An investigation on factors influencing the rearing and success of biological control agents of *Gonipterus* sp. n. 2 (Coleoptera: Curculionidae)

by

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SUMMARY

An investigation on factors influencing the rearing and success of biological control agents of *Gonipterus* sp. n. 2 (Coleoptera: Curculionidae)

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Gonipterus scutellatus species complex is a cryptic species that feeds on *Eucalyptus* leaves. Both the adult and larval stages cause high levels of defoliation. *Gonipterus* sp. n. 2 is the species present in South Africa and *Anaphes nitens* was released as a biological control agent. However, *Gonipterus* spp. populations have continued to cause economic damage and various countries continue to seek additional biological control agents. This dissertation focused on understanding how natural enemies interact if multiple species are released in a biological control programme. The biology of one potential parasitoid of *Gonipterus* sp. n. 2, *Centrodora damoni* was investigated. It was shown that the parasitoid's longevity, host age preference and developmental time are ideal for it to be a successful biological control agent. The success of the biological control of *Gonipterus* sp. n. 2 was also investigated by studying the reproductive development of the beetle and linking it to its seasonal egg production in the laboratory. The preoviposition period for *Gonipterus* sp. n. 2 was 30 days and oviposition occurred when the beetles were four weeks of age. Various factors that influence the rearing techniques were considered in this study including how the techniques affect preparation for releases. Four parasitoids that were reared in the laboratory, namely *Anaphes inexpectatus*, *Centrodora damoni*, *Cleruchoides noackae* and *Selitrichodes neseri* were measured to investigate the effect of rearing on parasitoid fitness using body size as a proxy across nine generations, six generations, nine generations and five generations respectively. Rearing reduced body size significantly over two generations for *C. noackae* and *C. damoni*. *S. neseri* had significant differences in body size between some generations but the first and last generations studied had no differences. *A. inexpectatus* did not exhibit any loss in body size across generations.

Declaration

I, Idea Albertina Makowe declare that the dissertation, which I hereby submit for the degree of Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Idea Albertina Makowe

May 2023

This is dedicated to my son Tafara Kaylum Zengenene, my siblings Tapuwa Cuthbert Makowe, Adelaide Tagwireyi, Angella Makowe, Chiedza Dawn Makowe and my friend Nancy Nyarai Matemera for supporting me through this dissertation.

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Preface

Gonipterus sp. n. 2., is an unidentified species of the *Gonipterus* species complex that falls within the *Gonipterus scutellatus* (Coleoptera: Curculionidae) cryptic species group. *Gonipterus* spp. are native to Australia but invasive in Africa, the Americas and Europe where they feed on *Eucalyptus* spp. In 1926, the egg parasitoid *Anaphes nitens* Girault (Hymenoptera: Mymaridae) was introduced into South Africa as a biological control agent for *Gonipterus* sp. n. 2. The introduction of *A. nitens* to manage populations of *Gonipterus* sp. n. 2. was initially reported as successful, but in the last decades there has been a resurgence in the pest population. This led to investigating other potential biological control agents, including another egg parasitoid, *Centrodora damoni* (Girault) (Hymenoptera: Aphelinidae).

There are several factors that need to be considered when introducing a biological control agent. If another biological control agent is already present, as is the case with *Gonipterus* sp. n. 2 in South Africa, then it is important to understand the potential interactions between the introduced biological control agents and how those interactions can influence the success of the biological control programme. In addition, knowledge on the biology of the potential biological control agent is needed, for example to inform the rearing and release of the agent. Other factors that will influence the rearing of the biological control agents, such as the effect of rearing over time on the fitness of the agent, should also be considered. For example, continuous rearing over a long period of time may perpetuate inbreeding depression due to population reduction of the quarantined parasitoid, and thus affect critical life history parameters such as sex ratio, longevity, adult size, fecundity, dispersal and patch location. If the biological control agent is reared on the insect host, then it is also important to understand the reproductive biology of the host insect in order to optimize the rearing of the host insect and inform the timing of the releases of the biological control agents.

This dissertation examines various factors that can influence the success of a biological control programme, with a focus on *C. damoni* as a potential biological control agent for *Gonipterus* sp. n. 2. **Chapter 1** is a literature review that discusses the interaction between natural enemies, including parasitoids and predators. In some cases, more than one natural enemy is found within the native range and more than one is released to control a pest (this phenomenon is common in the *Gonipterus scutellatus* spp. complex). It is therefore important to understand the various possible ways these biological control agents might interact, whether additive or adversative.

In **Chapter 2** the biology of *C. damoni,* a potential biological control agent of *Gonipterus* sp. n. 2, is examined. Parameters such as longevity, fecundity, developmental time, parthenogenesis, preoviposition period, sex ratio and host egg age preference were tested to assess *C. damoni* suitability as a potential biological control agent for release in South Africa. Egg capsules of *Gonipterus* sp. n. 2 (≤24 hours old) were exposed to a *C. damoni* breeding pair (also ≤ 24 hours old) in a vial to investigate the different parameters. A piece of honey paper, stuck on the side of the vial was used a food source. The adults were removed from the vial after 24 hours and the various observations were recorded.

Chapter 3 investigated the reproductive development of *Gonipterus* sp. n. 2 to develop a physiological age grading system for the insect pest. Physiological age grading systems of insects enable the development of a reproductive development model that will assist in timing parasitoid releases with peak oviposition periods of *Gonipterus* sp. n. 2 in the field. Beetles of different ages were dissected to determine the development of different reproductive structures over time. Structural changes of fat bodies, spermatheca, ovarioles, follicles, eggs, common oviducts and lateral oviducts were observed and compared.

In **Chapter 4**, four parasitoids reared for the control of different eucalypt insect pests were used to investigate the effect of laboratory rearing on the parasitoids body size, where body size was a proxy for fitness. The parasitoids used were *Cleruchoides noackae* Lin and Huber (Hymenoptera: Mymaridae), an egg parasitoid of *Thaumastocoris peregrinus* Carpintero et Dellape (Heteroptera: Thaumastocoridae); *Anaphes inexpectatus* Huber and Prinsloo (Hymenoptera: Mymaridae) and *Centrodora damoni* Girault (Hymenoptera: Aphelinidae), both egg parasitoids of *Gonipterus* sp. n. 2; and *Selitrichoides neseri* (Kelly and La Salle) (Hymenoptera: Eulophidae), a parasitoid of the eucalypt gall wasp, *Leptocybe invasa* Fisher and La Salle (Hymenoptera: Eulophidae). The body size of the parasitoids was measured by making temporary slides of the left hind tibia of each insect and taking a measurement using a Nikon SMZ1500 microscope. Measurements were taken across different numbers of generations per parasitoid depending on availability of generations as samples had been collected and placed in storage, prior to this study.

CHAPTER 1

Natural enemy interactions in classical biological control

1.1. Abstract

Natural enemies have been used to reduce pest impacts globally using biological control. The introduction of multiple natural enemy species is frequent in biological control programmes even though, in some cases, a single natural enemy can adequately suppress an invasive pest. The introduction of multiple natural enemies can reduce pest densities significantly, especially if they exhibit some niche segregation. However, introduction of additional agents does not ensure control since they often fail to establish and may cause antagonistic interactions. In this review, we consider the different natural enemy interactions in biological control. Case studies of significant multiple natural enemy introductions in biological control programmes and the resulting type of interactions between the natural enemies are considered.

1.2. Introduction

Insect populations within their native range are controlled to some degree by their natural enemies, including predators, parasites, pathogens, and antagonists (Sanda and Sunusi, 2016). In the invaded range they are normally free of their natural enemies and cause considerable damage to crop. However, natural enemies may be introduced into the invasive range of the pest unintentionally. Such unintentional introductions are favoured by factors such as the upsurge in trade throughout the globe; absence of surveillance for species that are not associated with live plants or animals; inability to intercept tiny organisms such as parasitoids; a lack of aggressive screening for pests that are already established, human vectors as transport system, and large invasive host populations at the source and/or receiving areas that allow rapid development (Weber et al. 2017). Most of the time, such introductions are discovered long after the host has established itself in new areas, sometimes even during, or after host range investigations have been started. (Mason et al. 2017; Wondafrash et al. 2021). For instance, in arthropod pest systems in Canada over the past 30 years, at least seven unintended introductions of natural enemies have taken place. Some of these introductions have had unanticipated advantages for the control of invasive alien species. (Mason et al. 2017).

Intentional natural enemy introductions for the management of insect pests, as a form of biological control, has often led to the successful management of introduced agricultural pests (DeBach & Rosen, 1991). Approaches to biological control are distinguished by how a biological control agent is used rather than the type of biological control agent (Eilenberg et al. 2001). There are three types/approaches of biological control that can be distinguished. Classical biological control (CBC), also referred to as introduction biological control, is defined as the introduction of a natural enemy of exotic origin to control a pest, usually also exotic, aiming at permanent control of the pest (Hajek 2004; Van Driesche & Hoddle, 2009). Augmentative biological control includes inoculative and inundative biological control. Inoculative biological control is the intentional release of a natural enemy as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not permanently. Inundative biological control is the use of a natural enemy to control pests where control is achieved exclusively by the released organisms themselves, not their progeny (Eilenberg et al. 2001; Hajek 2004). Conservation biological control is the protection of natural enemies against adverse effects of pesticides and incompatible cultural practices and improving their efficiency via providing food sources (Shields 2017).

Parasitoids and/or predators may be selected as potential biological control agents of CBC. Parasitoids are parasitic insects (that fall under the orders Diptera and Hymenoptera) that develop in a single host, usually killing it (Kenis et al. 2019). They parasitize all stages of insect development, from eggs to adults. Predators are free living entomophagous insects that can consume large numbers of prey in their lifetime and may consume their prey at any of the developmental stages. In CBC programmes used elsewhere, parasitoids are typically preferred over predators because predators have a wide host range which increases the risk of non-target effects. However, there are notable exceptions, and some of the most significant CBC achievements against tree pests have been produced by a few predators (Julien 2000; DeLoach et al. 2003; Landis et al. 2004; Lu et al. 2010).

Multiple introductions of different natural enemies targeting the same host can potentially improve the control of an insect pest. The natural enemies can form different interactions depending on the types of natural enemies introduced (predator or parasitoids). For example, natural enemies may not interact, and their combined effect will be cumulative to the sum of their separate effects on the prey population (Losey & Denno, 1998). If one natural enemy species kills (intraguild predation or superparasitism) or interferes with another natural enemy foraging behaviour (Jeffries 1990; Lima & Dill 1990), the interaction is antagonistic and the number of prey that is suppressed is by their combined action is reduced (Free et al. 1977; Polis et al. 1989; Rosenheim et al. 1993; Harmut et al. 1994). In contrast, natural enemies can also interact synergistically (Charnov et al. 1976). When natural enemies interact synergistically or facultatively, the natural enemies work together to kill more prey than the sum of their individual effects (Soluk 1993).

In biological control programs of invasive pest species, the introduction of more than one biological control agent is common. This can occur over time as new potential biological control agents are identified and released. In most cases, the impact of the introduction of a new biological control agent is not known (Valente et al. 2019), but the kind of interactions that may arise upon the release of additional natural enemies can be of importance to the success of the biological control programme and determine whether to use one natural enemy for the programme instead of multiple biological control agents. In this review we discuss the main types of natural enemies used in biological control programmes and examine the possible interactions associated with the use and release of multiple biological control agents. We further discuss the effects of releasing multiple biological control agents on the success of a biological control programme.

1.3. Types of natural enemies

1.3.1. Parasitoids

Parasitoids are the most common type of natural enemy that are introduced (Hall & Ehler 1979, Greathead 1986a). They are similar to a true parasite in that only a single host is required for the nutrients and energy for development, but they always kill their host making them different from true parasites (Vinson 1976; Vinson & Iwantsh, 1980; Waage & Greathead, 1988; Godfray 1994). Consumption of the host is not immediate but instead they oviposit on their host and their larvae use the host as a food source throughout their development,

1.3.1.1.Parasitoid life history traits

Parasitoid attack is spread across all insect life stages of their hosts. Egg parasitoids and larval parasitoids are those that attack the egg and larval life stages respectively (Van Driesche & Hoddle, 2009). The female lays eggs in, on or around the body of the host and the feeding eventually kills the host.

Egg maturation differs in different parasitoids and the processes involved encompass proovigeny and synovigeny. Proovigenic species have a full complement of eggs upon eclosion which enables rapid attack on hosts whilst synovigenic species have eggs that develop gradually over the adult female's lifetime (Van Driesche & Hoddle, 2009). Synovigenic species have been found to have longer lifespans than pro-ovigenic ones, and they are by far the most common egg maturation pattern (98.12% of species of Homopteran parasitoids) (Jervis et al. 2001). In some synovigenic parasitoids, maturation of eggs requires protein from nectar or honeydew, but others consume haemolymph. Haemolymph is obtained through the puncturing of the host's integument using the parasitoid's ovipositor and consuming the haemolymph as the hosts wound bleeds. This is known as host feeding and is a common behaviour in Hemipteran parasitoids (Jervis & Kidd, 1986; Van Driesche & Hoddle, 2009).

Parasitoids can either be endoparasitoids, that is the larvae of the parasitoid develop inside the host, or ectoparasitoids, where the larvae of the parasitoids develop externally on the host (Van Driesche & Hoddle, 2009). Many ectoparasitoid females lay eggs on hosts and the larva develop by feeding through small punctures in the host cuticle. Endoparasitoids inject eggs into the host's body and their larva feed internally. Endoparasitoids of stages other than eggs can suppress the host's immune system whereas egg parasitoids and ectoparasitoids do not (Van Driesche & Hoddle, 2009).

Female parasitoids sting mobile hosts into paralysis (Crawley 2009). Paralysis might occur temporarily, and the host will make a full recovery and continue to feed. Such parasitoids that permit hosts to grow after being parasitized are called koinobionts (Askew & Shaw, 1986). They are advantageous in that a female can attack smaller hosts and support a developing parasitoid without impeding the growth of the host (Harvey & Malcicka, 2016). The parasitoid maintains dormancy until the host has grown to a sufficient size to allow for the parasitoid's development, with a high adaptation to withstand cellular immune system attack from the host body (Crawley 1992). The koinobiont group includes the internal parasitoids that attack young larvae or nymphs and a few ectoparasitoids (Gauld et al. 1988). Some koinobionts attack one life stage and emerge from and kill another stage, such as an egg-larval parasitoid.

Idiobiont parasitoids are species that develop in non-growing hosts, such as eggs, paralyzed larvae, or pupae (Fortuna et al. 2012). The resources available to the developing stages of the idiobiont parasitoid are fixed because the host does not feed or continue to grow after parasitism (Otto & Mackauer, 1998). Because the resource system for idiobiont development is closed, they should always expand as quickly as they can to take advantage of a limited food supply (Mackauer & Sequeira, 1993). Because large hosts typically contain more resources for parasitoid development, they are typically considered to be of higher quality. Host size can therefore be used as a general indicator of host quality (Godfray 1994; Otto & Mackauer, 1998; Harvey & Malcicka, 2016).

According to the behavior and development of their larvae, parasitoid wasp species are frequently classified into solitary and gregarious species (Mackauer & Chau, 2001). The term solitary parasitoid denotes that only a single parasitoid develops to maturity in a single host. In contrast, a gregarious parasitoid is where several parasitoids develop in a single host (Crawley 1992). Superparasitism is defined as the occurrence of a second egg (or clutch of eggs, as in the case of gregarious parasitoids) being laid on a previously parasitized host by a second female of the same species (Crawley 2009). Multiparasitism would have taken place if the second female belonged to a different species than the first parasitoid (Hassell 2000).

Hyperparasitoids or secondary parasitoids have become specialised to develop on other parasitoids attacking the host (Crawley 1992) and these constitute obligate hyperparasitoids and facultative hypeparasitoids. Obligate hyperparasitoids reproduce only on primary parasitoids (Sullivan & Völkl, 1999; Nofemela 2013). Facultative hyperparasitoids are omnivores whereby a parasitoid act as a primary parasitoid and a hyperparasitoid thereby feeding on two trophic levels (Brodeur et al. 2000; Nofemela 2013). Hyperparasitism is generally detrimental in classical biological control except in special cases such as adelphoparasitism in whiteflies (Van Driesche & Holler, 2009). Adelphoparasitism is a type of hyperparasitism which is common in Hymenoptera in the family Aphelinidae. The males of this family develop as heteronomous hyperparasitoids that is they are hyperparasitoids of females of their own species, in contrast to the females, who develop as conventional endoparasitoids, primarily of Hemiptera (Williams 1991).

Polyembryonic reproduction makes it possible that 2000 wasps can emerge from the eaten-out husk of a moth caterpillar. These wasps have come about by the asexual division of one or two eggs laid by an adult female parasitoid into the egg of the moth. Polyembryony is a type of asexual reproduction which is common in four families of parasitic wasps, namely Braconidae (Macrocentrus), Platygasteridae (Platygaster), Encyrtidae

(Copidosoma), and Dryinidae (Ivanova-Kasas 1972). Polyembryony was investigated in the Encyrtidae wasp *Copidosoma floridanum* Ashmead (Hymenoptera: Encyrtidae). A noctuid moth's egg serves as the host for a female *C. floridanum*'s single or two eggs, which are always one male and one female. The moth egg hatches into a larva and during this time, the parasitoid egg splits to produce up to 1500 separate individuals. The host does not suffer majorly until the last instar when the parasitoids develop quickly, consume the whole caterpillar, and pupate within its skin (Godfray 1994).

1.3.2. Predators

Predators are insects that have a life stage where they kill and consume living creatures for growth, nutrition, and reproduction (Van Driesche & Holler, 2009). Insects that are predators are usually bigger than insects that are parasitoids. Predators feed on more than one prey item for their development to be completed. Insects can be predacious when they are immature or as adults or at both stages (Strand & Obrycki, 1996).

1.3.2.1.Predator Life History Traits

Predators have a wide prey range that varies from stenophagous species such as *Rodolia* beetles, whose larvae feed only on scales, to polyphagous groups such as lacewing larvae that feed on aphids, caterpillars, mites, scales, thrips and whiteflies. Many predators must find a series of prey hosts that they can subdue and feed on as they cannot complete their life cycles on a single host. It is common in some groups that diet changes as life stage changes, for example, lacewing larvae are predators while the adults are pollen or nectar feeders. In other species, when prey is scarce, there is dietary flexibility throughout all life stages, feeding on items like honeydew, nectar, sap, pollen and fungal spores (Symondson et al. 2002). Some predators are active hunters, and they chase their prey by searching through foliage or soil visually or tacitly or by catching prey in mid-air. Predators’ ability to suppress pests is affected by their dietary requirements as most of them must consume quite a number of prey before they reproduce. If reproduction is delayed, the effect is a slow numerical response to the increase in prey populations thereby reducing the rate of control by some predators (Sabelis 1992). If the predators are generalists whose range of hosts is broad, there might be no aggregative response to any individual prey species unless the species is dominant among all available.

The winged adults of predacious insects have higher mobility than nymphs or larvae (Van Driesche & Holler, 2009). The dispersing adults often have a well-developed sense of sight and olfaction that allow females to locate high density prey patches.

1.4. Natural enemy interactions

When there are multiple natural enemies present simultaneously, there are various possibilities for their combined effects on prey. Natural enemy effects can be additive in the absence of emergent impacts, and prey depletion by the total natural enemies' present can be determined having a total number of preys consumed by each individual predator species when present alone (Price et al. 2011). Alternatively, combined effects of multiple assemblages can be sub-additive or less than the prediction obtained by summing the impacts of individual predator species (Price et al. 2011). In this instance, the interaction is considered aggressive and lowers the prey's ability to gain control. Synergistic interactions occur when natural adversaries can cooperate to have a super-additive effect on their prey (Price et al. 2011). The result is an enhanced predation on prey when a collection of predators is present. Predicting the outcomes of prey-predator interactions within complex food webs is made easier by understanding how additive, antagonistic, and synergistic effects arise among natural enemies. This helps to characterize the relevant traits of predators and parasitoids that favour one type of interaction over the other.

1.4.1. Additive interactions

When the influence of one enemy species on the prey population is fully independent from the effects of the other enemy species, there is an additive effect of natural enemies on prey. In such cases, interspecific interactions among individuals are relatively insignificant and intraspecific interactions dominate. Resource partitioning is the most common contributor to this non-emergent effect of natural enemies on host is resource partitioning (Ives et al. 2005; Casula et al. 2006).

Resource partitioning arises due to enemy species attacking different subsets of host species and this is due to the interspecific differences in natural enemy traits that have an impact on how, where and when they attack. Natural enemies may divide up resources because of differences in the phenotypes of the host they prefer to attack (Wilby et al. 2005), their foraging habits (Tso et al. 2007), the microhabitats they exploit (Straub &

Snyder 2008), or their peak seasons (yearly). There is no chance for interspecific rivalry for hosts among predators when species entirely partition the prey population so that there is no overlap in their prey use patterns. Instead, cumulative influence occurs.

In a study conducted by Paine et al (2000) in California on the Eucalyptus longhorned borer, *Phoracantha semipunctata* Fabricius (Coleoptera: Cerambycidae) which is parasitized upon by several parasitoids, four parasitoids, namely *Syngaster lepidus* Brulle` (Hymenoptera: Braconidae), *Jarra phoracantha* Austin, Quicke and Marsh (Hymenoptera: Braconidae), *Jarra maculipennis* Austin, Quicke, and Marsh (Hymenoptera: Braconidae), and *Jarra painei* Austin and Dangerfield (Hymenoptera: Braconidae) were most commonly collected. There was a significant difference in terms of the preferred host size among the four parasitoids. This was due to *S. lepidus* preference of the smallest size of larvae. The three gregarious *Jarra* spp did not exhibit any significant differences in larval size preferences. Bark thickness was also used as a factor of host preference and there were significant differences in this pattern. *J. maculipennis* and *J. painei* females both have short ovipositors and exploit larvae under thinner bark. In contrast, *J. phoracantha* and *S. lepidus* females can exploit larvae that is underneath thicker bark due to their ovipositors that approach or exceed their body length.

1.4.2. Antagonistic interactions

Antagonistic interactions between the introduced natural enemies can occur (Pederson & Mills, 2004; Valente et al. 2018). The combined mortality from these non-additive impacts is smaller than the combined mortality from the natural enemies acting independently (Speight et al. 2008). When there is a disruption among natural enemies on the ability of either species to capture and consume prey, the interaction is termed antagonistic. Often, competition or, in the case of predators, when they kill one another (which is intraguild predation), results in antagonism. Predators may also change their behaviour to avoid intraguild predation or to avoid being displaced from prime forage owing to competition (Price et al. 2011).

Competition is described as a contact among individuals brought about by a shared need for a scarce resource that hinders the development, growth, reproduction, and survival of the individuals concerned (Begon et al. 2006). For competition to occur, there are important elements that are worth emphasising. The organisms in the system must overlap in their use of limiting resource and the interaction is viewed as negative-negative as both participants are adversely affected.

1.4.2.1. Intraspecific and interspecific competition

Competition occurs when resources are limited. Intraspecific competition will arise when members of the same species compete for the same resource. Interspecific competition, on the other hand, refers to interactions between members of different species. The interaction competition brought about between individuals has a regulatory effect on the size of a population (Bailey & Ridsdill- Smith, 1991). The conventional belief is that intraspecific rivalry is stronger than interspecific competition because members of the same species are more inclined to share resources than members of other species (Denno et al. 1995).

The gregarious ectoparasitoid *Goniozus nephantidis* Muesebeck (Hymenoptera; Bethylidae) provides an illustration of intraspecific competition (Hardy et al. 1992). On a single micro-lepidoteran caterpillar, female wasps lay up to 20 eggs. The size of the egg clutch grows with the larval host, indicating that body size may be a limiting factor in egg clutch size. Additionally, there is a parallel drop in female body size at maturity when the number of wasps on the larval host increases. Larger female wasps have longer lifespans and possibly lay more eggs. (Hardy et al. 1992).

Hymenopteran parasitoids appear to frequently undergo interspecific competition (Kato 1994; Monge et al. 1995; Speight et al. 2008). According to Mills (1999), interspecific competition is the harm that heterospecifics (two different species) cause by consuming or controlling access to a resource that is scarce or by being vulnerable to a common natural enemy. Interspecific competition can take one of three different forms: exploitative (scramble) competition, in which competitors interact by consuming a finite amount of a resource, interference (contest) competition, in which one species' activity restricts another from accessing a finite but crucial resource, or apparent competition, in which competitors face off against a common natural enemy (Alhmedi et al. 2009). An example of this type of competition is between *Encarsia noyesi* Förster (Hymenoptera: Aphelindae: Coccophaginae) and *Idioporus affinis* LaSalle and Polaszek (Hymenoptera: Pteromalidae) as the two parasitoids both attack the same instars of the Giant whitefly (Schoeller & Redak, 2018) (**Table 1.1**).

1.4.2.2. Extrinsic and intrinsic competition

When competition occurs between species, there is an influence on community stability, species structure and size (Force 1985). Parasitoid reproduction is directly affected by host availability and thus, if local hosts are scarce, the result will be intense intraspecific and interspecific competition (van Alebeck et al. 1993). This is the case for solitary endoparasitoid species. When competitive interactions occur by adults locating hosts, the interaction is known as extrinsic competition.

Reproductive capacity, differences in how efficient parasitoids are in finding hosts and phenological synchronization with the host are factors that influence the outcome of extrinsic competition (Lewis et al. 1990). If the first parasitoid manages to parasitize a host, the second parasitoid will render the host unsuitable for exploitation. When the local host density is low, the parasitoid with the most effective host location strategies may have a competitive advantage that is very significant (Godfray 1994).

On the other hand, intrinsic competition describes the competition between many parasitoid larvae growing inside of a single host individual (Godfray 1994). When numerous parasitoids are present, differences in parasitoid development rates, the number of eggs, the host's developmental stage, the order in which oviposition occurs, and the amount of time between the first and second oviposition all have an impact on the result of intrinsic competition (Tillman & Powell, 1992).

1.4.2.3. Indirect and direct competition

Competition among parasitoid species might be direct or indirect (Moretti and Calvitti, 2008; Xu *et al.* 2013). Indirect competition occurs when individuals respond not to each other but to the level of resource depletion that each produce (Bailey and Ridsdill-Smith, 1991). An example is when female parasitoids can interfere with each other directly by fighting, displaying, or hindering (Godfray 1994; Hardy & Blackburn, 1991; Goubault et al. 2005) to prevent attack or oviposition on a host. Direct competition occurs were individuals respond directly to each other rather than to the level of resource depletion each has caused (Begon et al. 1986). An example of indirect competition is when conspecifics or heterospecifics modify their host exploitation strategies (Visser et al. 1990; 1992a; Goubault et al. 2005).

1.4.2.4. Scramble or exploitative competition

Scramble competition occurs when each species receives the same amount of access to a scarce resource (Nicholson 1954; Price et al. 2011). Due to this competition, there is a high dependent death rate, unstable population, failure of reproduction, widespread overpopulation of resources, and potential population decline. Through exploitative competition among parasitoids, sequentially taking advantage of a shared host should be anticipated but nevertheless, surprisingly, formal proof of this type of competition in parasitoids is incredibly rare (Muller & Godfray, 1999; Hawkins 2000; Teder et al. 2013). One of the few studies has demonstrated that exploitative competition causes the inferior parasitoid species to be excluded (Luck & Podoler, 1985; Teder et al. 2013).

1.4.2.5. Contest or interference competition

This type of competition occurs when some individuals gain access to more than their share of resources and in so doing deprive other individuals of access, either conspecific or heterospecific species, for requisite resources (Nicholson 1954). Lethal interference competition, a crucial interaction occurring between parasitoids, refers to a direct interaction that leads to the mortality of the competitor (Mudorch et al. 1998; Xu et al. 2013). There is a direct killing, aggressive displacement behaviour and production of pheromones that hinder colonization, feeding or ovipositing (Denno et al. 1995; Kaplan & Denno, 2007).

Numerous herbivores, predators, and parasitoids can release chemical cues (marking pheromones) that indirectly deter future competitors from ovipositing or eating in the same host, host plant tissue, or neighbouring area (Denno et al. 1995). The effects of such pseudo-interference mechanisms are the same as indirect interference in that they rebuff or deny access to the competitor to shared resources without interacting aggressively. The assumption is that it leads to more stable population dynamics because through the reduction of resources, some individuals in the population will then access adequate resources for growth, reproduction, and survival (Hassell 2000).

1.4.2.6. Apparent competition

According to Van Veen et al. (2006), apparent competition is a sort of indirect interaction between two species that is facilitated by a third species. Since they do not exchange resources in this situation, species at the same trophic level have an indirect relationship. Sharing enemies rather than resources causes an antagonistic interaction that appears to be competition (Van Nouhuys & Hanski, 2002; Holt & Bonsall, 2017). When a prey species group's population increases, this directly results in the increase of predator species. This will in turn, also increase rate of predation of second prey species group. Apparent competition might result in the extinction of the apparent competitors (Rott et al. 1998; Van Nouhuys & Hanski, 2002).

Evidence of short-term seeming competition between two main parasitoids, mediated by a common secondary parasitoid (hyperparasitoid), was discovered in a manipulative field experiment. The generalist hyperparasitoid *Gelis agilis* Fabricius (Hymenoptera: Ichmeumonidae) parasitizes the cocoons of the specialized parasitoid *Cotesia melitaearum* Wilkinson (Hymenoptera: Braconidae), which attacks the Glanville fritillary butterfly in the land islands of SW Finland. For experimental purposes, *Cotesia glomerata* Linnaeus, a second host species for *G. agilis*, was added to the system; this species does not compete with *C. melitaearum* for resources. The natural populations of *C. melitaearum* in the treatment decreased after the one-time addition of the second parasitoid *C. glomerata* because the *C. glomerata* induced an increase in the hyperparasitoid density so that a larger portion of the summer population was hyperparasitoid (van Nouhuys & Hanski, 2002).

1.4.2.7. Symmetric and asymmetric competition

Competition can also be symmetric or asymmetric. Symmetric interspecific competition is when each species present will have a negative effect on the fecundity or mortality and growth of the other, although different processes may cause the interaction when resources are in limited supply. Asymmetric competition involves a negative effect on one species but no detectable effect on the other (Lawton & Hassel, 1981). Asymmetrical interspecific competition is common among parasitoids (Kato 1994; Monge et al. 1995) and can occur with unrelated taxa (Speight et al. 2008).

1.4.2.8. Intraguild predation

Intraguild predation (IGP) is one of the sophisticated interactions that predators are known to engage in (Polis et al. 1989). According to Polis et al. (1989), it happens when a top predator (the intraguild predator) eats an intermediate predator (the intraguild prey) with which it fights for the same prey resource. It is unknown how these interactions affect ecology, even though they are more frequent than previously believed (Denno & Fagan, 2003; Rosenheim 1998; Speight et al. 2008). Due to the intraguild predator's consumption of the competitively superior intraguild prey, as anticipated by intraguild models, the existence of the intraguild predator will increase the equilibrium density of the shared prey

(Holt & Polis, 1997). Therefore, in multispecies communities, the presence of intraguild predators could result in the increase of the shared pest population size.

Ferguson & Stiling (1996) found that the combined effect of a coccinellid predator *Cycloneda sanguinea* Linnaeus (Coleoptera: Cocillinidae) and a parasitoid *Aphidius floridaensis* Smith (Hymenoptera: Braconidae) on the mortality of an aphid *Dactynotus* sp. was less than that imposed by the parasitoid alone. This was because the predator fed on mummies or the parasitoids or both. The control of the herbivore population was hampered by the existence of the intraguild predator (Speight et al. 2008). The size of the herbivore population can be affected in a variety of ways by the existence of an intraguild predator (Rosenheim 1998; Eubanks & Denno, 2000). The intensity of the intraguild predator will most likely determine the impacts at the herbivore level (Moran et al. 1996). The population will be reduced if the intraguild predator prefers to feed on herbivores (Speight et al. 2008).

According to Snyder & Ives (2001); Snyder & Wise (2001); Finke & Denno (2004) and Prasad & Snyder (2004), IGP can also lead to relaxed predation. A study by Rosenheim et al. (1993) on heteropteran bugs (Zelus and Nabis) and lacewing larvae (*Chrysoperla carnea* (Neuroptera), predators of cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), serves as a good illustration. Due to lacewing predation, the aphid population decreases while the bugs are absent. However, when hemipterans are present in the system, they prey on the lacewing larvae which decrease its survivorship giving a partial refuge to the aphids from predation, thereby causing them to increase (**Table 1.1**). IGP is widespread in this system and creates significant interactions between the influence of the lacewings and the hemipteran bugs on aphid population suppression.

Colfer & Rosenheim (2001) showed that interactions between *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) and an aphid parasitoid, *Lysiphlebus testaceipes* Cresson (Hymenoptera: Aphidiinae), improved suppression of the cotton aphid populations. When given the option to eat on equal numbers of both prey types, the predatory coccinellid *H. convergens* favoured unparasitized aphids over aphid mummies. Almost all the aphid mummies on plants were devoured by *H. convergens* toward the conclusion of the experiment, drastically lowering the aphid mummy population while still suppressing the aphid population. The study's conclusion was that even if a predator produces large amounts of intraguild predation, we should anticipate the predator to increase herbivore suppression in situations where it exhibits both a partial preference for unparasitized hosts and high levels of predation on unparasitized hosts.

The impact of prey suppression by intraguild predation may be mediated by behavioural responses of prey to the presence of predators (Vance-Chalcroft et al. 2007). An important note to make is that intraguild prey (the intermediate predator) and shared preys do not passively wait to be preyed upon but that they can respond behaviourally to the threat of predation. It would be less likely for an intermediate predator to engage in hostile intraguild interactions, which would increase total prey suppression (Mueller et al. 2016). This avoidance could be brought on by predator emigration when intraguild predators are present. To prevent the threat of predation on possible prey, one should avoid patches with intraguild predators. According to research by Nakashima et al. (2006) and Senoo & Nakashima (2003), parasitoids can recognize the volatiles associated with coccinellid beetle trails and avoid patches where they are present.

1.4.2.9. Superparasitism

The condition known as superparasitism typically involves many conspecific parasitoid females parasitizing on the same patch of hosts at once (Godfray 1994). The host's limited resource may thus be the subject of intraspecific rivalry (Kaneka 1995; Morris & Caspard, 1996; Speight et al. 2008). Self-superparasitism, or laying two eggs in the same host, occurs when a parasitoid's female oviposits on the same patch. This is not ideal for solitary parasitoids because it wastes time and eggs because typically only one individual can emerge from each host (Goubalt et al. 2005).

It is known in some cases that the ovipositing individual destroys competitors that are on the host physically or already existing larvae in the host are killed by injecting in the host. It is known that, in some instances, the ovipositing individual kills competitors that are physically present on the host or kills existing larvae in the host by injecting them. The first female to leave a patch may lose offspring to larval competition if other females stay in the patch and continue to oviposit, hence an attrition war amongst parasitoid females is anticipated (van Alphen 1988; Goubault et al. 2005). In some circumstances, the behaviour of immature parasitoids controls the competition (Godfray 1994). Larvae of solitary parasitoids eliminate competitors through direct physical attacks when they encounter each other in the same host (Schroder 1974; Tillman & Powell, 1992), with one of the two parasitoids being physically or physiologically killed in a few days (Fisher 1971; Vinson & Iwantsch, 1980; Tillman & Powell, 1992; Bokonon-Ganta et al. 1996).

Due to the ovipositing female's historical inability to distinguish between already parasitized and non-parasitized hosts, superparasitism was once thought to be a mistake (Montoya et al. 2012). Although the fitness gains per host are lower when superparasitism occurs (van Alphen et al. 1990; van Alphen et al. 1996; Roseinhem & Hongkham, 1996; White & Andow, 2008), several authors have suggested that superparasitism may be an adaptive strategy. This is because it strikes a balance between the advantages and disadvantages of laying an egg in a host that has already been infected.

Superparasitism is modeled as an adaptation strategy in solitary species under the assumption that it has no fitness consequences for the larvae that survive (i.e., it does not lengthen larval development or shorten adult size) (Visser et al. 1990). For instance, substantial evidence supporting the claim made by Visser et al. (1992) that adults of *Leptopilina heterotoma* Thomson (Hymenoptera: Eucoilidae) that emerge from a single parasitized host are larger than adults emerging from superparasitized hosts has not been discovered. When it comes to *Diachasmimorpha longicaudata* Ashmead (Hymenoptera: Braconidae), superparasitism seems to be a common occurrence. The females of this species can distinguish between unparasitized and parasitized hosts, according to studies (Lawrence et al. 1978; Montoya et al. 2003), but they nevertheless show a tendency to superparasitize hosts even when there are a lot of unparasitized larvae present. Because a higher percentage of females are descended from superparasitism under mass-rearing settings, this characteristic supports the choice of this species as a natural enemy suited for augmentative biological control programs. (Montoya et al. 2012).

1.4.2.10. Multiparasitism

Female parasitoids often oviposit in or on hosts previously parasitized by other species, and this is called multiparasitism (Smith 1929; Kaneko 1995). The lack of ability of heterospecifics to discriminate each other and a resultant lack of oviposition restraint results in multiparasitism (Bokonon-Ganta et al. 1996).

It is usual for one adult of either species to emerge from the host when multiparasitism occurs and this is an effect of physical or physiological competition between larvae (e.g. Zwo1fer 1979; Vinson 1981; Kaneko 1995). In rare cases, two or more parasitoid species emerge from an individual host (Salt 1961; Ticehurst et al. 1978; Miller 1982; Weseloh 1983; Godwin & Odell, 1984) and this is called successful multiparasitism (Miller 1982; Kaneko, 1995). For heterospecific parasitoid populations to coexist there should be some differences in the niches between the species (Gause 1934; Hardin 1960) or presence of an aggregated distribution in competing species (Atkinson & Shorrocks, 1984; Ives & May, 1985; Bokonon-Ganta et al. 1996).

An example of multiparasitism is amongst three parasitoids of the pea aphid (**Table 1.1**). The parasitoid *Praon pequodorum* Viereck (Hymenoptera: Aphidiidae) produces an extraserosal envelope around its developing embryo and the envelope separates the chorion and trophamnion of the developing embryo and its production occurs only when eggs are laid in a previously attacked host by the heterospecific parasitoids, *Aphidius ervi* Haliday (Hymenoptera: Aphidiidae) and *Aphidius smithi* Sharma and Subba Rao (Hymenoptera: Aphidiidae). The envelope is not produced in singly or conspecific super-parasitised aphid hosts (Danyk & Mackauer, 1996). It is therefore suggested that the extraserosal envelope is for defense, and the embryos of *P. pequodorum* that are developing are protected from physical attack by mandibulate larvae of interspecific competitors by the envelope (Speight et al. 2008).

1.4.2.11. Hyperparasitism

Hyperparasitoids are parasitic wasps that attack another insect that is developing in or on another insect host (the larvae and pupae of primary parasitoids) (Poelman et al. 2012) and this sometimes impacts on biological control. The primary parasitoid attacks an insect host that is usually phytophagous, but which could also be a predator or scavenger. Although hyperparasitoids or secondary parasitoid species are plentiful among the Hymenoptera (Askew 1961), attempts to understand the dynamics of host-parasitoid-hyperparasitoid systems have been few (Jones et al. 1994).

Facultative hyperparasitoids are a type of parasitoid which feeds on two trophic levels by acting as a primary parasitoid and a hyperparasitoid (Brodeur et al. 2000). However, facultative hyperparasitism is only considered as detrimental to biological control when the hyperparasitoid attacks the primary parasitoids more than the insect pest (Moore & Kfir, 1995; Pérez-Lachaud et al. 2004; Nofemela 2013). In contrast, obligate hyperparasitism only affects primary parasitoids and because they impact primary parasitoids directly, they are the most studied hyperparasitoid group (Clausen 1972; Sullivan & Völkl, 1999).

Obligate hyperparasitoids fall into two categories: those that attack primary parasitoid pupae that are outside of the bodies of insect pests and those that attack the larvae of primary parasitoids growing inside insect pests (Sullivan & Völkl, 1999). If a species that attacks primary parasitoid pupae also parasitizes species that attack larval stages, the structure of the food web can change when both subcategories are present (Carew & Sullivan, 1993; Brodeur et al. 2000; Nofemela, 2013). Studies have revealed an increase in pest feeding when obligate parasitoids are present because insect pest populations are frequently liberated from the strong top-down influence of primary parasitoids (Rosenheim 1998; Brodeur et al. 2000; Schooler et al. 2011). Furthermore, obligate hyperparasitoids can leave cues on the plant surface that may increase dispersal probability of primary parasitoids, which further reduces mortality of insect pests (Höller et al. 1993; Van Veen et al. 2001).

1.4.3. Synergistic interactions

Prey suppression is generally improved by a high diversity of natural enemies, according to studies (Leteourneau et al. 2009; Greenop et al. 2018; Snyder 2019). Predator complementarity, one of which is synergistic interactions among predators, can have positive impacts on biological control. Predator-predator facilitation (Charnov et al. 1976, Losey & Denno, 1998) allows one adversary species to capture and consume more prey than would be achievable if foraging alone (Charnov et al. 1976; Losey & Denno, 1998) (Price et al. 2011). Because of the presence of numerous predators, prey often have paradoxical behavioural reactions, resulting in prey avoiding one species and being caught by another (Sih et al. 1998), resulting in the complex of predator species killing more prey than the sum of their individual impacts (Soluk 1993). Predator facilitation occurs when one predator species pushes prey from one habitat to another, and another predator pursues the fleeing or displaced prey (Zaret & Paine, 1973; Rahel & Stein, 1988; Soluk & Collins 1988; Soluk & Richardson, 1997).

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pest feeding when obligate parasitoids are present because insect pest populations are frequently liberated from the strong top-down influence of primary parasitoids (Rosenheim 1998; Brodeur et al. 2000; Schooler et al. 2011). (**Table 1.1**) (Losey & Denno, 1998; Prasad & Snyder, 2010; Roubinet et al. 2015). However, conflicting behavioural responses do not always result in facilitation among enemies.

1.5. Effects of natural enemy interactions on herbivore population dynamics

Parasitoids and their hosts are part of an essential complex web of multispecies interactions (Jones et al. 1994). Most species of parasitoids are polyphagous to a certain level and thus their populations are regulated from fluctuations by the abundance of any one of their host species (Jones et al. 1994). Zwolfer (1971) acknowledges the rarity of monophagous parasitoids (strictly specific) and that in some natural parasitoid complexes, they are non-existent. Specialist natural enemy population levels can show fluctuations which are influenced by the dynamics of the host (Jones et al. 1994). Generalists are less affected by the fluctuations of a single host as they can switch and forage somewhere else or on another host (Murdock 1969).

Numerous studies have begun to expand on single parasitoid-single host system to include three or more interacting species (Jones et al. 1994). The hypothesis has been that competition might occur amongst several introduced species and this might hinder the overall pest depression which will be less than that achieved by a single agent (Turnbull 1967). For example, a pest population that has overlapping generations will be caused to be discrete upon introduction of one agent thereby eliminating another agent whose developmental period depended on the continuous presence of a particular host stage (Godfray & Hassell, 1987). An analytical population model used by May & Hassell (1981) showed that the interaction between two parasitoids sharing a single host might result in eliminating one of the parasitoids or balanced competition, as in the above-mentioned argument. The resulting pest populations will however be less than they would have been if each species was acting alone. Later, Kakehashi et al. (1984) noted that this applied where the parasitoids distributed their attacks on the host independently of each other. If the subset of hosts the attacks were distributed to was similar, it was then possible to achieve lower pest numbers with both natural enemies than with one on its own (Godfray 1994).

Because complex systems are oversimplified, the guild approach is frequently contested (Polis & Strong, 1996). The interactions between members of predator guilds are one illustration of this complexity, as these interactions frequently do not lead to additive combined predation (Sih et al. 1998; Griffen & Byers, 2006). Common prey interactions between species of natural enemies frequently result in interactions that are less than additive. However, the non-additive effects of several natural adversaries do not always exclude the employment of a predator guild strategy. When numerous predator individuals are merged, non-additivity may happen, although this may happen regardless of whether conspecific or heterospecific predator species are mixed. For instance, when predator species compete with one another, predation rates decline, increasing the likelihood of failure to manage pests (Warfe & Barmuta, 2004; Griffen & Byers, 2006). The same predator species, though, may experience interference that lowers their rates of prey eating. Previous research has demonstrated that such interference between conspecific and heterospecific natural enemies may not only be non-additive but may also be redundant (Peckarsky 1991), resulting to comparable levels of risk reduction for shared host species (Griffen 2006; Griffen & Byers, 2006).

Other negative interactions among enemies such as facultative hyperparasitism, or behavioural interference, may have subtractive effects on herbivore suppression (Rosenheim 2007; Schmitz 2007). If agonistic interactions among natural enemies emerge instead of complementary ones, the addition of natural enemy species may release herbivore populations from predation or parasitism pressure and possibly lead to outbreaks (Perez-Lachaud et al. 2004; Rosenheim et al. 1999; Letourneau et al. 2009). Negative effects of predator diversity are shown by Michaud and Grant (2003) in the example of lacewing larvae and coccinellids that can be intraguild predators of each other and both can fall prey to *Pterostichus melanarius* Illiger (Coleoptera: Carabidae), a large ground dwelling beetle (Roubinet et al. 2015).

In some systems, intraguild predators are the dominating predators in the assemblage and in such systems, promoting predator diversity may inhibit prey suppression (Finke & Denno, 2005; Ives et al. 2005). If the presence of predators has a complementary effect through host resource partitioning or facilitation, then the increase of predator diversity can enhance prey suppression (Wilby et al. 2005; Snyder et al. 2006; Griffiths et al. 2008; Ramirez & Snyder, 2009). Where different predator species fill the same ecological roles in the community, their effects are redundant and prey suppression is unaffected by an increase in diversity (Straub & Snyder, 2006; Price et al. 2011).

Intraguild predation has the effect of preventing the three species from coexisting in a stable equilibrium. Coexistence is only possible when intraguild prey is a superior competitor for the base prey resource, according to models (Holt & Huxel, 2007). The probable consequences range from an extraordinarily high rate of death on the prey population to an incredibly low rate of mortality based on individual predator effects to a substantially lower rate of mortality than projected. The foes' synergistic impacts result in higher-than-expected death (Holt & Huxel, 2007).

Competition is a factor that influences community structure and Godfray (1994) reviewed the contribution of competition to the ecosystem. It is important to understand how competition as an interaction occurs between natural enemies as this is of relevance in selecting an effective biological control agent for importation and release (Mackauer 1990; De Moraes et al. 1999). An understanding of competitive interactions between biological control agents will improve the understanding and predictions of interactions between different guild interactions.

Competition is limiting to species. Interspecific competition is a major force affecting insect densities (Denno et al. 1995). An interaction web is a framework that develops from considering competition and trophic relationships among organisms (Wootton 1994). The growth of species in an interaction web may be restrained by either the upper or lower trophic levels and this is termed top down and bottom-up control respectively. Top-down influences are antagonistic or feeding relationships (predation, parasitism, herbivory, infection) in which a population is attacked or consumed in some way by a species in the upper trophic level (Van Driesche & Holler, 2009). Examples would include a caterpillar feeding on a plant, a parasitic insect attacking the caterpillar, a bird eating the parasitic insect, or a fungal pathogen attacking any of these organisms.

1.6. Conclusion

According to Van den Bosch (1971), "in the long history of natural enemy introduction, there has been no evidence that any serious adversity has resulted from multiple species introduction" is how the majority of papers address the issue of multiple species releases for biological control (Hokkanen 1985). Therefore, it could be preferable to use sympatric natural enemies that are attacking the same pest population for biological control rather than depending on new host relationships as suggested by Hokkanen & Pimentel (1984).

It should be addressed whether an exotic parasitoid species will compete with other parasitoid populations for shared hosts before importing and deploying it in a biological control system. It should also be considered how many parasitoids will affect the suppression of a shared host. The dynamics of competitive interactions between parasitoids and how competition may affect pest suppression require further research (Collier et al. 2002; Xu et al. 2013). Interspecific competition and its impact on community structure and dynamics are unfortunately poorly understood (De Vis et al. 2003; De Moraes & Mescher, 2005; Harvey et al. 2013; Xu et al. 2013).

Practitioners of biological control should look for natural enemies that have different foraging niches on the host when they plan for multiple introductions. This is to try and reduce competition and increase the impact factor on the host. Interactions between natural enemies attacking the same and different stages of a pest are particularly relevant when considering the introduction of exotic agents to control native pests.

The question of multiple introductions versus a single species is more complex than previously thought and deserves more scrutiny. However, negative effects appear to be rare in carefully planned biological control programmes, indicating that the benefits of multiple introductions far outweigh the alternative of trying to introduce the best agent the first time (Godfray 1994; Crawley 2009).

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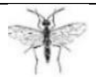
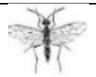
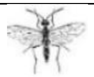
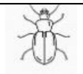
Table 1.1. Multiple Introductions of Natural Enemies (**Key**: Parasitoids- ○; Predators- ●; Hymenoptera  ; Coleoptera  ; Hemiptera  ; Diptera  ; Neuroptera  .

Pest	Order and family	Natural enemy	Type of NE	Order	Family	Type of introduction	Type of Interaction
Bemisia argentifolii	Hemiptera: Aleyrodidae	*Encarsia Formosa*	○		Aphelinidae	All intentional	Superparasitism and hyperparasitism by *Encarsia pergandiella* in *Encarsia formosa*
		Encarsia pergandiella	○		Aphelinidae		
		Delphastus pusillus	●		Coccinellidae		
		Encarsia transvena	○		Aphelinidae		
Paratrioza sinica	Hemiptera: Psyllidae	*Marietta picta*	○		Aphelinidae	All intentional	Hyperparasitism by *M. picta*, *T. lyciumi* has interspecific

		Tamarixia lyciumi	○		Eulophidae		discrimination to avoid multiparasitism by *P. arenarius*
		Psyllaephagus arenarius	○		Encyrtidae		
Leptocybe invasa (Blue gum chalcid)	Hymenoptera: Eulophidae	*Selitrichodes neseri*	○		Eulophidae	Intentional	*Megastigmus zebrinas* is a hyperparasitoid of *S. neseri*
		Selitrichodes kyceri	○		Eulophidae	Unknown	
		Megastigmus pretorianensis	○		Torymidae	Intentional	
		Megastigmus brasiliensis	○		Torymidae	Unintentional	
		Megastigmus zebrinus	○		Torymidae	Unintentional	
		Quadrasticus mendeli	○		Eulophidae		
		Megastigmus zvimendelli	○		Torymidae		
Aleurodicus rugioperculatus	Hemiptera: Aleyrodidae	*Encarsia guadaloupae*	○		Aphelinidae	Intentional	Unknown

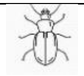
(Rugose spiraling whitefly)		*Encarsia noyesi* *Aleuroctonus spp* *Nephaspis oculate*	○ ○ ●	  	Aphelinidae Eulophidae Coccinellidae		
Aleurodicus dugesii (Giant whitefly)	Hemiptera: Aleyrodidae	*Encarsia noyesi,* *Entedononecremnus krauteri* *Idioporus affinis*	○ ○ ○	  	Aphelinidae Eulophidae Pteromalidae	Intentional	Interspecific competition between *E. noyesi* and *I. affinis* Possible scramble competition in *E. krauteri*
Acyrthosiphon pisum (Pea aphid)	Homoptera; Aphididae	*Aphidius ervi* *Praon pequodorum*	○ ○	 	Braconnidae Aphidiidae	Intentionally introduced	Interspecific competition between the parasitoids: Multiparasitism with *P.*

		Nabis and Orius bugs *Coccinella septempunctata* *Harpalus pensylvanicus*	● ● ●	  	Nabidae Oriudae Coccinellidae Carabidae		*pequodorum* being superior. Both parasitoids showed superparasitism with *P. pequodorum* exhibitating self-superparasitism. Synergistic interaction between the beetle predators (Predator facilitation)
Maconellicoccus hirsutus (Hibiscus mealybug)	Hemiptera: Pseudococcidae	*Anagyrus kamali* *Gyranusoidea indica* *Anagyrus dactylopii*	○ ○ ○	  	Encyrtidae Encyrtidae Encyrtidae	Native, accidentally and intentionally introduced	unknown

Bemisia tabaci (Sweet Potato Fly	Homoptera: Aleyrodidae	*Delphastus catalinae* *Encarsia Sophia*	● ○	 	Coccinellidae Aphelinidae	Intentional introduction	Intraguild predation of *E.sophia* by *D. catalinae* (Negative interaction)
Aleurocanthus woglumi (Citrus blackfly)	Homoptera: Aleyrodidae	*Encarsia perplexa* *Amitus hesperidum*	○ ○	 	Aphelinidae Platygastridae		Positive interaction
Diaphorina citri (Asian Citrus Psyllid)	Hemiptera: Lividae	*Tamarixia radiate* *Diaphorencyrtus aligarhensis*	○ ○	 	Eulophidae Encyrtidae	Both intentionally introduced	No competition as parasitoids attacks different host stages
Paracoccus marginatus (Papaya mealybug)	Hemiptera: Pseudociccidae	*Acerophagus papaya*	○		Encyrtidae	Intentional	Interspecific competition amongst the first 2 parasitoids

		Anagyrus loecki *Pseudleptomastrix mexicana*	○ ○	 	Encyrtidae Encyrtidae		
Phyllocnistis citrella (Citrus leafminer)	Lepidoptera: Gracillariidae	*Galeopsomyia fausta* *Ageniaspis citricola*	○ ○	 	Eulophidae Encyrtidae	Native to Brazil Introduced into Brazil	Unknown
Aulacaspis yasumatsui (Cycad aulacaspis scale)	Hemiptera: Diaspididae	*Coccobius fulvus* *Cybocephalus nipponicus*	○ ●	 	Chalcidoidea Cybocephalidae		Unknown
Siphoninus phillyreae (Ash whitefly)	Hemiptera: Aleyrodidae	*Encarsia partenopea* *Encarsia inaron* *Clitostethus arcuatus*	○ ○ ●	  	Aphelinidae Aphelinidae Coccinelliidae	All introduced into Egypt	Positive interaction

Sirex noctilio (Sirex woodwasp)	Hymenoptera: Siricidae	*Ibalia leucospoides*	○		Bethylidae	All intentionally introduced	Unknown
		Megarhyssa nortoni	○		Ichneumonidae		
		Rhyssa persuasoria	○		Ichneumonidae		
Cinara cupressi (Cypress aphid)	Hemiptera: Aphididae	*Pauesia juniperorum*	○		Braconidae	All intentional	Unknown
		Syrphus sp.	○		Syrphidae		
		Adalia bipunctata	●		Coccinellidae		
		Adalia angulifera	●		Coccinellidae		
Thaumastocoris peregrinus (Bronze bug)	Hemiptera: Thaumastocoridae	*Chrysoperla. Externa*	●		Chrysopidae	All intentional	Unknown
		Atopozelus opsimus	●		Reduviidae		
		Cleruchoides noacke	○		Mymaridae		

Glycaspis brimblecombei (Red Gum Lerp Psyllid)	Hemiptera: Psyllidae	*Psyllaephagus bliteus* *Anthocoris remoralis* *Atopozelus psimus*	○ ● ●	  	Encyrtidae Anthocoridae Reduviidae	All intentional	Intraguild predation between the first two natural enemies.
Heteropsylla Cuban (Leucaena Psyllid)	Hemiptera: Psyllidae	*Curinus coeruleus* *Psyllaephagus rotundiformis*	● ○	 	Coccinellidae Encyrtidae	All intentional	Unknown
Aphis gossypii (Cotton aphid)	Hemiptera: Aphididae	*Chrysoperla carnea* *Aphidius colemani* *Zelus renardii*	● ○ ●	  	Chrysopidae. Braconidae Reduviidae	All intentional	Synergistic interactions between *C. carnea* and *A. colemani*. Intraguild predation on lacewing by the bugs

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CHAPTER 2

The biology of *Centrodora damoni* (girault) (Hymenoptera: Aphelinidae)

2. Abstract

Centrodora damoni, native to Australia, is an egg parasitoid of *Eucalyptus* snout beetles, *Gonipterus* spp. It has the potential to be used as a classical biological control agent for the management of this beetle in countries planting *Eucalyptus* where this insect has been introduced and become a serious pest. However, outside of its description, very little is known on the basic biology of *C. damoni*. In this study, biological traits of *C. damoni*, namely fecundity, sex ratio, parthenogenesis, longevity, ovigeny, preoviposition, host age preference and mating behaviour, were investigated, using *Gonipterus* sp. n. 2 as a host. *C. damoni* developmental time was 19.05 +/- 1.557 days, longevity 34.25 +/- 5.19 days for females and 23.30+/- 5.759 days for males. The fecundity was 4.58+/- 1.19 eggs per female. *C. damoni* had no preoviposition period and exhibited synovigenic life history traits. Sex ratio was 1:2 males to females, and host egg age preference was not observed in *C. damoni* as it oviposited on all egg ages of *Gonipterus* sp. n. 2. The traits exhibited by *C. damoni* proved it to be a suitable parasitoid for field releases for a biological control programme.

2.1. Introduction

The high level of global travel, trade and an increase in the planting of non-native tree species is causing a subsequent introduction in the number and diversity of invasive organisms in new environments (Wingfield et al. 2008; 2013). This poses a threat to the sustainability of plantations of non-native species which have been planted at a global level (Wingfield et al. 2008). These non-native species comprise a small number of fast-growing species, largely pines, eucalypts, acacia, and poplars (FAO 2010).

Eucalyptus is one of the most planted trees across the globe. *Eucalyptus* is a genus under the myrtle family (Myrtaceae) and the trees are greatly valued in various countries for their fast growth, resistance to drought and attractive form (Pryor 1976; Grattapaglia et al. 2012). However, eucalypt-feeding insects, native to Australia, have been unintentionally introduced to different countries across the world where *Eucalyptus* species are grown, with some of them becoming serious pests (Hurley et al. 2016; Paine et al. 2011).

The Eucalyptus snout beetle (ESB), within the *Gonipterus scutellatus* species complex, are Australian weevils that have become important pests of *Eucalyptus* outside its native range (Garcia et al. 2019; Schröder et al. 2020). The genus *Gonipterus* Schoenhem comprises 20

Australian species of weevils, all of which are *Eucalyptus* leaf feeders (Mapondera et al. 2012; Oberprieler et al. 2014). Most of the species are native to Eastern Australia (Mapondera et al. 2012). Three of the species, namely *G. platensis* Marelli, *G. pulverulentus* Lea and *Gonipterus* sp. n. 2, have become pests in *Eucalyptus* plantations throughout the world (Mapondera et al. 2012; Garcia et al. 2019; Schröder et al. 2020). *Gonipterus* sp. n. 2 was first detected in South Africa in 1916 and has since been reported in other countries in southern and eastern Africa, as well as Italy and France (Arzone 1976, Mapondera et al. 2012; Rabasse & Perrin, 1979). *Gonipterus* sp. n. 2 is native to mainland Australia.

Gonipterus sp. n. 2 has both adult and larval phases that feed on leaves, but the larvae cause most of the damage (Mally 1924; Schröder et al. 2020). The edges of mature leaves develop a scalloped appearance as a result of the adults' preference to feed there (Mally 1924). Young leaves' epidermis and mesophyll are consumed by them, and they leave behind fibrous leaf tracks that are as wide as the larvae (Mally 1924). *Gonipterus* sp. n. 2 oviposit their eggs in clumps of four to twenty on young foliage and they are covered with frass excrement; these are called egg capsules (Mally 1924; Tooke 1955; Schröder et al. 2020).

A tiny wasp, *Anaphes nitens* Girault (Hymenoptera: Mymaridae), an egg parasitoid of *Gonipterus* sp. n. 2 in its native range (Tooke 1955), was introduced for the purpose of biological control in South Africa in 1926 (Tooke 1955) and subsequently to other countries where the weevil was causing significant damage (Valente et al. 2019; Schröder et al. 2020). Projections of wood loss indicate that 25% and 50% crown defoliation can result in over 20% and 85% loss in wood production, respectively, over a 10-year growth period (Reis et al. 2012). Since the 1960s, Australia has spent, or incurred *Eucalyptus* losses totaling at least US$298.58 billion (2017 value) (Bradshaw et al. 2021). The introduction of *A. nitens* into South Africa was initially considered successful (Tooke 1955; Tribe 2005). However, damaging infestations of *Gonipterus* sp. n. 2 have continued, especially over the past two decades (Rivera et al. 1999; Loch & Floyd, 2001; Valente et al. 2004; Loch 2008; Reis et al. 2012; Valente et al. 2017b; Schröder et al. 2020).

Classical Biological Control (CBC) is still the most sustainable, long-term tactic to manage many non-native forest insect pests (Hajek et al. 2016; Kenis et al. 2017). Different studies have thus investigated new and more suitable or specific biocontrol agents for *Gonipterus* spp. In surveys by Garcia et al. (2019) and Schröder et al. (2021) in the native range of *Gonipterus* spp. six egg parasitoids were identified. The parasitoids were *Anaphes inexpectatus* Huber and

Prinsloo (Hymenoptera: Mymaridae) (Valente *et al*, 2017a; 2017b; 2019; Schröder et al. 2021), *Anaphes tasmaniae* Huber and Prinsloo (Hymenoptera: Mymaridae) (Tribe 2003; Mayorga et al. 2013; Valente et al. 2017a), *Centrodora damoni* Girault (Hymenoptera: Aphelinidae) (Ward et al. 2016), *Closterocerus* Westwood (Hymenoptera: Eulophidae) *Cirrospilus* sp Westwood (Hymenoptera: Eulophiae). and *Euderus* sp. Haliday (Hymenoptera: Eulophidae). Valente et al. (2019) documented three larval parasitoids, namely *Anagonia cf. lasiophthalma* Malloch sp. (Diptera: Tachinidae) (Afonso et al. 2019; Gonçalves et al. 2020), *Entedon magnificus* Girault and Dodd (Hymenoptera: Eulophidae) (Gumovsky et al. 2015), *Oxyserphus turneri* Dodd (Hymenoptera: Proctotrupidae). *Podisus nigrispinus* Dallas (Hemiptera: Pentatomidae) is a predator of larvae and adults of *Gonipterus platensis* (Nascimento et al. 2017). A new entomopathogenic nematode *Steinernema diaprepesi* (Rhabditida: Steinernematidae) was recently added to the list of biological control agents after it was tested on *G. platensis* (Damascena et al. 2020).

Centrodora is a polyphagous genus of morphologically diverse species within the Aphelinidae family (Ward et al. 2016). The species associated with this genus are egg parasitoids (Polaszek 1991). Currently, the genus has 60 valid species, 12 of which are present in Australia (Noyes 2015). The first description of *C. damoni* was by A. A. Girault in 1922 from Queensland as *Aphelinus damoni* (Ward et al. 2016) and later Hayat and Fatima (1990) transferred it to the *Centrodora* genus. *C. damoni* was redescribed by Ward et al. (2016), who described the body colour of *C. damoni* as cream or off-white. The females and males are approximately 1mm and 0.8mm in length, respectively. The male and female are morphologically similar with the male antenna's scape slightly shorter and broader than the females. However, outside of its description, very little is known on the basic biology of *C. damoni*.

Knowledge on the biology of a potential biological control agent is important to determine its likely success. Certain parameters are investigated in biology studies of a parasitoid to evaluate how successful it will be if it is released for biological control. Sex ratio can influence the suppression of host populations or the stability of host–parasitoid interactions in the field (Nurkomar et al. 2021). Parasitoid developmental time is important as it can impact population dynamics of the parasitoid (Mody et al. 2012). Presence or absence of a preoviposition period primarily determines ovigeny as proovigenic parasitoids do not have a preoviposition period. Ultimately, the success of a predator or parasitoid in a biological control system depends on its ability to live long enough to reproduce in sufficient numbers to control a pest population

(Hentz et al. 1998). Longevity and fecundity are also vital history traits of a biological agent that will determine if it is the best potential biological control agent.

This study focused on determining biological characteristics of *C. damoni* relevant to its potential as a biological control agent of *Gonipterus* sp. n. 2. The aspects of the biology of *C. damoni* that were investigated were the duration of development from egg to adult, sex ratio, adult longevity, fecundity (average progeny per female), potential fecundity, determination of a preoviposition period, whether *C. damoni* is proovigenic or synovigenic, host egg age preference, mating behavior and parthenogenesis.

2.2.Material and Methods

2.2.1. Rearing

Gonipterus sp. n. 2 were reared in the biocontrol and insect rearing facilities of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. The weevil was reared at a temperature of 22°C and relative humidity of 80%. Egg capsules either collected from the field (field eggs) or from adult beetles reared in the laboratory (laboratory eggs) were placed in rearing containers (what type, size and manufacturing details) for hatching. After seven days, the eggs hatched into larvae (laboratory reared) which were fed with fresh new flush leaves of *Eucalyptus dunnii*. These were changed at least twice a week. Field collected larvae were also placed in boxes and followed the same rearing procedure as laboratory larvae depending on the sizes of the collected larvae. Larvae that were ready to pupate (fourth instar larvae) were placed in microboxes with autoclaved soil that had distilled water added to dampen it and left to wait for adult emergence. Newly emerged male and female beetles that were collected from pupae rearing microboxes were placed in a rearing plastic box of 10.5x17cm.

C. damoni was imported into South Africa from adults collected in southwest Australia in 2018. The wasps were reared at the FABI biocontrol and insect rearing facilities at the University of Pretoria, Pretoria, South Africa. The experiments were conducted using F16 and later generations. The ratio of egg capsules per female is 5:1 and therefore, each female was exposed to 5 egg capsules at a time. The experiments took over two years to run due to the unavailability of *Gonipterus* sp. n. 2 egg capsules. *Gonipterus* sp. n. 2 egg capsule production declines significantly in winter which subsequently affects parasitoid laboratory populations in the ensuing summer period as it will take time to boost the populations. In part, the experiments were also disturbed by the COVID-19 pandemic and subsequent restriction on field collections of beetles for egg production. Rearing was at 16ºC, 12:12 light and dark cycle and 80% RH. Parasitoids were reared in 24ml clear glass vials with polycaps. A 3 mm by 3 mm square piece of 100% concentrated honey-soaked paper towel was stuck onto the inside of the vial and used as a food source. The experiments below were undertaken under a different temperature of 23ºC, 12:12 light and dark cycle and 80%RD. A Nikon SMZ1500 microscope with an Olympus Stream Camera software were used for all experiments.

2.2.2.1 Female development time from egg to adult

The development time of *C. damoni* females was determined using a total of 20 replicates. For each replicate, a single breeding pair, newly emerged (≤24 hours) of *C. damoni* was allowed to mate and then exposed to five egg capsules on leaf discs and using forceps, the discs were put onto a sticky card that was 1×2 cm. Soil was sprinkled onto the card to cover up the sticky areas that were still exposed after placing the discs. Each female was placed in a vial with its egg card. After 24 hours, the females were taken out of the vial and the egg capsules were observed daily for adult emergence. The date the experiment was set up was recorded as the oviposition day and number of days until adult emergence was recorded.

2.2.2.2 Sex ratio

Adult parasitoids emerging from egg capsules of *Gonipterus* sp. n. 2 from the previous experiment (see 2.2.1) were counted and sexed under a microscope, and the sex ratio determined. The shape of the posterior end of the abdomen was used to determine sex. Males have a rounder “U” shaped posterior end whereas females have a pointed “V” shaped posterior end with the ovipositor showing along the bottom of the abdomen (Ward et al. 2016).

2.2.2.3 Adult longevity

Adults emerging from experiment 2.2.1 were used. 40 newly emerged adults, 20 males and 20 females (≤ 24 hours old) were each placed in a vial labelled with date of emergence and sex of the adult *C. damoni*. Observations were made daily, and mortality was recorded.

2.2.2.4 Mating behaviour

Two adult *C. damoni*, one male and one female aged ≤ 24 hours (newly emerged) were placed in a vial and observed under the microscope for 15 minutes. No egg capsules or honey paper were added to the vials. The time taken for the adults to mate, the courtship ritual occurring before mating, the number of times mating occurs, the length between mating events and the total length of mating was recorded.

2.2.2.5 Fecundity, pre-oviposition period and 'ovigeny'

The oviposition pattern of 15 females was observed. A mated breeding pair of *C. damoni* from experiment 2.2.4 was exposed to five egg capsules of *Gonipterus* sp. n. 2. The female was exposed to five fresh egg capsules daily until it died. Dead males were replaced to ensure continuous mating of the female. At each male replacement, the pair was allowed to mate before exposing them to the egg capsules. The exposed egg capsules, which were on a card, were placed in a separate vial according to the day of exposure to *C. damoni*. The number of adult parasitoids that emerged was recorded. When there was no more emergence of parasitoids (or *Gonipterus* larvae) after approximately 60 days or more, the egg capsules were dissected to count the number of parasitoids that did not emerge (if any) in the egg capsule. It took a period of 60 days during the rearing process for adults to emerge hence this period. The number of emerged and not emerged wasps per female were added to determine the realized fecundity. If eggs exposed to a newly emerged (≤ 24 hours) female produced progeny, that indicated that the female did not require a pre-oviposition period. The emergence of offspring from egg capsules exposed to the female parasitoid during her life span was used to determine if *C. damoni* is pro-ovigenic (all or nearly all its eggs mature prior to the start of oviposition) or synovigenic (continues to mature eggs throughout its reproductive life).

2.2.2.6 Host egg age preference

To test for host egg age preference, egg capsules were placed in three groups, namely young (1-day old), medium (2-days old) and old (3-days old) eggs. A total of 20 newly emerged mated females were used for each egg age treatment. For each treatment, five egg capsules of *Gonipterus* sp. n. 2. were exposed to a mated female (≤ 24 hours). Exposure occurred for 24 hours and thereafter the parent females were removed. Offspring emergence was observed for 60 days. The number of adult *C. damoni* emerging for each egg age (treatment) was counted and recorded. The developmental time for each egg age was also recorded upon eclosion of the offspring. An Analysis of Variance was tested using the Tukey HSD test (Posthoc Analysis) to compare the difference in means across the three age groups of the developmental time as well as number of adults emerging.

2.2.2.7 Parthenogenesis

10 virgin females were used to test if *C. damoni* was parthenogenetic. Each virgin female of *C. damoni* (age ≤ 24 hours) was placed in a vial and exposed to five egg capsules of *Gonipterus* sp. n. 2. The vials were observed for offspring emergence. The total number of offspring emerged and sex ratio of progeny per female was recorded. This experiment was completed before the other experiments were initiated, using from generations between F5 and F8 kept at 16ºC.

2.3. Results

2.3.1. Female developmental time from egg to adult

The average female *C. damoni* developmental time in the egg capsule (oviposition to adult emergence) was 19.05 (SE = +/- 1.56) days with the minimum development time of 17 days and maximum of 99 days (**Figure 2.1**). The developmental times of 76, 82 and 99 days (from three females) were outliers.

2.3.2. Sex ratio of *C. damoni*

The average number of males that emerged was 1.4 (SE = +/- 0.34) and the average number of females that emerged was 2.8 (SE = +/- 0.56) per egg capsule. Thus, the sex ratio of males to females was 1:2.

2.3.3. Adult longevity of *C. damoni*

C. damoni adult longevity was 34.25 (SE = +/- 5.19) days (range: 23 to 40 days) for females and 25.30 (SE = +/- 5.76) days, range: 17 to 34 days) for males. There was significant difference between the adult longevity of the two genders ($F_{1.38} = 26.66$, $p \leq 0.05$) (**Table 2.1**).

2.3.4. Mating behaviour

A courtship ritual was observed in *C. damoni*. In the ritual, the males exhibited a courtship ritual where the females' antennae were continuously caressed by the males. Time taken to commence mating ranged between 1 to 6 minutes with an average time of 3.25 (SE = +/- 0.64) minutes. Time between mating ranged from 1 to 10 minutes with an average time of 2.9 (SE = +/- 0.97) minutes between mating. Mating could occur from 1 to 6 times with an average of 3.25 (SE = +/- 0.67) times, with each mating lasting from 19 to 43 seconds. On average, mating lasted for 32.5 (SE = +/- 2.3) seconds.

2.3.5. Fecundity, pre-oviposition behaviour and 'ovigeny'

On average, each female produced 4.58 (SE = +/-1.19) offspring. *C. damoni* females have no pre-oviposition period as newly emerged females laid eggs within 24 hours of eclosion indicating that there was no pre-oviposition period. Most of the eggs were laid within four days of female emergence. However, one female laid eggs seven days after emergence, suggesting that *C. damoni* continued to produce eggs after emerging and is therefore proovigenic.

2.3.6. Host egg age preference

The number of adults that emerged varied across the different host egg ages (Figure. 2). The highest number of adults emerged from the 1-2-days old eggs which had an average of 6.05 (SE = +/- 0.64) adults, while the least number of adults emerged from 0-1-day old eggs, which had an average of 4.2 (SE = +/- 0.48) adults. The 2-3-days old eggs had an average of 5.25 (SE = +/- 0.57) adults emerging. However, there were no significant differences in adult emergence between the different egg ages ($F_{2.57}$ =1.97, $p \geq 0.05$) (**Table 2.1**).

The average development time from egg to adult increased with host egg age (**Figure. 2.2**). The average development time from egg to adult in 0-1-day old eggs was 19.05 (SE = +/- 0.34) days and in 1-2-days old eggs, the average developmental time from egg to adult was 21.75 (SE = +/-0.61) days. The average developmental time for eggs of the age 2-3-days old was 22.15 (SE = +/-0.75) days. However, there were no significant differences across the egg ages ($F_{2.57}$ = 1.82, $p \geq 0.05$) (**Table 2.1**).

2.3.7. Parthenogenesis

C. damoni was confirmed as a parthenogenic parasitoid as unmated females produced males.

2.4. Discussion

This was the first study to investigate the biology of *C. damoni*, a potential biological control agent for *Gonipterus* sp. n. 2. Information obtained from this study on the developmental time, fecundity, sex ratio, longevity, host age preference, preoviposition period and parthenogenesis is important to assess the suitability of *C. damoni* as a biological control agent. In addition, information from this study will help to improve the rearing of this parasitoid for future studies and potentially for release, if used as a biological control agent. *C. damoni* was confirmed as an egg endoparasitoid of the *Gonipterus* sp. n. 2 throughout its development. The occurrence of egg parasitism in aphelinids has been recorded in eight of the 38 valid genera (Polaszek 1991). Egg parasitism is particularly common in the genus *Centrodora*, which is known to be the most polyphagous in the family (Polaszek 1991).

Results from this study indicate that *C. damoni* has a relatively short developmental time, mostly between 20-25 days, although with some outliers. This is similar to the developmental time of *A. nitens* which ranged from 19-23 days (Santolammazza et al. 2009) and 18 days for *A. inexpectatus* (Valente et al. 2017b). The longest developmental time recorded for *C. damoni* was 99 days. This suggests that *C. damoni* has a life history strategy of delayed adult emergence. Due to unpredictable environmental conditions such as temperature and photoperiod, some insects have adapted by delaying adult emergence (Hanski 1988). It would thus be of interest to study the development time of *C. damoni* with temperature as a treatment. The delay in adult emergence enables a spread-out adult emergence of the parasitoid which can reduce pest population bursts (Corley & Bruzzone, 2009) as well as lowering the risk of substantial parasitoid losses during short term adverse conditions (Mody et al. 2012). Lastly, postponed emergence reduces negative effects of intraspecific competition (Ringel et al. 1998; Corley et al. 2004; Mody et al. 2012).

Understanding the female adult longevity of parasitoid wasps is important for their optimal use as biological control agents (Eliopoulos et al. 2003). Female *C. damoni* lived longer than males (average of 34.25 and 25.30 days, respectively). This has also been reported for other egg parasitoids of *Gonipterus* spp. (Santolamazza et al. 2009; Valente et al. 2017a). In this and other studies, the insects fed on a honey-based diet provided as a food source and no hosts were provided. Parasitoids commonly use a variety of sugar-based non-host foods, which increase their reproductive ability by prolonging longevity and providing more time to find hosts (Jones & Jackson 1990; Williams & Roane 2007; Wade et al. 2008; Lundgren 2009). Under field conditions, nectar is one of the most common food sources (Tooke 1955; Santolamazza et al. 2009). It has been argued that females live longer because of the energy present that has not been spent on oviposition (Sahad 1984; Santolamazza-Carbone et al. 2009).

The fecundity and the number of parasitised eggs determined in this study was likely underestimated. This is because parasitism of eggs does not always result in the development and emergence of the parasitoid, as the parasitoid may die before emergence. Such mortality can result from rearing conditions, low vitality or superparasitism (Tooke 1955; Hanks et al. 2000; Santolamazza & Rivera, 2003). Fecundity is a very important criterion for assessing the quality of reared parasitoids, because it determines the cost of mass production and can be influenced during long-term mass rearing (Sadat et al. 2021). Superparasitism refers to the ability of a parasitoid species to oviposit more than one egg in a single host (Van Alphen & Visser, 1990; Montoya et al. 2012; Chen et al. 2020). It is thus of importance to determine if superparasitism occurs in *C. damoni* and whether it is a gregarious parasitoid, or a solitary one, as these life history strategies can reduce negative effects of interspecific competition.

Immediate oviposition post emergence, which is the absence of a preoviposition period, as was observed in *C. damoni*, is associated with a weakly synovigenic (moderate synovigeny) life history in parasitoids, where no egg maturation period is required (Jervis et al. 2001; Mutitu et al. 2013). This is the case with most Aphelinids according to a study by Jervis et al. (2003) where 24 out of 25 Aphelinids studied were synovigenic. Work done by Valente et al. (2019) of the interaction between *A. nitens* and *A. inexpectatus,* the two parasitoids in the experimental design were less than 48hrs and both parasitoids exhibited parasitism. In extreme synovigeny, where no mature eggs are present, a preoviposition period of a few days is expected. An example is *Bracon mellitor* Say (Hymenoptera: Braconidae) which has a two days preoviposition period, but it is important to note that temperature might be an influencing factor (Ramalho et al. 2009). A few studies on ovigeny show that ovigeny index increased in parasitoids when temperature increased (Ramalho et al. 2009; Moiroux et al. 2018; Tabebordbar et al. 2022). In moderate synovigeny there can be a preoviposition period. For example, *Meteorus trachynotus* Viereck (Braconidae: Meteorinae) females emerge with some mature, and presumably ready to lay eggs but nevertheless have a preoviposition period of at least one day (Thireau & Régnière, 1995). Ovarian dissection studies were not carried out in this study, and these would be needed to confirm ovigeny in *C. damoni*.

In this study, there were no significant differences in developmental time and number of progenies emerging between all host (egg) ages. This suggests that *C. damoni* can develop successfully on a wide range of host egg ages. Lack of a specific host age preference is an advantage as it limits competition between parasitoid species with a similar host. It is a way of niche or resource partitioning that occurs in some parasitoids. Valente et al. (2019) studied competition between two *A. nitens* and *A. inexpectatus* and the results suggested that some eggs of *Gonipterus platensis* as old as 6 days were parasitized by both *A. inexpectatus* and *A. nitens,* however at very low parasitism rates. This is different in the case of *A. nitens* as findings from earlier studies showed that *A. nitens* particularly preferred eggs that were young and didn’t accept eggs older than four days (Williams et al. 1951; Santolammaza et al. 2004). Usually, most parasitoids prefer younger eggs (recently laid eggs) as observed by Queiroz et al. (2020) when they studied *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae), an egg parasitoid of *Anticarsa gemmatalis* Hübner (Lepidoptera: Eribidae). This might be due to the better nutritional value of young eggs (Molina et al. 2005). In larval parasitoids, host age preference studies showed that parasitoids are host age specific (Mattiacci & Dicke, 1995;

McGregor 1996) due to increased survival of their offspring or ease of parasitism. Different host ages have different host quality, and this may affect developmental performance of the larval parasitoid (Mackauer et al. 1996; Yazdani et al. 2015). Some hosts develop physical defense mechanisms as they grow, thus making parasitism more difficult with increasing host age (Pennacchio et al. 1992; Mattiacci & Dicke, 1995).

In this study, the sex ratio of males to females was 1:2. This may be owing to the pairing of male and female *C. damoni* in the study, which increased the likelihood of mating and led to a higher proportion of females because of the haplodiploid form of reproduction. The effort required to select acceptable egg capsules may also be to blame for the female biased sex ratio. A study by Ode & Hardy (2008) showed that host quality and size of parasitic wasps is correlated with the production of males and females being produced, and so males emerge from small, less viable hosts whilst the opposite is true for female progeny. The ideal sex ratio of most sexually reproducing species is 1:1 (Fisher 1958) as this affects the resources or energy to produce males versus females (Norkumar et al. 2021). However, female biased sex ratios are now common and are thought to have evolved in sexual individuals to compete with their asexual counterparts (Kobayashi & Hasegawa, 2016). A female bias of the sex ratio means more females available to mate and reproduce offspring which gives rise competition with asexually reproducing parasitoids, and this is consistent with favorable rearing conditions. Female based sex ratios have an advantage in biological control as females are the ones attacking the host and population growth depends on the females and not the males.

Arrhenotokous parthenogenesis was observed in *C. damoni*. Arrhenotoky is a mode of reproduction where unmated females will produce only male progeny, while mated females can produce both female and male progenies. Hymenopteran parasitoids frequently exhibit sex ratio variation, ranging from 100% males because of unmated females to female-biased ratios of up to 100% females because of maternally inherited symbionts (Nurkomar et al. 2021). Two significant variables that affect the field sex ratio in a population of hymenopteran parasitoids are the option of arrhenotokous parthenogenesis and the state of the females' mating (mated or unmated) prior to dispersal. The higher the proportion of virgin females, the higher the number of males that are produced, hence skewing the sex ratio to male bias in the population (Nurkomar et al. 2021).

The parasitoids used in this study were from a rearing culture under quarantine conditions, experiments were from F16 and higher generations since its importation to South Africa. The multiple generations in lab-reared conditions could possibly have resulted in inbreeding which can affect biological traits of the parasitoids. This was the case in *Mastrus ridens* Horstmann (Hymenoptera: Ichneumonidae) (Bueno et al. 2017; Sandanayaka et al. 2022) as well as *Venturia canescens* Gravenhorst (Hymenoptera: Ichneumonidae) (Vayssade et al. 2014). A highly inbred culture could affect various biological traits of the insects, such as longevity, fecundity, sex ratio, preoviposition behavior and ovigeny. Thus, it would be valuable to repeat the experiments from this study on a recent field collected *C. damoni* population, to confirm the biological traits presented in this study.

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Table 2.1. Analysis of Variance (ANOVA) results for *C. damoni* adult longevity between the sexes, egg age preference: developmental time, and egg age preference: number of adults emerged.

Parameters	**Df Value**	**F Value**	**Significant Difference**
C. damoni longevity comparison between the sexes	1.38	26.655	≤ 0.001
C. damoni egg age preference developmental time	2.57	1.965	0.149
C. damoni egg age preference total number of adults emerged	2.57	1.823	0.171

Figure 2.1. *C. damoni* development time from egg to adult

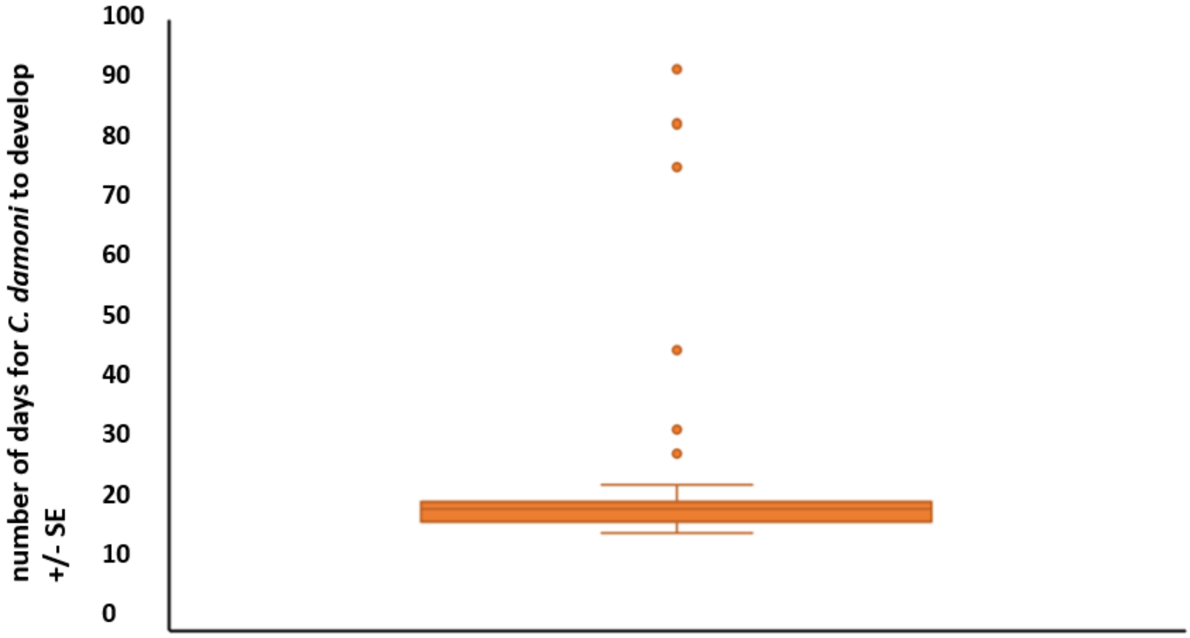


Figure 2.2. Mean number of *C. damoni* adults emerged, per egg capsule, for the different egg capsule ages

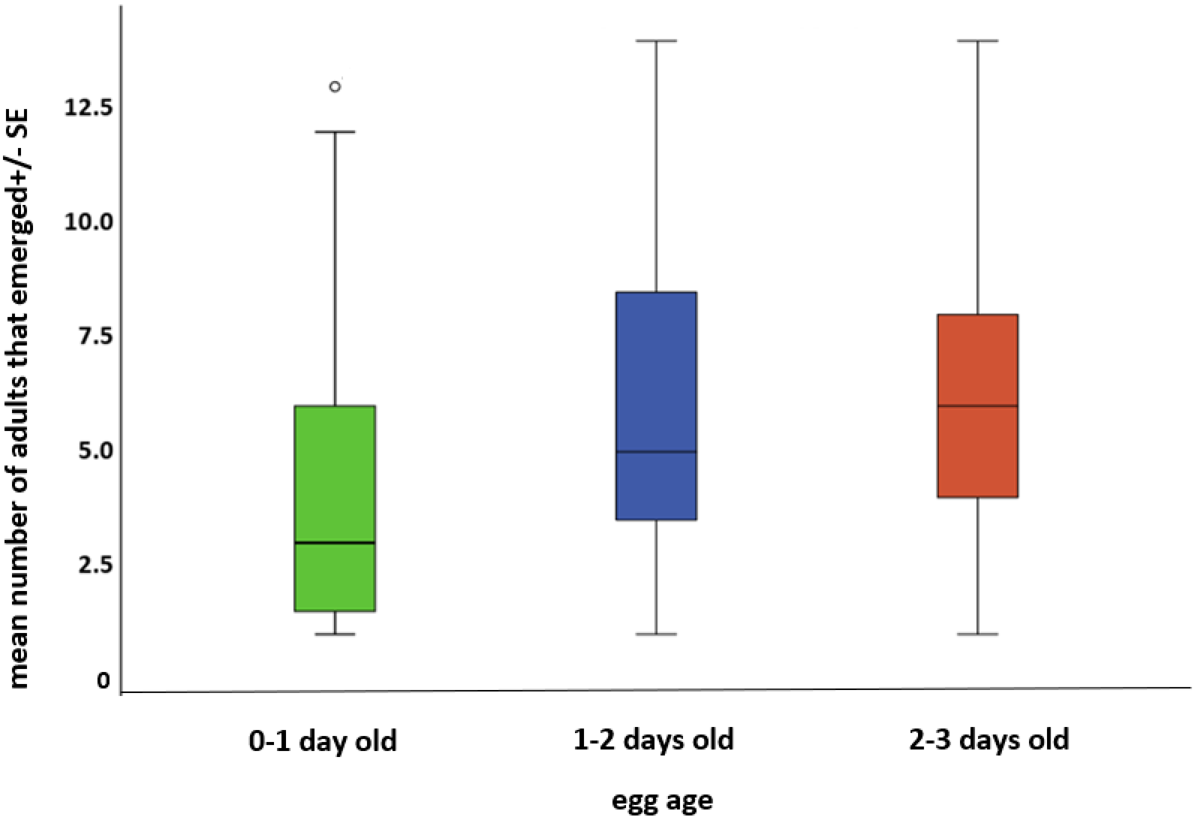
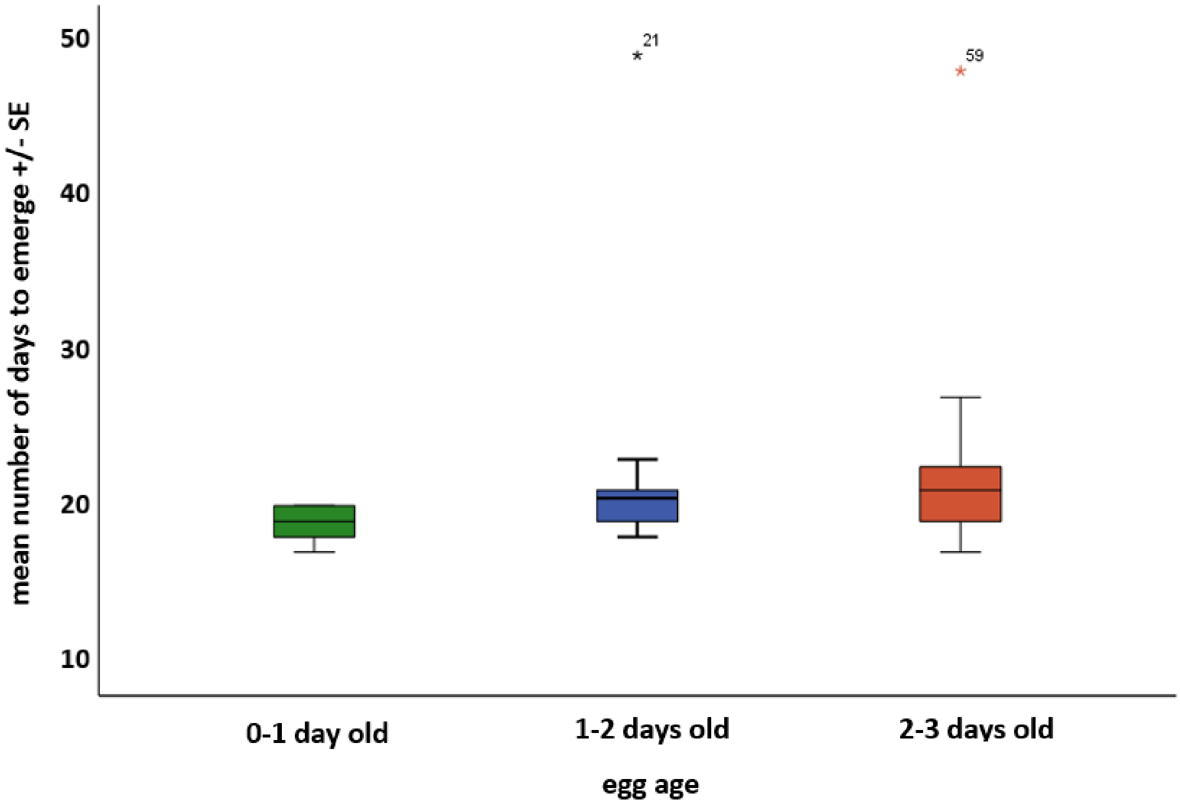


Figure 2.3. Mean number of days for adults to emerge for the different egg capsule ages.



CHAPTER 3

The reproductive development of the eucalyptus snout beetle, *Gonipterus* sp. n. 2

3.1. Abstract

Various characters define the reproductive status of insects. Understanding the changes in these characters assist in creating an age grading system which can inform management strategies, for example releases of biological control agents to coincide with the peak in the targeted life stage of an insect pest. In this study, the reproductive system of the *Eucalyptus* snout beetle, *Gonipterus* sp. n. 2, a serious pest of Eucalypts worldwide, was investigated so as to better understand how best biological control can be implemented. Lab-reared beetles from zero to eight weeks old were dissected and physiological changes in the beetles were noted. The beetles have a polytrophic-meroistic, a type of reproductive system that is unique to Coleoptera. They have two ovaries, each with two ovarioles. The ovarioles have the germarium region as well as the vitellarium. Ovarioles join into a lateral oviduct which then join into the common oviduct. The eggs that develop pass through the lateral and common oviduct in a process known as ovulation. Egg laying commences between four to six weeks. Around the same age, the lateral oviducts start expanding and follicles form and yolk is present in the follicles as the beetles grow older. Eggs and more developed follicles are present in older beetles. The yellow bodies are present in newly eclosed beetles and these remain yellow in nulliparous beetles and turn into a milky white colour in parous beetles. The development of a reproductive system enables an age grading system of laboratory reared *Gonipterus* sp. n. 2 adults which in turn assists in mass rearing parasitoids. It also assists in better understanding the timing of field releases of parasitoids to control the *Eucalyptus* snout beetle.

3.2 Introduction

Development of a physiological age grading systems has become common for different insects and these grading systems use a variety of characteristics which define the physiological status of the insects (Grodowitz et al. 2019). Changes in the ovarian system can give a measure of the physiological age of an insect. Knowledge on insect age structure is used to construct life tables that are time-specific, characterize factors that regulate population size fluctuations and monitor fertility and mortality (Perez-Mendoza et al. 2004). Studies of *Bagrada hilaris* Burmeister (Hemiptera: Pentatomidae), an invasive pest in the United States, examined the female reproductive system and identified the stages of ovarian development, which were used to develop a reproductive development age grading system based on distinct changes in the egg number and thus connect egg number to age classes (Grodowitz et al. 2019).

Various characters are used in the analysis of physiological changes in the reproductive system. These include physical changes in the ovary, appearance of the follicle, tracheation of the ovaries, and the presence or absence and appearance of follicular relic (which are commonly known as yellow bodies) (Tyndale-Biscoe 1984; Hayes & Wall, 1999; Grodowizt et al. 2019). Yellow bodies are part of the follicular tissue that is deposited and accumulates in the ovariole base during ovulation (Mendoza et al. 2002). In some studies, the germarium are studied as they increase in size as the insect gets older (Grodowitz et al. 2020).

The female reproductive system consists of a pair of ovaries, each composed of two to seven ovarioles depending on the insect, in which the oocytes develop and mature (Murray & Tiegs, 1935). The ovarioles are divided into two sections (**Figure 3.1a**). The first section is the germarium, the distal somewhat bulbous area, which contains trophic tissue, oogonia and oocytes that develop into follicles (**Figure 3.1a and 3.1b**) (Chapman 1998, Perez-Mendoza et al. 2004). The second section is the more proximal, tubular vitellarium **(Figure 3.1b),** which contains a series of follicles (oocytes with a surrounding follicular epithelium) in successive stages of development. The most mature follicles are located proximally in the ovarioles. The two ovarioles merge at the lateral oviducts which then merge the two ovaries at the common oviduct. The common oviduct is joined to the bursa copulatrix which is connected to the spermatheca also known as the receptaculum seminist **(Figure 3.1a and 3.1b)**. For beetles almost completing the preoviposition period and reproductively active females, the follicles in the vitellarium will become eggs once they pass through the lumen of the ovariole. During ovulation, a process that involves eggs moving through the lateral oviduct into the common oviduct, sperm stored within the spermatheca passes through the spermathecal duct into the bursa copulatrix where the eggs are fertilized (Khan and Musgrave, 1969).

An essential tool for assessing field populations and laboratory colonies destined for the application of various management strategies and experimental trials is the ability to assess reproductive health and reproductive status based on ovarian morphology (i.e., physiological age-grading) (Grodowitz et al. 2020). Understanding of the female reproductive system can assist in the rearing of insects and help to interpret host preferences and other factors, such as seasonal variation, that may influence the insect’s reproductive success. Studies by Herbert & Toews (2011); Grodowitz et al. (2019) and Grodowitz et al. (2020) support the theory that seasonal changes of insects to various plant hosts are due to the nutritional requirements at these various times for reproduction. Understanding reproductive development can also provide information on past and future reproductive potential, especially in field populations (Grodowitz et al. 2019; 2020). A population consisting of young females indicate a potential increase in numbers, whereas a population with older aged females could indicate a population decline (Grodowitz et al. 2019). For example, Grodowitz et al. (1987) assessed the reproductive morphology of *Neochetina eichhorniae* Warner (Coleoptera: Curculionidae) which led to an age grading system that was used to show that parous females were at their highest levels in March and decreased to low levels in June before rising again in September. Such information can aid in the determination of when to initiate management protocols.

There are different types of reproductive systems. The above-described system is typical in Coleoptera and Hemiptera and is known as the telotrophic-meroistic system **(Figure 3.2c)**. In this system nurse cells, or trophocytes, are present in the germarium (**Figure 3.2**) and are connected to oocytes in early stages of their development by trophic filaments called nutritive cords (King & Buning, 1985). Another type of reproductive system is the panoistic type **(Figure 3.2a)**, which is the common system in most basal insect orders. In this system all germ cells develop into oocytes. Each ovariole originates as a germarial region which usually persists into the adult. In the polytrophic-meroistic **(Figure 3.2b)** type of reproductive system, the germarium produces growing entities, the follicles, which undergo previtellogenic and vitellogenic growth and chronogenesis in the vitellarium (Büning 2005).

The *Eucalyptus* snout beetle (ESB), *Gonipterus* sp. n. 2, is part of a cryptic species complex represented by *Gonipterus scutellatus* Gyllynhal (Coleoptera: Curculionidae) (Schröder et al. 2020). The undescribed species, *Gonipterus* sp. n. 2, was introduced into South Africa, other parts of Africa, France, and Italy (Mapondera et al. 2012). Its first report in South Africa was in 1916 (Tooke 1955). The ESB adults and larvae defoliate trees by feeding on the leaves resulting in significant yield loss. Despite the introduction of an egg parasitoid, *Anaphes nitens* (Hymenoptera: Mymaridae) in 1926 (Tooke 1955; Tribe 2005; Schröder et al. 2020), and its establishment and initial success as a biological control agent, high infestations of ESB have occurred in the last decades (Schröder et al. 2020). This has sparked a continuous interest in the research of the beetle and various studies require the use of laboratory reared beetles of *Gonipterus* sp. n. 2. However, the beetle is difficult to rear as its reproduction activity, specifically oviposition behaviours is not fully understood. This has highlighted a lack of understanding of the reproductive biology of *Gonipterus* sp. n. 2.

This study provides the first detailed physiological age grading system for the *Gonipterus* sp. n. 2. This was done through the dissection of laboratory reared beetles of various ages, noting key physiological changes. The capacity to measure reproductive health and status based on ovarian morphology (i.e., physiological age grading) can be used to evaluate field populations and laboratory colonies, such as correlating changes to the reproduction system of *Gonipterus* sp. n. 2 to egg production. Such data would enable the creation of a physiological age grading system that would improve understanding of past, present, and future reproductive status in both field and mass raised colonies (Eisenburg et al. 2018). This can improve mass rearing of the *Eucalyptus* snout beetle's parasitoids as scientists are aware of when egg production is at its peak. It will also assist in detailed timing of field releases of the biological control agents.

3.3 Materials and Methods

3.3.4 Rearing

Gonipterus sp. n. 2 were reared in the biocontrol and insect rearing facilities of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. The weevil was reared at a temperature of 22℃ and relative humidity of 80%. Egg capsules either collected from the field (field eggs) or from adult beetles reared in the laboratory (laboratory eggs) were placed in rearing containers for hatching. After seven days, the eggs hatched into larvae (laboratory reared) which were fed with fresh new flush leaves of *Eucalyptus dunnii*. These were changed at least twice a week. Field collected larvae were also placed in boxes and followed the same rearing procedure as laboratory larvae depending on the sizes of the collected larvae. Larvae that were ready to pupate (fourth instar larvae) were placed in microboxes with autoclaved soil that had distilled water added to dampen it and left to wait for adult emergence.

Newly emerged male and female beetles that were collected from pupae rearing microboxes were placed in a rearing plastic box of 10.5x17cm. Tissue paper folded into four to five layers was placed at the bottom of the box along with fresh leaves of *E. dunnii*. The containers were cleaned, and old leaf material was replaced with new leaves twice a week. New beetles were added to the box as they emerged and were marked with nail polish of different colours to indicate the different beetle ages. The number of beetles in a box ranged from ten to fifty and upon reaching a total of fifty, a new rearing box was started. Beetles' ages were differentiated by weeks.

3.3.5 Dissections

Beetles of ages zero, two, four, six and eight weeks old were dissected in this study. Five beetles were dissected per age group. For the dissections, blue and purple coloured wax crayons were melted and left to dry in a 15cm petri dish. A 4mm rod inserted into a wooden handle was heated and used to melt the wax at the point touched by the hard pin and a beetle was pressed into the melted wax and left to dry. Distilled water was poured into the petri dish until the beetles were just covered and a few drops of ethanol were added to the water to break the surface tension. Using a dissecting pin, the elytra of the beetle were removed. The cuticle was removed by cutting into the cuticle using a dissecting pin, cutting along the lateral margins, round the abdomen starting from the right anterior side, down to the posterior of the abdomen and up the left anterior side of the abdomen. The cuticle was gently lifted and completely removed to expose the contents of the abdomen. The alimentary canal was removed and discarded, and the reproductive system was gently teased out of the abdomen, keeping it as intact as possible. A Nikon SMZ 1500 microscope with a Nikon LV-TV camera was used to take pictures at different magnifications.

The general reproductive system of *Gonipterus* sp. n. 2 was described, specifically noting the number of ovaries, number of ovarioles per ovary and the description of oviducts. The following characteristics were further noted for each adult age: whether the ovarioles were differentiated into distinct follicles; the number of follicles per ovariole; whether yolk was present in the follicles; the presence, colour and appearance of follicular relics; the change in the colour and appearance of the fatty bodies.

3.4 Results

The reproductive system of *Gonipterus* sp. n. 2 consists of two ovaries, each ovary having two tubular ovarioles. The ovaries are located dorsally in the abdominal cavity somewhat lateral to the medial line. The ovarioles combine proximally into the calyx region which then combine forming the lateral oviducts. The lateral oviducts from the two ovaries combine into the common oviduct. As the follicles pass through the lumen of the ovariole the resulting eggs often accumulate in the lateral and common oviducts until sufficient eggs are present for egg mass oviposition.

3.4.4 Zero-week-old beetles

Zero-week-old beetles were newly eclosed beetles (newly emerged). No egg or follicle formation was visible and because of this, there was no presence of yolk. The ovarioles are generally thin white tubular like structures surrounded by white filamentous strands that hold the beetles' reproductive system against the abdomen. Fatty bodies are present and are yellow in colour. The spermatheca is also visibly present. There is a golden yellow colouring around the area the follicular relics are assumed to deposit.

3.4.5 Two-week old beetles

Beetles that were two weeks old were very similar to the beetles that were zero weeks old. The ovarioles still look like thin tubular structures with no follicles, yolk or eggs present. The same filamentous strands are present, and the fatty bodies also have a similar colour (yellow) **(Figure 3.3a and 3.3b).**

3.4.6 Four-week-old beetles

At four weeks of age, the beetles were starting to exhibit changes in the reproductive system. The golden yellow colour was still present at this stage as in younger beetles and fatty bodies start to change colour from the yellow to a milky white **(Figure 3.4a).** At this stage, the follicles have started to distinctly form in the ovarioles making the germarium and vitellarium more distinct. We can identify them as follicles and not eggs as there was a distinct epithelium making up the white milky round structures. In some of the follicles, yolk can be seen because of the colour change from white to yellow. This is the case for the follicles that are closer to the proximal end of lateral oviducts **(Figure 3.4b).** The lateral oviducts start to exhibit an expansion in terms of size.

3.4.7 Six-week-old beetles

Six-week-old beetles have more developed follicles in terms of quantity as well as in some cases fully developed follicles forming in the lateral oviduct (at least two eggs). Development of the follicles is such that the ones closer to the distal end of the ovariole are better developed than the younger ones before them. All the "younger" follicles have yolk inside them (**Figure 3.5b)**. The fatty bodies in these beetles are a milky white and there are less stringy filaments holding the reproduction system down **(Figure 3.5a).**

3.4.8 Eight-week-old beetles

The beetles of the age of eight weeks have fully matured eggs stored in both lateral oviducts. There are more follicles than in younger beetles and follicles growing into eggs and progressively passing down the ovariole into the oviduct until they have fully matured. The filamentous strands are still present but like the six weeks old beetles, there are less of them. The oviducts look larger than in younger beetles especially in the case of the lateral oviducts and they contain more eggs than in younger beetles. The fatty bodies are now a distinct white colour and the spermatheca is also visibly present **(Figure 3.6a and 3.6b)**.

3.5 Discussion

The focus of the study was to create a physiological age grading system based on the reproductive system of *Gonipterus* sp. n. 2. The beetle ages that were dissected were from zero weeks through to eight weeks and there were notable changes in different characters making up the reproductive system. Fatty bodies were notably yellow in younger (zero to four weeks old) beetles but turned into a milky white colour as they aged. The younger beetles did not have any follicles, but these developed in beetles from the ages of two to four weeks and the lateral oviducts also enlarged as the beetles aged. There were eggs present in beetles of older ages (six to eight weeks old beetles).

Gonipterus sp. n. 2 anatomy is similar to that of the rice weevil (Perez Mendoza et al. 2004), the granary weevil, *Sitophilus granarius* Linnaeus (Richards 1947); the boll weevil, *Anthonomus grandis* Boheman (Grodowitz & Brewer, 1987); and the water hyacinth weevil, *Neochetina eichhorniae* Warner (Grodowitz et al. 1997). The weevils have a meroistic/telotrophic type of reproductive system which makes them similar to bugs (Maluf 1933; Kiritani 1963; Banerjee & Chatterjee 1985; Esquivel 2009; Fortes & Consoli, 2011; Grodowitz et al. 2020). Four ovarioles were present in all female *Gonipterus* sp. n. 2 dissected in this study. In the rice weevil, some atypical individuals may have only one, two, or three ovarioles, an anomalous trait that is genetically controlled and temperature sensitive (Grenier & Nardon, 1994), however this was not observed in *Gonipterus* sp. n. 2.

The continuum of ovarian development can be divided into distinct stages or physiological ages based on several characteristics associated with the reproductive system. Zero to four weeks old beetles fit into the nulliparous stages (i.e., preoviposition period). In this stage, follicles were not present or not distinctly formed, and yolk was absent (Grodowitz et al. 2020). Lateral oviducts start off as thin as the ovariole tubes. There were no follicular relics present.

Clear areas in the follicular relics are an indication that the follicular relics are not surrounding the ovariole base. During this stage fatty bodies are still a bright yellow and a thick mass.

The active reproductive period, the parous stage, was based on the presence of eggs in the lateral oviducts and more importantly presence and appearance of follicular relics (Grodowitz et al. 2020). The six and eight-week-old beetles fell into this stage. The fatty bodies change into a milky white colour and are now a cushion like mass that surrounds the reproductive system. There might be presence or absence of eggs in the lateral and common oviducts. Once ovulation occurs, follicular epithelium tissues peel off and are deposited as relics on the ovariole base. Less occurrence of ovulation will result in small follicular relics which are a pale-yellow colour. It is however difficult to determine if follicular relics are surrounding the ovariole base. Follicular relics can detach from the ovariole base through ovulation, so the quantity of follicular relics cannot be used to associate number of eggs produced to changes in reproductive system morphology (Tyndale-Biscoe 1984; Grodowitz & Brewer, 1987; Grodowitz et al. 1997; Hayes & Wall, 1999; Lenz et al. 2007; Eisenberg et al. 2018).

Fatty bodies in the nulliparous stage were a distinct deep yellow. However, in the parous stage, they turn into a milky white color and surround the lateral oviducts and the common oviduct. The use of these fat bodies in *Gonipterus* sp. n. 2 is not known. However, studies show that fat bodies assist in the synthesis of yolk protein precursors (e.g., vitellogenin) in the fat body (Skowronek et al. 2021). Oocytes synthesize vitellogenin at the start of oogenesis but after some time, it is extracted from the hemolymph. The protein synthesized in the body is sent to the hemolymph and used by the ovaries and the egg. The appropriate production and uptake of vitellogenin determines the proper development of the oocyte (Pan et al. 1969; Valle 1993; Sun et al. 1991; Skowronek et al. 2021).

Follicular relics occur in a wide range of insects and have been used in age determination of several Diptera (Vogt et al. 1974; Van Geem et al. 1983; Vogt and Walker, 1987; Wall et al. 1991). Coleopterans, in addition to the Dipterans have also had follicular relics studied in relation to age grading. These include the dung beetle, *Euoniticellus intermedius* Reiche (Coleoptera: Scarabaeidae) (Tyndale-Biscoe 1978); the Australian dung beetle, *Onthophagus granulatus* Boheman (Coleoptera: Scarabaeidae) (Tyndale-Biscoe et al. 1981); the boll weevil, *Anthonomus grandis* (Coleoptera: Curculionidae) Boheman (Grodowitz & Brewer, 1987); the weevil, *Neochetina eichhorniae* (Coleoptera: Brachyceridae)Warner (Grodowitz et al. 1997); the larger grain borer, *Prostephanus truncatus* (Coleoptera: Bostrichidae) Horn (Scholz et al.

1998), and the rice weevil *Sitophilus oryzae* Linnaeus (Coleoptera: Curculionidae) (Mendoza et al. 2004).

Another ovarian condition which was not observed in *Gonipterus* sp. n. 2 but observed in other beetles (Pratt et al. 2018), is the appearance of ovaries to be in a degenerative or senescing state. It is characterized by blackened areas within the ovarioles typically with none or only a limited number of maturing follicles. Older beetles have distinct darkened particles in the follicular relics. It has been suggested that as the follicles continuously pass through the small lumen of the ovariole, they are compressed, leading to the formation of darkened particles. Eggs may be present within the oviducts. In some species, senescence is a result of the decline in the reproductive state and cessation of oogenesis due to changes in nutrition or 'old age.' Pratt et al. (2018) found female *Oxyops vitiosa* Pascoe (Coleoptera: Curculionidae) with ovaries containing reduced follicles, clear areas within the ovarioles, and in most cases distinct follicular relics. Reproduction had seized in these females due to lack of suitable nutritional sources which occur during dry conditions when young leaf material of *Melaleuca quinquenervia* is not available. This condition was not observed in *Gonipterus* sp. n. 2 and a probable reason for this is that the beetles dissected were still too young to exhibit such sexual reproduction morphological characteristics. However, studies of other beetles like *Anthonomus grandis grandis* Boheman (Coleoptera: Curculionidae) (Grodowitz & Brewer 1987) and *Neochetina eichhorniae* Warner (Coleoptera: Curculionidae) (Grodowitz et al. 1997) exhibited this phenomenon.

The spermatheca (multiple spermathecae in some instances) is an invagination of the eighth abdominal segment (Snodgrass 1935); and its shape and number depend on the group of insects (Harterreiten-Souza & Pujol-Luz, 2012; Pascini & Martins, 2017). The spermatheca is C-shaped in *Gonipterus* sp. n. 2 which is the case in other studies of numerous Coleopteran spermatheca (Rodriguez-Mirón et al. 2017; Matsumura et al. 2020). The presence, absence, or changes of the following structures: spermathecal gland, spermathecal duct, and spermathecal capsule, distinguish the five forms of spermathecal morphology in Coleoptera (De Marzo 2008). A spermathecal capsule that stores sperm and is linked to the bursa copulatrix by a spermathecal duct constitutes the typical configuration. After copulation, the duct enables sperm transfer to the spermathecal capsule (Gack & Peschke 1994; De Marzo 2008). The migration of sperm from the bursa copulatrix to the spermathecal capsule is aided by the presence of a spermathecal gland (Aslam 1961; Grodner & Steffens 1978; Suzuki 1988; De Marzo 2008; Matsumura & Suzuki, 2008). Finally, the distal and proximal portions of the spermatheca are connected by a muscle, the contraction of which causes the sperm to be transferred to the bursa copulatrix (Rodríguez 1994). As eggs pass through the common oviduct, they are fertilized by sperm contained within the spermatheca, which is part of an elaborate spermathecal complex. In some beetles, it was experimentally confirmed that the spermatheca enables females to control sperm allocation and usage (Villavaso 1975; Rodriguez 1994; Bloch et al. 1998; Matsumura et al. 2020). It stores and maintains the viability of sperm until fertilization, a very important role in copulation and oviposition (e.g., Gschwentner & Tadler, 2000; De Marzo 2008, Harterreiten-Souza & Pujol-Luz, 2012; Pascini & Martins, 2017).

Several abiotic and biotic factors influence insect reproduction and abundance (Savopoulou-Suollani et al. 2012). Egg production is ultimately an aspect of conversion of ingested food (Muthukrishnan & Pandian, 1987) and thus food quality has an influence on reproductive development (Dittman & Biczkowski 1995; Wheeler 1996; Joern & Behmer, 1997; Adams 1999). Host quality can also be influenced by host species. A study by de Oliveira et al. (2022) showed that *Gonipterus platensis* Marelli (Coleoptera: Curculionidae) adult and larval development was shorter on *Eucalyptus urophylla* S. T. Blake (Myrtales: Myrtaceae) than other *Eucalyptus* species studied.

Host quality can also be influenced by climate, such as seasonal changes. For *Gonipterus* sp. n. 2, oviposition reduces in the field and in the laboratory in the winter season (unpublished data). This can be attributed to food quality as well as availability since the first rains are a cue to egg laying. With first rains comes new leaf flush and thus a restoration of food quality. The reduced oviposition of *Gonipterus* sp. n. 2 in winter might be attributed to oosorption which occurs when food reserves are depleted. This is a phenomenon observed in other beetles (Tyndale-biscoe & Watson, 1977; Lopez-Guerrero 1996; Ohgushi 1996; Rosenhem et al. 2000; Mendoza et al. 2004, Osawa 2005) and other insect orders (Lusis 1963; Bell 1971; Lum 1979; Santolamazza-Carbone et al. 2008; Grodowitz et al. 2020). Oosorption is resorption of developing oocytes in the ovary (Kotaki 2003). The process involves the interruption of vitellogenesis, occurring whilst they are still oocytes that have not yet been oviposited (Bell & Bohm 1975; Chapman 1998). Environmental stress brought about by starvation is usually the reason why resorption occurs. It is an adaptive mechanism to optimize fitness in hostile environments by recouping resources that might otherwise be lost (Bell & Bohm 1975; Papaj 2000; Barrett et al. 2008; Boggs 2009). The resources are then injected into other functions that increase life span and future reproductive potential. Egg resorption abilities provide the opportunity to react to different environmental stresses throughout the insect's reproductive phase (Moore & Attisano 2011). It can occur in virgin females, or when conditions are unsuitable for oviposition (e.g., at extreme climate conditions, seasonal changes, changes in photoperiod, or during diapause initiation) (Bell, 1971; Tyndale-Biscoe & Watson, 1977). In some cases, it results in the destruction of the oocyte while still enveloped in the follicle (Retnakaran & Percy, 1985). The influence of season (climate) and host species on the reproductive biology of *Gonipterus* sp. n. 2 is an area of research that requires further investigation.

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Figure 3.1. (**a**) Morphology of a mature (parous) reproductive system of *Gonipterus* sp. n. 2. and (**b**) right ovary of a young (nulliparous) *Gonipterus* sp. n. 2 (gm, germarium; vt, vitellarium; fo, follicle; sp, spermatheca; lo, lateral oviduct; co, common oviduct; me, mature eggs; ov, ovaries; ovl, ovariole) (Drawings by I.A. Makowe).

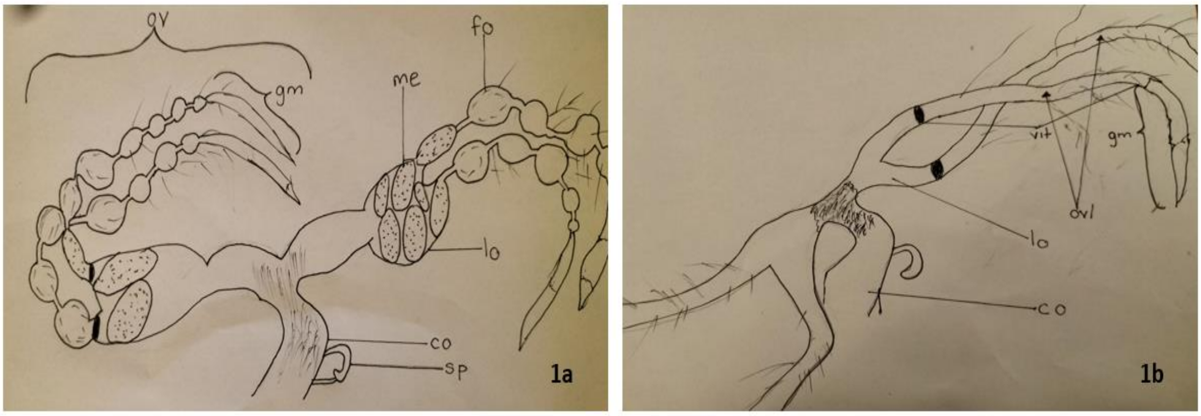


Figure 3.2. Key events in germ cell cluster formation and their impact on ovary types. Interaction of somatic apical cells (1/green) with germ cells maintains the latter as stem cells (2/red). Stem cells go through differential mitoses to form cytoblasts (3/purple) which directly transform into oocytes or undergo mitoses, generating clustered cytocytes (4/blue). Cystocyte clusters (5/turquoise) split or differentiate to generate nurse cells (black nuclei) and oocytes. In telotrophic meroistic ovaries, the character of apical cells (green) is extended to inner sheath cells and interstitial cells (green + yellow) by which all nurse cells will be kept in a terminal tropharium (Tr). As in panoistic ovaries, each follicle consists of the oocyte chamber only. Modified from Büning, (2005).

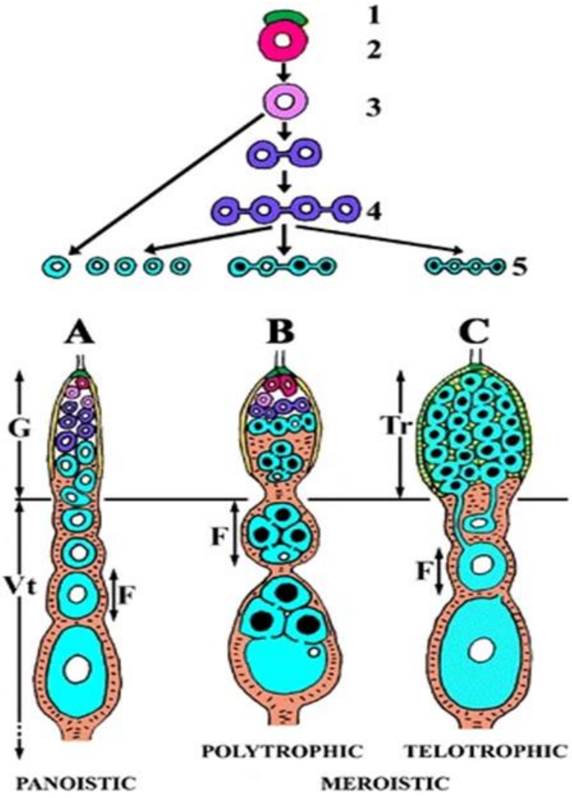


Figure 3.3. Reproductive system of two-week-old beetles of *Gonipterus* sp. n. 2 reproductive system. (**3a**), fat bodies changing colour to a light yellow; (**3b**), (ov, two ovaries; ovl, ovariole; sp, spermatheca; fs, filamentous strands).

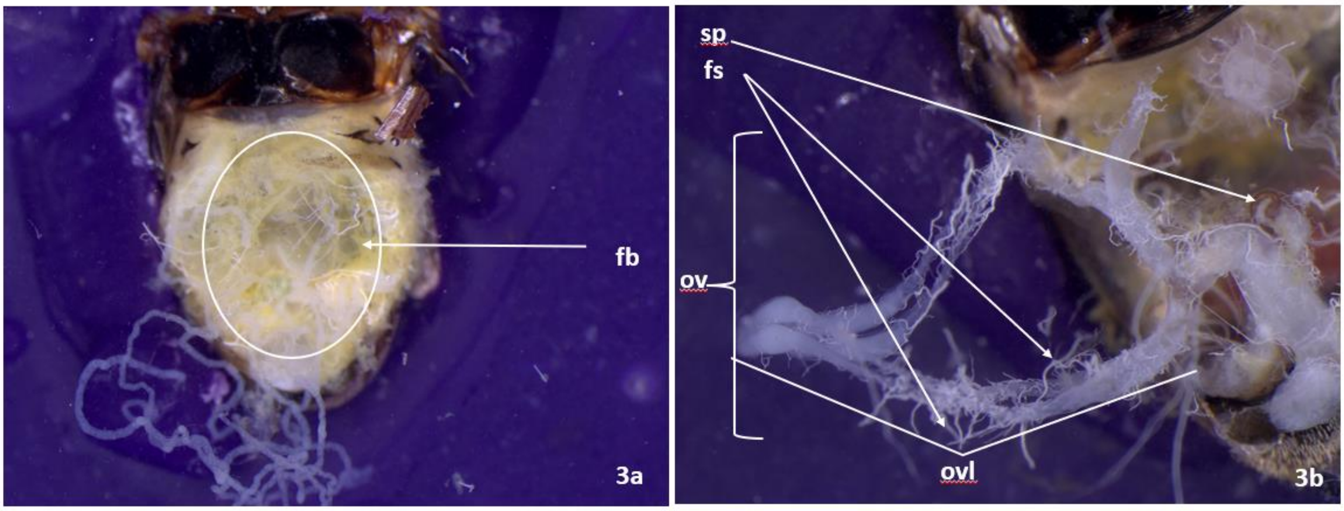


Figure 3.4. *Gonipterus* sp. n. 2 four-week-old beetles' ovarian morphology. (**4a**) fb, Fat bodies changing color to a milky white and (**4b**), left ovary of the female reproductive system of *Gonipterus* sp. n. 2 (gm, germarium; vit, vitelarium; lo, lateral oviduct; fo, follicles).

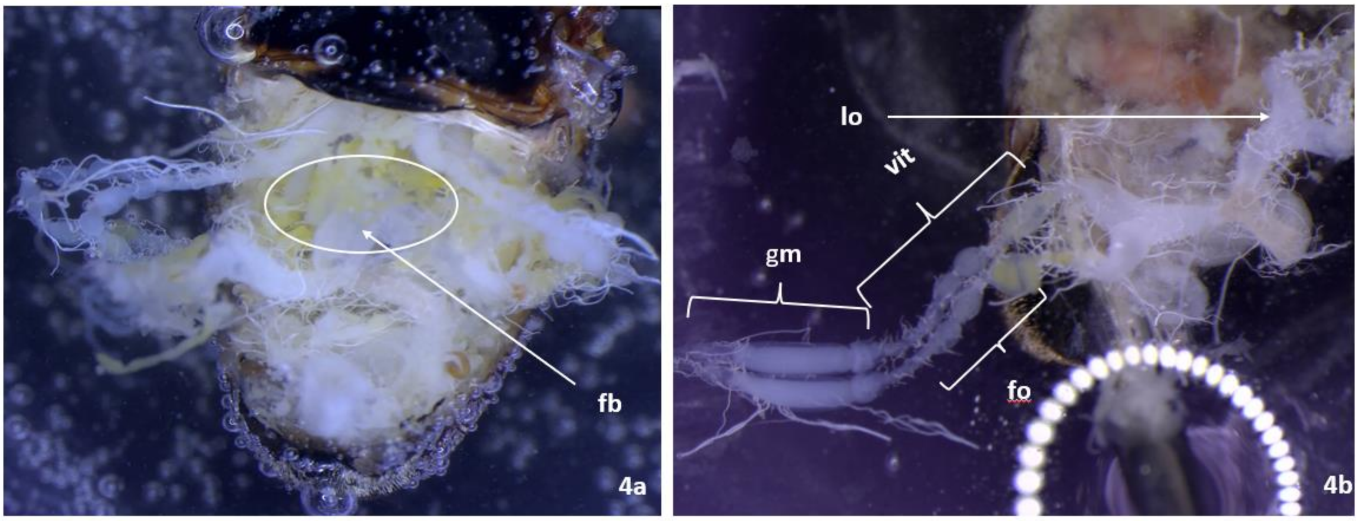


Figure 3.5. Reproductive system of six-week-old beetles of *Gonipterus sp. n. 2*. (**5a**), fat bodies mostly white in color and (**5b**) reproductive system of the beetle showing follicles and egg development (sp, spermatheca; fo, follicles)

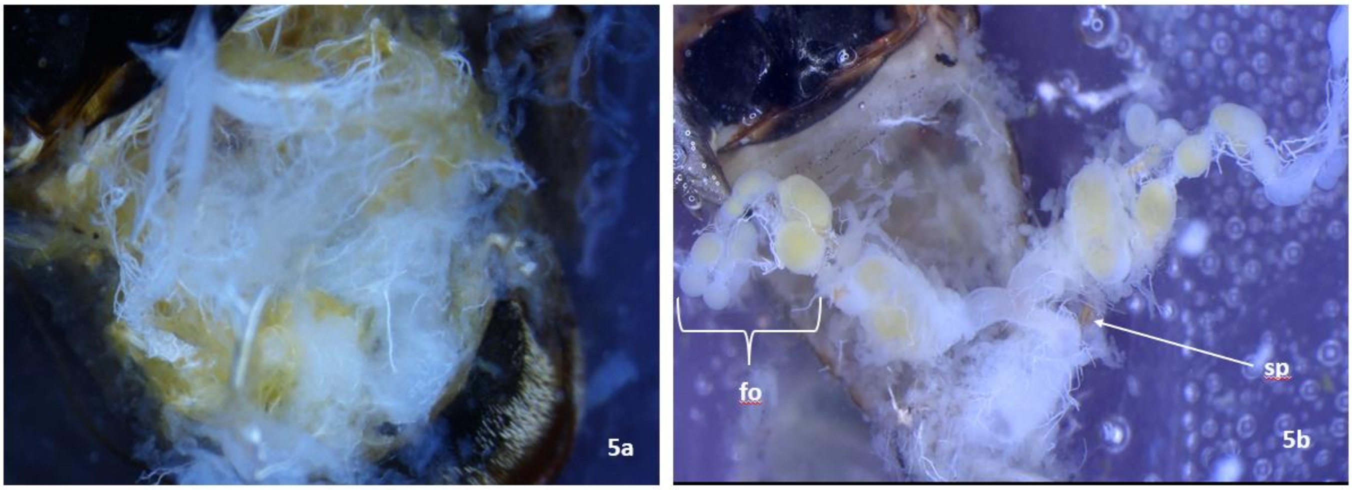
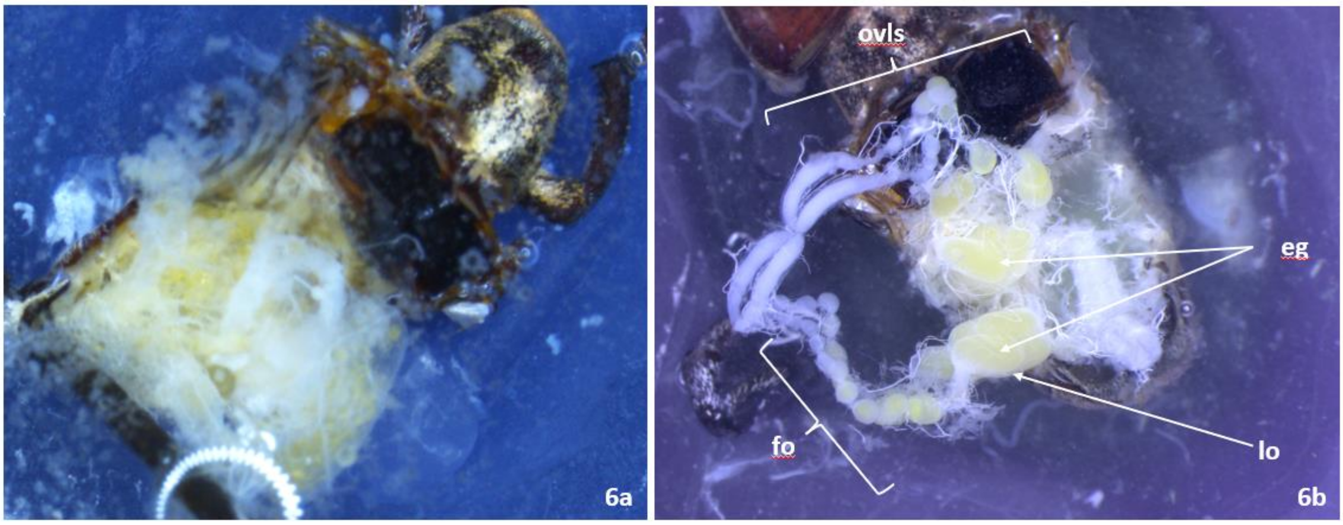


Figure 3.6. Reproductive system of eight weeks old beetles of *Gonipterus sp. n. 2* (fo, follicles, eg, eggs; lo, lateral oviduct; ovls, ovarioles)



CHAPTER 4

Effect of laboratory rearing on the body size of four parasitoid species used in biological control

4.1. Abstract

Insect biological control programmes are optimized to enhance parasitoid growth, especially in females. Larger females are correlated with better fitness performance. However, rearing techniques in biological control can give rise to inbreeding which can have a negative impact on parasitoid fitness. In this study, four parasitoids, *Anaphes inexpectatus* Huber and Prinsloo (Hymenoptera: Mymaridae), *Centrodora damoni* Girault (Hymenoptera: Aphelinidae), *Cleruchoides noackae* Lin and Huber (Hymenoptera: Mymaridae) and *Selitrichodes neseri* Kelly and La Salle (Hymenoptera: Eulophidae) were measured to understand the influence of rearing on body size using tibia length as a proxy. *C. noackae* and *C. damoni* generations showed that body size decreased significantly between the F0 and F8 for *C. noackae* and F2 and F9 for *C. damoni*. *S. neseri* had significant differences in body size between some generations but the first and last generations studied had no differences. *A. inexpectatus* did not exhibit any loss in body size across generations. These results support the prediction that some parasitoids maintained in laboratory conditions may undergo body size changes due to unintentional adaptation to the rearing conditions. Some will, however, not succumb to loss of body due to various factors which are both abiotic and biotic.

4.2. Introduction

Classical and augmentative biological control depend on successful laboratory rearing of natural enemies. However, initial population size, rearing methods and time in captivity can affect the success of a laboratory colony (Woodworth et al. 2002; Gilchrist et al. 2012; Francuski et al. 2014), and ultimately their success in controlling the pest once it is released in the field (Taylor et al. 2011; Fowler et al. 2015). Insect biological control programs can involve mass rearing of parasitoids over multiple generations within the confinement of a laboratory environment. Rearing practices, including how long the population is under laboratory conditions, can influence the quality of the insects by reducing their longevity, fecundity, sex ratio, preoviposition period, ovigeny and as well as adult body size and ultimately, the success of biological control programs (Bertin et al. 2017). A decline in fitness of *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) was observed after 15 generations of laboratory rearing (Ghaemmaghami et al. 2021). Biological control programmes are optimized to enhance female body size and/or fitness since they utilize female parasitoids to manage insect pest populations (Beukeboom 2018).

The ability of confined populations to reproduce and thrive in the natural environment may be reduced because of genetic adaptation to laboratory conditions through the selection of specific genotypes (Frankham 2008; Tayehet et al. 2012). Loss of genetic diversity occurs when a few individuals are utilized to start a colony or when populations remain small (Bertin et al. 2017)). This leads to inbreeding depression by mating among related individuals, causing an increase in homozygosity (Quaglietti et al. 2017). An increase in the number of homozygotes can result in dominance in once recessive alleles, which can affect fitness proxies such as egg size, dispersal, body size and patch location. Zaviezo et al. (2017) studied development of homozygosity in *Mastrus ridens* Horstmann (Hymenoptera: Ichneumonidae), a parasitoid of *Cydia pomonella* Linnaeus (Lepidoptera: Tortricidae), by crossing siblings and non-siblings. Inbreeding was observed to increase in inbred individuals and sex ratio became male biased over time. Genetic changes in individuals or a population can also occur through mutations which increase the expression of recessive harmful genes and can lower population fitness and field performance (Woodworth et al. 2002; Araki et al. 2007; Frankham 2008).

Parasitoid fitness can also be influenced by abiotic factors, including geographic location, temperature and humidity. Studies by Meister et al. (2018) showed significant differences in body size between two populations of the moth *Ematurga atomaria* Linnaeus (Lepidoptera: Geometridae) in different geographic locations. Temperature is however the main driver of fitness proxies such as sex ratio, ovigeny, preoviposition period, longevity, fecundity and body size (Romo & Tylianakis, 2013; Jerbi-Elayed et al. 2021). *Bracon vulgaris* Ashmead (Hymenoptera: Braconidae) has a longer preoviposition period in lower temperatures than in higher temperatures (Ramalho et al. 2009). Parasitoids are usually reared at optimum temperatures of 20-25˚C (Jerbi-Elayed et al. 2021; Chen et al. 2021) and they tend to exhibit smaller body size changes as an effect of high temperatures (Temperature-size rule) (Atkinson 1994; Colinet et al. 2007; Le Lann et al. 2011). A study on the effect of temperature on three flies, *Procladius rassinervis* Zetterstedt (Diptera: Chironomidae), *Tanytarsus nemorosus* Edwards (Diptera: Chironomidae) and *Polypedilum sordens* Maigen (Diptera: Chironomidae) showed that adult body size of the first two species and the males of the latter get smaller with increasing temperature (Wonglersak et al. 2021). It is thus important to optimize temperature and humidity to ensure female fitness does not decrease during the rearing process.

Body size is one of the most important drivers of life history strategies (Hirst et al. 2014; Gao et al. 2016) and it is positively correlated with fitness traits (Leather 1988; Gao et al. 2016;

Curtis et al. 2017). Large size individuals have more physiological advantages than small size conspecifics (Gao et al. 2016). There is an increase in the insects' ability to search and subdue large, high-quality hosts, lifetime fecundity, longevity, and mating success when the individuals are larger in size (Sagarra et al. 2001; Abram et al. 2016; Kasamatsu & Abe, 2015; Gao et al. 2016). Large size in insects also influence the outcome of numerous parasitoid–host interactions (Charnov 1982; Godfray 1994; Kazmer & Luck, 1995; Petersen & Hardy, 1996), especially in hymenopteran parasitoids (Liu et al. 2011). This fitness-size dependency seems to be more pronounced in females than in males (Charnov 1979; Visser 1994; Pandey & Johnson, 2005; King & Napoleon, 2006; Gao et al. 2016; Samková et al. 2022). In the field, large females have better dispersal capabilities (i.e., they disperse over longer distances) which reduces their risk of inbreeding as well as extinction if conditions become unfavourable (Wang & Messing, 2004). They can also locate the precise location of patches, and this leads to increased parasitism (Visser 1994; Kazmer & Luck 1995; West et al. 1996; Bennett & Hoffmann, 1998; Wang & Messing, 2004). Therefore, insect body size can be used as a good indicator of insect fitness.

In this study we investigated the changes in parasitoid fitness (using body size as a proxy) across generations of laboratory reared populations. We focused on four parasitoid species that attack invasive insect pests of *Eucalyptus* spp. and which were reared in the quarantine facility of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa: *Selitrichodes neseri* Kelly and La Salle (Hymenoptera: Eulophidae) is a parasitoid of *Leptocybe invasa* Fisher and La Salle (Hymenoptera: Eulophidae) that was released in South Africa in 2012 (Dittrich-Schröder et al. 2014); *Cleruchoides noackae* Lin and Huber (Hymenoptera: Mymaridae) is an egg parasitoid of *Thaumastocoris peregrinus* Carpintero et Dellape (Heteroptera: Thaumastocoridae) (Mutitu et al. 2013) that was released in South Africa in 2013. Two of the species, *Centrodora damoni* Girault (Hymenoptera: Aphelinidae) and *Anaphes inexpectatus* Huber and Prinsloo (Hymenoptera: Mymaridae), are egg parasitoids of *Gonipterus* spp. Gyllenhal (Coleoptera: Curculionidae) that were introduced into the FABI quarantine facility to assess their potential as biological control agents but have not yet been released in the country.

4.3. Materials and Methods

4.3.1. Collection and Importation of Parasitoid Species

S. neseri was imported into South Africa through unidentified galls on twigs, petioles and leaves of *Eucalyptus* spp. saplings collected in Nanango, Queensland, Australia in April 2010 (Dittrich- Schröder et al. 2014). *C. noackae* was imported into South Africa from New South Wales, Australia in 2009, by means of parasitized egg capsules of *T. peregrinus*. *C. damoni* was imported into South Africa as parasitized eggs of *Gonipterus* spp. collected in south-east Australia in 2018. *A. inexpectatus* was imported from Portugal through parasitized *Gonipterus platensis* egg capsules in 2016.

4.3.2. Rearing and Parasitoid Information

The insect material used in this study was collected throughout the rearing period of the parasitoids from the time of import to when the colony died out or field releases were done. Rearing was carried out at the Forestry and Agricultural Biotechnology Institute (FABI) certified quarantine facility at the University of Pretoria's Innovation Africa campus. They were preserved in 100% alcohol and stored in the FABI insect database. *S. neseri* were reared by exposing them to *L. invasa* galled GC540 plants at room temperature of 25.8 ± 0.03°C, relative humidity (RD) was 48 ± 0.35% with a photoperiod of 12:12 light: dark cycle (L:D). Five of the generations reared (F1 – F4 and F9) were stored in ethanol and used for the experiments carried out in this study. *C. noackae* was reared on *T. peregrinus* eggs oviposited on *Eucalyptus grandis* W. Hill ex Maiden clone TAG5 at 24 ± 2°C, 70 ± 10% RH, and a photoperiod of 12:12 hours (L:D). Nine generations were measured for *C. noackae* from F0 to F9. Data collected from *C. damoni* was from six generations (F2, F5 - F7, and F8) and *A. inexpectatus* from nine generations (F0 - F9). The two parasitoids were reared on *Gonipterus* sp. n. 2 egg capsules oviposited on *Eucalyptus dunnii* leaves. The egg capsules laid on the leaves were punched out into leaf discs and stuck on cards and exposed to the parasitoids in a vial. After two to three days, the adult parasitoids were taken out and the cards were incubated at 16°C and 80% humidity. There are gaps in some generations due to absence of these generations in the database. A sample size of ten replicates per generation, per parasitoid were measured except for *S. neseri* which had seven replicates measured due to unavailability of samples.

4.3.2. Measurements of hind tibia

The length of the hind tibia of the parasitoids was measured to determine insect body size as hind tibia length can be used as a body size index (Zhou et al. 2016). Temporary slides of the tibia were made by placing a drop of distilled water in the center of a glass microscope slide and positioning a detached insect leg on the liquid using forceps. A cover slip was placed against the slide at an angle and slowly lowered to avoid air bubbles. A Nikon SMZ1500 microscope at a magnification of 0.75× was used to take measurements of the tibia through an Olympus Stream (Version 2.4.2) Software. The mean tibia length was calculated and plotted against generations to assess if there was a change in adult body size across generations. The data was tested for normality and homogeneity of variance. Where the assumptions were met, an Analysis of Variance (ANOVA) was carried out with a Tukey HSD Posthoc test to determine significant differences in body size between generations. The data collected from *S. neseri, C. damoni* and *C. noackae* were not parametrically distributed and the data could not transform, therefore the non-parametric Kruskal-Wallis test was carried out and the Dunn-Bonferonni test were used for pairwise comparisons where generation had a significant effect on body size. All data analysis was carried out using IBM® SPSS® Statistics Software 28.0. (SPSS, Chicago, IL, USA).

4.4. Results

4.4.1. *Selitrichodes neseri*

The hind tibia length for *S. neseri* had a gradual decrease from F1 to F4 and the difference was significant between some generations (H (4) = 15.304, p = 0.004). F1 had the highest tibia length of 304.42 +/- 7.75 µm (mean +/- SE) whilst F4 had the smallest of 252.73 +/- 3.84µm (mean +/- SE). There was a slight increase in tibia length from generations F4 to F9 (266.61 +/- 7.098µm) (mean +/- SE) (**Figure 1**).

4.4.2. *Cleruchoides noackae*

C. noackae mean tibia length was the longest in F0 (152.06 +/- 0.58µm) (mean +/- SE) and the shortest tibia length was in F9 (126.87 +/- 0.9µm) (mean +/- SE). Tibia length fluctuated significantly between the generations with a significant decrease from F0 – F9 (H (8) = 76.235, p = 0.001) (**Figure 2**). There was a decrease in size from F0 (152.06 +/- 0.58µm (mean +/- SE)) to F2 (126.87 +/- 0.9µm (mean +/- SE)). Thereafter, tibia size increased to generation F4 (151.132 +/- 0.475µm) (mean +/- SE), followed by a second decrease in tibia length from F5 (146.149 +/- 0.593µm) (mean +/- SE) to F7 (137.123 +/- 0.878µm) (mean +/- SE) and then a slight increase in tibia length for F8 (140.353 +/- 0.828µm (mean +/- SE)).

4.4.3. *Centrodora damoni*

There was a gradual decrease in mean hind tibia length of *C. damoni* from F2 generation to the F9 generation. The tibia length of the F2 (266.14 +/- 1.97µm) (mean +/- SE) and F9 (245.43 +/- 3.67µm) (mean +/- SE) generation of *C. damoni* were the highest and the lowest respectively (**Figure 3**). There was evidence of significant differences (H (5) = 13.991, p= 0.016) amongst some pairs of generations compared.

4.4.4. *Anaphes inexpectatus*

Generation did not have a significant effect on tibia length of *A. inexpectatus* ($F_{8.81}$ = 1.525, p = 0.161), although a decrease from F0 to F9 was observed. The tibia length of *A. inexpectatus* was longest at generation F0 (252.28 +/- 5.83µm) (mean +/- SE). Tibia length decreased from F0 to F3 (235.658 +/-5.146µm) (mean +/- SE) and increased slightly to F5 (249.114 +/- 3.949µm) (mean +/- SE). There was another decrease in length from F6 (246.739 +/- 6.347 µm) (mean +/- SE) up to F9 (231.96 +/- 4.45µm) (mean +/- SE) which was the shortest tibia length measured (**Figure 4**).

4.5. Discussion

The results of this study indicate changes in insect body size in the generations of some of the parasitoids reared in the quarantine laboratory at the FABI biocontrol facility. *C. damoni* and *C. noackae* showed significant differences between generations. The general trend showed that there was a reduction between the first and last generations that were recorded for these two species. There were significant differences across some generations of *S. neseri* but there was no significant reduction in body size between the first and last generations. There was not a significant decrease in body size across generations for *A. inexpectatus*, although a general decrease was observed. The study supports the prediction that some parasitoids maintained in laboratory conditions may undergo body size changes due to unintentional adaptation to the rearing conditions.

A small parasitoid population in laboratory rearing is more likely to become extinct than a large population (Nouhuys & Kay, 2001) or to have body size altered in older generations. In laboratory rearing, emerging adults from different rearing containers are often mated together to avoid inbreeding depression (Zaviezo et al. 2018), but a small rearing population decreases the number of non-siblings crosses available. In this study, *C. damoni* was established using 180 adults. When there was no or low emergence from a breeding vial, related progeny (brothers and sisters) emerging was mated, sometimes for multiple generations, to keep the colony from dying out (unpublished data), thus increasing the probability of inbreeding. However, this is less likely to have been a factor for *C. noackae* and *A. inexpectatus*, which had over 500 adults each at the start of the colony or had more adults added into the colony.

Temperature may contribute to the fluctuations and eventual decrease in body size in parasitoids (Colinet et al. 2007; Le Lann et al. 2011). Le Lann et al. (2011) found temperature to cause a small change in parasitoid size, where an increase in temperature resulted in a decrease in parasitoid size. It is believed that a pause occurs in the physiological developmental processes in insects, a phenomenon known as diapause (Diniz et al. 2017). Diapause is common in insects and other arthropods, especially in areas with harsh winters (Diniz et al. 2017). A drop in seasonal temperatures affects insect life cycles regardless of the controlled temperatures in laboratory rearing (unpublished data). For example, *Gonipterus* sp. n. 2 ovulation and oviposition under laboratory rearing conditions as well as in the field is affected by seasonal changes, specifically the transition from summer to winter (unpublished data). Thus, seasonal changes may have resulted in a decrease in body size, or fluctuations in body size as observed with *C. noackae*. Seasonal changes can also affect host availability of laboratory reared colonies and thus impact parasitoid population fitness in the laboratory. This factor may have contributed to the changes in body size observed in *Gonipterus* sp. n. 2 parasitoids (*C. damoni* and *A. inexpectatus*). Egg capsule production of *Gonipterus* sp. n. 2 is affected by seasonal changes. In winter, egg capsule production decreases, a bottleneck that affects the parasitoid population size, and thus increasing the probability of inbreeding.

Changes in body size of parasitoids during rearing may require many generations. A study by Ghaemmaghami et al. (2021) of *Trichogramma brassicae* (Hymenoptera: Trichogrammatidae) rearing techniques showed a decline in fitness after 15 generations of laboratory rearing. Thus, it is possible that if the rearing period used in our study was longer, then greater differences across generations would have been observed for all four parasitoids. However, changes in body size or other fitness characteristics are not guaranteed to occur over rearing time. Martínez et al. (2018) studied two separate rearing populations of *C. noackae* for more than 30 generations and suggested that *C. noackae* exhibits a high tolerance to inbreeding depression in *in vitro* rearing, a condition that has been observed in other parasitoid wasps (Quaglietti et al. 2017).

The reduction in size in some of the parasitoids that were measured could be attributed to inbreeding which is brought about by a culture that has adapted to laboratory rearing (Frankham 2005). Individuals with an inbred background are more susceptible to environmental stress (such as changes in temperature, photoperiod, and humidity). Negative recessive alleles are more likely to exhibit themselves when there is environmental stress (Fox et al. 2011). This isn't always the case, though, as some studies have found a strong negative link between inbreeding depression and harsh surroundings, but other studies have found the exact opposite (Fox et al. 2011). The effects of variations in body size extend to ecosystem functions through environmental stress via size-dependent processes (such as connectedness of the food web and predator-prey dynamics) (Honk 1993). Consequently, a considerable ecological issue is presented by comprehending and eventually predicting body size variance with environmental variables (Curtis et al. 2017). In this study, environmental factors that affect insect body size were not investigated, but this would be important to investigate in future studies.

The study found that the rearing conditions within the quarantine facility had a variable effect on parasitoid body size. Challenges in optimizing conditions include general abiotic features that affect laboratory rearing (seasonal changes), the population size that is maintained during rearing, and host availability. A reduction in parasitoid fitness can have a negative impact on biological control efficiency. Thus, negative effects of laboratory rearing should be minimized for populations to be used in biological control programs. Laboratory rearing of parasitoids can be optimized by understanding how abiotic and biotic factors influence body size and related fitness parameters. This information can be used to optimize rearing conditions and thus parasitoid performance in biological control programs. It is recommended that when importing parasitoids, a large population size is preferred. It is best to always mate non- related adults once the colony is established to avoid inbreeding. If a parasitoid is not monophagous (host specific), it might be beneficial to consider use of factitious hosts for rearing of parasitoids to avoid host unavailability due to seasonal changes. In addition, the study of parasitoid body size can be bolstered by studying how it correlates with other fitness proxies like longevity, development time, sex ratio and fecundity.

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Figure 1: Length (in µm) (mean +/- SE) of the hind tibia of *Selitrichodes neseri* adults across five generations of a laboratory reared colony. Letters represent the generations that are significantly different (p = 0.004).

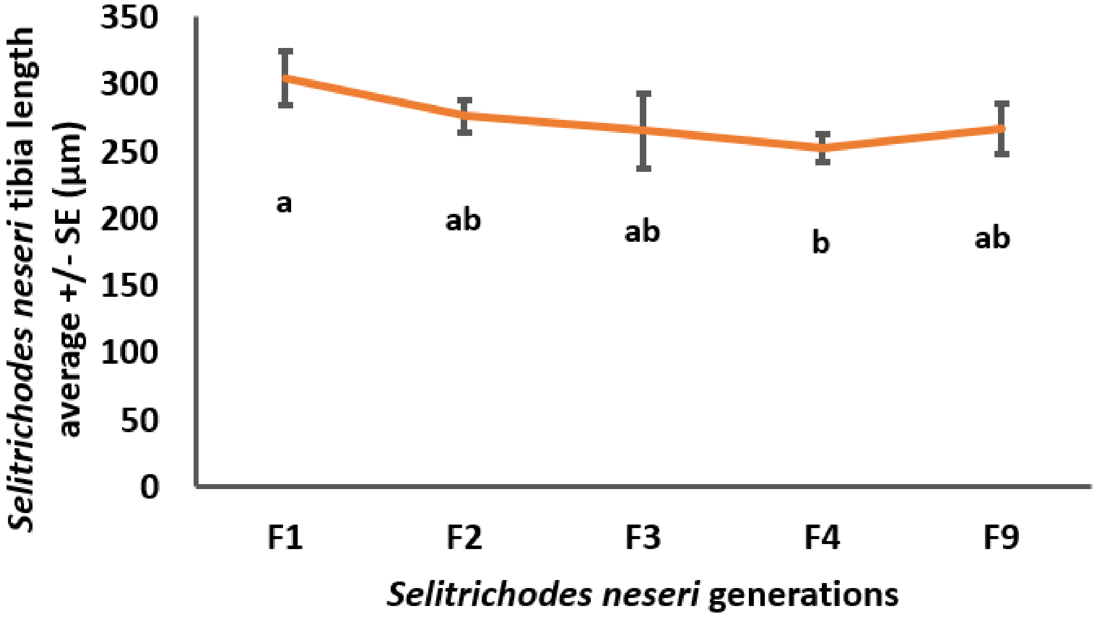


Figure 2: Size (in µm) (mean +/- SE) of the hind tibia of *Cleruchoides noackae* adults across nine generations of a laboratory reared colony. The letters are showing the significant differences in the generations (p = 0.001).

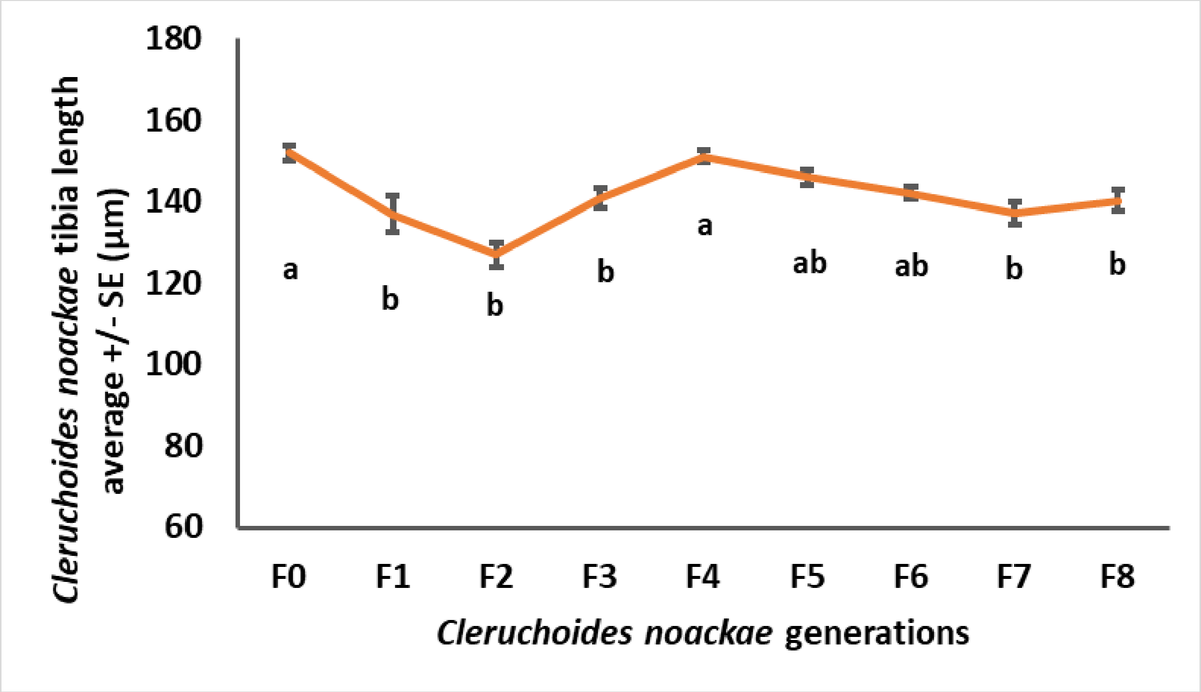


Figure 3: Size (in µm) (mean +/- SE) of the hind tibia of *Centrodora damoni* adults across six generations of a laboratory reared colony. Letters represent significant differences across generations (p = 0.016).

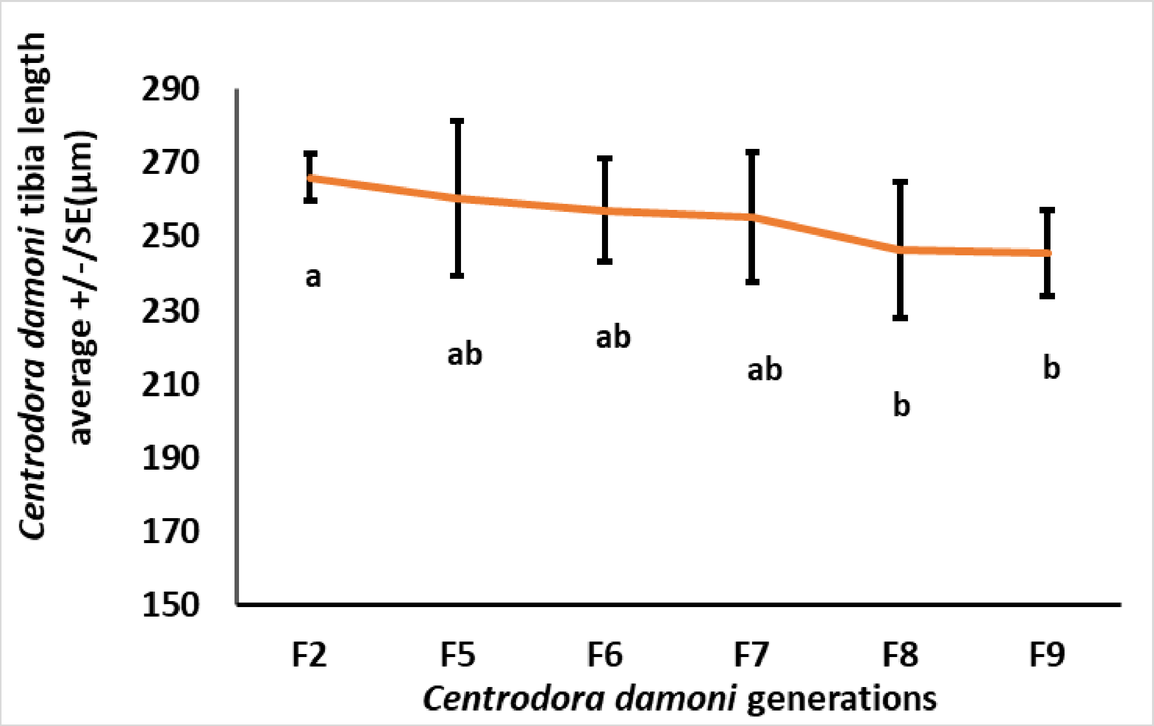
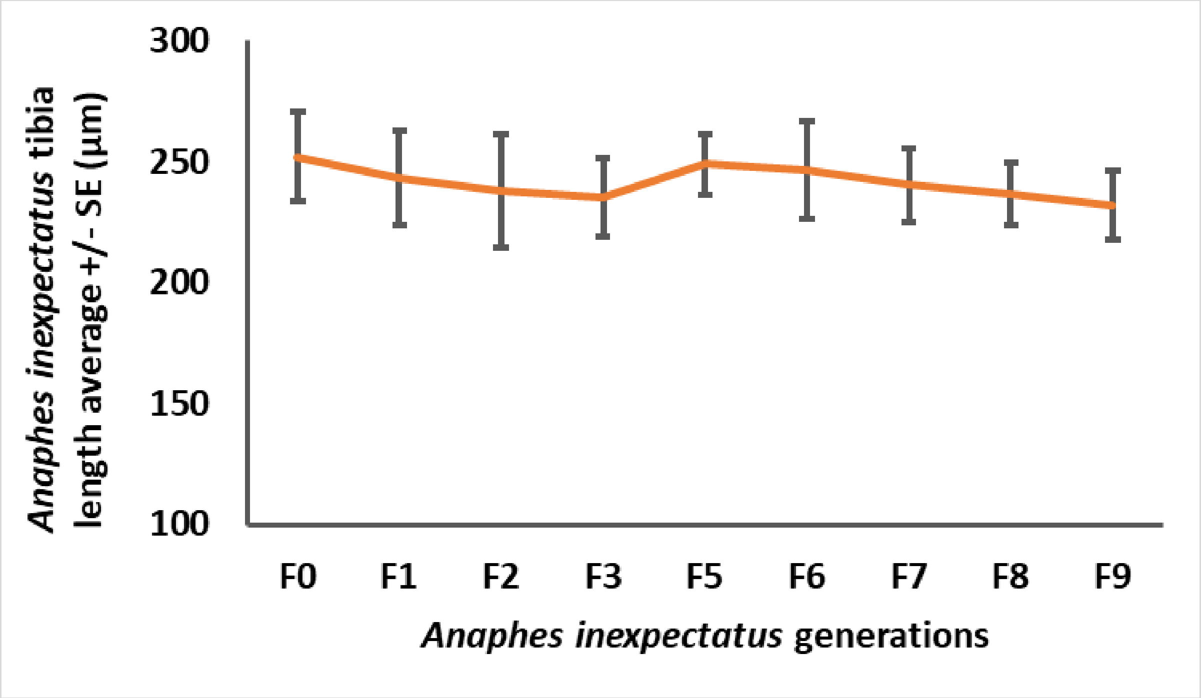


Figure 4: Average size (in µm) (mean +/- SE) of the hind tibia of *Anaphes inexpectatus* adults across nine generations of a laboratory reared colony (p = 0.161).



CHAPTER 5

General conclusion

Gonipterus sp. n. 2 has caused economic damage across the globe for decades. Even after the release of the egg parasitoid, *Anaphes nitens*, studies of the *Gonipterus* cyptic species complex are continuously undergoing in various parts of the world. These studies cover thermal requirements of the pest and its parasitoids, *Eucalyptus* host susceptibility to the pest, parasitoids biology and parasitoid interactions (Santolamazza et al. 2006; 2009; Valente et al. 2017a; 2017b; Valente et al; 2019; Schröder et al. 2020; 2021; Damascena et al. 2020; de Oliviera et al. 2022) including the redefining of parasitoids of this pest (Ward 2016). The feeding and reproductive behaviour of *Gonipterus* sp. n. 2 have been studied to a certain extent with a gap in the reproductive development of the pest.

The rearing of *Gonipterus* spp. egg parasitoids is onerous due to the fluctuating availability of egg capsules in varying seasons. *Gonipterus* spp. reproductive behavior has been studied but not its reproductive development. Studying the reproductive development of insect pests allows researchers to devise an age grading system which helped to understand the preoviposition period and the age at which egg production is highest. This will assist in pest guided management tactics such as timing rearing and releases of the parasitoids to when the highest egg load of the pest is available. Previous studies of female reproductive development have been on pests (Grodowitz & Brewer, 1987; Herbert & Toews, 2011; Grodowitz et al. 2019; 2020), predators (Grodowitz et al. 1997) and parasitoids (Nacro & Nénon, 2008; Xu et al. 2015; Mao et al. 2016). Understanding the reproductive development of an insect ensues after understanding the changes in reproductive system structures.

Centrodora damoni is one of the egg parasitoids of *Gonipterus* spp. Its importation into South Africa prompted an interest in its potential as a parasitoid through biology studies. This study was the first report on the biology of *C. damoni*, which enables a better understanding of the efficacy of the parasitoid through studying fecundity, longevity, host egg age preference, parthenogenicity, preoviposition period, sex ratio, and ovigeny. However, the presence of a previously released egg parasitoid, *Anaphes nitens*, in South Africa makes it of paramount importance that interactions between the two are studied and understood. It has become habitual across the world that more than one biological control agent is introduced to try and obtain a desirable outcome. However, introduction of additional agents does not ensure control since they often fail to establish and may cause antagonistic interactions.

Due to some rearing techniques such as prolonged rearing, siblings mating, as well as low parasitoid population upon introductions, parasitoids fitness can decrease due to inbreeding. Parasitoids such as *C. damoni* which were imported and studied under laboratory conditions might be affected by one or more of these rearing techniques. An increase in the number of homozygotes can result in dominance in once recessive alleles, which can affect fitness proxies such as egg size, dispersal, body size and patch location. This affects parasitoid performance and efficacy. Size is correlated to fitness, a loss in fitness is usually characterized by a reduction in body size. Large size individuals confer more physiological advantages than small size conspecifics (Gao et al. 2016).

Chapter 1 focused on the various associations that occur through the release of two or more natural enemies. Different natural enemy interactions in biological control were discussed in this review. The chapter discussed different types of life history traits of parasitoids and predators. Case studies of significant multiple natural enemy introductions in biological control programmes and the resulting type of interactions between the natural enemies were considered. Additive and synergistic interactions allow the natural enemies released not to hinder the function of each other. Interspecific and intraspecific competition, intraguild predation, superparasitism, multiparasitism as well as hyperparasitism fall under antagonistic interactions which obtrude in biological control programmes that have one or more parasitoid released. Releasing multiple biological control agents after understanding their foraging niches will reduce the chance of competition.

The general biology of *C. damoni* were studied in Chapter 2. We found out that at a temperature of 25 ° C, the results showed that *C. damoni*'s developmental time was 19.05 +/- 1.56 days. This is analogous to other *Gonipterus* spp. egg parasitoids. The developmental time of *Anaphes nitens* was 17 days at a similar temperature (Santolamazza et al. 2006) or 16.4 ± 0.11 days recorded in Portugal (Valente et al. 2017). Parasitoids with a shorter developmental time tend to reproduce faster and are preferred to those that take longer to develop. The longevity of *C. damoni* was longer in females than in males. These results are consistent with previous parasitoids longevity studies (Santolamazza et al. 2006; Valente et al. 2017). The studies focused on the *Anaphes* spp. parasitoids of *Gonipterus* spp. and showed that in both species, females lived longer than males, which is consistent with the results in this study. The host egg age preference was not observed in *C. damoni* as its oviposition on all egg ages of *Gonipterus* sp. n. 2. The parasitoid's reproductive potential is increased when there are no restrictions on its preferred host's age range.

Chapter 3 of this study focused on the reproductive development of *Gonipterus* sp. n. 2. It was observed that *Gonipterus* sp. n. 2 followed a polytrophic-meroistic, a type of reproductive system that is unique to Coleoptera. In this system, two ovaries are present, each with two ovarioles. Each ovariole has a germarium at its anterior end and the vitellarium at the posterior end which houses the follicles. Each pair of ovarioles joins at the posterior end into the lateral oviduct and the two lateral oviducts join into the common oviduct. In parous (younger) beetles, the structures are small (the lateral oviducts are shrunken in size), follicles form as the beetles grow older and there is no yolk in follicles and the fatty bodies are yellow in color. Nulliparous (older) beetles had follicle development progress into eggs and through a process known as ovulation, the eggs passed through the oviducts. The lateral oviducts expanded, and the fatty bodies changed to a milky white colour. It was also observed that *Gonipterus* sp. n. 2 egg capsule production reduced in winter, and this was possibly because the Eucalyptus Snout Beetle goes under oosorption.

Chapter 4 studied the effect of laboratory rearing on the size of parasitoids used in biological control. One of the parasitoids used in this study, *C. damoni*'s biology data, for example low fecundity are assumed to have been affected by prolonged rearing. Four parasitoids which were reared in quarantine were measured to understand the influence of rearing on body size using tibia length as a proxy. *C. damoni* and *Cleruchoides noackae* generations showed that rearing influenced parasitoid body size as body size decreased significantly between the first and last generations measured. *Selitrichodes neseri* had significant differences in body size between some generations but the first and last generations studied had no differences. *Anaphes inexpectatus* did not exhibit any loss in body size across generations. Generally, large female parasitoids have higher fecundity, longevity, greater reproductive potential because of larger volume of the spermatheca than small female (Gao et al. 2016), higher sex ratio, longevity, fecundity, as well as dispersal distance. The study supports the prediction that some parasitoids maintained in laboratory conditions may undergo body size changes due to unintentional adaptation to the rearing conditions.

For one to establish a biological control program that is effective, a full understanding of the insect's behaviour should be acquired. The reproductive development is one way of achieving this goal provides details on the reproductive behaviour of an insect. Understanding the current reproductive state of *Gonipterus* sp. n. 2 may help explain its movement from one host plant to another as well as behavioural activity on and around the plant, especially in relation to host *Eucalyptus* spp. and whether it goes into diapause and resorbs its eggs. Studies can continue to see the continuum of the reproductive development and how it affected by different dietary conditions as host species, effect of mating activity on reproduction and oviposition as well as the optimal thermal requirements for the reproduction development. Finally, developing reproductive life tables based on physiological age for field populations of *Gonipterus* sp. n. 2

should provide information on past reproductive activity (including a quantitative estimate of egg production), as well as future reproductive potential. For example, field populations consisting mainly of females in the early physiological ages may indicate the potential for substantial increases in the population as opposed to populations consisting of older physiological ages which would indicate a population in decline.

Choosing the ideal natural enemy to release for a biological control program is achieved by studying the biology of the parasitoid used in the system and choosing one that better suits optimum control of the pest. *Centrodora damoni* proved to be considerably promising as a parasitoid. This is due to its long adult life span, the ability to utilize different egg ages and development time which is like that of most parasitoids. However, some parameters could have been affected by the time *C. damoni* took in captivity (laboratory rearing period). Potential risks should be assessed before introducing an invasive parasitoid for biological control. Host specificity tests need to be carried to establish if *C. damoni* is monophagous which would make it ideal as a parasitoid. *Gonipterus* sp n 2 has multiple egg parasitoids that have been studied and released in various countries but only *A. nitens* has been released in South Africa. Studying how *C. damoni* interacts with *A. nitens* is of importance to time releases of the two parasitoids so that their effect in their control of *Gonipterus* sp. n. 2 is synergistic. However, the length of time *C. damoni* spent in captivity (laboratory rearing period) may have had an impact on several metrics. Before introducing an invasive parasitoid for biological control, potential dangers should be evaluated. *C. damoni* must undergo host specificity testing to determine whether it is monophagous, which would make it the ideal parasitoid. Numerous egg parasitoids from the *Gonipterus* sp. n. 2 species have been examined and released in different nations, but only *Anaphes nitens* has been introduced into South Africa. It is crucial to understand how *C. damoni* and *A. nitens* interact in order to time the release of the two parasitoids and maximize their combined ability to control *Gonipterus* sp. n. 2.

The time taken in captivity of biological control agents whilst they are being mass reared or studied can affect their fitness which in turn affects their performance in the field when they are released. Body size is one of the proxies used to determine fitness and it is affected by rearing techniques such as prolonged rearing as well as abiotic factors like humidity and temperature. Body size is correlated to life history traits such as sex ratio, fecundity, longevity as well as mating success. Since these life history traits differ between males and females, it is best to perform body measurements on both males and females in separate groups. Other parameters such as longevity, fecundity and sex ratio can be included in the study to better understand the trend of fitness changes in the parasitoid.

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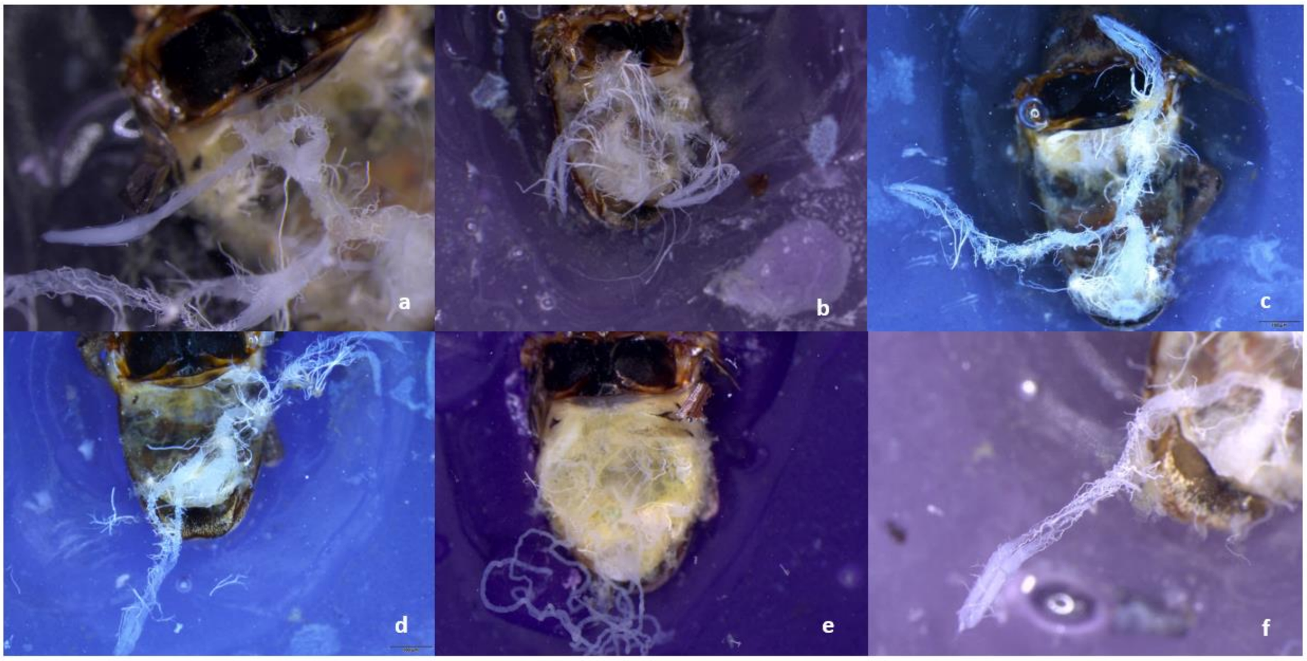
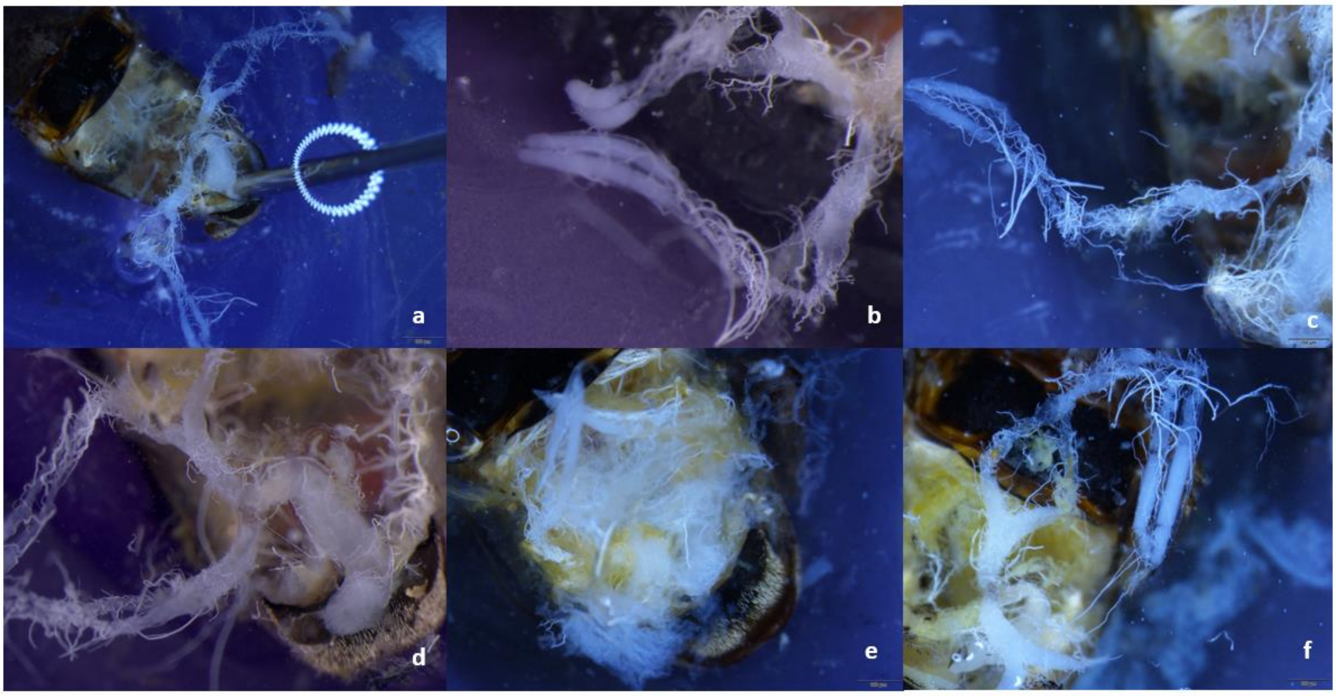
APPENDIX 1

Supplementary material for Chapter 3

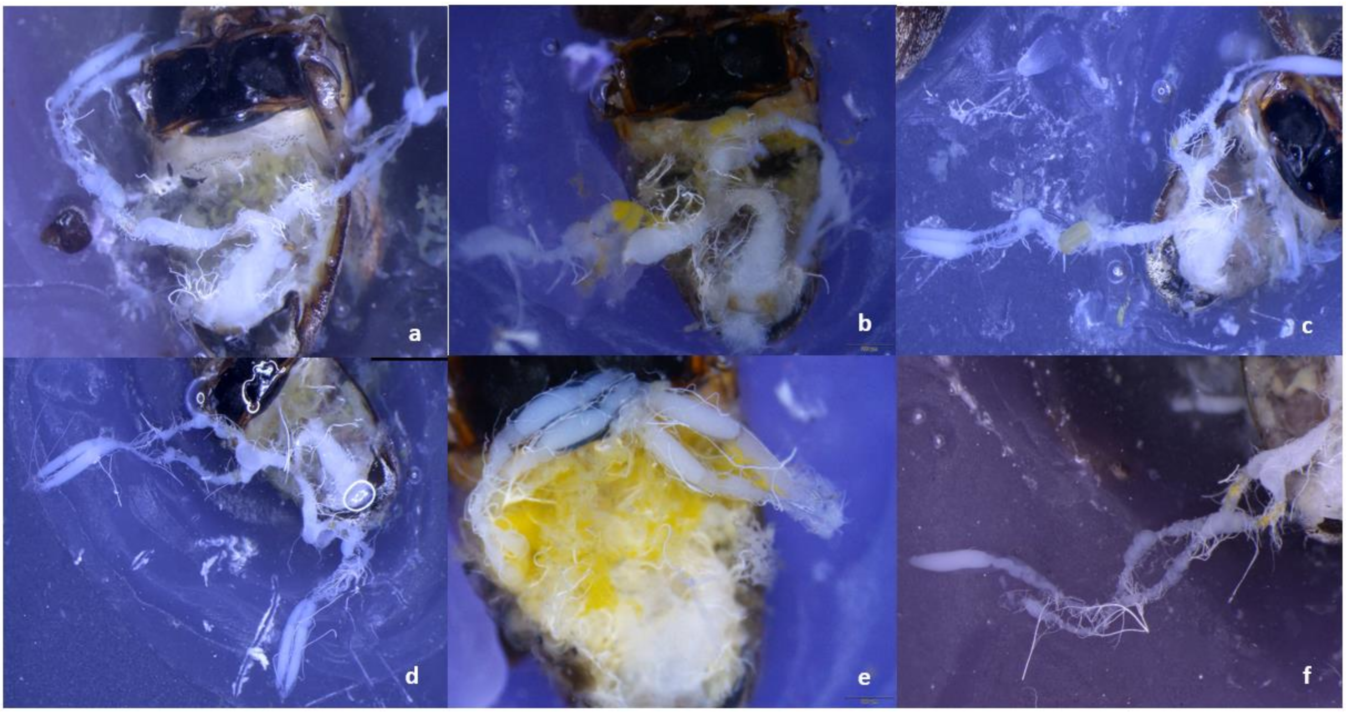
A1. Zero weeks old *Gonipterus* sp. n. 2 beetles



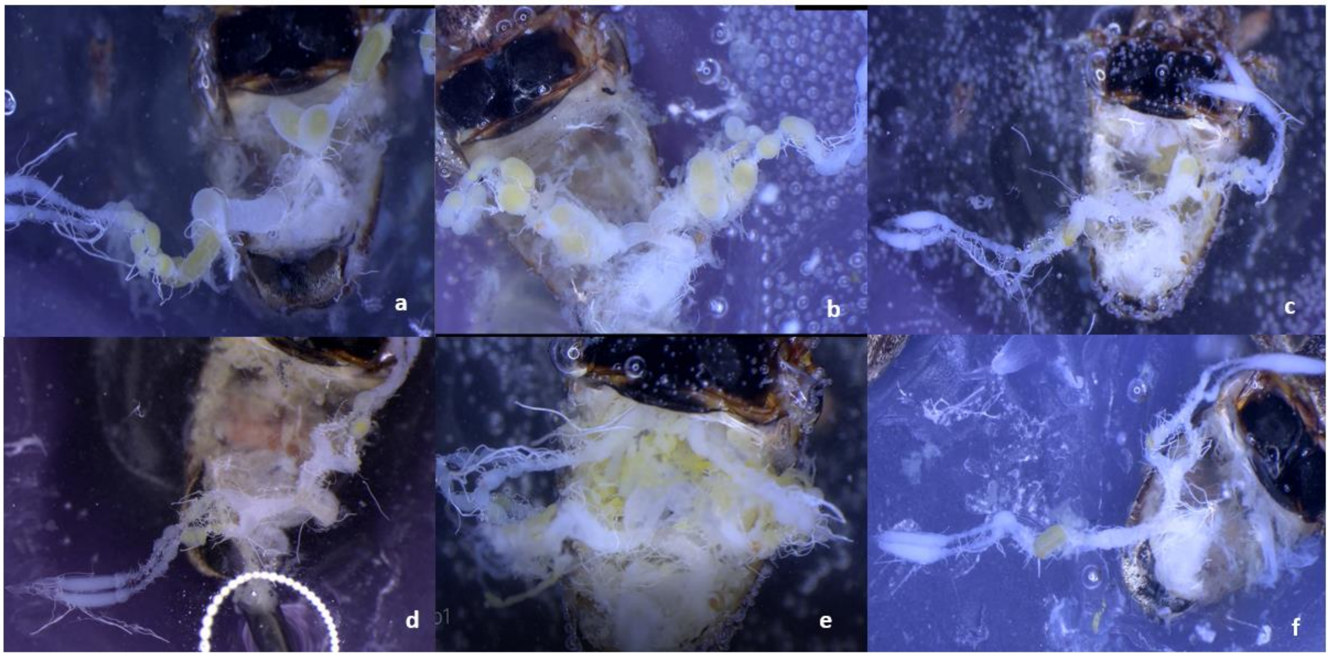
A2. Two weeks old *Gonipterus* sp. n. 2 beetles



A3. Four weeks old *Gonipterus* sp.n. 2 beetles



A4. Six weeks old *Gonipterus* sp.n. 2 beetles



A5. Eight weeks old *Gonipterus* sp.n. 2 beetles.