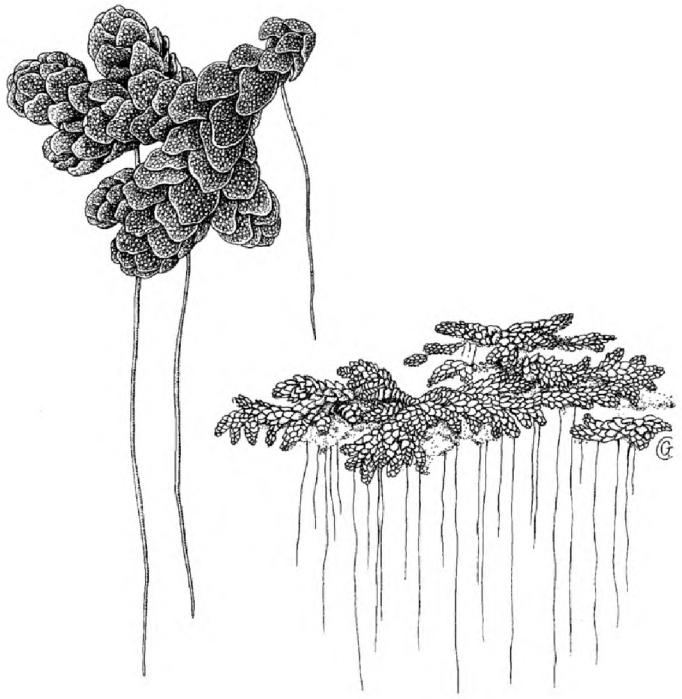

Figure 1.1 : *Azolla filiculoides*. (Drawn by G. Condy, first published in Henderson (1995).)

Azolla filiculoides is now widely distributed, having been introduced to a number of countries in which it is not indigenous (Ashton 1992) (Fig. 1.2). The plant has been dispersed by a variety of mechanisms, of which man has become the most significant (Lumpkin and Plucknett 1980). *Azolla filiculoides* was introduced to South Africa in 1948 as an aquarium plant (Oosthuizen and Walters 1961; Jacot-Guillarmod 1979). According to Szczęśniak *et al.* (2009), *A. filiculoides* was first recorded in Europe towards the end of the 19th century. The species may have been transported accidentally in ballast tanks of ships, in water with fry, or directly as an ornamental as Janes (1998a) noted the deliberate introduction of the plant as an ornamental into Europe through mainland Britain at the end of the nineteenth century. As a result the plant appeared independently in different places in Europe at about the same time. *Azolla filiculoides*

is able to reach new regions by natural transporting vectors, e.g. waterfowl. The species has been recorded as established in 21 countries in Europe, three African countries, six countries in Asia, and New Zealand and Australia (CABI 2018). One of the main reasons for the spread of the weed was as a model plant to study the *Azolla-Anabaena* symbiosis (Carrapiço 2010)

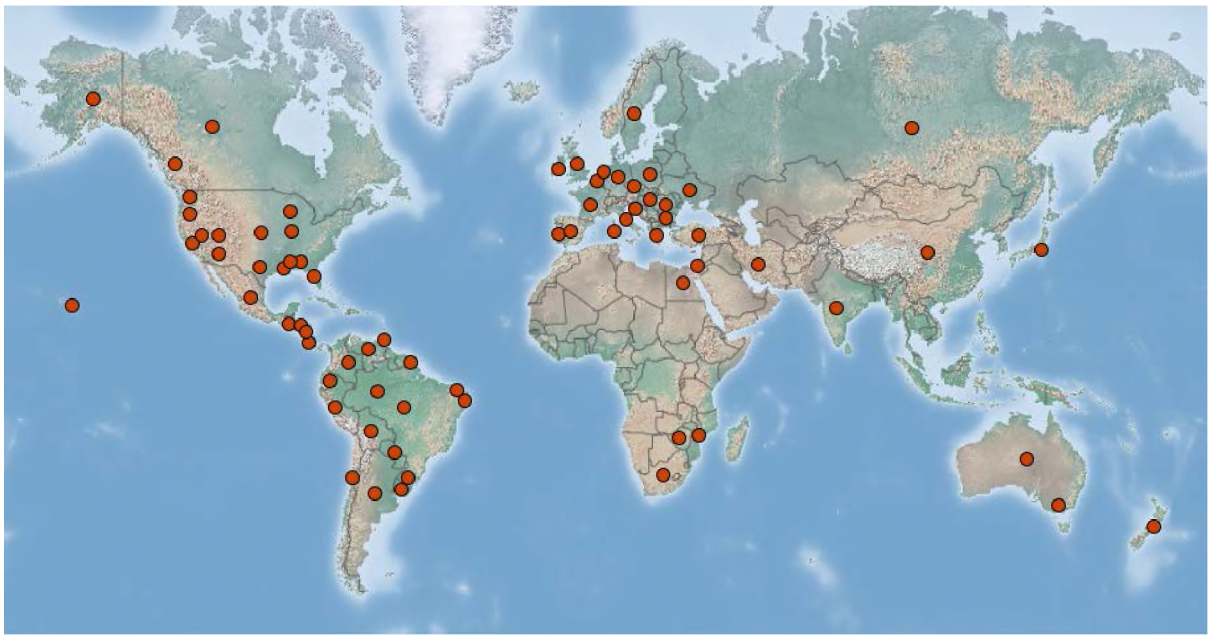

Figure 1.2. Global distribution (native and adventive) of *Azolla filiculoides* (www.cabi.org/isc/datasheet/8119 - accessed 8th January 2018).

Impact of *Azolla filiculoides*

Members of the genus *Azolla* are utilized throughout the world for a wide variety of purposes besides its widespread uses as an ornamental in fish ponds and tanks (Lumpkin and Plucknett 1980). *Azolla filiculoides* is used as a green manure in rice paddies, mainly in Asia, as an inhibitor of weed growth in rice cultivation in China and Vietnam (Kröck *et al.*1991), and as an alternative high protein fodder for cattle, swine, poultry and fish, and possibly as an alternative food source for humans, again, mainly in Asia. It has also been used as a nitrate-rich compost that potentially increases soil organic nitrogen levels and cation exchange

capacity. It is used for purification of water, removal of heavy metals (Sanyahumbi *et al.* 1998) and removal of nitrogen and phosphorous from wastewater (Forni *et al.* 2001). It has also been used variously as an ingredient in soap production, a cure for sore throats and as a control for mosquitoes in southern India as complete mats disrupt larval development (Rajendran and Reuben 1991). Nevertheless, a variety of biological factors have limited the usage of *A. filiculoides* for agricultural purposes (van Cat *et al.* 1989). These include low tolerance of high temperatures and insect damage (van Cat *et al.* 1989). In spite of this, its potential utilization means that this plant has been introduced to a number of countries around the world (see above), where it is now problematic, as it is able to reproduce both sexually through the production of spores, and asexually through fragmentation. Dispersed between water bodies and even catchments is possible through waterfowl (Hill 1998).

The economic impact of *A. filiculoides* in South Africa was studied by McConnachie *et al.* (2003). Thick mats on reservoirs and slow-moving water bodies caused economic losses to water-users. Among those water-uses most seriously affected were farming (71% of the impact), recreational (24% of the impact), and municipal (5% of the impact). On average, *A. filiculoides* was found to cause on-site damage of US$589 per hectare per year. In eutrophic water systems, *A. filiculoides* grows rapidly, easily outcompeting indigenous vegetation. Decaying root and leaf matter below a mat of *A. filiculoides*, and the lack of light penetration, creates an anaerobic environment. Not only can very little survive under such conditions, but the quality of drinking water is reduced, caused by bad odours, colour and turbidity (Hill 1998). Cases have been reported where both livestock and game farmers have lost animals due to them refusing to drink from infested water bodies or drowning as a result of mistaking the mat for solid ground. The weed also reportedly increases water loss through evapotranspiration and promotes the development of waterborne, water-based and water-related diseases (Hill 1998).

Azolla filiculoides infestations may form thick mats (5-20 cm thick) on water bodies of up to 10 hectares in size (McConnachie *et al.* 2003). Such infestations have been shown to severely impact the biodiversity of aquatic ecosystems and have serious implications for all aspects of water utilization (Gratwick and Marshall 2001). For example, one of the last remaining habitats of the endangered fish species, the Eastern Cape rocky (*Sandelia bainsii* Castelnau (Anabantidae)) in South Africa, had become so overgrown with the weed that had the biological control programme not been so successful (see below), *S. bainsii* faced extinction (Hill and McConnachie 2009). Primarily, social impacts of *A. filiculoides* have centred on the reduction of useful water surface area for recreation (fishing, swimming and water skiing) and water transport.

Control mechanisms for *Azolla filiculoides*

Chemical control

The recommended chemical control against *Azolla* is the mixture of surfactant with glyphosphate (Diatloff and Lee 1979; Steyn *et al.* 1979; Ashton 1992), kerosene as a spreader with paraquat and diquat (Axelsen and Julien 1988). Chemical control has a number of disadvantages: it is generally expensive, especially when considering the extensive follow-up programmes that are necessary to monitor and eradicate future plant germination from spores; treated plants remaining in the water, causing extensive deoxygenation within the system, thereby affecting water quality; the possibility of spray drift onto non-target vegetation; the water cannot be used for irrigation of crops or watering of stock until the breakdown of the herbicide is complete; and the need for trained personnel exists to monitor spray operations, which may be expensive (Hill and McConnachie 2009).

Mechanical control

Mechanical control traditionally involves physical labour where plants are collected by hand, after which they are dried on the bank of the system (Ashton 1992). More modern mechanical methods make use of machinery for the collection of plants (Lindsey and Hirt 2000). Ashton (1992) proposed a mechanical agitator as a control method for the weed, hoping that it would provide enough turbulence to fragment *A. filiculoides* stolons; however, cost of such a control method, even at a small scale, was prohibitive. According to Lumpkin and Plucknett (1980), use of a mechanical method for the control of *A. filiculoides* is ineffective, as the plant can double its population every 4-5 days under optimal field conditions, thus rapidly recovering from small fragments of plant left behind following mechanical removal.

The problems associated with mechanical control in general include finding suitable areas to dump the removed plants where they will not re-infest the water source, which may involve transport costs; labour costs; rotting weeds producing unpleasant smells; and health risks (Lumpkin and Plucknett 1980). However, an advantage of this method is that it is not ecologically harmful through knock-on or accumulative effects that are often associated with chemical control methods (Lumpkin and Plucknett 1980). Mechanical removal may also hinder the usefulness of biological control, especially when there are few plants left with agents (McConnachie *et al.* 2003).

Biological control

The failure and risk of mechanical control in the aquatic environment made *A. filiculoides* an ideal candidate for biological control in South Africa (Hill 1998). According to Ashton (1992), the use of biological control agents to control the plant was originally not approved due to insufficient research and its risks. Nevertheless, when compared with the expense, risks and variable results of mechanical control, biological control was a favourable long-term control

method (Hill 1998; McConnachie *et al.* 2003). Host records from around the globe show that the genus *Azolla* is attacked by generalist herbivores and that very few specialist insect species have evolved on these plants (Hill 1998). However, four beetle species, the weevils, *Stenopelmus rufinasus* and *Stenopelmus brunneus* (Hustache), and the two flea beetles, *Pseudolampsis guttata* (LeConte) and *P. darwinii* (Scherer) (Coleoptera: Chrysomelidae), have specialised on *Azolla* (Richerson and Grigarick 1967; Hill 1999) and were identified as potential biological control agents for *A. filiculoides* in South Africa (Hill 1998).

Following host range testing, *S. rufinasus* was released in 1997 (McConnachie *et al.* 2004). The biological control programme was highly successful (Coetzee *et al.* 2011). By 2004, nearly 25 000 weevils had been released throughout South Africa, and their feeding damage resulted in local extinctions of *A. filiculoides* from the majority of sites that were surveyed at the time. It took, on average, ten months for a site to be cleared after release of the weevils, which is remarkably quick for a biological control programme. The dispersal abilities of the weevils were originally underestimated, but they are capable of dispersing up to 350 km (Hill and McConnachie 2009). Just five years after the release of the weevils, *A. filiculoides* was no longer considered a threat to South African water bodies (McConnachie *et al.* 2004). Country-wide post-release evaluation surveys between 2008 and 2017 added further evidence about the success of this programme. In 2010, of the 102 *A. filiculoides* sites investigated, the weed was present at 19 of these sites, and *S. rufinasus* was recorded from 14 of the infested sites (Hill and Coetzee 2017). Interestingly, despite being originally collected in the warm regions of Florida, USA, this insect established and controlled the weed in high elevation regions of South Africa (above 1500m masl) in the centre of the country that are characterised by very cold winters where the temperature drops below 0°C for up to 30 nights of the year. This phenomenon serves as part of the rationale for this study.

Biology of *Stenopelmus rufinasus*

The biology of *S. rufinasus* was described by Hill (1998) and is summarised below. Adult *S. rufinasus* individuals are small, approximately 2mm long (Fig. 1.3). They are grey-black and covered with red, black and white scales in a variable pattern. The tip of the rostrum and legs are reddish. The sexes are superficially similar, but in the males, the first abdominal sternite is flat or slightly concave at the midline, while strongly convex in females. Both sexes are relatively long–lived (55-60 days), and copulation can occur immediately after eclosion (Hill 1998). Adult *S. rufinasus* females lay eggs singly in holes that they have chewed in the frond tip. The hole (and egg) is then covered with a cap of frass. The females will lay approximately 325 eggs during their lifetime. Three larval instars follow, all of which feed voraciously on the fronds of *A. filiculoides*. The first instar mines the upper frond lobes, while the second and third instars feed externally, and older larvae are each capable of consuming several plants per day. Pupation commences with larvae chewing out a depression in an *A. filiculoides* frond above the water surface. Larvae then surround themselves with an ovoid, black chamber made from anal secretions, in which they will pupate. The duration from hatching to adult eclosion ranged from 16 to 23 days at a constant temperature of 25°C in a quarantine glasshouse.

Figure 1.3: Adult *Stenopelmus rufinasus* Gyllenhal (Coleoptera: Curculionidae)

1.5.2 *Pistia stratiotes*

Description and distribution

According to Stuckey and Les (1984), *P. stratiotes* was first recorded in Florida by J. and W. Bartram in 1765 as a floating, herbaceous hydrophyte. That led to the belief that the plant was native to North America (Dray *et al.* 1993). On the contrary, Cordo and Sosa (2000) suggested that *P. stratiotes* rather originated from South America due to the presence of a suite of co-evolved herbaceous insects in the region, but earlier descriptions from antiquity suggest that the distribution of water lettuce might have been widely throughout the Northern hemisphere during the Eocene (Stoddard 1989).

The ancient Egyptian and Greek philosophers Diocorides and Theophrastus were the earliest to describe *P. stratiotes* as a perennial herb (Stoddard 1989). *Pistia stratiotes* was later identified as the solitary member of the subfamily *Pistiodea* in the Araceae (Bogner and Nicolson 1991). *Pistia stratiotes* became problematic throughout the world through rapid growth and dense mat formation as a result of lacking natural enemies. By the late 1970s, Holm *et al.* (1977) had recognised water lettuce as the second worst global water weed, following water hyacinth.

Water lettuce can establish in a variety of types of water bodies, including lakes, ponds, canals and slow flowing streams (Holm *et al.* 1977). In Florida, USA, the plant exhibits a seasonal growth pattern, reaching its highest densities in winter and spring and lowest densities during late summer and autumn (Dewald and Lounibos 1990). This is commonly seen at other sites too.

According to Neuenschwander *et al.* (2009), this plant consists of a rosette of obovate to speculate velvety, light green leaves, up to 40cm long in African and American clones (Fig. 1.4). These plants are covered in short hairs that trap air bubbles and thus enable flotation. The leaves range from 2-3.5cm in length (Neuenschwander *et al.* 2009), while roots are numerous, feathery and hang freely in the water (Cook *et al.* 1974). The underside of leaves display longitudinal ribs with embedded veins (Godfrey and Wooten 1981). A single cloned plant forms small colonies through stolons (Acevedo-Rodrigues and Nicolson 2005). Inflorescences are inconspicuous (7-12.5mm), with short peduncles in the centre of the rosette growing on a stem (Acevedo-Rodrigues and Nicolson 2005). The flowering spathe generally shows a constriction between the groups of male and female flowers (Cook *et al.* 1974), the latter of which opens first in the morning hours to expose the wet stigma, while the male flowers remain enclosed (Langeland and Burks 2008). Some hours later, the flowering spathe opens completely and exposes the part bearing male flowers for fertilization to occur (Langeland and Burks 2008). After fertilization, the peduncle bends and pulls the developing fruit (2mm long) underwater where the seeds are released (Godfrey and Wooten 1981).

Figure 1.4: *Pistia stratiotes*. (Drawn by G. Condy, first published in Henderson et al. (1987).)

Impact of *Pistia stratiotes*

Pistia stratiotes has been reported as interfering with agricultural production as it is a large sink of water through a high evapo-transpiration rate, and thus interferes greatly with any paddy crop production (Holm *et al.* 1977; Waterhouse 1993). Furthermore, water lettuce has direct economic impacts as a result of restricted water flow in irrigation and flood control operations (Ajuonu and Neuenschwander 2003). Dense populations of *P. stratiotes* can lead to lakes becoming thermally stratified, resulting in a decrease in dissolved oxygen (Yount 1963; Attiou 1976; Sridhar and Sharma 1980). This in turn increases the growth rate of the plant itself (Langeland and Burks 2008). Water lettuce also acts as a shelter for disease-carrying

mosquitoes, such as species of the malaria vectors *Anopheles* Meigen 1818 (Diptera: Culicidae) and *Mansonia* Walker 1901 (Diptera: Culicidae) (Holm *et al.* 1977).

Control

Chemical control

According to Vermeulen *et al.* (1998), Terbutryn is the only registered herbicide for *P. stratiotes* control in South Africa. Terbutryn is usually applied as a 3% mixture with water either from a boat or from riverbanks using backpack spray units (Cilliers *et al.* 1996). Glyphosphate, copper and diquat are registered as non-selective herbicides against water lettuce in the United States (Steyn *et al.* 1979; Axelsen and Julien 1988; Ashton 1992), but have not been used in South Africa. According to Madin (1984), chlorsulfuron is the only example of a sulfonylurea herbicide used to control water lettuce. However chemical control should be avoided as it endangers the ecosystems with its negative effect on animals and plants (Parsons and Cuthbertson 1992).

Mechanical control

According to Khan *et al.* (2014), water lettuce can be removed mechanically at small scales either by hand or nets where necessary. Mechanical control of water lettuce is practised in Florida (USA) using harvesters for floating water weeds, and booms are installed in slow flowing rivers to prevent spread of the plants (Habeck and Thompson 1994). In South Africa, Hill and Cilliers (1999) assessed the possibility of the use of mechanical control, and deemed it to be impractical, as *P. stratiotes* is known to be able to increase its surface area rapidly, thereby allowing plants that escape mechanical removal to quickly repopulate areas.

Biological control

To date, 46 species of phytophagous insects have been recorded on *P. stratiotes* (South America: 25 species, Asia: 13 species, Africa: 8 species) (Cordo and Sosa 2000). Most of these species are generalists that are not suitable for biological control, but 11 weevils species, belonging to the genera *Neohydronomus*, *Pistiacola*, and *Argentinorhynchus*, are assumed to be monophagous. Overall, only two species, a weevil, *Neohydronomus affinis* Hustache and a noctuid moth, *Spodoptera (Epipsammea) pectinicornis* (Hampson) have been given special attention as possible biological control agents (Winston *et al.* 2014). The moth was released in the USA, but failed to establish (Neuenschwander *et al.* 2009). *Neohydronomous affinis* has been released in 18 countries around the world, where it has resulted in excellent control of *P. stratiotes* (Cilliers *et al.* 2003), for example bringing *P. stratiotes* under complete control in Senegal in 18 months (Diop *et al.* 2010). Recently the weevil was released and has established in Morocco (Hill pers. comm.).

The first attempt at biological control of water lettuce in South Africa made use of *N. affinis*, a Brazilian agent first successfully used against the plant in Australia (Harley *et al.* 1984). It was released in South Africa in 1985, following importation of weevil populations from Australia which were released directly into the field, without host-specificity testing (Cilliers 1991). This initiative gave positive results within nine months on seasonal pools containing the plant, but results were not as quickly achieved on fast flowing rivers, where the insect took up to three years to achieve control of *P. stratiotes* (Cilliers 1991). Currently, *P. stratiotes* is considered under complete control in South Africa, where no other interventions are required (Coetzee *et al.* 2011). Countrywide surveys revealed that fewer than 7 % of the water bodies surveyed were infested with water lettuce, and the weevils were present at most of these sites (Coetzee *et al.* 2011).

In addition to South Africa, the introduction of the weevil has been highly successful around the world (reviews in Dray and Center 2002; Cilliers *et al.* 2003; Neuenschwander *et al.* 2009), where, according to Winston *et al.* (2014), it has been officially released in at least ten countries, including Australia, Benin, Botswana, Ghana, Papua New Guinea, South Africa, Senegal, the United States of America, Zambia and Zimbabwe.

Biology of *Neohydronomus affinis*

According to Moore (2005), adult *N. affinis* individuals are small (3mm long), and have a nearly straight rostrum that is strongly constricted ventrally at the base (Fig. 1.5). The weevil ranges in colour from uniform bluish grey to reddish brown (depending on the age) with a tan, lunate band across the elytra (Center *et al.* 2002). The specific colour pattern is associated with scales and may be difficult to distinguish if they are wet, dirty, or missing (Center *et al.* 2002). The eggs are cream coloured and sub spherical (0.33 mm by 0.40 mm) (Center *et al.* 2002). Females chew a hole of about 0.5mm in diameter in the water lettuce leaf (usually the upper surface near the leaf edge), deposit a single egg inside this puncture, and close the hole with frass (Center *et al.* 2002). The eggs usually hatch within 4 days (at temperatures above 24 °C) (Center *et al.* 2002). The young larvae, which are small with a head diameter of around 0.2mm, burrow under the epidermis and work their way toward the spongy portions of the leaf at a rate of about 1.5-2.0 cm/day (Center *et al.* 2002). Larval mines are often plainly visible in the outer third of the leaf where tissues are thin, but are less apparent in the central and basal portions of the leaf (Center *et al.* 2002).

Figure 1.4: The weevil, *Neohydronomus affinis* Hustache (Coleoptera: Curculionidae)

The first moult occurs when larvae are about three days old and the second, 3-4 days later (Center *et al*. 2002). The larval stages last 11-14 days in total (Center *et al*. 2002). Third instars are generally found excavating the spongy portions of the leaf where they moult to become naked pupae (Center *et al*. 2002). Under optimum temperatures, 4-6 weeks are generally required for *N. affinis* to complete the transition from egg to adult (Center *et al*. 2002). Adults chew holes (about 1.4 mm in diameter) in the leaf surface and burrow in the spongy tissues of the leaf to feed (Center *et al*. 2002). The characteristic round feeding holes are easily observed when weevil populations are large, but may be concentrated near leaf edges and more difficult to observe when weevil populations are small (Center *et al*. 2002).

1.6 Aims and objectives

The thermal physiology of several species of aquatic weed biocontrol agents have been tested in South Africa as both pre- and post-release evaluations to assess how agents will perform once released, and the reasons for failure or success of agents in different areas, respectively. Low winter temperatures in high-lying areas of South Africa have contributed to the lack of control of water hyacinth in South Africa (Hill and Olckers 2001). Investigations into the

thermal physiology of three species of control agents, *viz. E. catarinensis* (Coetzee *et al.* 2007), *M. scutellaris* and the moth, *Niphograpta albiguttalis* (Warren) (Lepidoptera: Pyralidae) (May and Coetzee 2013) went some way in explaining why biological control of water hyacinth is limited in these high lying areas that receive winter frost. While biological control of water hyacinth has been met with varied success, biological control of water lettuce and red water fern has been extremely successful in South Africa (Coetzee *et al.* 2011). It therefore seems likely that the control agents have not been limited by their thermal requirements. According to Center and Pratt (2004), thermal physiology experiments have not been conducted on *N. affinis*, while studies on *S. rufinasus* in South Africa were conducted as part of a PhD, but never published (McConnachie 2004). Thus, in this thesis I tested whether adaptation to the different environments in which two biological control agents have established has influenced their thermal physiology. Throughout South Africa, prior to control, red water fern was distributed mostly in cooler interior region, while water lettuce was mostly distributed throughout the warmer subtropical coast and low-lying interior. This study aims to determine if the insects are fulfilling their full potential range throughout South Africa.

Thus, the aims of this thesis were:

Aim 1: To compare the thermal limits of both weevil species, testing four parameters (CT_{min}, CT_{max}, LLT_{50}, and ULT_{50})

Aim 2: To compare developmental rates of both weevils, from egg to adult.

Aim 3: To evaluate degree-day models of both weevils, predicting the number of generations each weevil can complete at given sites in South Africa, and compare these to the actual distribution.

CHAPTER 2

METHODOLOGY

2.1 Introduction

This chapter presents the development of the research design and materials and methods used to determine the thermal requirements of *N. affinis* and *S. rufinasus*, in conjunction with a review and a rationale for why each parameter was selected and is therefore referred to as methodology.

2.2 Rearing of study organisms

Cultures of insect-free *P. stratiotes* and *A. filiculoides* were maintained in separate paddling pools (diameter 3 m x 0.67 m high, 3000l) in a polyurethane tunnel on the campus of Rhodes University, Grahamstown, South Africa where the temperatures varied between 25°Cand 36°C in summer and 13°C and 19°C in winter. The pools were filled with tap water to which nutrients in the form of nitrates and phosphates were added monthly at a rate of 5 mg N. L^{-1} and 0.5 mg P. L^{-1}which is representative of eutrophic water in South Africa (Coetzee and Hill 2012). The nutrient concentration was chosen because Holmes (1996) showed that, according to South African Water Quality standards, nitrogen and phosphorus concentrations of these levels are found in impoundments in South Africa, and these concentrations are similar to those used by Coetzee *et al.* (2007) in water hyacinth studies. To prevent chlorosis of the plants, 2mg L^{-1} of commercial iron chelate was added to each pool monthly. *Azolla filiculoides* grows in symbiotic association with *Anabaena azolla* within the dorsal leaf lobe cavities (Ashton and Walmsley, 1984). *Anabaena azolla* can fix atmospheric nitrogen and is able to fulfil nitrogen requirements of *A. filiculoides*, making it able to thrive in nitrogen-deficient waters (Ashton, 1992). In spite of this, and in order to keep the experiment controlled, the same amount of

nitrogen was added to both of the plant cultures. Plant material was sourced from these insect-free pools for the experiments, as required.

In a separate polyurethane tunnel, two identical pools were set up and stocked with *P. stratiotes* and *A. filiculoides* and fertilized as described above, but 100 adult *N. affinis* and *S. rufinasus* were added to the pools onto their respective hosts at the beginning of the trials (March 2014). The weevils were allowed to build up until the first thermal trials began in August 2014. Adult weevils could then be sourced from these pools as required. *Stenopelmus rufinasus* is a voracious feeder on *A. filiculoides*, and due to its short generation time, has the ability to build up large populations that crash mats of its host plant. Therefore, this population had to be managed by removing and destroying adults periodically when numbers started to build up.

2.3 Thermal limits

Comparison between the thermal limits of *Stenopelmus rufinasus* and *Neohydronomus affinis*

There are a number of thermal parameters that may be used when assessing the thermal profiles of insects. These include critical thermal minima (CT_{min}) and maxima (CT_{max}); upper or lower lethal limits (ULT_{50} and LLT_{50}); and degree day models (DD). According to Hoffmann *et al.* (1997) and Berrigen and Hoffman (1998), there are different ways for measuring upper and lower thermal limits, which include mortality assays and estimates of debilitating temperature.

Critical thermal limits (CT_{min} and CT_{max}) are specific temperature thresholds above and below which insects lose their locomotive capabilities (Hazel and Bale 2011). CT_{min} and CT_{max} define the ecological, or behavioural, temperature tolerance limits of a species, and are points short of death where locomotory impairment occurs, but from which recovery is possible (Klok and

Chown 1997). The lower critical tolerance (CT_{min}) is a measure of cold tolerance (MacMillan and Sinclair 2011). CT_{min} is defined as the measure of the starting point of chill coma (chill coma onset [CCO]) (Huey *et al.* 2012). The cold tolerance of insects are generally investigated to describe how the insect can survive temperatures where the body fluids should freeze (Sinclair *et al.* 2015). This could occur through the accumulation of cryoprotectants or the prevention or tolerance of ice formation (Sinclair *et al.* 2015). The upper temperature reached at the loss of righting, onset of muscle spasm is defined as the CT_{max} (Terblanche and Chown, 2006).

Sinclair *et al.* (2015) state that "cold tolerance strategy is not a substitute for understanding lethal limits, and cannot be used on its own to predict survival". Lethal limits were thus also determined for the purposes of this thesis. Lethal limits (LT_{100}) are a quantification of the temperature under which all individuals in an experiment are killed (Powell and Bale 2004; Terblanche and Chown, 2006). According to Addo-Bediako *et al.* (2000) and Kimura (2004), the LT_{50} is defined as the temperature where 50% of individuals survive acute hot or cold exposure. Beyond these temperatures, insects are unable to recover as the organism's physiological tolerance limit has been reached (Addo-Bediako *et al.* 2000; Kimura 2004). The lowest temperature at which no mortality occurs is known as the upper limit of the cold injury zone (ULCIZ) and indicates the lowest temperature that a species can tolerate without suffering fatal losses to the population (Nedvěd 1998; Sinclair *et al.* 2015). Even though mortality may not occur at the ULCIZ, most studies do not acknowledge the potential sub-lethal effects that adverse temperature exposure may have on the behaviour or fitness of the insects post-exposure (Sinclair *et al.* 2015).

The experiments completed as part of this study were conducted using procedures laid out by Mitchell *et al.* (1990), while the temperature ranges used were selected according to the methods of Coetzee *et al.* (2007). The critical thermal limits of adult *S. rufinasus* and *N. affinis* were measured by placing 10 weevils of both sexes individually into separate air-filled vials for each trial, which were then sealed with cotton wool. The vials were submerged in a water bath (Haake F8) connected to a programmable temperature controller (Haake C25, 0.1 accuracy) such that just the openings emerged from the liquid to prevent flooding. A 50% distilled water – glycol mixture was used in the bath, as glycol has a lower freezing point than distilled water and allows for sub-zero equipment operation. For the CT_{min} trials, the insects were first given an acclimation period from 20°C to 10°C at a rate of 4°C/min , after which they were held at 10°C for five minutes to stabilise. The temperature was then progressively lowered from 10°C until the insects could not self-right at a rate of 1°C/4min. For determination of the CT_{max}, temperatures were raised from 20°C to 30°C at a rate of 4°C/min. They were then held at 30°C for five minutes to stabilise them. After that, the temperature was progressively ramped depending on the trial conducted at a rate of 1°C/4min (see table 2.1 below).The above experiment was repeated three times using different individuals in each, for a total sample size of 30 insects from each species. The experiment was constantly monitored, and the ability to self-right was checked at each 1 degree change by flicking the vials so that the insects fell on their backs.

A temperature ramping rate of 0.25°C/min was used in this study, as is the current standard because ramping rates between 0.1°C/min and 0.5°C/min are considered to be the most ecologically relevant while still considering appropriate time investments in experimentation (Sinclair *et al.* 2015). Initially, a ramping rate of 1°C/min was used in these types of experiments (Sinclair *et al.* 2015), but this rate is now considered to be too fast to accurately

represent natural conditions (Sinclair *et al.* 2015), and may lead to shock in the insects, which may obscure their actual thermal physiologies (Sinclair *et al.* 2015). On the other hand, a particularly slow ramp rate will make experiments impractically long and allow the insects too much time to acclimate and harden to the experimental temperatures, also altering their thermal physiologies (Sinclair *et al.* 2015). Due to these potential effects that the temperature ramp rate can have on the results of an experiment for determination of both LTs and CTs (Sinclair *et al.* 2015), it is vital that the rate, whatever the researcher chooses it to be, is kept consistent throughout all experiments to keep them comparable (Sinclair *et al.* 2015). Here, the ramping rate was kept at a consistent 0.25°C/min (Table 2.1).

Table 2.1. The temperature steps used to complete critical thermal experiments.

Step	**CT_{min}**			
	Starting Temperature (°C)	**Final Temperature (°C)**	**Rate of Change (°C/min)**	**Time for Step (min)**
Ramp 1	20	10	0.25	40
Acclimation	10	10	0	5
Ramp 2	10	Trial Dependent	0.25	Trial Dependent
	CT_{max}			
Ramp 1	20	30	0.25	40
Acclimation	30	30	0	5
Ramp 2	30	Trial Dependent	0.25	Trial Dependent

The CT_{min} and CT_{max} for *N. affinis* and *S. rufinasus* were compared using two independent Students *t*-tests in Statistica 13 (Statistica, 2017). For each test, species was used as the

grouping variable with the temperature at which each individual lost muscle control as the dependent variable.

Lethal temperatures

Lethal temperature experiments were conducted on adult *S. rufinasus* and *N. affinis*. Groups of 10 weevils per test temperature were placed individually into plastic vials (as above in the CT trials), with the vials submerged in water bath that contained a water-glycol mix for the LLT_{50} and distilled water only for the ULT_{50}. The temperature of the vials was systematically lowered (LLT_{50}) or raised (ULT_{50}) from the room temperature to the experimental temperature, in a programmable water bath. The following temperature ranges were tested: 5°C to -10°C (in 1°C increments) for LLT_{50}s, and 35°C to 48°C (in 1°C increments) for ULT_{50}s following the temperature ranges used by McConnachie (2004). Different groups of weevils were used for each replicate and each temperature range. Following 2 hours exposure at the experimental temperature, the vials were removed from the water bath and the weevils were placed in petri dishes with moist filter paper and a single *A. filiculoides* frond, or *P. stratiotes* leaf depending on the insect species. These petri dishes were kept at room temperature, and recovery to normal behaviour was checked at a one and 24 hour interval following cessation of the experiment. Survival of each individual was recorded as a binomial factor (dead or alive).

The LT_{50} was calculated using probit regression analysis in Statistica v13, to determine the temperature at which 50% died. For comparison of the lethal temperatures between the two species, generalized linear models (GLZ) with binomial distributions and a logit link function were used. One model was run for each limit, the ULT and the LLT, to compare the species, as well as one comparing the number of insects that did not recover after one and 24 hours following completion of the experiment, for each species, resulting in a total of six models. All models were run in Statistica v13.

Developmental rates and Degree Day models

Temperature is one of the most influential abiotic factors for insect physiology. Insects require a certain amount of heat units to develop from one life stage to another (egg-adult). Insects have predictable development patterns depending on heat accumulation**.** The rates at which eggs develop and hatch, larvae feed and grow, and pupae metamorphose to adults is reflected in the time between oviposition and adult emergence known as "Total Development Time" (TDT) (Sweeney 1984). According to Sweeney (1984), temperature, food availability, photoperiod and genetically determined physiological processes, such as diapause and rates of growth, all influence developmental rates.

A description of the number of life cycles or generations that an organism can complete in one year is called voltinism (Tauber and Masaki 1986). Voltinism is divided into univoltine, (one generation per year), bivoltine (two generations per year), and multivotine or polyvoltine (more than two generations in a year) strategies. Insect life cycles are mostly univoltine or multivotine. Most tropical insects have fast development and most reproduce throughout the year (multivotine), i.e. have many life cycles, due to warmer temperatures and low seasonality in biotic and abiotic conditions.

Larval development times are often affected by food quality and quantity too, whereby an increase in developmental time and decrease in adult size are a result of low food quality or quantity (Sweeney 1984). Kumar *et al.* (2011) identified protein as one of the main requirements for biological activities during development, metamorphosis and the maintenance

of various physiological functions in different tissues. Hence proteins, carbohydrates and lipids, are major biomolecules which play an important role in biochemical processes on both insect growth and development (Ito and Horie 1959).

The experimental methods used here follow those of McConnachie (2004). One hundred and twenty adult *S. rufinasus* and the same number of adult *N. affinis* individuals were collected from the relevant cultures maintained at Rhodes University mass-rearing facility. Pairs of five males and five females were placed on fresh host plants for 12hours in a controlled environment room in the Department of Zoology and Entomology at Rhodes University, where temperatures were maintained at 24˚C, with a L12:L12 photoperiod. After 12 hours, any eggs that were oviposited on the plant were removed and placed singly onto the relevant plant frond/leaf discs. The eggs were then divided equally in petri dishes lined with filter paper with few a few drops of water added to provide humidity. These were then enclosed in a plastic sealable bag to ensure that moisture levels were retained throughout the experimental period. Each set of different experimental temperatures in five controlled environmental rooms were chosen using the methods of May and Coetzee (2013) for thermal physiology experiments on water hyacinth: 18˚C, 20˚C, 24˚C, 27˚C and 29˚C. The temperature in each chamber was recorded using Thermochron iButtons (DS19121G#F5) (Maxim/ Datas iButton Products), which have an operating range of -40 to + 85˚C, and capacity over 2000 digital readings. The petri dishes were inspected every 12 hours for evidence of larval hatching; moulting; pupation and adult eclosion, during which time the plants were replaced daily to ensure that food availability and quality were not limiting. It was found that at 16˚C and 30˚C, all the eggs died before hatching, and at 20˚C and 24˚C larvae did not survive after hatching. Egg mortality was suspected to be the result of temperature incompatibility, while larval death may have been caused by handling

during the daily plant changes. This resulted in a methodological change in all following experiments.

The new methodology involved pairing five adult males and five adult females in small 120ml dishes to mate (three replicates were used, for a total of 15 males and 15 females) on fully grown host plants with three to four leaves on each plant for *N. affinis,* and three to four *S. rufinasus* fronds. These insects were left to mate for 12 hours in the controlled environment rooms mentioned above at 24°C, with a L12:L12 photoperiod. Each of the 10 plants was then placed individually in tubs filled with water. Different growth chambers were maintained at five different temperatures, ranging from 18°C, 20°C, 24°C, 27°C and 29°C. The temperature in each chamber was confirmed using the means described above. The plants were checked daily for signs of oviposition and fresh plants were added to tubs exhibiting evidence of hatched 1st to 4th larval instars when necessary to ensure good food quality. These plants were repeatedly added when necessary to ensure that food availability and quality were not limiting without touching or removing the larvae until they reached the adult stage. The developed adults were removed on the day that they were first recorded before mating could occur, and the total number of days taken from egg to adult was recorded. The average number of days taken to develop from egg hatch to adult emergence was calculated for each of the experimental temperatures, then the number of days to complete full development (DT). The lower developmental threshold (t_0) and the thermal constant (K) were calculated using the reduced major axis method (Ikemoto and Takai, 2000). This method gives greater precision in thermal summation and the lower developmental threshold than traditional linear modes because the critical temperatures, i.e. the lower and upper developmental thresholds, are not estimated from the linear equation. In the linear regression method, a small range of temperatures is plotted, and the line is then extrapolated to estimate t, increasing the possibility of error occurring. In

the reduced major axis regression method, the product of development time and temperature (DT) is plotted against development time (D). This model also follows the equation for a straight line, y = a + bx, where y = DT, a =K, and b = *t*. This method does not require the estimation of standard error because its line parameters are the direct parameters K and t (Ikemoto and Takai, 2000).

The regression models for each species were then compared using a Homogeneity of Slopes test in Statistica v13 to determine whether the development and the product of development time and temperature were significantly different between the species.

Degree day models

There are two approaches that have been used in previous studies to estimate developmental times for insects in the field. The first is estimation of larval developmental times from instantaneous growth, which was used by Benke *et al.* (1992), while the second simulated field conditions in the field or laboratory while insect rearing from egg to adult (Sweeney 1984). The number of generations that insects can complete at a given locality can be predicted through a degree day (DD) model that makes use of temperature and time as factors (McClay and Hughes 1995). These models are developed to be species-specific, taking only the active temperatures within a species-specific range into account (Damos and Savopoulou-Soultani 2012). A DD is the unit measurement of physiological time where one degree is equal to one degree above the lower or below the higher developmental threshold of an insect over 24 hours (Damos and Savopoulou-Soultani 2012). These measures make the DD models universally applicable to living organisms (Damos and Savopoulou-Soultani 2012). Degree day models are particularly useful mathematical tools for predicting the change-over between insect life stages based on their development rates at certain temperatures. The results of these models

affect pest management decisions, as an insect DD model helps to prevent economic losses and excess use of pesticides (McClay and Hughes 1995). Degree day models help to predict possible areas of post-release establishment in biological control systems, as well as assisting in avoiding repeated agent release into an area with mismatched local climatic conditions (Damos and Savopoulou-Soultani 2012). McClay and Hughes (1995) showed that maps of the number of generations produced by an insect species in a year using estimated DD totals could be used to predict insect success and failure of establishment at a given locality. For example, fewer *Eccritotarsus catarinensis* generations were completed at high altitudes in the field, as Coetzee *et al.* (2007) expected from the results of their study.

Degree days are measured using daily maximum and minimum temperatures to provide a measurement of heat units over time at a given locality. Because insect development occurs only between an upper and lower temperature threshold, development stops when the temperature drops below the lower threshold and resumes when it rises above it. Degree days are calculated using the lower temperature threshold *t* as the base temperature. The maximum and minimum temperature data for the period 2014 to 2016 were obtained from the South African Weather Bureau for a total of 16 red water fern location sites and 20 water lettuce location sites throughout South Africa. The thermal constant K and developmental threshold *t* estimated from the reduced major axis regression models for both species were used to calculate accumulated degree-days for each year and each location according to the equation:

$$\text{Equation 1: } K = \Sigma \frac{Tmax+Tmin}{2} - t$$

(if $Tmin < t$, t was used)

This gives the number of generations that *S. rufinasus* and *N. affinis* are capable of producing at each site per year, by calculating the mean accumulated degree days of development (K) for both insect species. The Geographic Information Systems (GIS) programme, ARCVIEW (version 3.2, Environmental Systems Research Institute Inc., Redlands, CA, USA), was used to create maps that indicate the number of generations that *S. rufinasus* and *N. affinis* can potentially complete in a year at the sites presented in Table 3.2.

A particular disadvantage of this method, however, is that days where the temperatures drop below the species-specific lower limit are converted to equal this lower limit (Damos and Savopoulou-Soultani 2012). This means that the final developmental results may be underestimated, although often only mildly so (Damos and Savopoulou-Soultani 2012). For the needs of biological control, underestimation is not as big a concern as overestimation, indicating that this method is still applicable.

Chapter 3

RESULTS

3.1 Thermal limits

Both of the weevil biological control agents tested in this study, *N. affinis* and *S. rufinasus* displayed relatively wide thermal tolerance limits, with some sub-zero survival for limited periods of time.

3.1.1 Critical thermal limits

Adult *N. affinis* had a CT_{min} of 5.58 ± 0.31˚C (±SE), with full locomotory function at 10°C, and complete loss of locomotory function at 0°C, for all individuals; while the CT_{max} was 44.52 ± 0.27˚C (±SE), over a range of 40 °C to 49°C (Figure 3.1).

Adult *S. rufinasus* showed a similar critical thermal temperature range with a mean CT_{min} of 5.38 ± 0.33˚C (±SE), over a range of -1 °C to 10°C; and a mean CT_{max} of 44 ± 0.17˚C (±SE), over a range of 40 °C to 47°C (Figure 3.1). There was no significant difference in either CT_{min} ($t_{118} = 0.256$, $P = 0.798$), or CT_{max} ($t_{118} = 1.613$, $P = 0.109$) between the two species.

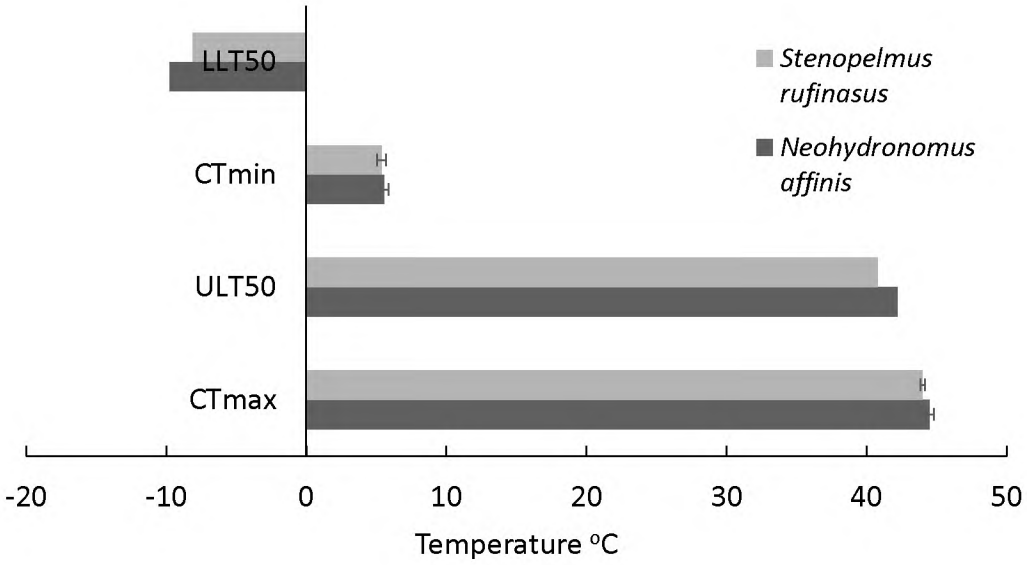

Figure 3.1: Mean lower lethal (LLT_{50}), minimum critical thermal (CT_{min}) (±SE), upper lethal temperatures (ULT_{50}), and maximum critical thermal (CT_{max}) (±SE) of *Neohydronomus affinis* and *Stenopelmus rufinasus.*

3.1.2 Lethal temperatures

For the lower lethal temperature experiments, all *N. affinis* adults survived the two hours of exposure at 4°C, but mortality was recorded from 3°C, after both 1 and 24 hours recovery, with 100% mortality at -12°C after both recovery intervals. The LLT_{50} for this species was -9.85°C after 1 hour recovery, and -9.73°C after 24 hours recovery (Figures 3.1, 3.2A).

All adult *N. affinis* weevils survived two hours exposure to temperatures between 35°C and 38°C, while mortality was recorded from 39°C, with 100% mortality recorded at 46°C for both recovery intervals. The ULT_{50} of *N. affinis* was calculated to be 42.7°C after 1 hour recovery, and 42.2°C after 24 hours recovery (Figures 3.1, 3.2B).

A. B.

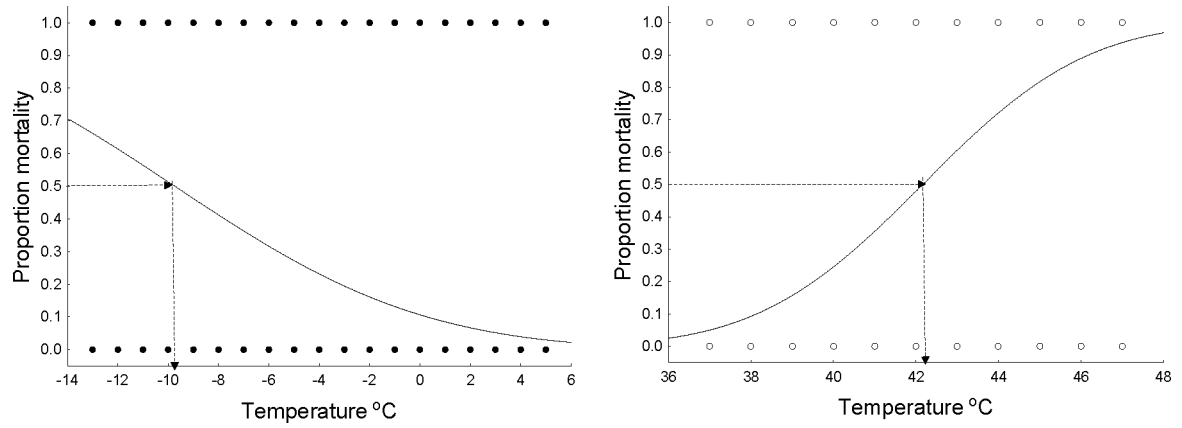

Figure 3.2: Probit regressions of temperature vs. mortality of *Neohydronomus affinis*, indicating (A) the lower lethal temperature (LLT_{50}) (y=(-1.244-0.128)*x,0,1) (χ^2 = 420.77, P<0.001) and (B) the upper lethal temperature (ULT_{50}) (y=(-13.405+0.318)*x,0,1) (χ^2 = 474.13, P<0.001).

There was no mortality of *S. rufinasus* adults after two hours of exposure to temperatures between 0°C and -3°C in LLT_{50} trials, and 100% mortality at -13°C. The mean LLT_{50} was recorded as -6.85°C after 1 hour recovery, and -8.1°C after 24 hours recovery (Figures 3.1, 3.3A). There was no significant difference in LLT_{50} values after 24 hours recovery between *S. rufinasus* and *N. affinis* (Wald χ^2 = 1.810, df = 1, P = 0.179).

At the upper temperature range, adult *S. rufinasus* survived temperatures between 35 and 39°C after 1 hour recovery time, but this was reduced to 36°C after 24 hours recovery time, in ULT_{50} trials. 100% mortality occurred at 44°C after 1 hour recovery, and 43°C after 24 hours recovery, while ULT_{50} of *S. rufinasus* was 41.9°C after 1 hour recovery, and 40.8°C after 24 hours recovery (Figures 3.1, 3.3B), which is significantly lower than the ULT_{50} of *N. affinis* (Wald χ^2 = 36.566, df = 1, P < 0.0001).

A. B.

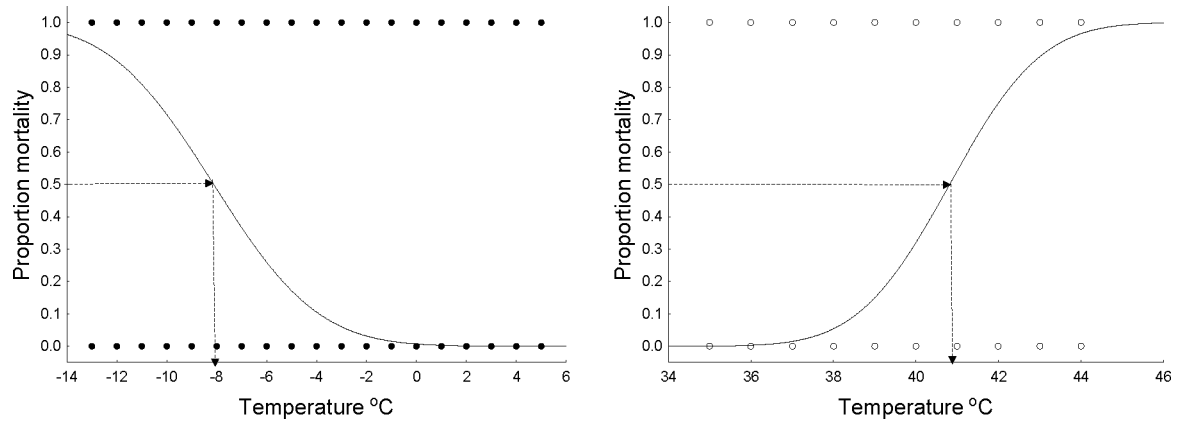

Figure 3.3: Probit regressions of temperature vs. mortality of *Stenopelmus rufinasus*, indicating (A) the lower lethal temperature (LLT_{50}) (y=(-2.464 -0.307)*x,0,1)) (χ^2 = 1149.07, P<0.001) and (B) the upper lethal temperature (ULT_{50}) (y=(-23.280+0.570)*x,0,1) (χ^2 = 699.15, P<0.001)

3.2 Degree Day Models

Developmental period was successfully measured between egg and adult stage for both *N. affinis* and *S. rufinasus,* at all experimental temperatures. The duration of development for both weevil species decreased as the rearing temperature increased, although the development time of *S. rufinasus* was approximately half that for *N. affinis* (Table 3.1).

Table 3.1: Total developmental time from egg to adult for *Neohydronomus affinis* and *Stenopelmus rufinasus* reared at five constant temperatures.

Rearing Temperature (°C)	**Mean duration of *N. affinis* development from egg to adult (Days ± SE) (n)**	**Mean duration of *S. rufinasus* development from egg to adult (Days ± SE) (n)**
18	82±1.01 (39)	48±1.37 (11)
20	61±0.42 (40)	32±0.16 (18)
24	41±0.94 (30)	19.8 ± 1.83 (38)
27	29.7 ± 0.64 (31)	16 ± 0.37 (69)
29	30 ± 0.99 (19)	16 ± 1.28 (54)

Using the developmental time and temperature data, the regression equation returned by the reduced major axis regression model for *N. affinis* was:

$$y=12.976x+435.24$$

indicating that 435.24 days are required for development (K), above a threshold of 12.98°C (t), for total development (Figure 3.2). Similarly, the equation of development time for *S. rufinasus* was:

$$y=13.6x+222.45$$

indicating that 222.45 days are required for development, above a threshold of 13.6°C (Figure 3.4). The relationship between duration of development and the product of development time and temperature is significantly different between the species ($F_{1, 345} = 467.96$, $P < 0.001$), with *S. rufinasus* requiring less time to complete development.

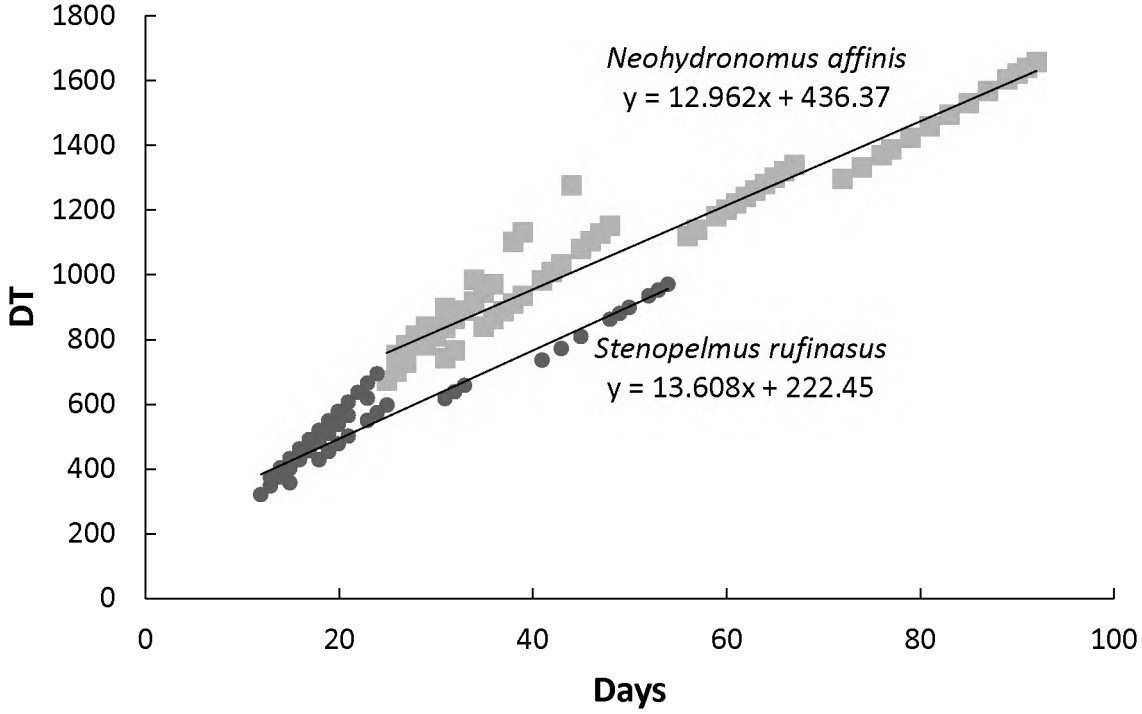

Figure 3.4: Temperature dependent development of *Neohydronomus affinis* and *Stenopelmus rufinasus,* using the reduced major axis regression method (Ikemoto and Takai, 2000). DT is the product of development time (days) and temperature**.**

3.4 Degree Day Accumulation

Maximum and minimum temperatures from weather station data from 20 sites around South Africa close to where *P. stratiotes* and *A. filiculoides* has been recorded, were downloaded from the South African Weather Service (Table 3.2). The weather station data does not cover the entire distribution of the weeds and their respective agents in the country, but does represent the eco-climatic zones in which the weeds occur. These data were then correlated with the

degree day model developed for each of the agents (above) in order to predict how many generations the agents would go through. *Neohydronomus affinis* was predicted to be able to complete between 4 and 9 generations per year (Figure 3.5). In contrast, *S. rufinasus* was predicted to complete more generations, between 5 and 14 generations at 16 sites around the country (Table 3.2), including the high altitude interior sites of the Free State Province (Figure 3.5). Although the interior of the country is charaterized by cold winter temperatures, these are not below the critical minumum temperatures for *S. rufinasus*, and the hot summer tempertures in these areas allows for reduced duration of development and thus shorter generation times. The cooler summers in the more temperate climate in the Eastern Cape and Western Cape, although they experience warmer winters, would result in far fewer generations.

Table 3.2: South African Weather Stations most closely matched to distribution records of *Pistia stratiotes* and *Azolla filiculoides* in South Africa.

Pistia stratiotes			***Azolla filiculoides***		
matched sites	**Latitude**	**Longitude**	**matched sites**	**Latitude**	**Longitude**
Port Alfred	-33.559	26.880	Dohne Agric	-32.527	27.460
Belfast	-25.691	30.034	Somerset East	-33.049	25.719
Lydenburg	-25.11	30.476	George	-34.004	22.384
Thohoyandou	-23.079	30.383	Strand	-34.141	18.848
			Cape Town-Slangkop	-34.148	18.319
Durban-Virginia	-29.772	31.055	Bloemfontein	-29.120	26.187
Richards-Bay	-28.737	32.093	Bethelehem	-28.249	28.334
Polokwane	-23.857	29.451	Vrede	-27.422	29.169
Nelspruit	-25.387	31.099	Frankfort	-27.267	28.494
Komatidraai	-25.514	31.910	Kroonstad	-27.666	27.313
Noupoort	-31.186	24.960	Welkom	-27.994	26.666
Mtunzini	-29.947	31.707	Bloemhof	-27.651	25.621
Greytown	-29.083	30.603	Fauresmith	-29.753	25.323
Vereeniging	-26.569	27.958			
JHB-Botanical Gardens	-26.156	27.999	Lydenburg	-25.111	30.383
Grand-Central	-25.983	28.133	Riverview	-28.447	32.182
Charters Creek	-28.197	32.414	Struisbaai	-34.800	20.056
Makatini	-27.394	32.176			
Riverview	-28.444	32.182			
Cape Town-Slangkop	-34.148	18.319			

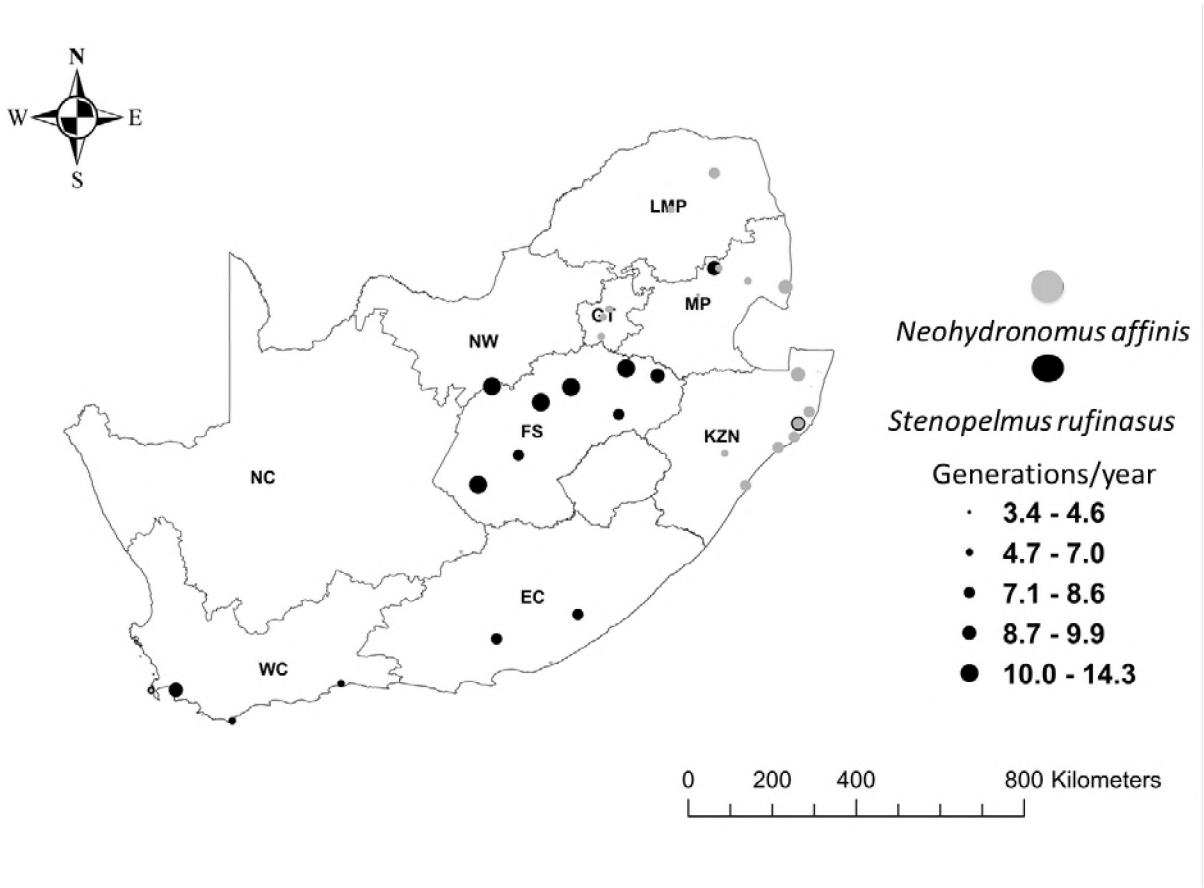

Figure 3.5: Number of generations that *Neohydronomus affinis* and *Stenopelmus rufinasus* can complete in a year estimated from the reduced major axis regression model, from mean monthly weather station data (provided by the South African Weather Service).

In summary, both weevil species have similar thermal limits after exposure to extreme temperatures, as well as similar lower developmental thresholds. However, *S. rufinasus* has a significantly faster developmental rate than *N. affinis*, allowing it to complete a greater number of generations per year in South Africa, including high elevation colder sites where the summers are considerably warmer than the temperate, coastal sites of the Eastern and Western Cape.

CHAPTER 4

DISCUSSION

4.1 Introduction

Byrne *et al.* (2004) stated that one of the main reasons for the lack of effectiveness of weed biological control agents was climate incompatibility (Chapter 1). The aim of this thesis was to undertake a retrospective analysis of the thermal physiology of two biological control agents for water weeds (*S. rufinasus* on *A. filiculoides* and *N. affinis* on *P. stratiotes* respectively) that have been considered highly successful agents (Coetzee *et al.* 2011), to see if their success could be linked to their thermal physiology, and ultimately to make predictions of their distributions and impact under climate change scenarios. Hill and Olckers (2001) stated that cool temperatures associated with high elevations reduced the effectiveness of the water hyacinth agents in South Africa, and this could also be the case with the other aquatic weed programmes, but the effect of higher temperatures have seldom been considered (see Tipping *et al.* 2014 for an exception). This lack of thermal compatibility has resulted in a number of biological control programmes around the world adopting an approach of trying to find more thermally tolerant genotypes of the agents from the source region (e.g. Foley *et al.* 2016).

4.1.1 *Stenopelmus rufinasus*

The results of this thesis showed that both *N. affinis* and *S. rufinasus* have broad thermal limits in the laboratory with the lower lethal temperature (LLT_{50}) for *S. rufinasus* of -8°C (allowing for 24 hour recovery period) and an upper lethal temperature (ULT_{50}) of 43°C (Chapter 3) which means that this insect is able to survive an approximate 50°C differential. The *S. rufinasus* culture that

was released in South Africa originated from 17 individuals collected in Gainesville, Florida, USA in 1996 (Hill 1998). The Gainesville climate is defined as humid subtropical, but does experience quite wide temperature fluctuations. Summer temperatures range between 21°C and 33°C, while in winter, the region averages some 6 nights of freezing, with an average winter temperature range of −1°C to 20°C. Thus the temperature range from the area of collection is considerably narrower than what the insect's thermal range in this study. However, *S. rufinasus* is widespread throughout its native range and has been recorded from Mexico, and the states of California, New Jersey and Oregon in the USA (O'Brien and Wibmer 1982). Further to this, *S. rufinasus* has been accidentally introduced with the weed to a number of European countries, including Spain (Florencio *et al.* 2015), France (Bredel 1901), Portugal (Carrapiço *et al.* 2011), England (Gassmann *et al.* 2006) and Ireland (Baars and Caffrey 2008). Thus this insect is not only able to survive, but develop and impact *A. filiculoides* in the cool conditions of Europe, and warm tropical conditions such as those in southern Florida, USA and Mexico, and its realized distribution is supported by the laboratory thermal physiology results presented in Chapter 3.

Several authors have suggested that insects might show far more thermal plasticity than originally thought (e.g. Sgrò *et al.* 2016; Buckley *et al.* 2017), and thus climate matching potential biological control agents between the region of origin and region of introduction might not be that important. McConnachie (2004) investigated the thermal physiology of *S. rufinasus* and the results of his study are compared with the results of this study (Table 4.1).

Comparing the duration of development of *S rufinasus* between the McConnachie (2004) and the current study presented in table 4.1, although the studies were not conducted at the same

temperatures, there were some marked differences in the developmental rates, with rates being about 20% higher (the insects developed quicker) in the McConnachie (2004) study. This could be ascribed to the origin of the populations used here (see below), but could also be due to differences in food quality. Although both studies do report on how the plants were grown, neither study analysed the carbon:nitrogen ratios in the fronds and rhizomes. Thus, if the quality of plants used by McConnachie (2004) was slightly higher, it is likely that the insects would have developed faster. The CT_{min} and CT_{max} values were also different between the two studies, with *S. rufinasus* showing a much narrower range in the current study. This could be due to methodological differences between the studies. In the McConnachie (2004) study, the rate of cooling and heating was 1°C per minute, while in the current study the rate was 0.25°C (see section 4.2 for discussion on this). The effect of this difference is that the duration of exposure to a particular temperature was far lower in the McConnachie (2004) study, therefore the insects were less stressed at the high and low extremes tested, and thus showed more tolerance for these temperatures. The LLT_{50} was also different between the two studies, and much lower in the McConnachie (2004) study. The McConnachie (2004) study was carried out using insects sourced from an outdoor pool during winter at Wits University in Johannesburg at an elevation of around 1800m above sea level where the winters are cold and characterized by frequent frosts (M. Hill pers. comm.), while the current study was conducted on insects sourced from a pool in a polyurethane tunnel during the summer months in the Eastern Cape Province. This suggests that there might have been some cold hardening (Teets and Denlinger 2013) of *S. rufinasus*. This difference is also expressed in the developmental threshold (t) and developmental constant (K), which is produced from the regression of the duration of development data. The developmental threshold and developmental

constant data suggest that the McConnachie culture could develop at lower temperatures, but it took them longer to develop.

Table 4.1 Comparison of several *Stenopelmus rufinasus* development parameters as influenced by temperature between the current study and McConnachie (2004).

Temperatures used (^{O}C)	**McConnachie 2004 Mean Duration of Development (Days ± SE)**	**Current Study Mean Duration of Development (Days ± SE)**
14.5	54.6 ± 3.5	
18.0		48.0 ± 1.4
18.9	28.3 ± 1.8	
20.0		32.0 ± 0.2
23.4	16.5 ± 1.5	
24.0		19.9 ± 1.8
27.0		16.0 ± 0.4
29.0		16.0 ± 1.3
29.4	12.8 ± 1.2	
31.9	12.0 ± 1.0	
CT $_{min}$	1.3 ± 0.2	5.4 ± 0.3
CT $_{max}$	47.5 ± 0.1	44.0 ± 0.2
LLT$_{50}$	-12.1	-8.9
UTL$_{50}$	36.5	43.0
t	9.2^{o}C	13.6^{o}C
K	256.4 days	222.5 days

McConnachie (2004) mapped his data over the known distribution of *A. filiculoides* in South Africa to predict the number of generations the agent might have at any one site as a proxy for how effective the weevil would be, as the current study did (Figure 3.3). A visual comparison of the maps shows while that *S. rufinasus* is predicted to establish throughout the distribution of *A. filiculoides,* the model presented here predicts fewer generations per year in comparison to the model presented by McConnachie (2004). McConnachie (2004) predicted up to 20.1 generations per year in the warmer parts the country (Musina, Limpopo Province), while the highest number of generations predicted in this study was in Mpumulanga Province (Figure 3.3). Both studies predicted between 10 and 14 generations at most sites of infestation by the weed.

4.1.2 *Neohydronomus affinis*

Neohydronomus affinis was also shown to be a thermally tolerant species with a LLT_{50} of -9°C and an ULT_{50} of 42°C, once again the differential in temperature range was approximately 50°C. The original collection site of *N affinis* was Pelotas, Brazil at sea level, 31°46S 52° W (DeLoach *et al.* 1976). The climate of Pelotas is once again characterized as humid subtropical with an average summer temperature of 23 °C, but the daytime temperatures frequently reach 32°C to 37°C, and an average winter temperature of 12 °C, while the temperature seldom drops below zero. This weevil has a fairly wide natural distribution having been collected in the Buenos Aires, Chaco and Formosa provinces of Argentina (DeLoach *et al.* 1976) north to the Amazon River (Cordo and Sosa 2000). *Neohydronomus affinis* was introduced into Australia from Pelotas in 1982, and then into quarantine in South Africa in 1985 (Winston *et al.* 2014). The insect thus spent approximately three years in quarantine, and if it is assumed that the quarantine temperatures were approximately 25°C, according to this study the insect would have gone through some 27 generation in quarantine.

The effect of these rearing conditions are unknown, but are likely to have been nullified by the some 32 years that this insect has been established in the field in South Africa (Klein 2011). *Neohydronomus affinis* has been released, and established in eleven countries in Africa, and Australia, Papua New Guinea, Puerto Rico, the USA and Vanuatu (Winston *et al.* 2014). Unlike *S. rufinasus,* the water lettuce weevils has mainly established in the more tropical and subtropical regions of the world, but this is where *P. stratiotes* is problematic and requires intervention. However, the fact that this insect occurs in the Buenos Aires Province of Argentina around 30°S (DeLoach *et al.* 1976) and has established in the higher elevation regions of South Africa (Coetzee *et al.* 2011) suggests that it might have a wide thermal tolerance. In this study, the developmental rate of *N. affinis* ranged from more than 80 days at 18°C to 30 days at 29°C. This compares favourably with the study of DeLoach *et al.* (1976) who recorded a total duration of development (egg to adult) of this species to be 25 to 30 days at 25°C, but once again methodology and food quality could have differed between the two studies.

The critical thermal parameters of *N. affinis* and *S. rufinasus* were very similar (see Figure 3.1), but the duration of development showed that *N. affinis* required significantly longer to develop, even at the higher temperatures and this resulted in a higher threshold for development (t) and duration of development at that temperature. The predictive modelling suggests that there could be as many as 8 to 10 *N. affinis* generations along the warm, subtropical to tropical east coast of South Africa and 4 to 7 generations in the cooler interior (Figure 3.3), suggesting that establishment in South Africa is not limited by climate.

4.2 Insect thermal physiology studies methodologies

Low temperatures are one of the main factors limiting the distribution and performance of many insect species, particularly those used in biological control programmes, however, it is not always clear which metrics should be measured to determine these limits. Choosing a single or few metrics as a proxy for cold tolerance is difficult as there are no set measures because these often depend on the life histories of the species being investigated. Because measuring low temperature performance is important to provide details of a species' cold tolerance, and to compare this with other species, in biologically relevant contexts such as weed biological control, no single measure will be appropriate for all species or situations (Sinclair *et al.* 2015).

For this reason, this study determined three metrics which have high ecological relevance, are relatively easy to measure, and directly relevant to the real world. Measurements of Critical Temperatures (CTs) require controlled heating or cooling equipment, and temperature measurement, but need continuous observation of active/mobile insects only. The advantage of these measures is that they can be correlated to other measures of thermal tolerance, they can be compared between species, as has been done here, and they can be compared to ambient temperatures. Similarly, LT studies also need heating/cooling equipment and temperature measurement, and are highly ecologically relevant because they are directly relevant to the real world. The only disadvantage is that they are time consuming. Developing degree days models are the most time consuming, but are also directly relevant to the real world because inferences on voltinism can be made for particular localities if maximum and minimum temperatures are known. In this study, it is clear that both *S. rufinasus* and *N. affinis* are able to complete multiple generations at sites throughout South Africa, indicating that their establishment should not be

limited by temperature. A shortcoming of this metric is that it does not take into account microclimates that the insects may experience, but Coetzee (2012) showed that this was not an issue when inferring the number of generations that a water hyacinth control agent could produce because there was no difference between weather station data or microclimate data from actual field sites.

4.3 Implications for the biological control of *Pistia stratiotes* and *Azolla filiculoides*

Undertaking thermal physiology studies on weed biological control agents has become increasingly popular over the last two decades (Byrne *et al.* 2004) and there have been a number of studies on aquatic weed agents. Some of these studies have been conducted prior to the release of the agent to determine if the agent will establish, and thus if it is worth the effort of screening for release (e.g. *Eccritotarsus catarinensis* on water hyacinth in the USA (Coetzee *et al.* 2009)). This is without doubt the most cost-effective use to thermal physiology studies in weed biological control, but they are seldom used for this purpose, and it is unclear, with the exception of the example cited above, if any agent has not been screened due to concerns of lack of thermal compatibility.

The most common use of insect thermal physiology studies in weed biological control is post-release of an agent, in an attempt to understand why the agent might not have established at all, or in certain geographic areas, or why it might not be as effective as anticipated (e.g. *Eccritotarsus catarinensis* on water hyacinth in South Africa (Coetzee *et al.* 2007); *Megamelus scutellaris* on water hyacinth in South Africa (May and Coetzee 2013) and the USA (Foley *et al.* 2016; *Agasicles hygrophila* Selman & Vogt on alligator weed (*Alternanthera philoxeroides* (Mart.) Griseb.) in

China (Zhao *et al.* 2015)). These studies are also very useful in that they inform weed biological control practitioners as to whether they should consider another, cold or heat adapted species of agent, or where to use other control interventions such as herbicide application and mechanical control.

More recently thermal physiology studies have been used to predict how the agent will respond to certain global change scenarios, and how this might affect the level of control achieved (e.g. *Cyrtobagous salvinae* on *Salvinia molesta* in South Africa (Allen *et al.* 2014)). The results presented in this thesis falls into this third justification for these studies. The biological control of the two weeds studied in this thesis have been highly successful and both weeds are considered to be under complete biological control and no other interventions are required to reduce their populations and impact (Coetzee *et al.* 2011). This study has shown that under the current climatic conditions, the agents have a wide thermal tolerance and are limited by the availability of their host plant and not extremes of temperature.

Pistia stratiotes grows best within the range of 15°C and 35°C, with optimum growth achieved between 22°C and 30°C. Seeds do not germinate at temperatures less than 20°C, but they survive at least two months in cold water at 4°C and several weeks in ice at –5°C (Neuenschwander *et al.* 2009). Although these conditions do not occur in South Africa, the cold tolerance of the seeds might have implications for the biological control of this weed elsewhere as although these temperatures are within the LLT_{50} of the insect, it is unable to survive at these temperatures for a prolonged period of time, and the insect does not have a diapause stage and tends to overwinter as late instar larvae in the leaves at the base of the plant (Neuenschwander *et al.* 2009).

The optimum temperatures for the growth of *A. filiculoides* are between 18°C and 28°C, but it is able to tolerant a wide range of temperatures and can survive in temperatures as low as -5°C. The growth of *A. filiculoides* is reduced above 35°C and mortality occurs if temperatures remain above 45°C for prolonged periods of time. *Azolla filiculoides* produces spores that are resistant to freezing and germinate during the early summer (Lumpkin and Plucknett 1980). Again these temperatures are within the thermal envelope of *S. rufinasus*, but as with *N. affinis,* the insect will not be able to survive for prolonged periods at these temperatures, and the agent also does not undergo diapause. There have been several reports from landowners in the higher elevation and thus cooler areas of South Africa that *A. filiculoides* becomes abundant at the end of winter (presumably from germinated spores), but that within a month, *S. rufinasus* has found these patches of the weed and controlled them (Hill and McConnachie 2009). McConnachie (2004) showed that *S. rufinasus* is a very good disperser, with individuals being able to fly up to 300km in a short period of time. This means that even where there is local extinction of the insect due to winter temperatures outside of their thermal capability, they are able to recolonize those sites when more favourable conditions return. In the United Kingdom, *S. rufinasus* dies out in the northern areas of England and Ireland during the winter and returns from the south during the summer (Baars and Caffrey 2008). So although *S. rufinasus* has a wide thermal tolerance of cold conditions, it also has a behavioural adaptation that adds to its success as a biological control agent.

4.4 Conclusion

Stenopelmus rufinasus and *N. affinis* are very effective agents against their respective aquatic weeds in South Africa. Both species display a wide thermal tolerance that will not preclude them

from establishing and impacting their host plants throughout South Africa and they are only limited by the presence of their hosts. Climate change scenarios in South Africa vary from the worst case scenario of very significant warming, as high as 5–8°C, over the South African interior by the end of this century, to a more moderate prediction of 2.5–3°C (DEA 2013). Even the higher prediction falls within the thermal envelopes of both weed and agent species, so we should thus not anticipate any change in the homeostasis of the interaction. Within South Africa, the biggest threat to the control of these two species remains phosphorous pollution for *A. filiculoides* and nitrogen pollution for *P. stratiotes*, which allow the plants to grow vigorously and compensate for herbivory.

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