Biology and Biological Control Potential of Bethylid Wasps

Sahand Kamal Khidr (MSc)

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Abstract

This thesis presents a series of experiments on the evolutionary ecology of the reproduction, behaviour, chemical and molecular characteristics of bethylid wasps in the genus *Goniozus*. Part One investigates host quality by varying host age after paralysis. The quality of paralyzed hosts declines with time since paralysis negatively influences life-history characteristics of *Goniozus nephantidis*. Assessment of host metabolomic profiles show which chemicals change as hosts age. Part Two investigates the effect of kin recognition on contest behaviour among adult females of *Goniozus legneri*. Competitive behaviour was thus used to study the basis of kin recognition mechanisms. Wasps that are genetic kin and wasps that are reared on the same host behave less aggressively towards each other than do non-kin and non-hostmates. It is likely that cuticular hydrocarbon profiles are used by wasps in kin recognition. The environmental and genetic influences on wasp cuticular hydrocarbon profiles were explored: chemical composition differed according to both wasp species and host species. Part Three investigates genetic characteristics of *Goniozus legneri* populations on kin recognition behaviour. A molecular genetic marker system was developed for *Goniozus* species. Microsatellites showed clear polymorphism in six primer pairs and are likely to be a valuable tool in the future for closely related species. One of these markers was utilized to assess sex allocation at oviposition, thus avoiding potentially biasing influences of developmental mortality. Developmental mortality does not differ between the sexes but mortality increased sex ratio variance across offspring groups and can obscure relationships between sexual composition and group size that are present at oviposition. A tendency for *Goniozus legneri* to lay male and female eggs in spatial separation was also observed. Although the focus of these studies is on fundamental aspects of bethylid biology, advances in all of these areas have potential to enhance the deployment of these parasitoids in biological pest control.

Impact statement

The research reported here could enhance the economy and agricultural research in various aspects because *Goniozus* is used in various biological programmes as well as in evolutionary ecology research. Thus exploring different behaviours, chemical and molecular aspects could further boost scientific knowledge and applied biology

potential. For instance the use of Nuclear Magnetic Resonance could help to save time and costs associated with using different tests to assess the nutritional properties of hosts used in mass rearing programmes. This methodology can be applied to many different host-parasitoid associations. The development of molecular markers and assessment of cuticular hydrocarbon profiles for *Goniozus* has the potential to facilitate insect identification (there are well over 100 species belonging to this genus) especially when species identification on the basis of morphology may require detailed taxonomic training and the number of qualified museum professions appears to be dwindling. Investigations on factors (e.g. host size, host age, female size) that affect clutch size decisions can form the basis for useful advice for biocontrol practitioners on how to increase the reproductive potential of parasitoids to control pests.

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Chapter 1 : General introduction

1.1 The aim of biological control

The practice of human agriculture is thought to have started around 10,000 years ago (Tudge 1998, Hunter 2007). It is to be expected that agriculturalists will strive to improve both quality and production. In the present day, demand for enhanced food production is as topical as ever due to human population growth (Zeigler & Mohanty 2010, Ronald 2011). There has been a 'Green Revolution', which started in the late 1960s, to increase agricultural production around the World through adopting new strategies, such as the development of high-yielding varieties of cereal grains (Gaud 1968); it is though that there is probably now need for a second phase to this revolution (Zeigler & Mohanty 2010, Lucas 2011). Global food security involved both improved pruduction and improved protection of agricultural products (Ronald 2011, Benton et al. 2011, Gregory & George 2011). High proportions of produce may be lost to agricultural pest, both pre- and post harvest. Strategies of pest control have thus attracted a great deal of attention. Chemical control for pests might achieve higher yields but a major problem is that their toxicity has a negative impact on both humans as well as the environment in general (Pimentel et al. 1980). There are further reasons for finding alternative methods of chemical pesticides. For instance if biodiversity in agroecosystems declines, many natural enemies as well as pest species will be killed. This can lead to a resurgence of pest problems in the absence of natural enemies. Secondly the poisonous residues on food raise a major concern for food safety. Finally applying of powerful chemical agents might cause the appearance of resistant strains making pesticide application a poor long term solution (Hajek 2004).

Due to problems associated with chemical control, emphasis has been placed on alternative such as biological control. Biological control is the deliberate use of living organisms, termed natural enemies, to reduce the undesirable effects of pests and disease through regulating their population densities (Debach & Rosen 1991, Eilenberg et al. 2001). Natural enemies include parasitoids, predators and pathogens. The latter category includes micro-organisms such as fungi, nematodes, protozoa, bacteria and viruses (Lacey et al. 2001, Hajek 2004). Parasitoid species such as *Bracon hebetor* and *Cotesia glomerata* while predators such as *Coccinella septeumpunctata* and *Rodolia cardinalis* have been frequently used. These natural enemies have the

potential to maintain the pest population below the economic damage threshold within the released environment.

The use of biological control began to attain wide attention when introduction of the vedalia beetle, *Rodolia cardinalis,* against cottony cushion scale (*Icerya purchase*) infestations saved the Californian citrus industry in the late 19th Centuary (Caltagirone and Doutt 1989). In general biological control can be considered a relatively cheap method in which the benefits far outweigh the costs when it is successful with potentially minimal effects on the environment and little or no health risk to humans.

1.1.1 Most selected organisms

Hymenoptera parasitoids appear to be the most effective natural enemies and more than half successful biological control programmes conducted by these organisms (Greathead 1986, Murphy & Moore 1990, Debach & Rosen 1991, Wajnberg & Hassan 1994, Mason & Huber 2001, Jervis 2005). Coccinellid beetles (order Coleoptera) are the most successful predators and have been introduced numerous times as a biological control agents to control arthropod pests in a range of agroecosystems (Caltagirone & Doutt 1989, Grill et al. 1997).

Regarding the pathogenic groups, the bacteria *Bacillus thuringiensis* is considered not only the most successful pathogen but also the most sold biopesticide (Kharbade et al. 1998, Hansen & Salamitou 2000, Lacey et al. 2001). Other microbial natural enemies such as fungus groups (especially, *Beauvaria bassiana* and *Metarhizium anisopliae*) have been used extensively due to their ability to penetrate the target insect's integument (Ihara et al. 2003, Akbar et al. 2004, Gindin et al. 2006).

1.1.2 Risks and requirements in biocontrol agents

Ecologists interested in the effect of biological control and its impact on the environment have shown that using this approach is not without its problems, especially during the implementation of classical biocontrol. In classical biological control, foreign natural enemies are deliberately imported from one region to another to control previously introduced, or native, pests. Indeed, the introduction of exotic natural enemies in order to control a specific pest species might have a negative outcome on the non-target species (Stiling & Simberloff 1999, Follett & Duan 2000) which in turn have intricate consequences on the ecosystem and food web linkages

(Simberloff 1992, McEvoy 1996, Thomas & Willis 1998, Louda et al. 2003, Memmott 2000, Henneman & Memmott 2001, McCoy & Frank 2010).

In order to evaluate the jeopardy associated with biocontrol programmes a series of tests was proposed to give an insight of how to establish a concrete strategy to make it more specific as well as safer (Frank 1998, McEvoy & Coombs 1999, Louda et al. 2003, van Lenteren et al. 2003). Thus, there are certain basic criteria that should be available in the organism that will be used as a biocontrol agent, such as short development life cycle in accordance to the pest and their efficiency in reproduction (Huffaker & Messenger 1976, Debach & Rosen 1991). Furthermore, when natural enemies selected should take in consideration how feasible it is to mass rear them in the laboratory in order to be ready for release into the field when necessary. However, to achieve satisfactory results, it is always recommended to execute a sequence of investigation before their release in to the novel ecosystem (Waage 2001, Arnett & Louda 2002, Kidd & Jervis 2005).

On the other hand, eventually if the natural enemies passed successfully through the tests another question will arise whether to release a single or multiple species to control the herbivorous pests (Smith 1929, Watt 1965, Myers et al. 1989, Denoth et al. 2002, Batchelor et al. 2005). Still it is not completely clear if expanding in the natural enemies' biodiversity will lead to stengthen or weakness in the method.

Several researchers have compared single and multiple species releases (Denoth et al. 2002, Matsumoto et al. 2003, Snyder et al. 2008). The supporters of single release justify their approach by stating that releasing multiple species will increase their interference and decrease their effectiveness (Myers et al. 1989, Ferguson & Stiling 1996, Wen & Brower 1995, Rosenheim et al. 1999, Snyder & Ives 2001, Denoth et al. 2002, Pedersen & Mills 2004, Batchelor et al. 2005, Batchelor et al. 2006). Consequently the combined effect of multiple species might negatively affect the biological control process rather than improving it.

Those in favour of multiple releases defend their attitude on the basis that utilising a range of enemy species promote for each other which enriches the community stability when they operate additively with each other to suppress herbivorous densities and tend to attack different life stage of the host (Kindlmann & Ruzicka 1992, Bogran et al. 2002, Wilby et al. 2005, Snyder et al. 2006). However, either type

of release might not be sufficient alone to suppress and regulate the pest's population below the economic threshold level. Thus, natural enemy release might usefully be combined with various pest management practices such as cultural and chemical control in a compatible manner. This offers long term solutions and helps to achieve the best management as a part of integrated pest management (IPM) (Hendricks 1995, Miranda et al. 2005, Subaharan & Ravindran 2009). Further, improving the success rate of biological control might be achieved via an understanding of the evolutionary ecology of life-history and behavioural characteristics such as, contest behaviour, clutch size, sex ratio, effects of host quality and their consequences for population biology.

1.2 Parasitoids and evolutionary studies

Parasitoids occur in several different insect orders the Hymenoptera, Lepidoptera, Diptera and Coleoptera (Clausen 1940). However, hymenopteran parasitoids are by far the most numerically dominant group, with more than 65,000 species described (Gordh et al. 1999).

Parasitoid wasps produce offspring by depositing their eggs on or in suitable host organisms. Female parasitoids usually paralyze the host prior to parasitism by injecting venom via the ovipositor in order to restrict the hosts' movement temporarily or permanently. In case of temporary paralysis (koinobiosis), the host continues to grow and feed so that the offspring's fitness (such as the adult size, longevity, searching ability and egg supplies) will be reliant on the host's growth. While with permanent paralysis (idiobiosis), the progeny must develop using the resource present at the time of attack (Askew & Shaw 1986, Godfray 1994). These parasitoids collectively are termed koinobiont and idiobiont respectively.

Ectoparasitoids lay eggs onto hosts; because their development is external and they commonly live in protected places. Endoparasitoids develop inside the hosts and the host may be found in more exposed places. Both kinds of development can be either 'solitary', when only one larva emerges from each host, or 'gregarious', when more than one offspring develop from a single host. Nevertheless, living alone or in groups the larvae would feed on the host to complete development and kill the host at the end.

Solitary parasitoids often display aggressive larval behaviour e.g. through the use of mandibles and high level of competition among progeny when more than one egg is laid into a host. Consequently only one larva can be successful and manage to survive to adulthood (Salt 1961, Godfray 1987, Marris & Casperd 1996, Mock & Parker 1997). In gregarious parasitoids within-brood competition is less intense and many individuals can develop successfully on the same host (Godfray 1994, Quicke 1997, Mayhew & Hardy 1998).

In most parasitoid species the number of the offspring developing in or on the host is under the mother control during the oviposition but later lethal contest might interfere between siblings during their development stages and their number might reduce significantly. There are also other sources of development mortality that may operate such as encapsulation by hosts, infanticide by adult parasitoids or death due to intrinsic genetic defects (Godfray 1994, Netting and Hunter 2000, Kapranas et al. 2011). An interesting case of limited maternal control occurs in polyembryonic wasps when the mother deposit a single egg or two eggs but later their numbers will increase clonally to several, hundreds or even thousands of genetically identical individuals (Doutt 1947, Ode & Strand 1995).

In accordance to the mode of egg production, parasitoids can be classified as: (1) Pro-ovigenic, in which the life-time complement of eggs is matured ahead of adult emergence (Jervis & Kidd 1986, Jervis & Copland 1996). The ecological condition behind this incidence such as the influence of the egg and adult size in relation to total resource allocation and travel costs has been tentatively investigated by mathematical modelling (Ellers & Jervis 2004). (2) Synovigenic, when females have few ripe eggs upon emergence and can mature further eggs and maintain their egg supply during the life span. The majority of parasitoid species are synovigenic (Jervis et al. 2001, Ellers et al. 2000).

Parasitoids can occur in different trophic levels of food chains with respect to the kind of host they attack. Primary parasitoids usually attack free-living hosts while secondary and tertiary parasitoids can hyper-parasitize both primary and secondary parasitoids subsequently (Godfray 1994, Harvey et al. 2009). In addition, facultative hyper-parasitoids can be distinguished from obligate hyper-parasitoids by their optional development on another parasitoid or on the free-living host, while the

obligate can develop only as hyper-parasitoids (Gordh 1981, Sullivan 1987, Pérez-Lachaud et al. 2004).

With respect to host development, the immature stages (e.g. egg, larva, pupa) are usually more sensitive to attack by parasitoids, although a combination of more than one stage may be utilised. However, rarely some species might attack the adult stage (Clausen et al. 1927, Espinosa et al. 2009). Similar to developing on different host stages, some parasitoids can develop on different host species and called polyphagus parasitoids, while oligophagus parasitoids can develop on limited number of host species. Further, monophagus parasitoid can attack a single specific host species only.

For all the aforementioned diversity in their behaviour, parasitoids are often regarded as good 'model' organisms in evolutionary studies. Thus, there has been enormous interest in examining the fitness consequences associated with variations in clutch size and sex ratio strategies in parasitoids.

1.2.1 Clutch size

Decades of research on clutch size in animals have provided valuable insights into both behavioural ecology and life-history strategies through testing hypotheses (Lack 1947, Godfray et al. 1991, Hardy et al. 1992, Wilson & Lessells 1994, Renison et al. 2002).

The pioneering studies of Lack (1947) on birds opened wide scope for further investigation on the evolution of clutch size. The well known concept of the Lack clutch size assumes that a mother depositing a clutch of eggs should maximise the fitness of each group of young. Also the resource quality will affect the clutch decision; as a result smaller clutches are predicted for lower quality resources. Consequently this hypothesis was later applied to other taxa such as parasitoids (Charnov & Skinner 1985, Parker & Begon 1986, Ives 1989, Godfray et al. 1991, Godfray 1994).

When there is a trade-off between current and future reproductive success, mothers will be selected to deposit smaller clutches than the Lack clutch size. For instance, when hosts were plentiful and there is a cost in time to depositing eggs, it is advantageous for the mother to spend less time producing each clutch and then seeks another host. As a result fewer eggs will be produced on each host (Charnov &

Skinner 1984, Skinner 1985). Reproduction of the parasitoids may also be constrained by the availability of eggs (Waage & Godfray 1985, Jervis 2005).

Cluch size decisions are important to all animals, because the optimal number of eggs laid by the mother is closely associated with the progenies quality (Smith & Fretwell 1974, Wilson 1994, Godfray 1994). Therefore, often when the number of eggs in a clutch is increased the individual's fitness (in terms of survivorship, body size and fecundity) will decrease, which indicates that better-provisioned offspring are fitter (Hardy et al. 1992, Stearns 1992, Mangel et al. 1994, Zaviezo & Mills 2000).

A positive relationship between fitness and body size is often observed among insect parasitoids (Hardy et al. 1992, Godfray 1994, Vet et al. 1994, West et al. 1996, Ellers et al. 1998, Rivero & West 2002) and in turn body size might be used as a correlates to components of fecundity.

1.2.2 Sex ratio

The evolution of sex allocation is one of the most prolific domains of behavioural ecology research and investigates various factors that might influence an organism's reproductive decisions with respect to producing male and female progeny (Charnov 1982, Hardy 2002, West 2009). Hymenopteran parasitoids have provided excellent opportunities to examine basic structure of sex determination due to (haplodiploid arrhenotoky) in which (in the majority of species) female offspring develop from fertilized (diploid) eggs inheriting both paternal and maternal genetic material, while males develop from unfertilized (haploid) eggs receiving only the maternal chromosomes (Flanders 1965, Crozier 1977, Cook 1993a, Ode & Hardy 2008, Mateo Leach et al. 2009). In view of the fact that mated female parasitoids store sperm in a spermatheca, as a result the decision to determine the sexes of their progeny to some extent will be mediated via opening or closing of the spermathecal valve to produce female or male offspring (e.g. Flanders 1965, Suzuki et al. 1984, Godfray 1994, Ode & Rosenheim 1998).

Within haplodiploid arrhenotokous species, the optimal investment into female and male offspring is a sophisticated problem due to the occurrence of complex life histories of some species (Cook & Crozier 1995, Butcher et al. 2000b, Ode & Hunter 2002, West 2009). Mating systems may be constrained by different genetic

mechanisms such as the presence of complementary sex determination (CSD) (Whiting 1939, Cook 1993a, Cook & Crozier 1995, Butcher et al. 2000a). Under CSD the sex of offspring is determined by allelic segregation either in single polymorphic locus termed (slCSD) or in multiple loci (mlCSD) (Whiting 1943, Crozier 1977, Cook 1993a, Ode & Hardy 2008, Zhou et al. 2006).

As a result, diploid individuals that are heterozygous at one or more loci would produce female progeny, while diploid fertilized and haploid unfertilized individual would develop into males if they are homozygous or hemizygous at all of the sex determining loci. In addition, diploid male embryos might not survive development or reproduce as adults; adult diploid males posses diploid sperm would generate triploid progeny, which have both low viability and fitness (Whiting 1961, Petters & Mettus 1980, Naito & Suzuki 1991, Ross et al. 1993, Cook & Crozier 1995, Liebert et al. 2005, de Boer et al. 2007).

Due to the fact that diploid males will occur only between matched mating (when the male and female share the same sex alleles) inbreeding for many generations can increase the chances of generating diploid males (Adams et al. 1977, van Wilgenburg et al. 2006, Heimpel & de Boer 2008, Ode & Hardy 2008). However, this is not the case for all parasitoid species, for instance continuously maintaining inbred lines of *Goniozus nephantidis* in laboratory for many generations did not establish any sign of diploid males or altered mortality which would indicate CSD (Cook 1993b); many hymenopteran species utilize different sex determination systems which appear to have replaced CSD (Cook 1993a). Therefore, diploid detection in population can be quite straightforward though comparing brood size and sex allocation to both sexes between inbreeds and non-inbreeds (Beukeboom et al. 2000).

Various hypotheses concerning what might influence sex ratios have been explored since Darwin's time. In fact, the first well known and formal contribution in to sex allocation theory was by Fisher in which he assumed that a balanced sex investment ratio should evolve in any panmictic population regardless the cost associated with the production of each sex (Fisher 1930). A key further advance was developed by Hamilton (1967) when he linked sex allocation optima to variation within a mating system and explained the divergence from equal investment ratios often seen in some (mainly) invertebrate systems. Hamilton's theory predicts female biased sex ratios under moderate or high degrees of local mating competition (LMC), due to the fact

that such bias reduces competition among related males as well as increasing the availability of mates for sons (Ode et al. 1998). As in patches with surplus sons, the male competition for access to their sisters might escalate to fatal injuries during the combat (Murray 1987, Abe et al. 2003). Further, fragmented habitats would promote LMC; for example, high dispersal of host patches may result in low foundress numbers which in turn would promote low probability of finding non-siblings. On the other hand, under LMC, sisters that emerge in patches lacking brothers have zero fitness because they are constrained to producing only sons. Thus, mothers are predicted to reduce the bias in their progeny sex ratios (approaching 1:1 ratio) when there are more mothers (foundresses) producing offspring in the patch (Hamilton 1967, Taylor 1981).

Another key sex allocation theory predicts the response to host quality (Charnov et al. 1981). This model can be applied to many aspects of the host quality, such as age, size, nutritional status and host species. Thus, in accordance to hosts' quality and the rate of fitness returns by each sex, on low quality resource, the male would bring more fitness to the mother than the female while on high quality hosts daughters more likely to increase the rate of fitness return the mother (Charnov 1982). Furthermore, the correlation between host size and sex allocation has been demonstrated in many parasitoid species (King 1993, Godfray 1994, Napoleon & King 1999, West 2009). Host age prior to parasitoid attack has also been shown to have a profound impact on sex allocation: a high proportion of females were deposited by the mother on younger hosts (King 1990, Ode & Strand 1995).

Despite the above mentioned theories, there are many genetic distorters that might shift sex ratio in hymenopteran parasitoids towards the transmitted sex in favour of females (Skinner 1982, Rousset et al. 1992, Stouthamer & Kazmer 1994, Ode & Hardy 2008 since the maternal genetic element would probably pass on to subsequent generation through egg cytoplasm (Bull 1983, Hurst 1993).

Thus, the most apparent mechanism for over production of female progenies is usually associated with maternity inherited microbes transmitted via cytoplasm and target male offspring (Hurst 1991, Stouthamer & Kazmer 1994, Hurst et al. 2003). Further, many selected male killing organims such as *Wolbachia* and *Arsenophonus nasoniae* would function mostly during embryonic stages and infected foundress recording high degree of differential mortality among the progeny (Huger et al. 1985, Hurst 1991,

Stouthamer et al. 1993, Balas et al. 1996, Groenenboom & Hogeweg 2002, Engelstädter & Hurst 2007).

The *Wolbachia* bacteria might cause another kind of distortion while present in the ovaries through sterile crosses termed as cytoplasmic incompatability (Breeuwer et al. 1992, Stouthamer et al. 1993, Stouthamer et al. 1999). Thus, this would not only detect when mating process occurs between the uninfected eggs and infected sperms but also when crosses occur between both infected sexes harbouring different *Wolbachia* strains (Hoffman & Turelli 1997, Werren & O'Neill 1997, Dedeine et al. 2001).

Alternatively, the inheritance of parental genetic material through sperm in few parasitoids might deviate the sex ratio or resource allocation towards male progenies only (e.g. Hunter et al. 1993, Stouthamer et al. 2001, Werren & Stouthamer 2003, Tram et al. 2006). Thus, diploid females would alter to haploid males due to losing the paternal chromosome during early development of fertilized eggs (Werren et al. 1987, 1988, Nur et al. 1988).

All the evaluation of parasitoid sex ratios in the majority of studies has relied on counting the adult offspring of each sex based on morphological characteristics. However, sex ratio at maturity (termed secondary sex ratio) might not be the same as the sex ratio at sex allocation (the primary sex ratio) due to mortality of offspring at the egg, larval or pupal stages. Developmental mortality may alter the variance in sex ratios or may affect one sex more commonly than the other (Smith & Shaw 1980, Nagelkerke & Hardy 1994, van Baaren et al. 1999, Krackow et al. 2002, Kapranas et al. 2011, Khidr et al. submitted, Chapter 6).

1.3 Parasitoid aggressive behaviour

Parasitoids may be aggressive as adults as well as as immature (Godfray 1994). The kinship between individuals might have potential impact on parasitoids behaviour in many aspects. Female parasitoids can evaluate and recognize their kin via both genetic and environmental signals (Gamboa 2004, Lizé et al. 2012, Chapter 3). Kin selection theory suggests that a genetic trait may be preferred even if it is not beneficial to the actors direct fitness, provided that the fitness of the recipient which

shared its genes would be adequately increased (i.e. indirect fitness) (Hamilton 1964b, Grafen 1984, Frank 1998, Griffin & West 2002).

The individual's relatives usually help each other to increase their fitness directly or indirectly in accordance to their level of relatedness. Thus, by assisting a close relative to reproduce, simultaneously an individual itself can increase its inclusive fitness indirectly due to the fact that its genes would be carried by the progeny of its kin to the next generation (Hamilton 1963, 1964). This suggests that individuals should be less aggressive, and more altruistic, towards closer relatives.

However, according to the model developed by (Taylor 1992, see also West et al. 2001, 2002), an excessively high level of kin competition might occur due to resource competition. This might reduce the advantage of being relatives and show more aggressiveness towards each other in comparison with unrelated species.

In general, to increase the survival rate or reproductive success in organisms, the contestants show agonistic behaviour towards conspecifics (Wilson 1975). The outcome of the contest might be resolved either when physical interaction involved in the behaviour such as biting, fighting or stinging in parasitoid wasps (Goubault et al. 2007a) or when sending different signals such as chemical to settle the contest without any direct bodily involvement (Bossert & Wilson 1963, Wilson 1975).

On the other hand, the ability to recognise and discriminate kin may influence the interference between related individuals competing for a given resource. Thus, the intensity of aggressiveness might depend on the degree of relatedness between individuals involved in the interaction. The influence of competition on altruism/selfish behaviour expression between related individuals has been addressed in few empirical studies (e.g. West et al. 2001, Giron et al. 2004, Segoli et al. 2009b).

According to Gadagkar (1985) and Pfennig and Sherman (1995) kin discrimination can be either inherited genetically through allele's recognition and phonotypical cues or via interaction between individuals within the environment. But it is worth mentioning that the mechanism behind how insects can discriminate conspecifics in accordance to genetic relatedness and environmental cues is still not completely understood for many species, though was well explored in some such as social *Polistes* wasps (Gamboa et al. 1986a). In general different cues might be associated with this

recognition. Chief among these are olfactory cues that can be transferred through the food resource (Downs & Ratnieks 1999).

The odour released from the host might assist parasitoids' orientation during their foraging and because of its small biomass they have capability to emit volatiles in the nanogram range (Turlings et al. 1995). Furthermore, parasitoids do not only display innate responses towards some food signals, but also they have capability to learn cues in order to improve their efficiency in finding foods and hosts (Takasu & Lewis 1995). Indeed, siblings developing on the same host can use smells associated with the host to recognise each other (Howard et al. 2001, Nash et al. 2008). Thus, discrimination between nestmate and non-nestmates did not occur when individuals were reared under similar controlled environmental conditions (Gamboa et al. 1986 b).

Cuticular hydrocarbons (CHCs), which primarily serve to reduce desiccation in insects (Edney 1977, Hadley 1981, Blomquist & Bagnères 2010), are considered as a vital element for kin recognition (Carlin & Hölldobler, 1986, Tsutsui et al. 2003, Dronnet et al. 2006). Consequently, CHCs can be used in communication system for species, gender and colony recognition (Haverty et al. 1990, Smith & Breed 1995, Singer 1998, Howard & Blomquist 2005, Lucas et al. 2005, Bagnères & Wicker-Thomas 2010).

The insect's integument (primarily the epicuticle) commonly consists of three major hydrocarbon compounds which are n-alkanes, olefins and methylalkanes (Lockey 1991). In social insects, cues used in kin discrimination rely on olfaction and are frequently associated with epicuticular hydrocarbons (Hölldobler & Michener 1980, Hepper 1986, Howard 1993, Lorenzi et al. 1997, Ozaki et al. 2005). The eusocial paper wasp *Polistes fuscatus* has the potential to distinguish nestmates from non-relatives through nests' odour secreted by the queen (Singer 1998).

Moreover, the learned odor would use by the wasp as a template to identify the relatives (Singer & Espelie 1992, 1996). The kin recognition between *Pachycondyla apicalis* takes place by transferring secretions either by sexual meeting or interaction with older nestmates (Soroker et al. 1998). In addition, old nestmates preferred communication with mates exposed to nest hydrocarbons rather than when they emerge while the chemical is not present (Singer & Espelie 1996).

Their major role as recognition cues can be associated with their chemical stability, low volatility and structural diversity allowing a great variability in the cuticular compound composition (Blomquist et al. 1987, Dani et al. 2001). It is generally the more common and abundant chemical surface found on the cuticle (Blomquist et al. 1987, Howard 1993).

In addition, the CHCs might play a dynamic role as chemical camouflage through imitating the cuticular hydrocarbons of the host by the insect attacker, which the organism either biosynthesis the cuticular hydrocarbons or obtain it through contact with the host (vander Meer et al. 1982, vander Meer et al. 1989, Dettner & Liepert 1994). Thus, some parasitoids hydrocarbons closely resemble the host they reared on and share majority of hydrocarbons in order to incorporate in to their life (Howard et al. 1980, Howard 1993, Howard et al. 2001). Nonetheless, both bethylid parasitoids *Cepalonomia waterstoni* and *Laelius utilis* to some extent illustrate different hydrocarbon compositions from their hosts (Howard 1992).

Furthermore, many researches revealed that the cuticular hydrocarbon composition of many parasitoid wasps was different between the sexes as well as different age classes (Howard 1992, Syvertsen et al. 1995, Howard & Infante 1996). Associations between social behaviour exhibition and family Bethylidae have been observed in some studies since early times (Evans 1964, Casale 1991). However, the profile changes of bethylid wasps in response to cuticular hydrocarbons observed more clearly when the wasp reared on different host larvae (Howard & Pérez-Lachaud 2002).

The aforementioned examples confirm that the cuticular hydrocarbon profile of insects is flexible and capable of transformation in accordance to environmental, ontogenetic, physiological and nutritional factors (Lockey 1988, Howard 1993, Howard et al. 1995, Howard & Pérez-Lachaud 2002).

1.4 Bethylid parasitoids

The Bethylidae is one of the most distributed families of aculeate wasps (order Hymenoptera) (Gordh et al. 1983, Gauld & Bolton 1988). The family is thought to include more than 4000 described and undescribed species (Gordh & Móczár 1990, Polaszek & Krombein 1994). Research by Evans (1964) suggests that those species

mostly belong to four broad subfamilies which are Bethylinae, Pristocerinae, Epyrinae and Mesitiinae. In addition, the relationships between these subfamilies have been studied in accordance to their morphological characters (Sorg 1988, Carpenter 1999). However, more recently (and possibly accurately) the higher level phylogeny of bethylid wasps has been estimated using molecular analysis and divided the family to five major subtaxa (Carr et al. 2010).

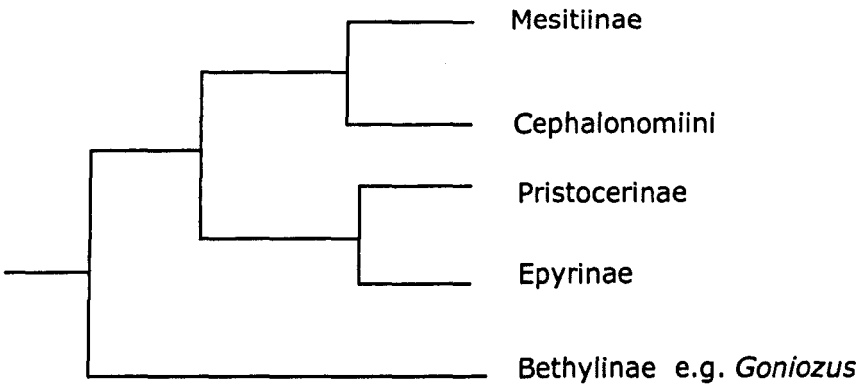

Figure 1.1 Bethylid phylogeny according to Carr et al. (2010).

Bethylid species mostly develop gregariously on the immature stages of Lepidoptera and Coleoptera (Evans 1978, Hawkins & Gordh 1986, Gauld & Bolton 1988, Gordh & Móczár 1990). Besides, they are considered as beneficial insects that have been used to attack a number of economically important crops and orchard pests worldwide; e.g. coffee, coconut, walnut, sugarcane, almond and palm. Consequently, bethylids have been used in a range of biological control programmes with some promising results (Legner & Silveira-Guido 1983, Greathead 1986, Murphy & Moore 1990, Smith et al. 1994, Lyla et al. 2006).

The females deposit eggs externally on paralyzed hosts, often with an attempt to lay them on precise locations (Gordh & Hawkins 1981, Peter & David 1991). Eggs develop to immotile larvae which feed via punctures in the host's integument and within few days pupate near the host. Soon the adult wasp emerges, varying in size usually from

1 to 10mm depending on the species. Thus, Bethylid wasps have relatively short life cycle. The sex ratio of emerging progeny is usually female biased (Hamilton 1967, Griffiths & Godfray 1988, Hardy & Mayhew 1998, Hardy et al. 1998). Moreover, the distribution of sex ratio variance is usually less than binomial (Green et al. 1982, Griffiths & Godfray 1988, Hardy & Cook 1995, Hardy et al. 1998).

Mothers of many bethylid species are distinguished by significant maternal care towards of their broods. They not only remain with the brood while it develops but also might aggressively defend their offspring against the action of both conspecific and allospecific females (Doutt 1973, Hardy & Blackburn 1991, Peterson & Hardy 1996, Goubault et al. 2007b). Maternal care probably contributes towards the relatively low offspring development mortality among broods (Gordh & Móczár 1990, Hardy & Blackburn 1991, Hardy et al. 1998).

1.4.1 Biology of *Goniozus nephantidis*

Goniozus nephantidis (Muesebeck) (Hymenoptera: Bethylidae) is a gregarious ectoparasitoid on the black headed caterpillar *Opisiana arenosella* (Walker). The caterpillar is a major pest that attacks different stages of coconut palms in India, Sri Lanka, Bangladesh and Myanmar (Cock & Perera 1987, Perera et al. 1988). The wasp can be reared on several substitute hosts; such as *Corcyra cephalonica* and *Galleria melonella* (Hardy & Blackburn 1991, Cook 1993b, Chandrika & Shameer 2003, Venkatesan et al. 2004). *Corcyra cephalonica* has been tested and used to maintain the parasitoid in culture effectively in many laboratory based experiments over the past 20 years (Kapadia & Mittal 1986, Hardy & Blackburn 1991, Hardy et al. 1992, Humphries et al. 2006). Thus, regarded as appropriate and economically valuable host for *Goniozus* mass production (Venkatesan et al. 2007).

On finding a suitable host, the female paralyzes the caterpillar and then lays between 3-18 elongate eggs 1-3 days later onto the integument of the larva (Dharmaraju & Pradhan 1977, Hardy et al. 1992, Peterson & Hardy 1996). Females are capable of parasitizing more than one host and total eggs laid might reach more than 100 during the female's life time under laboratory conditions (Antony & Kurian 1960, Hardy et al. 1992). After laying a clutch of eggs the female will typically guard the offspring during their development until the pupal stage (Antony & Kurian 1960, Cock & Perera 1987, Remadevi et al. 1981, Goubault et al. 2007b). Maternal care increases the

survivorship of developing offspring because *O. arenosella* larvae are susceptible to attack from different competitive pathogens, predators and parasitoids, including conspecific ovicide (Dharmaraju 1962, Cock & Perera 1987, Hardy & Blackburn 1991, Goubault et al. 2007b). The sex ratios of broods of *G. nephantidis* are typically highly female biased (proportion of offspring that are males is approximately 0.093) with low variance, almost certainly due to high levels of local mate competition (Hardy & Cook 1995, Hardy et al. 1999).

1.4.2 Biology of *Goniozus legneri*

The parasitoid wasp *Goniozus legneri* Gordh (Hymenoptera: Bethylidae) is an idiobiont gregarious ectoparasitoid of many important crop pests in several new world agro-ecosystems (Legner & Silveira-Guido 1983, Legner & Gordh 1992, Steffan et al. 2001, Zaviezo et al. 2007). *Goniozus legneri* are effective in attacking lepidopteran larvae especially of almond, pistachio, apple and walnuts (Steffan et al. 2001, Zaviezo et al. 2007).

On finding a host a female deposits 1-20 eggs onto the host's surface approximately 24h later after paralysis with larger clutches laid onto larger hosts (Gordh et al. 1983, Hardy et al. 1998). Females remain with their broods for several days post-oviposition to protect them and their offspriong against the detrimental actions of conspecifics (Goubault et al. 2006, Bentley et al. 2009). Development to adulthood takes approximately two weeks, development mortality in this species is estimated to be about 12% (Gordh et al. 1983, Hardy et al. 1998). Brood sex ratios are typically female biased (81-91% of adult offspring are female), with low variance, probably due to high levels of local mate compeition (Gordh et al. 1983, Hardy et al. 2008, 2000), but all-female broods are sometimes produced due to the development mortality of males (Hardy et al. 1998). Newly matured females usually disperse around 24h after eclosion to search for suitable hosts (Hardy et al. 2000).

1.5 Contest behaviour

Extensive evidence suggests that large numbers of animal species might compete with conspecific and allospecific individuals via direct agonistic interactions. Many game theoretical models have examined factors resolving the contest outcomes between individuals (Hammerstein 1981, Maynard Smith 1982, Mesterton-Gibbons 1992,

Sigmund 1993). Fighting behaviour might vary in response to the target resource whether it is food, territories or mates (Huntingford & Turner 1987, Blanckenhorn 1991, West at al. 2001, Kemp & Alcock 2003, Lindström & Pampoulie 2005, Kokko et al. 2006).

Moreover, when pairs of animal compete for an indivisible resource, contest decisions are expected to be based upon resource value, ownership status, costs and benefits, relatedness between rivals as well as fighting ability (Leimar & Enquist 1984, Enquist & Leimar 1987, West et al. 2001, Bentley et al. 2009). Usually there is positive relationship between fighting ability and body size, energy reserves, weaponry, acoustic signals that make it powerful fighter (Enquist & Leimer 1990, Mason 1996, Briffa & Elwood 2000, Taylor et al. 2001, Davidson & Wilkinson 2004, Hsu et al. 2006).

On the other hand, even if the individual has all the necessary requirements to be a good fighter, it seems sensible to consider carefully the relative costs and benefits behind this conflict. Thus, escalations of aggressiveness behaviour were envisaged in situation where the benefits of winning are potentially high relative to future expectation (Maynard Smith & Price 1973, Enquist & Leimar 1990). Some form of dangerous fighting might result in the death of one of the contestants (Hamilton 1979, Cook 2005). Other combats might cause injuries that affects individual's future fitness and posed it to the risk of predation (Clutton-Brock et al. 1979, Briffa & Sneddon 2007).

The combination of both ownership status (whether it is prior owner of the resource or intruder) and fighting ability components are termed resource-holding potential (RHP) (Parker 1974, Maynard Smith & Parker 1976, Hammerstein 1981). There are many models in response to RHP; for instance, the self-assessment model, which predicts that the individual will only assess the quality of their own RHP (Taylor et al. 2001, Prenter et al. 2006, Garland & Kelly 2006). In this case weaker contestants, with smaller RHP, will be inclined to give up the combat autonomously and without gathering information on the rivals RHP. While the mutual-assessment model predicts that decision whether to continue the fight or not would be based on not only information about its own abilities but also on the quality of their opponents (Enquist & Jakobsson 1986, Stuart-Fox 2006). Individuals appear to evaluate their rivals' capabilities through cues and direct contact with each other during the fighst as they

escalate. Thus, they might withdraw from the combat when they estimate that they are unlikely to win.

In addition, there is a cumulative assessment model which is similar to self assessment in which no direct strategies involved to evaluate opponent RHP, but they can still terminate their decision in accordance to information on opponents and greater RHP impose higher costs (Payne 1998, Briffa & Elwood 2000). So withdrawal and termination of the fight would depend on both contestants own RHP.

The method by which contestants evaluate each other's fighting ability might be associated with signals without direct physical interaction (Wilson 1975). In fact, repeating signals several times might enhance the individual's accuracy regarding its opponent potential via transferring information (Enquist & Leimer 1983, Enquist et al. 1990, Leimar et al. 1991). According to Payne and Pagel (1997), signals repeated by animals either to substitute earlier signals or to enhance the strength of previous signals.

Consequently, an individual in possession of higher total RHP will tend to win the competition (Maynard Smith & Parker 1976). So, whenever asymmetry in RHP between contestants increased, less intense fighting behaviour with shorter contest duration is predicted (Wells 1988, Englund & Olsson 1990, Jennions & Backwell 1996, Taylor et al. 2001, Gammell & Hardy 2003, Taylor & Elwood 2003).

Many other influential factors might interfere and alter the direction of the conflict settlement (Parker 1974, Grafen 1987), such as to what extent each contestant is ready to take risks and defend the resource against the rival (Resource value, RV) (Parker 1974, Maynard Smith & Parker 1976, Enquist & Leimar 1987). Hence, each contestant has a different perception of the available resource and the opponent that places greater value on the resource, either because they are particularly scarce or they yield higher fitness gains, is more likely to exhibit more agonistic behaviour during the encounter. Arising out of this when asymmetry in RHP is not present, the contest is more likely to terminate in favour of the individual that most value possession of the resource (Parker 1974, Hack et al. 1997).

1.5.1 Contest behaviour in *Goniozus legneri* and *G. nephantidis*

Studies of parasitoid contest behaviour have provided a quite comprehensive investigation of factors influencing animal contests as well as the influence of animal contests on further aspects of parasitoid life histories and the consequence of these for population and community ecology. Competition between adult females has been demonstrated in several parasitoids while foraging for suitable hosts (Hardy & Blackburn 1991, Field & Calbert 1999, Batchelor et al. 2005, Bentley et al. 2009, Mohamad et al. 2010). Contests between *Goniozus* females over host involve biting and stinging but only rarely result in death. (Humphries et al. 2006, S.K.K. pers. obs.): in the majority of fights no direct indication of physical injuries was observed (Petersen & Hardy 1996).

There is positive association between the female weight and contest resolution. If the variable size increased probably the contestant fighting ability would improve and consequently lead to won the combat (Peterson & Hardy 1996, Stokkebo & Hardy 2000, Humphries et al. 2006). In addition, contests between owners and intruders are influenced by prior ownership status (Goubault et al. 2006 Figure 1.2, Bentley et al. 2009). However, when both components are present simultaneously, ownership would be advantages and larger intruders might lose versus smaller owners (Peterson & Hardy 1996, Bentley et al. 2009). Though, the probability of intruder winning the resource from owner increases significantly with both intruder size and age (Humphries et al. 2006). The numbers of matured unlaid eggs possessed by contestants (termed egg load) can also influence contest resolution, at least when other asymmetries are minimized or absent (Stokkebo & Hardy 2000).

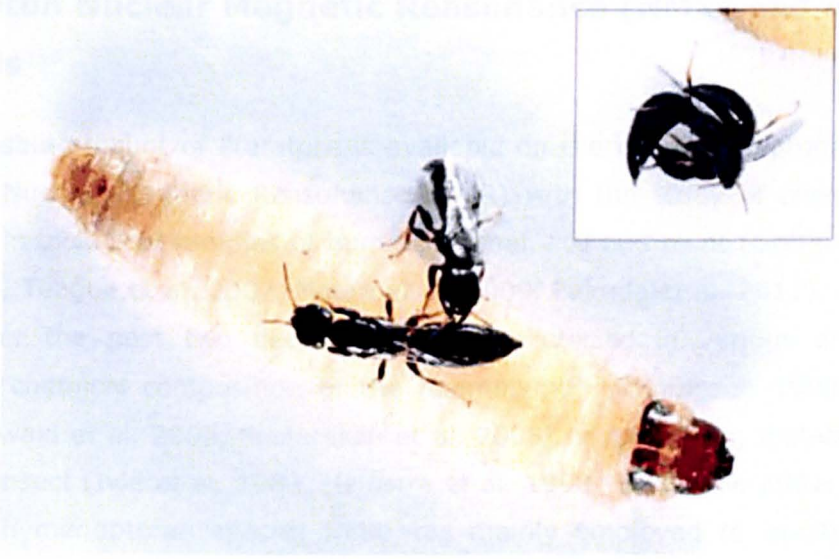

Figure 1.2 *Goniozus* females competing for *Corcyra cephalonica* host from (Goubault et al. 2006).

Previous studies have explored the value that contestant females place on the resource through varying contestant age, host size or offspring developmental stages at the time of agonistic interactions (Humphries et al. 2006, Goubault et al. 2007a & Bentley et al. 2009). Host size has an effect on contest outcomes, especially when asymmetries in ownership status are absent (Humphries et al. 2006). Among parasitoids in general, host size measurement can function as indicator for host's quality (Charnov et al. 1981, Godfray 1994). Hence, larger numbers of eggs are often laid on larger hosts (Hardy et al. 1992, Mayhew & Hardy 1998) and larger progeny can be produced on larger hosts (Hardy et al. 1992). When contests are over hosts already bearing broods, maternal defence was attuned to the vulnerability of the offspring to the actions of victorious intruders: for instance mothers of broods late-stage larvae did not defend them as effectively as when larvae were younger but intuders effected little damage on well-developed larvae compared to the larvicide and ovicide they committed when gaining access to recently oviposited broods (Bentley et al. 2009).

1.6 Proton Nuclear Magnetic Reasonance (NMR) and data analysis

A considerable amount of literature is available on the metabolite profiling technique known as Nuclear Magnetic Reasonance (NMR) with the study of chemical changes that occur in biological samples of human, animal, soil and plant (Griffin 2003, Harker et al. 2006, Teague et al. 2007, Moura et al. 2009, Fukuda et al. 2011). In the field of insects over the past two decades, NMR was utilized in various areas such as identifying chemical composition of the haemolymph (Thompson 1990, Lenz et al. 2001, Moriwaki et al. 2003, Phalaraksh et al. 2008) or generating metabolic profile of the whole insect (Ivie et al. 1983, Heinstra et al. 1990, Thompson 2001, Trivedi et al. 2010). In hymenopteran species NMR was mainly employed to isolate compounds from the wasp's venom and to characterize their molecular structure (Karst et al. 1990, Hori et al. 2001, Sforca et al. 2004, Saidemberg et al. 2010).

NMR spectroscopy is sophisticated technique that is performed on atomic nuclei; the most employed nuclei are ^{1}H, ^{13}C, ^{15}N and ^{31}P (Abraham et al. 1988, Ikura et al 1990, Thompson 1990, Adam et al. 2005, Arumugam et al. 2006). NMR spin-active nuclei have a characteristic magnetic moment when placed in a magnetic field will be absorbed and send a pulse with a range of radio frequencies (RF), generating signals that gives different peaks between magnetic energy levels (Norell 1984, Thompson 1990). ^{1}H NMR is based on detecting all proton-bearing compounds such as carbohydrates, amino acids, amines, esters, ethers, lipids and fatty acids present with different constituents in biological cells, fluids and tissue extracts (Sumner et al. 2003, Phalaraksh et al. 2008). Thus, chemical study of a variety of low molecular weight compounds found in the metabolic pathway can be measured (Sands et al. 2009).

^{1}H NMR is considered as a powerful dynamic tool because it is non-destructive to the sample integrity and multiparametric analytic techniques can be employed for data analysis, which allows nonspecific evaluation of a large number of molecular metabolites without pre-selection or bias (Jardetzky & Roberts 1981, Keun et al. 2002, van Dorsten et al. 2006). Even though ^{1}H NMR is an advanced analytical method to profile metabolites, it has some disadvantages due to comprising thousands of signals from various metabolites at high proton resonance frequencies, that might overlap and obscure the assignment procedure when applied to complex mixtures (Robertson

2005, Widarto et al. 2006). Thus, statistical data analysis techniques such as principal component analysis (PCA) have been frequently used in the analysis of metabolic profiles in a quantitative way (Gartland et al. 1991, Lindon et al. 2003, Robertson 2005, van Dorsten et al. 2006). Multivariate statistic analysis facilitates the reduction of data complexity and makes optimal use of the information present in the spectrum for sample classification (Holmes et al. 2000, Lindon et al. 2003, Robertson 2005, Robertson et al. 2011). Hence, samples that have close biochemical structure would cluster together due to their spectral similarity (Robertson 2005).

1.7 Microsatellite markers and parasitoids

Molecular markers have proved to be valuable tool in various areas of research and have revolutionized the entire scenario of biological sciences in organisms. In entomology, numerous markers are available today to study taxonomic, phylogenetic relationships and population genetics (Loxdale & Lushai 1998, Caterino et al. 2000, Behura 2006). In hymenopteran parasitoids, phylogenetic reconstruction using molecular markers has allowed clarification of the relationship between families and other taxa (Dowton & Austin 1994, Ronquist 1999, Carr et al. 2010).

One of the most important types of molecular marker is microsatellite markers which are also known as simple sequence repeats (SSRs). These basically consist of short DNA sequences that have tandem repeats of mono-, di-, tri-, tetra-, penta-, or hexa-nucleotide, with an array up to around 200bp long in the genomes of organisms (Tautz 1989, Chambers & MacAvoy 2000, Ellegren 2004). They frequently known as variable number tandem repeats (VNTR) because they belong to high mutable genomic sequence class (Tautz & Renz 1984, Buschiazzo & Gemmel 2006).

Microsatellite markers serve as prominent genetic markers for different applications due to their co-dominant, high level of allelic diversity per locus and Mendelian inheritance (Buford & Wayne 1993, Jarne & Lagoda 1996). More specifically microsatellites offer fascinating tools for studies on evolution and population genetic that can monitor the degree of inheritance and polymorphism in population, and could influence the success of biological control (Buford & Wayne 1993, Jarne & Lagoda 1996, Sunnucks 2000, Luikart et al. 2003, Selkoe & Toonen 2006, Katabuchi et al. 2008). Knowledge of the genetic variance of parasitoid species, native and imported, against target insect pest species is important in control programmes in the longer

term because this would provide information about parasitoid effectiveness in combating the insect below damage thresholds, dispersal distances and gene flow rates.

Moreover, they can identify high expected heterozygosity and high mutation rate (Jarne & Lagoda 1996, Hancock 1999, Katabuchi et al. 2008, Lavandero & Dominguez 2010). The mutation rate of microsatellites is usually influenced by both allele length (Xu et al. 2000, Whittaker et al. 2003) and the number of repeat units (Chakraborty et al. 1997, Brinkmann et al. 1998).

Despite numerous advantages of microsatellite markers, their development is still challenging; it requires the construction of genomic libraries, screening and sequencing of clones (Queller et al. 1993, Jarne & Lagoda 1996, Zane et al. 2002, Beukeboom & Zwaan 2005). However, bioinformatic analysis might be used to recognize the marker if adequate information of the species genome sequence is present (Toth et al. 2000, Vidal et al. 2009, Mikheyev et al. 2010). Markers developed for a particular species can be employed for closely related species, but the percentage of amplified loci decreases with increasing genetic distance making such markers less suitable for distantly related species (Hancock & Simon 2005, Barbara et al. 2007). Furthermore, non-amplifying alleles (termed null alleles) that are caused by mutation in the primer binding region and prevent amplification of affected alleles might be detected in some loci and can invalidate population studies because they create false homozygotes and affect population genetic analyses (Pemberton et al. 1995). Null alleles are commonly found in populations with high effective population sizes such as insects (e.g. Lehmann et al. 1997, Chapuis et al. 2005).

Thus, primers specific for the sequences of parasitoids flanking microsatellite loci required in order to amplify the marker with polymerase chain reaction (PCR), later the amplified fragments can be visualized by using a technique called gel electrophoresis that stained with chemicals such as ethidium bromide or silver stain (Vanlerberghe-Masutti & Chavigny 1997, Baker et al. 2003, Zhou et al. 2005, Douhovnikoff et al. 2006).

SSRs (simple, tandemly repeated di- to tetra-nucleotide sequence motifs flanked by unique sequences) have been developed to deal with different genetic aspects in a number of parasitoids (e.g. Zavodna et al. 2002, Baker et al. 2003, Lozier et al. 2006,

Anton et al. 2006, Katabuchi et al. 2008, Pannebakker et al. 2010). These markers have been used to investigate mating systems and the sex determination in some hymenopteran species (Ratnieks & Keller 1998, Zavodna et al. 2002, Zhou et al. 2005, Abe et al. 2009).

1.8 From behavioural ecology to population biology and biocontrol

There is substantial body of literature suggesting that behavioural ecology research on parasitoids will benefit biocontrol programmes. For instance, female biased sex ratio is valuable when parasitoids are mass released in the field because it is females that are responsible for finding and parasitizing suitable hosts (Luck 1990, Hardy & Goubault 2007, Ode & Hardy 2008). In addition, studies on parasitoids' genetic distorters that alter population and species sex ratios would help to provide possible solutions to adopt successful breeding strategies making biocontrol agents more efficient (Stouthamer et al. 1990, Werren & Stouthamer 2003, Dedeine et al. 2001, Ode & Hardy 2008). Likewise, studies on clutch size and factors influencing development mortality within each species are considered important from the biological control point of view as they enhance natural enemies' effectiveness in pest suppression (Hardy et al. 1992, Hardy et al. 1998, Kapranas et al. 2011).

Molecular markers have frequently been used to address population genetic structure in many hymenopteran parasitoids. For example, the dispersal ability of many species often revealed by utilizing genetic markers (Althoff & Thompson 2001, Zavodna et al. 2005). Further, microsatellite markers were developed to understand the influence of different geographic distribution on the population biology through genetic variation (Zavodna et al. 2005, Lozier et al. 2009).

Sex allocation and the presence of diploid males through breeding in population were shed light on by the markers (Zhou et al. 2005, Wu et al. 2003, Grillenberger et al. 2008). Therefore, from biological point of view diploid males reduce the efficiency of the population because emerged diploid male from fertilized eggs would either die or unable to reproduce during adulthood (El Agoze et al. 1994, Cook & Crozier 1995, de Boer et al. 2007). Thus, biological control might improve when more than one population from different geographic location is released in the field due to increasing allelic diversity of the population (Stouthamer et al. 1992, Cook & Crozier 1995).

In addition, host quality, especially host size, can be considered as another crucial factor which influences not only sex allocation but also clutch size decisions (Charnov et al. 1981, West 2009). Thus, in gregarious parasitoids fewer eggs are predicted on small size hosts with more tendencies to produce male progenies and vice versa (Charnov 1982, Hardy et al. 1992). Further, bigger progeny that have greater evolutionary fitness would usually emerge from bigger hosts (Hardy et al. 1992). Thus, releasing bigger females in the field would enhance biocontrol programmes through their better ability to find hosts (Kazmer & Luck 1995, West et al. 1996, Ellers et al. 1998).

Furthermore, larger females expected to have better fighting ability to take over the resource during contests (Peterson & Hardy 1996, Stokkebo & Hardy 2000, Humphries et al. 2006). Besides, contestant who placed more value on the resource is more likely to win the competition (Leimar & Enquist 1984). So, it is important to perform a series of tests in order to understand factors that might affect the contest outcome between and within species before introduced to the field. For instance, releasing different parasitoid species of coffee berry borers could result in lethal contests between species that might reduce their efficiency as biocontrol agents (Batchelor et al. 2005).

1.9 Summary of thesis structure

In this thesis various aspect of behaviour, molecular and chemical properties of different parasitoid species belonging to the genus *Goniozus* were investigated. Thus, Bethylid wasps are used as model organisms to test basic theories and to improve their efficacy in pest control. The structure of thesis is as follows:

1.9.1 Part one: Resource value, host quality and parasitoid age

Chapter 2. The effects of host age post-paralysis were explored. Laboratory studies were performed to test the reproduction and development of *Goniozus nephantidis* (Muesebeck) (Hymenoptera: Bethylidae) on hosts of different ages. Metabolic changes that occurred to the host after paralysation were evaluated by using proton Nuclear Magnetic Resonance (NMR). Caterpillars extracted then the NMR spectra of the extracts were used to identify the chemical composition of hosts.

1.9.2 Part two: Kin recognition, aggressive behaviour and chemical cues

Chapter 3. Kin recognition effects through both genetics and proxy cues on the aggressive behavior were explored by using different strains of *Goniozus legneri* Gordh (Hymenoptera: Bethylidae). The strains were reared on the facultative host *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae). Further, investigated the effect of resource value and degree of aggressiveness on contest resolution. An experimental movement of eggs between hosts were created non-siblings reared on same hosts and genetic siblings on different hosts. Later during the competition their aggressive and and non-aggressive behaviours were recorded.

Chapter 4. The cuticular hydrocarbon profiles (CHC) of different species and populations were inspected through using Gas Chromatography-Mass Spectrometry (GC-MS) while rearing on *Corcyra cephalonica*. Further, the CHC components of *G. legneri* U-strain developed on various hosts were tested to monitor profile changes due to host association cues and its influence on the chemosensory kin recognition.

1.9.3 Part three: Molecular genetics and sex ratios

Chapter 5. The effect of direct genetic kinship between both *G. legneri* populations (U-strain was obtained from a commercial insectary in the USA and C-strain collected from Chile) were addressed for relatedness [Chapter 3] by using molecular markers. Twenty four primers were designed for this species to test polymorphism between both U and C-strains. In addition, twelve other primers were developed for *G. nephantidis* in order to provide an insight of genetic variation within population.

Chapter 6. Precise sex ratio in *G. legneri* was examined by microsatellite markers. To avoid any biased maternal or paternal influence on the sex ratio reciprocal crosses were performed between both populations. The Polymerase Chain Reaction products of primer GISSR7 were run on MetaPhor agarose gel. The sex ratio was calculated at oviposition (primary sex ratio) as well as at emergence (secondary sex ratio). Direct assessment of primary sex ratios was thus coupled with comparison to secondary sex ratios and the influence of development mortality on sex ratio variance was examined.

Part one: Resource value, host quality and parasitoid age

Chapter 2 : Post-paralysis declines in host quality assessed by parasitoid behaviour, development and metabolomics

2.1 Abstract

An immature parasitoid obtains resources by feeding on a single host. The quality of a host can be defined in terms of how it affects the developmental success of immatures and their subsequent performance as adults. Similarly, the quality of an encountered host can affect the decisions of adult female parasitoids foraging for reproductive opportunities. Host quality may be determined by intrinsic properties of the host, such as its species, size and developmental stage, which further interact with extrinsic influences, such as the host's diet and any prior parasitism. Here we show that the quality of paralyzed but unparasitized hosts declines with time since paralysis, a little explored facet of host quality. We assess quality by observing the behavioural and life-history responses of parasitoids to variation in host age: older hosts are accepted less frequently for oviposition, accepted hosts have smaller clutches laid onto them, the survivorship of offspring developing on older hosts is lower and surviving adults are smaller. Adult females also compete less successfully for older compared than for younger hosts. We use Proton Nuclear Magnetic Resonance to investigate the underlying changes in the metabolomic state of aging paralysed hosts, for instance, biochemicals associated with energy decrease while metabolic waste products increase. While post-paralysis but pre-parasitism ageing is an unusual component in many host-parasitoid interactions, our metabolomic analysis serves more broadly as a methodological example that can readily be employed to understand the biochemical basis of a variety of host quality correlates across a wide range of host-parasitoid associations.

2.2 Introduction

Parasitoid wasps, by definition, obtain resources for immature development by feeding on just one host individual, usually another insect (Godfray 1994). Individual hosts vary in 'quality', which can be defined in terms of how properties of the host affect a parasitoid's evolutionary fitness. There is an extensive literature showing that many fitness-correlated aspects of the life-histories and behaviours of numerous parasitoid species are affected by host quality. Important among these are host acceptance decisions (van Alphen & Visser 1990, Thiel & Hoffmeister 2009), behavioural defence of hosts (Humphries et al. 2006), clutch size (Zaviezo & Mills 2000, Häckermann et al. 2007, Kapranas et al. 2011), sex allocation (Ode & Heinz 2002, West 2009), parasitoid development (Wright & Kerr 1988, Hochberg 1991, Hofstetter & Raffa 1998, Otto & Mackauer 1998, Pérez-Lachaud & Hardy 2001, Wang & Liu 2002, Cleary & van Ginkel 2004, Ueno 2004, Karsai et al. 2006, Häckermann et al. 2007, Hegazi & Khafagi 2008, Kapranas et al. 2011) and the size and subsequent longevity, fecundity and foraging success of parasitoid progeny (King 1998, Wright & Kerr 1988, Hardy et al. 1992, Vet et al. 1994, Otto & Mackauer 1998, Zaviezo & Mills 2000, Ueno 2004, Karsai et al. 2006, López et al. 2009, Aruna & Manjunath 2010, Fand et al. 2011).

Host quality itself usually has a multitude of contributing, and inter-connected, facets including the species of the host (Wright & Kerr 1998, Rivers & Denlinger 1995, Pérez-Lachaud & Hardy 2001, Cleary & van Ginkel 2004, Häckermann et al. 2007, Thiel & Hoffmeister 2009), its genetic composition (Kraaijeveld & Godfray 2009, Henry et al. 2010), its developmental stage (Kidd & Jervis 1991, Harvey et al. 1994, Vet et al. 1994, Otto & Mackauer 1998, Karsai et al. 2006, Thiel & Hoffmeister 2009, Fand et al. 2011), its diapause status (Rivers & Denlinger 1993, 1994), its size (Schmidt 1991, Hardy et al. 1992, Harvey et al. 1994, Otto & Mackauer 1998, Zaviezo & Mills 2000, Cleary & van Ginkel 2004, Ueno 2004) and its age prior to parasitoid attack (Ode & Strand 1995, Hofstetter & Raffa 1998, King 1998, Sousa & Spence 2001, Wang & Liu 2002, Ueno 2004, He & Wang 2006). Some of these 'intrinsic' quality components may interact with 'extrinsic' factors such as the quantity and chemical composition of the host's diet (Godfray 1994, Harvey et al. 1995, Vinson et al. 2001, Ode 2006), whether or not the host harbours endosymbionts (Cheng et al. 2010) or has been previously attacked by another (conspecific or allospecific) parasitoid (van Alphen &

Visser 1990, Goubault et al. 2007a, Moretti & Calvitti 2008, Thiel & Hoffmeister 2009) or by a pathogen (Hochberg 1991).

In this study, I explore how the quality of envenomated and paralysed hosts is affected by the time since paralysis using a host-parasitoid association in which stinging and oviposition are temporally separate events. I assess quality by observing the behavioural and life-history responses of parasitoids to variation in host age and also by using Proton Nuclear Magnetic Resonance to investigate the underlying changes in the metabolomic state of aging paralysed hosts.

2.2.1 Biology of the host-parasitoid study system

Goniozus nephantidis (Muesebeck) (Hymenoptera: Bethylidae) is a gregarious larval ectoparasitoid for which many aspects of behaviour and life-history have been documented (e.g. Hardy et al. 1992, Cook 1993b, Hardy & Cook 1995, Humphries et al. 2006, Goubault et al. 2007a,b, 2008). It is naturally associated with the coconut pest, *Opisina arenosella* Walker (Venkatesan et al. 2007), but can be reared on the larvae of several factitious hosts, such as the wax moth *Galleria mellonella* L. (Lepidoptera: Galleridae) (Mohan & Shameer 2003) and *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae) (Cook 1993b, Venkatesan et al. 2007). On encountering a host larva, the adult female parasitoid attacks it by injecting venom. Host larvae become paralysed within minutes and their development is arrested (idiobiosis). Rather than laying eggs onto the host immediately, *G. nephantidis* females oviposit after 1-3 days (Jayaratnam 1941, Dharmaraju & Pradhan 1977), possibly using the intervening period to mature eggs (Stokkebo & Hardy 2000, but see Goubault et al. 2007a). Eggs hatch about 1 day after oviposition and the larvae begin to feed on the host through punctures in its integument.

During the period between paralysis and oviposition, females remain in close physical association with their hosts, aggressively guarding them against intruding conspecific females which would otherwise utilize the unguarded host (Petersen & Hardy 1996). Although prior-ownership is an advantage in such host-ownership contests, other factors, especially body size differences, contribute to determining the outcomes of agonistic encounters (Petersen & Hardy 1996, Humphries et al. 2006) and prior owners can thus be driven away from hosts they have paralysed. As the host that the intruder has won may have been paralysed for three days and the intruder may not be

physiologically ready to oviposit for several further days, hosts may have been paralysed for a considerable time before they are fed on. Further mutual interference interactions, which may be more likely when parasitoid population density is higher, could delay host exploitation repeatedly. The empirical investigations presented here explore change in the quality of unparasitized hosts as they age following paralysis.

2.3 Material & Methods

All cultures and experiments were carried out in a climate room at 27°C, 12 L: 12D, with relative humidity maintained by evaporation from a water bath. *Corcyra cephalonica* was used as the host and was reared on a diet of glycerol, honey, corn meal, wheat bran and yeast, as reported by Lizé et al. (2012).

2.3.1 Part 1: Longevity of paralysed hosts

The number of days that paralysed hosts are likely to live was explored. This facilitated subsequent investigation (Parts 2-4) of the effects of host age across a wide age-range and allowed us to classify, in subsequent experimental design, hosts that had been paralysed for a given time as young, middle-aged and old relative to their life-expectancy.

Fifty hosts of known weight were individually exposed to a female *G. nephantidis* in a vial until stung and paralysed. The wasp was removed before any eggs were laid. Paralysed hosts were inspected daily for signs of life (movement of the body in response to gentle stimulus with paint brush, movement of mandibles or legs, visible pumping of haemolymph) until the host died or when the investigation was stopped. We stopped the investigation after 26 days when only 5 hosts were alive: these were treated as censors in subsequent survival analysis. A further censor was generated after 3 days when a host was accidentally killed during inspection.

2.3.2 Part 2: Effects of host age on host weight, parasitoid reproductive behaviour and life-history

Hosts of known weight (range: 17.71-50.95mg, to an accuracy of 0.01mg) were placed individually in vials and allowed to be stung by an initial female (the 'stinging wasp') which was then removed before laying eggs on to the host (as in Part 1). Because had have observed that older *Goniozus* individuals attack presented hosts

more rapidly than do younger individuals, we used as stinging wasps the oldest females that were available in the culture at the time.

Each paralysed host was then held in the vial for 1, 8 or 16 days, so generating three experimental treatment groups for host age ('young', 'middle-aged' and 'old'). Because substantial proportions of hosts would be likely to die before 8 or 16 days (Part 1, Figure 2.1), Iinitially allocated greater numbers of hosts to the middle-aged and, in particular, old treatment groups but the final sample sizes in this experiment inevitably had a stochastic element (39 young, 29 middle-aged, 74 old). At the appointed age, each (surviving) host was reweighed and presented to a second female (the 'laying wasp') in a vial. The response of these 142 laying wasps, and their offspring, to host age was the prime focus of the experiment.

All laying wasps were females that had emerged from their pupal cocoons within 3-5 days (the age at which most females disperse from their natal broods, Hardy et al. 1999) and was confined in the vial until its death (long associations with each host are normal in *G. nephantidis*, Goubault et al. 2007 b). The contents of vials were then inspected daily and the presence and numbers of parasitoid eggs, larvae and pupae noted. The numbers, sexes and combined dry weight of any adult offspring developing from the host were also recorded.

2.3.3 Part 3: Effects of host age on parasitoid contest behaviour

I followed methods for assessing factors influencing female-female contest behaviour in *G. nephantidis* that are given in detail elsewhere (e.g. Petersen & Hardy 1996, Humphries et al. 2006). We set up 66 owner-owner contest dyads (Humphries et al. 2006) in which closely weight-matched and age-matched (3-5 day old) paint-marked females (red or yellow), from different natal broods and with no previous experience of contest interactions, had each been presented, 15-20 hours before the contest, with a host that had been stung and paralysed by a different wasp (as in Part 2) either 1 day or 10 days previously, generating two host age classes. At the time of stinging, hosts weighed 30-60mg and were reweighed on presentation to the second female. At the start of each contest, one female was the owner of a younger host and the other female was the owner an older host and the effect of the host age difference on contest outcome was the focus of the experiment.

Contest behaviour was recorded on videotape for 90 minutes, then the winner of the interaction was noted (winning females were usually in close association with the hosts with losing females distant within the confines of the apparatus; however, there was no clear winner in 27/66 replicates and these were excluded from subsequent analysis). Both females were then reweighed immediately after the contest and these data were used to assess influences of contestant size (Humphries et al. 2006).

Analysis of the data generated from the above experiment suggested an effect of host age might be obscured by the effects of host weight differences (within the clearly-won replicates mean±SD: 0.456±0.399 mg, see Results). We therefore repeated the experiment using hosts aged either 1 or 12 days and paying extra attention to minimising the weight difference between the young and the old host within each replicate: there were 60 dyads of which 49 had a clear winner and host weight differences within these clearly-won replicates were an order of magnitude smaller than in the initial experiment (0.047 ±0.039 mg).

2.3.4 Part 4: Effects of host age on metabolomic state

Living but paralysed hosts aged 1, 8 and 16 days (n = 15, 12 and 12, respectively) were prepared as in Part 1 and then snap-frozen in liquid nitrogen and stored at -20°C overnight to halt metabolic change. Extraction was then performed using a chloroform/methanol method based on Folch et al. (1957), during which hosts were transferred individually into eppendorf tubes, methanol (1 ml) was added and samples were homogenised with a ball mill (2 min, 30 revolutions per second). The resultant homogenate was transferred to glass tubes, water (0.4 ml) and chloroform (2.5 ml) were added and samples were vortexed for 10 minutes. The resultant mixture was centrifuged (20 min, 10,000xg) until two layers formed. The two layers were separated with a Pasteur pipette and the aqueous extract dried down using a centrifugal evaporator (Thermo Scientific SpeedVac).

Proton Nuclear Magnetic Resonance (^{1}H NMR) spectra of extracts were obtained using a Bruker Avance spectrometer, operating at 400.13 MHz ^{1}H resonance frequency and equipped with a 5mm quadruple nuclei probe (QNP), a BACS-120 autosampler and z-axis gradients. All spectra were measured with 298K internal probe temperature. Prior to acquisition of spectra, each sample (0.6 ml) was mixed with D_2O (0.1 ml) and vortexed to ensure thorough mixing prior to being placed into 5mm o.d. NMR tubes.

In order to suppress the large water signal, spectra were acquired using a presaturation solvent suppression pulse sequence (NOESYPRESAT) with a relaxation delay of 1.5 s, during which the water resonance was selectively irradiated. Typically 128 transients were collected into 32k data points with a spectral width of 6000 Hz. Prior to Fourier Transform, exponential line broadening of 0.3 Hz was applied to free induction decays (FIDs) which were also zero-filled by a factor of 2. Spectra were manually phase and baseline corrected and chemical shifts were referenced internally to the lactate methyl doublet at δ 1.33 in TopSpin version 2.1 (Bruker, GMbH).

Many of the ^{1}H NMR spectral peaks could be assigned by inspection based upon comparison with existing literature (Gibb et al. 1997, Bundy et al. 2001) in combination with an in-hose spectral database. Further confirmation of metabolite identification was made using a two-dimensional correlation (COSY) NMR spectrum that provides cross-peaks in a contour plot between the chemical shifts of spin-coupled nuclei. Acquisition and processing parameters for COSY spectra included a relaxation delay of 1.86 s, a spectral width in F1 and F2 of 5995.2 Hz, 2-k time domain points, 128 F1 increments, 64 transients per increment, and qsine apodization in F1 and F2 and with 2-k points in F1 and F2.

2.3.5 Data analysis

Data generated in Parts 1-3 were analysed using generalized linear modelling with Genstat statistical package (Version 8, VSN International, Hemel Hempstead, UK). Parametric cohort survival analysis (Crawley 1993) was used to assess survival time, age-dependency and the effect of host weight on the longevity of paralysed hosts (Part 1). Effects of host age on parasitoid behaviour and developmental biology (Parts 2 and 3) were explored using logistic analyses for proportional response variables (host acceptance, developmental mortality, contest outcome), log-linear analysis for integer response variables (clutch size) and standard analysis for numerical responses (offspring weight) (Crawley 1993, Hardy and Field 1998, Wilson and Hardy 2002). In logistic analysis of contest outcome the (ungrouped binary) response variable was defined as 1=red wasp won or 0=red lost (Petersen & Hardy 1996, Humphries et al. 2006) and differences in wasp weight, host weight and host age and their interactions were fitted as explanatory variables. In log-linear analysis and analyses of grouped binary data, quasi-Poisson and quasi-binomial distributions of residuals were adopted, using empirically estimated scale parameters, to take potential overdispersion into

account (Crawley 1993, Wilson and Hardy 2002). Formal evaluations of deviation from binomial variance in sex ratio and developmental mortality used the Meelis test (Krackow et al. 2002).

Metabolomic data from Part 4 in the spectral region of δ 0.5-10.0 were reduced to ASCII format using AMIX (Analysis of MIXtures, version 3.9.2, Bruker GmbH, Germany). Each NMR spectrum was reduced to 238 discrete regions of equal width (0.04 ppm), and the integral of each region was determined (Holmes et al. 1998) resulting in a table of intensity. The region δ 4.3-5.1 was removed from all data because it contains the large water peak. Each remaining region was then normalized to the total spectral area ('block normalisation') to minimise the effects of concentration differences between the samples.

Umetrics SIMCA-P (version 11) was used for multivariate analysis of data from Part 4. Two types of scaling were used, mean-centring (subtracting the calculated average of a variable from the data so that the mean for each variable is 0) and mean-centring followed by autoscaling (division of each variable by the standard deviation for that variable). The use of mean-centred, but not autoscaled, data in basic multivariate analyses such as Principal Component Analysis (PCA) often results in an emphasis on perturbation of the metabolites that are present in high concentrations, whereas autoscaled data convert each variable to a standard variable with equal weight and are more sensitive to changes in the levels of minor metabolites. For the purpose of illustrating the results of this study, autoscaling of the data is reported. PCA was used for initial visualization of the ^{1}H NMR spectra (Part 4); this reduced the dimensionality of a dataset, which had a large number of variables, while maintaining as much variation within the data as possible (Massart et al. 1988). The results of PCA are discussed in terms of component score vectors (observation coordinate along a PC) and loading vectors (direction coefficient of a PC).

Partial Least Squares (PLS) regression modelling (Geladi & Kowalski 1986) was applied to determine the covariance between the NMR spectra and host age: a regressive model was built by maximizing the covariance between a set of variables, X, and a dependent variable, Y and then used to predict values of Y for a given set of X variables. The number of latent variables (three) used to predict Y was chosen from when the least error in prediction was observed. The PLS model was validated by dividing the data set and using half of the spectra from each group of samples as a

'training set' for the model and the remaining half as a 'test set'. The process was repeated four times using different combinations of samples to form the training and test sets in order to evaluate stability of the model.

2.4 Results

2.4.1 Part 1: Longevity of paralysed hosts

Some hosts lived for more than three weeks post-paralysis, but most had died by this age (Figure 2.1). We fitted two alternative initial models of survival time: an exponential model (estimating the rate of mortality but assuming to be constant over time) and a Weibull model (additionally estimating variation in mortality rate). The Weibull gave a significantly better fit ($G_1 = 21.2$, $P < 0.001$) indicating that the daily probability of mortality increases significantly as hosts age (Figure 2.1). Adding host weight to this statistical model did not improve the fit, indicating that there is no relationship between host size and longevity ($G_1 = 0.02$, $P > 0.1$).

These survival results were then used to choose and define three age classes of hosts for Part 2: as ≈90% of hosts died by 16 days, we defined hosts of 16-days since paralysis as 'old'. All hosts survived for at least one day post paralysis and 1-day old hosts were defined as 'young'. At the intermediate age of 8-days ≈70% of hosts were alive and these were defined as 'middle aged'. For Part 3 we similarly defined 'older' hosts as those at 10- or 12-days post-paralysis, when ≈50% of hosts were alive, 'younger' were defined as 1-day post paralysis. It was thus the age classifications, rather than the hosts themselves, that were used in subsequent experiments.

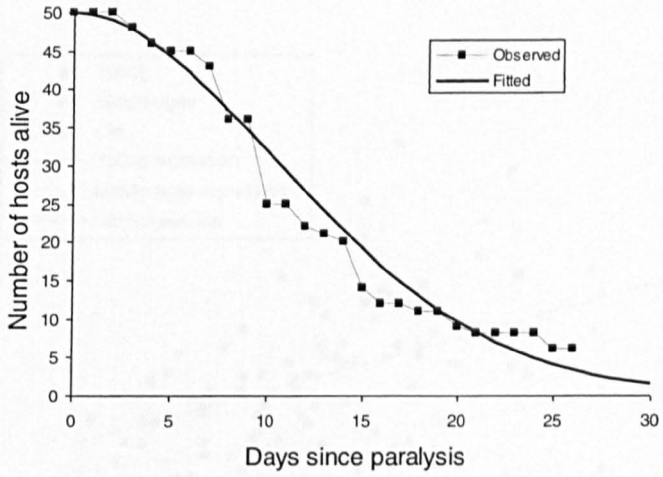

Figure 2.1 Cohort survival of paralysed hosts. The arrows show the age-classes used as host age treatments in subsequent experiments. By week one ≈14% of the hosts had died and by week two ≈76% of the hosts died.

2.4.2 Part 2: Effects of host age on host weight, parasitoid reproductive behaviour and life-history

2.4.2.1 Host weight change

The weight of individual hosts generally decreased between paralysis by the stinging wasp and presentation for oviposition (Figure 2.2). Weight loss was significantly greater for initially heavier hosts ($F_{1,136}$= 17.99, P<0.001) and for hosts that were stored for longer before, and were still alive at, presentation ($F_{2,136}$ = 74.53, P<0.001); these effects also interacted significantly ($F_{2,136}$ = 3.70, P=0.027) i.e. the graphs are not parallel.

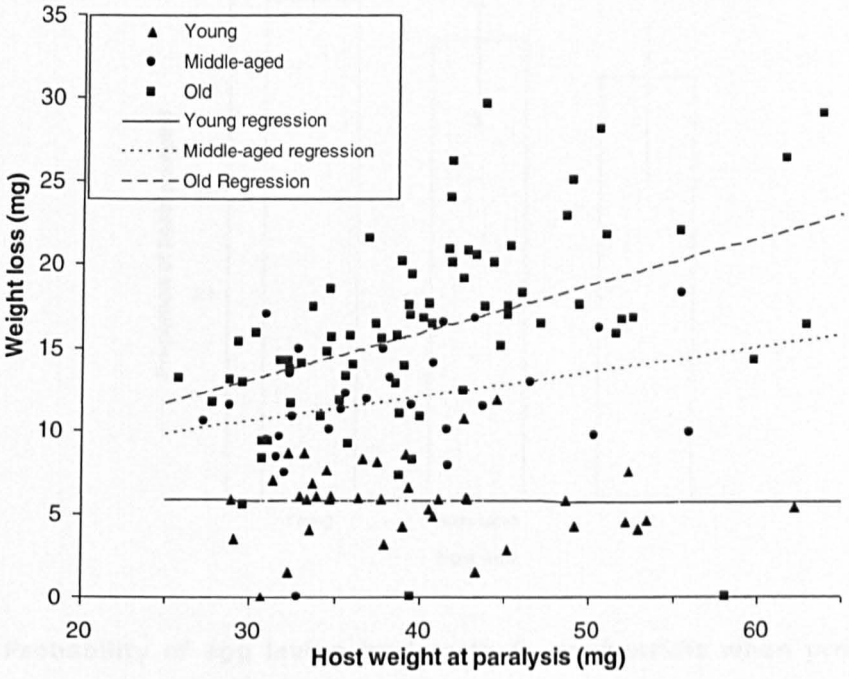

Figure 2.2 The effects of initial weight and time since paralysis on the weight lost by surviving hosts.

2.4.2.2 Host acceptance

The probability that a female oviposited on a given host was not significantly affected by the weight of the host at presentation (G_1 = 1.79, P = 0.181) or by an interaction between host weight and age (G_2 = 2.73, P= 0.065) but was affected by the age of the host (G_2 = 3.33, P = 0.036, all results from logistic analysis) with the probability of egg laying declining as host age increased (Figure 2.3).

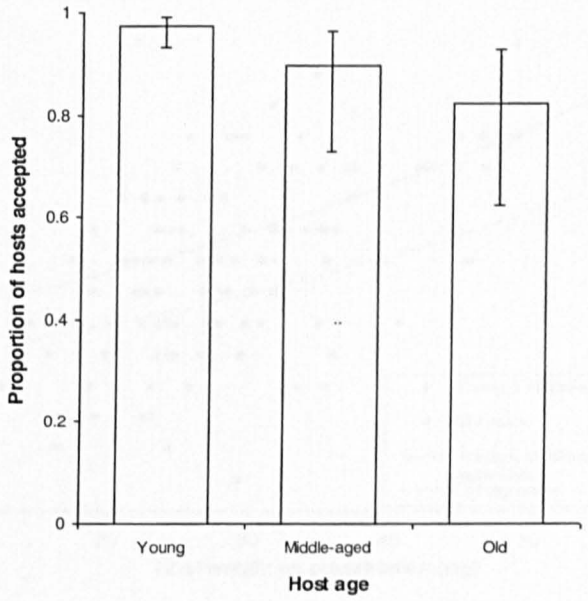

Figure 2.3 Probability of egg laying by female *G. nephantidis* when presented with hosts at different ages since paralysis. Means ± (asymmetric) S.E.s are back transformed from logit-scale estimates.

2.4.2.3 Clutch size

The number of eggs laid on the host (Figure 2.4) by the 125 females that accepted the host for oviposition was positively related to host weight (log-liner analysis corrected for overdispersion: $F_{1,121}$ = 21.10, P<0.001) and negatively to host age ($F_{2,121}$ = 22.87, P<0.001) with no significant interaction between these variables $F_{2,119}$ = 0.66, P=0.516). The sizes of clutches laid on young and middle-aged hosts were, however, statistically indistinguishable (aggregation of factor levels: $F_{1,121}$ = 0.0005, P=0.979).

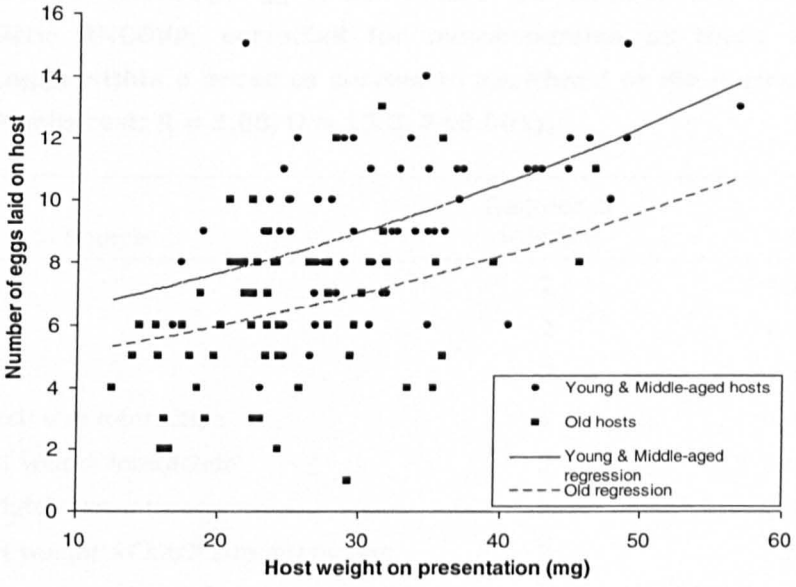

Figure 2.4 Clutch size decisions in relation to host age and weight.

2.4.2.4 Developmental mortality

The probability of egg survival to adulthood was negatively influenced by host age and positively influenced by host weight and also by clutch size via an interaction with host age (Table 2.1). These results derive from 124 of the 125 clutches laid, as an unknown number of offspring escaped from a vial containing a brood of initially 11 eggs. The mean probability of an egg developing successfully was 0.586 (S.E. ±0.06) on young hosts, 0.430 (±0.08) on middle-aged hosts and 0.273 (±0.06) on old hosts. Survival was unaffected by the weight of the host divided by the number of eggs laid, a rough index of the quantity of resource available to each offspring ($F_{1,122}$ = 0.006, p = 0.978).

Table 2.1 Factors affecting egg-to-adult survival. Results are from backwards stepwise logistic ANCOVA, corrected for overdispersion as there was a strong tendency for eggs within a brood to survive to adulthood or die during development collectively (Meelis test: R = 3.88, U = 15.0, P<0.001).

Source	Degrees of freedom		*F*	*P*
Host age	2		4.54	0.013
Host weight	2		4.69	0.032
Clutch size	2		0.39	0.535
Host age × Clutch size interaction	1		4.30	0.016
Host age × Host weight interaction	2		0.17	0.843
Host weight ×Clutch size interaction	1		0.00	0.997
Host age × Host weight ×Clutch size interaction	2		1.84	0.164
Residual deviance	112	678.16		
Total deviance	123	896.09		

2.4.2.5 Sex ratio

The sex ratios (proportion of adult offspring that were male) of broods that produced adult offspring (N=69) were unrelated to the age of the host at oviposition (logistic analysis corrected for overdispersion: $F_{2,68}$ = 0.06, P = 0.94) and qualitatively the same result was found if broods containing only male offspring (which are likely to have been produced by unmated mothers, Hardy & Cook 1995) were excluded ($F_{2,50}$ = 0.47, P = 0.63). The mean sex ratio of broods containing females (indicating the mother had mated) was 0.129 (S.E. ± 0.2) and sex ratio variance was not significantly different from binomial (Meelis test: R= 0.833, U = -0.44, P>0.05).

2.4.2.6 Offspring size

Adults developing from broods containing females had greater dry weight than those in broods containing males only ($F_{1,65}$ = 5.80, P = 0.019). As males are known to be smaller than females when developing on hosts of unmanipulated quality (e.g. Hardy & Mayhew 1998) and are usually the much rarer sex in broods produced by normal, mated, mothers (Hardy & Cook 1995 and see above), further analysis was restricted to the 44 broods containing entirely or largely females (sex ratios ranged between 0.0 and 0.2).

Dry weight was positively influenced by host weight ($F_{1,38}$ = 8.51, P = 0.006) and brood size ($F_{1,38}$ = 24.35, P <0.001) and was negatively influenced by the age of the host at presentation ($F_{2,38}$ = 3.49, P = 0.04) but there was no significant difference between the dry weights of wasps developing on young and middle aged hosts ($F_{1,39}$ = 0.01, P = 0.918, Figure 2.5). There were no significant interactions between the effects of host age, brood size and host weight.

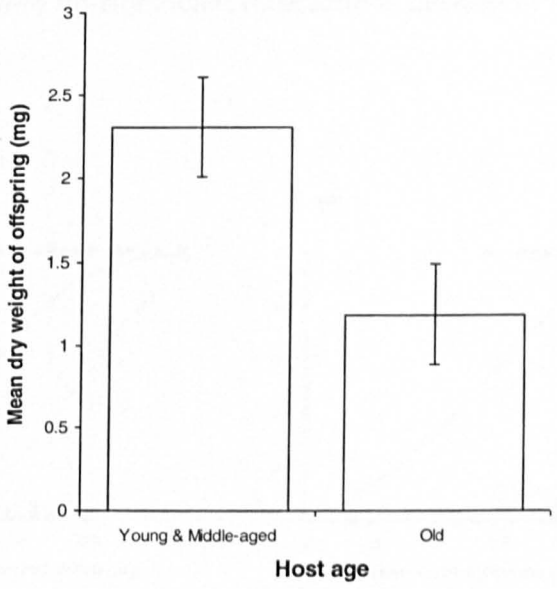

Figure 2.5 Effect of host age on adult parasitoid mean dry weight (± S.E.D). Parasitoids developing on 1- and 8-day old hosts had similar weights but those developing on 16-day old hosts were smaller. Parasitoid weight was also influenced by brood size and host weight (see text).

2.4.3 Part 3: Effects of host age on parasitoid contest behaviour

The probability of winning a contest was increased by both being the larger contestant and by being in initial possession of the larger host (wasp weight difference × host weight difference interaction: G_1=6.18, P=0.013) but not by the age of a contestant's host (G_1=1.44, P=0.231). However, a tendency for owners of younger hosts to win

contests was detected when host age was fitted in a model with wasp weight difference or fitted alone (host age: G_1=4.62, P=0.032). These latter indications lead to the repeat of the experiment with host weight differences minimised as far as practicably possible. This first series of replicates also generated one instance of fatal fighting between contestant females.

Analysis of data from the second series of replicates indicated that probability of winning a contest was increased by being the larger contestant (G_1=18.36, P<0.001, Figure 2.6a), by being in initial possession of the larger host (G_1=19.77, P=0.002, Figure 2.6b) and by being the initial owner of the younger host (G_1=17.58, P<0.001, Figure 2.6a,b). There were no significant interactions between these main effects.

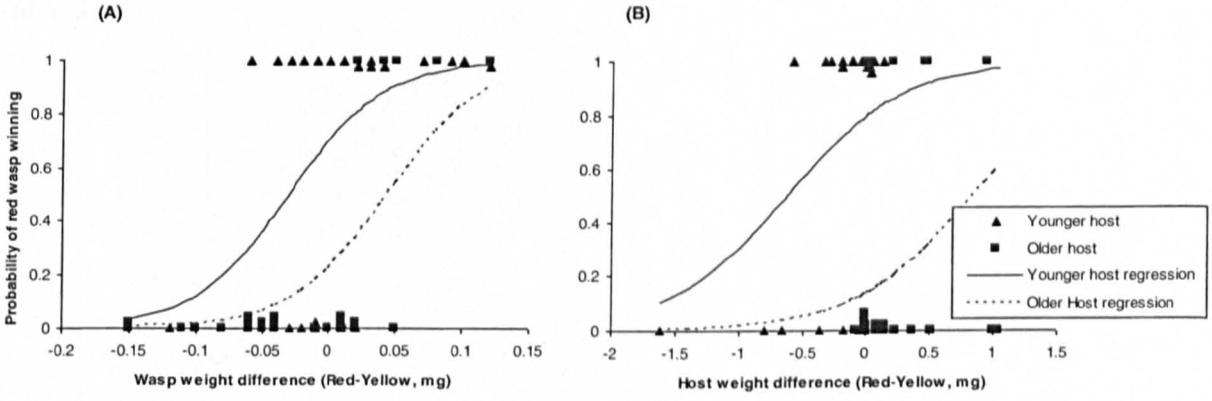

Figure 2.6 The influences of differences in wasp weight, host weight and host age on the outcomes of owner-owner contests. Panel (a) shows effects of wasp weight and host age differences while panel (b) shows effects of host weight and host age differences. The curves are the estimated probabilities of the red-marked wasp winning when it was the initial owner of either the young or the old host (see text section 2.3.3). The parameters for these graphs were estimated by separate logistic analysis of size and age and weight and age; the main text reports results from analysis of these three effects simultaneously. In cases of overlap, data points are shown vertically displaced to illustrate sample sizes.

2.4.4 Part 4: Effects of host age on metabolomic state

The compounds identified within paralyzed hosts included free primary amino acids, sugars (such as glucose), citric acid cycle intermediates (such as citrate, a-ketoglutarate and fumarate) and other organic acids including lactate, formate and acetate. A nucleotide derivative was observed and tentatively assigned as ATP on the basis of peak multiplicity: the downfield singlet observed at δ 8.61 was found to be present at δ 8.54 in the reference spectrum. Similarly, the resonance at δ 5.20 was tentatively assigned to trehalose (a sugar) on the basis of comparison with literature (Gibb et al. 1997) but due to peak overlap with glucose in the region δ 4-3, this identification is unconfirmed. Many other compounds within the spectrum remain unassigned, as is common with NMR spectra of biological samples. Figure 2.7 shows a typical 400 MHz ^{1}H NMR spectrum of an aqueous extract from a host 16 days post-paralysis and the assignments of all identified peaks within the spectra are given in Table 2.2.

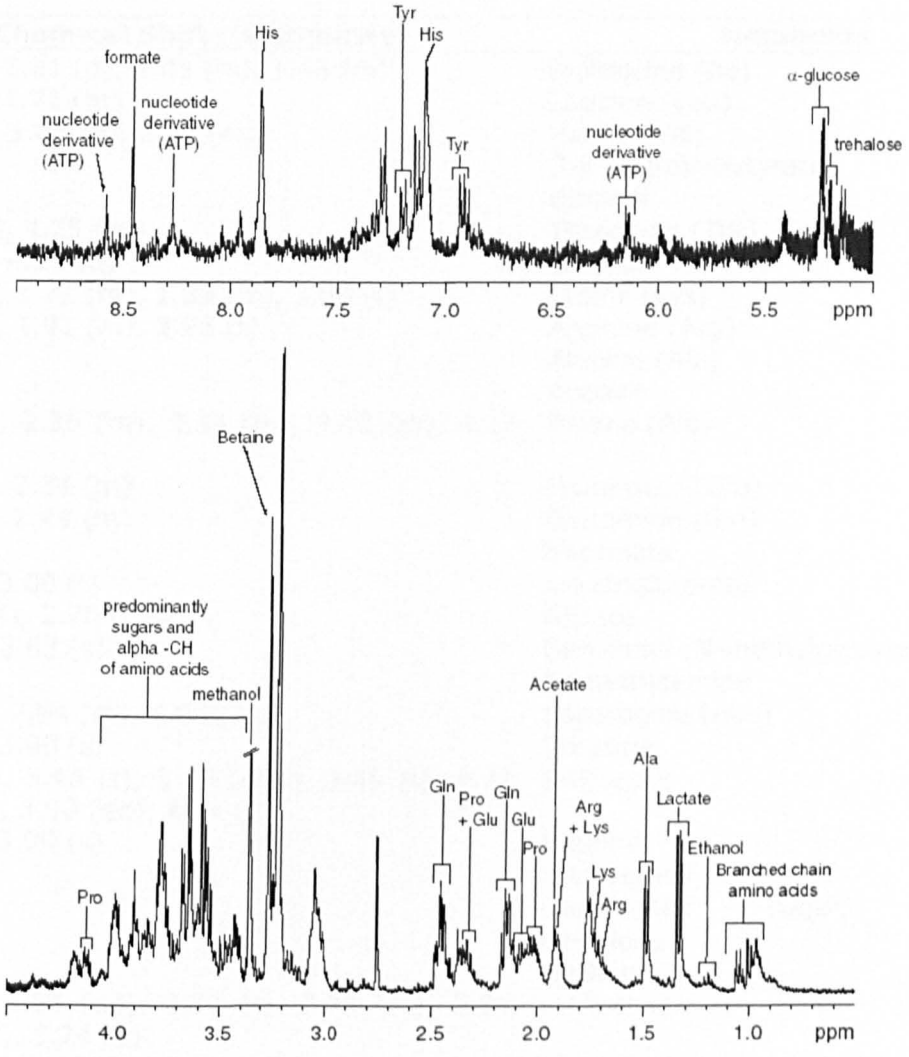

Figure 2.7 Typical 400 MHz NMR spectrum of aqueous caterpillar extract, 16 days-post paralysis. The lower figure shows the aliphatic spectral region (δ 4.5 – 0.5), whereas the upper figure shows the aromatic spectral region (δ 9.0-5.0). Amino acids are labelled according to the 3-letter code.

Table 2.2 Assignment of resonances observed in the 1H NMR spectra of caterpillars.

Chemical Shift (Multiplicity)	Metabolite
0.94 (t), 1.01 (d), 1.25 (m), 1.45 (m)	Isoleucine (Ile)
0.96 (t), 1.71 (m)	Leucine (Leu)
0.99 (d), 1.04 (d), 2.28 (m)	Valine (Val)
1.19 (d)	3-β-hydroxybutyrate
1.19 (t)	Ethanol
1.32* (d), 4.25 (m)	Threonine (Thr)
1.33 (d), 4.11 (q)	Lactate
1.46 (m), 1.71 (m), 1.89 (m), 3.00 (t)	Lysine (Lys)
1.69 (m), 1.91 (m), 3.23 (t)	Arginine (Arg)
1.48 (d)	Alanine (Ala)
1.92 (s)	Acetate
2.02 (m), 2.36 (m), 3.34 (m), 3.42 (m), 4.13 (m)	Proline (Pro)
2.07 (m), 2.34 (m)	Glutamate (Glu)
2.13 (m), 2.44 (m)	Glutamine (Gln)
2.40 (s)	Succinate
2.44 (t), 3.00 (t)	α-ketoglutarate
2.57 (ABX), 2.70 (ABX)	Citrate
2.75 (s), 3.63 (s)	Sarcosine (N-methylglycine)
2.87 (s)	Trimethylamine
2.88 (m), 2.94 (m), 4.00 (m)	Asparagine (Asn)
3.01 (s), 3.90 (s)	Creatine
3.25 (dd), 3.40 (t), 3.47 (ddd), 3.49 (t), 3.72 (dd), 3.90 (dd), 4.64 (d)	β-Glucose
3.27 (s), 3.90 (s)	Betaine
5.14 (d)	Unassigned sugar
5.20 (d)	Unassigned sugar, possibly trehalose
3.36 (s)	Methanol
3.41 (t), 3.54 (dd), 3.71 (t), 3.74 (m), 3.84 (ddd), 5.24 (d)	α-Glucose
3.53 (s)	Glycine (Gly)
6.51 (s)	Fumerate
6.92 (d), 7.19 (d)	Tyrosine (Tyr)
7.05 (s), 7.77 (s)	Histidine (His)
7.21* (t), 7.54 (d), 7.74 (d)	Tryptophan (Trp)
6.15 (d), 8.28 (s), 8.61 (s)	Nucleotide derivative, probably ATP
8.46 (s)	Formate

*Resonance partially obscured due to peak overlap. Letters t, d, m & s stand for peak multiplicity, where t=triplet, d=doublet, m= multiplet & s=singlet.

Using three principal components, the PCA model had R^2X (cum) of 0.383, where PCs 1, 2 and 3 explained 20.3, 9.5 and 8.55 of the total spectral variation, respectively. The scores plot (Figure 2.8) shows clear separation between all three host-age groups across the first three principal components. Cross-validation of the partial least squares (PLS) regression resulted in R^2X and R^2Y of 0.38 and 0.99, respectively; Q^2(cum) was 0.74 (Table 2.3). The root mean square error of estimation (RMSEE) was 0.72 ±0.2, and the root mean square error of cross validation (RMSECV) was 4.74 ±1.9.

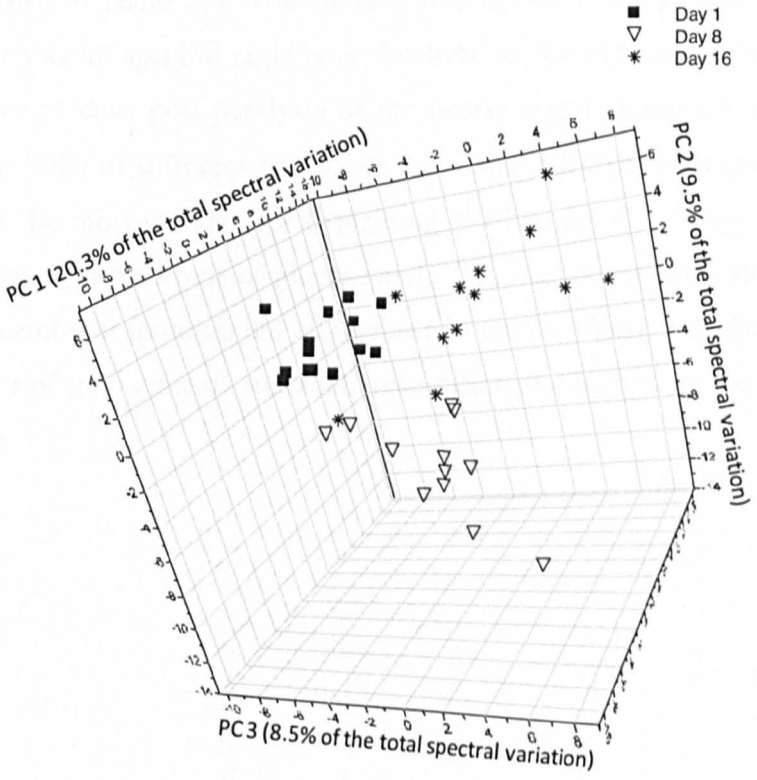

Figure 2.8 Scores plot from principal component analysis illustrating clustering of host metabolic composition according to host age.

Table 2.3 Cross-validation results from partial least squares regression

Actual days post-paralysis	Predicted days post-paralysis (training set)	Predicted days post-paralysis (test set)
1	1.09 ± 0.5	3.40 ± 1.9
8	7.94 ± 0.8	9.78 ± 5.8
16	15.92 ± 0.5	15.76 ± 5.9

The spectral regions in which the largest metabolomic changes between days were observed are shown in Table 2.4. The variable importance in the projection, VIP, values reflect the importance of spectral regions or 'buckets' in the PLS model both with respect to Y (the number of days post-paralysis of the hosts) and with respect to X (the NMR spectra), and the VIPs of different terms can be compared. Spectral regions with a VIP larger than 1 are the most relevant for explaining Y (the number of days post-paralysis). There were decreases in the levels of glucose and ATP, metabolites associated with energy release through aerobic respiration but an increase in citrate. There was also an increase in ethanol, a product of (non-animal) anaerobic respiration and in most of the detected amino acids (Table 2.4).

Table 2.4 Spectral regions with the largest between-day metabolomic changes

Spectral 'Bucket' (VIP coefficient)[1]	Metabolite	Mean Fold Change[2] from 1 to 8 days ± standard deviation	Mean Fold Change[2] from 1 to 16 days ± standard deviation
1.16 (1.328) 1.2 (1.527)	Ethanol	1.08 ± 0.00	1.36 ± 0.05
3.2 (1.595) 3.24 (1.562)	Unassigned	NC[3]	1.43 ± 0.02
2.0 (1.149) 2.04 (1.013) 2.36 (1.096)	Proline	1.20 ± 0.01	0.82 ± 0.03
2.52 (1.495) 2.56 (1.696)	Citrate	2.31 ± 0.17	2.15 ± 0.24
2.84 (1.696) 2.88 (1.824) 2.92 (0.859) 2.96 (0.772)	Asparagine	NC[3]	1.48 ± 0.30
3.16 (1.596) 4.0 (1.652) 7.08 (1.650) 7.8 (1.002)	Histidine	1.38 ± 0.16	2.13 ± 0.50
3.4 (1.792) 3.48 (1.791) 3.72 (1.466) 3.76 (1.689) 3.84 (1.239) 3.88 (1.468) 5.24 (1.508)	Glucose	0.86 ± 0.11	0.69 ± 0.10
6.92 (1.757) 7.2 (1.279)	Tyrosine[4]	1.86 ± 0.67	3.36 ± 1.49
7.0 (1.268) 7.6 (1.645)	3-methyl histidine[5]	<LOD	3.56 ± 0.05
6.16 (1.112) 8.28 (1.1921) 8.64 (0.939)	Nucleotide derivative (ATP)	0.70 ± 0.17	0.77 ± 0.06

[1]There is more than one spectral bucket value for each metabolite because more than one peak per metabolite is usually obtained.
[2]Mean fold changes were calculated taking into account all peaks for a particular compound and all caterpillars in the group: e.g. a fold change of 1.0 indicates no change, 2.0 indicates a doubling of the metabolite and 0.5 indicates the metabolite has halved.
[3]NC denotes no change. This has been noted where metabolite levels changed by ≤ 1 standard deviation from their levels at day 1.
[4]The intensity of the tyrosine resonances are above the limit of detection (defined as 3x the noise level) in all samples but in some samples are below the limit of quantification (defined as 10x the noise level) hence the high S.D. of the measured fold change.
[5]Peak is below the limit of detection (LOD) and quantification in extracts from hosts 1 and 8 day old hosts.

2.5 Discussion

There is clear evidence that the quality of a host is influenced by time since paralysis. Specifically, *G. nephantidis* eggs that were laid onto older hosts had a lower probability of developing to adulthood than eggs laid onto younger hosts and the surviving offspring were smaller as adults. Increased mortality is of obvious detriment to offspring fitness and smaller body size is associated with lower longevity and fecundity in adult *G. nephantidis* females (Hardy et al. 1992) and also with reduced success in 'dyadic' contests for resources that are vital for reproduction (Petersen & Hardy 1996, Humphries et al. 2006, this study). Further, adult *G. nephantidis* females are able to assess age-related components of host quality and attune their behaviour accordingly: older *C. cephalonica* larvae were accepted less frequently for oviposition and those accepted had smaller clutches laid onto them than did younger hosts of similar size. In addition, females guarding an older host tended to lose contests against females guarding a younger host, suggesting that the importance that contestants place on retaining the host they are defending (resource value) declines with its age; this is in direct analogy to interpretations of the effect of host-weight differences in owner-owner contests in this species (Humphries et al. 2006). Our results (Figure 2.6) also show that contest outcomes are influenced simultaneously by two aspects of resource value asymmetry (host age and size) plus asymmetries in resource holding potential (contestant size). Similarly, Figure 2.4 shows that clutch size decisions are influenced by host age and size simultaneously.

Sex ratios in this study (mean = 0.129, variance ratio = 0.833) were quantitatively similar to previous estimates for *G. nephantidis* (e.g. 0.093 and 0.743, Hardy & Cook 1995) and were the only assessed aspect of *G. nephantidis* reproductive biology that was unaffected by host age. This suggests that host age affects the mortality of both sexes equally and/or that ovipositing mothers do not attune their sex allocation decisions to the host age component of host quality: this is not unexpected since, as a gregarious species, typically laying clutches of around 5-15 eggs, sexually differential investment returns in *G. nephantidis* are likely to be more strongly affected by population mating structure than by host quality (Godfray 1994, Hardy & Cook 1995, Mayhew & Godfray 1997, West 2009). Sexually differential developmental mortality also appears to be absent in this and other *Goniozus* species (Hardy & Cook 1995, Khidr et al. submitted, Chapter 6).

The assessment of host size by *G. nephantidis* has been shown in several previous studies, for instance Hardy et al. (1992) found that clutch size is adjusted to host size, whether experimentally measured as host length, width or weight, and there have been many other demonstrations of host-size dependent oviposition decisions in parasitoids (Schmidt 1991, Godfray 1994). The mechanisms by which parasitoids assess size include measuring the host by walking along it (Schmidt 1991) and *G. nephantidis* females spend the vast majority of the period between stinging a host and laying eggs on it, either motionless on or next to the host or moving slowly along its length on the host (e.g. Goubault et al. 2007b).

In contrast, the assessment of host age since paralysis by *G. nephantidis* is a novel finding. There have been many prior reports of host age, prior to attack by parasitoids, influencing host quality and oviposition decisions (e.g. van Alphen & Drijver 1982, Ode & Strand 1995, King 1998, Husni & Honda 2001, Ueno 2004, Hegazi & Khafagi 2008). However to our knowledge, this is the first study documenting the influence of the age of paralyzed but unparasitized hosts on the behaviour of adult parasitoids. For the majority of parasitoids there is little scope for post-paralysis age to affect host acceptance and oviposition decisions as these are usually made within minutes or even seconds of host encounter. Nonetheless, there are several thousand species of bethylids, and possibly other taxa, for which assessment of post-paralysis host age may have adaptive value.

The post-paralysis host age effects we have found indicate that biochemical changes occur within paralysed hosts and that these are detected by adult *G. nephantidis* and influence the development of their progeny. The nutritional status of the host can be considered as of a central importance to parasitoid life-history strategies and the energy metabolism associated with these (Rivers & Denlinger 1995, Mackauer et al. 1997). It is likely that *G. nephantidis* assesses host nutritional state via chemical sense organs on, for instance, its ovipositor, mandibles, tarsai and/or antennae, which are able to respond to amino acids and other chemicals: such structures and abilities are present in other parasitoid species (Schmidt 1991, Vinson 1991, Quicke 1997). Observations *G. nephantidis* may non-destructively host feed and/or malaxate paralysed hosts suggest that it assessed host chemistry via taste.

Idiobiont parasitoids tranquilize their larval hosts with venom. In *Goniozus legneri*, a congener of *G. nephantidis*, venom consists of low molecular weight proteins and

polypeptides including polyamines (e.g. putrescine), proline and dopamine (Skinner et al. 1990). Venom frequently disrupts host physiology and development by evoking paralysis, through inhibition of host moulting, and in some cases, by disrupting calcium homeostasis in specific host tissues (Rivers & Denlinger 1994, Thompson & Dahlman 1998, Parkinson & Weaver 1999, Rahbé et al. 2002, Rivers 2004). The impact of venom produced by ectoparasitoids on the nutrient composition of host haemolymph has been investigated in several parasitoid species. In general, venom induced changes to the host's metabolism involve freeing nutrients (such as fatty acids, Nakamatsu & Tanaka 2004, or free amino acids Guerra et al. 1993, into the host's haemolymph which enhances uptake by developing parasitoids and frees them from substantial metabolic costs of chemical breakdown (Garrett & Grisham 1999). The simple fact that envenomated *C. cephalonica* hosts cease to feed is probably a major influence on their metabolomic profiles. Harvey et al. (1995) found that smaller parasitoids emerged from lepidopteran hosts that had been starved prior to parasitism, and in *Drosophila* flies, starvation induces autophagy, whereby non-essential proteins and organelles are recycled to generate amino acids for other purposes (Scott et al. 2004), a similar process is likely to account for our observations of increased amino acid levels in older *C. cephalonica* hosts (Table 2.4). Thompson (1981 and Quicke 1997) showed that parasitoids drawn from a wide array of hymenopteran taxa (though not including the Bethylidae) had absolute requirements for 10 amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, typtophan and valine. Eight of these were found in *C. cephalonica* (the exceptions are methionine and phenylalanine, Table 2.2) along with seven other non-essential amino acids. Amino acids can be used by wasps to synthesise carbohydrates and thus a general increase in amino acid levels might be expected to enhance, rather than decrease, the quality of *C. cephalonica* as hosts for *G. nephantidis* (see also Yazgan 1972). However, there were also large changes other (non-amino acid) metabolites as paralysed *C. cephalonica* aged: glucose and ATP, both closely associated with energy, decreased and dietary glucose has been found to positively affect parasitoid growth (Thompson 1979, Hu et al. 2001). Citrate levels increased as hosts aged from 1 to 8 days post-paralysis and were also higher in 16-day old hosts compared to 1-day old hosts, but not so much as in 8-day hosts. Citrate is important as an intermediate in the citric acid cycle, and therefore occurs in the metabolism of all organisms and might also be produced by fermentation (see below).

In larvae of another lepidopteran species, the activity of midgut citrate synthase decreased when feeding ceased (Chamberlin & King 1998): in my study levels of citrate were higher after 8 days of starvation but then were not as high after a further 8 days, suggesting a dome shaped relationship for the citrate component of host quality with time. The levels of ethanol in hosts also increased with age. Ethanol is a waste product of metabolism of microbes, such as yeast, and some plants, but not of animals. While some parasitoid species inject yeast-like organisms into their hosts which then develop in the intestines of feeding parasitoid larvae (Quicke 1997) we regard it as more likely that increased ethanol levels we detected are due to fermentation by yeast in the host's diet (see Materials and Methods). As ethanol acts as a toxin, levels of ethanol are likely to be negatively correlated to the nutritional quality of hosts.

Post-paralysis but pre-parasitism ageing is a relatively unusual component of host-parasitoid interactions, but our metabolomic analysis serves more broadly as an illustration of methodology that can readily be employed to understand the biochemical basis of a variety of host quality correlates across a wide range of host-parasitoid associations (see also studies that used NMR before: Thompson 1990, Thompson & Dahlman 1998, Thompson 2001) for instance, hosts of different species are likely to have different metabolic profiles, making them suitable hosts for some parasitoid species but not for others. Similar arguments can be made for host developmental stage and most, if not all, of the facets of host quality listed in the Introduction. An advantage of NMR as an analytical approach is that it allows exploration of the biochemical changes occurring without *a priori* focus on given classes of molecular nutrient metabolites (Jardetzky & Roberts 1981, Keun et al. 2002, Rahbé et al. 2002, van Dorsten et al. 2006) or the need to assay different sub-sets of the organisms sampled for different chemical classes. For instance, Rivers and Denlinger (1994) carried out separate tests, on separate sets of hosts, for amino acids, proteins, keto acids, lipids, glycogen and trehalose to investigate the association between various changes in host's physiology with nutrient availability. In addition, nutrient identification into different sub-classes (e.g. lipids, carbohydrate and protein) might not be used equivalently for reproduction and maintenance (Strand & Casas 2008). Thus, NMR has the potential to assess all of these biochemical components on the same time from a single host.

2.6 Conclusions

Paralysed but unparasitized *C. cephalonica* larvae are unable to feed but may not die for several weeks: during this period they have a 'shelf-life' in that their quality as hosts declines with time. This decline in quality is manifest in its effects on key life-history components of *G. nephantidis* development (immature mortality and body size of surviving adults), and adult behavioural decisions (host acceptance, clutch size and contest outcomes). This decline in quality appears largely nutritionally based with decreases in energy-associated metabolite and increases in waste products playing the most likely roles. While post-paralysis but pre-parasitism ageing is an unusual component of host-parasitoid interactions, NMR analyses can be employed to understand the metabolomic basis of a variety of host quality correlates, such as developmental stage and species, across a wide range of host-parasitoid associations and are predicted to play an important future role in both biocontrol applications of parasitoids and further studies of their evolutionary and behavioural ecology.

Part two: Kin recognition, aggressive behaviour and chemical cues

Chapter 3 : Two components of kin recognition influence parasitoid aggression in resource competition

3.1 Abstract

Kin recognition, defined as the ability to differentiate genetically related individuals from unrelated individuals, plays a key role in a range of biological processes ranging from mate choice to altruistic behaviours but kin-based altruism may be overridden by competition for resources. Here we explore kin recognition in a gregarious parasitoid wasp, *Goniozus legneri*, which exhibits adult female-female contests for hosts. Contest behaviour was less aggressive when competitors were more closely related and also when females had developed on the same host (in nature, brood-mates will almost always be siblings). *Goniozus legneri* appears to be the only parasitoid species utilizing both genetically based (phenotype matching) and environmentally based (familiarity) mechanisms of phenotypic kin discrimination. While perceived resource value affects aggression in *Goniozus*, resource competition did not completely override kin recognition effects.

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[1]These authors contributed equally to the study

3.2 Introduction

Kin recognition, defined as the ability to differentiate genetically related individuals from unrelated individuals, plays a key role in a range of biological processes ranging from mate choice (Ode et al. 1995, Pusey & Wolf 1996, Enigl & Schausberger 2004, Lihoreau et al. 2007, Metzger et al. 2010) to altruistic behaviours (Hamilton 1964a, Mateo 2004, Lizé et al. 2006, Gardner & West 2007). Behaving altruistically toward relatives by definition involves costs to the altruist. Kin selection theory predicts that a genetic trait may be selected even if it does not generate an advantage to the actor (i.e. direct fitness), provided that it increases sufficiently the fitness of other individuals sharing the actor's genes (i.e. indirect fitness) (Hamilton 1964b, Grafen 1984, Frank 1998, Griffin & West 2002). This suggests that individuals should be less aggressive (more altruistic) towards closer relatives when conflicts of direct-fitness-interest arise.

Broadly, two categories of kin recognition mechanism exist; some species utilize phenotypic kin recognition, ultimately based on individuals' features while others may use non-phenotypic kin recognition (Waldman 1987, 1988, Holmes 2004). Non-phenotypic kin recognition relies on cues associated with a common environment such as the nest or spatial location, rather than on the perception of phenotypic traits expressed by individuals themselves (Waldman 1987, Hepper 1991, Holmes 2004, Ruf et al. 2010). Phenotypic kin recognition relies on learning the traits expressed or borne by individuals and can operate by different mechanisms such as familiarity and phenotype matching. Familiarity refers to interactions between individuals regardless of genetic relatedness: any individual will become familiar if it is encountered often, whether or not it is a relative. By contrast, phenotype matching involves the phenotypic cues of its siblings or its own cues (Gadagkar 1985). Disentangling phenotype matching from familiarity would require related individuals that have never met before to recognize each other as kin.

Behavioural aggression is often manifested when individuals compete for resources. However, influences of kin recognition on competition have been generally ignored in ecological studies (Waldman 1988, Lizé et al. 2006). Moreover, recent studies have suggested that an excessively high level of kin competition may cancel-out the benefits of being less aggressive toward related individuals rather than non-relatives (West et al. 2001, 2002, Segoli et al. 2009a). While the ability to treat kin

differentially may influence interference between related individuals competing for a given resource, only a few studies have tested this empirically: across fig wasp species, the level of fighting between adult males shows no correlation with the relatedness of interacting males (West et al. 2001) but is negatively correlated with future mating opportunities (the contested resource). In contrast, in the polyembryonic wasp genus *Copidosoma* attack by the larval soldier caste is unaffected by levels of resource competition but inversely correlated to competitor relatedness (Giron et al. 2004, see also Segoli et al. 2009b) and is mediated by kin recognition based on properties of the larval extra-embryonic membrane (Giron & Strand 2004, Segoli et al. 2009a). However, in the parasitoid wasp genus *Mellitobia* neither the value of the contested resource nor the relatedness of competitors influence levels of aggression (Innocent et al. 2011).

In this study, we explore the effect and basis of kin recognition among adult females on contest behaviour in the parasitoid wasp *Goniozus legneri* Gordh (Hymenoptera: Bethylidae). Individual *G. legneri* females paralyze each host by stinging and, around 24 h later, lay 1-20 eggs onto its surface (Hardy et al. 1998). The developing larvae then feed externally (ectoparasitically) and thus potentially encounter both kin and host cues. Maturing females tend to disperse around 24h after eclosion from the cocoon (Hardy et al. 2000). On finding and paralysing a host, a female typically remains with it for several days and defends it, and the developing brood, against conspecific intruders (sub-social behaviour, Choe & Crespi 1997, p3). Contests in *Goniozus* involve chases and escalated fights but are very seldom fatal. As predicted by game-theory, the outcomes of owner-intruder contests are influenced by asymmetries in both body size (fighting ability) and prior ownership status (Goubault et al. 2006, Bentley et al. 2009). Two non-owner females encountering an undefended host simultaneously will also engage in direct competitive behaviour (see below). Such host-ownership contests may take place between closely or distantly related conspecifics. We therefore investigated the importance of competitor relatedness, alongside the influence of ownership and female size asymmetries, on agonistic behaviour and contest outcomes, and used larval transfer between hosts to explore any mechanisms of kin recognition.

3.3 Material & Methods

3.3.1 Parasitoids

This study employed two strains of *G. legneri*. One was obtained in 2003 from a commercial insectary in the USA (the original material is believed to have been collected from southern Uruguay in 1978, Gordh 1982). This strain, which we term 'U', has been used in previous studies of contest behaviour (Goubault et al. 2006, Bentley et al. 2009) and was the only strain used in our first experiment. The other strain, 'C', was collected in May 2009 around Santiago, Chile, where a natural population had previously been found (Zaviezo et al. 2007). These two strains will readily interbreed in the laboratory and are thus conspecific but are also genetically distinct (evaluation of 24 microsatellite markers found inter-strain polymorphism at 6, S.K.K. unpublished data). Our second experiment used both strains.

All parasitoids were reared on the facultative host *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae), following methods given in Stokkebo and Hardy (2000). *Corcyra cephalonica* was reared on a diet of glycerol, corn meal and wheat bran and yeast, as reported by Cook (1993), with the addition of honey in equal quantity to the glycerol. All cultures and experiments were carried out in a climate room at 27°C with and constant illumination and with high relative humidity maintained by evaporation from a water bath.

3.3.2 Experiment 1: Identifying determinants of aggression

Contests were staged between pairs of females over possession of a host larva. In a 3-way factorial design we varied independently the relatedness between contestants, whether they had developed on the same or on a different host (termed 'host of origin') and whether the contest was between a prior owner and a non-owner (intruder) or between two non-owners (termed 'ownership'), each experimental factor had two treatment levels. In an attempt to achieve high statistical power (Smith et al. 2011), we carried out between 16 and 24 replicates of each of the 8 combinations of the three factors (not including replicates that failed due to wasp developmental mortality, the individuals being males or adult females not interacting during the observation period [≈10% of replicates]), giving an overall sample size of 161.

Relatedness between contestants was varied by using either sibling females from the same clutch (theoretical relatedness ≈0.75 due to haplo-diploidy and maternal sib-mating, Hamilton 1964a) or non-sibling females (from within strain U). Siblings developing on different hosts were created by removing, with fine seekers (Hardy et al. 1992), around half of the unhatched eggs from a parasitized host initially bearing 10-14 eggs and placing them, in a separate vial, on another host that had been previously paralysed, but not parasitized (no eggs laid), by another female (which had been removed). We created broods of non-siblings by placing 5-7 eggs, each from a different clutch laid by a different mother, onto a previously paralysed but unparasitized host (prior to egg laying). Developing broods were checked frequently and as soon as adults were observed to have eclosed from their cocoons they were isolated from each other; post-eclosion association of adult females rarely exceeded six hours. Owners were created by placing 1-day old female wasps with a healthy host larva 24h prior to the contest, whereas intruders were females that had not been given a host (Bentley et al. 2009).

All contestant females were of the same age (*ca.* 48h post-eclosion) and pairs of contestants were selected to be of similar size: individual females were weighed to an accuracy of 0.01mg prior to contests (the mean [±SD; range] size of females in this experiment was 1.128mg [±0.243; 0.24 to 1.81]) and within-replicate size differences ranged from 0.00 to 0.93mg (mean±SD: 0.144±0.172). None of the contestants had had previous experience of contest interactions (but all of them had experienced sibling or non-sibling cues and the same or different host cues). Within each replicate, individual females were marked by painting a dot of yellow or red acrylic paint on the dorsal surface of their thorax (Driessen & Hemerik 1992, Petersen & Hardy 1996); to achieve this, and for weighing, an anaesthetic (CO_2 gas) was utilized a day before the contest to minimize its effect on subsequent behaviour (Nicolas & Sillans 1989).

Contests were staged in an opaque plastic block covered with clear Plexiglas and with three chambers connected by a slot filled with movable barriers (Petersen & Hardy 1996, Goubault et al. 2006). In Owner-Intruder replicates, an owner and its host were placed into the central chamber and an intruder into a peripheral, and initially separated, chamber. In Intruder-Intruder replicates, the host was placed in the central chamber while the two intruder females were initially separated in a different peripheral chamber. After 30 minutes, the barriers were withdrawn sufficiently to

interconnect the three chambers. Wasp behaviour was recorded from above for 60 minutes using a digital video camera, starting when both females were first present in the central chamber. Behavioural interactions, classified as non-aggressive interactions, chases, bites, attacks with a stinger and fights (Goubault et al. 2008), were counted and the identity of any winner, defined as the wasp that remained in the vicinity of the host while the other, the loser, had been excluded (Petersen & Hardy 1996), was noted.

3.3.3 Experiment 2: Focussing on kin recognition determinants of aggression

Analysis of data from Experiment 1 suggested that the presence of ownership asymmetries influences the aggressiveness of behavioural interactions and also that size differences between contestants interacted with both relatedness and the host-of-origin. These results suggest that sharing a host during larval development may act as a proxy for genetic relatedness. To focus more directly on whether less related individuals are more aggressive than more related ones, we carried out a second experiment in which relatedness and 'host-of-origin' varied. This followed the methods of Experiment 1 with four differences: (1) All contests were staged between pairs of non-owners (ownership asymmetries were thus absent). (2) We made greater efforts to minimise the size differences between females, resulting in within-replicate differences ranging from 0.0 to 0.05mg (mean±s.d.=0.0172±0.0149), an order of magnitude smaller than in Experiment 1 (the mean size of females in this experiment was 1.081mg (SD±0.155; range 0.77 to 1.45). (3) We increased the likely variation of within-replicate relatedness by collecting and establishing the second, Chilean, strain of *G. legneri*: contestant females either belonged to different strains (C *vs.* U) or to the same strain in one of two ways (C *vs.* C, or U *vs.* U). Relatedness was thus initially treated as a factor with three levels. (4) Broods of wasps from different strains were created by placing just one egg from each strain onto the previously paralysed, but unparasitized, host (each host thus received two eggs): this was necessary to ensure that a contest could subsequently be staged between wasps known to be from different strains. We carried out 12-14 (successful) replicates of each of the 6 experimental combinations of relatedness and host of origin, giving an overall sample size of 80.

3.3.4 Statistical analysis

Data were analysed using generalized linear modelling available in the Genstat statistical package (Version 12, VSN International, Hemel Hempstead, UK). Logistic analyses were employed for proportional response variables, where possible using empirically estimated scale parameters to account for potential overdispersion, and also with the caveat that hypothesis testing with generalized linear models is inexact when significance is marginal and small proportions of the deviance are explained (Crawley 1993, Wilson & Hardy 2002, Warton & Hui 2011). Because size differences between contestants are effectively impossible to eliminate completely and have consistently been found to influence *G. legneri* contest outcomes in prior studies (Goubault et al. 2006, Bentley et al. 2009), the absolute weight difference between the females in each replicate was fitted as a continuous variable alongside the categorical factors. Analyses initially included these main effects and first order interaction terms in a maximal model and then established the minimum adequate model by backwards, stepwise model simplification, using deletion tests and factor-level aggregation (Crawley 1993, Hardy & Field 1998, Wilson & Hardy 2002). All tests were 2-tailed.

3.4 Results

3.4.1 Experiment 1: Identifying determinants of aggression

There were 1 to 91 (mean±SD: 17.78±16.83) behavioural interactions between individual pairs of females during the 60-minute observation period. The proportion of interactions that were aggressive varied greatly between replicates and was influenced by relatedness, by ownership asymmetries and by whether the females had developed on the same or different hosts, via an interaction with body size (absolute difference in weight); size difference also interacted with relatedness while other interactions were non-significant (Table 3.1).

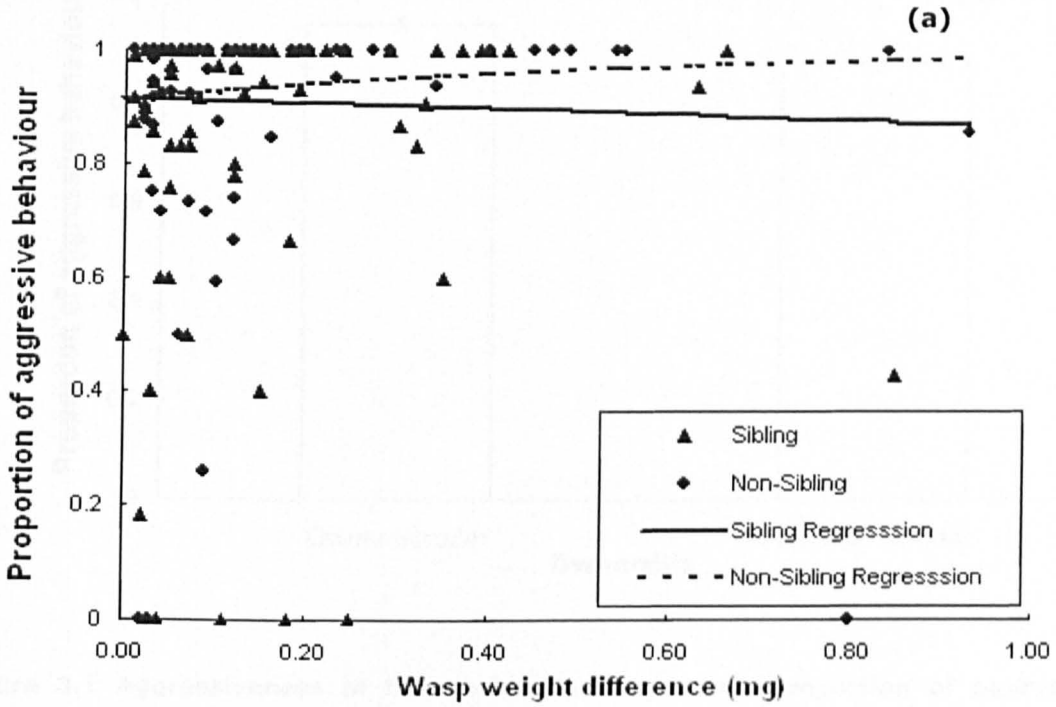
Table 3.1 Influences on the proportion of behaviours that were aggressive

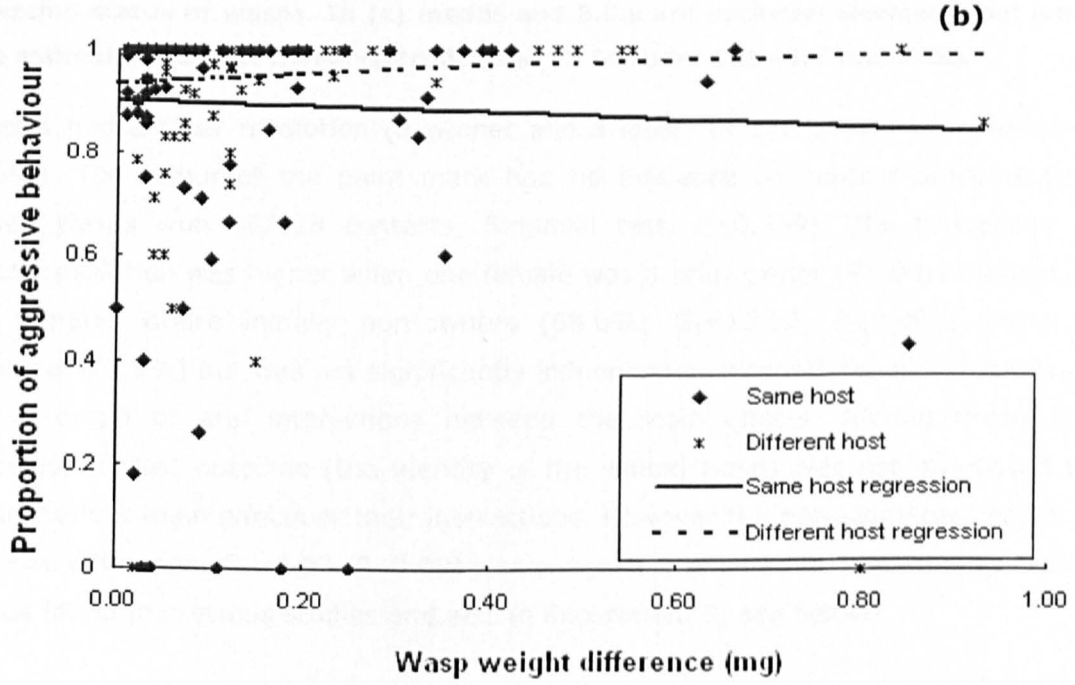
Source	d.f.	Deviance	F-ratio	P	%Deviance explained
Relatedness	1	20.566	5.40	**0.021**	2.89
Host of origin	1	1.929	0.51	0.478	0.27
Ownership	1	64.231	16.87	**<0.001**	9.02
Size difference	1	0.001	0.00	0.987	0.00
Relatedness × Host of origin interaction	1	0.003	0.00	0.978	0.00
Relatedness × Ownership interaction	1	10.111	2.66	0.105	1.42
Host of origin × Ownership interaction	1	7.650	2.01	0.158	1.07
Size difference × Relatedness interaction	1	26.178	6.87	**0.010**	3.67
Size difference × Host of origin interaction	1	30.275	7.95	**0.005**	4.25
Size difference × Ownership interaction	1	0.169	0.04	0.834	0.02
Residual	150	571.258			
Total	160	711.577			

P-values of significant explanatory variables are shown in bold font.

Around 20% of the variation in aggressiveness was explained by the significant terms in the statistical model. The proportions of behavioural interactions that were aggressive were higher when females were non-siblings than when they were siblings (Figure 3.1a), when females had developed on different rather than the same hosts (Figure 3.1b) and when one female was a prior owner than when both were non-owners (Figure 3.1c). Increasing size differences increased the difference in the

proportions of aggressive behaviour based on relatedness (Figure 3.1a) and host of origin (Figure 3.1b).





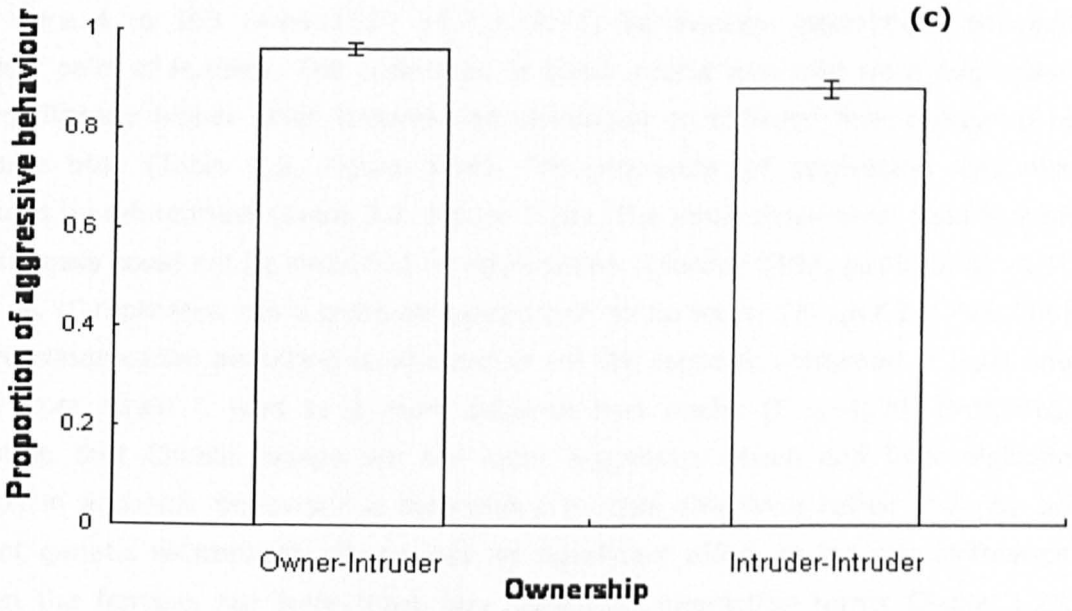


Figure 3.1 Aggressiveness of female-female encounters. Proportion of aggressive behaviour in relation to (a) relatedness and body size difference, (b) whether contestants had developed on the same host and body size difference, and (c) ownership status of wasps. In (c) means and S.E.s are back-transformed from logit-scale estimates from 78 Owner-Intruder and 83 Intruder-Intruder replicates.

Contests had a clear resolution (a winner and a loser) in 128 of the 161 replicates (79.5%). The colour of the paint mark had no influence on contest outcome (red marked wasps won 66/128 contests, Binomial test, P=0.759). The probability of contest resolution was higher when one female was a prior owner (91.0%) than when both females where initially non-owners (68.6%; G_1=13.02, P<0.001, Deviance explained = 7.9%) but was not significantly influenced by size difference, relatedness, host of origin or any interactions between the main effects. Among these 128 replicates, contest outcome (the identity of the wining wasp) was not influenced by any of the four main effects or their interactions. However the non-significant effect of body size difference (G_1=2.87, P=0.09) was suggestive of an advantage of larger body size (as found in previous studies and also in Experiment 2, see below).

3.4.2 Experiment 2: Focussing on kin recognition determinants of aggression

There were 4 to 103 (mean±SD: 24.71±19.71) behavioural interactions between individual pairs of females. The proportion of these interactions that were aggressive was significantly higher when females had developed on different host compared to the same host (Table 3.2, Figure 3.2a). The proportion of aggression was also influenced by relatedness (Table 3.2, Figure 3.2b). The initial three-level classification of relatedness could not be simplified by aggregation (Crawley 1993, p190) of 'C vs. C' and 'U vs. U' replicates into a common category of 'same strain' ($F_{1,76}$=7.14, P=0.009) but a re-classification according to whether or not the replicate contained at least one female from strain C lead to a more parsimonious model ($F_{1,76}$=0.28, P=0.596), suggesting that Chilean wasps are the more aggressive strain and inter-replicate variation in agonistic behaviour is determined by this difference rather than by an effect of genetic relatedness. There was no significant effect of the size difference between the females nor were there any significant interaction terms (Table 3.2). Around 14% of the variation in the proportion of behaviour that was aggressive was explained collectively by relatedness and host of origin (Table 3.2).

Table 3.2 Kin recognition components influenced the proportion of behaviours that were aggressive

Source	d.f.	Deviance	F-ratio	P	%Deviance explained
Relatedness	2	56.419	3.85	**0.026**	9.03
Host of origin	1	30.636	4.18	**0.045**	4.90
Size difference	1	0.359	0.05	0.826	0.06
Relatedness × host of origin interaction	2	21.592	1.47	0.236	3.45
Size difference × relatedness interaction	2	1.366	0.09	0.911	0.22
Size difference × Host of origin interaction	1	1.068	0.15	0.704	0.17
Residual	70	513.174			
Total	79	624.404			

P-values of significant explanatory variables are shown in bold font.Note that the 3-way categorization of relatedness can be further simplified according to the geographical origin of the contestant females (see text).

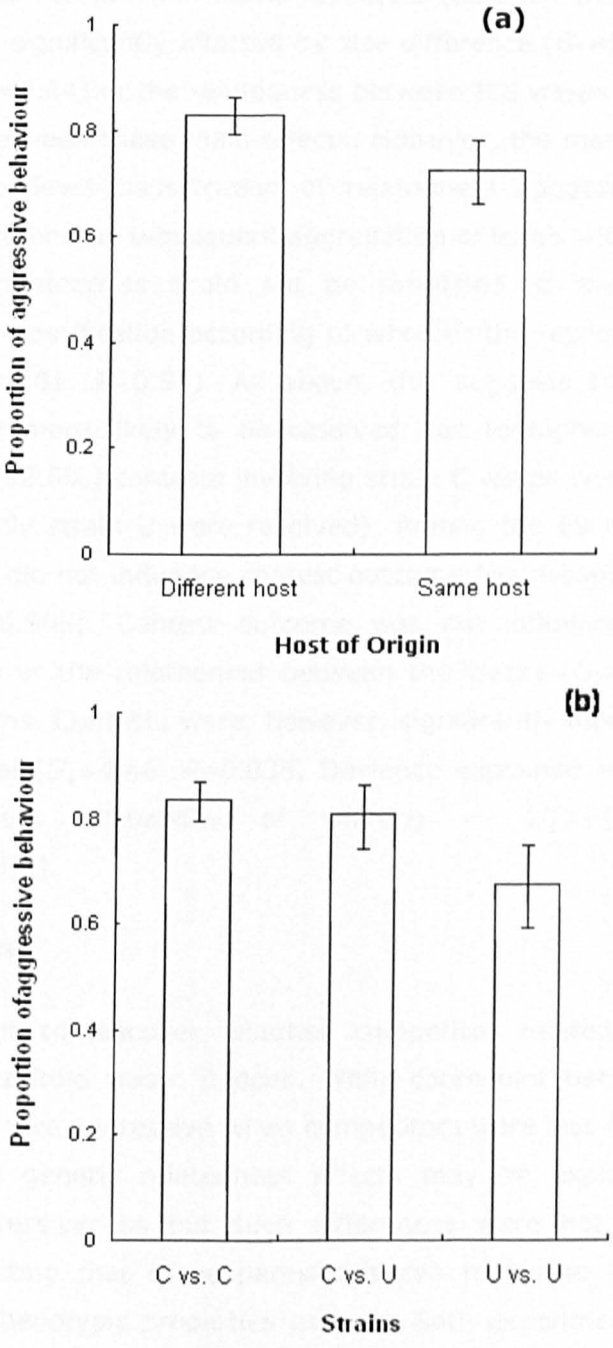


Figure 3.2 Effects of host sharing and genetic strain on the aggressiveness of female-female encounters. Panel (a) illustrates how aggressiveness is influenced by whether contestants had developed on the same host (38 different host replicates and 42 same host replicates) and Panel (b) shows how aggressiveness is influenced by the strains involved (28 C vs. C replicates, 26 C vs. U replicates and 26 U vs. U replicates). Means and S.E.s are back-transformed from logit-scale estimates. Strain C derived from a population in Chile and strain U was obtained from laboratories in the USA with probable origins in Uruguay.

Contests had a clear resolution in 69/80 replicates (86.2%). The probability of contest resolution was not significantly affected by size difference (G_1=0.66, P=0.42), host of origin (G_1=0.60, P=0.44) or the relatedness between the wasps (G_2=2.63, P=0.07) or any interactions between these main effects. However, the marginally non-significant effect of the three-level classification of relatedness suggested the possibility of between-strain differences: subsequent aggregation of levels within this factor showed that, as above, relatedness could not be simplified to 'same strain' (G_1=3.96, P=0.047) but a re-classification according to whether the replicate contained strain C was possible (G_1=0.01, P=0.94). As above, this suggests that contests involving Chilean wasps are more likely to be resolved due to higher levels of aggressive behaviour (50/54 [92.6%] contests involving strain C wasps were resolved and 19/26 [73%] involving only strain U were resolved). Among the 69 resolved contests, the colour of the mark did not influence contest outcome (red wasps won 35/69 contests, Binomial test: P=0.905). Contest outcome was not influenced by host of origin (G_1=0.05, P=0.82) or the relatedness between the wasps (G_2=0.38, P=0.68) or by any interaction terms. Contests were, however, significantly more likely to be won by the larger individual (G_1=4.46, P=0.035, Deviance explained =4.66%, fitted logistic regression equation: probability of winning = 1/[1+{1/(exp([23.4×Weight difference]+0.111))}]).

3.5 Discussion

This study set out to discover whether competitor relatedness affects contest behaviour in a parasitoid wasp: it does. While contestant behaviour was generally aggressive, it was more aggressive when competitors were less closely related. In the second experiment genetic relatedness effects may be explained by inter-strain differences in aggressiveness but such differences were not present in the first experiment, suggesting that these parasitoids can recognise kin using individuals' genetically-based phenotypic properties as cues. Both experiments also indicate that environmentally-based properties are used by interacting females as a proxy cue for kinship.

Our results thus provide evidence that adult females of a sub-social parasitoid species are able to recognize other females as kin, whether or not they developed on the same host, and are also able to recognize brood-mates, whether or not they are kin.

In nature, individuals that have developed on the same host will almost always be full siblings, due to brood guarding by mothers and infanticide or host rejection by successful intruders (Bentley et al. 2009), so brood-mate recognition will serve as an effective means of recognizing kin. These results are similar to those for the gregarious parasitoid *Bracon hebetor* in which adult females recognize brood-mates and discriminate against them to avoid genetic disadvantages of inbreeding, but in *B. hebetor* there appears to be no genetic component of phenotypic kin recognition (Ode et al. 1995). The opposite is reported from a solitary species, *Venturia canescens*, in which there are no brood-mates and mate choice is influenced by genetic kin recognition (Metzger et al. 2010). Several other recent empirical studies of adult parasitoid wasps have found no evidence for kin recognition effects on mate choice (Bourdais & Hance 2009, Ruf et al. 2010), sex allocation behaviour (Reece et al. 2004) or male-male combat (Innocent et al. 2011). At present, *G. legneri* appears to be the only parasitoid wasp reported to employ both genetic and environmental mechanisms of phenotypic kin discrimination. However, *G. legneri* is classed as sub-social due to brood care (Choe & Crespi 1997) and the use of both phenotypic and non-phenotypic kin recognition cues as determinants of aggression by social *Polistes* wasps (non-parasitoids) is well known (Gamboa et al. 1986a,b, Bura & Gamboa 1994).

Because *G. legneri* contests for hosts occur after dispersal from the common developmental site, reduced aggression towards prior brood-mates is unlikely to be mediated by non-phenotypic kin recognition but instead fits the definition of phenotypic kin recognition via familiarity. Two possible, and mutually non-exclusive, means of achieving this are the use of cues deriving from the host and of cues deriving from brood-mates. Our results cannot distinguish between these potentials but a simple mechanism would be for females to learn the cues of conspecific brood-mates to form a recognition template and then match it to labels expressed by females encountered during contests (phenotype matching). Learning the cues of brood-mates (which normally are siblings) rather than hosts would also lead to the recognition of genetically related females that have not previously been encountered (having developed on a different host), and *G. legneri* does behave less aggressively towards unfamiliar kin than towards unfamiliar non-kin. However, females may learn several cues related to their host and/or brood-mates leading to the formation of different templates being used when they encounter familiar and unfamiliar females. Individuals could also learn their own cues (self-referent phenotype matching, Lizé et

al. 2006, 2007, Metzger et al. 2010) for the recognition of unfamiliar but related females. According to Newey (2011), colonial insects may use two types of cues to distinguish nest-mates from non-nest-mates: the individual's innate odour and the shared colony odour. While further experiments are needed to identify which kin recognition cues are used by *G. legneri* females, these cues must have been learned during the pre-imaginal stages and/or within the few hours after adult emergence from the cocoon (in our experiments females were isolated within ≈6h post-emergence and, under laboratory conditions, disperse within 24h, Hardy et al. 2000).

Our results indicate that *Goniozus legneri* females use genetically-based recognition as well as familiarity, because related but unfamiliar females were less mutually aggressive than were unrelated unfamiliar females. Although recognition alleles ('greenbeard effects', Dawkins 1976) could also explain our results, they are in general expected to be rare due to invasion by cheaters and also, when they do occur, to be difficult to detect due to having gone to fixation in a population (Gardner & West 2010). For these general reasons it seems unlikely that our results are mediated by greenbeard effects. Phenotype matching relies on the genotype of individuals being expressed through their phenotype, in contrast to familiarity in which phenotypic cues may derive from genotypes and/or the environment. Familiarity and phenotype matching are thus considered different perceptual mechanisms but phenotype matching could be an extension of familiarity (Holmes 2004, Mateo 2004, Schausberger 2007). The former requires an exact match to an individual template, whereas the latter generalizes from individual templates that have been formed from individuals sharing phenotypic traits to a common representation of kin (Mateo 2004). However, the lack of significant interactions between genetic relatedness (suggestive of phenotype matching) and host-of-origin (familiarity) suggests that female *G. legneri* utilize two different recognition mechanisms, rather than the phenotype matching mechanism being an extension of familiarity. Different recognition mechanisms may be used in different social contexts (Mateo 2004, Schausberger 2007). Both mechanisms are used by the same individuals in Belding's ground squirrels (Holmes & Sherman 1982, Mateo & Johnston 2003, Mateo 2004), lambs (Ligout & Porter 2003), social paper wasps (Gamboa et al. 1986b, Bura & Gamboa 1994, Gamboa 2004). Among invertebrates, mainly social Hymenoptera have been shown to use recognition mechanisms based on familiarity and phenotype matching (reviewed in Gamboa 2004). However, an individual may use different ways of

assessing relatedness in the same context as for female *G. legneri*. To our knowledge, *G. legneri* is the only example of a sub-social wasp able to use two mechanisms of kin recognition in the same context (i.e. during contests for resources). Understanding the functional advantage of the use of these two mechanisms would be valuable for our knowledge of social evolution.

Favouring related individuals at the expense of less related or unrelated ones might be overridden by a high level of competition between siblings (West et al. 2001). In *G. legneri,* and in other host-guarding parasitoid species, prior ownership of hosts is associated with a greater value being placed on the resource and also higher levels of aggression (Petersen & Hardy 1996, Stokkebo & Hardy 2000, Humphries et al. 2006, Goubault et al. 2006, 2008, Mohamad et al. 2010, this study). Although the presence of ownership asymmetries explains more deviance than any other fitted variable (Table 3.1) aggressive behaviour is still tempered by kin recognition. In other wasp systems resource competition appears to override kin selection effects completely (West et al. 2001). Theory suggests that the evolution of kin-based altruism, in this case less aggressive behaviour, may be suppressed in populations unless dispersal occurs which allows the beneficiaries of altruism to compete for resources against non-relatives (Taylor 1992, Queller 1994, West et al. 2002) or unless mistakes in kin discrimination are evolutionarily costly (Segoli et al. 2009a). These concepts in turn suggest that *G. legneri* has imperfect kin discrimination abilities and/or that populations are not so viscous that competing females are frequently close relatives but there is almost no information available on patterns of dispersal beyond that from the remains of the natal host (Hardy et al. 2000).

3.6 Conclusion

Goniozus legneri females appear able to recognize kin via two phenotypic mechanisms, one genetically-based (phenotype matching) and the other environmentally-based (familiarity) which operate together additively rather than interactively. Kinship and perceived kinship are both associated with lower levels of inter-contestant aggression. This is expected from classic theory (Hamilton 1964a,b) but more recent theory and comparative evidence (West et al. 2001, 2002) suggests that in hymenoptera with viscous population structures, resource competition may suppress the evolution of less aggressive, more altruistic, behaviours. In other hymenopteran systems resource competition is less important and altruism operates apparently due to a mixture of pre-competition dispersal from the natal patch and (possibly limited) kin recognition. *Goniozus legneri* appears to be intermediate between these extremes, possibly suggesting an intermediate degree of population viscosity, as females behave more aggressively when resource competition is more manifest but the level of aggression is also mediated by the recognition of kin.

Chapter 4 : Cuticular hydrocarbon profiles of *Goniozus* species and of hosts influence wasp aggressiveness

4.1 Abstract

The cuticular hydrocarbon profiles of insects are well known to be highly variable. Variation may be due to genetic or environmental influences, or both. The majority of prior studies have focused on social insects, mainly those in the Hymenoptera, and have shown that hydrocarbons play an important role mediating social behaviour, particularly via kin recognition. Here we assess the cuticular hydrocarbon profiles (CHC) of three species of parasitoid wasps in the genus *Goniozus* (Hymenoptera: Bethylidae), some of which are known to attune their behaviour according to both environmentally based and genetic based recognition of kin. We find that CHC profiles vary according to both genetic background (wasp species) and the developmental environment (host species) thus showing that kin recognition is likely to be based on CHC profiles in these parasitoids as it is in social hymenoptera. Because the CHC profiles of different species within the genus *Goniozus* are dissimilar, we also conclude that chemical analysis can be used as a taxonomic tool alongside morphological and molecular genetic identification for *Goniozus* and other species.

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4.2 Introduction

Insects' exoskeletons consist of many layers of cuticle secreted by the epidermal cells (Hillerton et al. 1982, Reynolds 1987). Cuticular hydrocarbons (CHCs) are present in the exoskeleton of the majority of insect taxa (Blomquist et al. 1987, Howard 1993, Blomquist & Bagnères 2010) and the integument commonly consists of complex mixture of three major hydrocarbon compounds, which are n-alkanes, olefins and methyl alkanes (Lockey 1991, Martin & Drijfhout 2009). An insect's fitness might be associated with its cuticular hydrocarbon composition as CHCs serve as a prime barrier against desiccation (Edney 1977, Hadley 1981, 1984, Blomquist & Bagnères 2010), microorganism penetration and parasitoid and predator attack (Koidsumi 1957, David 1967).

Cuticular hydrocarbons can also affect insect fitness via influence on behaviour: due to their chemical stability, low volatility and structural diversity, variability in CHC composition has great potential to convey information and CHCs act as semiochemicals, serving as pheromones, kairomones and allomones (Blomquist et al. 1987, Howard 1993, Gamboa et al. 1996, Hölldobler 1999, Dani et al. 2001, Denis et al. 2006, Grillet et al. 2006, Rani et al. 2006, Ruther et al. 2011, van Wilgenburg et al. 2012). For instance, some parasitic wasps have the ability to distinguish gender-specific host CHCs which assists in the identification of hosts suitable for oviposition (Colazza et al. 2007, Lo Giudice et al. 2011). Further, CHCs function importantly in insect communication systems, serving as cues for species-, colony- and gender-recognition (Howard & Blomquist 2005, Haverty et al. 1990, Bagnères & Wicker-Thomas 2010, Lahav et al. 1999, Smith & Breed 1995) and the social behaviours, such as aggression and altruism, of many organisms have been correlated with the cuticular hydrocarbon profiles of actors and recipients (Jutsum et al. 1979, Lahav et al. 1999, Lenoir et al. 2001, Howard & Blomquist 2005, Dalecky et al. 2007, Ugelvig et al. 2008, Guerrieri et al. 2009, Drescher et al. 2010, El-Showk et al. 2010). For example, experimental work has shown that the intensity of aggression can be affected by the application of synthetic hydrocarbons to nest-mates in social insects (Lahav et al. 1999, Guerrieri et al. 2009) or by manipulating inert materials with

artificial or natural hydrocarbons (Akino et al. 2004, Greene & Gordon 2007, Martin et al. 2008).

The cuticular hydrocarbon profiles of individual insects usually have a genetic component, meaning that they can be used in kin-recognition by insects (Gamboa 2004, Howard & Blomquist 2005, Dronnet et al. 2006, El-Showk et al. 2010) and also as taxonomic indicators by researchers (Carlson & Yocom 1986, vander Meer 1986, Bagnères et al. 1990, Uva et al. 2004). CHC profiles usually also have an environmentally determined component; this means that they can be used by insects as indicators to determine relationships within and between populations (e.g. as a proxy for kinship, Lahav et al. 1999, Howard & Blomquist 2005, Dronnet et al. 2006, El-Showk et al. 2010, Helanterä et al. 2011) and also by researchers as indicators of the geographical or developmental origin of examined specimens (Dapporto et al. 2009, Perdereau et al. 2010).

In this study we investigate cuticular hydrocarbon profiles of three species of bethylid wasps. The CHC profiles of 5 bethylid species, belonging to the sub-family Epyrinae, have been reported previously: there are well over 100 different hydrocarbons found across these species, with most hydrocarbons occurring only in one species and with around 30-70 hydrocarbons present per species (Howard 1992, 1998, Howard & Infante 1996, Howard & Pérez-Lachaud 2002). Here we examine three species belonging to the sub-family Bethylinae, *Goniozus legneri* Gordh, *G. nephantidis* (Muesebeck) and an unidentified *Goniozus* species from Oman, and also examine whether these vary according to genetic and environmental factors.

The reproductive and behavioural biologies of *G. nephantidis* and *G. legneri* are relatively well known. Both are gregarious ectoparasitoids of lepidopteran larvae, both exhibit maternal care of their progeny via aggressive brood guarding and both have been deployed as agents of biological pest control; *G. legneri* in the New World and the middle-east and *G. nephantidis* in and around the Indian sub-continent (e.g. Legner & Silveira-Guido 1983, Gothilf & Mazor 1987, Goubault et al. 2007b, Bentley et al. 2009, Venkatesan et al. 2009). The species from Oman, which for convenience we refer to here tentatively as *Goniozus* sp. indet., is less well known but exhibits similar life-history and behavioural characteristics to *G. legneri* and *G. nephantidis*, and is also a natural enemy of agricultural pests (Abbas et al. 2008; ICWH pers. obs.). As this species has not been formally described it could be more closely related to, or

even synonymous with, either *G. legneri* or *G. nephantidis.* While these two described species are congeners, *G. nephantidis* was previously classified as *Paraseriola nephantidis* before the genera were merged by Gordh & Evans (1976) and it may be that further taxonomic revision based on new (e.g. molecular, Carr et al. 2010) evidence re-establishes an intra-genus distinction. One of the motivations for this study was thus to attempt to utilize CHC profiles as a species identification characteristic. Identifying the likely taxonomic position of *Goniozus* sp. indet.would help to establish whether it is a species for which the biology is already well known or a relatively unexplored species with biology still needing to be investigated, for instance, to assist with developing its potential as an agent of biological pest control.

Behavioural research on *Goniozus legneri* has found that adult females in situations of resource competition attune their aggression according to competitor relatedness, with relatedness apparently assessed both via actual genetic similarity and via sharing the same individual host during development (which serves as a proxy for genetic relatedness because in nature brood-mates will almost always be siblings) (Lizé et al. 2012, Chapter 3). It seems likely that such kin-recognition operates via the expression and detection of cuticular hydrocarbons, and that the CHC profile expressed is influenced by both the genetic composition of the wasps and the chemical composition of the developmental environment (Gamboa et al. 1986b, Dronnet et al 2006, Nehring et al. 2010). Another motivation for this study was to explore whether *Goniozus* CHC profiles correlate with relatedness within species (using different strains of *G. legneri* and also different sexes of *G. legneri* and *G. nephantidis*) and according to the identity of the host (using four different host species).

4.3 Material & Methods

4.3.1 Parasitoids

Goniozus legneri Gordh attacks a number of species of lepidopteran larvae which are pests of walnuts, pistachio nuts, almonds and apples (Steffan et al. 2001, Zavlezo et al. 2007). In 2003 we obtained a laboratory strain from a commercial insectary in the USA but the original material is believed to have been collected from a population in southern Uruguay in 1978 (Gordh 1982, Gordh et al. 1983, Legner & Silveira-Guido

1983): we term this strain 'U'. Two other strains of *G. legneri* were brought to the UK from Santiago, Chile, in 2009. One strain was collected directly from walnut trees, and is termed 'Chile field', while the other strain, termed 'Chile lab', had been maintained in a Chilean insectary since the discovery of the natural populations in apples and walnuts in 2003 (Zaviezo et al. 2007). *Goniozus nephantidis* (Muesebeck) attacks the coconut pest *Opisina arenosella* Walker in the Indian sub-continent (Venkatesan et al. 2007) and our strain has been maintained in culture for more than 10 years after having been obtained from an Indian insectary. The unidentified species, *Goniozus* sp. indet. attacks the larvae of the lesser date moth *Batrachedra amydraula* (Meyrick) in the Sultanate of Oman (Abbas et al. 2008).

4.3.2 Culturing

Goniozus legneri and *G. nephantidis* were maintained on a factitious host, the rice moth *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae) on an artificial diet consisting of glycerol, corn meal and wheat bran and yeast, following methods reported in Lizé et al. (2012). We did not culture *Goniozus* sp. indet. in our laboratory: hexane extractions (see below) were delivered from Oman where the parasitoid had been reared on larvae of the wax moth, *Galleria mellonella* L (Galleridae: Lepidoptera).

In further work on strain 'U' of *G. legneri*, we reared it on three host species additional to *C. cephalonica*: the Indian meal moth *Plodia interpunctella* (Hübner), the Mediterranean flour moth *Ephestia kuehniella* Zeller and the tropical warehouse moth (almond moth) *Ephestia cautella* (Walker). These three moth species were all reared on the same diet as *C. cephalonica*.

All cultures were maintained in a climate room at 27°C with relative high humidity maintained by evaporation from a water bath.

4.3.3 Sampling programme

To compare CHC profiles of female parasitoids across strains and species reared on *C. cephalonica*, we analysed extracts from a total of 20 individuals (4 from each strain or species). To explore inter-sexual differences in CHC profiles we analysed extracts from a total of 18 individuals (3 males and three females from *G. nephantidis* and *G. legneri* strains U and C lab). Further, two pooled samples of concentrated extract from eight

individuals of each of *G. nephantidis* and *G. legneri* U strain were to ascertain the peak identity of the chemical compounds. To examine the effect of host species on the CHC profiles of females within a strain we analysed extracts from total of 16 *G. legneri* U strain individuals (4 from each host species).

4.3.4 Sample extraction

Cuticular hydrocarbon extractions were obtained using methods based on Howard et al. (1978): adult wasps that had been reared on separate hosts (*i.e.* from different broods) were placed individually in 1.5ml glass vials containing 500µl of n-hexane, 100µl of decane (Sigma Aldrich) and 1µg/sample of n-alkane C24 as an internal standard. Vials were shaken gently and the insects were removed after 10 minutes and discarded. Extractions in solvent were then either concentrated under a gentle stream of N_2 or left to evaporate at room temperature, in either case resulting in volumes *ca.* 50µl. These samples were transferred to 250µl vials for analysis by gas chromatography-mass spectrometry.

4.3.5 Cuticular hydrocarbons analysis

All chemical analysis used gas chromatography - mass spectrometry (GC-MS). Sample analysis was performed using Trace GC Ultra equipped with ZB-5 capillary column (30m long, 1µm film thickness)(Phanomenex, Macclesfield) connected to DSQ11-Mass Spectrometer (Thermo, Hemel Hempstead) and Xcalibur data system. The injector port and transfer line were set at 280°C and 1µl of the sample was injected in splitless mode (split closed for 10s) using an AS3000 autosampler. The oven temperature was programmed with an initial temperature of 100°C then increased to 330°C at a rate of 6°C min^{-1}. Helium was used as a carrier gas at a linear flow rate of 1.2ml min^{-1} constant flow.

The DSQ11-MS was used in the total ion scan mode with scan mass ranges from *m/z* 45-500 in order to detect individual components (Wack 1976, Nels 1978). For each specimen, the relative abundance of each compound was normalized by dividing the area of each compound by the area of the internal standard in the same chromatogram. Compound identification was based on mass spectral comparison of spectra with those of a standard library of mass spectra (NIST MS Search Version

2.0). Further, the chemical identities were confirmed by comparison to the profiles of other bethylids, as published in Howard (1992) and Howard & Pérez-Lachaud (2002).

4.3.6 Statistical analysis

Principal Components Analysis (PCA), based on sums of squares and products, was used to explore the influences of host species and parasitoid sex, strain and species on the CHC profiles of parasitoids (Quinn & Keough 2002, Martin & Drijfhout 2009, Kather & Martin 2012). Formal evaluation of the effects host species, parasitoid species and strain was carried out using MANOVAs (Everitt & Dunn 1991, Quinn & Keough 2002). Because results of multivariate analyses combine the effects of CHC profile components and the prevalence of individual components may be biologically important (Martin & Drijfhout 2009), we also present the results of ANOVAs carried out on the quantity of each individual hydrocarbon, with significance thresholds adjusted due to multiple comparisons to control Type I error rates (Quinn & Keough 2002). PCA and ANOVAs were carried out in the Genstat statistical package (Version 12, VSN International, Hemel Hempstead, UK) and MANOVAs were carried out in StatView (version 5.0.1).

4.4 Results

Cuticular hydrocarbon profiles were determined for the three species; *Goniozus nephantidis*, *G. legneri* ('U' and both 'C' strains) and the unidentified species here termed *Goniozus* sp. indet. (Figure 4.1). There were inter-specific differences in CHC profiles in terms of both composition (qualitative differences) and relative abundances (quantitative differences) (Figure 4.1, Table 4.1). There was one compound (C23 alkene) found in *G. nephantidis* but not in *G. legneri* and *G.* sp. Indet. and there were three compounds found in *G. legneri* but not in the other two species (Table 4.1). While no detected compound was unique to *G.* sp. Indet., one (C27 alkane) was absent from this species and present in the other two (Table 4.1). The cuticular hydrocarbon composition of the adult female was mainly n-alkanes (C23, C25, C27 and C29) and alkenes. Principal components analysis generated a separate cluster for each *Goniozus* species (Figure 4.2a) illustrating these interspecific differences. C25 alkene 2 had a large influence on the first principal component (Figure 4.3a) and its prevalence of in *G. nephantidis* (Table 4.1, Figure 4.1a) was important in separating *G. nephantidis* from the other species (Figure 4.2a). Similarly, the C27 alkene 2

particularly influenced the second principal component (Figure 4.3a) and its high presence in *G. legneri* (Table 4.1, Figure 4.1c) was important in separating *G. legneri* from *Goniozus* sp. indet. (Figure 4.2a).

For *G. legneri* there was no qualitative variation across strains and no significant quantitative difference overall (MANOVA) or in seven of the individual hydrocarbons (ANOVAs); also the two compounds that differed significantly according to separate ANOVA analysis (C25 alkene 2 & C29 alkene) were not interpreted as different when multiple comparisons corrections were applied (Table 4.1). PCA also illustrates that *G. legneri* strains cluster together (Figure 4.2a). There were, however, significant differences between the CHC profiles of *G. legneri* males and females within both evaluated strains (Table 4.2). In contrast, *G. nephantidis* males and females have similar CHC profiles (Table 4.2). PCA generated separate clusters male and female *G. legneri*; while in *G. nephantidis* females clustered closely to the males (Figure 4.2b).

The CHC profiles of female *G. legneri* ('U' strain) were significantly quantitatively affected by the species of host on which they had developed (Table 4.3) but qualitative differences were absent (i.e. the same chemicals were present in all females). PCA generated a separate cluster for females that had developed on *C. cephalonica* and for females from *E. kuehniella* hosts but females that had developed on *P. interpunctella* or *E. cautella* mainly clustered together (Figure 4.2c). Both principal components were strongly influenced by variation in the composition of C25 hydrocarbons (Figure 4.3b).

Table 4.1 Inter-specific and inter-strain CHC profile variation. Mean relative abundance ± SD of cuticular hydrocarbon composition of adult females belonging to different *Goniozus* species and strains, reared on *Corcyra cephalonica* hosts. Cross-strain MANOVA (*G. legneri*): Wilks' Lambda = 0.001, $F_{(18,2)}$ = 3.520, P=0.2611. Cross-species MANOVA: Wilks' Lambda = 0.001, $F_{(20,16)}$ = 25.995, P<0.0001. Because up to 10 ANOVA tests were carried out within cross strains and species, we adjusted the significance criterion according to the Bonferroni procedure to be 0.05/10, i.e. <0.0050: P-values less than this value are indicated with an asterisk.

Compound, (Carbon chain length)	Molecular weight, Da	Retention time (min)	*G. nephantidis*	*G.* sp. indet.	*G. legneri*			*G. legneri* cross-strain ANOVAs		Cross-Species ANOVAs	
					U	C field	C lab	$F_{(2,9)}$	P	$F_{(2,17)}$	P
Alkene (C23)	322	30.61	0.15±0.04	0.00	0.00	0.00	0.00			176.616	**<0.001***
Alkane (C23)	324	30.85	0.26±0.04	0.06±0.02	0.15±0.05	0.12±0.03	0.11±0.02	1.460	0.282	34.285	**<0.001***
Alkene 1 (C25)	350	33.49	0.00	0.00	0.69±0.19	0.69±0.13	0.62±0.05	0.403	0.679	1022.758	**<0.001***
Alkene 2 (C25)	350	33.59	3.26±0.95	0.53±0.09	0.97±0.16	0.75±0.08	0.59±0.22	5.365	**0.029**	54.111	**<0.001***
Alkane (C25)	352	33.79	0.11±0.04	0.05±0.01	0.34±0.09	0.33±0.07	0.32±0.01	0.076	0.927	60.237	**<0.001***
Alkene 1 (C27)	378	36.24	0.00	0.00	0.30±0.06	0.43±0.11	0.39±0.05	2.737	0.117	67.216	**<0.001***
Alkene 2 (C27)	378	36.34	0.11±0.04	0.08±0.04	1.32±0.36	1.39±0.25	1.30±0.50	0.061	0.941	47.042	**<0.001***
Alkane (C27)	380	36.48	0.09±0.03	0.00	0.11±0.04	0.09±0.03	0.09±0.04	0.835	0.465	17.916	**<0.001***
Alkene (C29)	406	38.90	0.00	0.00	0.13±0.04	0.26±0.05	0.26±0.09	6.091	**0.021**	23.323	**<0.001***
Alkane (C29)	408	39.01	0.07±0.04	0.02±0.01	0.07±0.05	0.08±0.03	0.08±0.03	0.111	0.896	4.310	**0.030**

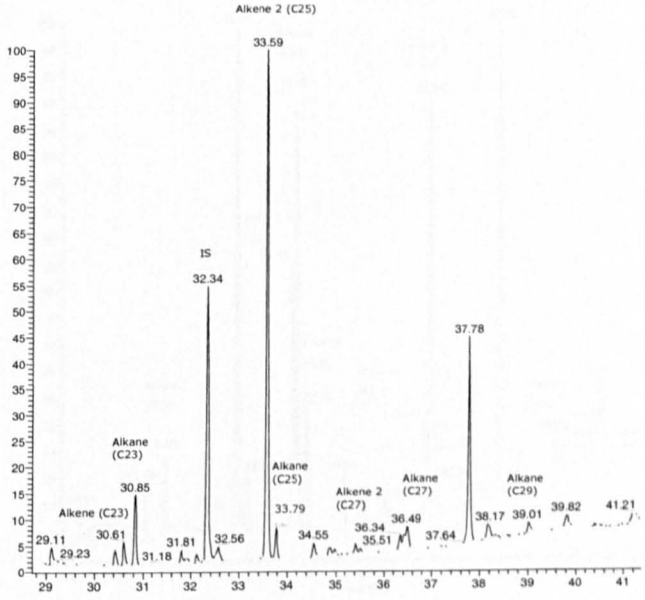
Table 4.2 Inter-sexual CHC profile variation. Mean relative abundance ± SD of cuticular hydrocarbons of adult male and female *Goniozus nephantidis* and *Goniozus legneri* (U & C lab strains) reared on *Corcyra cephalonica*. Because 9 ANOVA tests were carried out within each species, we adjusted the significance criterion according to the Bonferroni procedure to be 0.05/9 = 0.0055: P-values less than this value are indicated with an asterisk.

	G. nephantidis				*G. legneri* U				*G. legneri* C lab			
	Female	Male	ANOVAs		Female	Male	ANOVAs		Female	Male	ANOVAs	
Compounds			$F_{(1,4)}$	P			$F_{(1,4)}$	P			$F_{(1,4)}$	P
Alkene (C23)	0.14±0.03	0.10±0.03	3.250	0.145	0.00	0.00			0.00	0.00		
Alkane (C23)	0.26±0.05	0.30±0.03	1.714	0.260	0.16±0.05	0.06±0.04	6.00	**0.070**	0.12±0.02	0.04±0.03	15.55	**0.016**
Akene 1 (C25)	0.00	0.00			0.63±0.16	0.10±0.00	31.60	**0.004***	0.61±0.06	0.11±0.01	235.06	**<0.001***
Alkene 2 (C25)	2.85±0.58	1.98±0.13	6.298	0.066	0.91±0.14	0.38±0.03	43.71	**0.002***	0.52±0.21	0.20±0.04	6.59	0.062
Alkane (C25)	0.10±0.03	0.10±0.01	0.32	0.866	0.31±0.08	0.09±0.01	24.97	**0.007**	0.32±0.01	0.08±0.01	504.10	**<0.001***
Alkene 1 (C27)	0.00	0.00			0.32±0.06	0.02±0.00	79.02	**<0.001***	0.38±0.05	0.04±0.01	123.34	**<0.001***
Alkene 2 (C27)	0.10±0.05	0.03±0.03	4.809	0.093	1.09±0.14	1.07±0.01	151.76	**<0.001***	1.01±0.24	0.11±0.01	41.82	**0.002***
Alkane (C27)	0.09±0.03	0.05±0.01	2.793	0.170	0.10±0.03	0.02±0.01	24.04	**0.008**	0.07±0.02	0.02±0.00	36.12	**0.003***
Alkene (C29)	0.00	0.00			0.13±0.05	0.00	21.55	**0.009**	0.23±0.08	0.01±0.01	23.12	**0.008**
Alkane (C29)	0.06±0.04	0.04±0.01	0.364	0.579	0.07±0.05	0.02±0.01	2.08	0.222	0.07±0.02	0.02±0.01	15.00	**0.017**

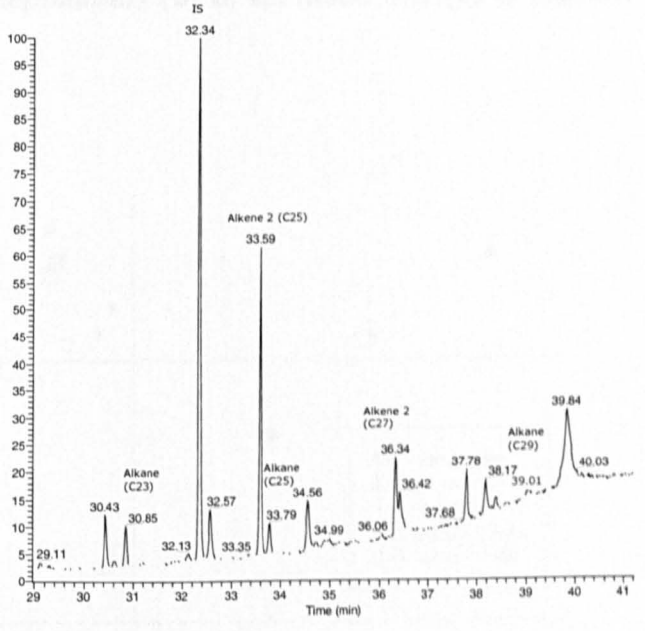
Table 4.3 Variation in CHC profile according to host species. Mean relative abundance ± SD of cuticular hydrocarbons of adult female *Goniozus legneri* (strain U) reared on different host species. MANOVA: Wilks' Lambda = 0.001, F (27,12) = 4.156, P=0.0048. Because 9 ANOVA tests were carried we adjusted the significance criterion according to the Bonferroni procedure: P-values less than 0.0055 value are indicated with an asterisk.

Compound (Carbon chain length)	Molecular weight Da	Host species				ANOVAs	
		Corcyra cephalonica	*Plodia interpunctella*	*Ephestia cautella*	*Ephestia kuhniella*	$F_{(3,12)}$	*P*
Alkane (C23)	324	0.20±0.03	0.17±0.01	0.17±0.02	0.15±0.01	4.348	**0.027**
Alkene 1 (C25)	350	0.55±0.07	0.29±0.06	0.35±0.10	0.45±0.11	6.466	**0.007**
Alkene 2 (C25)	350	0.80±0.09	0.38±0.02	0.40±0.12	0.29±0.10	24.304	**<0.001***
Alkane (C25)	352	0.25±0.02	0.60±0.03	0.54±0.13	0.37±0.13	11.525	**0.008**
Alkene 1 (C27)	378	0.17±0.04	0.14±0.04	0.13±0.03	0.16±0.06	0.792	0.521
Alkene 2 (C27)	378	0.77±0.14	0.68±0.19	0.62±0.11	0.44±0.14	3.970	**0.035**
Alkane (C27)	380	0.09±0.02	0.11±0.03	0.08±0.02	0.08±0.01	2.381	0.120
Alkene (C29)	406	0.10±0.02	0.13±0.06	0.10±0.03	0.09±0.02	1.152	0.368
Alkane (C29)	408	0.04±0.00	0.06±0.01	0.06±0.01	0.04±0.01	4.736	**0.021**

(a)


Alkene 2 (C25)
33.59
IS
32.34
37.78
Alkane (C23)
30.85
Alkane (C25)
Alkene 2 (C27)
Alkane (C27)
Alkane (C29)
Alkene (C23)
30.61
33.79
36.49
39.01
39.82
41.21
29.11
29.23
31.18
31.81
32.56
34.55
36.34
35.51
37.64
38.17
Time (min)

(b)


IS
32.34
Alkene 2 (C25)
33.59
39.84
Alkene 2 (C27)
36.34
Alkane (C23)
Alkane (C25)
Alkane (C29)
37.78
38.17
40.03
30.43
30.85
32.57
34.56
36.42
39.01
29.11
32.13
33.35
33.79
34.99
36.06
37.68
Time (min)

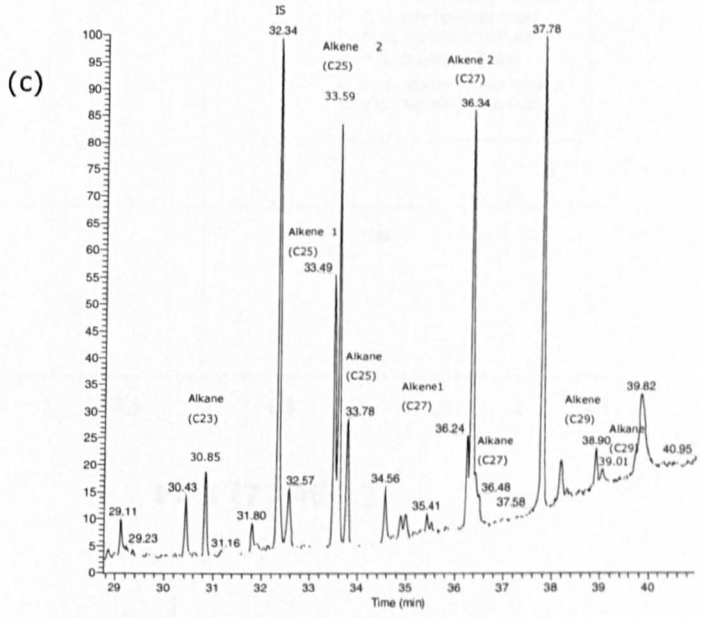
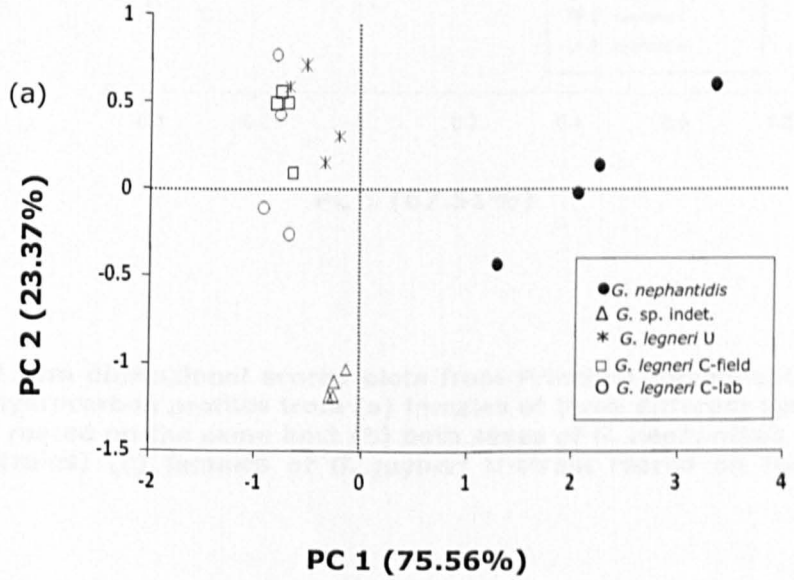


Figure 4.1 Total ion chromatogram examples showing the cuticular hydrocarbons of an adult female of (a) *G. nephantidis* (b) *G.* sp. indet. and (c) *G. legneri*. 'IS' denotes internal standard.



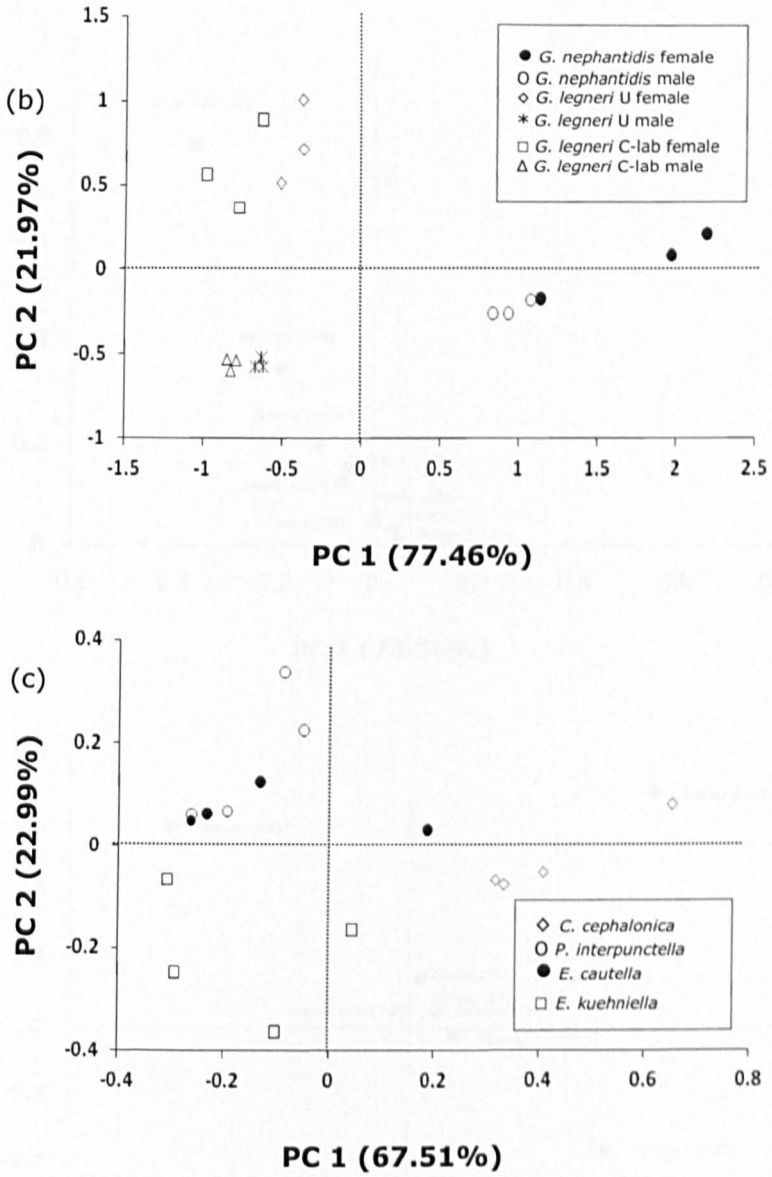


Figure 4.2 Two dimensional scores plots from Principal components analysis of the cuticular hydrocarbon profiles from (a) females of three different species of *Goniozus* parasitoid reared on the same host (b) both sexes of *G. nephantidis* and *G. legneri* (U & C-lab strains) (c) females of *G. legneri* U-strain reared on four different host species.

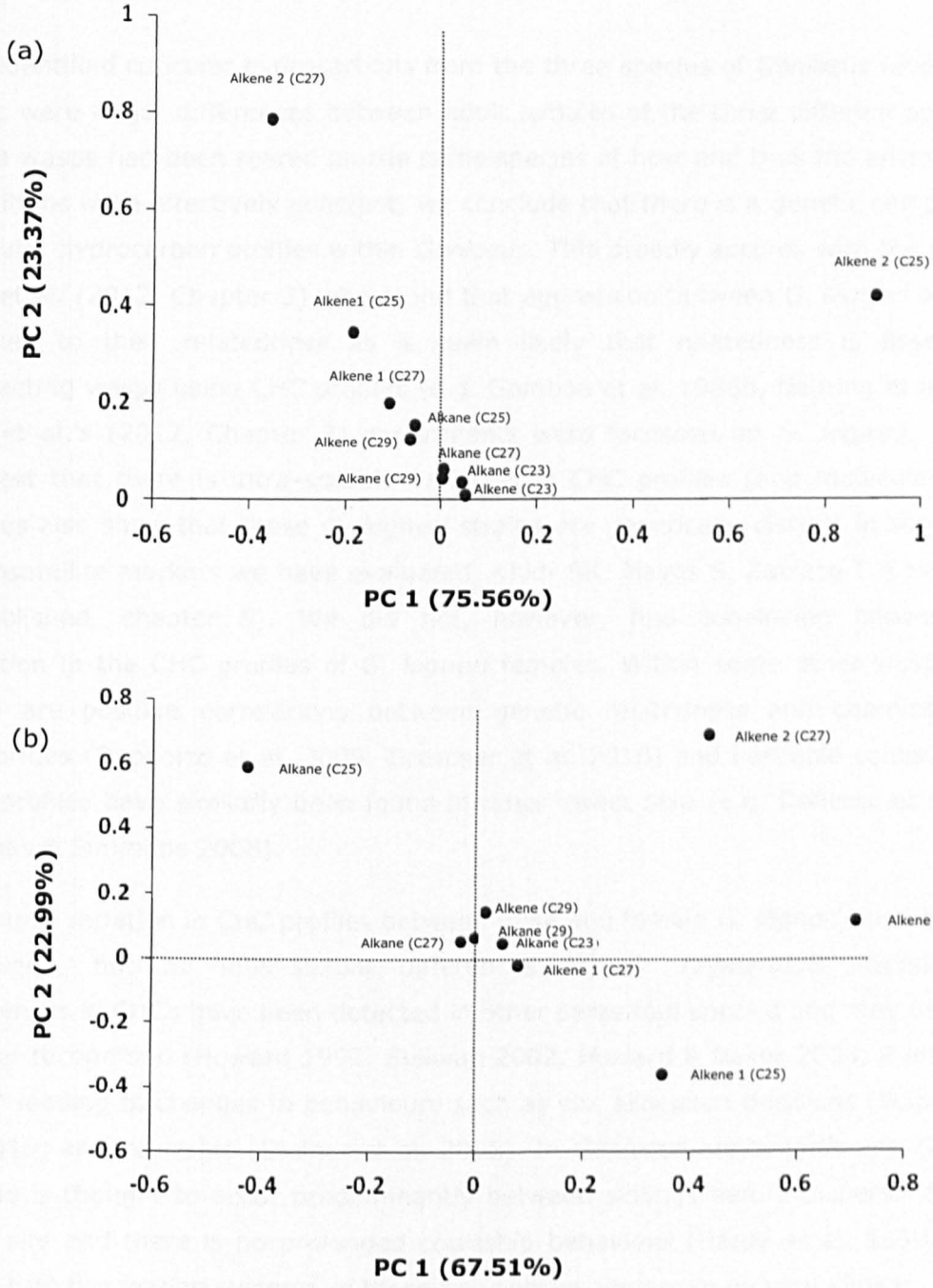


Figure 4.3 Two-dimensional loading plots from principal components analysis of the cuticular hydrocarbon profiles from (a) females of three different species of *Goniozus* parasitoid reared on the same host (b) females of *G. legneri* U-strain reared on four different host species. Panels (a) and (b) correspond to the scores plot shown in Fig. 2a and 2c respectively. The loading plot associated with Fig. 2b is not shown as it is essentially the same as panel (a) of this figure.

4.5 Discussion

The identified cuticular hydrocarbons from the three species of *Goniozus* revealed that there were major differences between adult females of the three different species. As these wasps had been reared on the same species of host and thus the environmental conditions were effectively constant, we conclude that there is a genetic component to cuticular hydrocarbon profiles within *Goniozus*. This broadly accords with the results of Lizé et al. (2012, Chapter 3) who found that aggression between *G. legneri* females is attuned to their relatedness as it seem likely that relatedness is assessed by interacting wasps using CHC profiles (e.g. Gamboa et al. 1986b, Nehring et al. 2010). Lizé et al.'s (2012, Chapter 3) experiments were focussed on *G. legneri*, and thus suggest that there is intra-specific variation in CHC profiles (and molecular genetic studies also show that these *G. legneri* strains are genetically distinct in some of the microsatellite markers we have evaluated, Khidr SK, Mayes S, Zaviezo T & Hardy ICW unpublished, chapter 5). We did not, however, find convincing between-strain variation in the CHC profiles of *G. legneri* females. Within some other wasp species there are positive correlations between genetic relatedness and chemical profile similarities (Dapporto et al. 2009, Drescher et al. 2010) and heritable components of CHC profiles have similarly been found in other insect taxa (e.g. Dallerac et al. 2000, Thomas & Simmons 2008).

We found variation in CHC profiles between male and female *G. legneri*, in both strains examined, but no inter-sexual differences for *G. nephantidis*. Gender-based differences in CHCs have been detected in other parasitoid species and may be used in gender recognition (Howard 1992, Sullivan 2002, Howard & Baker 2003, Ruther et al. 2011) leading to changes in behaviours such as sex allocation decisions (Darrouzet et al. 2010) and courtship (Steiner et al. 2006). In *Goniozus nephantidis* and *G. legneri* mating is thought to occur predominantly between siblings before dispersal from the natal site and there is no prolonged courtship behaviour (Hardy et al. 1999, 2000): given that the mating systems of these two species appear to be very similar, we have no current explanation for the absence of gender differences in CHC profiles in one species and the presence in the other.

The host species on which female *G. legneri* developed also influenced their CHC profiles. As these females belonged to the same strain and thus genetic influences were effectively constant, we conclude that there is an environmental component to

Goniozus cuticular hydrocarbon profiles. Again, this accords with Lizé et al.'s (2012, Chapter 3) result that aggression between *G. legneri* females is attuned to whether or not they had developed on the same or different individual hosts and the notion that females use host derived chemical cues to assess relatedness (e.g. Ross & Gamboa 1981, Shellmann & Gamboa 1982, Pfennig et al. 1983, Gamboa et al. 1986b, Ode et al. 1995, Liang & Silverman 2000, Florane et al. 2004). The strength of correspondence between parasitoid CHC profiles and the chemical composition of their hosts can vary greatly: in the bethylid *Cephalonomia hyalinipennis* the degree of total cuticular hydrocarbon composition of the parasitoids which resemble their hosts is less than 40% (Howard & Pérez-Lachaud 2002) but is more than 90% in the Eucharitid *Kapala sulcifacies* (Howard et al. 2001). Lizé et al.'s (2012, Chapter 3) experiments used only *C. cephalonica* larvae as hosts and thus suggest that the influence of host identity on parasitoid CHC profiles operates in a finer scale than just the species of the host fed upon (as also suggested by Ode et al.'s, 1995, study on parasitoid mating behaviour): further work will be required to assess how individual hosts of a given species influence CHC profiles.

While analysis of cuticular hydrocarbons has greatly assisted the understanding of social behaviour, particularly in hymenopterans, it has also been suggested and utilized as a tool for taxonomic identification of species (Kather & Martin 2012). Our CHC analyses revealed that the unidentified species of *Goniozus* from Oman, *Goniozus* sp. indet., showed closer resemblance to *G. nephantidis* than to *G. legneri.* This species is, however, chemically distinct, both qualitatively and quantitatively, and thus very unlikely to be synonymous with either congener studied here. Further research on the application of *Goniozus* sp. indet. to pest control problems should proceed on this basis. There are around 160 described species of *Goniozus* (Gordh & Móczár 1990) and further work on their chemical profiles may help establish the taxonomic identity of *Goniozus* sp. indet. as well as potentially revealing currently cryptic distinctions and synonymies within the genus.

4.6 Conclusion

We carried out this investigation for two reasons: to discover whether *Goniozus* CHC profiles varied according to genetic relatedness and environmental influences and also to evaluate whether CHC profiling might be utilized as a tool for taxonomic identification of *Goniozus* species. We find both genetic (wasp species) and environmental (host species) influences on CHC profiles. While we have examined cross-species differences in both wasps and hosts, rather than differences between siblings and non-siblings reared from the same or different individual hosts of the same host species, our findings support the expectation, arising from the results of Lizé et al. (2012, Chapter 3), that *Goniozus* CHC profiles would vary according to both genetic background and developmental environment. Because the CHC profiles of different species within the genus *Goniozus* are dissimilar, we also conclude that chemical analysis can be used as a taxonomic tool alongside morphological and molecular genetic identification and thus aid researchers who need to obtain an identity for parasitoids that they have collected from the field.

Part three: Molecular genetics and sex ratios

Chapter 5 : The development of molecular genetic markers in bethylid wasps

5.1 Abstract

Genetic markers work as indicators to reveal differences between genotypes by focussing on the presence of alleles of target loci. Detecting association between phenotype and the genotype of the markers involves dividing the population into different groups depending on a particular marker locus and testing for significant differences between groups with respect to the trait being measured. Markers can also be used to randomly sample the genome and infer information regarding the genetic relationships between individuals. Twelve primer pairs for the south Asian bethylid wasp *Goniozus nephantidis* and 24 for its New World congener *Goniozus legneri* were designed to investigate polymorphism between and within populations, using samples of 85 individuals of both species and including three putatively different strains of *G. legneri*. Annealing gradient tests (50-65°C) were conducted for these primers, to study the quality of the PCR amplification across an annealing temperature gradient using a mixed genotype DNA template from each species separately. Seven primer pairs which amplified clear products of approximately the expected size of *G. nephantidis* and 18 of *G. legneri* were then selected for capillary analysis for fragment size determination on a Beckmann CEQ 8000. Neither *G. nephantidis* nor *G. legneri* were polymorphic within populations. However, there were 6 primer pairs that show polymorphism between *Goniozus legneri* populations that originate from different geographical areas within South America; Uruguay and Chile. Further, one primer pair showed diversity between the two strains collected within Chile. Knowledge of these genetic polymorphisms is potentially useful to investigations of a range of questions in evolutionary and applied ecology and one of the makers developed here has already been used to provide unbiased assessment of primary sex ratio in *G. legneri*.

5.2 Introduction

Among the natural enemies of agricultural pest species, parasitoid wasps are considered as one of the most important classes of biological control agent and are also widely used in studies of evolutionary ecology and basic population biology (Godfray 1994, Jervis 2005, Hochberg & Ives 2000, Wajnberg et al. 2008). The distributions and population structures of parasitoids may be influenced by a wide range of factors, such as geological and geographical components, ecological processes, evolutionary and genetic aspects (Zink 2002, Bond & Stockman 2008). Successful population genetic, ecological and evolutionary studies can be achieved through the availability of suitable molecular markers as they are regarded as important indicators of relationships between both individuals and populations (Carvalho 1998). These markers have the ability to reveal differences between genotypes through the application of a range of random markers, not linked *a priori* to traits.

Among the classes of genetic markers are 'microsatellite' markers, which are also known as simple sequence repeats (SSRs). Microsatellites essentially consist of short repeated units of around two to six base pairs in length with an array up to around 200bp long and can be found in both coding and non-coding regions in all prokaryotic and eukaryotic genomes (Tautz 1989, Arcot et al. 1995, Beukeboom & Zwaan 2005). Frequently, microsatellite loci have been isolated from partial genomic libraries of the species of interest through screening several thousand clones through colony hybridization with repeat containing probes (Rassmann et al. 1991). SSRs (simple, tandemly repeated di- to tetra-nucleotide sequence motifs flanked by unique sequences) have been developed in a number of parasitoids (Baker et al. 2003, Anton et al. 2006, Lozier et al. 2006). Microsatellite markers have many advantages over other marker types because not only do they generally have a high number of alleles per locus which can identify polymorphism but they also have high expected heterozygosity and high mutation rates (Hancock 1999).

Thus, microsatellite markers have been used to determine the genetic diversity and differentiation between populations through measuring the degree of heterozygosity in parasitoid species (e.g. 0.378-0.063 in *Cotesia melitaearum*, 0.171-0.629 in *Neotypus melanocephalus* and 0.170-0.367 in *Lysiphlebus hirticornis;* Kankare et al. 2005, Anton et al. 2007, Nyabuga et al. 2010) and the degree of gene flow and dispersal

between populations (Avise 1994, McCoy et al. 2001, Molbo et al. 2003, Kankare et al. 2005, Zavodna et al. 2005, Drescher et al. 2010, Nyabuga et al. 2010).

This study set out to design a microsatellite marker system for screening two species of bethylid wasps for genetic polymorphisms within and between populations. In principle such markers could prove useful for pest control applications (Aebi et al. 2008, Ugelvig et al. 2008, Lozier et al. 2009, Zygouridis et al. 2009, Nicholls et al. 2010, Lavandero et al. 2011), evaluating the effect of kinship on social behaviours (Lizé et al. 2012, Chapter 3) and for measuring population parameters, such as levels of inbreeding, which have not been directly evaluated but are important in the understanding of reproductive decisions (Hardy & Cook 1995, Hardy et al. 1998, 1999, 2000). However, the first direct application of these markers has been to provide assessment of the sex of individual eggs to evaluate maternal sex allocation without the biasing influence of developmental mortality (Khidr et al. submitted, Chapter 6).

5.2.1 Biology of bethylids

The Bethylidae is a family of parasitoid wasps which has been thought to comprise four extant subfamilies: Bethylinae, Epyrinae, Pristocerinae and Mestitiinae (Evans 1964) with over 2000 described species (Gordh & Móczár 1990). Recently the higher level phylogeny of bethylids has been estimated using molecular data of 33 species resulting in a split of the sub-family Mestitiinae into two separate sub-families; the Mestitiinae and the Cephalonomiini (Carr et al. 2010). Bethylid wasps attack almost exclusively the immature stages of coleopterans and lepidopterans, many of which are pests of important agricultural commodities such as coffee, coconut, sugarcane, apple, walnut and almonds (Gordh 1982, Batchelor at al. 2005, Venkatesan et al. 2007, Zaviezo et al. 2007). In this study we focus on *Goniozus nephantidis* (Muesebeck) a parasitoid of the lepidopteran larvae the coconut pest (*Opisina arenosella* Walker), and *G. legneri* Gordh, a parasitoid of several lepidopteran pests. Both G. *nephantidis* and *G. legneri* have each been used in biocontrol programmes (Dharmaraju 1963, Legner & Silveira-Guido 1983, Gothilf & Mazor 1987, Lyla et al. 2006) and in a range of behavioural ecological studies (e.g. Hardy & Cook 1995, Goubault et al. 2006, 2007a, Humphries et al. 2006, Bentley et al. 2009, Lizé et al. 2012, Chapter 3). Their basic life-histories are similar; both are gregarious idiobiont ectoparasitoids exhibiting sub-social behaviour, such as maternal care and defence of the developing brood (Hardy & Blackburn 1991, Bentley et al. 2009), and appear to conform closely, but probably not

exactly, to single foundress Local Mate Competition (Hamilton 1967, Hardy & Cook 1995, Hardy et al. 1998, 1999, 2000). Males usually emerge before females and have effectively unlimited mating capacity to inseminate their sisters (Hardy et al. 1999, 2000) and, as with many other bethylids, the sex ratios of these species are generally female biased with low variance (Green et al. 1982, Hardy & Mayhew 1998, Hardy et al. 1998, Khidr et al. submitted, Chapter 6).

5.3 Material & Methods

5.3.1 Parasitoid origins and cultures

Goniozus nephantidis (Muesebeck) is a natural enemy of the coconut pest *Opisina arenosella* Walker in the Indian sub-continent (Venkatesan et al. 2007). Our culture has been maintained in the laboratory for more than 20 years on the facultative host *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae), following methods given in Lizé et al. (2012).

We used three strains of *Goniozus legneri* Gordh. One, termed strain 'U', was obtained from a commercial insectary in the USA and kept in our laboratory for more than eight years. The original material is believed to have been collected from a population in southern Uruguay in 1978 (Gordh 1982, Gordh et al. 1983, Legner & Silveira-Guido 1983). Two further strains of *G. legneri* were brought to our laboratory in May 2009 from Santiago, Chile. One strain was collected directly from walnut trees and is termed 'C field' while the other strain is termed 'C lab' as it had been maintained in a Chilean insectary for several years following collection from the field near to Santiago (Zaviezo et al. 2007). *Corcyra cephalonica* was used as a facultative host for all three strains of *G. legneri*. In the field it is known to attack a range of lepidopteran species that are pests of walnuts, pistachio nuts, almonds and apples (Steffan et al. 2001, Garrido et al. 2005, Zaviezo et al. 2007). All cultures were maintained in a climate room at 25-27°C, 12 L: 12D with high relative humidity maintained by a water bath.

5.3.2 Design and preparation of the primers

Microsatellite-enriched genomic libraries were created essentially according to Kloda et al. (2004) with the final sequencing step being carried out through use of barcoded

adaptors and a 1/16th run of non-titanium reagents Roche 454 Pyrosequencing (as part of a mixture of 9 different libraries). The generated Fasta files were separated *in silico* to identify the individual libraries and those for *G. legneri* and *G. nephantidis* were searched for microsatellite motifs using the MISA.pl script (pgrk.ipk-gartersleben.de/misa/misa.html). Primer pairs flanking the simple sequence repeats were designed either by Primer 3 (Rozen & Skaletsky 2000) and/or WebSat (Martins et al. 2009) for *G. legneri* (Table 5.1) and for *G. nephantidis* (Table 5.2). Primers were synthesised by MWG Eurofins with a forward primer 5' extension consisting of the M13 sequence, to allow labelling of the final product through a three primer reaction (Schuelke, 2000) and prepared to 1000× concentration using Sigma molecular biology grade water (to create primer stocks of 200pmol/µl). After vortexing and spinning, tubes were placed on ice for 30 min. Primers were kept in a freezer at -20°C and to produce a 10× primer stock; 5µl of the 1000× stock was mixed with 495 µl sterile distilled water (SDW) for both Forward and Reverse primers into separate tubes on ice. The third primer (M13) was ordered from Sigma Aldrich and labelled with dye D4 (blue; WellRed dyes) and the sequence used was 5'-TGTAAAACGACGGCCAGT-3'.

Table 5.1 Primers designed for *G. legneri*

Name	product size	SSR	length	sequence F	length	sequence R
GlSSR1	183	(AG)18	24	GCAGAAAGTTTTCACGAGCGATTT	18	TACCCGGTACCCCGTTCC
GlSSR2	142	(CT)6	25	CCCTTAAATCGACATCGGTTATCCT	24	CGAAAACAAAGCTCGCTCCATTAC
GlSSR3	158	(AG)9aaagg(GA)8	24	CGCCGAGTTCTTTCTCTCGTTTTA	24	TCGAAGTTATACGCATCCCGAAAC
GlSSR4	113	(CA)13	24	TCTTGCTTACGGGTGGACTAACAA	22	AACGCTCCACCTCGTGTGTGTG
GlSSR5	205	(GA)11	24	GGCTTCAACCTTGCGATTCTATTG	27	CCTTGCATAATAATAACGTACACTCTC
GlSSR6	118	(GA)12a(AG)7	23	GGTAGCTGCGAGCGAAAAGAGAG	23	GTCCCGTCTCACTAACCCCTCCT
GlSSR7	120	(GA)13	24	AGGGTATCATTACGCGAGACCGTA	24	CCACTCTCTCGTTACACCGCGTAT
GlSSR7*	142	(GA)13	19	CGAGGGTATCATTACGCGA	20	GGCCACTCTCTCGTTACACC
GlSSR8	178	(GA)14	24	TACACACACGCTGCATTGTGACTT	24	TAGCGAAACCTACGCGTCTACCTC
GlSSR9	138	(GA)14	22	CATTATCGCTGCGCCGAAAGTC	24	ACGCTCGGTGCTCTCTCATTCTAC
GlSSR10	80	(GA)9	24	ATGAGAATGCGTAGAGGGGGTAGA	24	GCGCTATCGGACGAACTACTCTCA
GlSSR11	107	(GC)8	27	ACCCTCGATGCTCGTTTGATTG	24	GCGCGAGACTGTATGAGCTTGTAA
GlSSR12	101	(TG)8	25	CAATGTAAGATGCGGTAATCGATGAAT	24	TTACGAGATGCACGGAGAGAAAAA
GlSSR13*	121	(AG)10	20	CGCCACGGTTTTGATTAAGT	19	GCGCTATTCGGCACTCTCT
GlSSR13	151	(AG)10	19	GCGATATGCGATTGACAGG	19	GCGCTATTCGGCACTCTCT
GlSSR14*	134	(AG)11	20	CGTGAACAAATCGAACGAAA	22	GACCGAACGTACTAACCAACCT
GlSSR14	138	(AG)11	20	CGTGAACAAATCGAACGAAA	20	TCCCGACCGAACGTACTAAC
GlSSR15*a	162	(GA)12	22	TCAGCGAAATCGAGAGCTAAAT	22	GGCTAATTGCGTTATACTCCGT
GlSSR15*b	117	(GA)12	19	AGTCTCCGGTTTATGCCGT	22	GGCTAATTGCGTTATACTCCGT

GISSR16*a	152	(GA)13	18	ATTATGCCCTCGTTGCCT	22	CTCTTTCTCTCCTTCTCTCCGT
GISSR16*b	141	(GA)13	20	CCACCGGCATGGATTTCTTT	27	GGGAGGTCGCTTATTCTAACTCTTTCT
GISSR16	176	(GA)13	19	GCTGCATTATGCCCTCGTT	26	GGGAGGTCGCTTATTCTAACTCTTTC
GISSR17*	164	(GT)8	18	CAGAAGGGGCATCCTTGA	25	CTTGAAACTTACTGCGCTAATACAC
GISSR17	155	(GT)8	20	ATCCTTGACGACGGCCTAAC	26	GCTTGAAACTTACTGCGCTAATACAC
GISSR18*	102	(Tc)11	21	ACGTAGTCCTGCATCACGAAA	22	AGACGAAGATACGAAGAGTCGG
GISSR18	201	(Tc)11	20	AGGTGAGCCGAGCTTTATTG	22	GGATTCCTTCGAGAGAGAGAGA
GISSR19*a	125	(Tc)11	18	GACGCAACGCCATCCATA	22	CACCGAGTAGAGTTTCATTCCG
GISSR19*b	102	(Tc)11	22	ACCAAATAGAGTCGAAAATGCG	22	CACCGAGTAGAGTTTCATTCCG
GISSR19	179	(Tc)11	22	CGAGTCGATGATAAATCCCTGT	21	CACCGAGTAGAGTTTCATTCC
GISSR20*	112	(AC)6	22	TGTCACGTTGCCAGTTAGAAGA	18	CGTGTGTGTGCGTGTGTG
GISSR20	182	(AC)6	20	TTTCAGGTGCGGGAAAGAAC	20	GTGCGTGTGTGCAATCATCT
GISSR21*	162	(AG)9	18	GGTGTCCCAGGCGTCTTT	18	GTCTCCCTCCCCTCCACC
GISSR22*	108	(AG)15	22	ACGCGACAATTTCTTTCTTCTC	20	CCCGACGTGTCTTCTCTCTT
GISSR22	168	(AG)15	20	CGTTCCTCACTCCTCTCATC	20	CCCGACGTGTCTTCTCTCTT
GISSR23	126	(AG)22	20	GCTCGAGATAATTGCCGTCT	20	GTCCGTCTCGTTCGTCTCTC
GISSR24	102	(GA)9	20	AGCAATAACATTGCGGAGGA	21	GCGCTATCGGACAACTACTCT

*Denotes primers designed using WebSat, in some cases there were two versions designed for a particular fragment length denoted by [a] or [b]. The remainder of the primers were designed using Primer 3.

Table 5.2 Primers designed for *G. nephantidis*

Name	product size	SSR	length	sequence F	length	sequence R
GnSSR1	173	(AC)13	24	GCACGTGAATTTATGAACGAG GAA	24	CTAGGGACCGTGCAGAAAACT ACG
GnSSR2	89	(AC)17	24	TTCTGAGGGTTATCTCGGTGTT CG	23	TCCGTCGGACGTAACTACACCT C
GnSSR3	104	(AC)8	24	GGATAAGCTCGTGAAAGCTTC GTC	24	GATCATAGGAACGGACGAACG AAC
GnSSR4	114	(AC)8	26	CGGGTAACGTGATTAATTCCTC TTTC	24	GGCAATTTCACGGGGTTACAG TTA
GnSSR5	138	(AC)9	24	AGCAGCAGCATACTCACACACA GA	24	CGCGCTTGAATCGCATATAAAT CT
GnSSR6	111	(AC)9	23	ACCGAGCAGCGTTGTATGATGT C	24	ACCATTGTAAAATCTTCGCGGG TA
GnSSR7	163	(AC)9	24	GATTGTCGGTAAGGGGACAAT GAG	23	TGGACTAGGCTCGAATCGTTCA C
GnSSR8	158	(ACGA)5tagaa(AG)10	24	GATCATAGGAACGGACGAACG AAC	24	TATATCTGGACGACGATGGGG AAC
GnSSR9	136	(AG)8	24	ACGAGGATTGGAAGAGAGTCG AAG	26	CCTACAGTTTACGTACCCACTC TCTC
GnSSR1 0	173	(AG)10	24	CCCTGTTTCAGGCTTACAGATA GA	24	GTTCCCGCGTGGACTAACAATT AC
GnSSR1 1	97	(AG)10	24	GGGTGGTAAAGCAAGAAGAAA GCA	21	AAGACACGACAATTCATTACG
GnSSR1 2	188	(AG)10	24	AGCGGTATAGAGGACTTCGGG AAC	24	CGATAAAGTCGCACACGCAAAT AC

5.3.3 DNA extraction

A sample size of 85 individuals was used for capillary testing (section 5.3.7). We examined 17 individuals of *G. nephantidis* and a total of 68 individuals for the different strains of *G. legneri* (25 of 'U' strain, 22 of 'C lab' strain and 21 individuals of the 'C field' strain). In addition, five pooled samples of 20 individuals were used for the annealing gradient test.

Individual females or pooled samples were placed in 1.5 ml eppendorf tubes (Sarstedt) then immersed into liquid nitrogen and crushed using a mini pestle to start the extraction. Genomic DNA was then extracted either by using a GenElute plant Genomic kit (Sigma Aldrich) or by following, with some modifications, methods given

in Sambrook et al. (1989) and Vogler and Desalle (1993) before elution/resuspension into 50-60µl of sterile distilled water and storage at -20°C.

5.3.4 Polymerase Chain Reaction (PCR)

PCR reactions were carried out in either a Thermo Hybaid Express PCR machine (Electron Corporation, Milford, MA, USA) or in an ABI PCR 9700 Thermocycler machine (ABI, Carlsbad, CA, USA). The Thermo Hybaid Express PCR was used for annealing gradient tests and run with a 15°C gradient by using a total volume of 20µl for one reaction through mixing different components consisting of 2µl of 10× Forward primer & 2µl of 10× Reverse primer (2 pmol/µl final);2µl of 10X PCR buffer; 0.16µl of dNTP's (mixed dNTP 25mM final concentration per nucleotide); 2µl of DNA template (mixture of many individuals); 0.10µl of Taq DNA polymerase (5 units/µl) and finally 11.74µl of SDW. Thus, the optimal annealing temperature was determined for each pair of primers according to following program:

Initial denaturation:	94°C for 3 min	
Main cycle (denat):	94°C for 1 min	} 35 cycles
Annealing temp grad:	50°C – 65°C for 1 min	
Polymerase extension:	72°C for 2 min	
Final Extension:	72°C for 10 min	

Hold at 4°C

For samples amplified in the ABI PCR 9700 Thermocycler the aforementioned programme was used but the determined optimum temperature of the annealing used was 60°C for the majority of the primers unless stated otherwise (section 5.4.2). PCR reactions consisted of 0.2µl of 10X Forward primer and 2µl of 10× Reverse primer (2 pmol/µl final), 2µl (10×) PCR buffer, 0.16µl dNTP's (each in a 25mM final concentration), 0.04µl M13 Blue Taq of 1000× (53.8 nM concentration), 2µl of individual genomic DNA (approximately 5ng/µl), 0.10 Taq DNA polymerase (5 units/µl) and 13.5µl SDW. Thus, a fluorescently-labelled M13 tail sequence was added to the 5'-end of the Forward primer (Schuelke 2000) to be used for capillary sequencing.

5.3.5 Agarose Gel Electrophoresis

Samples to be loaded on the gel were mixed with 6x gel loading blue buffer (Promega) in the ratio of one part sample to one part loading buffer. The mixture was added to each well of the plates from the PCR machine and was spun briefly. Then 10µl from each well were loaded onto a submerged gel that consisted of a 2% concentration of agarose (Molecular Grad, Bioline) prepared in 0.5× TBE (Tris- Borate- EDTA) buffer, followed by addition of 2µl of ethidium bromide stock before pouring (10mg/ml; Promega corporation). Each primer pair reaction was loaded onto one row of the gel (each primer pair having 12 reactions across a 15oC annealing gradient). Alongside appropriate size marker (5µl of 2-log DNA ladder; New England Biolabs, Ipswich, MA, USA) was loaded in the first lane of each primer pair, then the gel was run at 90V for approximately 1 hour. After electrophoresis, results were visualised and photographed under UV-light in a Bio-RAD Gel Doc 2000 gel box.

5.3.6 Quantitation test of DNA templates and *Goniozus* individuals

DNA extractions were quantified by comparison with known uncut lambda DNA (BioLabs) (50ng/µl) loaded in the following amounts; 10µl, 5µl and 2.5µl and 1.5µl, to represent fluorescence comparison with the unknown samples. Thus, the 1% agarose gel was run at 90V for 75 min then different individuals of both species were quantified, tested for DNA integrity by ensuring that genomic samples largely ran at limiting mobility. Good quality samples were used as DNA templates.

5.3.7 Capillary sequencing: Preparing fragment samples for analysis

The CEQ 8000 Fragments Analysis Software Version 8 was used to measure and analyse the fragment sizes of the PCR products. The preparation of the sample in half-reactions was described as below:

First, for each row of 8 samples 215µl of SLS (sample loading solution) was added to 2µl of SS 400 (Standard size) mixed by vortexing and spun briefly. Then 27µl of this mixture was added to each well in the row and 2µl of multiplexed PCR product was added later. Finally the mixture in each well was overlaid immediately with a drop of mineral oil and placed in the CEQ machine. Later, cluster analysis between different populations of *Goniozus legneri* was generated by Multi Variate Statistical Package (MVSP; Kovachs) version 3.2.

5.4 Results

5.4.1 Primer design in the Microsatellite library

Genomic libraries consisting of 273 sequences containing microsatellite motifs were screened to design primers for *Goniozus legneri*. Among the best 24 microsatellites were considered to be clearly unique sequences with adequate flanking sequence length to design primer pairs flanking the repeat unit. The remaining fragments shared the same SSR sizes, or the SSRs were located at the end of the fragments leaving insufficient flanking sequences for primer design, or the SSRs appeared in compound formation. The dinucleotide $(GA)_n$ was the predominant marker followed by $(AG)_n$ and $(TC)_n$. While the tri- and tetra-nucleotide microsatellites were frequently in complex forms.

In G. *nephantidis* there were 3356 SSRs of which 12 were chosen to design primers for the investigation of polymorphism within the population. The dinucleotide microsatellite repeat motifs $(GA)_n$ and $(AG)_n$ were the most common repeat category in the library.

5.4.2 Annealing gradient tests

PCR analysis was performed to optimise annealing gradients for the 12 new *G. nephantidis* primer pairs and 24 primer pairs for *G. legneri* strains. Sometimes a number of primer pairs were designed to the same microsatellite repeat sequence to increase the probability of success. The process was repeated several times to test the reliability of the new primers. Representative results of the annealing electrophoresis gels are shown in Figure 5.1 and Figure 5.2.

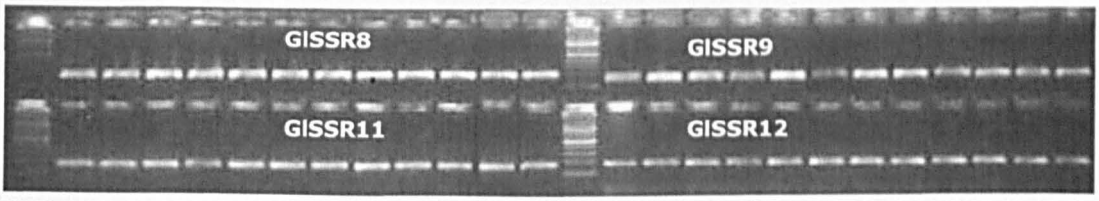


Figure 5.1 Annealing gradient for *G. legneri* primers (primer labels correspond to those in Table 5.1)

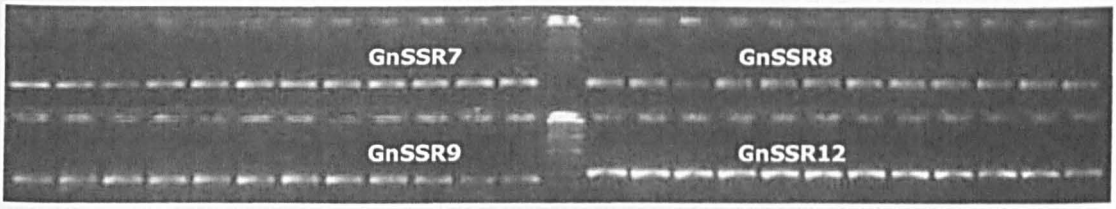


Figure 5.2 Annealing gradient for *G. nephantidis* primers (primer labels correspond to those in Table 5.2)

Primers showing clear bands in annealing tests were selected for PCR amplification and polymorphism testing. In addition the best annealing temperature for each primer was recorded in order for this to be used for the PCR. According to the results of the annealing tests, the best temperature for all *G. nephantidis* primers was 60°C except for primer GnSSR11 at 56°C. No amplification was observed for primers GnSSR1, 2, 3, 4 and 10 in the test. In *G. legneri,* the optimum temperatures were 54°C, 56°C and 58°C for primers GlSSR 14, 5 and 22, respectively. The remainder of the primer pairs had optimal annealing temperatures close to 60°C, allowing simultaneous amplification in the same thermoblock. The following primers were excluded from further work GlSSR1, 4, 6, 10, 19^{*b}, 20*, 21*, 22* and 23.

5.4.3 DNA quality test

DNA quality tests were conducted for *Goniozus* that were to be used as a template and diluted for annealing tests (Figure 5.3). However, DNA preparations from individual wasps for use in PCR did not need dilution because they were less than 62.5 ng lambda DNA (Figure 5.4).

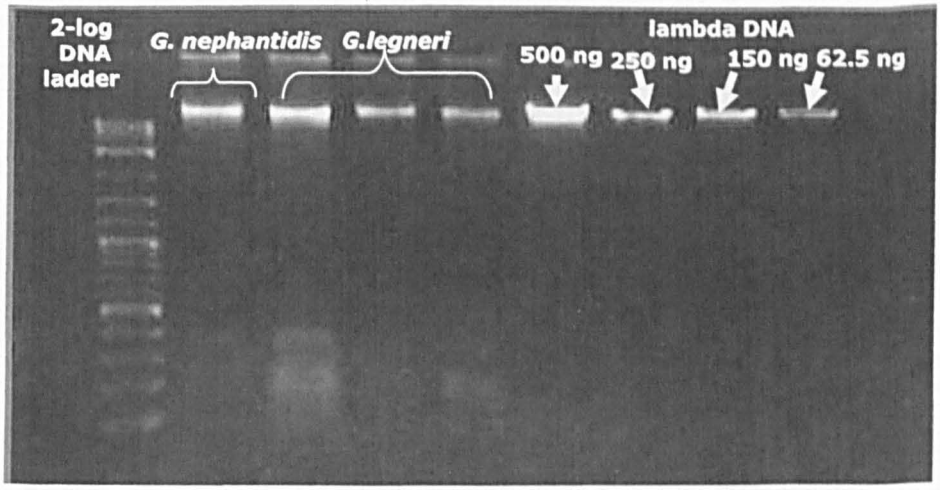


Figure 5.3 Quality and quantity test for *Goniozus* DNA templates

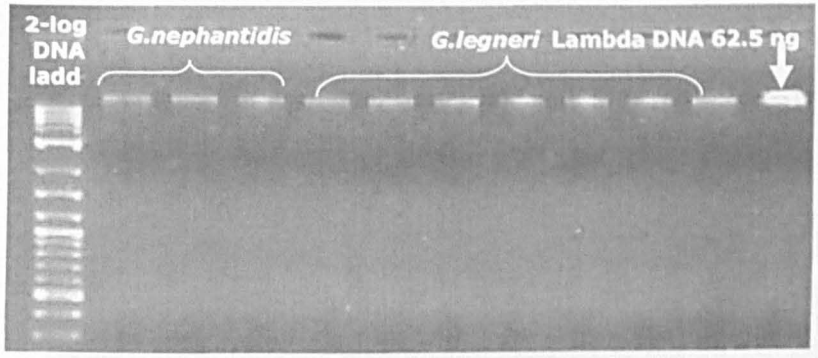


Figure 5.4 Quality and quantity test for individual *Goniozus* DNA templates

5.4.4 Second round of PCR runs

Primers chosen in the annealing test were amplified on the ABI PCR machine at different temperatures according to their annealing test optima. The gel electrophoresis results were visualized using UV light. Some primers were rejected before capillary test due to not amplifying in an annealing test or, if they amplified, not showing clear/discrete single bands on the gel. Representative results of the PCR electrophoresis gels are shown in Figure 5.5 and Figure 5.6.

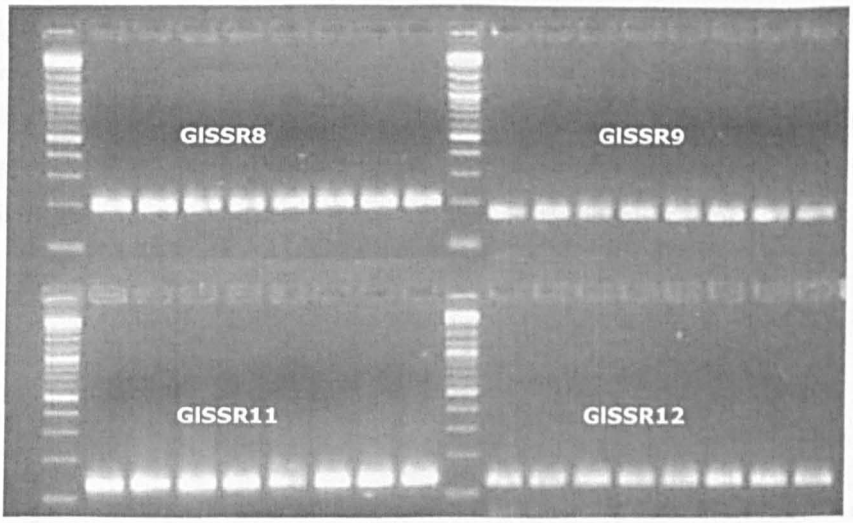


Figure 5.5 Gel plate of PCR product for different primers of G. *legneri*

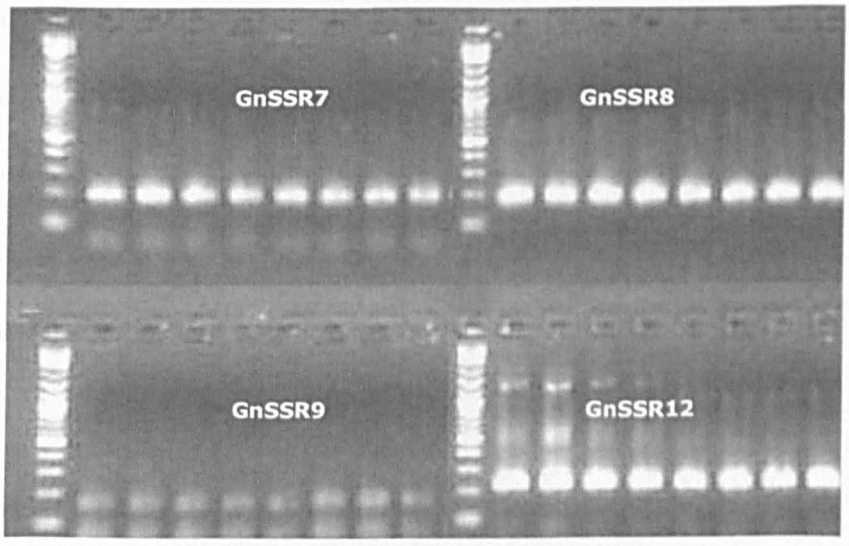


Figure 5.6 Gel plate of PCR product for different primers of *G. nephantidis*

5.4.5 Capillary sequencing

The results of the capillary fragment analysis were processed using the Beckmann CEQ 8000 software to determine fragment size. Neither *G. nephantidis* nor *G. legneri* were polymorphic within strains. Nonetheless, there were six primers that showed clear inter-strain polymorphism in *G. legneri* (Table 5.3). For instance, primer GISSR7

showed a large size difference between strains: while U-strain was 137bp both Chilean populations were 153bp. Further, primer GISSR5 showed a different size allele at 228bp for both U and 'C lab' strains, with 224bp for the 'C field' strain. Variability in individuals between G. *legneri* strains ranged between two and 16 bp (e.g Figure 5.7 and Figure 5.8) the product sizes from all the PCR amplifications were less than 300bp.

Table 5.3 The six polymorphic primers for *G. legneri* strains

Primers	Strength	T range	Best T	'U' size	'C lab' size	'C field' size	expected size	Polymorphism test
GISSR3	Medium	50°C-65°C	60°C	176	174	174	158	Yes
GISSR5	Strong	50°C-65°C	58-60	228	228	224	205	Yes
GISSR7	Medium	50°C-65°C	Any	137	153	153	120	Yes
GISSR8	Strong	50°C-65°C	Any	193	195	195	178	Yes
GISSR13	Strong	50°C-65°C	Any	178	182	182	151	Yes
GISSR22	Medium	50°C-65°C	58-60	190	192	192	168	Yes

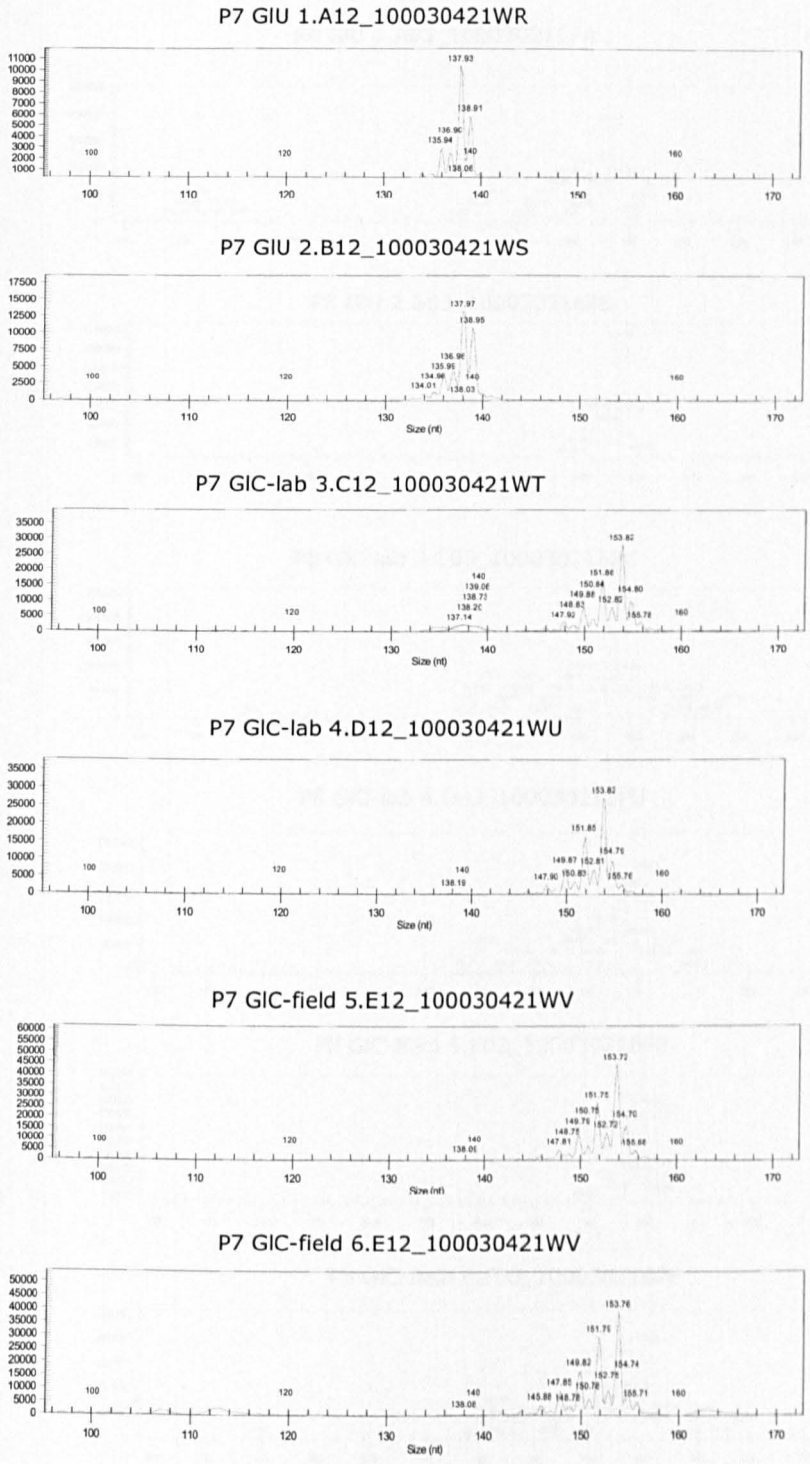
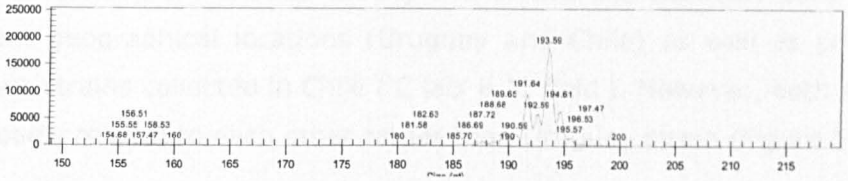
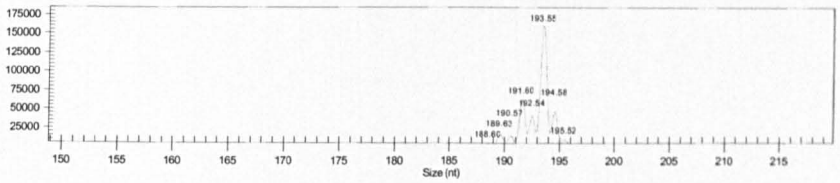


Figure 5.7 Examples of capillary tube analysis of primer 7 for different genotypes of *G. legneri* strains (2'U' +2'C lab' + 2'C field').

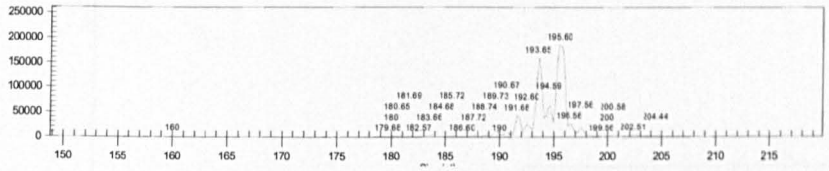
P8 GlU 1.A03_100030216PA



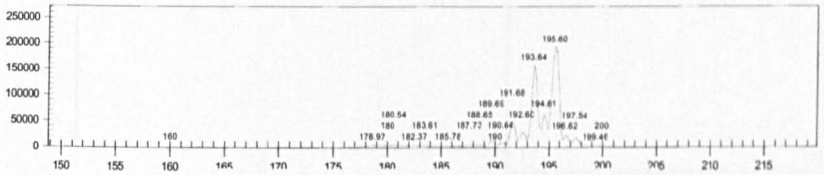
P8 GlU 2.B03 100030216PB



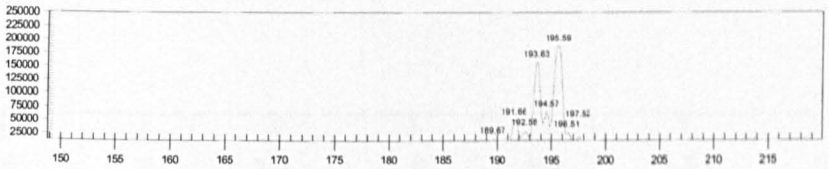
P8 GlC-lab 3.C03_100030216PC



P8 GlC-lab 4.D03_100030216PD



P8 GlC-field 5.E03_100030216PE



P8 GlC-field 6.F03_100030216PF

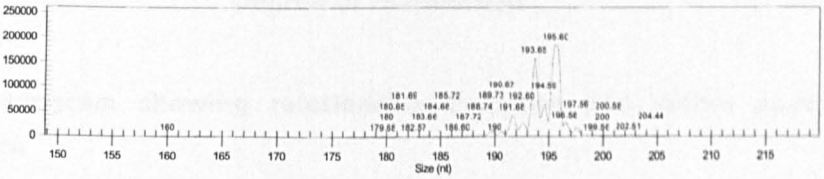


Figure 5.8 Examples of capillary tube analysis of primer 8 for *G.legneri* strains (2'U' +2'C lab' + 2'C field').

Moreover, the dendogram showed clearly the differences between both populations from different geographical locations (Uruguay and Chile) as well as polymorphism within the two strains collected in Chile ('C lab' & 'C field'). However, both strains were still more closely related to each other rather than Uruguay strain (Figure 5.9).

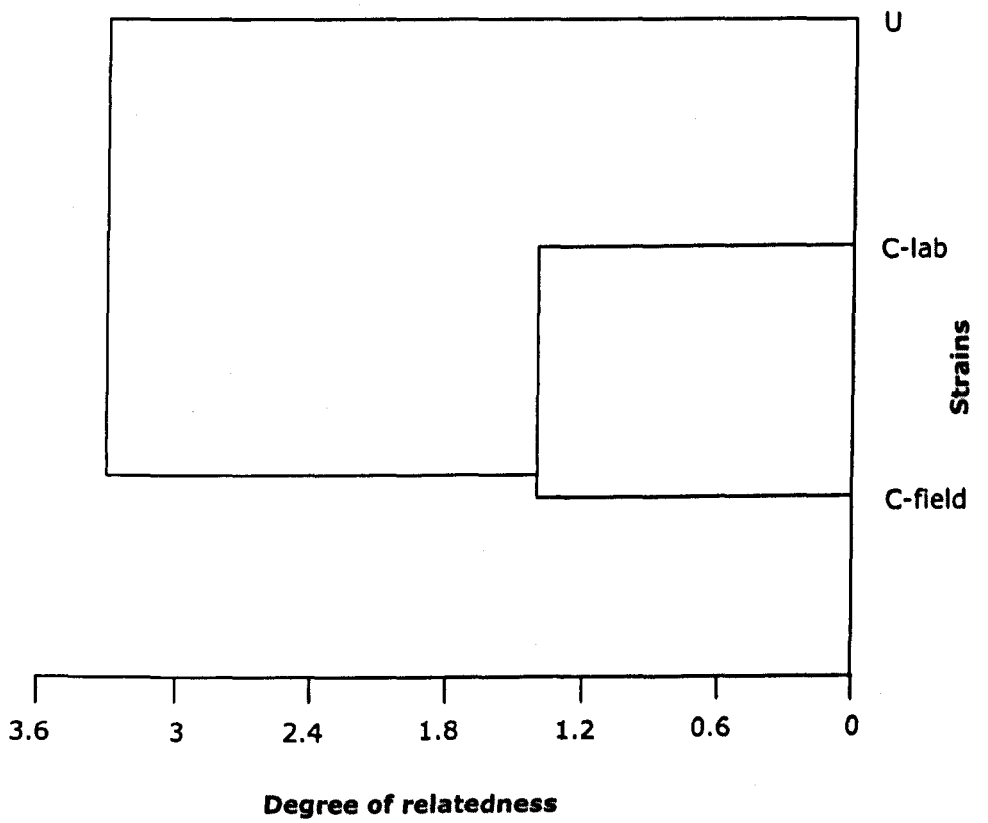


Figure 5.9 Dendogram showing relationship between and within populations of *Goniozus legneri*.

5.5 Discussion

This study set out to develop molecular markers for bethylid wasps with the idea of subsequently using these markers in evolutionary ecology and agricultural research. In general, the percentage of amplified loci decreases with increasing genetic distance making such markers most suitable for closely related species, such as congeners (Hancock & Simon 2005, Barbara et al. 2007). We note that there are well over 100 described species of *Goniozus* (Gordh & Móczár 1990).

Our molecular data show a lack of variation within our strains of *Goniozus nephantidis* and *G. legneri*. Potential reasons for this include a loss of genetic diversity during laboratory maintenance (e.g. due to relatively small populations in culture or occasional population crashes) (Unruh et al 1984, Shields 1993, Cook 1993b, Henter 2003). However, this explanation would most likely apply to *G. nephantidis* and the U-strain of *G. legneri* as these have been maintained in culture for many more years than the strains of *G. legneri* from Chile (C lab and C field). The fact that we did not find polymorphisms within the much more recently collected Chilean strains thus does not fit with a genetic drift due to small population and 'time in culture' effect. An alternative explanation is that there is limited genetic variation within each of the populations from which field collections were made. Genetic homozygosity can result from inbreeding because relatives mate more frequently than expected by chance given the overall size of the population (Henter 2003, Elias et al. 2010, Mazzi et al. 2011) and both species of *Goniozus* are known to exhibit high levels of pre-dispersal sibling mating in the laboratory (Hardy et al. 1999, 2000) and have sex ratios that largely conform to theoretical expectations under such 'local mate competition' (Gordh et al. 1983, Hardy & Cook 1995, Hardy et al. 1998, Khidr et al. submitted, Chapter 6). It is further known that *G. nephantidis* does not exhibit inbreeding depression in terms of effects on developmental mortality or sex ratio control (Cook 1993b). Nonetheless, the post-dispersal mating behaviour of neither species has been directly evaluated and the details of the natural mating systems of these wasps are likely to have a large effect on the evolution of their sex ratios (Hardy 1994, Hardy & Cook 1995, Hardy & Mayhew 1998, Hardy et al. 1998). The lack of within-strain genetic polymorphism we observed provides a degree of evidence that sibling-mating is the predominant feature of the natural mating system of these *Goniozus* species.

For *G. legneri* strains collected within the same geographical region of Chile, 'C lab' and 'C field', we observed a genetic difference in just one developed primer. This could relate to the fact that the field strain was collected from carob moth larvae (*Ectomyelois ceratoniae* Zeller, Lepidoptera: Pyralidae) feeding on walnuts while the laboratory strain was derived from a mixture of individuals collected from both carob moth on walnuts and codling moth larvae *(Cydia pomonella* L., Lepidoptera: Tortricidae) feeding on apples (Zaviezo et al. 2007, T.Z. pers. obs., I.C.W.H. pers. obs.). Genetic diversity in several other parasitoid species has been found to be associated with host and host plant species (e.g. Kavallieratos et al. 2004, Stireman et al. 2006).

There were six primers that showed clear microsatellite polymorphism between *G. legneri* strains (U and C field). Genetic differences between these strains could arise due to differences in the species of insect host or host plant they were collected from (see above) and also to differences between the geographical regions of their collection (Menken 1981, Ruiz-Montoya et al. 2003, Stireman et al. 2006, Pannebakker et al. 2008, Phillips et al. 2008, Lozier et al. 2009, Lavandero et al. 2011). Various biological traits and genetic diversity might associate with populations from different geographic localities due to being geographically isolated, influenced by different climatic effects and thus experienced different selection pressures (Diehl & Bush 1984, Hopper et al. 1993, Thompson 1994, Goodisman et al. 2001, Hufbauer et al. 2004).

Establishing that there is genetic polymorphism within parasitoid species opens up a number of possibilities for the use of genetic markers. For instance, molecular markers have been used to show that the host searching behaviour of different *Agathis* sp. populations was not affected by geographical structure and they have the ability to disperse for long distances (Althoff & Thompson 2001) and reciprocal crossing of two geographically and host-species distinct strains of *Aphelinus albipodus* showed reproductive compatibility and no reduction in fecundity (Wu et al. 2004). Further, genetic relatedness is a crucial factor in the evolution of social behaviours between individuals (Hamilton 1964a,b, Mateo 2004, Lizé et al. 2006, Gardner & West 2007) and relatedness between insects can usefully be assessed using microsatellite markers (Buczkowski et al. 2004, Trindl et al. 2004, Jaquiery et al. 2005, Drescher et al. 2010) leading to key insights into behaviours such as kin-based altruism and aggression

assays (Giraud et al. 2002, Tsutsui et al. 2003, Drescher et al. 2010, El-Showk et al. 2010). In my own study system the developmental of microsatellite markers provides useful support for empirical work on kin recognition mechanisms (Lizé et al. 2012, Chapter 3), as they confirm the assumption that females from different strains of *G. legneri* derive from populations with different genetic backgrounds, and are thus not as closely related as are females from within the same strain. In general, genetic recognition cues will usually associate with the level of polymorphism (Ratnieks 1991, Buczkowski et al. 2004) and the degree of aggressive behaviour between encountered individuals is attuned to the level of genetic diversity recognition loci (Giraud et al. 2002, Drescher et al. 2010).

A further use of microsatellite markers has been to identify the sex of eggs in several studies of wasp sex ratios (Ratnieks & Keller 1998, Abe et al. 2009). Assessment of the primary sex ratios of the parasitoid *Melittobia australica* showed that sex allocation is under precise control with the sexes produced in a regular sequence throughout the period of oviposition (Abe et al. 2009). The microsatellites we have developed have also been directly applied to the molecular-genetic detection of haploid (male) and diploid (female) eggs in *G. legneri* (Khidr et al. submitted, Chapter 6). This provides an evaluation of primary sex ratios that is unbiased by developmental mortality (a longstanding obstacle in sex allocation research on many species, e.g. Fiala 1980, Hardy & Cook 1995, Hardy et al. 1998, Krackow & Neuhäuser 2008, Abe et al. 2009). We used the consistent between-strain polymorphisms (U and C field) and cross-mated mothers, such that haploid and diploid eggs had different marker compositions. This work showed, for instance, that relationships between sex ratio and group size can be obscured by developmental mortality when the sex of eggs is not assessed directly and also that male and female eggs may tend to be laid in spatial separation (Khidr et al. submitted, Chapter 6).

5.6 Conclusions

In summary, the *G. nephantidis* laboratory culture evaluated here was not polymorphic in terms of the 12 microsatellite markers developed. This may be a reflection of limited genetic variability within this population or due to a prolonged period in laboratory culture. For *G. legneri*, no polymorphisms were found within strains using the 24 designed markers; as some strains were recently collected from the field this suggests natural genetic variation is locally limited. However, there were six primers that showed clear between-strain marker polymorphism in *Goniozus legneri*: six markers differed between strains collected recently in Chile and strains believed to originate from Uruguay several decades ago, while the two Chilean strains differed in only one microsatellite marker. These markers are useful for experimental work on kin recognition mechanisms, as they show that females from different strains genuinely derive from populations with a different genetic background, and also for studies on sex allocation strategies as consistent between strain polymorphisms allow the molecular-genetic detection of haploid (male) and diploid (female) eggs of cross-mated mothers.

Chapter 6 : Primary and secondary sex ratios in a gregarious parasitoid

6.1 Abstract

Haplodiploid sex determination affords female parasitoids mechanistic control over the sex ratios of their progeny. Strongly female-biased sex ratios, with low between group variance, are selected for when single mothers produce groups of sib-mating progeny. While the sex of progeny is determined at oviposition (primary sex ratio) the selective value of given sexual compositions is often only apparent when offspring mature and mate (secondary sex ratio). As developmental mortality can alter the sexual composition of given offspring groups its occurrence can select for mothers to adjust their (primary) sex allocation strategies in insurance. Empirical assessment of primary sex ratios is problematic when male and female eggs are indistinguishable. Here we apply DNA microsatellite markers to evaluate primary sex ratios in the gregarious parasitoid *Goniozus legneri* Gordh (Hymenoptera: Bethylidae) and compare these with secondary sex ratios. We find that sexually differential mortality is absent or weak but mortality acts to increase sex ratio variance and to obscure initially present relationships between sex ratio and group size. In some groups of offspring, there is a tendency for males and females to be laid in spatial separation. Our direct assessments of the sex of eggs avoids problems inherent in utilizing sub-sets of matured offspring groups with no mortality as representative of overall primary sex ratios but in this instance also confirms interpretations made by studies constrained to employ methods which are strictly incorrect.

Submitted as Khidr SK, Mayes S & & Hardy ICW. Primary and secondary sex ratios in a gregarious parasitoid. (*Behavioral Ecology*). 2012.

6.2 Introduction

Sex allocation is one of the most productive domains of behavioural ecology and has led to a sophisticated understanding of factors that influence an organism's reproductive decisions (Charnov 1982, Hardy 2002, West 2009). Parasitoid wasps have proven to be useful study systems for sex allocation research due, in particular, to variation around a relatively simple core life-history (Godfray 1994) and the fact that in these arrhenotokous species female offspring usually develop from fertilized (diploid) eggs while males develop from unfertilized (haploid) eggs (Flanders 1965, Crozier 1977, Cook 1993a, see Ode and Hardy 2008 and Mateo Leach et al. 2009 for details on some exceptions). Mated females, which store sperm in their spermathecae, can thus have a large degree of behavioural control over the sexes of their progeny at oviposition, via the release or retention of sperm (e.g. Flanders 1965, Suzuki et al. 1984, Godfray 1994, Ode and Rosenheim 1998).

There are several major classes of circumstances when it might be evolutionarily advantageous to control progeny sex ratios (Godfray 1994, West 2009). One of these is offspring developing in relatively isolated and ephemeral population sub-groups, and tending to mate with each other before dispersing as adults: strongly female-biased sex ratios are selected when small numbers of mothers contribute progeny to each group (Local Mate Competition theory: Hamilton 1967, West 2009). In the extreme case that each offspring group is produced by a single mother, males develop successfully and have effectively unlimited mating capacity, and all mating occurs prior to dispersal, the optimal sexual composition of groups is one male with the remainder of offspring being female: *i.e.* the optimal sex ratio (the proportion of offspring that are male) is the reciprocal of offspring group size (Green et al. 1982, Griffiths and Godfray 1988). It also follows that the distribution of sex ratios across offspring groups is selected to have low (≈ zero) variance, termed sex ratio precision (Green et al. 1982, Hardy 1992, Morgan and Cook 1994, Nagelkerke 1996).

While precise and strongly female-biased sex ratios may combine to maximize the number of mated daughters dispersing from each group (≈ maternal fitness), maternal decisions are more complicated when sex ratio at offspring maturity and mating (termed 'secondary sex ratio' or 'brood sex ratio') might not be the same as at sex allocation (the 'primary sex ratio' or 'clutch sex ratio') due to developmental mortality at the egg, larval or pupal stages. Stochastically acting mortality is expected

to increase initially precise sex ratio variance during development (Hardy et al. 1998) and, as insurance against broods containing no males and thus producing only unmated daughters (with ≈ zero fitness), mothers should increase their allocation to sons when male mortality is more common and/or clutches of eggs are larger (Green et al. 1982, Heimpel 1994, Nagelkerke and Hardy 1994). For a given mean probability of mortality the maternal primary sex ratio response should, however, be reduced when the distribution of mortality across offspring groups has higher variance (Nagelkerke and Hardy 1994).

Empirical assessment of parasitoid primary sex ratios is often problematic because the sexes of immature offspring are generally not morphologically distinct (see Discussion). Moreover, the sex ratio of surviving gender-distinct adults cannot correctly be used to estimate the primary sex ratio because any sexually differential mortality will bias the sample of surviving offspring in favor of the sex with lower mortality (Fiala 1980, Krackow and Neuhäuser 2008). Sexually differential developmental mortality may be expected due, for instance, to exposure of all deleterious mutations among haploids but not diploids (Smith and Shaw 1980) or to sexually asymmetric resource competition under some parasitoid life-histories (e.g. Ode and Rosenheim 1998, Giron et al. 2007, Kapranas et al. 2011). Separate assessment of male and female developmental mortality is thus challenging yet desirable as, for instance, it is only male mortality that is predicted to influence maternal primary sex ratio optima under extreme Local Mate Competition (Nagelkerke and Hardy 1994). Only a few prior studies have been able to provide direct assessment of parasitoid primary sex ratios coupled with comparison to secondary sex ratios (e.g. van Dijken et al. 1993, Ueno and Tanaka 1997, Hardy et al. 1998, Ode and Rosenheim 1998, Abe et al. 2009).

Here we apply microsatellite markers to investigate the primary sex ratio of *Goniozus legneri* Gordh (Hymenoptera: Bethylidae) a gregarious parasitoid of lepidopteran pests in several new world agro-ecosystems (Legner and Silveira-Guido 1983, Legner and Gordh 1992, Steffan et al. 2001, Zaviezo et al. 2007). The behavioural and reproductive biology of *G. legneri* has been relatively thoroughly explored (e.g. Gordh et al. 1983, Legner and Warkentin 1988, Lee 1992, Hardy et al. 1998, 2000, Goubault et al. 2006, Bentley et al. 2009, Lizé et al. 2012, Chapter 3). Each host is stung and paralyzed by an adult female which guards it against utilization by other females

(Goubault et al. 2006, Lizé et al. 2012) and lays eggs externally onto the host approximately one day later. Larger clutches of eggs are laid on larger hosts (Gordh et al. 1983, Lee 1992, Hardy et al. 1998), with ca. 72% of eggs on the host's dorsal surface, ca. 26% on the lateral surface and ca. 2% on the ventral surface (Gordh et al. 1983). Brood guarding and conspecific infanticide (Bentley et al. 2009) lead to groups of offspring developing on the same host being the offspring of a one mother (single 'foundress' broods, Hamilton 1967). Development to adulthood takes approximately two weeks, with around 88-93% of eggs surviving to maturity (Gordh et al. 1983, Hardy et al. 1998). Sibling mating is strongly prevalent prior to dispersal from the remains of the host but some degree of outbreeding may occur (Hardy et al. 2000). Brood sex ratios are female biased (9-19% of adult offspring are male) and have low variance (Gordh et al. 1983, Hardy et al. 1998) qualitatively conforming to expectation under single foundress Local Mate Competition (Hamilton 1967, Krackow et al. 2002, West 2009). Mated females are able to produce around 17 broods before becoming sperm depleted (Gordh et al. 1983), thereafter producing all-male broods. The genetic mechanism of haplo-diploid sex determination is unevaluated but is likely to be similar to that of *Goniozus nephantidis* which has been shown not to operate by complementary sex determination (which is thought to be ancestral to the Hymenoptera), probably as an evolved response to inbreeding (Cook 1993b, Asplen et al. 2009).

6.3 Material & Methods

6.3.1 Host and parasitoid rearing

Two strains of *Goniozus legneri* Gordh (Hymenoptera: Bethylidae) were utilized in this study. The first strain, which we term 'U' had been cultured in our laboratory for at least seven years and prior to this it had been maintained in commercial insectaries in the USA (the original material is believed to have been collected from southern Uruguay in 1978 Gordh et al. 1983, Legner and Silveira-Guido 1983). While the other strain, which we term 'C', was brought into our laboratory in 2009, following field collections in Santiago, Chile, where a natural population had previously been reported (Zaviezo et al. 2007).

Both strains were reared on a facultative host, the rice moth *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae), following the method reported in Stokkebo and

Hardy (2000). *Corcyra cephalonica* was reared on a diet of glycerol, corn meal, wheat bran and yeast, following Cook (1993b) but with the addition of honey in equal measure to the glycerol. All cultures and experiments were carried out in a controlled climate room at 27°C with relative humidity maintained by evaporation from a water bath.

For primary and secondary sex ratio evaluation, eight to ten virgin C-strain females were placed with one to two U-strain males for 2-3 days to allow mating and C-strain males were similarly allowed to mate with U-strain females. We obtained 186 cross-mated females which were then individually provided with a host weighing 30-50mg then monitored for 1-2 days until eggs were laid on the host's integument. Fifty five of these 186 replicates were then used for molecular genetic evaluation of the primary sex ratio and the remaining 131 were allocated to one of two secondary sex ratio evaluation treatments (see below).

6.3.2 Primary sex ratio

6.3.2.1 Egg laying and DNA extraction

Once clutches had been laid I counted the eggs and noted their positions on the hosts. DNA was extracted separately from each egg (N = 639 eggs) using methods based on those of Abe et al. (2009): under a dissecting microscope, the contents of each egg were squeezed with the rounded tips of insect pins onto parafilm (Pechiney Plastic Packaging, Chicago, IL, USA) and 2µl of 10 mg/ml Proteinase K (Macherey-Nagel, MN, USA) was placed onto the egg contents then mixed with 45µl of buffer (10 mM Tris, 1mM EDTA, 25 mM NaCl) followed by incubation overnight at 55°C. The extracted DNA was stored in a -20°C freezer.

6.3.2.2 Establishment of a microsatellite marker for egg sexing

Six polymorphic primers for *G. legneri* had previously been designed and assessed for polymorphism via a Beckmann CEQ 8000 capillary sequencer (S.K.K. unpublished data). Primer GlSSR7 was selected due to having the largest between-strain variation in allele size (16bp) at a given locus, such that alleles could be differentiated on MetaPhor agarose (Bio Whittaker Molecular, Rockland, ME, USA). The primer sequences were GlSSR7F 5'-CGAGGGTATCATTACGCGA-3' and GlSSR7R 5'-

GGCCACTCTCTCGTTACACC3-' and the original microsatellite repeat sequence had a $(GA)_{13}$ repeat motif.

An examination of the alleles sizes present in a large sample (n > 100) of individuals of the U-strain and C-strain revealed that the U-strain carried only the 143bp allele of GISSR7, while the C-population carried the 159bp allele of GISSR7. Thus, by making crosses between the two strains it was possible to determine which eggs were female (heterozygotye diploids) and which were male (hemizygotes). Note that while diploid males occur in some Hymenoptera due to complementary sex determination mechanisms, these are extremely unlikely to operate in *G. legneri* (Cook 1993a,b).

6.3.2.3 Polymerase Chain Reaction and gel preparation

PCR reactions were carried out in a 20µl reaction mixture containing forward and reverse primer (2 pmol/µl final), 2µl (10x) PCR buffer, 0.2µl dNTP's (each at a 25mM final concentration), 6µl genomic DNA (approximately 3ng/µl), 0.2 *Taq* DNA polymerase (5 units/µl) and 7.6µl Sterile Distilled water.

Samples were amplified in an ABI PCR 9700 Thermocycler (ABI, Carlsbad, CA, USA) with an initial 3 minutes denaturation at 94°C, followed by 35 cycles of 1 minute at 94°C (denaturation), 1 minute at 60°C (annealing) and 72°C for 2 minutes (extension) with a final extension of 72°C for 10 minutes at the end of the program.

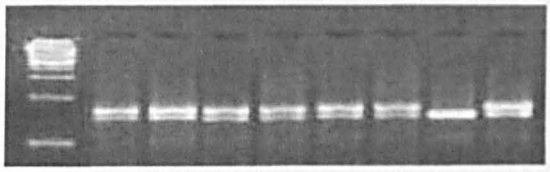
The PCR products from the first clutch of eggs were also sized on a Beckmann CEQ 8000 capillary sequencer, using standard techniques. For routine determination of alleles from GISSR7, PCR products were run on 4% MetaPhor agarose (Bio Whittaker Molecular, Rockland, ME, USA) prepared in 0.5 × TBE and run for 2 hours at 90V on a BioRad sub-cell GT gel kit. Bands were visualized under UV-light in a BioRad Gel Doc 2000 gel box after adding 2µl of Ethidium bromide stock (10mg/ml; Promega corporation) before the gel was poured.

6.3.2.4 PCR products visualized on MetaPhor gel electrophoresis

While the results of the capillary analysis confirmed that females and males can be determined using GISSR7 if crosses are made between the U- and C-strains, the cost of running capillary analysis on large numbers of offspring were prohibitive. For this reason, we determined conditions for MetaPhor agarose gels which would allow an

unambiguous determination of heterozygotes from hemizygotes, which allowed the presence of hybrids between the strains to be determined (Figure 6.1) On the basis of the ploidy and strong inference on mating system and genetic sex determination (Cook 1993b, Hardy et al. 2000), eggs were determined to be male or female. In a small number of cases PCR needed to be repeated before clear results were obtained.

(a)



(b)

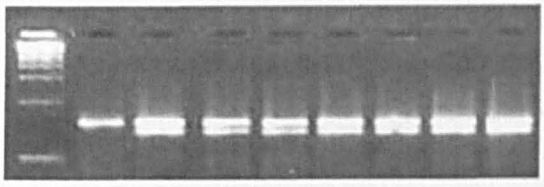


Figure 6.1 Molecular evaluation of clutch sexual composition. Two clutches from reciprocal crosses visualized on MetaPhor gels. Each clutch contains 7 females and 1 male egg laid by (a) a U-strain female mated with C-strain males (b) a C-strain female crossed with U-strain males. Females are inferred by the presence of two bands (heterozygotes) and males by the presence of a single band (hemizygotes). Strain U carries the 143bp allele of GlSSR7R and Strain C the 159bp allele. The left hand column shows the 2-log DNA ladder (New England Biolabs, Ipswich, MA, USA).

6.3.3 Secondary sex ratio

For the majority of the clutches used for secondary sex ratio evaluation (92/131), the mother was removed and laid eggs counted after 1-2 days (as with the replicates used for evaluation of the primary sex ratio) and the offspring were allowed to develop to maturity. For the other 39 replicates, the mother was allowed to remain with the host for a further 24 hours, then removed, and the number of eggs on the host was recounted: this served as a check that oviposition decisions by mothers were complete at the time that mothers were removed from their clutches during evaluation of

primary sex ratios and also in the other secondary sex ratio evaluation treatment. The sexual composition of all 131 broods (secondary sex ratio) was determined after the emergence of adults from their cocoons based on external morphology (males are smaller than females and do not possess stingers).

6.3.4 Data analysis

Offspring group composition data were analyzed using generalized linear modelling within the Genstat statistical package (Version 12, VSN International, Hemel Hempstead, UK). Log-linear analyses were utilized to explore influences on clutch size and male number (small integer response variables) and logistic analysis was used for factors influencing proportional response variables (proportion developmental mortality = proportion of eggs that failed to become adults, sex ratio = proportion of offspring that were male) (Crawley 1993, Wilson and Hardy 2002). Logistic Generalized Linear Mixed Modeling (Bolker et al. 2008) was used to explore sex ratio differences between sub-groups of offspring laid onto the same host. In log-linear analysis and analyses of grouped binary data, quasi-Poisson and quasi-binomial distributions of residuals, using empirically estimated scale parameters, were adopted, respectively, to take potential over- and under-dispersion into account (Crawley 1993, Wilson and Hardy 2002) we also note that these statistical models do not return exact probability estimates (Crawley 1993). The Meelis test statistic, U, was used to assess the significance of any deviation from binomiality for sex ratio and mortality data and the variance ratio, R, was used as a quantification of variance (Krackow et al. 2002).

6.4 Results

6.4.1 Clutches laid

Larger clutches were laid on larger hosts ($F_{1,183} = 31.27$, $P < 0.001$) but clutch size was unaffected by whether the mother belonged to the U-strain or the C-strain ($F_{1,182} = 1.35$, $P = 0.246$). Of the 39 mothers left with hosts after oviposition was apparently complete, one subsequently added eggs to the clutch: 5 eggs were added to an initial clutch of 6. As this suggests that only ≈1% (5/477) eggs would have been omitted from our remaining primary and secondary sex ratio evaluations, we conclude that data on the sexual composition when mothers were removed after 1-2 days are a very

close match to underlying maternal decisions and we do not consider the distinction between the two secondary sex ratio evaluation treatments further.

6.4.2 Primary sex ratio

Gel analysis indicated that six of the 55 clutches evaluated contained males only: as this indicates that the mother probably had not mated, these clutches were excluded from further sex ratio analysis (following Hardy and Cook 1995). In two mixed sex clutches DNA extraction for egg sexing was not completely successful so these data were also excluded from analysis. Consequently, data from 47 clutches were used in the analysis of primary sex ratio.

The mean sex ratio (proportion of offspring that were male) of the 47 clutches was 0.118 (+S.E. = 0.012, -S.E. = 0.011). Overall distribution of sex ratio across clutches was significantly under-dispersed (R = 0.439, Table 6.1). Overall, primary sex ratios were significantly greater in larger clutches ($F_{1,45}$ = 9.71, P = 0.003, Figure 6.2A) but sex ratio was unaffected by the strain of the mother ($F_{1,44}$ = 0.06, P = 0.807) or by an interaction between strain and clutch size ($F_{1,43}$ = 0.08, P = 0.781). The mean number of males per clutch was 1.362 (+S.E. = 0.181, -S.E. = 0.160) and increased significantly with clutch size ($F_{1,45}$ = 24.80, P < 0.001, Figure 6.2B) but was not influenced by strain (strain: $F_{1,44}$ = 0.03, P = 0.862; strain × clutch size interaction: $F_{1,43}$ = 0.05, P = 0.826).

In 21 clutches the mother laid eggs in spatially separate batches on the host. In the majority of these cases most eggs (8-15) were on the dorsal and lateral surfaces of the host larvae with a much smaller number (1-3) laid on the ventral surface: we term these 'major sub-clutches' and 'minor sub-clutches' respectively. Sex ratios within minor sub-clutches were significantly higher than among major sub-clutches (Logistic GLMM with host identity fitted as a random effect: $F_{1,38}$ =15.53, P < 0.001, Figure 6.3). Sex ratios of major sub-clutches increased significantly with increase in the size of the sub-clutch while those of minor sub-clutches decreased (clutch type × sub-clutch size interaction: $F_{1,38}$ = 8.61, P < 0.006): eggs in the smallest minor sub-clutches were usually male.

6.4.3 Secondary sex ratio

Among the 131 broods allocated to evaluate secondary sex ratio, the mean proportion of eggs that developed to adulthood was 0.924 (+S.E. = 0.009, -S.E. = 0.011). Developmental mortality was uninfluenced by clutch size ($F_{1,129}$ = 0.12, P = 0.73) but there was a weak tendency for the offspring of C-strain females to survive better when developing on larger hosts while survivorship in U-strain broods decreased slightly (Host size × strain interaction: $F_{1,127}$ = 7.52, P < 0.007, Deviance explained = 0.05%). Developmental mortality was highly overdispersed across broods (R = 8.677, U = 39.2, P < 0.001) but all members failed to mature in just one of these broods.

In 17/130 replicates with surviving offspring, all eclosed adults were male, indicating maternal virginity and these were excluded from further sex ratio analysis. A significantly higher proportion of eggs survived to adulthood among these all-male broods than among the broods containing mainly females (respective means: 0.971, +S.E. = 0.012, -S.E. = 0.020; 0.923, +S.E. = 0.009, -S.E. = 0.011; $F_{1,128}$ = 4.67, P = 0.033); while suggestive of sexually differential mortality, it should be noted that this distinction accounted for only 3.5% of the deviance and the P value, estimated from logistic analysis, is both inexact and close to the conventional significance/non-significance threshold.

The mean sex ratio of the remaining 113 broods (containing at least one female at adult eclosion) was 0.133 (S.E. ±0.008) and the overall distribution of brood sex ratios was significantly underdispersed (R = 0.572, Table 6.2). Secondary sex ratio was not significantly affected by brood size ($F_{1,111}$ = 1.01, P = 0.318, Figure 6.2C), strain ($F_{1,110}$ = 0.01, P= 0.936) or by an interaction between strain and brood size ($F_{1,109}$ = 0.07, P = 0.795).

To explore relationships between developmental mortality and brood sexual composition, we classified the 113 broods according to whether or not all eggs survived to adulthood. We note that sex ratio comparisons between sub-sets of data with and without mortality must be made with caution but can generate useful insights (Fiala 1980, Krackow and Neuhäuser 2008). The mortality distribution among these broods was overdispersed (R = 2.684, U = 13.9, P < 0.001), further suggesting a categorical distinction might be informative. There was no significant sex ratio difference between broods with some mortality (n = 53) and broods in which all eggs

survived to adulthood (n = 60; $F_{1,111}$ = 0.02, P = 0.897) nor was there a significant interaction between mortality occurrence and brood size ($F_{1,110}$ = 2.13, P = 0.147) but we illustrate the observed trends in these sub-sets of data on Figure 6.2C to aid the comparison of primary and secondary sex ratio patterns (see below). The same conclusions were reached when exploring mortality in terms of the number of eggs that died in each clutch rather than whether or not it occurred. These results collectively imply that *G. legneri* developmental mortality is not dependent on the primary sex ratio (Krackow and Neuhäuser 2008). The sex ratio variance of the sub-set of broods in which some mortality occurred was, however, greater than in the sub-set without mortality, such that the variance among broods with mortality was not significantly different from binomial (without mortality: R = 0.467, U = 2.5, $P < 0.05$; with mortality: R = 0.678, U = 1.26, $P > 0.05$).

In terms of number of males among the 113 broods containing at least one female at adult eclosion, there was a significant increase with brood size ($F_{1,111}$ = 24.60, $P <$ 0.001, Figure 6.2D) with no influence of strain ($F_{1,110}$ = 0.00, P = 0.952) or strain × brood size interaction ($F_{1,109}$ = 0.00, P=0.963). The mean number of males per brood was 1.513 (+S.E. = 0.111, -S.E. = 0.103).

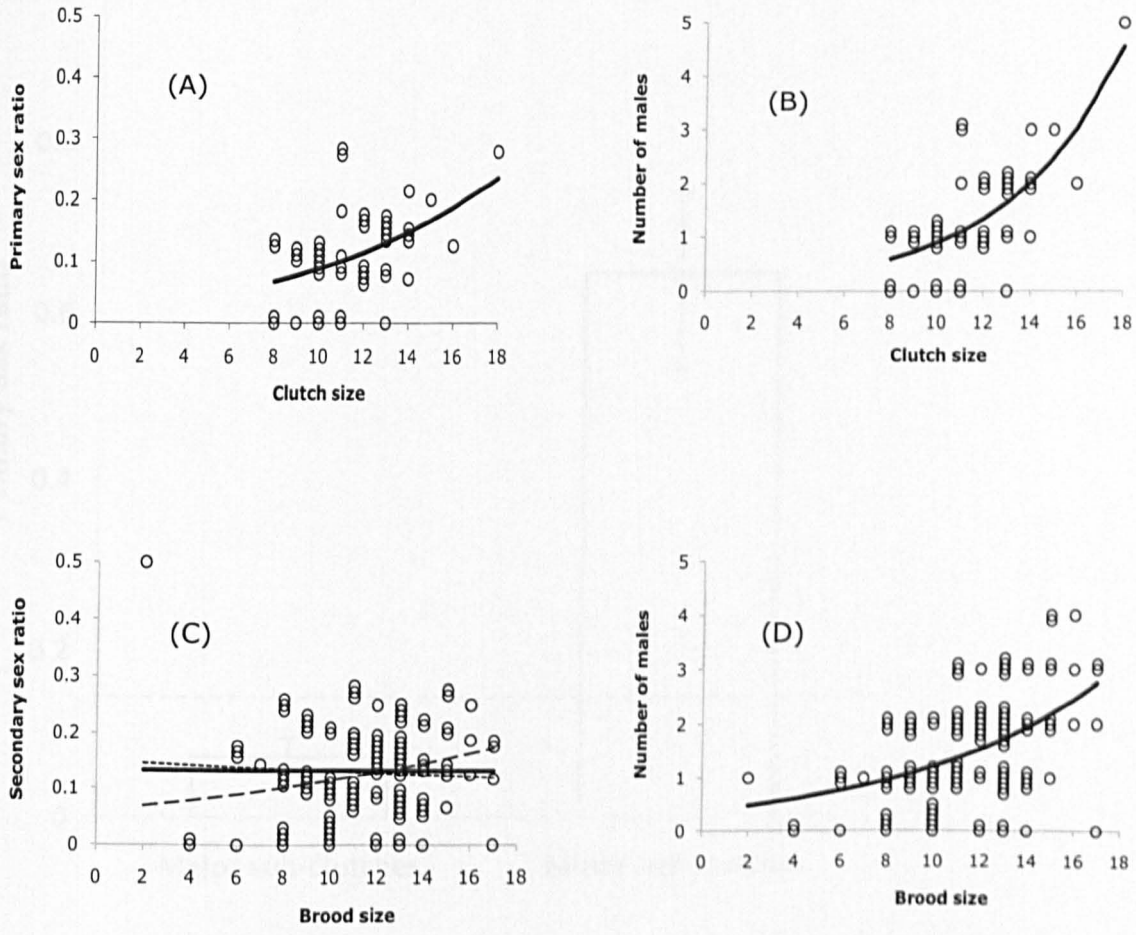


Figure 6.2 Relationships between sexual composition and offspring group size at oviposition (A & B) and at adult eclosion (C & D). Fitted solid lines show the minimal adequate models from logistic (A & C) or log-linear (B & D) regressions. In panel C the dotted line shows the best fit regression line for broods in which there was some developmental mortality and the dashed line is for broods without mortality, although these illustrated trends are not significantly different from each other or from a slope of zero (see main text). Overlapping data points are slightly vertically displaced to illustrate sample sizes.

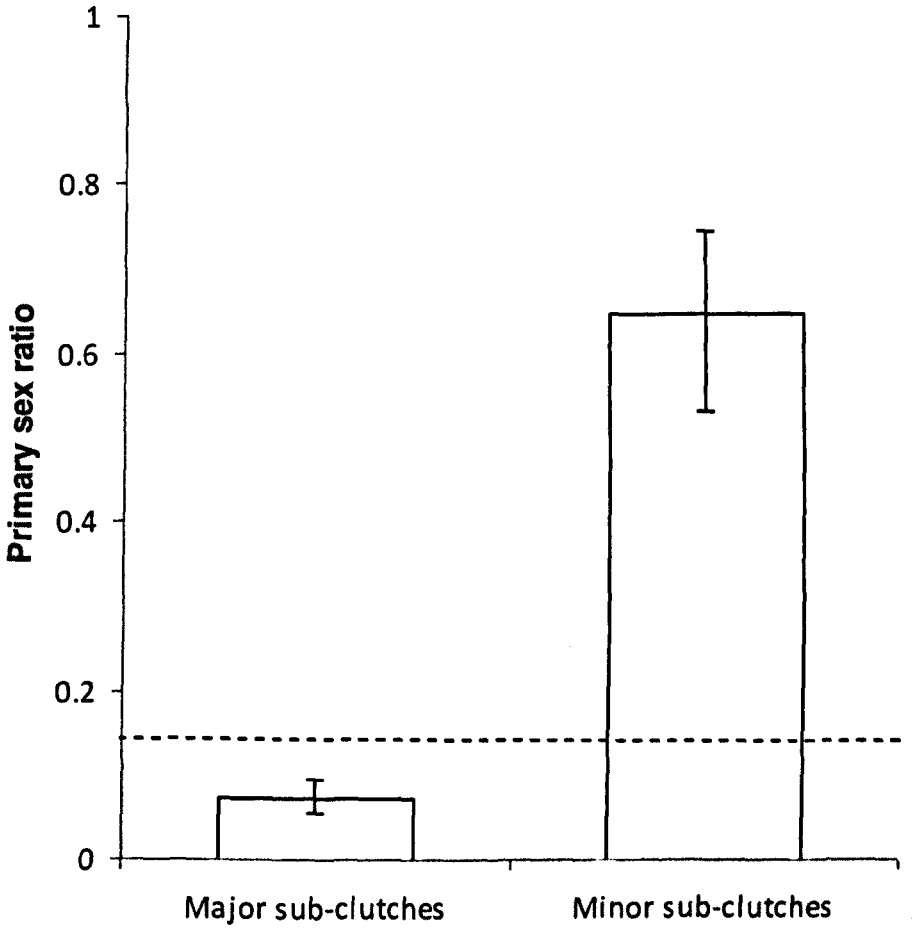


Figure 6.3 Sex ratios within major and minor sub-clutches (±S.E). The dashed line shows the mean sex ratio (proportion of males) across the 21 clutches analyzed.

Table 6.1 Primary sexual composition of clutches laid by mated *Goniozus legneri*

		Frequencies of number of males per clutch						*R*	*U*
Clutch size	Frequency	0	1	2	3	4	5		
1								–	–
2								–	–
3								–	–
4								–	–
5								–	–
6								–	–
7								–	–
8	4	2	2					0.711	-0.540
9	4	1	3					0.364	-0.972
10	8	2	6					0.309	-1.464
11	8	2	3	1	2			1.173	0.320
12	7		4	3				0.227	-1.445
13	8	1	2	5				0.431	-1.145
14	5		1	3	1			0.292	-1.072
15	1				1			–	–
16	1			1				–	–
17	0							–	–
18	1						1	–	–
Totals	47	8	21	13	4		1		
Approx. proportion		17%	45%	28%	9%		2%		
								Overall: R=0.439, U=2.39, p<0.01	

Table 6.2 Secondary sexual composition of broods produced by mated *Goniozus legneri*

		Frequencies of number of males per brood						*R*	*U*
Brood size	Frequency	0	1	2	3	4	5		
1								–	–
2	1		1					–	–
3								–	–
4	2	2						–	–
5								–	–
6	4	1	3					0.381	-0.957
7	1		1					–	–
8	10	4	3	3				0.960	-0.122
9	10		6	4				0.226	-1.775
10	13	6	5	2				0.875	-0.354
11	14		6	5	3			0.430	-1.544
12	13	2	2	8	1			0.541	-1.197
13	20	2	6	8	4			0.577	-1.374
14	10	1	4	3	2			0.659	-0.776
15	8		1	3	2	2		0.519	-0.947
16	3			1	1	1		0.410	-0.629
17	4	1		1	2			1.133	0.153
18								–	–
Totals	113	19	38	38	15	3			
Approx. proportion		17%	34%	34%	13%	3%			

Overall: R=0.572, U=2.87, p<0.01

6.4.4 Comparison between primary and secondary sex ratios

Broods in which no mortality occurred have identical primary and secondary sexual compositions (and brood size = clutch size): with the caveat that these broods are a self-selected sub-set of secondary sex ratio data, we compared the sex ratios of the 60 broods with no mortality to those of the 47 clutches used for primary sex ratio evaluation. The significant increase in sex ratio with clutch size ($F_{1,105}$ = 12.27, P < 0.001, as expected from Figure 6.2A,D) did not differ between these data sets ($F_{1,104}$ = 0.41, P = 0.522) nor was there a significant interaction between data origin and clutch size ($F_{1,103}$ = 1.52, P = 0.210). In this instance, primary sex ratios appear to be closely represented by the self-selected sub-set of broods without mortality, suggesting the absence of sexually differential mortality.

Heuristically combining all 113 broods used for secondary sex ratio analysis with the 47 primary sex ratio replicates indicated that sex ratio increases with offspring group size among broods with no mortality and among primary clutches but appeared to decline weakly among broods with some mortality (brood size × data sub-set interaction: $F_{2,154}$ = 3.24, P = 0.042). As above, there was no significant distinction between broods with no mortality and primary replicates (aggregation of factor levels: $F_{2,156}$ = 0.89, P = 0.414) but sex ratios of these were significantly different from those of broods with some mortality ($F_{1,157}$ = 5.39, P = 0.022). Although this analysis suggests that the non-significant distinction illustrated in Figure 6.2C may be biologically present, the interpretation relies on the inclusion of an outlying brood of one male and one female adult offspring (upper left hand corner of Figure 6.2C) that survived from an initial clutch of 10 eggs: repeating the analysis without this brood suggested no significant distinction between the sex ratios of the three sub-sets of data ($F_{2,155}$ = 0.40, P = 0.668) and an overall increase in sex ratio with offspring group size ($F_{1,157}$ = 6.77, P = 0.01). As mortality has no convincing effect on sex ratio in this analysis or in those above (beyond increasing sex ratio variance) we conclude that sexually differential mortality is absent or weak.

6.5 Discussion

Several methods have been developed to assess primary sex ratios directly. In some species maternal movements during egg-laying make it apparent which sex of egg is being laid (Cole 1981, Suzuki et al. 1984, van Dijken and Waage 1987, Strand 1989, Luck et al. 2001) and hence primary sex ratios can be assessed visually (Ode and Rosenheim 1998, Yamada and Kawamura 1999). In other species, male and female eggs are laid in, or migrate after hatching to, different locations within the host (Flanders 1950, Luck et al. 1982, Walter 1983, Espinoza et al. 2009). Other developed methods have been genetic. Dijkstra (1986), van Dijken (1991) ,van Dijken et al. (1993) and Ueno and Tanaka (1997) have identified the sexes of laid eggs through counting stained chromosomes within cells in the metaphase of mitotic division. This methods is reliant on the presence of twice as may chromosomes being present in fertilized (diploid) female eggs in comparison to unfertilized (haploid) male eggs and thus cannot distinguish females from the diploid males that are occasionally produced in some species with complimentary sex determination (CSD: Cook 1993a, 2002, Ode and Hardy 2008) and can also be difficult to apply if the tissue to be sexed has developed somatic polyploidy (Aubert 1959) and when parasitoid eggs have thick chorions (van Dijken 1991). More recently, DNA genetic markers, such as microsatellites, have been used to identify the sex of eggs in a small number of wasp species (Ratnieks and Keller 1998, Abe et al. 2009). Genetic markers have the potential to distinguish between diploid males and females under CSD as males will be homozygous for the sex allele while female are heterozygous. Furthermore, genomic DNA can be extracted from different types and ages of eggs, making genetic markers powerful and accurate tools for evaluating sex ratios in haplo-diploid organisms.

Using a genetic-marker method has allowed us to evaluate primary sex ratio patterns while avoiding the problems inherent in using a sub-set of offspring groups in which all offspring have survived (Fiala 1980, Krackow and Neuhäuser 2008). Our primary sex ratio data show that sex ratios also have significantly lower than binomial variance across offspring groups (R=0.439) and are female biased overall, but less female biased in larger groups of offspring (because the number of male eggs per clutch increases rapidly with increase in clutch size, Figure 6.2B). Using equations provided by Heimpel (1994) for optimal sex allocation under single foundress strict Local Mate Competition, we calculate that, with the observed mean developmental mortality of

7.6%, the optimal number of males per clutch will not exceed 1 until clutch sizes exceed 18 eggs. In fact, under the assumptions of these calculations male mortality would have to exceed 50% before ≥4 males should optimally be laid in clutches of 18 eggs (the fitted regression on Figure 6.2B predicts *ca.* 4.5 males per 18 egg clutch). The observed increase number of males is thus unlikely to be due to insurance against male mortality (Green et al. 1982, Heimpel 1994, Nagelkerke and Hardy 1994) but could be due to the occurrence of non-local mating in *G. legneri* populations (further discussed by Hardy et al. 1998, 2000).

Primary sex ratio analysis also shows, for the first time, that when *G. legneri* mothers lay offspring in spatially discrete sub-clutches, the sex ratios of major sub-clutches are female biased while those of minor sub-clutches are male biased. Although the sequence of sex allocation during oviposition of a clutch is unknown for *G. legneri*, in several other gregarious species of bethylids male eggs tend to be laid last (reviewed in Hardy 1992). Such comparative evidence suggests that minor sub-clutches are laid after major-sub clutches in *G. legneri*. This would also accord with Lee's (1992) observation that female *G. legneri* females sometimes add extra eggs to a clutch (in Lee's study this occurred when some eggs had been removed). Further, the ventral surface of the host is reported to be the least frequent location for *G. legneri* egg deposition (Gordh et al. 1993) potentially implying that males tend to be laid in 'inferior' locations. As developing bethylids do not usually engage in aggressive inter-larval competition (Mayhew and Hardy 1998), which could be reduced by physical separation and result in the less strongly competitive sex developing in an inferior location (e.g. Espinoza et al. 2009), we currently have no clear adaptive explanation for the tendency towards spatial separation between male and female offspring on some hosts. We do, however, note that as our data are weakly suggestive of lower mortality among males when developing on separate hosts from females the possibility that males in mixed sex *G. legneri* broods are laid spatially separately to reduce intersexual competition deserves future attention.

Our data on secondary sex ratios also show that sex ratios are female biased overall but, in contrast to data on primary sex ratios, sex ratio is unrelated to offspring group size (because the number of male eggs per broods increases insufficiently rapidly with increase in brood size; compare panels B and D of Figure 6.2): similar results have been found in previous studies on *G. legneri* (Gordh et al. 1983, Hardy et al. 1998).

We further found that sex ratio variance is precise, with a variance estimate (R=0.572) almost identical to that obtained by previous evaluation based on more than three times as many broods (R=0.56, Hardy et al. 1998).

Comparing our results for primary and secondary sex ratios indicates that developmental mortality, with a mean of 7.7% (as estimated from mixed-sex broods), obscures initially present relationships between sex ratio and offspring group size. Mortality also increased sex ratio variance (from R=0.439 at oviposition to R=0.572 at eclosion) but not so much that initially precise sex ratios would be classified as binomial on offspring maturity. Note however, that when secondary sex ratio data were separated according to whether or not any members of the brood died during development, sex ratio variances were significantly lower than binomial with no mortality and not significantly different from binomial when mortality occurred). Higher levels of mortality (ca. 57%) obscure initially precise sex ratios in the gregarious parasitoid *Colpoclypeus florus* (Hardy et al. 1998), in which primary sex ratios have been evaluated by cytology (Dijkstra 1986). In accord with both of these results, further within-species and cross-species evidence shows that sex ratio variance is correlated with the frequency of parasitoid developmental mortality (Hardy et al. 1998, Kapranas et al. 2011). Such empirical observations do not contradict Fiala's (1980) and Krackow and Neuhäuser's (2008) predictions that sex ratio variance will be unaffected by developmental mortality, as these authors assume that primary sex ratios are binomially distributed. In contrast, it is intuitively to be expected (Hardy et al. 1998) that initially precise sex ratios in *G. legneri*, *C. florus* and other parasitoid species will be assessed as less precise after the stochastic action of mortality.

One reason for estimating primary sex ratios directly is to evaluate potential sexually differential mortality while avoiding methodological bias (e.g. Ueno and Tanaka 1997, Abe et al. 2009). Mortality itself is often straightforward to assess in ectoparasitoids but separating this into male and female components usually is not. Our comparison of independently estimated primary and secondary sex ratios indicated little or no difference between male and female developmental mortality. Our additional comparison of mortality of all-male broods produced by virgin mothers and female-biased broods produced by mated mothers (which approximates a test for sexually differential mortality, Hardy and Cook 1995, Ueno and Tanaka 1997) suggested that males survive better than females but the distinction was very weak. We conclude,

overall, that sexually differential mortality is effectively absent: previous estimates for *G. legneri* and other species of bethylids have drawn similar conclusions (Legner and Warkentin 1988, Hardy et al. 1998), as have other studies that provide direct comparison of primary and secondary sex ratios in further parasitoid taxa (e.g. Ueno and Tanaka 1997, Abe et al. 2009) although there is less direct evidence for sexually differential mortality in some parasitoid species (reviewed in Nagelkerke and Hardy 1994).

Finally, we return to the problem that the sex ratio of surviving offspring cannot correctly be used to estimate the primary sex ratio because any sexually differential mortality will bias the sample of surviving offspring in favor of the sex with lower mortality (Fiala 1980): this has proven worrisome in the sex ratio literature either because authors are unaware of the problem (reviewed by Krackow and Neuhäuser 2008) or because they are aware but do not possess a fully valid method to assess primary sex ratios. Authors in the latter category have compared sex ratios of broods with no mortality with those of broods in which some developmental mortality occurred, along with the stated caveat that their conclusions may not strictly be valid (e.g. Morgan and Cook 1994, Hardy et al. 1998, Kapranas et al. 2011). Our data allowed us to evaluate sex ratio patterns both correctly (using a direct estimate of primary sex ratio) and, strictly, incorrectly (using sub-sets of secondary sex ratio data with and without mortality). Secondary sex ratios from broods with no mortality were similar to primary sex ratios in terms of the relationship between sex ratio and clutch size and sex ratio variance ($R = 0.467$ and 0.439 respectively) and comparisons between sub-sets of secondary sex ratio data with and without mortality lead to similar conclusions to comparisons between primary and secondary sex ratios. We make no claim that the concerns raised, correctly, by Fiala (1980) and Krackow and Neuhäuser (2008) are unimportant; rather we note that direct assessment of *G. legneri* primary sex ratios has not led us to conclusions that differ greatly from those tentatively obtained using strictly incorrect methods (Hardy et al. 1998): this situation would, however, likely be different if mortality in *G. legneri* were strongly differential between the sexes.

6.6 Conclusions

Independent assessment of primary and secondary sex ratios in *G. legneri* indicates that sexually developmental mortality does not differ between the sexes but does increase sex ratio variance across offspring groups between oviposition and maturity and can lead to initially present relationships between sex ratio and offspring group size being obscured. Direct assessments of primary sex ratios are desirable as these are methodologically more correct than indirect methods and can reveal new facets of sex ratio biology and may also serve to confirm tentative conclusions drawn from indirect analyses.

Chapter 7 : General Discussion and Conclusions

7.1 Summary of results

This project has investigated various aspects of metabolomic, behavioural, chemical and molecular properties of *Goniozus* species. The results of these investigations can be summarised into a number of main points.

- The metabolomic properties of hosts change with time after paralysis. Nuclear Magnetic Resonance revealed that the metabolites associated with energy, such as glucose, decreased while amino acids were liberated from proteins and ethanol, a waste product, increased (Chapter Two).

- Host age since paralysis negatively influenced parasitoid reproductive behaviour and life-history characteristics, such as progeny survivorship (Chapter Two).

- Resource value appears to influence contest outcomes: contest interactions were more often resolved in favour of wasp in possession of high-quality hosts (younger and bigger) (Chapter Two).

- Resource holding potential affects contest outcomes: the probability of winning a contest was higher for the larger contestant (Chapters Two and Three).

- Kin recognition behaviour in *Goniozus* females appears to operate via both genetically-based (phenotype matching) and environmentally-based (familiarity) cues, which revealed during contest interactions (Chapters Three, Four and Five).

- Chemical analysis revealed that various species of *Goniozus* exhibit dissimilarity in the cuticular profiles and also that the host species on which female *G. legneri* developed on also influenced their CHC profiles (Chapter Four). These results accord with those from kin recognition studies (Chapter Three), in that the mechanism of kin recognition is likely to be CHC profile similarity with both genetic and environmental components.

- Molecular genetic markers were developed for *G. legneri*. These can be a useful tool to address many questions of interest in the future and can be employed across closely related species as well (Chapter Five).

- The assessment of primary sex ratio, using genetic markers developed in Chapter Five, revealed that that secondary sex ratio variance is higher than primary sex ratio variance due to developmental mortality and also that relationships between sex ratio and offspring group size that are present at oviposition can be obscured by developmental mortality (Chapter Six).

Each of the aforementioned empirical chapters has their own discussion, thus in the remainder of this chapter I briefly recap the objectives of the work and suggest directions for future research.

7.2 Post-paralysis host age influence on *Goniozus nephantidis* fitness

When host quality varies, parasitoid wasps are expected to oviposit selectively in high-quality hosts. Whilst the influence of resource value e.g. host age prior to attack by parasitoids has been frequently studied (King 1998, Husni & Honda 2001, He & Wang 2006), no investigation has assessed the impact of envenomated and paralysed host age on parasitoid behavioural and life-history strategies. In accordance to our expectations the host quality declined significantly with time since paralysis: this is directly reflected in parasitoid behaviour, as females in possession of younger hosts fight more aggressively to win the contests for hosts. This behaviour indicates that they value younger hosts more greatly than older hosts.

Hosts started to lose weight continuously after paralysis and metabolic profiling showed that levels of glucose and ATP decreased with time, and waste products increased with time. These nutritional changes probably make older hosts less reproductively valuable to the wasps because, for instance, glucose is considered as an important factor associated with parasitoid growth (Thompson 1979, Hu et al. 2001). Hence, the host's quality started to deteriorate and the required metabolic processes to continue their survival began to decrease.

Further studies would involve chemical manipulation of the nutritional profile of young hosts via injection or diet supplements of glucose and/or ethanol. Then the female's

fitness can be explored in terms of offspring production and mortality while reared on hosts receiving different treatments. Further, contest behaviour between females in possession of glucose-enhanced hosts or ethanol enhanced hosts *versus* control (unmanipulated) hosts can be performed. In fact, such studies have already been carried out (but are not presented in this Thesis) and show that ethanol-enhanced hosts are treated similarly to older hosts by female parasitoids, but glucose treatment has no discernable effect.

Another suggested research area is that *Goniozus nephantidis* can be reared successfully on *Corcyra cephalonica* but it cannot be reared on *Plodia interpunctella*. Thus, hosts of different species are likely to have different metabolic profiles, making them suitable hosts for some parasitoid species but not for others. Chemical profile differences and similarities between different host species can be screened through Proton NMR. This might be especially revealing for ectoparasitoids that do not have intimate immunological interactions with their hosts, because they cannot be encapsulated, but may have co-evolutionary interactions via hosts 'fighting back' via their metabolomic profiles. Thus, this might provide insight into the molecular metabolites which make some host less suitable for female parasitoids than others.

Similar to studies on host age, the technique can be employed to explore the wasp's metabolic composition during different ages but first the method needs optimization to obtain a profile of the individual wasp which might be challenging due to their small sizes.

7.3 Agonistic behaviour in association with recognition and resource value in *Goniozus legneri*

Despite the tremendous amount of research that has focussed on the influence of relatedness on animal contests, few experiments have assessed the influence of competition on aggressiveness between related individuals (e.g. West et al. 2001, Giron et al. 2004, Innocent et al. 2011). Thus, we explored the effect and basis of kin recognition among adult females of *Goniozus legneri* on contest behaviour and the mechanism involved in this recognition.

The sub-social female wasp *Goniozus legneri* was able to employ two mechanisms to assess relatedness for recognition behaviour. Genetically-based recognition between

relatives as well as environmentally based cues between individuals whether or not it is a relative. The previous researches usually suggested that recognition either was based on genetic components that affected mate choice decision in *Venturia canescens* (Metzger et al. 2010) or was environmental based properties for brood mate recognition in *Bracon hebetor* species (Ode et al. 1995). However, might depend on both properties in social insects such as the paper wasps (Gamboa et al. 1986b, Bura & Gamboa 1994, Gamboa 2004).

Previous studies of *G. legneri* contests have minimised variation in relatedness asymmetries between competing females (competitors were always non-sibling females from strain U) and have found effects of asymmetries in both body size and prior ownership status on aggression and on contest outcomes (Goubault et al. 2006, Bentley et al. 2009). As with prior studies, we found effects of size and ownership asymmetries on aggression and that larger contestants tend to win contests.

The evaluation of recognition behaviour cues in *G. legneri* was based on behavioural observations during female contests in the laboratory. Therefore, future investigation should direct towards assessing the role of these cues on *Goniozus* population in general and on contest interaction particularly within field environment. Hence, we can build more solid interpretation for recognition behaviour.

In addition, further studies are required to identify the possible environmental chemical cues in *G. legneri* females and whether it is individual's innate odour or the shared host odour or both. Also inspection in the laboratory can be performed to find out whether the crossed strains of *G. legneri* (U and C) can enhance wasp's efficiency and fitness such as, increasing clutch size or decreasing mortality rates in the subsequent generation.

7.4 Cuticular hydrocarbons in *Goniozus* sp

The main objective of this study was to explore how environmental cues impact on resource competition behaviour in parasitic wasps. In *Goniozus* species it is likely that odours are acquired from the host during development and used by interacting females as a proxy for relatedness (Chapter Three): this was presupposed because kin recognition is mediated by cuticular hydrocarbon biosynthesis in a diversity of taxa

(Carlin & Hölldobler 1986, Lahav et al. 1999, Tsutsui et al. 2003, Gamboa 2004, Howard & Blomquist 2005, Dronnet et al. 2006, El-Showk et al. 2010).

The surface chemistry profiles of *Goniozus legneri* U-strain females differ when they develop on different host species. Also, the three investigated species of *Goniozus* have different chemical profiles even when developing on the same host species, supporting the assertion that chemical cues are used to assess relatedness. Hence, our finding might show that sister wasps growing up on different hosts 'smell' different, which is what our aforementioned experiment would suggest. However, this study was performed on a broader scale (cross-species differences rather than cross-brood differences) but in the future the same technique, or more refined versions of it, can be applied to evaluate differences between siblings and non-siblings.

Since CHCs can be used as a reliable taxonomic tool for species identification (Carlson & Yocom 1986, Uva et al. 2004, Kather & Martin 2012), an attempt to identify a species of *Goniozus* from Oman which we termed *Goniozus* sp. Indet. confirms that it was neither *G. nephantidis* nor *G. legneri*. However, because of the limited samples we received from Oman we only examined CHC profiles of few individual females. Therefore, obtaining further samples from Oman and investigating further aspects of the biology of this putative species would be useful.

Further, it is very important to find a suitable factitious host for this unknown species so it can be used for mass rearing. Currently in Oman the wax moth *Galleria mellonella* L has been utilized. However, there are some problems with using this host in the laboratory: wax moth larvae are quite aggressive and larger larvae often kill the parasitoid rather than *vice versa* (I.C.W. Hardy pers. comm.). I have tried to rear a few females on *Corcyra cephalonica* Stainton but was not successful. Therefore, testing further hosts such as *Plodia interpunctella* (Hübner), *Ephestia cautella* (Walker), *Pectinophora gossypiella* (Saund.), and *Phthorimaea operculella* (Zeller) would be useful in the future to maintain the species more efficiently in culture. Later, the next potential step would be collecting samples from different *Goniozus* species to compare the hydrocarbon profile in an attempt to establish a general idea of the chemicals that correspond with species identity.

7.5 Microsatellite markers and *Goniozus* species

The specific aim of the study was to elucidate the genetic cues that impact on resource competition behaviour in parasitic wasps. A further aim was to develop a microsatellite marker system for bethylid wasps as, to my knowledge, there is no previous marker present for this genus and only few designed for the family Bethylidae (Carr et al. 2010). The occurrence of polymorphic primers between *Goniozus legneri* populations ('U' and 'C') provides supporting information for the recognition behaviour experiment (Chapter Three), in essence that the crossed strains are genetically different from each other.

The primers designed for *Goniozus* species can be applied in further studies such as investigations of correlates of genetic diversity of *G. legneri* populations: whether diversity is based only on geographical distance, host plant species, continuous inbreeding, genetic elements or an interaction between these factors, as found in other species (e.g. Kavallieratos et al. 2004, Stireman et al. 2006, Pannebakker et al. 2008, Phillips et al. 2008, Lozier et al. 2009, Lavandero et al. 2011). This can be achieved by collecting this species from different localities and on various host plant species.

The genomic DNA sequence enriched library for *G. legneri* microsatellites, constructed by 454 Pyrosequencing, contained inadequate fragments that restrict us to design only 24 primers to this species with ultimately six molecular markers. Thus, either an attempt to develop another library by using different sequencing, such as standard (Sanger) sequencing, though this might be expensive, or perhaps testing *G. nephantidis* primers on *G. legneri* might be worth research attention in the future. Designing primers for *G. nephantidis* and *G.* sp. Indet. may also be useful. Another option would be to try using the primers that are already designed on the other *Goniozus* species on *G.* sp.Indet. as finding molecular markers for this species might further assist better understanding the species identity and phenotypic traits.

7.6 Precise sex allocation in parasitoid wasp

This study set out to evaluate offspring sex ratio at oviposition (primary sex ratio) of the parasitoid wasp *Goniozus legneri* (using molecular markers), compare this with the sex ratio at emergence (secondary sex ratio) and thus to test the influence of developmental mortality on the gender composition of *Goniozus* wasp broods. Similar comparisons have been made in only a few other species (e.g. Ueno & Tanaka 1997, Abe et al. 2009). Mortality increased sex ratio variance across offspring groups but sexual differential mortality was not observed. Further observation regarding the position of eggs on the host showed that eggs laid spatially separately, in 'minor clutches', tend to be male. It seems likely that these are also the temporally last laid eggs. The exact sequence of sex allocation during oviposition of a clutch in this species would require further observation during eggs deposition and perhaps by recording it on video tapes. Further it would be useful to use molecular markers to evaluate the relative contributions, in terms of numbers and sex ratios of eggs, of different mothers to broods in bethylid species that have a more communal breeding system, such as *Scelodermus guani* in which several wasps collaborate to sting the host then guard the broods while their offspring develop. These investigations would apply techniques developed in this thesis for studying sub-social *Goniozus* to the understanding of the biology of bethylids that exhibit more advanced degrees of social behaviour.

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