

**Investigating thermal physiology as a tool to
improve the release efficacy of insect biological
control agents.**

THESIS

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Abstract

Biological control is commonly used for the control of invasive aquatic weeds, which often involves the release of multiple host-specific agents. Releasing multiple agents has inherent safety concerns as the introduction of each new agent is associated with risks, but is often required to improve control where establishment is limited. Climatic incompatibility between the agent's thermal physiology and its introduced range often causes agents to fail to establish. However, it has been suggested that the thermal physiology of insects is plastic. Therefore, the potential to manipulate their thermal physiologies before releasing them into the field needs to be explored; reducing the need to release additional agents, thereby ensuring the safety of biological control. This thesis therefore aimed to firstly, determine whether season and locality influenced the thermal physiology of two field populations of a water hyacinth (*Eichhornia crassipes*) control agent, the mirid *Eccritotarsus catarinensis*; one collected from the hottest establishment site, and one collected from the coldest establishment site in South Africa. Their thermal physiology was significantly influenced by season and not by the sites' climate, suggesting their thermal physiology is plastic under field conditions. Secondly, the classical method of determining the lower critical thermal limit (CT_{min}), and a new respirometry method of determining this limit, compared the thermal physiology of two *Eccritotarsus* species reared in quarantine. *Eccritotarsus catarinensis* was significantly more cold tolerant than the more recently released *Eccritotarsus eichhorniae*, despite similar maintenance conditions, and as such, was used to establish whether cold hardening under laboratory conditions was possible. Successfully cold hardened *E. catarinensis* had a significantly lower CT_{min} compared to the field cold acclimated population, suggesting that cold hardening of agents could be conducted before release to improve their cold tolerance and increase their chances of establishment, allowing

for further adaptation to colder climates in the field to occur. Increasing establishment of the most effective agents will decrease the number of agents needed in a biological control programme, thus encouraging a more parsimonious approach to biological control.

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Chapter 1: General introduction

1. 1 Biological control

In 1964, DeBach defined biological control (biocontrol) as “the actions of parasites, predators, and pathogens in maintaining another organism’s density at a lower average than would occur in their absence” (DeBach 1964; McFadyen 1998). Weed biological control is the process whereby a natural enemy from the native range of an invasive alien plant is released onto the populations of the plant in the introduced range. Agents are only approved for release after they have undergone extensive host specificity testing. Only agents that are host specific to the target weed, and are therefore not a threat to close relatives of the target weed, indigenous or commercially important plant species, are accepted for release (Briese 2000). Under ideal conditions, once the biological control agent is released, it will be sufficiently damaging to reduce plant populations. Shortly after plant populations decrease, the control agent population will decrease because the target plant is the only source of food available to the agent (Briese 2000). The agent and plant populations will continually oscillate. But if the agent is effective, then plant populations will be permanently reduced below the damage threshold (Figure 1) (Briese 2000).

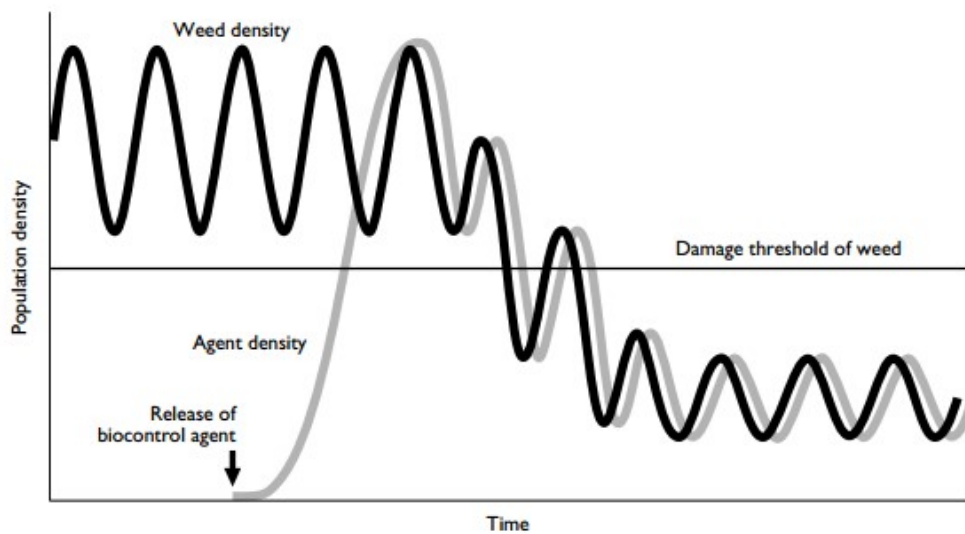


Figure 1.1: Relationship between the biological control agent and the invasive alien plant (Briese 2000).

Biological control programmes have been initiated against a variety of weeds in South Africa, including water weeds (Zimmermann *et al.* 2004; Coetzee *et al.* 2011). Water weeds are problematic as they cause an increase in siltation, evapotranspiration and deoxygenation of water bodies they have invaded (Cilliers 1991; Hill 2002). Biological control programmes against water weeds were initiated in South Africa in 1974 when the weevil, *Neochetinaeichhorniae* Warner (Coleoptera: Curculionidae), was released onto water hyacinth, *Eichhornia crassipes* (Mart.) Solms (Pontederiaceae) (Coetzee *et al.* 2011). Since then, biological control of several floating invasive water weeds, native to South America, have been successfully controlled in South Africa using biological control. Some of the invasive water weeds that have been controlled are *Salvinia molesta* D.S. Mitch. (Salviniaceae), *Pistia stratiotes* L. (Araceae), *Myriophyllum aquaticum* (Vell.) Verdc. (Haloragaceae) and *Azolla filiculoides* Lam. (Azollaceae), while only partial control has been obtained against *E. crassipes* (Coetzee *et al.* 2011). In some cases, a single agent is sufficient

to control the target weed, but many biological control programmes, including the *E. crassipes* programme, require multiple agents.

1. 1. 2 Predicting the efficacy of an agent

There are however, numerous risks when releasing multiple biocontrol agents. Although biological control has an excellent safety record worldwide (Suckling & Sforza 2014), there is still an innate risk in the release of any agent, no matter how small that risk is (Denoth *et al.* 2002; Suckling & Sforza 2014). So, the release of ineffective agents should be avoided if possible (Hoelmer & Kirk 2005). Predicting the efficacy of an agent prior to release is an essential part of any biological control programme. Yet, it is often overlooked (McClay & Balciunas 2005). Although there are arguments against the ability to predict the efficacy of an agent, there are criteria which an agent needs to fulfil before release. Finding the most appropriate agent for release can be challenging with no guarantee that the agents released will result in establishment or control (McClay & Balciunas 2005). The focus of pre-release studies has been on host-specificity testing to ensure that no non-target feeding effects occur once agents are released. Agents may successfully establish and become abundant, but may not necessarily provide the level of damage needed to control the weed (McClay & Balciunas 2005).

Thus, more emphasis should be placed on predicting the efficacy of agents prior to release to ensure that only the most effective agents are released and to avoid potential failures. There are many reasons why insects fail in the field; however, one of the main reasons for biological control failure is climatic incompatibility (Byrne *et al.* 2002). Establishment and success rates may be improved if we had a better understanding of the agent's thermal physiology before release.

1. 2 Climate incompatibility & thermal tolerance of agents.

Occasionally released agents fail to establish in the field and one possible reason for many of the failures is climatic incompatibility between the agent and the climate in the new introduced range (Stewart *et al.* 1996). This incompatibility causes about 44% of biological control agents to fail once released (Byrne *et al.* 2002). For example, control of *Solanum sisymbriifolium* Lamarck (Solanaceae), wild tomato, which is native to South America, and invasive in South Africa is limited due to incompatibility of its control agent with the South African climate (Byrne *et al.* 2002). The native range of *S. sisymbriifolium* experiences a warm temperate climate, allowing the plant to survive very cold temperatures by dying back and re-establishing in spring from root stock and seed banks (Byrne *et al.* 2002). A leaf-feeding biological control agent from South America, *Gratiana spadicea* (Klug) (Coleoptera: Chrysomelidae) (tortoise beetle), was released against this weed in 1994 (Hill & Hulley 1995). It was released at seven sites in the Mpumalanga Highveld, however establishment of the beetle only took place at two of these sites. It was suggested by Olckers *et al.* (1999) that establishment failure may have been due to low temperatures experienced during the winters in these areas. It was then proposed that if beetles were collected from cooler sites in their native range, they would be cold adapted and therefore able to survive the cold winters (Olckers *et al.* 1999). Climatic incompatibility was later confirmed by Byrne *et al.* (2002) who suggested that although the insect is cold tolerant, repeated exposure to cold temperatures at night may cause an accumulation of stress on the insects, resulting in mortality. The temperatures in the introduced range often do not go above the developmental threshold of the agent, resulting in fewer generations and therefore it is more difficult for the populations to persist and build up to high enough numbers to control the target weed (Byrne *et al.* 2002).

Environmental temperature is one of the most important ecophysiological variables that affects the performance of insects. Locomotion, foraging ability, reproductive output, immune function and developmental time are all governed by the insect's body temperature, which is directly influenced by the environment (Angilletta *et al.* 2002). Thermal tolerance is also thought to influence an insect's range size and geographical variation (Klok & Chown 2003). Insects that inhabit colder climatic regions have lower temperature thresholds compared to insects which inhabit warmer climatic regions (McClay & Hughes 1995; Bryant *et al.* 2002).

In cool temperate climates, the insect's ability to tolerate winter low temperatures and to continue important processes, such as reproduction and development, determines whether establishment of populations is possible (Hughes *et al.* 2011). For example, *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae) has a developmental threshold of 5.8°C which would allow for some development during winter months in the cool temperate climate of northern Europe where it is a biocontrol agent for aphids. The low developmental threshold, along with its low lower lethal limit (LLT₅₀) (from -10.1°C to -22.1°C) allow for the production of approximately 9.8 generations in a year, resulting in enough generations for successful establishment of *L. testaceipes* (Hughes *et al.* 2011). Biocontrol agents therefore need to have thermal tolerances which match the range of the weed in the invaded range. Understanding biocontrol agent's thermal tolerance could reduce the number of failed agents that are caused due to climatic incompatibility.

1. 3 Parameters used to determine thermal physiologies.

Experiments to determine thermal thresholds of biological control agents are usually conducted in laboratories under controlled conditions. These experiments include determining critical and lethal thermal limits, as well as developmental times at constant temperatures. All limits described have value in determining the agent's potential distributions as they provide information on the cold or heat tolerance of the agent.

1. 3. 1 Development time and degree-days.

One of the most commonly used measures of insect thermal physiology, particularly for constructing potential distribution maps, is determining the influence of temperature on the developmental rate of a species. Development occurs within a range of temperatures, starting at its lower developmental threshold (t), increases to a maximum at its optimal temperature and then decreases back down to zero after its upper lethal temperature is reached. The 'physiological time' (K) is defined as the heat accumulation that is required for a species to complete development at the lower developmental threshold (Campbell et al. 1974).

The t and K values determined during developmental assays can be used to determine how many generations agents will be able to go through at a particular release site using weather station data for that particular site. This then gives an idea of whether releasing an agent in that area will allow for enough generations in a year to make its release viable.

Furthermore, this is often used to determine post-release potential distributions (McClay and Hughes, 1995). Degree-days ($^{\circ}\text{D}$) is the unit used to represent the number of days an agent needs to complete development from egg to adult. Insects that are warm adapted will have higher degree-days as they need more heat to complete development. One degree-day is equal to 24 hours when the insect is exposed to 1°C higher than their developmental threshold, t (the lower temperature where development ceases) (Campbell *et al.* 1974).

1. 3. 2 Critical thermal minima and maximum

Although developmental rates are the most commonly used parameter for thermal physiology studies in biocontrol, there are other limits which may determine potential distribution of agents. Critical thermal limits (CTs) are the temperatures that indicate the point at which behaviour is impaired (e.g. loss of locomotory function), but from which recovery is possible (Mitchell *et al.* 1993). Testing CTs is a dynamic method which involves the constant increase or decrease rate of temperature change until the insects “knockdown” temperature is reached (Addo-Bediako *et al.* 2000), defined as the temperature at which an individual either cannot right itself when turned on its back or the insect can no longer adhere to the side of the experimental chamber.

1. 3. 3 Lethal limits

Lethal limits (LTs) are physiological tolerance limits and are the temperatures beyond which recovery is not possible (Mitchell *et al.* 1993). Methods to determine these limits are static, which usually involves insects being exposed to a range of temperatures for a set duration of time. The LT_{50} (upper lethal limit ULT_{50} ; lower lethal limit LLT_{50}) is the temperature at which 50% of the population dies (Addo-Bediako *et al.* 2000).

Although the above methods are used for determining an insect’s thermal physiology, it has been proposed that insect thermal limits may vary depending on their environment, and may therefore increase or decrease depending on the temperature they are exposed to, known as phenotypic plasticity (Gavrilets & Scheiner 1993).

1. 4 Thermal plasticity

Thermal plasticity is defined as a direct change in the phenotype/ physiological trait of each individual within a particular population. These plastic traits only appear due to external stimuli when the particular trait increases fitness and are not based on natural selection. These traits are not expressed or are even reversed when the stimuli are absent (Gotthard and Nylin 1995).

All organisms, including insects, respond to their environment and any changes that may occur within their environment. Insects have the ability to change particular traits, including physiology, life history traits and even development when a change in their environment occurs (Beaman *et al.* 2016). This change is phenotypic and, as mentioned above, does not change the organisms' genotype. This phenomenon is known as phenotypic plasticity (Beaman *et al.* 2016). Phenotypic plasticity may increase an individual's (and population's) overall fitness greatly. In a heterogeneous environment, it is unlikely that the same traits will be beneficial for all changes in the environment (e.g. climate) (Beaman *et al.* 2016) and therefore being able to change certain phenotypic traits allows individuals to persist in changing environments (Via *et al.* 1995).

Plasticity is a very old term used for any change during development or for environmentally sensitive traits which are changed within an individual's life time (Beaman *et al.* 2016). Phenotypic plasticity may result in traits which are reversible and traits which are non-reversible (although not heritable). The process where an offspring's phenotype is changed due to the environmental conditions its parents experienced is known as transgenerational plasticity and is thought to be non-reversible (Beaman *et al.* 2016). Inherited genes cannot solely explain the effect of the parental phenotype on offspring phenotype through the transmission of non-genetic developmental factors and plasticity. Transgenerational plasticity is favoured when the environment is unpredictable (Uller 2008). A non-transgenerational plastic trait, for example, the ability of an insect to be rapidly cold

hardened, thereby decreasing the insect's critical thermal minima (CT_{min}), is thought to be a reversible plastic trait and not determined by the parent's environment as it is not heritable, but rather a change experienced by the individual. This relationship can be envisioned as a reaction norm, which describes how a single genotype is phenotypically expressed across a range of environments (Gavrilets & Scheiner 1993).

Although the phenotypic trait which has changed is not heritable, the genes which can be plastic are often selected for and can be passed down (DeWitt *et al.* 1998). One mechanism suggested for phenotypic plasticity is known as allelic sensitivity where some alleles are expressed depending on the environment in which the individual occurs; these alleles have varying effects on the phenotype (DeWitt *et al.* 1998). For example, rearing two *Tetrix* species, *Tetrix ceperoi* Bolívar and *T. subulata* Linnaeus (Orthoptera: Tetrigidae) on different colour substrate resulted in a change in their phenotype colour (Hochkirch *et al.* 2008). Dark substrate typically produced dark morphs of these species and light substrate, light morphs. These colour changes increased with each moult, suggesting it was a change dependent on the colour of the substrate. The colour changes were also reversible, suggesting that the morphs are not purely determined by genetics (Hochkirch *et al.* 2008). The ability to change between the different colour morphs could be crucial for predator avoidance by camouflaging with the substrate (Hochkirch *et al.* 2008). All organisms will be born with the genetic capability of expressing each of the phenotypes; however, the environment to which they are exposed will determine which phenotype is expressed for each individual.

Most of the studies that manipulate insects' thermal physiology to test phenotypic plasticity theories have been conducted using a single family of fly, the Drosophilidae. This family was used as a model organism because individuals are easy to rear and have short lifespans and it is therefore easier to experiment with transgenerational changes (Klok & Chown 2003). As thermal plasticity has become an increasingly popular topic of research

around how insects will handle changing environments due to climate change, other insects are being tested for plastic responses to various external stimuli, such as temperature changes. The most common method of testing phenotypic plasticity is determining an insect species' thermal limits before and after a temperature change to determine whether exposure to varying temperatures can cause a shift in their thermal tolerance.

Klok and Chown (2003) set out to test phenotypic plasticity on a non-model species which is not chosen for extensive study because it does not have model organism characteristics (i.e. difficult to rear in the laboratory, has low fecundity, long life cycle or poor genetics). This was done to test whether phenotypic plasticity theories can be more broadly applied to insects. A study to test thermal variation among populations was conducted on the *Ectemnorhinus* group of weevils which are restricted to the islands in the Southern Indian Ocean. Weevils were collected along a temperature gradient along two islands, Marion Island and Heard Island. Results showed that critical thermal limits varied between islands as well as between populations on each island. This variation is thought to be a consequence of thermal plasticity rather than an adaptive change. Thermal plasticity was suggested as a reason behind the variation in thermal limits due to the populations of weevils maintaining the same responses to acclimation when exposed to different climates in the field (Klok & Chown 2003).

1. 5 Phenotypic plasticity and biocontrol

The implications of phenotypic plasticity for biological control is that if an insect does exhibit phenotypic plasticity in its thermal physiology, then the rearing temperature should become a lot more important depending on where the insects are to be released. It has been shown in a few cases that rearing insects at a higher/ colder temperature increases their heat/ cold resistance. For example, this has been found with rearing *Drosophila* species at constant

colder, winter temperatures which resulted in an increased cold resistance (Hoffmann *et al.* 2005). The implication for biocontrol is that the temperature at which the agents are reared may influence their thermal physiologies and therefore the climatic areas in which they can successfully establish.

One way that phenotypic plasticity has been shown in insects is through cold hardening, which involves exposing insects to cold temperatures for an extended period of time which usually results in a decrease in the insects CT_{min} (Teets & Denlinger 2013). Cold hardening was tested in a study on the Antarctic chironomid, *Belgica antarctica* Jacobs (Diptera: Chironomidae) (Lee *et al.* 2006). Larvae of *B. antarctica* were collected during summer and were referred to as summer-acclimatised insects. Larvae were then collected from ice samples and referred to as cold-acclimated. A sample of larvae, both summer and cold-acclimated, were subjected to rapid cold hardening (RCH) trials and another sample were kept as controls and were not subjected to RCH trials. Survival for both the RCH sample and the control sample were then tested under different temperature treatments. Larvae which were cold hardened had a higher survival rate at colder temperatures compared to the controls. The cold-acclimated sample were more cold tolerant than the summer acclimatised sample both with the control trials and the RCH trials. Larvae that survived the coldest temperature however, showed limited mobility which suggests that these larvae did experience some freezing injury and that RCH did not protect the larvae completely from injury (Lee *et al.* 2006). If the cold hardening could be induced in biological control agents in a similar way to *B. antarctica*, then this may suggest that populations of agents could be cold hardened before being released into the field to potentially reduce climatic incompatibility failures. This thesis will explore the potential of thermal plasticity and cold hardening of two biological control agents released against *E. crassipes* in South Africa in an attempt to improve establishment and control.

1. 6 Invasion Ecology and Control of *Eichhornia crassipes*

Eichhornia crassipes one of the most difficult water weeds to control in South Africa and is very damaging to the aquatic ecosystems it invades (Cilliers 1991). Initial control measures included chemical herbicides as well as mechanical removal of smaller invasions, but these methods are expensive, damaging to the environment and non-target organisms; and only provides temporary relief for the water body (Cilliers 1991). Biological control is therefore considered the only long-term and sustainable solution. Successful control has been achieved for *E. crassipes* in some areas in South Africa; however, there has been limited control in other more temperate areas of invasions (Coetzee *et al.* 2011).

Eichhornia crassipes, remains South Africa's worst aquatic weed despite attempts to control it over the last 50 years (Coetzee *et al.* 2007). It originates from the Amazon Basin in South America and was brought to South Africa as an ornamental plant in the 1900s (Cilliers 1991). Because water hyacinth is found in many climatic conditions throughout South Africa, including areas of high altitude which experience summer rainfall and winter frost; the coastal areas which experience winter rainfall and no frost, as well as the coastal areas which receive summer rainfall, the biological control agents need to be able to establish and reproduce under all these different conditions (Julien *et al.* 2001). Hill and Olckers (2001) stated that the lack of control of *E. crassipes* in some parts of South Africa is due to the climate being too cold and harsh for the biological control agents in areas that experience winter frost. This means that biological control agents cannot establish permanently as they cannot survive through winter, or populations crash in winter and cannot build up to large enough population to cause sufficient damage to reduce the plant populations below the damage threshold. Although biocontrol success has been patchy in South Africa due to climatic incompatibility (Coetzee *et al.* 2007), complete control has been obtained in the tropical areas where water hyacinth has invaded, such as Papua New Guinea and Uganda (Coetzee *et al.* 2007).

Because of the difficulty in attaining acceptable levels of control in South Africa, eight species of biological control agents have been released on water hyacinth which is more than anywhere else in the world. The agents include; a moth, *Niphograptia albiguttalis* Warren (Lepidoptera: Pyralidae), two weevils, *Neochetina bruchi* Hustache (Coleoptera: Curculionidae) and *N.eichhorniae* Warner (Coleoptera: Curculionidae); a mite *Orthogalumna terebrantis* Wallwork (Acari: Galumnidae); a pathogenic fungus *Cercospora rodmanii* Conway (Conway and Cullen 1978); a planthopper *Megamelus scutellaris* Berg (Hemiptera: Delphacidae), a grasshopper, *Cornops aquaticum* Brünner (Orthoptera: Acrididae) and two bugs *Eccritotarsus catarinensis* (Carvalho) (Stanley & Julien 1998; Julien *et al.* 2001; Coetzee *et al.* 2011; Coetzee & Hill 2012) and *E. eichhorniae* Henry (Hemiptera: Miridae) (Henry 2017).

1. 7 Thermal physiology and plasticity of *Eccritotarsus catarinensis* and *Eccritotarsus eichhorniae*

Eccritotarsus catarinensis and *E. eichhorniae* are cryptic species and have both been released on *E. crassipes* in South Africa. At high densities, both the nymphs and the adult cause chlorosis in the leaf which then reduces reproduction and growth of the weed, eventually killing the plant (Hill *et al.* 1999). The populations of *E. catarinensis* used for biocontrol in South Africa was collected in Florianopolis in southern Brazil, which has an average temperature of 22 °C. It was then brought back to South Africa and kept in quarantine for host specificity studies. While in quarantine, the population experienced a drastic genetic bottleneck and was reduced to only one gravid female (Taylor *et al.* 2011). It was first released in 1996, and has since established at a number of sites in the warmer parts of South Africa. (Coetzee *et al.* 2011) *Eccritotarsus eichhorniae* was collected on the Yarapa River near Iquitos in Peru in 1999, which has an average temperature of 27 °C. At the time that it was collected, the population from Peru was thought to be the same species as the population from Brazil, so a

collection was made in Peru and imported into quarantine in the hopes that they would interbreed and increase the genetic diversity of the biological control agent population (Taylor *et al.* 2011). After importation they were found to be cryptic species that were reproductively isolated (Paterson *et al.* 2016).

Due to the agents' native climate influencing their thermal tolerance and because the two species come from climatically different areas, they may have different thermal physiologies, which will therefore have an impact on the success of each species as a biocontrol agent (McClay & Hughes 1995; Mopper *et al.* 1995; Hill & Olckers 2000; Coetzee *et al.* 2007; Taylor *et al.* 2011). This suggests that close consideration of the thermal tolerance of the agents needs to be taken into account when releasing them in to the wild. Studies of their development at different temperatures showed that the thermal physiologies of the two populations were significantly different and the *E. eichhorniae* had a higher developmental threshold (Voogt *et al.* 2010). These results suggest that *E. eichhorniae* establish more readily at warmer sites and *E. catarinensis* at cooler sites. In the field, however, the distribution of *E. catarinensis* is limited by colder temperatures (Coetzee *et al.* 2007). Invasions of *E. crassipes* in temperate areas which experience winter frost are a challenge for biological control programmes because the frost causes plant die back which causes a knock-on effect on the population numbers of *E. catarinensis*. The plant is able to reach high densities in spring; however, the insect only reaches high enough densities in the middle of summer. This lag results in at least three months of unregulated growth of *E. crassipes* with control agent populations not reaching high enough numbers fast enough to regulate *E. crassipes* populations (Hill *et al.* 1999).

Although establishment has failed in most temperate sites of *E. crassipes* invasions in South Africa, one population of *E. catarinensis* has persisted. This population was first released on the Kubusi River (Stutterheim, Eastern Cape, South Africa) in 1997, and has

persisted for the last 20 years (pers. obs.). This area experiences winter frosts as well as plant die back. It is not known whether this population has become cold adapted after extended exposure to the unfavourable conditions or if its survival at this site can be explained by thermal plasticity. Unlike phenotypic plasticity, populations that have become cold adapted show genetic differences for thermal tolerances which corresponds to their local climate and is not a reversible change (Yampolsky *et al.* 2014). However, there may also behavioural changes which allow the Kubusi population to survive (Bale 2002), for example, hiding inside a curled leaf for warmth during colder temperatures.

Since being brought into South Africa, both *Eccritotarsus* species have been reared in similar constant climatic conditions at Rhodes University. The quarantine and mass-rearing facility where these two populations are mass-reared are kept at a constant 26°C with a 14:10 photoperiod cycle. Due to these similar rearing conditions, both species' thermal physiologies were re-evaluated to determine if their physiologies were still representative of their native climates and if one species was cold/ warm adapted. Preliminary results in 2015 showed that the thermal physiologies of the two *Eccritotarsus* species had converged after the extended period under the same climatic conditions. It is possible that if the species thermal physiologies have converged, it is because their thermal physiologies are plastic.

It was shown by Porter *et al.* (in review) that *E. catarinensis* does express phenotypic plasticity through the process of cold hardening. However, phenotypic plasticity could not explain all the changes in the insect's thermal physiology after cold hardening as the two populations' thermal tolerances did not completely converge, suggesting that both phenotypic plasticity and local adaptation to the climate has occurred.

1. 8 Research aims

The aims of this thesis were to investigate the potential to manipulate the agent's thermal physiology to improve thermal tolerance and therefore improve establishment success in temperate areas. This was done by firstly, investigating whether *E. catarinensis* expresses seasonal phenotypic plasticity by determining the thermal limits (CTs and LTs) and degree-day models of populations of *E. catarinensis* found at the warmest and coldest site of establishment in South Africa. Secondly, it was determined whether *E. catarinensis* or *E. eichhorniae* is more adapted to the conditions in their native distribution or if quarantine conditions have caused their thermal physiologies to converge. Thirdly, it was determined if cold hardening has an effect on the thermal physiology (plasticity) of the more cold adapted species of *Ecritotarsus* under laboratory conditions. An alternate method for determining thermal limits, using respirometry, was also briefly explored.

If agents' thermal physiology can be manipulated under laboratory conditions prior to release, it may increase the success of establishment through a decrease in climatic incompatibility. Increasing the success of agents will prevent the release of ineffective agents which will enhance the success and safety of biological control programmes.

Chapter 2: Thermal physiology of two Eccritotarsus catarinensis field populations.

2.1 Introduction

For a biological control agent to be effective, it needs to have high fitness in the field (Morin *et al.* 2009). Due to the ectothermic nature of insects, their metabolism is dependent on the environmental temperature; thus, the agent's fitness, which is regulated by the insect's ability to feed and reproduce, is affected by temperature and can be reduced towards extreme low and high temperatures which ultimately reduces the agent's performance in the field. Most insects' optimal performance and developmental temperatures fall within a wide range of temperatures (Neven 2000). The time taken for an insect to develop is also directly dependent on temperature. Developmental time decreases as temperature increases, up to a certain temperature, after which it is fatal for the individual (Bale 1991; Bryant *et al.* 2002). This has implications for the rate of population growth and hence control of the target weed (Bryant *et al.* 2002).

Understanding insect thermal physiology is therefore crucial for biological control as it is important to know whether the chosen control agent will be able to survive and perform optimally under the climatic conditions in the area into which it is to be released (Bryant *et al.* 2002; Terblanche *et al.* 2007; Hazell *et al.* 2008). Although some studies have investigated the thermal physiology of agents prior to release (Hatherly *et al.* 2005), most are performed retrospectively to explain an agent's failure to establish following release (Ramanand *et al.* 2017). Both pre- and post-release studies are important. Thermal studies are often performed post-release as these studies can be time consuming and need economic resources and are therefore often not conducted unless there is evidence of climatic incompatibility (May &

Coetzee 2013). Post-release studies are important as they allow for determining a possible reason as to why an agent may have failed to establish or thrive in the introduced range. For example, post release studies revealed that the distribution pattern of the five *Eretmocerus* spp (Hymenoptera) which were released as biological control agents for *Bemisia tabaci* biotype “B” Gennadius (= *Bemisia argentifolia* Bellows and Perring) in the USA was thought to be due to climatic incompatibility. North America, particularly east of the Rocky Mountains experiences what is called a “climate trumpet” caused by a funnelling of cold air along the mountain ranges which are north to south orientated. This phenomenon causes a dramatic fluctuation in temperatures which can produce deep freezes as far south as Texas. The Western US does not experience this as drastically because of the buffering effect of the Rocky Mountains. Therefore, agents released in Texas have to be cold tolerant to survive these extreme freezes. Agents were however collected from areas in Sharjah, UAE and Awash, Ethiopia, that do not go below 5 or 10°C, but can reach 50°C, which could explain why the wasps cannot survive the cold temperatures in Texas, but can establish in more buffered areas such as Yuma, Arizona, which also experiences very hot temperatures. Agents collected from northern Pakistan are thought to be more cold tolerant as they experience temperatures near 3°C and were able to establish in the Rio Grande Valley in Texas (Goolsby *et al.* 2005). A weed biological control example is the release of *Anthonomus santacruzi* Hustache (Coleoptera: Curculionidae), native to Argentina, in several areas around South Africa as a biological control agent for the invasive alien plant, *Solanum mauritianum* Scopoli (Solanaceae). Establishment was successful in Umkomaas (KwaZulu-Natal Province) and Sabie (Mpumalanga Province) but failed in Pretoria and Johannesburg (Gauteng Province) (Cowie *et al.* 2016). CLIMEX (a computer program which uses climatic and biological data to predict a potential distribution of organisms (Sutherst & Maywald 1985)) modelling revealed that the sites of failed establishment did not fall within the climatically suited range for A.

santacruzi. The native range climate is similar to the successful sites of establishment. Thermal studies revealed that *A. santacruzi* had a fairly high CT_{\min} of $4.1 \pm 0.2 \pm C$ ($n = 20$) which was representative of its native range's warm climate (Cowie *et al.* 2016). The effect of climate on the establishment success on *A. santacruzi* was confirmed through field observations (Singh & Olckers 2017).

Pre-release studies can help to determine optimal release strategies, as well as allow for prioritization of candidate agents due to climate compatibility. Pre-release studies often focus on the critical thermal minima of an insect as this is thought to be the limit that will be most critical to the agent's distribution range. Although both CT_{\min} and CT_{\max} are important in determining agents' potential distributions, generally when an agent fails to establish, it is because temperatures are too cold for the agent (Hill & Olckers 2000; Byrne *et al.* 2002; Byrne *et al.* 2004). These pre-release studies would make biological control more efficient and cost effective as areas which will be climatically suitable for the biological control agent can be prioritised (May & Coetzee 2013).

Although determining thermal thresholds under laboratory conditions does not exactly mimic natural conditions, it does give a good indication as to what would be expected in field conditions (Bryant *et al.* 2002; Overgaard *et al.* 2012). Climate-based physiological models are often used to map an insect's distribution, as well as to map possible shifts in distribution caused by climate change as projected distributions may shift with temperature changes (Bryant *et al.* 2002).

The ability for insects to survive at high or low temperatures is related to seasonal changes in their habitat temperature, as well as their native ranges climate (McClay & Hughes 1995; Hu & Appel 2004). *Calophasia lunula* Hufnagel (Lepidoptera: Noctuidae), a stenophagous European moth, was released into the northern United States as well as Canada as a biological control agent for *Linaria vulgaris* Miller (common/ yellow toadflax) and

Linaria genistifolia subsp. *dalmatica* (L.) Maire & Petitmengin. (Dalmatian toadflax) (Plantaginaceae) (McClay & Hughes 1995). Laboratory thermal studies, using temperature dependent developmental data to determine degree-days, were conducted on two strains of *C. lunula*, one strain was from former Yugoslavia and the other strain was collected in Montana (which was originally found in Switzerland and released in Montana). The thermal studies revealed that a population collected from former Yugoslavia had a higher degree-day requirement compared to the Montana population (McClay & Hughes 1995). Yugoslavia has a higher average temperature in both summer and winter compared to Switzerland which is reflected in the insects' thermal physiology. Distribution models (using degree-days determined in the laboratory as well as weather data) of the invaded range were produced for *C. lunula*. Most of the insects realized distribution falls within the area which was predicted by the models; however, the insect's actual distribution extends further north in Scandinavia than originally predicted. This suggests that long term acclimatization of this population has resulted in a field population which has adapted to lower summer temperatures. The model also showed that central and northern Alberta, Canada, was out of the insect's possible distribution range. This may explain why previous releases in those areas was unsuccessful (McClay & Hughes 1995).

The thermal physiology of insects is also affected by season (Teets & Denlinger 2013). Seasonal cold hardening is defined as cold hardening brought on by the slow change in season and requires at least days to weeks of exposure in order to be induced. An example of seasonal cold hardening is seen with the larvae of *Eurosta solidaginis* Fitch (Diptera: Tephritidae), a gall fly. At the end of summer and beginning of autumn, the larvae cannot survive when exposed to temperatures of -6°C , however as the season progresses, the larvae are able to withstand -20°C which is thought to be due to the slow accumulation of the cryoprotectants,

glycerol and sorbitol, which is triggered by the decrease in temperature and drying up of the gall tissue (Williams *et al.* 2004).

Coetzee *et al.* (2007) investigated the potential distribution of *E. catarinensis* in South Africa using thermal physiology parameters including; degree-day models, lower critical thermal limits and lower lethal temperatures. CLIMEX modelling illustrated that the only area that has a 75% climate match with Florianopolis and Rio de Janeiro where native populations of the insect were sourced, was the north-east coast of South Africa. An ecoclimatic index (EI) generated in CLIMEX showed that the limiting factor for potential distribution in other parts of the country was cold stress (Coetzee *et al.* 2007). Despite the model restricting the establishment of *E. catarinensis* to the warmer more subtropical areas of South Africa, there has been establishment on the Kubusi River in the Eastern Cape Province, where it was predicted to be unable to establish due to the cold climate. This suggests that there has been some degree of acclimation in this population to the colder temperatures, necessitating the re-evaluation of the thermal physiology 15 years after the initial studies were conducted.

A study conducted in 2015 at Rhodes University suggests that the population found at Kubusi has become adapted to the cold temperatures and frost experienced in winter at this site (Porter *et al.* in review). The authors showed that the Kubusi population had a different thermal tolerance to the population kept in culture at Rhodes University and that the difference could not completely be explained by phenotypic plasticity as introducing Kubusi individuals to a warmer climate for 5 days did not increase their thermal limits significantly, suggesting that the change was a genetic adaptation which could be passed on to next generations (Porter *et al.* in review).

This study was conducted on two field populations of *E. catarinensis* from South Africa, one from the hottest site of establishment (Enseleni River, KwaZulu-Natal) and one from the coldest (Kubusi River, Eastern Cape Province) site of establishment. Gene flow would be

limited between these two populations due to the geographical distance of approximately 800km between them.

Therefore, the experiments conducted here were performed to determine, (1) the thermal biology of *E. catarinensis* populations from two field sites that are climatically different, one cold, temperate site (Kubusi) and one warm, tropical site (Enseleni), and (2) to observe any differences between the thermal physiologies of the populations which could suggest that thermal plasticity has occurred. The thermal biology of *E. catarinensis* was investigated by calculating the critical thermal minima and maxima (CT_{min} and CT_{max}), upper and lower lethal limits (LLT_{50} and ULT_{50}), and degree-day requirements for the two field collected populations in South Africa in order to determine whether there were differences between populations due to differences in climate, as well as within population effects caused by seasonal changes in temperature.

2. 2 Materials and methods

2. 2. 1 Sites

Weather station data were collected from 2014-2017 from stations closest to each of the two sites. Dohne weather station (32°52'70"S 27°46'00"E) was used for Kubusi and Richards Bay Airport weather station (28°73'70"S 32°09'30"E) was used for Enseleni (South African Weather Service (SAWS)).

Kubusi River (32°35'33.25"S 27°25'19.38"E), which leads into Wriggleswade dam, is situated 64.8km outside of Stutterheim, Eastern Cape, South Africa. It is considered the coldest site in South Africa where *E. catarinensis* has established (Byrne *et al.* 2010). It experiences extreme temperature fluctuations between summer and winter, and frost during the winter months (Figure 2.1).

Enseleni River (29°48'19"S 30°39'53"E), situated in the Enseleni Nature Reserve, is approximately 23km outside of Richards Bay, Kwa-Zulu Natal. This site is the warmest site where *E. catarinensis* has established (Byrne *et al.* 2010) and experiences no winter frost and relatively warm temperatures throughout winter, and extreme high temperatures in summer months (Figure 2.1).

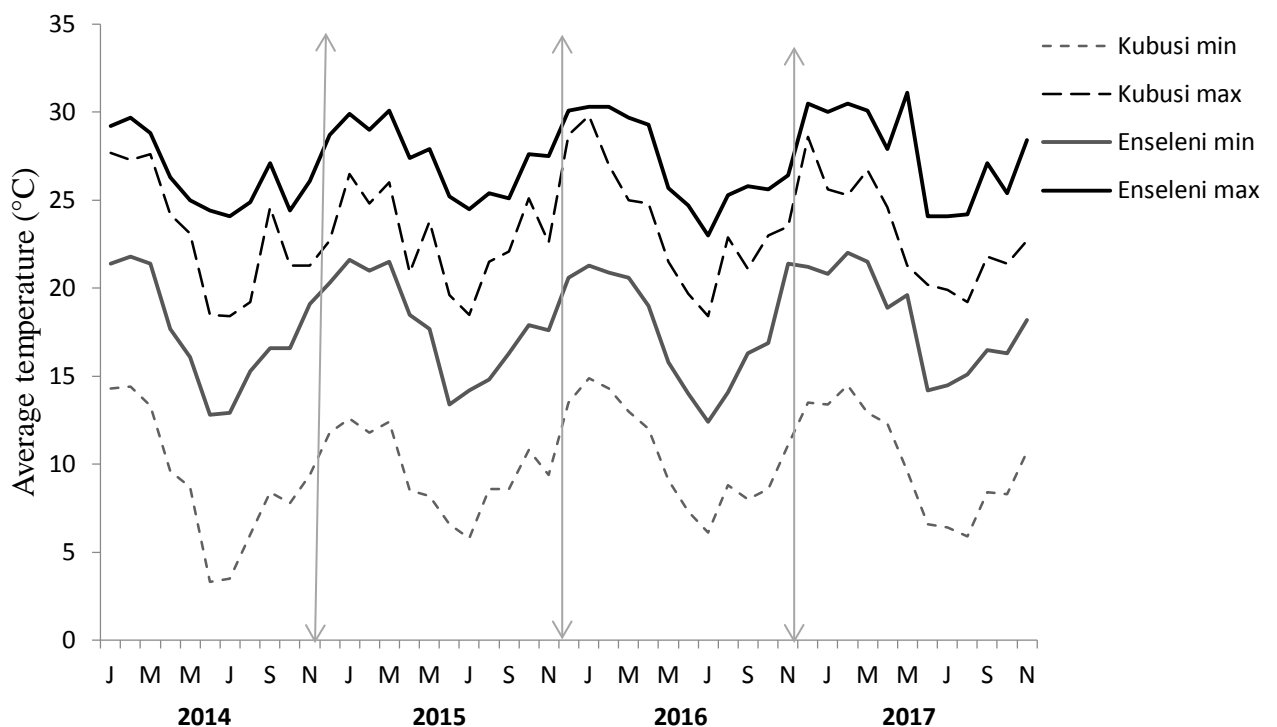


Figure 2.1: Mean monthly temperature data for Kubusi and Enseleni River for 2014, 2015, 2016 and 2017 sourced from SAWS.

Live *E. catarinensis* adults were field collected during August 2016 for winter populations and February 2017 for summer populations at Enseleni and Kubusi. The insects were collected using aspirators filled with tissue paper to prevent them being damaged as they entered the vial. The insects were then placed into polystyrene containers and kept in cooler boxes with ice packs for transportation to the laboratory at Rhodes University.

2. 2. 2 Critical Thermal minima and maxima

Critical thermal minima (CT_{\min}) and Critical thermal maxima (CT_{\max}) were determined for both summer and winter populations from both collection sites. CT_{\min} were determined following the methodology of Mitchell *et al.* (1993) and Terblanche *et al.* (2007). Ten insects from each population were placed into separate air filled 2 ml Eppendorf tubes, sealed with damp cotton wool. High density foam was then used to keep the opening of the tubes above the water to preventing water getting into the tube and drowning the insects. The insects were then cooled from 20°C to 10°C over 15 minutes in a programmable water bath (model: Grant GP200- R4 refrigerated water bath and TXF heating circulator®, Grant Instruments). The temperature was then dropped from 10°C to -2°C by 1°C every four minutes (Terblanche *et al.* 2007), which is considered the standard rate of temperature decrease for determining these limits and therefore allows for comparison of results. A slower rate of temperature change may allow the insects to acclimatise to the decrease in temperature, a short term form of phenotypic plasticity, and therefore could confound the results (Chown and Nicholson, 2004; Terblanche *et al.* 2007). Locomotory function was monitored at each temperature and the temperatures where an individual experienced impaired locomotory functioning was recorded; this was defined as an insect's inability to stand on the side of the tube (Mitchell *et al.* 1993; Klok & Chown 1997). This was done by briefly removing the tubes from the water bath at each temperature treatment and visually observing each individual in the tubes. The experiment was repeated three times with new individuals for each repetition to avoid pseudo-replication and to avoid using stressed insects.

To determine the CT_{\max} , a similar procedure was followed as above, however the water bath was heated from 20°C to 37°C and thereafter, increased by 1°C every four minutes until all insects lost locomotory functioning. $CT_{\min s}$ and $CT_{\max s}$ were determined by calculating the mean temperature at which the insects lost locomotion. Differences in $CT_{\min s}$ and $CT_{\max s}$

between the populations, and the seasons were tested using a generalised linear model in Statistica V13.2 (StaSoft Inc. 2013).

2. 2. 3 Lower and upper lethal temperatures

The upper (ULT_{50}) and lower (LLT_{50}) lethal limits are the temperatures at which 50% of the population cannot recover after being exposed to a particular temperature (Mitchell *et al.* 1993). To test the lower lethal temperatures for each population and season, 10 insects were placed into individual Eppendorf tubes and closed with damp cotton wool. The insects were then cooled from 20°C to 0°C over 20 minutes in a programmable water bath (model: Grant GP200 - R4 refrigerated water bath and TXF heating circulator®, Grant Instruments). The insects were then left for two hours at each experimental temperature. The experimental temperatures were every degree between 0°C and -7°C. Ten different insects were used for each temperature treatment. After the two-hour period, the insects were removed and placed into a clear Petri dish with a water hyacinth leaf for the insects to feed on once they had recovered. They were then checked one hour, 24 and 48 hours later for recovery/ self-righting. Self-righting was defined as the individual being able to right itself after being turned onto their dorsal surface, the number that had died was noted. Insects with very little locomotory functioning were recorded as dead as they were unable to recover.

The same handling procedure was then followed for determining the ULT_{50} , except that the programmable water bath was set to increase in temperature from 26°C to 30°C over 16 minutes. New insects were then used for each experimental temperature (31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C and 45°C).

The upper and lower LT_{50} s were then calculated using probit analysis. A generalised linear model was conducted on the data to test if season and population had a significant effect

on LT_{50s} . All analyses were conducted using the statistical software Statistica V13. 2 (Statsoft Inc., Tulsa, Oklahoma, USA).

2. 2. 4 Development of degree-day models

The method for developing the degree-day model was adapted from Coetzee *et al.* (2007). Summer and winter populations from both field sites were placed on five water hyacinth plants in a container with a gauze cover creating an enclosure. The enclosure was left for 24 hours in a constant environment (CE) room at 27°C to ensure oviposition at a constant temperature. After the 24-hour period, all adults were removed and plants with the eggs were placed into new enclosures. Each enclosure was then placed into a CE room at five different temperatures (16°C, 20°C, 24°C, 27°C and 29°C). All CE rooms had a 12L: 12D photoperiod.

The plants were closely monitored, and records were made of the date that the nymphs hatched, and from this, the time that the eggs took to hatch was calculated. Individual first instars were placed into a petri dish with moist filter paper and a water hyacinth leaf disk of approximately 4cm². These were checked every day for exuviae which marked a change in instar and allowed for the time to each developmental stage to be calculated. This was continued until adult emergence. The reduced major axis regression model adapted by Ikemoto and Takai (2000) was used to determine the thermal constant (K) and developmental threshold (t). The developmental temperature, t , is the temperature at which development ceases, and K is the effective cumulative temperature.

The regression models for each population and each season were then compared in Statistica V13.2 (Statsoft Inc., Tulsa, Oklahoma, USA) using homogeneity of slopes to test for significant differences between the slopes.

3. 3 Results

3. 3. 1 Critical thermal limits and lethal temperatures

Although population did not have a significant effect on critical thermal limits, populations from Enseleni had significantly higher CTs in summer and winter compared to the Kubusi population (Table 2.1 & 2.2). Population was found to have a significant effect on the insects LLT_{50} with Kubusi having a significantly lower LLT_{50} than the insects from Enseleni (Table 2.1 & 2.2). The CT_{min} , CT_{max} and ULT_{50} for both populations were found to be strongly influenced by season with values being significantly higher in summer compared to winter (Table 2.2). CT_{mins} and CT_{maxs} were significantly higher in summer compared to winter for both the Enseleni and Kubusi population. The trend continued with lethal temperatures. The summer population from Kubusi and Enseleni had a higher LLT_{50} and ULT_{50} compared to the winter population.

Table 2.1: Thermal limits of *Eccritotarsus catarinensis* in summer and winter from two populations, Enseleni and Kubusi. Values presented as mean \pm S. E, CTs N=30 and LTs N=10.

	Enseleni summer	Enseleni winter	Kubusi summer	Kubusi winter
CT_{min}	$5.20 \pm 1.9^{\circ}C$	$3.7 \pm 0.36^{\circ}C$	$4.77 \pm 0.26^{\circ}C$	$3.60 \pm 0.33^{\circ}C$
CT_{max}	$45.57 \pm 0.25^{\circ}C$	$40.80 \pm 0.49^{\circ}C$	$42.57 \pm 0.31^{\circ}C$	$40.60 \pm 0.51^{\circ}C$
LLT_{50}	$-3.62 \pm 0.06^{\circ}C$	$-3.49 \pm 0.04^{\circ}C$	$-4.00 \pm 0.04^{\circ}C$	$-4.69 \pm 0.13^{\circ}C$
ULT_{50}	$39.80 \pm 0.06^{\circ}C$	$36.96 \pm 0.13^{\circ}C$	$40.10 \pm 0.1^{\circ}C$	$36.35 \pm 0.13^{\circ}C$

Table 2.2: Outcome of the generalised linear model showing the effect of population, season and the interaction of population and season on the CT_{\min} , CT_{\max} , ULT_{50} and LLT_{50} of the field populations of *E. catarinensis*.

	Wald X^2	Population		Season		Population*	
		d.f	P	d.f	P	Season	
						d.f	P
ULT_{50}	14.67	1	0.52	1	<0.001	1	0.04
LLT_{50}	24.85	1	<0.001	1	0.41	1	0.89
CT_{\min}	74.58	1	0.41	1	<0.001	1	0.67
CT_{\max}	13.56	1	0.09	1	<0.001	1	0.26

3. 3. 2 Degree-day models

The developmental rates at different temperatures for *E. catarinensis* are shown for the Kubusi winter and summer population, as well as the Enseleni summer and winter populations in Table 2.3-2.6. No nymphs survived passed first instar at 29°C for the Kubusi Winter population and no eggs hatched at 18°C or 20°C for the Enseleni summer population. Developmental models and degree-days for each population for both summer and winter were calculated using the reduced major axis regression (Ikemoto and Takai 2000) (Figure 2.2) The gradient of the four models was significantly different ($F_{3, 162} = 6.77$, $P < 0.01$), indicating that the Enseleni summer population had a significantly higher t , and therefore lower degree day requirements. Kubusi summer and winter populations had the lowest developmental threshold of 12.3°C and Enseleni winter population had a slightly higher threshold than Kubusi (winter and summer) at 13.3°C.

Table 2.3: Developmental rates (days) at each life stage of *Eccritotarsus catarinensis* from Kubusi during winter, at four fixed temperature treatments.

Mean duration (days) \pmSE at indicated temperatures, N = 10					
Rearing Temperatures	18	20	24	28	29
Stage Egg-					
1st	26.1 \pm 0.57	22.00 \pm 0	15 \pm 0.00	10 \pm 0.00	
1st- 2nd Instar	4.5 \pm 0.27	3.4 \pm 0.16	3.6 \pm 0.16	2.2 \pm 0.20	
2nd-3rd Instar	5 \pm 0.30	3.1 \pm 0.18	2.1 \pm 0.28	1.4 \pm 0.16	
3rd- 4th Instar	4.4 \pm 0.22	3 \pm 0.21	2.1 \pm 0.18	1.6 \pm 0.16	
4th- 5th Instar	6.6 \pm 0.40	3.5 \pm 0.17	2.2 \pm 0.13	2.0 \pm 0.15	
5th-Adult	2.6 \pm 0.16	4.5 \pm 0.31	2 \pm 0.26	1.5 \pm 0.17	
Total (egg- adult)	49.2 \pm 0.44	39.5 \pm 0.43	27 \pm 0.30	18.7 \pm 0.37	

Table 2.4: Developmental rates (days) at each life stage of *Eccritotarsus catarinensis* from Kubusi during summer, at four fixed temperature treatments.

Mean duration (days) \pmSE at indicated temperatures N = 10					
Rearing Temperatures	18	20	24	28	29
Stage					
Egg- 1st Instar	24 \pm 0.00	18 \pm 0.00	13 \pm 0.00	11 \pm 0.00	6.4 \pm 0.16
1st- 2nd Instar	3.5 \pm 0.27	3.2 \pm 0.33	1.4 \pm 0.16	1.6 \pm 0.16	1.8 \pm 0.20
2nd- 3rd Instar	3.2 \pm 0.2	3.9 \pm 0.31	1.5 \pm 0.22	1.5 \pm 0.17	1.4 \pm 0.16
3rd- 4th Instar	3.4 \pm 0.27	3.6 \pm 0.22	2.5 \pm 0.31	1.6 \pm 0.16	1.7 \pm 0.15
4th-5th Instar	2.9 \pm 0.31	2.4 \pm 0.16	2.1 \pm 0.10	1.5 \pm 0.17	No 5 th instar
5th-Adult	2 \pm 0.15	1.7 \pm 0.15	2.5 \pm 0.22	1.5 \pm 0.17	1.2 \pm 0.13
Total (egg- adult)	39 \pm 0.68	32.8 \pm 0.55	23 \pm 0.33	18.7 \pm 0.37	12.5 \pm 0.22

Table 2.5: Developmental rates (days) at each life stage of *Eccritotarsus catarinensis* from Enseleni during winter, at four fixed temperature treatments.

Mean duration (days) \pmSE at indicated temperatures, N = 10					
Rearing Temperatures	18	20	24	28	29
Stage Egg-					
1st Instar	22 \pm 0.00	22 \pm 0.00	21 \pm 0.00	11 \pm 0.00	6.3 \pm 0.15
1st- 2nd Instar	4.5 \pm 0.45	3.2 \pm 0.36	2.6 \pm 0.40	1.9 \pm 0.18	1.8 \pm 0.25
2nd- 3rd Instar	3.7 \pm 0.34	3.5 \pm 0.52	3.3 \pm 0.26	1.6 \pm 0.16	2.1 \pm 0.18
3rd- 4th Instar	4.6 \pm 0.34	3.7 \pm 0.37	2.4 \pm 0.31	1.5 \pm 0.17	2 \pm 0.21
4th-5th Instar	2.1 \pm 0.18	2.7 \pm 0.26	1.5 \pm 0.17	1.4 \pm 0.16	No 5 th instar
5th- Adult	5.2 \pm 0.20	4.9 \pm 0.38	2.8 \pm 0.33	1.8 \pm 0.20	3 \pm 0.30
Total (egg-adult)	42.1 \pm 0.31	40 \pm 0.60	26.6 \pm 0.16	19.2 \pm 0.25	13.2 \pm 0.42

Table 2.6: Developmental rates (days) at each life stage of *Eccritotarsus catarinensis* from Enseleni during summer, at four fixed temperature treatments.

Mean duration (days) \pmSE at indicated temperatures, N = 10					
Rearing Temperature	18	20	24	28	29
Stage Egg-					
1st Instar			19 \pm 0.00	12 \pm 0.00	6 \pm 0.00
1st- 2nd Instar			2 \pm 0.15	1.8 \pm 0.13	22.2 \pm 0.13
2nd- 3rd Instar			2 \pm 0.21	2.7 \pm 0.15	2.1 \pm 0.23
3rd-4th Instar			2.3 \pm 0.15	1.9 \pm 0.18	2 \pm 0.15
4th-5th Instar			2.6 \pm 0.27	1.9 \pm 0.10	2.3 \pm 0.15
5th-Adult			2.1 \pm 0.23	2.3 \pm 0.21	2.1 \pm 0.23
Total (egg- adult)			30 \pm 0.47	22.6 \pm 0.27	16.7 \pm 0.30

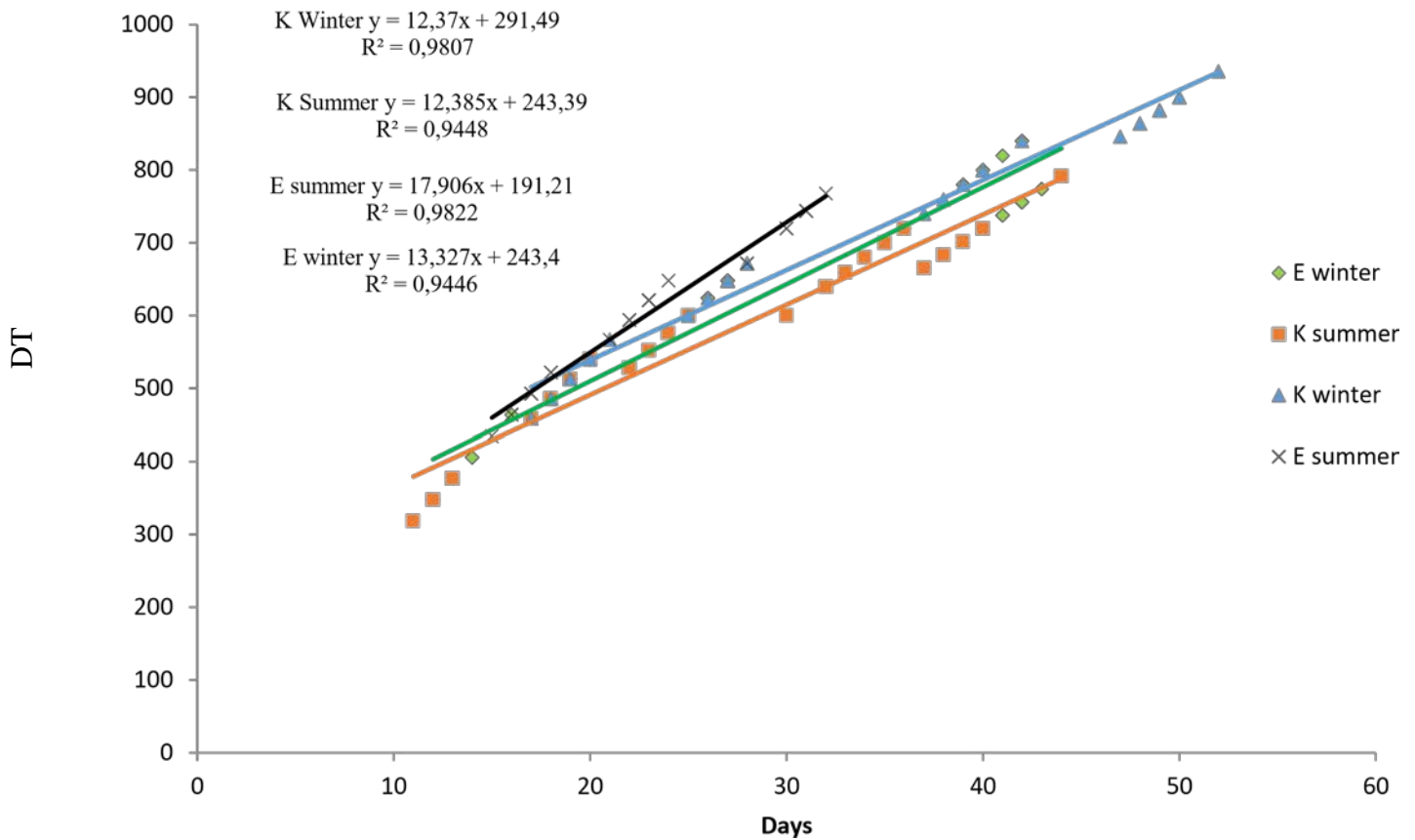


Figure 2.2: Temperature-dependent development of two field populations of *Eccritotarsus catarinensis*, from egg to adult, for summer and winter using the reduced major axis regression method (Ikemoto & Takai, 2000). E = Enseleni, K = Kubusi. DT is the product of developmental time and temperature.

3. 4 Discussion

The LLT_{50} of both populations of *E. catarinensis* were affected by location and not season which suggests that LLT_{50} s may not be plastic, like their CT_{mins} were shown to be by Porter *et al.* (in review). The Kubusi population had a slightly lower LLT_{50} ($-4.69 \pm 0.13^{\circ}C$) compared to the quarantine population of *E. catarinensis* examined by Coetzee *et al.* (2007), which had an LLT_{50} of $-3.5^{\circ}C$. This suggests that there has been some form of adaptation to the unfavourable conditions experienced in winter at the Kubusi site, however repetition of this experiment would be worthwhile to confirm these results.

The Enseleni population had a slightly higher thermal threshold (13.3°C) than the Kubusi populations (summer and winter: 12.38°C and 12.37°C, respectively). The Kubusi winter population had higher developmental days (K) at the developmental threshold. Kleynhans (2014) suggests that a slower developmental time in a population adapted to cold temperatures results in a trade-off which allows for more energy to be reserved for cold tolerance and therefore higher activity, such as feeding during or predator avoidance, under cold temperatures in the field. This may explain the survival of the *E. catarinensis* population during the unfavourable conditions experienced on the Kubusi River. Because it was predicted that the Kubusi River was too cold for *E. catarinensis* to establish at, and that Enseleni River had favourable conditions for *E. catarinensis* (Coetzee *et al.* 2007), thermal plasticity/adaptation would have occurred in the Kubusi *E. catarinensis* population and not in the Enseleni population.

Other studies confirm that the thermal physiology of an insect may shift between seasons. *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae) and *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae) are two termite species which have invaded the United States and have demonstrated seasonal fluctuation of their CTs and ULTs, with a significant interaction between season and their CT_{min} values (Hu & Appel 2004). However, there was no significant interaction between season and their CT_{max} and ULT₅₀ values. Regression analyses on those data confirmed the relationship between the thermal tolerance capabilities of those termites and the fluctuating seasonal temperatures within their habitat (Hu & Appel 2004). This study supports what was found for the *E. catarinensis* populations as season had a significant effect on their thermal limits (Table 2.1 & 2.2).

Another study conducted by Tereblanche *et al.* (2006) found that season had a significant effect on the thermal limits of *Glossina pallidipes* (Diptera: Glossinidae), the tsetse fly. Adult flies were collected from two climatically different areas in Kenya, East Africa. The CT_{min} of

both populations was found to be higher during summer and decreased during winter months. Geographic variation was also found for *G. pallidipes* thermal tolerances; CT_{min} and CT_{max} declined with an increase in elevation. High elevation sites are representative of colder climatic areas and therefore the insects are exposed to cooler temperatures compared to lower elevation sites (Singh & Olckers 2017). The insects' CT_{min} showed a steeper slope and therefore a stronger relationship with altitude compared to their CT_{max} . Laboratory acclimation studies provided evidence that variation among these populations was due to phenotypic plasticity and not genetic differences (Tereblanche *et al.* 2006). Like *G. pallidipes*, *E. catarinensis* populations examined in this study were also found to have a seasonal shift in their CT_{min} , with a shift downwards during winter seasons.

The similar thermal physiologies of both *E. catarinensis* populations' both contrasts and supports other studies reported in the literature. Studies comparing species have shown constant variation in thermal limits linked to geographic variation; however, conflicting results have been found when these same comparisons are made within a particular species (MacLean *et al.* 2016). Studies that have shown within-species geographical variation in thermal tolerances include a study on *Drosophila melanogaster* Meigen (Diptera: Drosophilidae). The results showed that recovery time from chill coma (the temperature where insects lose all locomotory function), brought on by exposing insects to 0°C, was dependent on the native climate of the population in question, with the populations from cold climates having a shorter recovery time than those from warmer areas (Hoffmann *et al.* 2002a). Populations which lived at high altitudes which experienced a more temperate climate had a faster recovery time and those living at lower altitudes in tropical climatic areas had a slower recovery time. It was suggested that the populations living at higher altitudes therefore had lower lethal (LLT_{50s}) and critical limits (CT_{min}) which allowed them to recover faster from the induced chill coma (Hoffmann *et al.* 2002a; Rako & Hoffmann 2006). This however, suggests that the cold tolerant

populations have become cold adapted and it may not be attributed to thermal plasticity as Anderson *et al.* (2005) showed that chill coma recovery time is heritable and therefore is selected for in the field in colder climates. Kimura (2004) looked at the thermal tolerance of various drosophilid species. The thermal tolerance of strains of the same species was examined between strains living in subtropical climates and strains living in temperate climates. The thermal tolerance of the different species living in these two climatically different sites was also compared. It was found that the variation between the species was a lot higher than the variation between the strains. Although some strains showed a small difference, some did not show a difference at all between the two sites. When differences were found, the strains or species from the temperate site were more cold adapted than those from the subtropical sites (Kimura 2004). This suggests that populations or strains of the same species conserve their thermal tolerance which is what was shown for the two *E. catarinensis* populations here. Ayrinhac *et al.* (2004) showed that survival of cold temperatures by *D. melanogaster* was more dependent on phenotypic plasticity than genetic variability between populations from different climatic regions and that phenotypic plasticity accounted for 80% of the variation between populations.

Other studies have shown that insects conserve thermal limits across geographical ranges and survival is thought to be more dependent on thermoregulatory behaviour and not a shift in their thermal limits. The use of both strategies (physiological as well as behavioural mechanisms) is seen in the invasive termites example mentioned above of *R. flavipes* and *C. formosanus*, which have the ability to survive lower temperatures in cold seasons compared to in warmer seasons. Behavioural studies, both in the laboratory and the field, demonstrated that when temperatures drop below freezing, the termites actively dig deeper into the soil where temperatures are warmer and more constant, thus avoiding the very cold temperatures. Both

the physiological and behavioural changes allow for these termites to survive the decrease in temperature in winter (Esenther 1969; Hu & Song 2007).

Although the temperature differences are not as extreme at Enseleni as they are at Kubusi, summers are extremely hot. Summer temperatures range from 25°C to 40°C with an average of 30°C and winter temperatures ranging from 17°C to 27°C with an average of 22°C. Winter populations do however fall near the CT_{min} of the summer Enseleni population; the *E. catarinensis* winter population at Enseleni would therefore need to experience a decreased CT_{min} in order to survive the winters even though they do not experience frost like at Kubusi.

It was unexpected to see no difference between the CT_{mins} for summer and winter populations at Kubusi as it is predicted that insects exposed to fluctuating conditions may be more prone to select for genes which allow for phenotypic plasticity compared to insects which experience favourable conditions for many years (Sørensen *et al.* 2016; Klok & Chown 2003). It would therefore be more likely to see plasticity in the Kubusi population where there is a definite summer (temperatures range from 20°C to 29°C) and winter season (temperatures range from 8°C to 18°C), and not in the Enseleni population. This suggests that the Kubusi populations may also adopt behavioural changes in winter to avoid the harsh temperatures. During unfavourable cold temperatures the insects aggregate in folded up leaves of water hyacinth plants and emerge to open leaves during warmer parts of the day (pers. obs).

The similarities and differences found between the two populations have implications for biocontrol. The results suggest that season has more of an effect on the thermal tolerance of agents compared to local adaptations. So, although thermal studies may predict limits agents may experience, they are not always robust, as plasticity may allow agents to survive in areas thought to be out of their thermal range.

Chapter 3: Manipulating agent thermal physiologies: an approach to reducing climatic incompatibility of biological control programmes.

3.1 Introduction

Field populations of have *E. catarinensis* experienced changes in their thermal physiologies since release in South Africa (Chapter 2). This suggest that it might be possible to manipulate the agent's thermal physiology under laboratory conditions to improve their establishment success and impact in the field. Climatic incompatibility is one of the main reasons why biological control agents fail to control their target weed and this often results in the release of more biological control agents with the hope of achieving an acceptable level of control. Although host specificity testing ensures that all agents are safe for release, there is still an innate risk in releasing biological control agents, so the release of ineffective or unnecessary agents should be avoided (McClay & Balciunas 2005). Creating cold acclimated populations in the laboratory could allow biological control practitioners to utilise the agents that are already released in South Africa better and therefore reduce risks of biological control by releasing fewer biocontrol agents. As previously mentioned, more emphasis needs to be placed on also predicting the efficacy of agents to ensure that a successful control programme is possible (McClay & Balciunas 2005). Thermal physiology studies should be included in pre-release efficacy studies to prevent releasing agents into climatically unsuitable environments.

McEvoy and Coombs (1999) suggest a parsimonious strategy to biological control programmes as the best way to reduce non-target effects by reducing the number of agents released. They suggest that a few compatible agents should be released which represent

different functional groups which will disrupt the weed's life cycle. The parsimonious strategy emphasizes interaction strength as well as avoiding redundant agent releases (McEvoy & Coombs 1999). Often multiple agents are released without knowing just how effective each agent will be or how the agents will interact once released (Pearson *et al.* 2003). A better understanding of agent thermal physiologies and the plasticity of these thermal physiological traits could improve selection of effective agents and allow for ineffective agents to be utilised better through manipulating their thermal physiologies.

When agents fail due to climatic incompatibility, biological control practitioners will generally review the invasive plant's native range and select a more climatically suited agent (van den Bosch *et al.* 1970; Robertson *et al.* 2008). The new agent can either be an entirely new species or a consignment of the current agent which may be found in a more suited environment. For example, Ragwort, *Senecio jacobaea* L. (Compositae) is an invasive weed in Australia and California, USA. Control of *S. jacobaea* has been successful in California due to the release of two biological control agents, a flea beetle *Longitarsus jacobaeae* Waterhouse (Coleoptera: Chrysomelidae) and a moth *Tyria jacobaeae* L. (Lepidoptera: Arctiidae). Due to the success of *L. jacobaeae* in California, it was considered for release in Australia. The strain of *L. jacobaeae*, initially collected in Italy, which was released in California, however was predicted to not be climatically suitable for release in Australia, due to its diapause characteristics in summer. Annonay in central France was climatically similar to the areas in Australia where *S. jacobaea* has infested. A strain of *L. jacobaeae* was successfully found in this area, and after host specificity tests, was released in Australia. It successfully established which confirmed that it was climatically suited to the Australian climate (Wapshere 1983).

Climate suitability is potentially important for all classes of biological control agents, including pathogens. For example, climate matching was done between the areas in Hawaii where mist flower, *Ageratina riparia* (Regel) R. King and H. Robinson (Asteraceae) is

successfully controlled, to areas in New Zealand where mist flower needed controlling. This climate matching allowed for prioritisation of agents that would be climatically suited for New Zealand. The two agents that were chosen for release in New Zealand were a smut fungus, *Entyloma ageratinae* Barreto and Evans (Ustilaginomycetes) and a gall fly *Procecidochares alani* Steyskal (Tephritidae). Due to the attention given to their climate compatibility, both agents successfully survived the first winter and were able to establish and disperse successfully (Barton *et al.* 2007).

It is important to understand the thermal physiology of agents that are going to be released to avoid climatic incompatibility (Byrne *et al.* 2002). Knowing the thermal physiology of the candidate agents could reject certain agents' pre-release that are doomed to fail due to climatic incompatibility (May & Coetzee 2013) and will provide a better understanding if new agents will actually be better suited for the area.

When collecting new consignments of the same agent species from more climatically suitable areas, there are also potential risks. Releasing new agent populations could result in hybridization which may have unpredictable consequences for the host range of offspring (Volchansky *et al.* 1999). It is also possible that the new consignment will have a different host range and in extreme cases, may be a cryptic species (Toševski *et al.* 2001; Smith *et al.* In Press), as well as more general challenges which are caused by releasing too many biological control agents. Two geographically separated populations of *Eccritotarsus* were found to be two cryptic species only after being released into the field (Paterson *et al.* 2016; Henry 2017). As mentioned in Chapter 1, *E. catarinensis* and *E. eichhorniae* were brought in to South Africa separately, but released as a single species. Because it was thought that they were the same species, extensive research was not done on *E. eichhorniae* before being released; however, host-specificity testing was done and both species are safe for release in South Africa (Paterson *et al.* 2016.). Although host specificity testing of the new consignment was conducted in this

case, it is not a legal requirement in most countries (Paynter *et al.* 2008; Paterson *et al.* 2016). *Eccritotarsus eichhorniae* was imported, with the intention of increasing the genetic variability within the species (*E. catarinensis*) and therefore possibly increasing the agent's ability to adapt to the climate in South Africa which was believed to be limiting the agent's performance (Coeztee *et al.* 2007). However, the two species are reproductively isolated and do not interbreed under field conditions (Paterson *et al.* 2016). *Eccritotarsus eichhorniae* was found in the Yarapa River near Iquitos, Peru, and is therefore exposed to higher temperatures than *E. catarinensis* which originates from Florianopolis (Santa Catarina), Brazil, which experiences a lower average temperature (Paterson *et al.* 2016). It is important to do thermal physiological research on *E. eichhorniae* to determine whether one species is more suited for colder/warmer areas compared to the other, which will allow for prioritisation of species for release into areas where they will be most climatically suited.

An alternative to releasing new or unnecessary agents, and therefore improving the safety of biocontrol, is to try and improve the effectiveness of agents which have already been released, especially if they are one of the most effective agents. Thermal physiologies of insects have also been found to be plastic which could potentially be beneficial for biological control programmes. Although not currently common for biocontrol agents, cold hardening or acclimating is used in other fields. For example, *Sitophilus granaries* Linnaeus (Coleoptera: Dryophthoridae) and *Cryptolestes ferrugineus* Stephens (Coleoptera: Laemophloeidae) are both stored grain pests. Both species were subjected to cold acclimation for two weeks at 15, 10 and 5°C for two weeks. Acclimation increased survival of *S. granaries* (which has a higher lower temperature threshold compared to *C. ferrugineus*) from 12 to 40 days at 0°C. Cold acclimations also increased survival of *C. ferrugineus* at -10°C from 1.4 days to 24 days (Fields *et al.* 1998). Furthermore, the time of survival of *Pyrrhocoris apterus* L. (Heteroptera: Pyrrhocoridae) at -5°C was also increased after a four week long acclimation of a gradual

decrease in temperature from 25°C – 0°C (Šlachta *et al.* 2002). These results showed that the insects could be cold hardened through cold acclimation which increased the insect's survival at cold temperatures. If the thermal physiology of the *E. catarinensis* species is plastic, then the use of cold hardening a population before release should be explored which should potentially increase its chance of establishing in colder sites where establishment may have failed previously. Creating a cold adapted population may allow for a single agent release strategy as the need for a more climatically suited agent will not be necessary, thus avoiding potential problems caused by multiple agent releases. If climate incompatibility is the main reason for searching for a new biological control agent, further research, such as cold hardening of existing agent, could be conducted before introducing new biological control agents.

Thermal plasticity has become an increasingly popular topic of research, with particular interest in determining how insects will respond to climate change (Gabriel *et al.* 2005). More and more research is showing that insects are able to shift their thermal physiologies according to their surrounding temperatures. Many studies have been on rapid cold hardening, which show a shift in thermal physiology in insects exposed to very cold temperatures for a short amount of time (usually a few hours) (Overgaard *et al.* 2007). However, these studies do not show if thermal physiologies continue to shift when the insects are exposed to these conditions for longer periods of time. Furthermore, *Sitobion avenae* Fabricius (Homoptera: Aphididae), an aphid, was exposed to cold hardening. *Sitobion avenae*, like many other insects have a short generation time. Winter survival therefore depends on the insect's ability to survive cold temperatures, but more importantly to continue to perform, i.e. feed and reproduce. Rearing a population of *S. avenae* at 10°C as opposed to its normal 20°C rearing temperature resulted in a decrease of 0.7°C in its CT_{min}. Populations were reared at 10°C for six months which was considered as long-term acclimation and not rapid cold hardening (Powell & Bale 2006). This has implications for biocontrol programmes as agents with short generation times need to be

able to reproduce during winter months in order to establish successfully, therefore their survival at cold temperatures needs to be increased.

A previous study by Porter *et al.* (in review) showed that after only five days, there was a change in *E. catarinensis* thermal physiology after cold hardening. A population collected from Kubusi was exposed to temperatures that are typical of quarantine conditions, 26°C and a population was collected from the quarantine culture and exposed to 6°C for five days. Both populations experienced thermal physiology shifts and therefore five days was chosen as the time intervals for this study in order to make studies comparable.

This chapter evaluates the thermal physiology of *E. eichhorniae* as well as re-evaluating the *E. catarinensis* thermal physiology to determine if one species is more cold-adapted which will allow for prioritisation of that species for release in the cooler areas of South Africa. It also investigates whether the cold adapted species' thermal physiology could be manipulated to further increase its cold tolerance to improve establishment success in temperate areas to avoid having to release an additional agent.

3. 2 Materials and methods

All insects used in this study were obtained from the populations of the two species of *Eccritotarsus* which are reared at Rhodes University, Grahamstown, Eastern Cape in quarantine.

3. 2. 1 Critical thermal limits and lethal temperatures

Critical thermal limits as well as lethal temperatures were determined for both *E. catarinensis* and *E. eichhorniae* to determine whether one is more cold tolerant than the other. Ten insects from each species were placed into separate air filled 2 ml Eppendorf tubes, sealed

with damp cotton wool. High density foam was then used to keep the opening of the tubes above the water to prevent water getting into the tube and drowning the insects. The insects were then cooled from 20°C to 10°C over 15 minutes in a programmable water bath (model: Grant GP200- R4 refrigerated water bath and TXF heating circulator®, Grant Instruments). The temperature was then dropped from 10°C to -2°C by 1°C every four minutes (Terblanche *et al.* 2007), which is considered the standard rate of temperature decrease for determining these limits and therefore allows for comparison of results. A slower rate of temperature change may allow the insects to acclimatise to the decrease in temperature, a short term form of phenotypic plasticity, and therefore could confound the results (Chown and Nicholson, 2004; Terblanche *et al.* 2007). Locomotory function was monitored at each temperature and the temperatures where an individual experienced impaired locomotory functioning was recorded; this was defined as an insect's inability to stand on the side of the tube (Mitchell *et al.* 1993; Klok & Chown 1997). This was done by briefly removing the tubes from the water bath at each temperature treatment and visually observing each individual in the tubes. The experiment was repeated three times with new individuals for each repetition to avoid pseudo-replication and to avoid using stressed insects.

To determine the CT_{max} , a similar procedure was followed as above, however the water bath was heated from 20°C to 37°C and thereafter, increased by 1°C every four minutes until all insects lost locomotory functioning. CT_{mins} and CT_{maxs} were determined by calculating the mean temperature at which the insects lost locomotion.

Statistical analyses were performed in Statistica® V13. 2 (2013 Statsoft Inc., Tulsa, Oklahoma, USA). First the data were tested for normality using a Shapiro- Wilk test. To determine whether species had an effect on the critical thermal limits (CT_{min} and CT_{max}), a Mann-Whitney U test was performed. A logistic regression model was used to determine both the ULT_{50} and the LLT_{50} (the temperature at which half the population did not survive) for

both species. A generalised linear model, using a Poisson error structure, in Statistica® V13.2 was then used to test whether *E. catarinensis* and *E. eichhorniae* had significantly different LTs. Only *E. catarinensis* was used for further thermal studies and the cold hardening experiment as it was found to be more cold adapted than *E. eichhorniae*.

3. 2. 2 Degree-days

A degree-day model was determined for the quarantine culture of *E. catarinensis* to determine if its physiology has changed after being reared for many generations under controlled climatic conditions. The method for developing the degree-day model was adapted from Coetzee *et al.* (2007). Forty adults from the *E. catarinensis* population was placed on five water hyacinth plants in a container with a gauze cover creating an enclosure. The enclosure was left for 24 hours in a constant environment (CE) room at 27°C to ensure oviposition at a constant temperature. After the 24 hour period, all adults were removed and plants with the eggs were placed into new enclosures. Each enclosure was then placed into a CE room at five different temperatures (16°C, 20°C, 24°C, 27°C and 29°C). All CE rooms had a 12L: 12D photoperiod.

The plants were closely monitored, and records were made of the date that the nymphs hatched, and from this, the time that the eggs took to hatch was calculated. Ten replicates were done for each experimental temperature. Using a fine paint brush, individual first instars were placed into a petri dish with moist filter paper and a water hyacinth leaf disk of approximately 4cm². These were checked every day for exuviae which marked a change in instar and allowed for the time to each developmental stage to be calculated. This was continued until adult emergence. The reduced major axis regression model adapted by Ikemoto and Takai (2000) was used to determine the thermal constant (K) and developmental threshold (t). The

developmental temperature, t , is the temperature at which development ceases, and K is the effective cumulative temperature.

3. 2. 3 Cold hardening

A sample of 300 *E. catarinensis* was taken out of the culture kept at Rhodes University and was placed into a controlled environment chamber (BINDER growth chamber model: KBW 240, Germany) and kept at a temperature of 12°C (photoperiod cycle of 18:6) which is just above this species' thermal threshold ($t = 10^{\circ}\text{C}$) (Coetzee *et al.* 2007). This temperature was chosen as *E. catarinensis* should still survive at 12°C and should still be able to perform activities such as feeding, but it is cold enough to explore what happens when *E. catarinensis* is exposed to extreme cold temperatures. Every five days (up until 25 days), a sub-sample of 60 individuals was used to test their CT_{\min} (30 individuals) and CT_{\max} (30 individuals) using the water bath method described in Chapter 2.2.2. A One-way ANOVA was performed in Statistica V13.2 to evaluate the effect of the cold hardening on critical thermal limits (CT_{\min} and CT_{\max}), where the thermal limit was the dependent variable and the number of days the insects had been exposed to 12°C was the grouping factor. A post-hoc Tukey test was performed to see where significant differences occurred. Due to the insects in this experiment being exposed to 5°C for longer than just a few hours (typical cold hardening), it is considered to be more acclimation; however, it is still a form of plasticity that resulted in a decrease in their cold tolerance, and will therefore be referred to as cold hardening for this chapter (Sgró *et al.* 2016).

3. 3 Results

3. 3. 1 Critical thermal limits and lethal limits

Critical and lethal limits were determined for both species (Table 3.1). *Eccritotarsus eichhorniae* had a significantly higher CT_{min} ($5.87 \pm 0.33^{\circ}C$) compared to *E. catarinensis* ($2.9 \pm 0.38^{\circ}C$), however there was no difference in CT_{max} between the species. The CT_{max} of *E. eichhorniae* is $42.77 \pm 0.27^{\circ}C$ and the CT_{max} of *E. catarinensis* is 42.93 ± 0.26 . Similarly, *E. eichhorniae* had an LLT_{50} of $-2.65 \pm 0.11^{\circ}C$ which was found to be significantly higher than the $-5.74 \pm 0.06^{\circ}C$ LLT_{50} of *E. catarinensis*, but no difference was found between the ULT_{50} (*E. eichhorniae* $ULT_{50} = 43.66 \pm 0.027^{\circ}C$ and *E. catarinensis* $ULT_{50} = 43.66 \pm 0.042^{\circ}C$). This data was used to justify not including *E. eichhorniae* in further experiments in this chapter as *E. catarinensis* was more cold adapted.

Table 3.1: Values for critical thermal limits (CT_{min} and CT_{max}) and lethal limits (ULT_{50} and LLT_{50}) for both *Eccritotarsus eichhorniae* and *E. catarinensis* populations kept at Rhodes University. Values are in degree Celsius. Values are presented as mean \pm SE.

	<i>E. eichhorniae</i>	<i>E. catarinensis</i>	<i>P</i>	<i>d.f</i>	Test statistic
CT_{min}	5.87 ± 0.33	2.9 ± 0.38	< 0.01	1	$U = 130.5$
CT_{max}	42.77 ± 0.27	42.93 ± 0.26	0.69	1	$U = 422.5$
LLT_{50}	-2.65 ± 0.11	-5.74 ± 0.06	0.91	1	$X^2=0.011$
ULT_{50}	43.66 ± 0.027	43.66 ± 0.042	0.92	1	$X^2=0.011$

3. 3. 2 Degree-days

The reduced major axis regression estimated the parameters K and t for *E. catarinensis*, to be 297.48 days to develop at their temperature threshold (t) of 11.94°C (Table 3.2 and Figure 3.1).

Table 3.2: Duration of development for *Eccritotarsus catarinensis* at five constant temperatures, where $N = 10$ for each temperature.

Rearing Temperatures	Mean duration (days) \pm SE at indicated temperatures				
	18	20	24	27	29
Stage					
Egg- 1st Instar	27 \pm 0	18 \pm 0	12 \pm 0	9 \pm 0	6.6 \pm 0.16
1st- 2nd Instar	5.8 \pm 0.83	3.7 \pm 0.26	2 \pm 0	2.4 \pm 0.16	2.6 \pm 0.22
2nd- 3rd Instar	3.5 \pm 0.31	3 \pm 0.15	2.8 \pm 0.33	2.1 \pm 0.18	2 \pm 0
3rd- 4th Instar	3.6 \pm 0.27	3.6 \pm 0.27	4 \pm 0.21	2 \pm 0.15	1.6 \pm 0.16
4th-5th Instar	3.5 \pm 0.17	3 \pm 0.26	2.3 \pm 0.15	2.5 \pm 0.31	1.5 \pm 0.17
5th- Adult	5 \pm 0.26	4.2 \pm 0.2	2.7 \pm 0.15	2.1 \pm 0.23	1.8 \pm 0.13
Total (egg-adult)	48.4 \pm 0.96	35.5 \pm 0.37	25.8 \pm 0.47	20.1 \pm 0.43	16.1 \pm 0.38

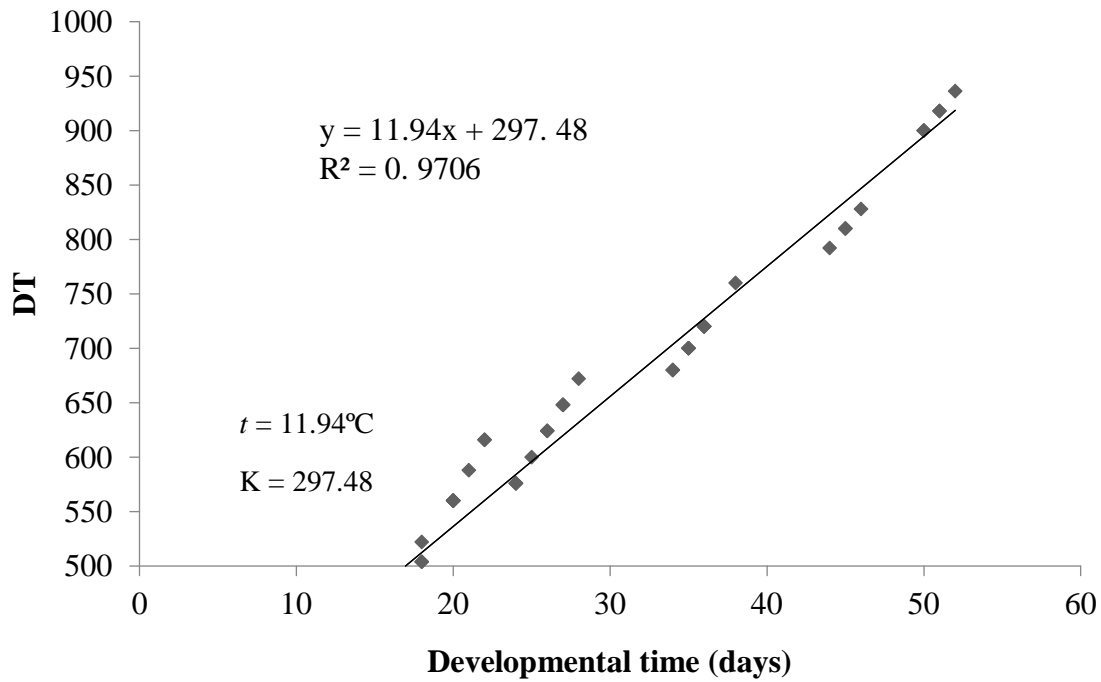


Figure 3.1: Temperature dependent development, from egg to adult, of *E. catarinensis* using the reduced major axis regression method (Ikemoto & Takai 2000). DT is the product of temperature and developmental time.

3. 3. 3 Cold hardening

Exposure to 12°C resulted in cold hardening of *E. catarinensis* where both the CT_{\min} and CT_{\max} were reduced significantly (Figure 3.2 & 3.3). The CT_{\min} s decreased from 2.9°C to -0.3°C within 15 days, but began to increase after 15 days. The CT_{\max} decreased from 42.9°C to 40.23°C within 15 days. CT_{\max} s were stopped at 15 days due to a shortage of insects; therefore, it is unknown if there is a continual decrease of CT_{\max} after 15 days.

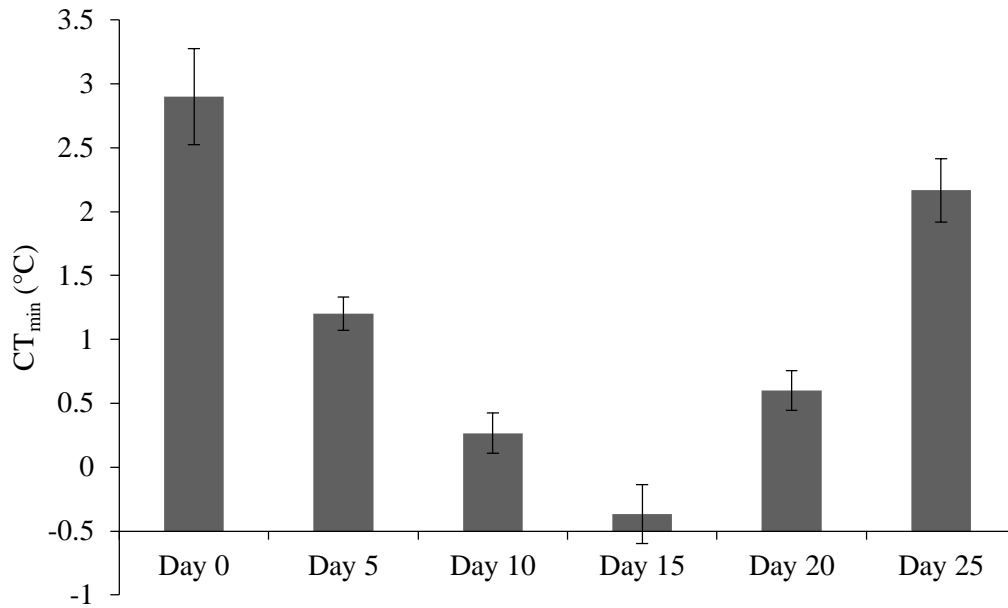


Figure 3.2: The change in CT_{min} of *E. catarinensis* exposed to 12°C, at five day intervals.

Different letters above the bars indicate significant differences. Error bars represent ± S. E.

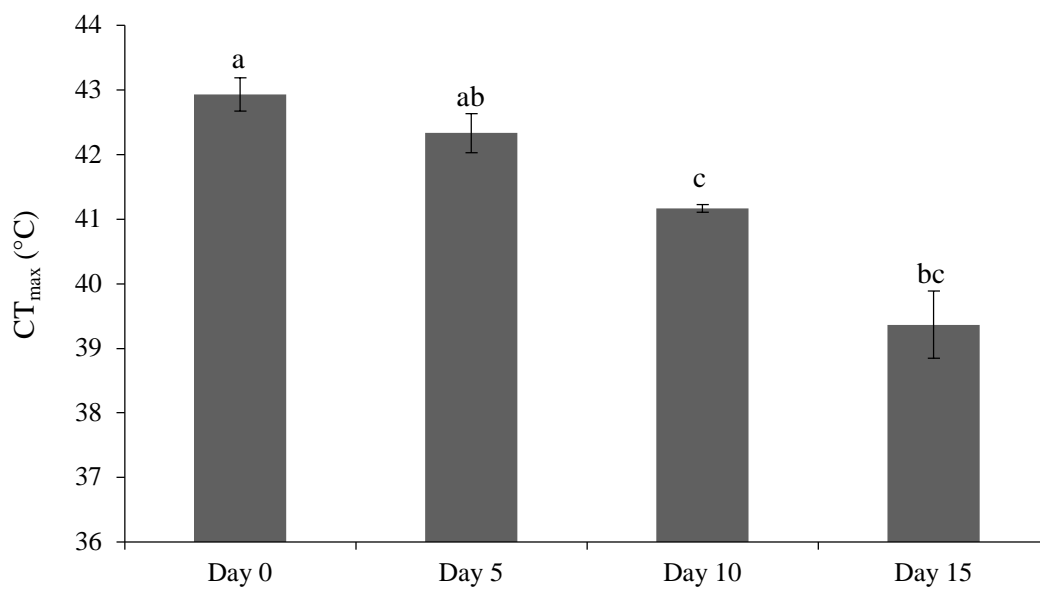


Figure 3.3: Change in CT_{max} of *E. catarinensis* exposed to constant 12°C for 15 days.

Different letters above the bars indicate significant differences. Error bars represent ± S.E.

3. 4 Discussion

This chapter aimed to determine whether agents can be cold hardened to increase establishment success or if a field acclimated population should be used when climatic incompatibility prevents establishment of the agent that has already been released. The comparison of the two species' thermal physiologies revealed that *E. eichhorniae* has a very similar CT_{max} and ULT_{50} to *E. catarinensis*, but a significantly higher CT_{min} suggesting that it is not as cold tolerant as *E. catarinensis*. Although the LLT_{50s} were not found to be significantly different between the two species, it is possible that the high variance of the data influenced the significance as the two LLT_{50} values differed by $3.09^{\circ}C$, with *E. catarinensis* having a lower LLT_{50} .

These results suggest that it may be beneficial to focus on *E. catarinensis* for biocontrol of *E. crassipes* in the temperate areas in South Africa and that releasing *E. eichhorniae* into temperate areas could result in the release of an "unnecessary agent" whose chances of establishment are reduced due to climate incompatibility. Although both species have similar upper limits, *E. eichhorniae* has been shown to have higher reproduction rate and therefore outcompetes *E. catarinensis* at warmer temperatures (Paterson *et al.* 2016), it is therefore suggested that *E. eichhorniae* should be released into warmer sites in South Africa instead of *E. catarinensis*. If the original agent can be improved, in this case through manipulation of their thermal physiology, then this may be more beneficial for biocontrol programmes than finding new agents to release.

Thermal physiology shifts for *E. catarinensis* were recorded in this study since it has been kept in quarantine, suggesting that its thermal physiology is plastic. Limits found by Coetzee *et al.* (2007) were $1.2^{\circ}C$, $49.6^{\circ}C$, $-3.5^{\circ}C$ and $37^{\circ}C$ and the limits found in this study were $2.9^{\circ}C$, $42.93^{\circ}C$, $-5.74^{\circ}C$ and $43.66^{\circ}C$ for CT_{min} , CT_{max} , LLT_{50} and ULT_{50} respectively. Thermal physiology data are unavailable for *E. eichhorniae* from when it was first brought into

South Africa, therefore it is unknown if this species has experienced a change in its thermal physiology. Results of this research show that the CTs of *E. catarinensis* have become narrower; however, the LT range has increased. It would be expected that the thermal limits become narrower in warm stable environments (Colinet *et al.* 2015). It was unexpected that the LTs range increased, however this could be due to having a constant high quality food source which decreased possibilities of trade-offs. During starvation at cold temperatures, energy reserves, particularly fat, are consumed. Insects with a higher stored fat percentage are expected to have an advantage for survival at low temperatures (Colinet *et al.* 2007). Feeding status of an insect is known to have an impact on that individual's CTs (Nyamukondiwa & Terblanche 2009). This quarantine population of *E. catarinensis* has a thermal physiology (CT_{\min} and CT_{\max}) very similar to what was found for the summer Kubusi population of *E. catarinensis* ($CT_{\min} = 4.77 \pm 0.26$; $CT_{\max} = 42.57 \pm 0.31^{\circ}\text{C}$) in Chapter 2. The developmental graphs indicate that the quarantine culture of *E. catarinensis* and the Kubusi population of *E. catarinensis* have a similar developmental threshold ($y = 12.385x + 243.39$). This further supports what was said in Chapter 2, that the change in thermal tolerance occurs in winter at Kubusi as the summer population has similar thermal tolerance to the quarantine culture.

The cold hardening study on *E. catarinensis* shows that if the agent was kept at a temperature close to its developmental threshold, over time, the critical thermal thresholds shift down towards what would be expected of a species living in a cooler climate (Hoffmann *et al.* 2003). The results found in this study show that it is possible to manipulate the thermal tolerance of *E. catarinensis* through cold hardening. Although the mechanisms underlying increased cold tolerance in insects exposed to cold temperatures either through RCH or acclimation is largely unresolved (Shintani & Ishikawa 2007), one suggestion is that it is due to an increase in glycerol production when the insects are exposed to a sudden decrease in temperature (Teets & Denlinger 2013). The ability for *E. catarinensis* to cold harden has

implications for its use as a biological control agent. The CT_{\min} of the laboratory cold hardened population of *E. catarinensis* decreased further than what was found for the Kubusi winter populations ($CT_{\min} = 3.60 \pm 0.33^{\circ}\text{C}$), suggesting that it may be more beneficial to cold harden the populations before release instead of re-releasing field cold acclimated populations.

Before biological control practitioners investigate a new agent to release on the *E. crassipes* infestations which are too cold for *E. catarinensis*, cold hardened populations of *E. catarinensis* should first be released and monitored for successful establishment to determine whether cold hardened populations can in fact establish in the field. These results also suggest that *E. catarinensis* may be better suited for prioritisation in South Africa as it already has a wider thermal tolerance compared to *E. eichhorniae*.

Further studies that determine the most effective way to cold-harden biocontrol agents is needed. Short term hardening versus long term acclimation (rearing conditions) needs to be investigated. While the CT_{\min} of *E. catarinensis* decreased significantly within the first 15 days of cold hardening, the CT_{\min} then increased suggesting that the insects experienced some physiological damage (indirect chilling injury) during long exposure to such an extreme temperature. Adult *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae) that were exposed to 0°C for 20 days experienced an increase in mortality with an increased duration of exposure time, suggesting that they experienced indirect chilling injury (Chen & Denlinger 1992). The same was found for *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae) where short term exposure to 5°C was not lethal for any of its life stages, however longer exposure to this temperature resulted in retarded larval development and low pupation rates caused by indirect chilling injury (Kim & Song 2000). Insects exposed to their developmental threshold or temperatures close to it can experience retarded growth and even mortality (Teets & Denlinger 2013). This indicates that there is some sort of physical damage to the insects when exposed to such low temperatures, indicating that survival at cold temperatures is also time

dependent (Renault *et al.* 2002). Feeding habits were not observed during the cold hardening study, but it has been reported that cold temperatures may reduce feeding and therefore death may occur once the insects' energy resources are depleted (Renault *et al.* 2002). Personal observations showed that the longer the insects were exposed to the 12°C, the more their locomotory ability was impeded, and they often fell off of leaves into the water when disturbed. It may therefore be more beneficial to rear populations at less extreme temperatures or for shorter durations at extreme temperatures. Fitness costs were not measured during the cold hardening study, it is important to optimise the cold hardening of agents to avoid any potential fitness costs that may occur as agents that are released still need to be viable to increase populations and ensure persistence in the field.

Releasing cold hardened populations in Autumn, before temperatures are too extreme, will hopefully provide the population the physiological limits (CT_{min}) needed for survival during the first winter. Population survival through winter will allow for further adaptation to the cold climate. The Kubusi population shows that if establishment is successful, agents can physiologically adapt to the colder climates. Field studies would need to be conducted to ensure that establishment is successful after releasing a cold hardened population. It is not suggested to release cold hardened agents during warm seasons as the exposure to warm weather may result in a reverse of the cold hardening.

The cold hardening study was done at a constant 12°C for the duration of the experiment, with no fluctuations. In the field however, insects experience fluctuating temperatures throughout their life span. Under experimental conditions, constant temperature environments (CTEs) and fluctuating temperature environments (FTEs) have different effects on fitness traits of insects such as morphology, life span, fecundity, as well as thermal tolerances. If the temperatures of FTEs are kept within the optimal range of temperatures, then there is a positive effect on these fitness traits. FTEs have varying effects on these fitness traits, which is

dependent on the level of fluctuation and whether the conditions reach deleterious temperatures (Colinet *et al.* 2015). Depending on the regime of the FTEs, they have been shown to increase development (Kingsolver *et al.* 2009), morphological asymmetry (Bradley 1980), life span (Fischer *et al.* 2011), fecundity, and cold tolerance (Arias *et al.* 2011), as well as mitigating prolonged low- temperature stress (Colinet *et al.* 2011). Exposing the insects briefly to warmer temperatures during the cold hardening experiment may have increased their survival and ability to withstand the 12°C for a longer period of time. The use of FTEs in biological control, with particular focus on mitigating low-temperature stress, will be discussed in detail in Chapter 5.

Cold hardening agents could be a practical alternative to finding new field cold acclimated biocontrol agents or new consignments of agents from climatically suitable areas. Finding new agents, however, can be costly and time consuming as searching the native country, host specificity tests as well as other experiments will need to be conducted. An example is with *Cyrtobagous salviniae* Calder and Sands (Coleoptera: Curculionidae), a biological control agent of *Salvinia molesta* Mitchell (Salviniales: Salviniaceae) which has been very successful in controlling *S. molesta* in tropical and sub-tropical regions. Due to climatic incompatibility, establishment has been inconsistent in the temperate areas, indicating the need for a more cold adapted population/ agent. Areas of South America which are more temperate and climatically match Louisiana, USA, were determined (Russell *et al.* 2017). The Lower Paraná-Uruguay Delta region (LPUD) was found to have a similar climate to Louisiana. The thermal tolerances and phylogenetic relationships of populations of *C. salviniae* found in these areas were tested. It was found that the populations found in this area were a different biotype to the population found in Brazil which was the original site of collection of *C. salviniae* released in the USA in 2001. The LPUD biotype was found to be more climatically suitable for release in the temperate areas due to its ability to withstand colder climates.

However, due to it being a different biotype, host- specificity tests and cross- breeding experiments needed to be conducted to ensure that no non-target effects or hybridization occurs in the field (Russell *et al.* 2017). The resources used for this research may have been utilised better in manipulating the original agent's thermal physiologies to suit the climate in Louisiana. If climate is the main concern and a suitable agent has already been found, then the potential to use thermal plasticity, as suggested with *E. catarinensis* to the advantage of the biological control programme could reduce the need to find a more climatically suitable biotype.

More focus should be placed on improving agents that are already released, as well as conducting more extensive pre-release thermal studies so that only climatically suitable agent populations are imported for host specificity testing. Improving current agent's thermal physiology could mean that rearing conditions need to be investigated more closely as this has been shown to affect agent thermal physiologies. Further research also needs to be done to determine whether the CT_{min}/LLT_{50} of an insect needs to be decreased or if survival time at the low temperatures needs to be increased. Field experiments also need to be conducted to determine if cold hardening does in fact increase their chances of establishment in the field.

This study suggests that an alternative approach to searching for a new biological control agent, the plastic nature of biocontrol agents should be taken advantage of and that agents that have already been released should be cold hardened. With each new release of an agent comes the possibility of non-target impacts, it is therefore suggested that a more parsimonious approach is adopted (Cory & Myers 2000, McEvoy & Coombs 2003).

Chapter 4: Using respirometry as an alternate method for determining thermal limits of insects.

4. 1 Introduction

Understanding the thermal physiology of an agent prior to release, as well as understanding the plastic nature of released agents, holds great value for improving the safety of biological control through reducing the number of agents that are required to be released. This relies on accurate and ecologically relevant data collection during thermal physiology studies. Although standardised techniques exist, thermal physiology studies can be time consuming and require resources that could be used for host specificity testing of new agents (McEvoy & Coombs, 1999; May & Coetzee, 2013). For this reason, thermal physiology studies are usually conducted only after climatic incompatibility is thought to reduce agent efficacy.

The classic thermal physiology methods, such as those used in this thesis, are utilized for determining an insect's thermal limits, namely critical thermal ranges and lethal limits and are widely used in the scientific literature. Generally, these classic methods determine thermal limits based on an observed change in behaviour deviating from normality (Hazell *et al.* 2008), and they rely on human observations of these changes, and are therefore not free from observer bias (Chown *et al.* 2004).

Each experiment relies on the researcher's perspective of what indicates the "knock down" of an insect. From personal observation during this study, a small tap on an Eppendorf may cause the individual insect to fall off, and this may indicate that the insect was in fact not adhering to the Eppendorf intentionally but may have been "stuck" in place until it was tapped. The method also relies on briefly removing the insects from the water bath and exposing them to ambient temperature, therefore insects may be able to self-right because

they experience a sudden increase in temperature, which could lead to incorrect readings. These disturbances could potentially affect the body temperature and physiological status, which in turn can affect the trait being observed, such as the insect no longer being able to adhere to the vial (Hazell *et al.* 2008).

Including observer bias, there are a few other methodological concerns associated with the classic method of determining critical and lethal temperatures, which are particularly apparent when using smaller insects. Some studies look at the insect's ability to self-right instead of not being able to adhere to the chamber. The knocking of the tube to test righting response may cause the insects to experience stress, which could potentially affect their behaviour (Hazell *et al.* 2008). Terblanche *et al.* (2007) showed that the rate of temperature change during the temperature ramping, as well as starting temperature, affected results. The ramping of temperatures (up or down) can allow insects to acclimatise when ramps are too slow, or they do not allow for compensation by the individual when ramps are too fast (Terblanche *et al.* 2007). The concerns relating to the classic method raise the question whether this standardized method should be continued to be used, or if a custom-built protocol should be developed for each species tested (Chown *et al.* 2009).

Pre-release, insect thermal physiologies, as determined by these classical methods, as well as climate matching are often used to determine post-release potential distributions for potential biological control (biocontrol) agents (Hoelmar & Kirk 2005). Thermal studies are also often conducted as part of a post-release investigation into possible causes of any establishment failures which may be caused by climatic incompatibility (Coetzee *et al.* 2007).

There have been cases, however, where biocontrol agents have established in areas where it was previously thought they could not be based on their thermal physiologies.

Megamelus scutellaris (Berg) (Hemiptera: Delphacidae) is a biological control agent for

Eichhornia crassipes (Mart.) Solms (Pontederiaceae), that was released in South Africa in 2013 (Ray & Hill 2016). Its thermal physiology was investigated post-release and possible distribution maps were then created to determine the best sites for future releases (May & Coetzee 2013). The Kubusi system, Eastern Cape, South Africa (32°35'33.25"S 27°25'19.38"E), which falls within a high-lying, temperate area, was predicted to be too cold for *M. scutellaris* to establish (May & Coetzee 2013, Coetzee *et al.* 2011). *Megamelus scutellaris* unexpectedly established on the Kubusi River (Pers. Obs.). *Eccritotarsus catarinensis* is another example of the same post-release distribution mismatching. As mentioned in Chapter 2 of this thesis, it was predicted that *E. catarinensis* would not establish on the Kubusi River due to climatic incompatibility, but it nonetheless has (Coetzee *et al.* 2007). There are also examples where insects may not establish in areas where climate modelling had predicted a suitable environment for establishment (Zachariades *et al.* 2011, Coetzee *et al.* 2011). For example, *Pareuchaetes insulata* Rego Barros (Lepidoptera: Arctiidae), a biological control agent of *Chromolaena odorata* (L.) R.M. King & H. Rob (Asteraceae: Eupatorieae), failed to establish at 17 sites along the coast of KwaZulu-Natal, South African. Pre-release thermal physiology modelling predicted that establishment should be possible (Zachariades *et al.* 2011). The same mass-rearing and release techniques had been used in other areas, also predicted to be climatically matched, where establishment of *P. insulata* had been successful (Zachariades *et al.* 2011).

Thermal plasticity may be a reason behind some of these post-release distribution mismatches, as the thermal physiology of many insects is plastic and therefore can cause range expansions due to the ability to acclimate to the environmental temperatures (Klok & Chown 2003, Klok & Terblanche 2007 as cited in Calosi *et al.* 2009). This is a similar theory as to why invasive species are so successful, as they can expand their ranges quickly due to thermal plasticity (Nyamukondiwa *et al.* 2010). Using the classic method, three different

studies on *E. catarinensis* produced three different CT_{mins} (Coetzee *et al.* 2007; Porter 2015; Chapter 3 in this thesis). The studies were however not done on the same population, suggesting this is further support for plasticity rather than error from using the classical method as *E. catarinensis* has been shown to exhibit plasticity in their thermal tolerances (chapter 2 & 3).

Differences in thermal tolerances can be subtle, and small changes to an agent's thermal physiology can have large effects in the field. While the classical methods are useful, they are rather robust and other methods that can more accurately measure thermal tolerance are desirable. Examples like those given above suggest that it is possible that these insects may have different functional thermal tolerances to what is identified when using the classic methods, which test the behavioural response to temperature that may be more realized than functional. It is possible that insects may have a narrower physiological thermal tolerance than anticipated by the classical behavioural methods, and can therefore not establish in some areas that are thought to be climatically suitable (C. Owen, Pers. Comm.). Insects may experience physiological stress before their knock down temperature, a common measure used to predict thermal physiology in the classical methods, which may inhibit successful establishment (C. Owen, Pers. Comm.). It may thus be beneficial to look more closely at the methodologies used to determine the thermal physiologies of insects.

The classical method that is used to test thermal physiologies of insects is practical and convenient, but may not be as ecologically relevant as other methods (Rezende *et al.* 2011). Although insect thermal limits are generally tested using knock down temperatures, it is a common practice to use the organism's respiration as a determinant of thermal tolerances amongst fish and copepods (Klok *et al.* 2004). In ectotherms, temperature directly affects their metabolism and therefore affects their respiration (Verberk *et al.* 2016). With an increase in temperature, there is an increase in both metabolism and respiration until a

thermal threshold is reached, after which respiration decreases again and usually the death of the insect follows (Neven 2000).

The oxygen and capacity limitation of thermal tolerance (OCLTT) proposes that organisms' thermal limits are set by a shift from aerobic to anaerobic metabolism, causing upper and lower limits to be coupled (Verberk *et al.* 2016). This hypothesis has been thought as a general principal for all ectotherms whether aquatic or terrestrial (Verberk *et al.* 2016). However, insects upper and lower thermal tolerances is uncoupled (Klok *et al.* 2004), suggesting the principal is not as generally applicable as previously thought. Furthermore, unlike other organisms, the thermal tolerance of insects is not affected by oxygen concentration, and respiration rate can thus be used to calculate CTs in insects, as a decrease in oxygen will not cause a shift in insects CTs. Oxygen concentrations can affect CT_{max} , even in air-breathing arthropods, but only when extreme hypoxia is reached (Verberk *et al.* 2016). A comparison between a terrestrial isopod, *Armadillidium vulgare* Latr. (Crustacea: Isopoda) and a tenebrionid beetle, *Gonocephalum simplex* Fabricius (Coleoptera: Tenebrionidae), showed that only the isopods thermal maximum had a strong relationship with oxygen consumption (Klok *et al.* 2004). Due to the conserved structure of an insect's tracheal system across most species, it is thought that the results found for the tenebrionid beetle can be widely assumed as the normal state for all insects (Klok *et al.* 2004).

It is always beneficial to improve experimental designs to ensure accurate data is always obtained. This chapter investigates a method that may allow for physiological changes (changes in respiration) to be observed, rather than changes in behaviour (knock down/self-righting) during temperature changes. The aim of this chapter is to look at a method which will allow for a performance range to be observed, which will provide more information on the effect of temperature of *E. catarinensis* between the upper and lower limits, compared to the classical method, which only provides two points (the upper and lower thresholds). The

use of respirometry has not been used for many insects, and has not yet been done for biological control agents.

4. 2 Methods and materials

All testing was conducted on live *E. catarinensis* and *E. eichhorniae* individuals from the same quarantine cultures used for other experiments in this thesis. These cultures have both been kept under the same conditions, with a constant controlled temperature of 26°C. The rate of oxygen consumption at various temperatures was determined for each species using a closed system GmbH respirometer (PreSens® Germany). All trials were conducted at the Aquatic Ecophysiology Research Platform (Department of Ichthyology and Fishery Sciences, Rhodes University). Single adults were placed into individual sealed chambers containing atmospheric air. The chamber tray was then submerged in a flow-through unit that was positioned on top of a SDR- 436 respirometer sensor reader, and the unit was then attached to PolyScience® circulating water bath (PP20R-30-A12E, USA) using plastic tubing to allow consistent flow-through. The experimental temperature was monitored constantly within this flow-through unit using a thermocouple connected to the water bath, to ensure that the insects were exposed to the correct temperatures.

An acclimation period of 10 minutes at 26°C (the temperature at which both species were reared) prior to the commencement of the study was used for each trial, after which the temperature was either lowered or raised to the experimental temperature at a rate of 0.25°C/min. This rate of temperature change was chosen as it is the standard rate to use and therefore makes studies comparable (Terblanche *et al.* 2007). The experimental temperatures were run in 5°C intervals deviating from the acclimation temperature until none of the insects in the trial survived. Once each experimental temperature was reached (5°C, 10°C, 16°C, 20°C, 26°C and 30°C for *E. catarinensis*, and 10°C, 15°C, 20°C, 26°C and 30°C for *E.*

eichhorniae), the respirometer was calibrated for that temperature using a built-in calibration system to ensure correct readings. Consistent ambient light was ensured in the room during the trials to prevent mis-readings of the colour changing respirometry sensor dots.

Twenty individuals were used for each trial, with four control chambers that contained no insects per trial. Seven trials were completed for *E. catarinensis*, and five trials were completed for *E. eichhorniae* with only one species used per trial. Trials were allowed to run until the oxygen value in the chambers reached just under 90% of their initial reading. Values used for further analyses did not fall below this 10% reading, to ensure that individuals did not become oxygen-deprived during experimentation (Klok *et al.* 2004). Oxygen readings (cO₂ (μml/L)) were read instantaneously in each chamber sequentially, and recorded automatically every minute using the PreSens SDR version 38 software. The data recorded for any individuals that were dead at the end of the trial were disregarded during the analysis.

The volume of each insect was determined on removal from the respirometry equipment by using the equation for an ellipsoid (Equation 1). The length, breadth and depth were determined for each individual using an Olympus SZX16 stacker microscope (Olympus SDF Plapo 1XPF camera) with the associated Steam Motion 1.9 software. Equation 1:

$$\text{volume} = \left(\frac{4}{3}\right) \times \text{PI} \times \left(\frac{1}{2}\text{length}\right) \times \left(\frac{1}{2}\text{breadth}\right) \times \left(\frac{1}{2}\text{depth}\right)$$

The equation used to determine oxygen concentration for each insect requires oxygen concentration to be in ml, therefore oxygen concentration readings were converted from μmol to ml by dividing each value by 44.661 for analysis (From: <http://ocean.ices.dk/Tools/UnitConversion.aspx>). Each insect's oxygen concentration for the duration of the trial was graphed using scatter plots in Microsoft® Excel 2010. A line of best fit was plotted for each graph and the slope was then determined. Slopes were also determined for control chambers. To remove experimental outliers, the slopes of the data were compared using a Thompson Tau

test. A delta value was then determined for each individual by finding the absolute value when the average of the trials oxygen consumption is subtracted from each chambers oxygen consumption. These values were then used along with the volumes of each individual for further analysis.

Using the data obtained from the PreSens SDR and the volume of the insects, the oxygen consumption was then determined for each temperature using equation 2.

$$\text{Equation 2: } VO_2 = ((mw_{100} - mc)) \times (V_c - V_w) \times \beta O_2$$

Where VO_2 is the oxygen consumption rate per volume of insect per unit of time ($O_2 \text{ml} \cdot \mu\text{ml}^3 \cdot \text{min}^{-1}$); mw is the slope of the line of the change in oxygen during each trial for each chamber ($cO_2 \cdot \text{min}^{-1}$); mc is the mean slope of the lines of the change in oxygen during the experimental for all controls in the same trial ($cO_2 \cdot \text{min}^{-1}$); V_c is the chamber volume (μm); V_w is each insect's volume; and βO_2 is the oxygen capacitance of each temperature.

At 20°C, atmospheric air is cited as having a 54.73 $\mu\text{mol} \cdot \text{L}^{-1} \cdot \text{Torr}^{-1} \beta O_2$ (Truchot 1987). The βO_2 for the other experimental temperatures were calculated using equation 3 (Owen 2015).

$$\text{Equation 3: } PV = nRT$$

Where P is pressure (Pa); V is volume (m^3); n is the number of mols of the medium; R is the universal gas constant (8.31451 J/K.mol); and T is the temperature ($^{\circ}\text{C}$).

The above analysis results in oxygen consumption values for each unit volume of each insect at each temperature. The mean \pm SE is then determined for each species at each temperature. Graphs were then plotted for the oxygen consumption of each species throughout the range of their temperature treatments in Excel® 2010. A generalised linear model was

conducted in Statistica® V13.2 to compare the consumption between the different temperatures and between the species.

4.3 Results

A total of 120 *E. catarinensis* and 72 *E. eichhorniae* individuals were used in the final oxygen consumption determinations. Each trial was run for a different length of time, for *E. catarinensis* trials were run for 2, 3.5, 4, 7.5, 5, 12, 20 and 37 hours for the trials from 40°C-5°C respectively. For *E. eichhorniae* trials were run for 6, 6, 14, 8.5 and 17 hours for the trials from 30°C – 10°C respectively. Mortality of all *E. catarinensis* individuals occurred at 0°C and 40°C, and these temperatures were thus excluded from the analysis. *Eccritotarsus eichhorniae* samples experienced complete mortality at 5°C and 35°C, so these trials were also excluded from the analysis. The oxygen consumptions at different temperatures for each species are shown in Figure 4.1. Oxygen consumption for *E. catarinensis* plateaus until about 26°C, thereafter increases drastically to 35°C, and then decreases again. *Eccritotarsus eichhorniae* is a little less straightforward, as oxygen consumption stays relatively low across all temperatures with a gradual increase to 26°C, and then oxygen consumption decreases rapidly thereafter.

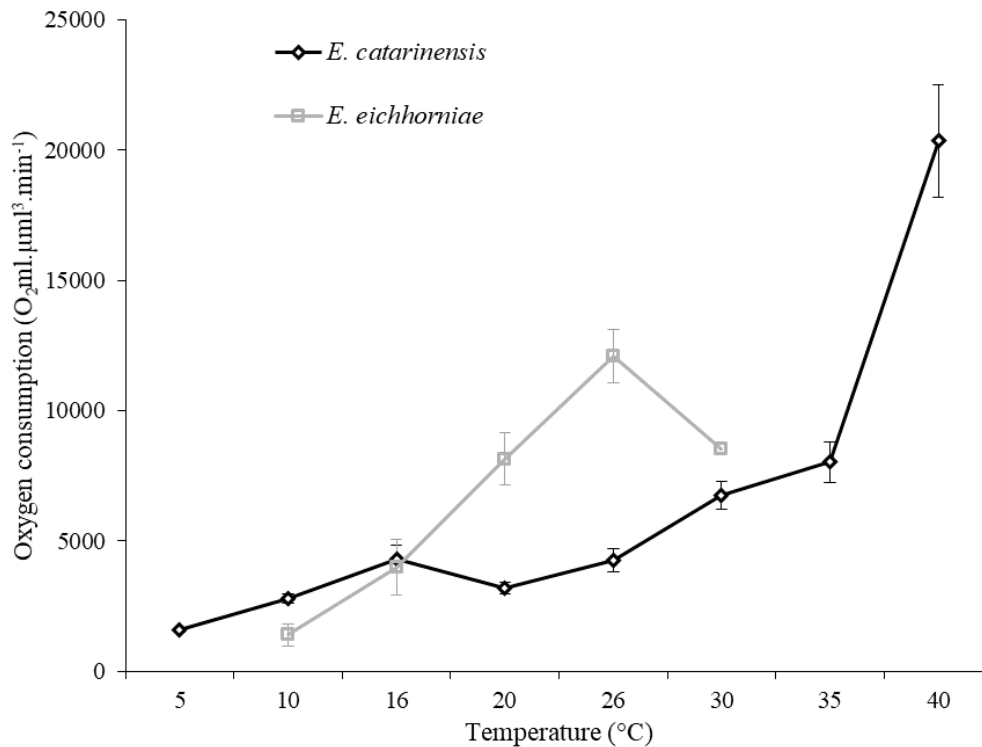


Figure 4.1: Mean oxygen consumption (\pm SE) for *Eccritotarsus catarinensis* and *Eccritotarsus eichhorniae* at various temperatures. The overall O₂ consumption between the species did not differ significantly ($P = 0.94$, t -value = 0.07) but, temperature had a significant effect on O₂ consumption ($P < 0.001$, t -value = 9.96).

4. 4 Discussion

The use of respirometry techniques here proved that thermal physiology may be explained through physiological measures rather than behavioural ones, which is expected to give a more accurate representation of the true limits of a species. This method shows a common trend to what was found using the classical methods, where *E. catarinensis* has a wider thermal range and can tolerate both colder and hotter temperatures compared to *E. eichhorniae* (Chapter 3).

The oxygen consumption for *E. eichhorniae* produced a graph that followed the general pattern of a performance graph (Figure 4.2), where there is an increase in oxygen consumption to a point, and then a steady decrease again (Angiletta *et al.* 2002). A general performance

curve increases to a peak, T_o (Temperature Optimal), which is the temperature where performance is maximised, then just below T_o on either side is the range of temperatures where performance is $\geq 80\%$, B_{80} . The points where the graph touches the x-axis usually represent the CT_{min} and CT_{max} of the species, which indicate the minimum and maximum temperatures, respectively, where performance is possible (Figure 4.2, Angiletta *et al.* 2002).

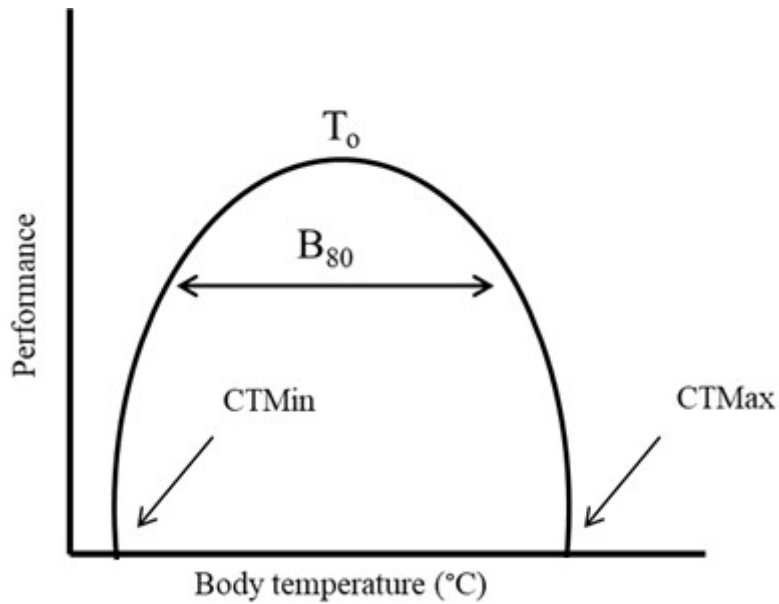


Figure 4.2: A standard performance graph (from Angiletta *et al.* 2002).

The fact that *E. catarinensis* did not survive long exposures to temperatures below 5°C and above 40°C indicates that the CT_{min} and CT_{max} of the species lies around these points, although behavioural changes would need to be monitored during the respirometry trials to determine their actual CT_{min} . Use of the classical method in Chapter 3 of this thesis illustrated a CT_{min} and CT_{max} of 2.9°C and 42.9°C , respectively for this species. The CT_{min} and CT_{max} of *E. eichhorniae* using the classical method was 5.87°C and 42.77°C , respectively. This method however, suggests that the optimal temperature range for *E. eichhorniae* was only between 10°C and 35°C , indicating a narrower performance range compared to what is was found with the classical method.

These results illustrate that the two methods are relatively comparable, more for *E. catarinensis* than *E. eichhorniae*. It is important to note that nothing is known about performance of the agent between the indicated limit temperatures when using the classical methods. The implication for biological control is that although insects may survive at their CT_{min} and CT_{max} , they may stop performing at a much narrower thermal range. Performance ability of a biocontrol agent is what makes an agent effective or not, and needs to therefore remain in the range of temperatures that allow for optimal performance. For example, although the *E. eichhorniae*'s CT_{min} is 5.9°C and CT_{max} is 42.8°C, based on the respirometry results they may only be performing (e.g. eating, mating and ovipositing) between 10-35°C.

The newer methodology used here indicates that *E. catarinensis* performs best between 26°C and 40°C, following which metabolic performance rapidly declines as death of all insects occurred at 45°C. If more temperature trials between 40°C and 45°C were conducted, a decrease in oxygen consumption would have occurred, producing a more ‘typical performance’ graph. *Eccritotarsus eichhorniae* performs much better than *E. catarinensis* in cooler conditions, but with a maximum performance at 26°C. Performance of *E. eichhorniae* decreases when temperatures reach above 26°C. Whether this translates into a difference in the action of the agents, such as feeding rates and oviposition factors, is unknown and still needs to be assessed. Although these results are contradictory to what would be expected as they are from different climatic native ranges, quarantine populations were used for the respirometry, and therefore this may not be completely reflective of the thermal physiologies of populations collected from their native ranges may experience.

Eccritotarsus eichhorniae reached a peak in oxygen consumption at a lower temperature than *E. catarinensis*. Furthermore, complete mortality was reached at 5°C for *E. eichhorniae* and at 0°C for *E. catarinensis*. These patterns may indicate that *E. catarinensis* exhibits a much wider thermal tolerance than *E. eichhorniae*, with the former tolerating lower temperatures for

survival, but also higher temperatures for optimal performance. In other words, *E. catarinensis* is more eurythermic than *E. eichhorniae*, which exhibits more stenothermic traits. These results suggest that it may be beneficial to focus on releasing *E. catarinensis* in South Africa where the area experiences fluctuating temperatures, especially when very high temperatures are reached. However, in areas which experience less extreme, stable temperatures *E. eichhorniae* may be a better candidate for release as it does not require very high temperatures to perform optimally. It has also been shown that *E. eichhorniae* is more fecund and outcompetes *E. catarinensis* at standard greenhouse temperatures (Paterson *et al.* 2016).

As mentioned above, respirometry has been used to determine thermal tolerances in other taxa. Respiration, using CO₂ emission as opposed to O₂ consumption was used to determine the CT_{max} of a terrestrial isopod, *Armadillidium vulgare* (Latreille) (Isopoda: Armadillidiidae) (Klok *et al.* 2004). Both CO₂ concentrations and motor activity were monitored, and the study showed that both methods produced the same CT_{max} for *A. vulgare* (Klok *et al.* 2004). CO₂ production increased, due to an increased respiration rate, drastically until the CT_{max}, after which the isopods were no longer active (motor activity had ceased), after which respiration rate, and therefore CO₂ production, decreased steadily until the death of the isopod occurred (Klok *et al.* 2004). They also showed that a lack of oxygen supply results in a decrease in the CT_{max} of the isopods (Klok *et al.* 2004).

The current classic laboratory assays for determining thermal tolerances of insects has practical convenience, but although a useful tool for determining a robust overview (Terblanche *et al.* 2007) may not provide the detailed ecologically relevant data that respirometry could provide. The need for more ecologically relevant assays is increasing and new methods are being developed and used more widely to determine thermal physiologies both in the laboratory (Hazell *et al.* 2008, Mitchell & Hoffmann 2010) and the field (Chidawanyika and Terblanche 2011). Hazell *et al.* (2008) suggested a new method for

determining CT_{mins} , where converted condensing tubes were used and the temperatures where insects fell to the bottom was recorded. However, this was found to be only suitable for flying insects as non-flying insects tended to crawl out of the tube, particularly at high temperatures (Hazell *et al.*, 2008). However, to make results comparable, a method is needed which can be used for most, if not all insects.

Both methods have their advantages and like the classical method, there are also a few confounding factors involved with respirometry. Firstly, each trial is run for different lengths of time, as the respiration rate changes depending on the temperature the insects are exposed to. The colder temperatures for *E. catarinensis* ran for 37 hours whereas the 40°C trial only ran for 2 hours. Acclimation, exposure time, to colder temperatures has an effect on thermal physiology (Terblanche *et al.* 2007). Because the insects are only removed from trials once 90% of the initial oxygen consumption is reached, trials do not have a set time that they run for. This resulted in trials run at colder and very hot temperatures running for longer times than those at the middle temperatures. As previously mentioned, a 90% oxygen reading is used as the threshold for an experiment to end as the CT_{max} can be influenced by extreme hypoxia (Klok *et al.* 2004). The extended length of time that the insects are exposed to the experimental temperature, particularly for cold temperatures, could result in acclimation, which could affect their thermal tolerances. The respirometry method is more similar to the way lethal limits (LTs) are tested, as insects are exposed to constant temperatures for an extended period of time.

Furthermore, feeding status is known to affect respiration rate (Bennett *et al.* 1999) and insects are not starved before the trial is run, which means they are digesting during the trial, which would influence their metabolic readings. Age, gender, whether females were virgins or mated and time of day all have an effect on metabolism of insects (Denlinger *et al.* 1983; Rogowitz & Chappell 2000). Furthermore, the analysis of the data for the newer method is

more labour intensive and requires expensive specialised equipment. The most beneficial part of this technique is that there is no need to continuously disturb and monitor each specimen as the oxygen readings are recorded automatically using software, resulting in robust, more detailed results, which are not influenced by human error.

Chapter 5: General discussion

5.1 General

This study has shown that *E. catarinensis* exhibits phenotypic plasticity which can help it survive at sites near the edge of its thermal limits. Although their thermal physiology is plastic, both *Eccritotarsus* species seem to have conserved some thermal differences and still have parts of their thermal physiologies which are representative of their native climates, *E. catarinensis* having a thermal tolerance which is more adapted to a temperate climate compared to *E. eichhorniae*. *E. catarinensis* seems to be able to tolerate both colder and higher temperatures compared to *E. eichhorniae* and therefore should be the main focus when trying to improve the efficacy of this insect as a biological control agent in areas where control of water hyacinth is limited by cooler temperatures.

A previous study on *E. catarinensis* and *E. eichhorniae* looked at the effect of temperature on various fitness traits of both species such as lifetime fecundity, egg hatching rate, realised fecundity, nymphal survival rate, sex ratio, female and male longevity were studied (Ismail and Brooks 2016). In most cases, temperature had an effect on each fitness trait observed, however differences were only found for realised fecundity and nymphal survival rate at 30°C for both species. The second generation was observed to determine the potential for adaptation. Once exposed to higher temperatures, the second generations were found to have increased life fecundity, egg hatching rate and realised fecundity. The two species responded differently, especially at the higher temperatures, in some traits (Ismail and Brooks 2016). This suggests that each has still maintained these differences even after being reared for many years at the same temperature. These traits show an adaptation each species has to their

native climates, with *E. eichhorniae* being more adapted to higher temperatures due to its more tropical native climate which correspond with their thermal limits found here.

5. 2 Reducing climatic incompatibility and improving the efficacy of biocontrol agents

This thesis has provided evidence that supports the need for improved pre-release data on agents, with particular reference to understanding biocontrol agent's thermal physiology. Also, that agents should be cold hardened before releasing into the field if climate is thought to be a possible limiting factor in that area.

5. 2. 1 Pre-release cold hardening

The CT_{min} of *E. catarinensis* can be decreased drastically when exposed to an extreme low temperature (chapter 3). However, this effect was reduced after long term exposure to 12°C, a temperature close to its lower developmental limit. It is suggested that short exposures to warmer temperatures during long term exposure to cold temperatures can reduce or even mitigate the injuries sustained through long term exposure. As mentioned in chapter 3, fluctuating temperatures (FTs) can reduce the injury caused by exposure to low temperatures (Colinet *et al.* 2015). It may therefore be beneficial to look at the effect of long term cold acclimation when short temperature increases are introduced. This may prevent the increase in CT_{min} that was found in this study after 15 days which is presumed to be due to physiological injuries that the insects accumulated.

Chen and Denlinger (1992) acclimated *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae) at 0°C for 10 days and then exposed them to -10°C. No insects could survive 2 hours of exposure to -10°C. The 0°C acclimation was then redone, but this time short increases in temperature were introduced into the regime. On day 10, the temperature was increased

once to 15°C for six hours. With this single temperature increase breaking the cold acclimation at 0°C, it allowed for 53% of the insects to survive exposure to -10°C for ten hours (Chen & Denlinger 1992). Fluctuating temperature regimes have since been shown to increase thermal physiology across a range of insect taxa including Hemiptera, Coleoptera (Renault *et al.* 2004), Lepidoptera and Diptera (Colinet *et al.* 2015).

Survival of *Alphitobius diaperinus* Panzer (Coleoptera: Tenebrionidae) was slightly increased when a population that had been reared at the standard temperature of 30°C was exposed to a fluctuating regime of 5°C with daily two-hour temperature increases to 10°C. The survival was further increased when the insects were reared at 15°C and then exposed to the FT regime of 5/ 10°C (Renault *et al.* 2004). It is thought that the increases in temperature during cold acclimation allowed for a reversal of chill injury that the insect experienced which made it possible for it to handle colder temperatures. The use of FTs in developing cold tolerant insects for releases in areas on edge of agent's thermal limits should be investigated further. Alternatively, insects could be exposed to extreme cold temperatures for a very short period of time, known as rapid cold hardening. Larvae of *Belgica antarctica* Jacobs (Diptera: Chironomidae) were exposed to rapid cold hardening (RCH) trials. The summer-acclimatised larvae survived freezing at -5°C for one hour (Lee, Jr, *et al.* 2006). However, when larvae were directly exposed to -10°C, survival fell to <25%. Exposing the larvae to -5°C for one hour prior to being exposed to the various treatments, increased survival rates to >97%. Larvae were also cold acclimated where they were kept at sub-zero temperatures for 7 days and then exposed to -15°C and -20°C. This trial was done before and after RCH of the larvae. Few larvae survived -15°C and none survived -20°C, however after RCH larvae survival increased at -15°C to 90% survival and -20°C to 75% survival. Larvae that did survive exposure to -20°C had only limited mobility, suggesting that these larvae did experience some freezing injury. The larvae that were RCH showed an increase in cell survival by 24% compared to the larvae exposed directly

to 20°C. The low adult survival rate during RCH is thought to be due to the adults having an intrinsic cold tolerance. This was shown by their high survival at -5°C for 24 hours (Lee *et al.* 2006).

Although it is thought that cold hardening is a reversible trait, cold hardening agents before release could increase initial establishment and survival of the first winter, allowing for some adaptation, to cold temperatures, in the field to occur. This study has showed that it is possible to increase agent's thermal physiology and that field acclimation does occur if agents are able to survive the first few winters. Although the adaptation in the field is still limited by the agent's thermal physiology that evolved in their native range, the combination of cold hardening and field acclimation could increase success of biological control programmes by increasing agent's cold tolerance.

5. 2. 2 Difference in thermal tolerance of different life stages

Only adults were investigated for this thesis; however, many studies suggest that different life stages experience different thermal limits (Chown 2001). It may therefore be beneficial to determine the thermal tolerance and cold hardening abilities of all life stages. If one life stage can survive colder conditions than the others, it may suggest that releasing that particular life stage initially may increase their chances of surviving any initial and unpredicted cold spells in the release site. For example, CT_{min} of both larvae and adults of a brachypterous kelp fly *Paractora dreuxi* Séguy (Diptera: Helcomyzidae) were included in a thermal physiology study (Klok & Chown 2001). The larvae had a lower CT_{min} compared to the adults ($-5.1 \pm 0.09^\circ\text{C}$ and $-2.7 \pm 0.19^\circ\text{C}$) and the larvae were also able to recover from their CT_{min} at a lower temperature compared to adults ($-1.3 \pm 0.32^\circ\text{C}$ and $1.9 \pm 0.40^\circ\text{C}$, respectively). This suggests that the larvae are more cold tolerant compared to the adults. The different thermal tolerance of the larvae and adults is thought to have developed due to differences in behaviour between the two stages,

with the larvae being far less mobile than the adults and therefore unable to escape unfavourable temperatures as readily as the adults (Klok & Chown 2001). It is possible that the nymphs of *E. catarinensis* may be more cold tolerant as they are not as mobile as adults because they are smaller and have not developed wings. If the nymphs of *E. catarinensis* are more cold tolerant, then it is suggested that nymphs are initially released instead of adults as it may increase the chances of survival of the first winter allowing for a quicker establishment. However, the adults may still be better suited as they have an increase ability to avoid the unfavourable conditions through behavioural changes. Furthermore, both the nymphs and adults have different colouring with the adults being black and the nymphs being white, which could also potentially affect their ability to withstand certain temperatures and would need to be researched further.

5. 2. 3 Quarantine programmes

Many biological control agents are sourced from warm, tropical climates such as South America (Hoffmann 1991). Candidate biological control agents are then reared under artificial conditions in a quarantine facility, with rearing conditions typically set at relatively warm temperatures to encourage reproduction. Many quarantine facilities are kept at constant temperatures. Both *Ecritotarsus* species were kept in similar rearing conditions and neither population experienced cold temperatures or any extreme fluctuations in temperature over the 24 years for *E. catarinensis* and 19 years for *E. eichhorniae* that they were in quarantine and the following years in the mass-rearing facility. The increase in CT_{min} , from $2.9 \pm 0.38^{\circ}C$ found in this study to $1.2 \pm 1.17^{\circ}C$ found by Coetzee *et al.* (2007) may therefore be due to long term acclimation from being kept in the warm constant rearing conditions. Similar results have been found with other insects. It has been shown that thermal physiology of Diptera is

significantly influenced by rearing temperature and long term acclimation (Jensen *et al.* 2007). Further research will need to be done in order to observe the effects of rearing conditions on biocontrol agents which will give better insight into whether agents should be reared at colder temperatures if they are to be released in colder climates. Insects in more stable environments may increase their reproductive output instead of focusing energy on producing the proteins involved in cold hardiness. It has been found that there is a trade-off between reproductive rate, body size and cold hardiness (Colinet *et al.* 2015).

The quarantine temperature regime should therefore be re-examined, with the possibility of including a fluctuating temperature (FT) regime instead of using only constant temperatures. Due to phenotypic plasticity having a genetic basis, it is assumed that the selection for “plastic genotypes” will occur in populations exposed to varying climates (Overgaard *et al.* 2011), therefore a FT regime could potentially increase the selection for phenotypic plasticity or at least avoid the selection against this trait, resulting in an increased thermal tolerance (Colinet *et al.* 2015). Some insects, like *E. catarinensis* are kept in quarantine for many years and this may eventually lead to populations which do not select for the plastic genotypes, resulting in decreased thermal plasticity in the field. This could also be avoided by employing a FT regime in the quarantine facilities to encourage increases thermal plasticity and therefore the insect’s ability to quickly adapt to the changing environment once released into the field. Studies to test the effect of both fluctuating and constant temperatures were conducted on *Drosophila melanogaster* and revealed that insects acclimated to fluctuating temperatures which simulated “outside” temperatures experienced a decrease in CT_{min} by $\sim 2^{\circ}C$ and an increase in CT_{max} of $\sim 1.5^{\circ}C$ compared to the insects that were acclimated to a constant temperature of $24^{\circ}C$ (Overgaard *et al.* 2011). The temperatures fluctuate much more widely at the Kubusi river than at Enseleni river. This could explain the successful establishment of *E. catarinensis* on the

Kubusi River; the outside fluctuating temperatures may have allowed for an increase in their cold tolerance.

5. 3 Parsimonious biological control

If undertaken correctly, many invasive weeds can be controlled by a single, or in some cases two, biological control agents.

However, introducing multiple agents, especially if they are new consignments of the already released agent may increase the potential for challenges in the field. Cryptic species may cause problems in biological control programs due to differences in host specificity, thermal tolerances and hybridization. *Opuntia stricta* var. *stricta* and *Opuntia stricta* var. *dillenii* are two subspecies of *Opuntia stricta* Haworth (Cactaceae) and are highly invasive in Australia and South Africa. *Dactylopius opuntiae* (Cockerell) (Hemiptera; Dactylopiidae), a cochineal agent, was released in Australia in 1920 and is highly damaging to *O. stricta*. *Dactylopius opuntiae* was subsequently collected in Australia for release into South Africa against another prickly pear species, *O. ficus-indica* (L.) Miller in 1937 and was presumed to be from the same stock that was so successful in Australia against *O. stricta* and therefore it was thought that *D. opuntiae* would control both species. However, *D. opuntiae* never established on *O. stricta* in South Africa. Upon further investigation, it was found that during 1921 and 1935, 15 consignments of different strains of *D. opuntiae* were brought into Australia from North America for testing (Volchansky *et al.* 1999). During host specificity testing, records indicating collection site and host plant were lost. It is thought that the insects released in South Africa were actually collected from a prickly pear species (the exact species is unknown) which would explain the establishment on *O. ficus-indica* and the lack of establishment on *O. stricta*. A new lineage of cochineal which was more effective on *O. stricta* was sourced and released in South Africa which successfully established and controlled *O.*

stricta, unlike the first lineage of *D. opuntiae* released (Volchansky *et al.* 1999). This study shows the importance of ensuring correct identification of newly found strains to ensure that the introduced and new agent are the same strain to avoid non-target feeding on native or economically important plants. New consignments of biological control agents that are thought to differ in terms of their thermal tolerances are likely to differ in other regards too, and it is therefore essential to treat each consignment as a new agent which must undergo all the relevant genetic testing and then based on the genetic results further host specificity testing and impact studies will need to be performed (Paynter *et al.* 2008; Paterson *et al.* 2016). The possibility of hybridisation with other lineages of the agent and the effect of this hybridisation must also be determined (Hoffmann *et al.* 2002b). These studies can be expensive and time consuming, so it is important that the best possible climatically suited population of an agent is collected at the initiation of biological control programmes and that the possibility of improving existing agent's thermal tolerances should be investigated before new, and supposedly more climatically suitable, consignments are sourced in the native range.

5. 4 Improving thermal studies for use as prioritisation tools for selection of biocontrol agents

Finding climatically suited agents through climate matching has been given a lot of attention and has proven to be beneficial in the past (Robertson *et al.* 2008). Climate matching along with thermal physiology studies should be incorporated more readily into future agent selection and prioritisation. Understanding agent's thermal physiology pre-release can result in the rejection of agents which are not compatible and allow for prioritisation of agents which are climatically suited, thus allowing for fewer agents to be released. A French biotype of *Trioxys pallidus* Haliday (Hymenoptera: Aphidiidae) failed to establish as an agent for

Chromaphis juglandicola (Kaltenbach) (Hemiptera: Aphididae) due to climate incompatibility in northern California. A successful biotype was found in Iran which was more climatically suited for release in this region (van den Bosch *et al.* 1970). If thermal studies had been conducted pre-release, resources could have been focused on prioritising and releasing the biotype from Iran, avoiding the expenditure on sourcing and releasing the incompatible French biotype.

More ecologically relevant thermal physiology studies are sought after and being used for a variety of reasons (Chapter 4). The standard method is practical and does not require advanced equipment, however it may not always be ecologically accurate (Terblanche *et al.* 2007). Thermal limits can be influenced both by ramping rate and the starting temperature. Slower ramping rates generally produce the lowest thermal limits (Terblanche *et al.* 2007; Klok *et al.* 2009). Respiration is suggested as a good tool to understand physiological changes that insects may experience under different temperatures and once developed fully, may provide a more ecologically relevant understanding of insect's thermal physiology and better predicting where agents are likely to establish and thrive.

5.6 Future research

Although this study provides a suggestion for cold hardening insects before release, field studies will need to be conducted to ensure populations establishment success is increased due to cold hardening. A cold hardened population should be released during Autumn with post-release studies conducted during spring and summer to ensure successful establishment. Long term post-release studies will also need to be conducted to ensure the population survives future winters and continues to be established in the area. Because cold hardening effects can be

reversible, field studies are important to ensure the cold hardening effects are not reversed during the warm seasons, preventing survival during the following winter.

5. 5 Conclusion

It is encouraged to take a more parsimonious approach to the release of biological control agents. This encourages practitioners to do more pre- release evaluations in order to find the most suited biological control agent for a particular program. The pre-release experiments should include thermal physiology studies so that the new potential biological control agent will be climatically suited to the areas where it will be released. If biological control programs are already in place for weeds and there is failure to establish in colder climates, then whether their thermal tolerance can be manipulated and improved should be considered before the search for a new biological control agent is undertaken. It is vital that biological control receives as little bad exposure as possible because many biological control programmes, especially in agriculture, rely heavily on the cooperation of the community. It is therefore important that biological control programmes fail as little as possible to ensure that they are successful and are received willingly by the community. This thesis suggests a few alternatives to consider when improving a biological control programme where failure to establish is primarily due to climatic incompatibility.

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