Investigating the influence of endosymbionts and population genetics on the predacious ladybird *Chilocorus nigritus* – Implications for biocontrol

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Project abstract

The scale insect predator *Chilocorus nigritus* is considered as one of the most successful and important biological control agents in classical biocontrol. The ladybird is currently a commercial product, used for scale insect control in glasshouse environments, but despite widespread success in wild field releases, use of the predator in glasshouses has achieved only moderate success. This study aimed to find out which factors may affect the success of *C. nigritus* in glasshouse pest control. Two key factors with potential to have a dramatic impact on the predator were identified; genetic variability within and between insect populations, and male killing endosymbiotic bacteria, known to have a diverse range of effects on up to 70% of all insect species.

Beetle strains were sourced from several insectaries and geographical locations. DNA sequencing determined significant genetic differences between biotypes of *C. nigritus* from different localities, indicating that *C. nigritus* exists as a series of functional biotypes across its range. All biotypes were tested for *Wolbachia*, *Rickettsia* and *Spiroplasma* infections, identifying *Rickettsia* and *Wolbachia* presence in most populations. Uninfected sub-lines of these strains were therefore created via tetracycline treatment. A number of bionomic characteristics of the beetle were compared across these strains and infection types in order to assess the influence of genetics and bacterial endosymbionts.

Significant effects of endosymbionts were noted in fecundity and prey consumption, and genetically distinct biotypes varied in their prey consumption. A combination of biotypes and infection types also provided successful suppression of scale insects in glasshouse trials at Royal Botanical Gardens, Kew, and beetles were shown to produce a defence pheromone similar to hippodamine, which could potentially be another strong influence on the species' ecology. This thesis suggests that variability within a predator population may be an asset, rather than a hindrance.

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1. Introduction.

Wolbachia (Hertig), *Rickettsia* (da Rocha Lima) and *Spiroplasma* (Saglio) are bacterial taxa which 'manipulate' arthropods' reproductive physiology in order to accrue an evolutionary benefit. These intracellular endosymbionts have received increased attention recently due to the implications of their actions for evolutionary biology (Telschow *et al.* 2007) and medicine (Pfarr & Hoerauf 2005). Until very recently however it has been suggested that the role these bacteria play in other areas of science has been undervalued, especially amongst the agricultural sciences. Floate *et al.* 2006 highlighted the lack of research in the area of biocontrol in particular. Given the potential of these bacteria to increase or decrease the reproductive success of both pests and beneficial insects, it is unusual that little research has been carried out in this field; especially as these micro-organisms could potentially have a large economic impact. With increasing world population, changes in dietary habits, climate change, and financial instability, food security has become a world issue (FAO 2009), and thus agricultural science is unquestionably a sector worthy of research. Since these bacteria are thought to inhabit up to 70% of all insect species (Kozek & Rao 2007), and can lead to significant changes in insect reproduction, it is appropriate to investigate their role in agricultural systems.

1.1 – The evolutionary benefit of killing male hosts.

Male killing bacteria are almost entirely vertically transmitted, and rely on oocytes from females to pass their offspring from the primary host (the mother) to the secondary host (the daughter) (Hurst & Jiggins 2000). This mechanism is favoured over male gamete transmission because the significantly higher volume of the female sex cells makes them much more suitable as transmission vessels for intracellular bacteria (Hurst & Jiggins 2000). The evolutionary benefit of having a female host is therefore much greater for these bacteria, and as a result it has been theorised that mechanisms of killing male hosts have evolved to maximise vertical transmission. By killing male hosts the bacteria ensure that there is increased survival of female siblings, ergo more oocytes will successfully develop into infected daughters, increasing the bacterium's own reproductive success (Hurst & Jiggins 2000). It is thought that this mechanism has led to the development of a sustainable method of pathogenicity; hence why these bacteria have become so successful, with up to 70% of the world's insects thought to be infected (Kozek & Rao 2007).

1.2 - Effects of 'Male Killing' endosymbionts on arthropods.

Generally (but not always) the end-product of these intracellular infections is a female-biased sex ratio, caused by mechanisms such as genetic male-feminization, male-killing and parthenogenesis. In addition, these bacteria have been known to cause an effect called cytoplasmic incompatibility (CI), which usually leads to a more 'normal' 1:1 sex ratio (Werren 1997). Whilst the majority of literature focuses on *Wolbachia* infections, effects related to *Rickettsia*, *Spiroplasma*, *Cardinium* and *Flavobacteria* are also discussed in this chapter. Although the biological niches of these bacterial taxa are similar, the implications for arthropods' health and reproductive success can vary dramatically.

Drosophila melanogaster (Meigen) is a case which illustrates a neutral effect on the host (a commensal relationship). One strain of *Wolbachia* identified in one population of *D.melanogaster* was considered almost avirulent, showing no reduction in fecundity or lifespan in infected individuals and only a minor mortality by cytoplasmic incompatibility (0-30%) (Werren 1997). In this case bacteria were also only found at low densities.

For some organisms *Wolbachia* infections can be regarded as mutualistic. The genomes of *Brugia malayi* (Brug) (a nematode responsible for lymphatic filariasis) and its *Wolbachia* endosymbiont were sequenced and compared. It was discovered that the co-evolution of the two organisms had led to a reliance on one another for essential molecules. *Wolbachia* provides the nematode with heme, riboflavin and nucleotides, whilst the host in turn provides the *Wolbachia* with essential amino acids. As a result, neither partner can successfully live independently from its symbiotic associate (Foster *et al.* 2005).

In addition to the symbiotic relationships, some endosymbionts also have direct negative (and sometimes pathogenic) effects on particular hosts. In addition to commensal strains, *D. melanogaster* can harbour a highly virulent strain of *Wolbachia*. This particular strain has been shown to reduce the lifespan of adult flies by causing deterioration in brain and retinal tissues. Infected flies examined via electron microscopy showed a high bacterial density, which could possibly be related to the aggressive symptoms these insects encountered (Werren 1997).

Another common effect of endosymbiont infection is Cytoplasmic Incompatibility, which is considered a more complex symptom caused by these bacteria. This effect can be either unidirectional or bidirectional. Unidirectional CI results in incompatible matings (reduced or halted fecundity) between infected males and uninfected female insects. Mating with the same infection type will however produce viable offspring, as will crosses between uninfected males and infected females, thus the interference with mating is *uni*directional. This was observed in

Aedes albopictus (Skuse) where crosses were incompatible only when the males harboured a *Wolbachia* strain that was not present in the females (Dobson *et al.* 2004).

Bidirectional CI results in incompatible matings between individuals harbouring different strains of bacteria. An example is the divergent wasp species complex *Nasonia.* This complex contains three species (*N. Vitripennis, N. longicornis,* and *N. Giraulti* (Walker)), each harbouring a different lineage of *Wolbachia* (Bordenstein & Werren 2007). When uninfected insects from each species were crossed they showed no indication of CI whatsoever. However, when the infected strains were crossed, all species showed incompatibility with one another. Furthermore, the crosses were incompatible in both directions between genders (e.g. ♂ *N. vitripennis* vs. ♀ *N. longicornis* = Incompatible, and ♀ *N. vitripennis* vs. ♂ *N. longicornis* = Incompatible), hence the term *bi*directional CI.

1.3 - Effects of 'Male Killing' endosymbionts amongst coccinellids.

Male killing bacteria in coccinellids demonstrate a relatively narrow range of effects on their hosts. The most common consequences of an endosymbiotic infection in ladybirds (with virtually no exceptions) tend to be low hatch rate, reduced fecundity and female sex-bias. The following is a list of the effects found amongst the Coccinellidae;

In *Adalia bipunctata* (L.) four species of male-killing bacteria were found in the wild population collected in Moscow. The majority of this population produced low hatch rates (<0.55) and significantly biased sex ratios (for example, one *Rickettsia*-infected line named 'Mos 3' produced a sex ratio of 0.147, determined by the proportion of males) (Majerus *et al.* 2000). Some *Spiroplasma* infected lines of *Anistosticta novemdecimpunctata* (L.) showed a hatch rate exactly half that of uninfected siblings (0.24 compared to 0.48 in uninfected lines), and sex ratios close to 0.00 (with almost no male offspring) (Tinsley & Majerus 2006). *Coleomegilla maculate* (de Geer) produced significantly biased sex ratios as

well as a reduction from a normal 80% hatch rate to 32% (Hurst *et al.* 1996) in lines infected with what was later found to be a member of the *Flavobacteria* (Hurst *et al.* 1997). *Rickettsia* in *Adalia decempunctata* (L.) caused hatch rates significantly lower than 60% (0.298 – 0.477) and significant female bias where in some cases *no* males were found (von der Schulenburg *et al.* 2001). Infected *Harmonia axyridis* (Pallas) populations showed sex ratios of 0.394 with some lines again producing only female offspring. These biased lines also showed a low hatch rate, sometimes as limited as 0.23 (Majerus *et al.* 1998). Finally *Adonia variegata* (Goeze) was regarded as having no beneficial physiological effects of infection by a strain of *Flavobacteria*, and evidence of a weak cost to the host in the form a reduced fecundity (Hurst *et al.* 1999).

In all of these cases the bacterial endosymbionts could be considered somewhat pathogenic, having a direct negative effect on the host's reproductive fitness.

In an independent screening of 28 populations of ladybirds (covering 21 species), 11 were found to harbour 'male-killers' (Weinert *et al.* 2008). Eight of these showed a female biased sex ratio, and one uninfected species *Exochomus quadripustulatus* (L.), had a male bias. From all of these data it could be postulated that most coccinellids are affected similarly by this kind of male-killing endosymbiont, perhaps due to the similar host morphology or physiology. This assumption must be approached with some caution however. The wide range of different effects in other insects suggests that alternative effects could still be encountered. Literature reviews have so far revealed no record of cytoplasmic incompatibility in coccinellids, nor was there a record of bacteria significantly *increasing* reproductive fitness in the form of higher hatch rates. However, this may simply be a result of the limited data we have on this family with only 20-30 species screened from a possible 475 described species (Majerus 1994).

Regardless of the apparent negative effects amongst ladybirds, the wider consequences of reduced reproduction and sex bias are more complex when considering ladybird populations as a whole. Whether this direct negative effect on fecundity is harmful in a biological control context still needs to be clarified by looking into population effects of endosymbionts.

1.4 – Effects of endosymbionts on whole populations.

The seemingly straightforward 'pathogenic' effects of male-killers on ladybirds become more complicated when the overall population effects are considered. Male killers are known to be transmitted maternally, but this process is often imperfect. Transmission efficiencies in coccinellids are around 87% (Majerus 1994) with some variation within populations (*Adalia bipunctata* efficiencies ranged from 0.71 to 0.99) (Majerus *et al.* 2000). Factors preventing perfect vertical transmission can include host genetics, but are strongly related to environmental fluctuations. An example is given by Hurst *et al.* (2001) in *Drosophila bifasciata* (Pomini), in which *Wolbachia* transmission efficiencies were reduced by temperature changes of as little as 1.5ºC (from an optimal temperature of 23.5°C). Under optimal conditions (particularly in lab cultures) host-endosymbiont relationships can reach 'near' perfect transmission, such as *Wolbachia* in the butterfly *Acraea encedon* (L.) (Jiggins *et al.* 2002), but normally transmission efficiencies over 95% are rare.

Over time the consequence of imperfect transmission should theoretically be the gradual reduction in prevalence of bacteria in the population (to the point where the bacteria are eliminated altogether). In practice however it seems that bacteria are maintained within the host species over many generations. In order for a stable population to occur from generation to generation, there must be some kind of contributing factor which compensates for this imperfect transmission. Majerus (1994) suggested that this compensation would be in the

form of selection advantages found in infected females, thereby allowing them to accrue greater total fitness than the uninfected females (Majerus 1994).

It has also been suggested that the increased fitness comes indirectly via inbreeding avoidance and resource reallocation due to male-killing (Majerus 1994). The former would mean that consequences of male killing could potentially offset the effect of homogeneity and lethal recessive genes brought on by inbreeding. Put simply, the killing of male siblings will also reduce potential inbreeding opportunities. In this situation it is easy to see how inbreeding avoidance could be of overall benefit to the population. However, this may be inconsequential in wild populations of coccinellids if they are highly panmictic, and will disperse to other plants before mating. In lab cultures male-killing might be of more benefit for inbreeding avoidance, as inbreeding depression in lab cultures is more common (Majerus & Kearns 1989).

The second selective advantage infected females could accrue is related to resource reallocation. This involves the freeing-up of resources for female larvae upon emergence. These resources can come in the form of infertile male eggs which can provide an important initial food source for emerging female siblings (Majerus 1994). Additionally, upon emergence, females from infected lines will encounter reduced intraspecific competition as the prey resource is not being consumed by male siblings (Majerus 1994). This increase in fitness was demonstrated by Hurst *et al.* in *Adalia bipunctata*. Beetles of this species lived twice as long when given an egg meal than if they had none (in a situation where there was no other food source). Infected daughters also developed into second instars more quickly. It was concluded that the infected females therefore had a survival advantage over the uninfected, especially at low food densities (Majerus 1994).

Underpinning the pathogenic effects of these bacteria, there are benefits to male-killing infections that balance out the imperfect transmission efficiency, allowing the bacteria to be successful for several generations. How this affects the rearing of biological control agents is still unclear. Reduced hatch rates (if found in commercially available predators like *Chilocorus nigritus* (Fabricius)) might be seen as a drawback when producing large numbers of insects in a lab culture. In this case, curing male-killing infection would be of use to the industry. There is also evidence about fitness compensation that might lead to an alternative outcome (Majerus 1994). Even *with* reduced hatch rates in a lab culture, released individuals may have a fitness advantage due to reduced competition and additional egg meals. Also, the fitness compensation through inbreeding avoidance might be more prominent when culturing beetles in vitro, but of less benefit in the wild, where beetles disperse after pupation. Therefore it might be beneficial to maintain infections during the rearing process, since inbreeding avoidance might improve the quality of the culture (even if fecundity is reduced). This project aims to clarify some of these issues by looking into the direct and indirect effects more closely.

1.5 - Mechanisms of reproductive manipulation.

For a biological control agent to be optimally productive in vitro or in the field, its whole reproductive process from mating, through sperm and egg production and embryo formation must be at optimum efficiency. Previous sections in this chapter already demonstrate the many ways in which endosymbionts and parasites can alter an insect's sexual health, sometimes to the animal's detriment. This section shows some of the mechanisms used by male-killing bacteria to manipulate their host's reproduction at stages of the life-cycle.

1.5.1 - Interference with mating and behaviour.

In populations of two-spotted spider mites *Tetranychus urticae* (Koch), *Wolbachia* infections cause cytoplasmic incompatibility (CI) between uninfected females and infected males (Vala *et*

al. 2000). In this way, mating with an infected male creates a fitness cost for uninfected females, as they produce no offspring when mated with a male harbouring *Wolbachia*. Through this scenario, female mites are thought to have adapted to discriminate between infected and uninfected mates. As a result, uninfected females show a preference for other uninfected mates over those harbouring infections, resulting in higher mating success for them by avoiding CI (Vala *et al.* 2000).

Although there is currently little evidence for CI in coccinellids, it has been noted in other members of the Coleoptera such as *Hypera postica* (Gyllenhal) (Hsiao & Hsiao 1985) and *Callosobruchus chinensis* (L.) (Kondo *et al.* 2002). For biological control species, this kind of CI could have negative implications, causing infection types to avoid one another and reducing the potential number of matings within a species. Where it is found in pest species, this effect could be used to the grower's advantage, keeping pests at a lower baseline level.

Where CI is encountered, the ability of females to discriminate between an infected and uninfected mate could improve natural breeding success by avoiding incompatible mating. *Tetranychus urticae* females employ pre-copulatory mate choice, but the mechanism for the choice is unknown (Vala *et al.* 2000). It is suggested that there must be a symptom or phenotype displayed which shows the female whether a male is infected or clean of bacteria. Since no obvious physical attributes were found to be different between infected and uninfected mites, pheromonal and/or gustatory cues are thought to play a part in 'marking' infected males (Vala *et al.* 2000). If these bacterial species can influence pheromone production in some way (as this suggests) this could have implications for biological control agents, especially mobile predators such as coccinellids, which often rely on chemical cues to locate mates (Hemptinne *et al.* 1998).

1.5.2 - Interference with predator dispersal

The success of many predators relies on their ability to disperse. The predatory spider *Erigone atra* (Blackwall) normally undergoes ballooning in order to disperse, using silk as a 'sail' to allow the spider to be lifted by air currents (Goodacre *et al.* 2009). It has also been shown that *Rickettsia*-infected individuals show a reduced ability to disperse in this manner. *Rickettsia* infections in *E. atra* are found to be present in the sub-oesophageal ganglion, but not the super-oesophageal ganglion responsible for motor functions. This suggests a form of behavioural alteration by *Rickettsia*, and also demonstrates that the pathogenic effects of these bacteria are not limited to the reproductive system (Goodacre *et al.* 2009). Notably, such an effect is likely to limit gene flow and cause reproductive isolation, as well as the ability to colonise new heterogeneous environments.

1.5.3 Direct interference with sex cells.

Another aspect of reproductive success in insects is sperm viability. In many cases, male gametes can be affected by *Wolbachia* both in terms of sperm production and viability. Work carried out on infected *Drosophila simulans* (Sturtevant) showed that flies carrying *Wolbachia* sired 71.5% of offspring on the second mating (competing with an initial male's sperm) (Champion de Crespigny & Wedell 2006). The uninfected males sired a significantly higher 82.3%. This is at least in part attributed to the lower production of sperm previously recognised by Snook *et al.* 2000, where infected males were shown to produce 40% less sperm in total. Conversely *Tribolium confusum* (Du Val) beetles experience a benefit from *Wolbachia* infections, demonstrated by increased sperm competition in infected males (Wade & Chang 1995).

1.5.4 Endosymbionts and reproductive isolation – The link between host genetics and male killing endosymbionts.

The kinds of speciation which lead to formation of biotypes can often be caused by the environment and indeed host plants with which an insect interacts. This kind of event is often referred to as ecological speciation (Funk 2010). Evidence of host-specific speciation is apparent amongst the leaf beetle *Neochlamisus bebbiana* (Brown). On the whole this species of beetle has been considered oligophagous, but evidence of specialization towards several host plants has been observed, suggesting the existence of more intricate species complex (Funk 2010). This evidence came from studies on prey preference and reproductive traits of the beetle. The experiments were designed to measure the acceptance of several forms of the beetle for multiple host plants. Upon examination of the results it became clear that six host 'forms' existed, each form being adapted to a monophagous lifestyle on one of the six different host plants (Funk 2010). This level of specialization *within* the species is usually attributed to reproductive isolation brought on by divergent selection pressures, such as the pressures caused by several different host plants.

Speciation events in insects are not always driven by the environment or by host species on which they feed. Endosymbiont-driven speciation is thought to be particularly prevalent in many arthropods. A prime example of this is found in the *Tetranychus urticae* species complex. These mites suffer from cytoplasmic incompatibility between mites from two different host plants (Rose and Cucumber), despite being considered as the same species (Vala *et al.* 2000). It is thought that this separation of sympatric populations is a result of adaptation (whereas in allopatric population speciation might be incidental). In this example it initially seems that any speciation was a result of adaptation of *T. urticae* to the different host plants (Vala *et al.* 2000). However, this was shown not to be the case, since previous experiments had shown no evidence of adaptation to novel host plants causing reproductive isolation (Fry 1999). In *T.urticae* the isolation event is therefore considered a result of incompatible endosymbiont

types causing CI between the Rose and Cucumber strains, and not as an adaptation to the host plant.

1.6 The current role of *Chilocorus nigritus* in scale control.

Scale insects are an increasing pest problem worldwide, most prominently on citrus (Grafton-Cardwell *et al.* 2006) and ornamental plants (Peronti *et al.* 2001). Their worldwide economic cost was estimated to be at least $5 billion per year when the cost of control was taken into consideration (Kosztarab and KozAr 1988). Due to their sedentary lifestyle, and waxy scale covering, scale insects are notoriously difficult to suppress with chemical applications (Donahue & Brewer 1998). Previously growers have had some success with a range of organophosphates, carbamates and pyrethroid pesticides (Rill *et al.* 2007), but in most cases significant levels of resistant build up in these pest populations soon after their use (Grafton-Cardwell & Vehrs 1995). With growing popularity of the 'organic food' market, and increased concerns about public health linked to pesticides, many restrictions on their use have been put in place, including more stringent Maximum Residue Levels (MRLs) for food crops (European Parliament 2005). As a result, alternative methods of pest control are being sought, particularly in the field of biocontrol, and use of natural enemies. This section focuses on one model scale-predator, *Chilocorus nigritus*, which is currently used in biocontrol programmes targeting both diaspid (armoured) and coccid (soft) scale insects.

1.7- Bionomics of *Chilocorus nigritus*

The following section details those bionomic characteristics of C. nigritus relevant to its use in biological pest control.

1.7.1- A note on ambiguities in the bionomics of *Chilocorus nigritus*.

There is some ambiguity in the literature over the particular bionomics of *Chilocorus nigritus* as a species. These ambiguities lie mainly in the climatic requirements and prey relations, and are apparent when beetles from different locations are examined by different authors (Omkar & Pervez 2003). Some have suggested that the variations occurring between ladybirds from different places indicate multiple 'biotypes' around the world (possibly representing the beginnings of speciation) (Omkar & Pervez 2003, Ponsonby 2009). The material in this section aims to describe *C. nigritus*' role in biological pest control, but also demonstrates that the bionomics of this species are highly variable between cultures.

1.7.2 - Climatic requirements and temperature relations

C. nigritus' natural range spreads from Pakistan through the Indian Subcontinent into South East Asia (Ponsonby 2009). Within this range, reports of their preferred climatic biotope are contradictory, with some reporting preference for cooler hilly areas (Ghani & Muzaffar 1974), and others assuming preference for warmer coastal locations (Ahmad 1970). Other sources state the beetles' preference for the whole range of climates from the coast up to 1524m altitude in the Shevaroy Mountains in India (Tirumala Rao *et al.* 1954).

The preferred climate of this species therefore remains a point of controversy. Some authors have regarded *C. nigritus* as a dry savannah specialist in East Africa (Greathead and Pope 1977) and others consider it to be better suited to introductions in arid deserts such as those in Oman (Kinawy 1991). Samways (1989) suggests that *C. nigritus* favours climatic conditions provided by Zonobiomes I and II (as described by Walter 1973, 1984). These consist of tropical evergreen rainforest (Zonobiome I) and wet tropical deciduous forest or savannah with a dry cool season (Zonobiome II).

In the past, classical biocontrol efforts using *C. nigritus* have had varying degrees of success, perhaps in part due to unsuitable climates. There have been many failed attempts at introduction in the southern coasts of the USA (Hutson 1933, Smith & Flanders 1949), Bermuda (Bennett & Hughes 1949) and Eastern and Western Cape of South Africa (Samways 1984). These areas are now considered to be unsuitable for *C. nigritus*, with most sites experiencing long hot summers and cool wet winters, far from the ladybird's optimum environment. In more suitable regions such as Israel (Argov & Russler 1988) and Turkey (Senal 2006), the beetle has experienced varying degrees of success. In both of these cases the cold winters killed overwintering populations after successful summer colonisation of citrus crops. In a more successful attempt, full colonisation by the predator was achieved in the citrus farms in Malelane, South Africa (Samways 1984). In Malelane, the beetle appeared of its own accord, reducing the red scale populations of *Aonidiella aurantii* (Maskell) from severely high levels (those leading to 48% crop loss) to much lower and more sustainable ones (virtually free of red scale) (Samways 1984). The beetle is generally unsuccessful in these warm low-veldt areas of South Africa, but in this case managed to successfully colonise regardless of this climatic preference.

More recently work has been carried out on precise climatic requirements in a laboratory setting. Ponsonby & Copland (1998) focused on bionomic characteristics that might influence

the species' reproductive success upon release. Looking at oviposition, egg viability and egg cannibalism it was concluded that the range of suitable temperatures for reproduction was between 20 and 30ºC, with the optimum egg viability between 26 and 30ºC. No significant effect of humidity was found on reproductive numerical responses, suggesting that temperature is the main limiting factor for the beetle's reproduction. In another study it was found that feeding rate of *C. nigritus* peaks at a constant 26ºC, whilst cyclic temperatures between 14 and 30ºC result in a mean of 9.78 scales being consumed in a 24 hour period (Ponsonby & Copland 2000). The data in these experiments were confounded by extremely high variability between individual beetles, which itself poses the question of "what causes individuals to behave so differently under such similar conditions?" Whilst this variability could go some way to explaining the ambiguities in the literature, Omkar & Pervez (2003) suggest further research, stating that the "optimisation of abiotic factors is needed to enhance its reproductive performance [referring to *C. nigritus*]."

Potentially biotic factors such as *C. nigritus'* genetic background could also have an impact on reproductive success and climatic preferences. The relationship between these two factors is fairly well established in insects. In an example from Sands *et al.* (1986), two similar species of parasitic hymenoptera from the same location in South Africa were released on the Eastern coast of Australia. Despite their similar habitats in their original location, the two species showed very different climatic adaptations upon release. *Anicetus communis* (Annecke) was able to colonise most of the coast of Queensland, where it now provides significant control of *Gascardia destructor* (Newstead). The second scale parasitoid, *Paruceraptrocerus nyasicus* (Compere) provides similar control along the eastern seaboard, although this time much further South along the coast of New South Wales (Sands *et al.* 1986). This provides some evidence for the genetic basis of climate preference, but also illustrates the vast amount of plasticity insects can display in that preference.

1.7.3 - Prey preference

Interspecific variation in coccinellids has often been noted in connection with prey preference, including varying preferences amongst the genus *Chilocorus* (Sloggett & Majerus 2000). In *Chilocorus nigritus*, Samways (1984) identified 46 species of Homopteran hosts, and further research on prey relations also established several other economically important species as suitable for mass rearing (Ponsonby & Copland 2007). With this and other research taken into account the known range is now approximately 63 species of Homoptera (Ponsonby & Copland 2007, Ponsonby 1995 & Omkar & Pervez 2002). As a result, *C. nigritus* can be considered as a polyphagous/generalist predator, but within its wide host range there is a degree of *intra*specific variation regarding its preferred hosts.

Essential prey species	**Supplementary prey species***
Coccidae	**Coccidae**
Coccus hesperidum Linnaeus	*Chloropulvinaria psidii* (Maskell)
Coccus viridis (Green)	*Coccus colemani* Kannan
Megapulvinaria maxima (Green)	*Drepanococcus chiton* (Green)
Saissetia coffeae (Walker)	*Drepanococcus cajani* (Maskell)
	Eucalymnatus tesselatus (Sign)
	Parasaissetia nigra (Nietner)
	Pulvinaria polygonata Cockerell
Asterolecanidae	**Asterolecanidae**
Bambusaspis miliaris (Boisduval)	*Bambusaspis bambusae* (Boisduval)
Diaspididae	**Diaspididae**
Abgrallaspis cyanophylli (Signoret)	*Acutaspis umbonifera* (Newstead)
Aonidiella aurantii (Maskell)	*Aonidiella citrina* (Cochquillet)
Aonidiella orientalis (Newstead)	*Aonidiella simplex* (Grandpré and
Aonidomytilus albus (Cockerell)	Charmoi)
Aspidiotus destructor Signoret	*Aulacaspis citri* Chen
Aspidiotus nerii Bouché	*Aulacaspis vitis* (Green)
Aulacaspis tegalensis (Zehntner)	*Chrysomphalus dictyospermi* (Morgan)
Aulacaspis tubercularis Newstead	*Chrysomphalus diversicolor* (Green)
Chrysomphalus aonidum Linnaeus	*Diaspis bromeliae* (Kerner)
Hemberlesia lataniae (Signoret)	*Diaspis echinocacti* (Bouché)

Lepidosaphes cornuta Ramakrishn Ayyar	*Hemiberlesia palmae* (Cockerell)
Melanaspis glomerata (Green)	*Ischnaspis longirostris* (Signoret)
Parlatoria blanchardii (Targioni Tozzetti)	*Insulaspis gloverii* (Packard)
Pinnaspis buxi (Bouché)	*Parlatoria crypta* McKenzie
Quadraspidiotus perniciosus (Comstock)	*Parlatoria zizyphi* (Lucas)
	Pinnaspis strachani (Cooley)
	Pseudaulacaspis pentagona (Targioni-Tozzitti)
	Selenaspidus articulatus (Morgan)

Table 1.1 – Essential and Supplementary Prey Items of C. nigritus (adapted from Ponsonby 2009).

*Prey species that have reliable records of having been fed upon but no experimental evidence yet found to show that they are 'essential' prey

For example, larvae originating in Pakistan show a different ability to consume armoured scale insect (diaspid) prey from those of South African origin. South African larvae were able to manipulate and consume only first instar *Aspidiotus nerii* (Bouche) (Samways & Wilson 1988), whilst larvae from Pakistan were capable of consuming first and second instars of a similar diaspid, *Abgrallaspis cyanophylli* (Signoret) (Ponsonby & Copland 2000). Furthermore, beetles from South Africa and Pakistan show differing abilities to adapt to novel prey items. South African *C. nigritus* showed very little ability to switch between two armoured scale hosts, *A. nerii* and *Aonidiella aurantii* (Maskell) (Samways & Wilson 1988), or between armoured (*A. nerii.*) and soft (*Bambusaspis miliaris* Boiduval.) scale insects (Hattingh & Samways 1992). Conversely, beetles from Pakistan were fairly promiscuous, switching between armoured and soft scale hosts (*A. cyanophylli* to *Coccus hesperidum* L. and vice versa) with little or no effect on their reproductive success (Ponsonby & Copland 2007).

The argument for geographically separated biotypes is further supported by observations of predation made in the field. *C. nigritus* from India, Pakistan, New Hebrides and the Seychelles have been seen to feed consistently on various members of the Coccidae (soft scales) (Tirumala Rao *et al.* 1954, Mani & Krishnamoorthy 1997, Puttarudriah & Channabasavanna 1993, Puttarudriah & Channabasavanna 1995, Ghani & Muzaffar 1974, Chazeau 1981, Vesey-Fitzgerald 1953). This is perhaps unsurprising, as in lab conditions many coccid species do

have properties of an essential prey item, i.e. a host that supports full development of the predator's larvae, and allows further reproduction to occur (Hodek & Honěk 1996, Ponsonby & Copland 2007) (see table 1.1). As such they are regarded as a suitable food source for mass rearing of the predators.

In South Africa, *C. nigritus* is rarely thought to accept soft scale insect prey, even in cases where the predators are given no choice in the matter. In caged avocado crops for example, *C. nigritus* showed no signs of predation on plants infested with *Protopulvinaria pyriformis* (Cockerell) (Samways 1984), even in the absence of any other food source. In one exception, Chazeau (1981) found beetles from the New Hebrides to have a similar ability to raise eggs and develop on both *Coccus viridis* (Green) (a species of coccid) and *Aspidiotus destructor* (Signoret) (a diaspid), still suggesting a fondness for diaspids. In fact, many diaspid species such as *Chrysomphalus ficus* (Ashmead) (Dixon 2000) and *Abgrallaspis cyanophylli* (Ponsonby & Copland 2007) are also considered as essential prey, allowing for high oviposition and high survival rates. As such we are able to use these hosts as a food source for mass rearing. A full list of essential prey species is shown in table 1.1, and includes those prey suggested to be 'supplementary' (accepted as a food source, but not supporting full development of *C. nigritus* larvae or reproductive activity).

In field situations the preferences of coccinellid species could depend on numerous and often intangible factors, making prey preferences difficult to pinpoint precisely. For example, the presence of multiple scale insect species (as opposed to a single host) can influence *C. nigritus*' choice of prey. This relationship between population structure and prey preference was apparent in beetles from the Seychelles, which initially were found feeding entirely on diaspids (Vesy-Fitzgerald 1953). However, when two coccids *Eucalymnatus tessellatus* (Sign.) and *Vinsonia stellifera* (Westw.) were found together, *C. nigritus* would accept soft scale as prey

(Vesy-Fitzgerald 1953). So evidently there are also effects on prey preference where an assortment of different hosts is present, further complicating the issue.

Prey choice changes with the conditions and circumstances (e.g. *C. nigritus* is opportunistic and will feed on less preferable prey as a temporary alternative), but it is also possible that the predator's geographical origin might influence prey preference. Additionally host specificity is thought to lead to speciation amongst coccinellids where sympatric species adapt to alternative food resources (Kuwajima *et al.* 2010). Sympatric populations of phytophagous ladybirds *Henosepilachna niponica* (Lewis) and *Henosepilachna yasutomii* (Katakura) are thought to have diverged through adaptations to different host plants, *Cirsium alpicola* (Nakai) and *Caulophyllum robustum* (Maxim) respectively (Kuwajima *et al.* 2010). There is already evidence of divergent dietary habits in *Chilocorus nigritus* as this section denotes, but study is needed to determine the differences between these putative 'biotypes' spanning the Indian subcontinent. Whilst it is clear that *C. nigritus* is predominantly coccidophagous, geographical variations in prey preference still appear within its host range. This evidence seems to support the theory that multiple biotypes of the beetle exist, but this hypothesis remains largely untested.

1.7.4 Other important characteristics of *Chilocorus nigritus*.

1.7.4.1 Sex ratio.

The sex ratio of this species is to some extent dependent on the age of the individuals. Young cultures have been observed with highly female-biased ratios (see table 1.2), but often become male biased as the beetle population ages. Samways and Tate (1984) recognised this pattern in their insectary cultures which reached 1:0.42 (male:female sex ratio) in beetles aged 2-6 months. This is accredited to higher female mortality, even though the older females are better adapted to stress and more resistant to pesticides (Omkar and Pervez 2002). A potential reason for this phenomenon could be the high reproductive stress put on females in captivity as a contributory factor to this early mortality.

Despite the age-dependent nature of sex ratio in *C. nigritus*, most authors suggest an inherent female bias in wild and captive populations. Table 1.2 shows the recorded sex ratios from several authors. Whilst female bias is indicative of male-killing infections in coccinellids, the link between infection and sex-ratio bias has yet to be made in *C. nigritus*. Notably there is considerable variation between authors, which may be a result of the different ages sampled, or potentially relative infection status of the insects.

Table 1.2 – Sex ratios of Chilocorus nigritus

Author	*Sex ratio range (m:f)*
Erichsen *et al.* 1991	1:1.44 - 1:3.55
Samways at Tate 1984	1:2.07 and 1.171 (from 2 prey-sites)
Burman & Ponsonby (in prep.)	1:2.85
Ponsonby & Copland 1996	1:1.44

1.7.4.2 Prey consumption.

Methods and host scale species used vary between researchers, so comparable information about the beetle's prey consumption is difficult to summarize and data on functional response are limited to those in table 1.3. These studies are hard to equate however since different host species would be different weights and likely would have different nutritional value. So these findings are merely a statement of current knowledge.

Table 1.3 – Prey consumption of C. nigritus.

Author	Prey species	Consumption
Ponsonby & Copland 2000	*Abgrallaspis cyanophylli*	0.097 mg/day at 13˚C, 1.432 mg/day at 30˚C (mean of 7 individuals per day.
Jalali & Singh 1989	A complex of 9 diaspid species	20.9 diaspids/day

Ponsonby & Copland (2000) found that males and females differed significantly in voracity, with females consuming the most prey. The only exception to this was at 30°C, where oviposition peaks with a mean of 7.76 eggs/day (Ponsonby & Copland 1998), at which stage females appeared not to consume more food than males, suggesting their efforts are focussed on oviposition.

1.7.4.3 Fecundity

Table 1.4 shows the mean daily oviposition rates of *C. nigritus* as described by various authors. The beetle demonstrates high levels of variability between individuals as previously noted in the chapter (section 1.7.2 - Ponsonby & Copland 2000). As such, the mean values give the best general overview of our current knowledge.

Table 1.4 – Mean daily fecundity of C. nigritus fed on Aspidiotus nerii and Abgrallaspis cyanophylli.

Author	Mean Daily Fecundity (eggs/day)	
	A. nerii	*A. cyanophylli*
Samways 1986		2.9
Samways 1989	3.1	2.9
Muralidharan 1993		6.3
Ponsonby & Copland 1995	3.5	5.9
Omkar & Pervez 2003	3.4	7.8

It is worth noting that fecundity can be highly dependent on rearing method. Maximum fecundity in South African beetles was observed when beetles were provided with large flight cages (Samways & Tate 1986). Pakistani beetles however managed to provide the optimum levels of egg oviposition when reared in small ventilated boxes (Ponsonby 1995). Rearing methods may go some way to explaining variability between fecundities measured by different authors. Nevertheless, it is noteworthy that again, some level of variability can be seen between *C. nigritus* obtained from different locales.

1.8 Research Aims and Objectives

Endosymbionts can have numerous significant effects on a biocontrol agent. If endosymbiotic bacteria such as *Wolbachia* can drive speciation through cytoplasmic incompatibility, it may be that different 'biotypes' of *C. nigritus* show different genotypes and phenotypes which distinguish them from one another *below* the species level. These genetic differences may also be a result of geographical or ecological separation however, and therefore this thesis aims to clarifywhich factors (ecological speciation, reproductive isolation from endosymbionts or direct effects of endosymbionts) are leading to some of the ambiguous characteristics that *C. nigritus* displays. Thus the following research objectives will be addressed in the forthcoming chapters;

Objective 1 - To determine underlying genetic variation within *Chilocorus nigritus* populations from various world locations. This will determine whether population genetics is a possible source of bionomic variation within the predator population, which may have led to the unpredictable characteristics observed in this species (section 1.7.1).

Objective 2 – To determine the range of endosymbionts present in different populations of *C. nigritus*. This is another potential source of variation, because endosymbiont infection has such a heavy impact on an individual's bionomics (section 1.3).

Objective 3 – The determine the effect of endosymbionts and genetics on bionomic characteristics relevant to *C. nigritus* and its use as a biocontrol agent. This includes the following aspects;

a) **Fecundity**

b) **Functional response and prey consumption**

c) **Pheromone production and chemical ecology**

Objective 4 – To determine the efficacy and establishment of multiple *C. nigritus* biotypes and infection types in a glasshouse environment. This will allow us to determine whether observations made in a lab environment are reflective of observations made in practical applications.

These objectives will help to determine what influence the genetic variability and endosymbiont infection status of a biocontrol agent has on its use. This will allow us to provide guidelines for scale insect management and the treatment of this economically important biocontrol agent by producers and end-users.

1.9 References

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Chapter 2 – Population genetics of *Chilocorus nigritus*.

2.1 Abstract

CO1 and ITS2 regions from *C. nigritus* were sequenced to determine population level genetic variation amongst the putative 'strains' of the beetle (with *Chilocorus baileyi* samples and GenBank sequences used as the comparative out-groups). Upon examination of phylogenetic differences, *C. nigritus* populations from South Africa and India formed distinct clades, as did the two lab strains from Wyebugs and Entocare, re-enforcing their separate lineages. The implications of these different genetic backgrounds are discussed, as well as the effects of endosymbiont infection types on the different beetle populations. It is suggested that bionomic variation between these populations may be affected by a combination of host genetics and bacterial infection type, and as such these findings may have implications for how the insect is used as a biocontrol agent (BCA).

2.2 Introduction

Within an insect species there can be considerable bionomic variation (variation in traits relating to the insect's use as a BCA) when different geographical populations are studied under the same environmental conditions. For example, the Mexican bean weevil, Zabrotes subfasciatus (Boheman) has 5 different biotypes, all with differing reproductive and developmental characteristics depending on where they originated (Credland & Dendy 1992). Coccinellids such as Harmonia axyridis can also show notable genotypic and phenotypic adaptation when exposed to direct and indirect environmental pressures (such as dietary constraints) (Astles et al. 2005). This can lead to specific adaptations to a particular host plant (Astles *et al.* 2005). This phenotypic plasticity could become more prominent when populations of an insect are geographically or physically separated, as they are likely to encounter different environmental selection pressures (climates, prey items, host plants etc.). In this way, direct and indirect environmental conditions (and geographical origin) can have a significant influence of the evolution of an insect (Astles *et al.* 2005), perhaps even leading to speciation.

Chilocorus nigritus portrays many defined bionomic characteristics vital to its success as a BCA. Many of these characteristics can show noticeable differences when physically and geographically separate populations are studied. Such variation is detailed in Chapter one, but includes various perceived differences in climatic requirements (Omkar and Pervez 2003, Greathead & Pope 1977, Kinawy 1991, Ghani & Muzaffar 1974, Ahmad 1970) and prey preference (Sloggett & Majerus 2000, Vesey-Fitzgerald 1953, Tirumala Rao *et al.* Chazeau 1981, Ponsonby & Copland 2007). There are also more tangible variations between populations, such as age-specific fecundity, recorded during lab-based studies (Ponsonby & Copland 1998, Omkar & Pervez 2003). Unlike field studies, these lab-based methods allow critical variables such as temperature and humidity to be controlled to produce what should be

reproducible data. However, it seems that in practice, even with similar lab conditioning of *Chilocorus nigritus*, characteristics such as mean oviposition and total fecundity can still vary greatly between authors and individual animals (see Chapter one). The cause of this variation between separate 'biotypes' remains largely unexplored. Whether these differences are a result of slight variations in rearing methods and methodology between authors, or the result of something related to the biology of the insects themselves, has yet to be determined.

The potential biological sources of variation can be divided into those originating from *Chilocorus nigritus* itself (host factors), and the influences from other organisms which it must interact with in order to live (trophic interactions). In lab-controlled rearing conditions these trophic interactions are limited, and occur for the most-part with host-plants, scale insects and micro-organisms. Since the narrative of this thesis is focussed on bacterial endosymbionts, bacteria will remain a consideration when discussing variations related to trophic interactions, whilst allowing for the fact that any number of biological interactions could affect how *Chilocorus nigritus* functions. Regardless, it remains important to explain whether inherent factors in the host's biology are at all responsible for bionomical variation between strains, or if trophic interactions with bacterial endosymbionts can have an effect.

The life history of *C. nigritus* can be traced back to the Indian subcontinent (Samways 1984). From here the ladybird's natural range stretches as far East as Sri Lanka, Sumatra, Vietnam, and parts of China (Samways 1984). The Western range ends in North-West Pakistan, limited by the highlands and semi-desert (Ponsonby 2009). The range currently extends further due to a mixture of classical biocontrol introductions and accidental migration from Mauritius and the Seychelles, to mainland Africa during the 20th century (Ponsonby 2009). Since then a number of institutions have obtained cultures from different regions in order to mass-rear the insects, but have encountered varying bionomic traits depending on the point of origin (as detailed in Chapter one).

In this chapter the underlying genetics of various *Chilocorus nigritus* cultures are examined, to determine to what extent any genetic variation amongst populations might affect their ability to work efficiently as a BCA. In order to determine sub species level population variation a variety of comparative DNA sequences can be used. The 'internal transcribed spacer 1' (ITS1) region of ribosomal DNA is potentially useful in the taxonomy and identification of the Coccinellidae (von der Schulenburg *et al.* 2001). There is significant variation in the sequences of ITS1 between ladybird species (as well as variation in fragment length of this region) (von der Schulenburg *et al.* 2001). Furthermore base-pair differences can be observed between individuals from different populations of the same species. This variation below the species level might allow for the identification of different biotypes/ecotypes, but research on this particular target region comes mainly from von der Schulenburg *et al.* (2001) and as such is limited to a small sample group and cannot be considered as overly reliable for wider applications.

Other established and more commonly used regions for genomic sequencing are ITS2 (a non-coding rDNA region) and CO1 (a mitochondrial DNA sequence coding for Cytochrome Oxidase) (Baldwin 1992, Kress & Erickson 2008). Given their widespread use and the high availability of comparative data from online databases, these target regions can be considered suitable for comparative genomics of this kind. Ultimately, a combination of several diagnostic target sequences can be used to provide the most conclusive results possible for the taxonomy and recognition of any putative *C. nigritus* biotypes/ecotypes. Additionally, due to the influence of maternally inherited endosymbionts on mtDNA, conclusions on evolutionary history can be confounded (Hurst & Jiggins 2005). Thus the use of solely mitochondrial DNA markers is not recommended, and the inclusion of CO1 and ITS2 limits any source of error in this respect.

The aim of investigating the evolutionary relationship and life history of different populations of *Chilocorus nigritus*, was to test the hypothesis that there is underlying genetic variation between biotypes/ecotypes. This may go some way towards explaining the variations observed in the insect's phenotypes. Although mitochondrial DNA is considered to evolve separately from other genes, there can be some correlation between morphological changes from non-mitochondrial genes and CO1 evolution. Marko & Moran (2002) showed that evolution of egg size in a number of bivalve molluscs correlated significantly with the rates of CO1 evolution. Whilst mitochondrial DNA evolves up to 10 times faster than nuclear DNA in general (Human *et al.* 2009), the CO1 gene still evolves relatively slowly compared to most other non-mitochondrial genes due to its conserved nature (Yasuda *et al.* 2006). Therefore it can be postulated that genes responsible for reproduction and functional response (traits vital to the predator's role as a BCA) will have undergone more evolutionary change than the CO1 gene (providing selection pressures have been applied). If this is the case, then divergence in CO1 will imply potential differences in bionomic traits between *C. nigritus* strains, albeit at a different rate of evolution.

Alternatively, differences in these traits might be attributed to the differing endosymbiont infections identified in the *C.* nigritus populations (see Chapter four), or perhaps even phenotypic plasticity within the same species. A previous study has also shown strong interaction between host genotype, endosymbiont genotype and other factors (Kondo et al. 2005), which provides support for this argument. In this study, the relationship between Chilocorus nigritus' genotype and its infection status was studied by sequencing CO1 and ITS2 genes of individuals from different provenances whilst simultaneously using diagnostic PCR results to determine bacterial infection status.

In assuming the existence of biotypes, identifying the presence or absence of genetic differences is important for several reasons. Firstly it will establish whether the biotypes *are*

genetically different, and therefore whether those genetic differences may account for variability in phenotypes. Secondly, sequencing data can act as a diagnostic tool for identifying different strains of beetle in future (especially where one strain is found to be more effective in certain situations).

2.3 Methodology

2.3.1 DNA extraction and sequencing.

Specimens were collected from several locations, including 30 insectary reared *Chilocorus baileyi* from BugsforBugs™, Queensland, Australia in 2009 (CbaileyiAUST) and 30 lab cultured *Chilocorus nigritus* from Wyebugs™, Kent, England, originally sourced from the Institute for Biocontrol, Rawalpindi, Pakistan in 1992 (LS1). These individuals were immediately frozen prior to DNA extraction at -20°C. Wild caught *C. nigritus* came from the Nelspruit, Republic of South Africa in 2009 (RSA, *n=30*) and also from the Indian subcontinent sourced from Bangalore in 2009 (INDIA, *n=30*). The wild-caught individuals travelled without freezing or preservation in alcohol, but were immediately frozen upon arrival in the UK.

DNA extraction and sequencing were carried out at the Natural History Museum in South Kensington, London. Total DNA was extracted from individual ladybirds using the QIAgen® Biosprint 96 DNA extraction robot and the QIAgen® BioSprint 96 DNA Tissue Kit. Amplification of the nuclear ribosomal spacer (ITS2) was achieved using 5.8SF and 28SR primers (Collins and Paskewitz 1996), and partial mitochondrial cytochrome oxidase 1 (CO1) with Cl-J-1718 and Cl-N -2191 primers (Linton *et al.,* 2001). PCR products were amplified using the reaction and thermocycler parameters described by Linton *et al.* (2001). Products were cleaned using ExoSAP-IT PCR Clean-up Kit (GE Healthcare UK Ltd, Buckinghamshire, England) before sequencing of all successfully amplified samples. The sequencing reaction

was run bidirectionally using dyes from a Big Dye Terminator Kit (PE Applied Biosystems, Warrington, England), via an ABI 377 automated sequencer to read chromatograms (PE Applied Biosystems).

2.3.2 Sequence Analysis.

Sequences were edited using Sequencher™ version 4.8 (Genes Codes Corporation, Ann Arbor, MI) to replace misread base pairs and remove primer segments. Alignments were made in Clustal X (Larkin *et al.* 2007) followed by analysis of intraspecific variation using Mega 4.0 (Tamura *et al.* 2007). Comparative sequences from GenBank were also used from an independent lab-strain from Entocare (Pasquer *et al.* 2010) and two out-group species, *Chilocorus politus* and *Chilocorus rubidus* (Fu & Zhang 2006). A bootstrap neighbour-joining phylogenetic tree was drawn up using 10,000 resamplings.

All sequenced DNA samples were also run through a diagnostic PCR protocol to ascertain infection status of three endosymbiotic bacteria; *Wolbachia* (as described by Westbrook 2006), *Rickettsia* and *Spiroplasma* (Majerus *et al.* 2000). Negative controls were performed, using double distilled dH_2O in place of template DNA, and to determine successful DNA extraction, the 28s arthropod primer set was used as a positive control (Morse *et al.* 2009).

Isolation by distance (IBD) analysis was performed using the method described by Bohonak (2002) in order to ascertain whether genetic differences were a result of isolation by geographical distance. This was achieved by using a Mantel test on the pairwise genetic distance matrix regressed against a pairwise geographical distance matrix.

2.4 Results

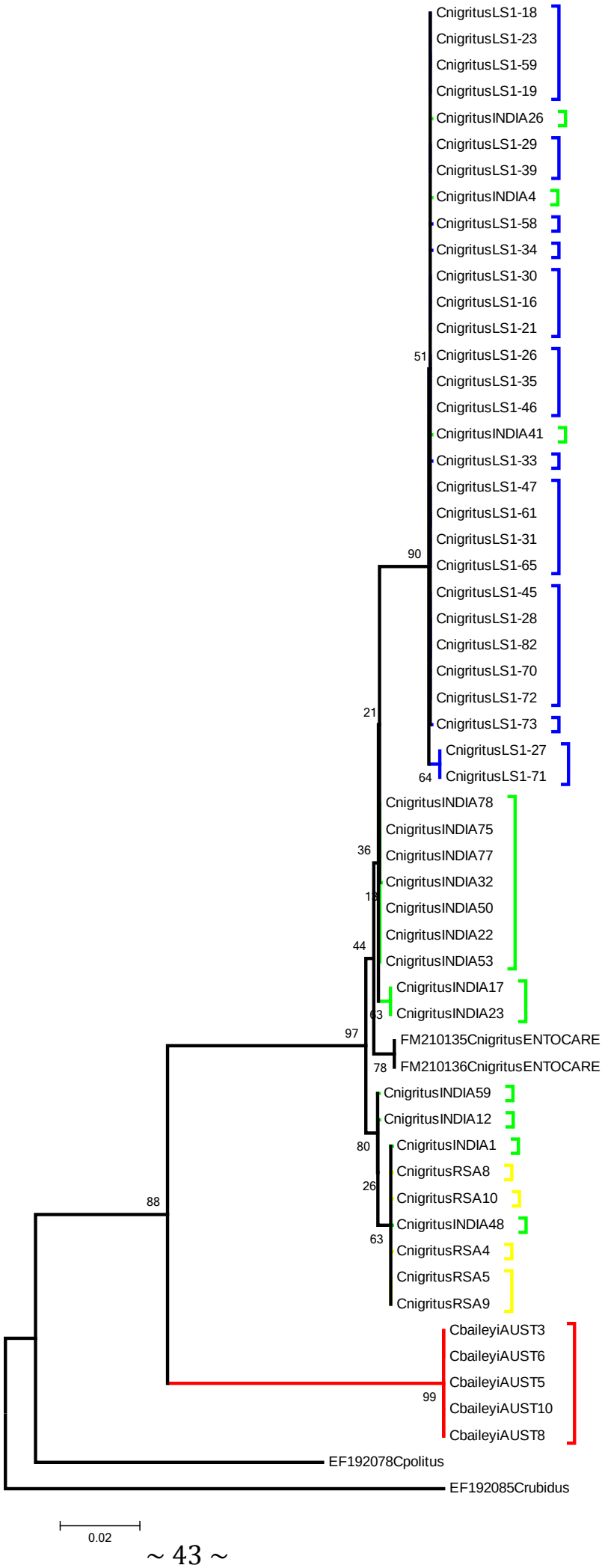

Figure 2.1 - CO1 Phylogeny of Chilocorus nigritus. Blue = LS1, Green = INDIA, Yellow = RSA. Red clade shows the Chilocorus baileyi outgroup.

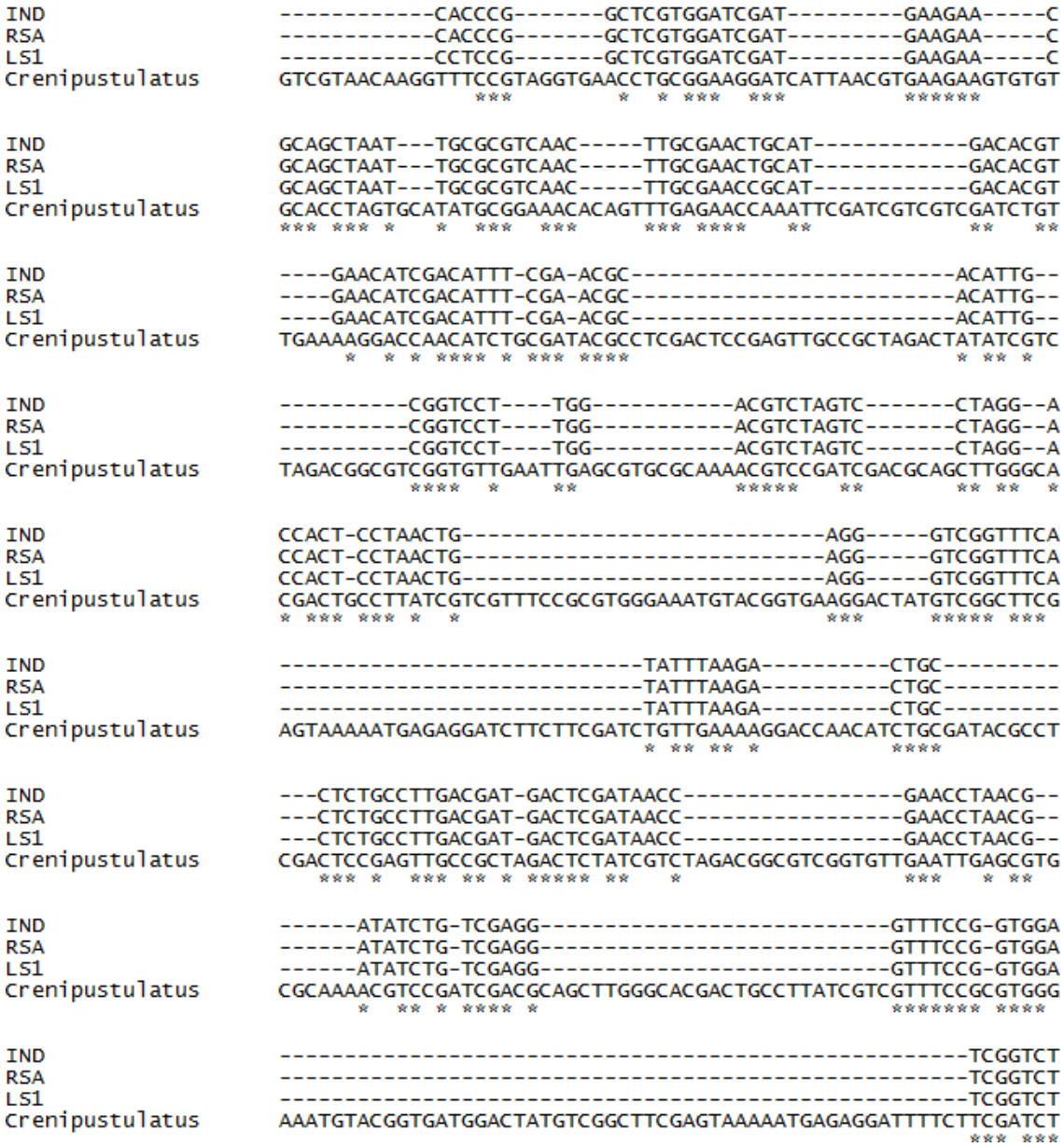

Figure 2.2 – Example of ITS2 alignments for three biotypes of C. nigritus (C. renipustulatus as an outgroup). ITS2 sequences for IND, RSA and LS1 are identical throughout, in comparison to variation seen between C. nigritus and C. renipustulatus.

Table 2.1 – Mean genetic distances between Chilocorus nigritus biotypes.

	1. *Chilocorus baileyi*	2. *Chilocorus nigritus* (IND)	3. *Chilocorus nigritus* (LS1)	4. *Chilocorus nigritus* (RSA)
1. *Chilocorus baileyi*				
2. *Chilocorus nigritus* (IND)	0.123			
3. *Chilocorus nigritus* (LS1)	0.137	0.013		
4. *Chilocorus nigritus* (RSA)	0.118	0.010	0.022	

Table 2.2 – Mean genetic distances within taxonomic groupings of Chilocorus nigritus.

	Mean within group variability
1. *Chilocorus* baileyi	0.000
2. *Chilocorus nigritus* (IND)	0.008
3. *Chilocorus nigritus* (LS1)	0.000
4. *Chilocorus nigritus* (RSA)	0.000

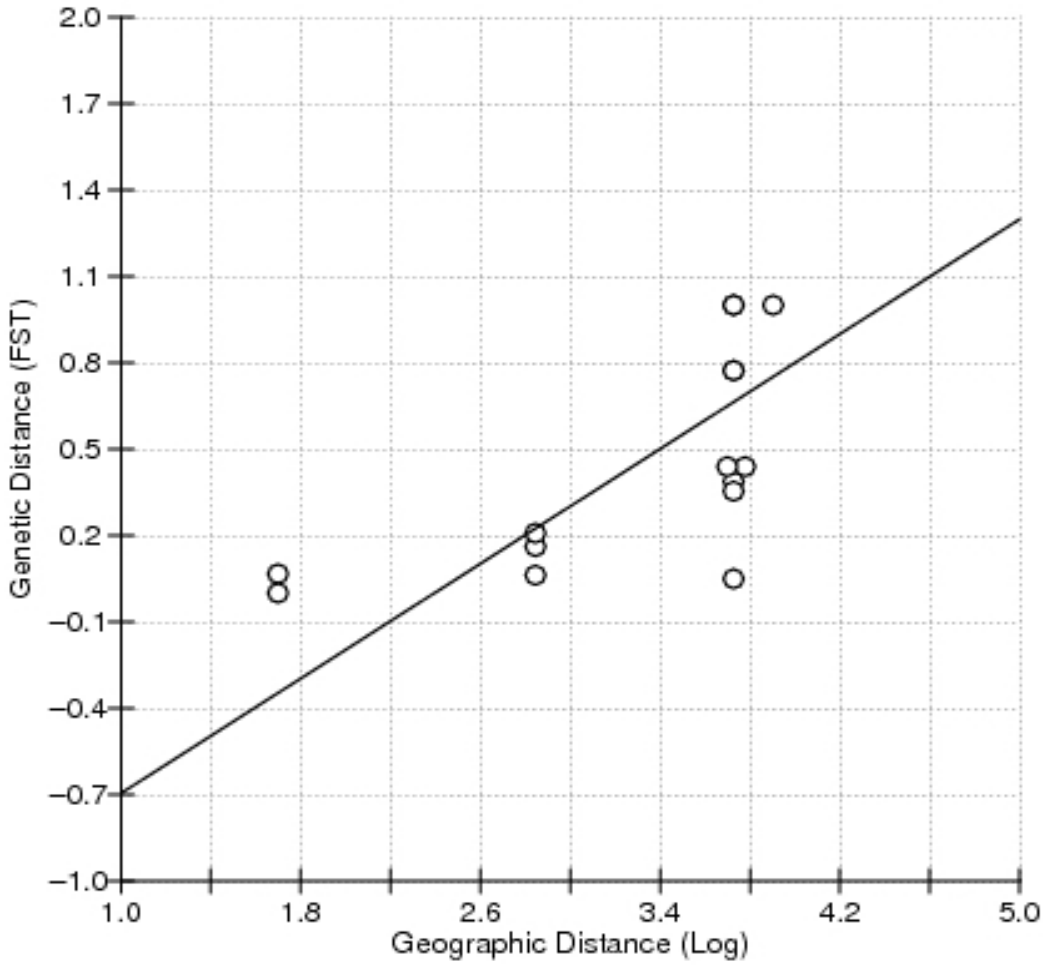

Figure 2.3 - Results of Isolation By Distance Test. A strong positive correlation is shown between genetic distance and geographical distance, suggesting distance based divergence.

Partial CO1 sequences showed expected outgrouping of *Chilocorus baileyi* (Blackburn), *Chilocorus politus* (Mulsant) and *Chilocorus rubidus* (Hope) when displayed as a phylogenetic tree supported by bootstrap values of 100 for the species clades (fig.2.1). Results showed no intraspecific variation in the ITS2 region (fig.2.2), with all sequences from *Chilocorus nigritus* individuals being identical in length and sequence. Between-group variation amongst *Chilocorus nigritus* showed strong separation of LS1 from the wild caught beetles from RSA and INDIA as well as ENTOCARE sequences from the two intraspecific GenBank entries, suggesting different lineages. Three wild caught individuals shared the same haplotype as the LS1 clade (India4, India26 and India41). Table 2.1 shows high levels of genetic variation in the CO1 gene between putative biotypes of this species, but low genetic variation within groups (Table 2.2). The isolation by distance analysis (fig.2.3) provides evidence of a significant relationship between geographical separation and genetic difference between samples ($R^2 = 0.66$, $P = 0.0220$). Figure 2.4 shows a small number of Rickettsia infected individuals in the LS1 clade, and individuals were not noted in other samples in which CO1 genes were successfully amplified.

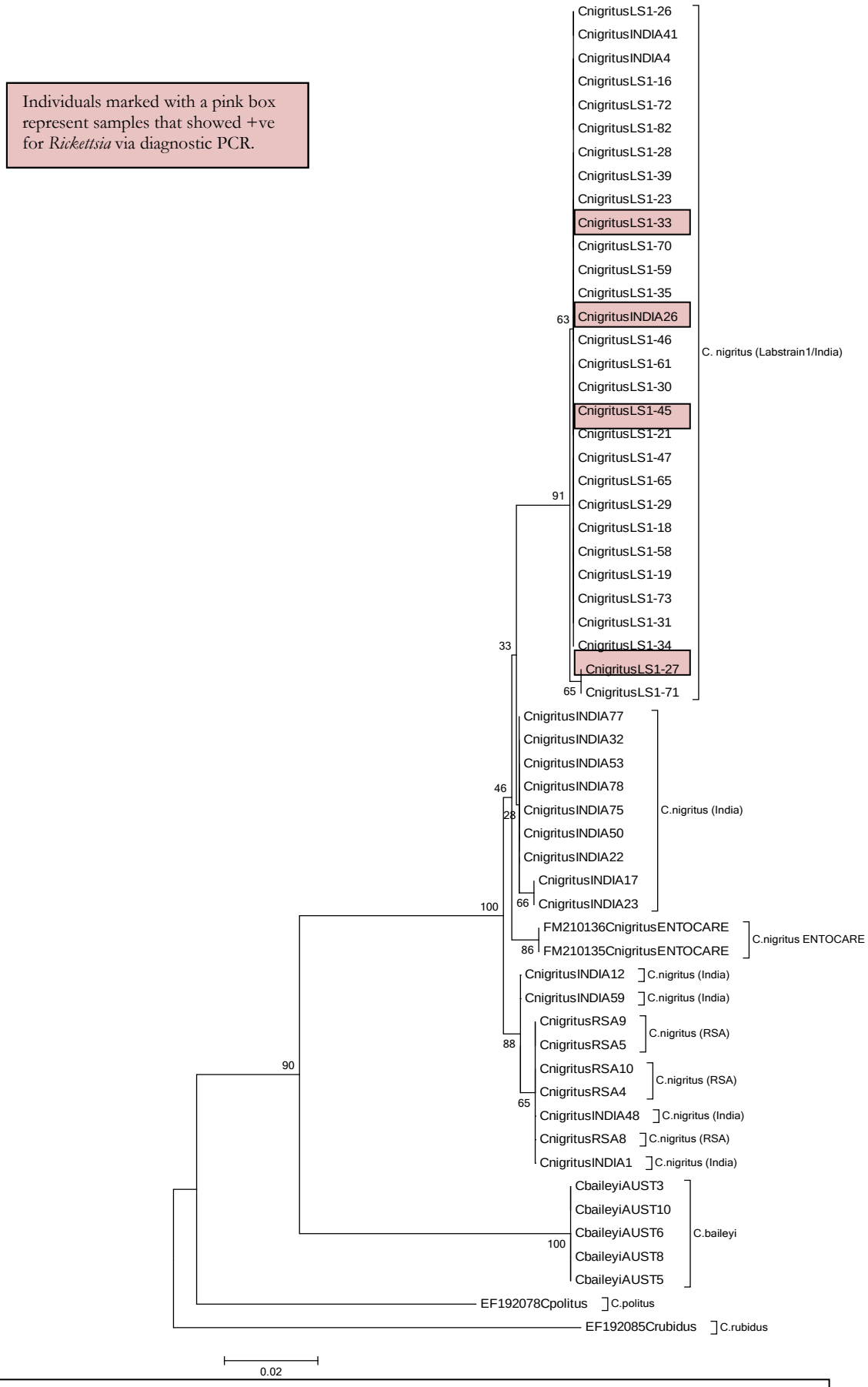

Figure 2.4 – Rickettsia Infection amongst Chilocorus nigritus populations. Only four individuals showed positive for Rickettsia in the LS1 lab culture.

2.5 Discussion

The long term lab culture (LS1) showed high sequence similarity between all samples, forming a distinct clade containing all LS1 individuals as well as the three Indian samples. Within the LS1 clade there were only 2 haplotypes, indicating low genetic variability within this group. The low variability within this group is indicative of homogeneity, possibly due to genetic bottlenecking after wild samples were acclimatized to the lab. In addition, the intrusion of the Indian beetle haplotypes in the LS1 clade may suggest that the lab strain was originally obtained from the Indian subcontinent or that beetles from this geographical region were introduced into the culture at some point. Although precise sourcing information for the LS1 biotype is ambiguous, these data suggest that the likely source would be local to Rawalpindi.

The wild caught specimens (INDIA & RSA) show sub species level differences from LS1, in some cases genetic differences were as high as 2.2% between LS1 and RSA individuals (Table 2.1-2.2), which is considered high for intraspecific mitochondrial variation in animals (Hebert *et al.* 2003). However, the theoretical threshold set by Hebert *et al.* of 3% divergence for separation of insect taxa was not met, and thus these populations can be considered as the same species, but showing strong genetic isolation due to their geographical separation. In any case, such high levels of intraspecific variation could have many implications for the use of *Chilocorus nigritus* as a BCA, and might explain some of the different phenotypes that these strains can demonstrate.

The degree of separation between clades supports what is known of *C. nigritus*' recent history. The beetle originated in the Indian subcontinent, and was only introduced recently into the Seychelles and Mauritius in 1938 and 1939 respectively (Vesey-Fitzgerald 1941, Jepson 1941). From there further colonisation of Eastern Africa occurred via introduction or invasion, with early reports beginning in Tanzania in 1966 (Ponsonby 2009). However, CO1 divergence

between the groups suggests a far more substantial period of divergence between beetles from Africa and India when assuming the generally accepted rate of change in the CO1 gene of approximately 1.4%/myr (Marko 2002). Greater still is the average phylogenetic difference between LS1 individuals and the South African/Indian populations (2.2%). This may indicate that the LS1 individuals originated somewhere other than India/Pakistan but is more likely the result of 17 years of in-breeding in laboratory culture conditions.

However, isolation by distance analysis on the samples suggests that this level of divergence is concurrent with the theory of distance-based divergence (fig.2.3) (Bohonak 2002). The regression provides evidence of a significant relationship between geographical separation and genetic difference ($R^2 = 0.66, P = 0.0220$). This kind of effect is likely to have come about due to localised mating restricting gene flow, i.e. Beetles from South Africa will breed much more frequently with nearby individuals and are far less likely to mate with Indian beetles, creating further separation of those two genotypes. This combined with the bottlenecking effect of inbreeding in the LS1 culture is likely to have amplified the differences between these individuals, and potentially give an inflated estimate of divergence time.

Additionally, large phylogenetic differences must be approached with caution when considering the range of endosymbiotic bacteria found in *C. nigritus*. Endosymbionts are usually found in linkage disequilibrium with their host (von der Schulenburg *et al.* 2001). That is to say that the bacterial genome is maternally transmitted along with maternal genes found in the host's mitochondria. As such, there is an interaction between the two separate genomes, to the point where the CO1 gene itself can be subject to selection pressure from the endosymbiont. As a result, certain haplotypes are favoured by this selection pressure, and *Rickettsia* infection can result in higher than normal variation in CO1 genes (von der Schulenburg *et al.* 2001). The presence of *Rickettsia* (and other endosymbionts) within these populations could explain the minor discrepancies between *C. nigritus*' known history and the

phylogenetic history implied by this study. The data suggest that there is sub-species level divergence occurring across *C. nigritus*' geographical range, potentially responsible for some of the differences observed in the species' prey preference and climatic requirements. More work will need to be carried out to establish a link between genotype and phenotype in this case.

Despite previous records of *Rickettsia*, *Wolbachia* and *Spiroplasma* in these populations (see Chapter three), the only male killing endosymbiont found was *Rickettsia*. *Rickettsial* genes were present in 14 beetles in total but amplifiable CO1 DNA was obtained from only four of those individuals. This discrepancy could be due to different DNA extraction methodologies to some extent, or perhaps the timeframe over which the beetles were sampled (PCR results in Chapter three were sampled from cultures in January and November 2008, whereas DNA samples in this chapter were obtained May 2009). IND and RSA samples were directly wild-caught, and had not been exposed to long term lab culturing. Bacterial prevalence in a population is known to fluctuate over time (Kyei-Poku *et al.* 2003), and can become especially high when wild insects are laboratory conditioned. This fluctuation could potentially have affected the bacterial titre in the insects, and thus the likelihood that our PCR method would have highlighted an infection. Also, there is the possibility of DNA degradation, due to repeated freezing and thawing of samples. The likelihood of this is somewhat re-enforced by the low proportion of CO1 products that were amplified (approximately 68% of samples generated a CO1 sequence).

Initially, figure 2.4 seems to show a tendency for *Rickettsia*l infection to be associated with the long term lab culture from Wyebugs™ (LC1). This would seem reasonable given the tendency of lab cultures to build up high infection levels within the population (Kyei-Poku *et al.* 2003). However, several samples that are absent in figure 2.4 (those that failed to amplify a CO1 product) still showed positive for *Rickettsia*. Notably there were 2 x Indian, 3 x Entocare

and 2 x South African individuals which were infected, in addition to the 4 x LC1 individuals (fig 2.4) suggesting that *Rickettsia* infection has no association with one particular biotype of *Chilocorus nigritus*.

It is worth noting that many geographically separate insect populations with the same infection type may harbour genetically distinct endosymbionts. For example, two divergent strains of *Wolbachia* are found in sympatric populations of the butterfly *Acraea encedon* from Tanzania and Uganda (Jiggins *et al.* 2001). Future research might aim to describe the phylogeny of inherited bacteria amongst the populations by means of *gltA* sequencing (von der Schulenberg *et al.* 2000), since genetically distinct endosymbionts can also have different effects on the host (Bordenstein & Werren 2000, Werren 1997).

Diagnostic PCR also found no evidence of male killing bacteria in *Chilocorus baileyi*. The small sample sizeprevents any firm conclusions being made on the absence of male killers in this species (Weinert 2008). Nevertheless, it is notable that not even *Rickettsia* were found in *C. baileyi*. Since *C. nigritus* and *C. baileyi* are both predators of armoured scale, especially *Aspidiotus nerii*, a comparative study between the two might provide a valuable assessment on endosymbiont effects on BCAs.

Due to the interaction between the host genotype and endosymbiont infection, phylogenetic comparison of *Chilocorus nigritus* may be somewhat confounded by linkage disequilibrium. However, there does appear to be a high level of variation in mtDNA which could imply further genetic differences between beetle populations (especially reproductive genes, which could undergo similar selection pressure from endosymbiont infection). Whether these differences are a result of geographical separation or bacterial selection pressures is largely irrelevant to their application as a biocontrol agent. This level of genetic variation could potentially affect important bionomic characteristics of *C. nigritus* such as its fecundity and

egg-viability, and may go some way to explaining bionomic variations observed by other authors, as well as those observed in further chapters.

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Chapter 3 - Establishing infection status of *Chilocorus nigritus*.

3.1 Abstract

Wolbachia, Spiroplasma and _Rickettsia_ are bacterial genera that cause male killing and sexual disorders in insects. Today the implications of such infections in a biological pest control context remain largely unexplored. This chapter details the screening of two wild and two laboratory cultures of _Chilocorus nigritus_ (a scale insect predator) for these three bacterial endosymbionts, which were previously undiagnosed in this species. Beetles from Bangalore, India and Nelspruit, Republic of South Africa harboured only low level single _Rickettsia_ infections. The lab strain LS1 (originally from Rawalpindi, Pakistan) and Entocare cultures harboured both _Wolbachia_ and _Rickettsia_ spp, indicating an infection polymorphism within the population. In the groups where multiple infections occurred, some individuals were superinfected with all bacterial genera, others had double infections of _Wolbachia_ and _Rickettsia_, and the remaining insects were singly infected with either _Wolbachia_ or _Rickettsia_. The potential effects of these different infection types on beetle populations are discussed in relation to how they could affect _C. nigritus_' ability to reproduce and control scale insect pests.

3.2 Introduction

Male-killing endosymbiont species and strains can act together upon a host insect population in two distinct ways. Firstly, multiple endosymbiont strains can inhabit individual insects resulting in a superinfection. The adzuki bean beetle *Callosobruchus chinensis* is super-infected with three distinct *Wolbachia* strains (Ijichi *et al.* 2002). In *C.chinensis* the three strains of *Wolbachia* have evolved different 'strategies' for successful infection of a single host insect (Ijichi *et al.* 2002). These *Wolbachia* strains exhibit different growth rates, and spread into different tissues, characteristics that are presumed to be the product of interspecific competition amongst the bacteria. Each strain also expressed different levels of cytoplasmic incompatibility (CI) which is attributed to the varying bacterial densities found in the reproductive organs (Ijichi *et al.* 2002).

In addition to infection of individual insects, a *population* of insects can also play host to several endosymbiont strains and/or species. This occurrence (a polymorphic population) is apparent in coccinellids, and results in each bacterial species inhabiting a different individual host insect. A prime example is *Adalia bipunctata* which was shown to harbour two strains of *Wolbachia,* a *Spiroplasma* species, and a *Rickettsia* endosymbiont in different individuals taken from a single population in Moscow (Majerus *et al.* 2000). The existence of these population polymorphisms associated with several male-killers has been studied at a theoretical level using mathematical models. Randerson *et al.* 2000 showed with a basic model that a male killing bacterium should not co-exist in equilibrium with other male killers in the same host population. In practice it is suggested that the only way in which equilibrium could be reached is with other factors, namely host resistance, playing a role. If the insects in a population are displaying resistance to endosymbionts, then theoretically a 'weaker' endosymbiont can be successful in hosts that have resistant genes to the 'stronger' endosymbionts. This in turn will

allow multiple strains to stably infect a population. Thus, such population polymorphisms might indicate the presence of resistance genes within the host population and a potentially shared evolutionary history between host and endosymbiont.

Resistance to *Wolbachia* is a relatively new topic for study, having been brought to light initially in 2007 (Charlat *et al.* 2007). Movement of resistant alleles through populations has been suggested recently in the butterfly Hypolimnas bolina (L.). Two island populations of the butterfly showed dramatic changes in sex ratio over as little as ten generations, linked to expression of resistance genes which suppressed the male-killing symptoms (Charlat et al. 2007). Although the presence of these resistance mechanisms has been demonstrated, the mode of action has yet to be discovered. However, polymorphic populations can indicate a long evolutionary relationship between host insect and bacterium, and suggests a strong and persistent selection pressure having been put on the host insect (Jaenike & Dyer 2008). However, many insects do not build up resistance, even after many thousands of years of infection (Jaenike & Dyer 2008). This unusual absence of resistance has been attributed to cases where prevalence of bacteria has been low, or where there has been a short co-evolutionary history between the two organisms. In the latter case, favourable (Wolbachia-resistant) mutations are less likely to have arisen in the population. In these situations, infected individuals could still lack resistance genes, despite being infected with Wolbachia.

It is also suggested that environmental factors can lead to infection polymorphisms, and thus a particular bacterial strain may be suited better to insects feeding on a particular host for instance (Majerus *et al.* 2000). This theory is only tenable when other characteristics such as host immobility prevent population panmixia. It is notable that neither *Adalia bipunctata*, the aphidophagous coccinellid studied by Majerus *et al.* (2000) nor *Chilocorus nigritus* demonstrate this characteristic, and are therefore both highly panmictic. It is therefore unlikely that any

polymorphisms in bacterial infection are due to environmental constraints in coccinellids, and are more likely to be the result of variable host resistance.

Wild caught insects can show an increase in bacterial prevalence when acclimatizing to lab conditions (Kyei-Poku *et al.* 2003) (*Wolbachia* in *Urolepis rufipes* (Ashmead) reached 100% infection in lab cultures and only 10-45% in field populations). The direct consequences of prevalent bacterial infections in coccinellids have been discussed in Chapter one, although endosymbiotic infection in *Chilocorus nigritus* cultures have yet to be established. Male-killing infections can potentially cause many complications in the insects' breeding and population dynamics. Cytoplasmic incompatibility can result in incompatible or reduced fecundity matings within a population where infection types vary (Bordenstein & Werren 2007). The numerous accounts of reduced oviposition and hatch rates amongst coccinellids (Chapter one) also suggest that male-killers could be detrimental to the biocontrol industry. Thus for commercially important predators such as *C. nigritus* it is important to establish the range of endosymbiont infections within the host population. Once infection status has been defined, the specific effects of bacteria on these insects can be examined.

The aim of this experiment was therefore to establish the range of endosymbionts present in *Chilocorus nigritus*, including variation between separate populations from India, South Africa, and in two long term lab cultures. Determination of superinfected individuals or polymorphic populations could be used to imply host resistant genes and possible breeding complications in pest management systems.

3.3 Methodology

3.3.1 Sourcing beetles

Cultures originated from four different sources, Nelspruit, Republic of South Africa (RSA), Entocare Biological Crop Protection (whose cultures were confirmed as part South-African) (ENT), wild caught Indian beetles from Bangalore (INDIA) and one culture from Wyebugs Ltd. (originally sourced from Rawalpindi, Pakistan) (LS1). 10 female *C. nigritus* were taken from each of the four different cultures (40 in total) and were starved for 48 hours before DNA extraction.

3.3.2 DNA extraction

Under sterile conditions the abdomen of each beetle was removed and the DNA from each beetle was extracted using a Qiagen DNeasy® tissue extraction spin column kit. The scalpel and working area were cleaned thoroughly with disinfectant wipes containing chlorhexidine between each beetle to avoid cross-contamination. Removed abdomens were also inspected for scale insect crawlers and were rinsed with distilled water. Eluted DNA samples in buffer solution were labelled and frozen along with the spin columns at -20˚C.

3.3.3 Diagnostic PCR

Diagnostic primers used were *Rickettsia* (*fwd* CATCCGGAGCTAATCCTTTTGC *Rev* CATTTCTTTCCATTGTGCCATC) (Majerus *et al.* 2000), *S.ixodetis* group (*Fwd* AGACGGTTTAGCAAGTTTGGG *Rev* AGCACCGAACTTAGTCCGACAC) (Majerus *et al.* 2000) and *Wolbachia* 16s rDNA *(fwd* CATACCTATTCGAAGGGATAG *Rev* AGCTTCGAGTGAAACCAATTC) (as described by Werren and Windsor 2000). Each

primer was diluted to 50μM. PCR tubes each contained 4μl dH_2O, 1μl Fwd primer, 1μl Rev primer, 10μl PCR mix and 4μl extracted DNA, and were run with a pre dwell of 2 minutes, followed by 38 cycles of 94˚C for 30 seconds, 55˚C for 45 seconds and 72˚C for 90 seconds. The final extension period was 72˚C for 10 minutes.

Negative controls were used with double distilled H_2O in place of template DNA, and to determine successful DNA extraction, the 28s arthropod primer set was used as a positive control (Morse *et al.* 2009) (*fwd* TACCGTGAGGGAAAGTTGAAA *Rev* AGACTCCTTGGTCCGTGTTT). Positive bacterial DNA samples from the three bacterial 'species' (obtained from University of Edinburgh) were tested for amplification with the relevant primers.

3.3.4 Gel electrophoresis

Products were run through a 2% agarose gel at 50V for 15 minutes followed by 1.5 hours at 70V. Gels were loaded with EZload molecular markers (20-1000 b.p.) in order to ascertain the approximate size of amplified products. All gels were then stained for 15 minutes in Ethidium Bromide, followed by 10 minutes de-staining in distilled H_2O. Gels were then viewed under UV and imaged using Polaroid film.

3.4 Results

Table.3.1 – Table showing infection status of each C. nigritus population/ biotype.

Individual insect	INDIA		LS1		ENT		RSA	
	Wolbachia	*Rickettsia*	*Wolbachia*	*Rickettsia*	*Wolbachia*	*Rickettsia*	*Wolbachia*	*Rickettsia*
1		•	*	•	*			
2		•	*	•	*			
3		•	*		*	•		•
4			*		*	•		•
5		•		•				
6		•	*	•		•		
7		•		•				
8		•	*	•	*	•		
9			*	•		•		
10		•	*			•		

• *Rickettsia*-positive PCR result

* *Wolbachia*-positive PCR result

Due to the small sample numbers, a more stringent William's correction was applied to test differences in prevalence, resulting in an insignificant X^2 value of 0.369, *P>0.05*.

Figure 3.1 – Sizes of amplified PCR products from Rickettsia. Size of products appears different in different strains of Chilocorus nigritus.

Wolbachia and *Rickettsia* showed positive in both the LS1 and ENT samples, whilst the INDIA and RSA samples showed positive results for *Rickettsia only*. Overall prevalence varied somewhat between the host populations (Table.3.1). For example, 80% (*n=10*) of the LS1 strain harboured *Wolbachia* compared to 0% in IND and RSA, but William's adjusted x^2 results showed the overall differences to be insignificant. Noticeably infection types varied between individuals within the LS1 and ENT samples, where some individuals harboured double-infections of *Rickettsia* and *Wolbachia*, others harboured just one species of bacterium, and some showed no infection at all. The RSA insects had relatively low infection status, with only 2 out of 10 samples showing positive for *Rickettsia*, but again (possibly due to the small sample numbers) the differences between populations were deemed non-significant. The amplified products from each endosymbiont genus appeared to be of different sizes, especially noticeable amongst *Rickettsia* products (see figure 3.1)

3.5 Discussion

The infection status of each biotype appears to be quite dissimilar in terms of prevalence and diversity of the endosymbiont species, although insignificant chi-squared results suggest they do not differ in overall numbers of infected individuals. Whilst Entocare and Wyebugs strains both appeared to harbour *Rickettsia* and *Wolbachia* genera, the amplified products from each endosymbiont genus appeared to be of different sizes (see figure 3.1). Considering the citrate synthase gene used for *Rickettsial* identification is well conserved amongst bacterial genera (a property that makes it suitable for evolutionary comparison of species), the different length fragments may suggest that the different host insect populations are harbouring different bacterial strains. The suggestion that the taxonomy of the bacterial strains in *C. nigritus* is divergent is substantiated by the fact that the host populations appear to have diverged quite significantly as well (see Chapter two). This could be an indication that the geographically and

genetically different host populations have been evolving alongside the endosymbionts for some time. In order to ascertain the true nature of this relationship, sequencing of bacterial genes will be necessary. Comparison of the sequences obtained from the PCR products with sequences from previously obtained isolates will also serve to validate the PCR results.

At this point it is worth bearing in mind the limitations of the PCR as a diagnostic tool for low level infections. If the gene number is too low in the samples, the primers may not have picked up low density infections. It is difficult to find the threshold bacterial titre at which PCR would fail to amplify a particular bacterium's DNA (Weinert *et al.* 2007). To determine this threshold would require serial dilution of bacterial colonies and subsequent PCR; however the difficulty of maintaining these endosymbionts in culture outside of the host restricts the viability of carrying out such experiments (Gamston & Rasgon 2007).

Another limitation of using PCR as a diagnostic tool for low level infections stems from host population sample size. In this particular experiment, for logistic reasons a sample of 10 female individuals was taken from each host population. Clearly the smaller the size of the sample, the greater the risk of missing low prevalence endosymbionts. Larger sample groups have been known to identify prevalences as low as 1% (Weinert *et al.* 2007), a level which could easily be diagnosed as negative in smaller sample groups. The same study (Weinert *et al.* 2007) also highlighted the potential influence of sampling strategy as well as sample size on endosymbiont detection.

The low prevalence shown especially in RSA individuals may be in part due to this inability of PCR to pick up low level infections. This sampling bias will be amplified if *C. nigritus* builds up a higher infection titre under lab conditioning, similar to *U.rufipes* (Kyei-Poku *et al.* 2003). Assays to determine the fluctuations in level of infection during lab rearing would help to

establish this as a potential source of error. As such, overall time spent in captivity might be a contributing factor towards the variation seen between populations.

The *C. nigritus* RSA and INDIA populations appeared to be singly-infected with *Rickettsia*, and in the latter the infection was present in all individuals sampled. This may indicate either host resistance to *Wolbachia* or that these host populations have never come into contact with *Wolbachia* bacteria at all. Either way, this suggests that the Indian and South African populations in particular could have a different evolutionary history with male killing bacteria. This is again re-enforced by the CO1 phylogeny in Chapter two, where Indian individuals form a distinct clade and are shown to diverge from the insects from South Africa and Pakistan.

The Entocare and Wyebugs populations are infected with both *Wolbachia* and *Rickettsia*, and contain superinfected individuals (harbouring both *Wolbachia* and *Rickettsia*). Given that horizontal transmission of *Wolbachia* is believed to be rare (Dobson *et al.* 2004), the presence of both *Wolbachia* and *Rickettsia* superinfected individuals suggests that two evolutionary endosymbiont invasions may have occurred in which bacteria have been transmitted into *C. nigritus* for the first time. Again, it is worth mentioning that a potentially high titre found in these long term lab cultures could be exaggerating this superinfection compared to the wild strains, making it more visible to PCR.

The interaction between two endosymbionts within a superinfected beetle requires further investigation, for instance to determine the distribution of the bacteria within the host, and the possibly implications on virulence. Ijichi *et al.* have shown different endosymbionts inhabiting different tissues in the Adzuki bean beetle (Ijichi *et al.* 2002), presumably reducing the competition for resources between the endosymbionts. Multiple endosymbionts living sympatrically in the same tissues of the host presumably have a more antagonistic relationship

as they compete for resources. It has often been suggested that pathogens that use resources for competition will be less virulent (Brown *et al.* 2002), but the alternative view, that competition increases virulence is also supported in many cases (Nowak & May 1994).

The issue of whether superinfection leads to virulence is ultimately a complex one, but the effect of a superinfection (like the one found in LS1 and ENT *C. nigritus*) is likely to be different from effect of a single infection (found in the Indian and South African populations). In *C. nigritus*, the cumulative effect of two symbionts on the superinfected host individual requires further examination, but should be relatively easy to determine given that the population contains singly-infected and uninfected sub-populations with which to compare. The presence of singly-infected and uninfected individuals in the Entocare and Wyebugs populations suggests that host genetics might influence susceptibility to bacterial infection or inheritance. In this case, the lab cultured populations from ENT and LS1 could be polymorphic for susceptibility to *Wolbachia* and *Rickettsia*. This could have important implications for biological pest control as some infection types might be better suited to scale insect control than others through increased fecundity or longevity. Such a hypothesis could be tested through genetic crossing experiments between so-called "resistant" and "susceptible" individuals for instance.

With a differing distribution of infection types throughout the beetle populations, there is also a potential for Cytoplasmic Incompatibility. The results obtained so far shed little light on whether or not cytoplasmic incompatibility (CI) plays a role in the *C. nigritus* populations under study, although there is the possibility that multiple strains of bacteria present might lead to incompatible mating between infection types. If CI were present it could result in incompatible populations forming between biotypes, and even within the Entocare and Wyebugs populations. If this reduces compatible matings and the overall population size of beetles in a given area, it may also reduce the potential for pest suppression. CI has been

tested as a means of biological control since it effectively reduces male fertility (Charlat *et al.* 2002), so is potentially detrimental to populations of beneficial insects as well.

The consequences of these novel bacterial infections need to be tested in order to give them some relevance to the bionomics of *C. nigritus*. As such, further research needs to be done to answer the following questions in order to put this new discovery of male killing endosymbionts in *C. nigritus* into context;

1. Are the bacterial species shared between biotypes actually different?
2. Do these endosymbionts cause CI between any particular mating combinations?
3. What effects do these different infections have on the fecundity and bionomics of *Chilocorus nigritus*?

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Chapter 4 – The effects of endosymbionts on fecundity of *Chilocorus nigritus*

4.1 Abstract

Chilocorus nigritus is infected with _Wolbachia_ and _Rickettsia_, two of the most prolific bacterial endosymbionts found in insects. These endosymbionts have the potential to influence the reproductive output of numerous biocontrol agents of economic importance. This study aimed to determine the effects of endosymbionts on the fecundity of _C. nigritus_ by creating an uninfected sub-line via antibiotic curing. Oviposition and egg-viability was recorded for both uninfected and infected beetles in order to establish the effects of endosymbionts on fecundity. Tetracycline dosage was shown to be an important consideration in the curing of bacterial endosymbionts, and some deleterious effects of high dosages were shown, potentially due to Tetracycline having antiphagant properties. However, 10% dosages of Tetracycline were shown to be effective in reducing bacterial infection, and led to higher oviposition in antibiotically cured ladybirds. This is the first observation of deleterious effects of endosymbionts associated with _C. nigritus_, the consequences of which are discussed in this chapter.

4.2 Introduction

In chapters two and three it was established that cultures of *Chilocorus nigritus* differ genetically and in terms of the compliment of the endosymbionts they play host to. Both of these factors have the potential to affect the functioning of these ladybirds as an agent for biocontrol. It has been shown that beneficial predators acquired from different locations around the world can vary in their bionomic characteristics, including survival and oviposition (Chang *et al.* 2000) and overall reproductive success (Messing *et al.* 1988). These characteristics could in turn give varying success with regards to classical and augmentative biocontrol introduction.

However, in addition to effects emerging from the host's biotype (discussed further in Chapter five), bacterial infection in the host can affect a predator's bionomics. The interplay between host biotype and infection status makes the issue more complex. Host and endosymbiont put strong selection pressures on one another, to the point where it is thought that both organisms can drive speciation in the other (Shoemaker *et al.* 2002). Endosymbionts like *Wolbachia* are thought to drive speciation in the host by creating a barrier to gene flow (cytoplasmic incompatibility) (Shoemaker *et al.* 2002). Also, the host itself is often able to evolve a resistance mechanism, which can put a strong selection pressure on the bacteria to adapt. Resistance and susceptibility to *Wolbachia* have been shown to spread remarkably quickly through some insects (Charlat *et al.* 2007) in just a few generations.

When *Wolbachia* susceptibility spreads through some insect populations, it can carry other more favourable traits with it. Many of these result in increased fecundity and oviposition (detailed in Chapters one and three). There are also numerous accounts of other beneficial traits associated with endosymbiont infection, including pathogen and pesticide resistance. For example, in *Drosophila melanogaster, Wolbacha* infection confers direct resistance to several

RNA viruses (Teixera *et al.* 2008), as well as resistance to Dengue fever in *Aedes aegypti* (L.) (Bian *et al.* 2010). In the latter case, this increased resistance not only reduced the potential for mosquitoes to vector the pathogen, but also provided increased longevity to the mosquitoes themselves (Bian *et al.* 2010). Other benefits to the host include endosymbiont-associated pesticide resistance to organophosphates (OPs) such as the association found in *Culex pipiens* (L.)(Berticat *et al.* 2002). Notably the causative direction of this relationship appears to be different from the previous examples of induced pathogen resistance. In *C. pipiens*, increased *Wolbachia* susceptibility is an associated cost of increased OP-resistance. Nevertheless, this evidence shows that *Wolbachia* infected insects may also carry beneficial traits which are related to their infection status.

These added benefits of male killing infection add a new layer of complexity to the nature of trophic interaction in a biocontrol system. Along with the selective advantage offered to endosymbiont-infected females upon emergence (Chapter one, pp Majerus 1994), these beneficial endosymbiont-associated traits make it difficult to class male killing endosymbionts as 'pathogenic' *per se*. The true effects of infection therefore need to be determined in controlled lab conditions, as well as being tested in the field.

Whilst the direct consequences of bacterial infections in coccinellids have been discussed in Chapter one; the effects on *Chilocorus nigritus* as a biocontrol agent have yet to be established. Establishing effects of bacteria on fecundity (e.g. oviposition and egg viability), may prove to be useful to producers and users of biocontrol agents. For example, ladybirds in general are fairly labour intensive to culture, requiring scale insects or aphids for food in large quantities. Each individual *C. nigritus* requires 16.24 mg of *Abgrallaspis cyanophylli* in order to reach its adult stage, which equates to over 500 scale insect prey items each (Ponsonby & Copland 2000). In addition, the scale insects themselves require a host plant, high labour costs associated with maintenance, and the whole process requires incubation: so production can be costly. Any

improvement that can be made to increase the efficiency of production would benefit producers and users.

In order to determine the direct effect of bacteria on *C. nigritus* (and thus determine if such an infection is beneficial) a comparison must be made between beetles with bacteria and those without. In coccinellids this comparison can be carried out by curing beetles to create an uninfected sub-line (whilst the remaining beetles remain infected). Elimination of male killing bacteria has been demonstrated by repeated heat treatment over several generations at 32˚C (van Opijnen & Breeuwer 1999) and by treatment with the antibiotic Tetracycline in golden syrup of approximately 10% concentration (Tinsley & Majerus 2006). The first experiment in this chapter aimed to establish the effect of curing *Wolbachia* and *Rickettsia* infection on the fecundity of *C. nigritus* using Tetracycline treatment. Experiment 2 addressed an additional aim, to determine the efficacy of Tetracycline treatment in order to create an uninfected sub-line of *C. nigritus*.

4.3 Methodology

4.3.1 Experiment 1

Beetles from long term lab strain LS1 were raised at 26˚C (±1˚C) with a photoperiod of twelve hours light and twelve hours darkness and a relative humidity of 55% (±10%). They were fed on a mixture of uni-parental and bi-parental *Aspidiotus nerii* raised on potato tubers (cv. Desiree). Twenty pairs of six-seven week old beetles were sexed using criteria described by Samways & Tate, 1984.

Each pair was placed in a controlled humidity unit (CHU) containing a saturated ammonium nitrate solution in the bottom section to maintain a stable humidity of 65% (±5%). The top

section of each CHU contained a potato tuber covered with biparental *A. nerii* of overlapping generations (approximately eight weeks old) and a 4cm^2 piece of surgical gauze for oviposition. Insects were maintained at 26˚C (± 1°C) under the same 12:12 light regime.

The gauze, tubers and CHUs were examined for eggs daily and the total numbers of eggs laid by each pair was recorded. Eggs were then removed to separate containers. The same treatment was given to all twenty pairs, with potatoes in the CHUs being replaced when necessary.

During the treatment period each pair received a syrup meal for four days. Ten pairs received approximately one gram of golden syrup on a filter paper, whilst the other ten pairs received one gram of 10% Tetracycline hydroxide in syrup (also on filter paper). After the syrup and Tetracycline treatments, normal feeding with *A. nerii* was resumed for a further seven days, and all eggs were again counted and removed.

4.3.2 Experiment 2

Rearing and methodology was performed as in Experiment 1, except using four different levels of Tetracycline dosage (0.0%, 3.3%, 6.6% and 10% Tetracycline in golden syrup). Each test group consisted of five sexed pairs of *C. nigritus*, fed on biparental *A.nerii* for five days, followed by 3 days of treatment with the Tetracycline treatment. All insects were then returned to *A.nerii* for a further five days. Eggs were removed and kept at 26˚C in separate containers. Larvae were counted and removed daily once emerged, allowing egg viability to be calculated for each pair's offspring.

DNA was extracted from 24 offspring from subsequent maternal lines used in the 0% and 10% Tetracycline groups as a comparison. The extraction was performed using Qiagen

DNeasy tissue extraction kit. The samples were subsequently tested for the presence of *Rickettsia* and *Wolbachia* via the same PCR protocol detailed in Chapter three.

4.3.3 Data Analysis

Repeated measures tests comparing mean daily oviposition between different Tetracycline treatments and "before vs. after treatment" were carried out using Minitab 16. The oviposition of *Chilocorus nigritus* follows a cyclic pattern and age-specific fecundity is a noticeable trait of this species (Ponsonby and Copland, 1998, 2007). As such, the data contained un-equal variances throughout the treatment period, and samples were dependent. Therefore repeated measures ANOVA was deemed as the most suitable test for differences between means. This analysis was achieved through the use of a General Linear Model in Minitab 16.

Differences in total hatch and total oviposition pre and post-treatment were analysed using Student's t-test for testing differences between two groups, and ANOVA for comparisons of multiple groups. These data were tested for normality using an Anderson-Darling normality test and displayed normal distributions (Total hatch $P = 0.281$, Total oviposition $P = 0.139$).

Egg viability was calculated from the ratio of the number of the eggs which produced larvae to the total oviposition. These data displayed a non-Gaussian distribution ($P < 0.005$) and were therefore treated with non-parametric statistics. Differences between egg viability of Tetracycline treatment groups were tested with a Kruskall Wallis test. Differences between two groups were tested with a Mann-Whitney test.

Differences between the number of individuals infected with *Wolbachia* and *Rickettsia* were tested with a Chi-squared Goodness-of-fit test to determine successful elimination of endosymbionts by antibiotic curing.

4.4 Results

4.4.1 Experiment 1

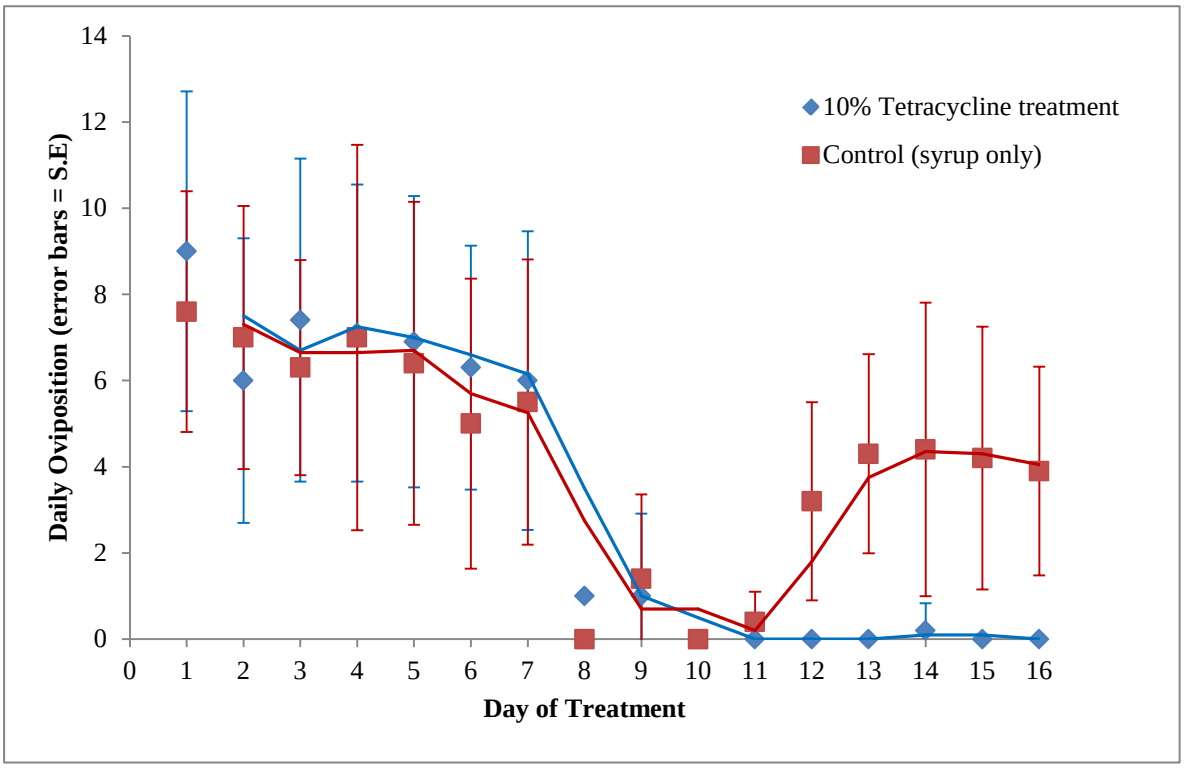

Figure 4.1 – The effects of 10% Tetracycline treatment on Chilocorus nigritus (LS1 biotype). The graph shows oviposition over 16 consecutive days of treatment, with Tetracycline feed (blue) and without (red). Antibiotics/control syrup were given to the insects between days 8-11. Tetracycline treated insects appear to reduce their oviposition in response to antibiotic treatment, whilst the control group returns to normal baseline oviposition rate.

There was no significant difference between the two control groups in the pre-treatment period (*d.f.=1, F = 0.95, P = 0.330*) (fig. 4.1), but after treatment the oviposition of the control

group was significantly higher than the 10% Tetracycline treated group, which displayed little to no oviposition immediately after treatment (d.f. = 1, F = 122.66, $P < 0.001$).

4.4.2 Experiment 2

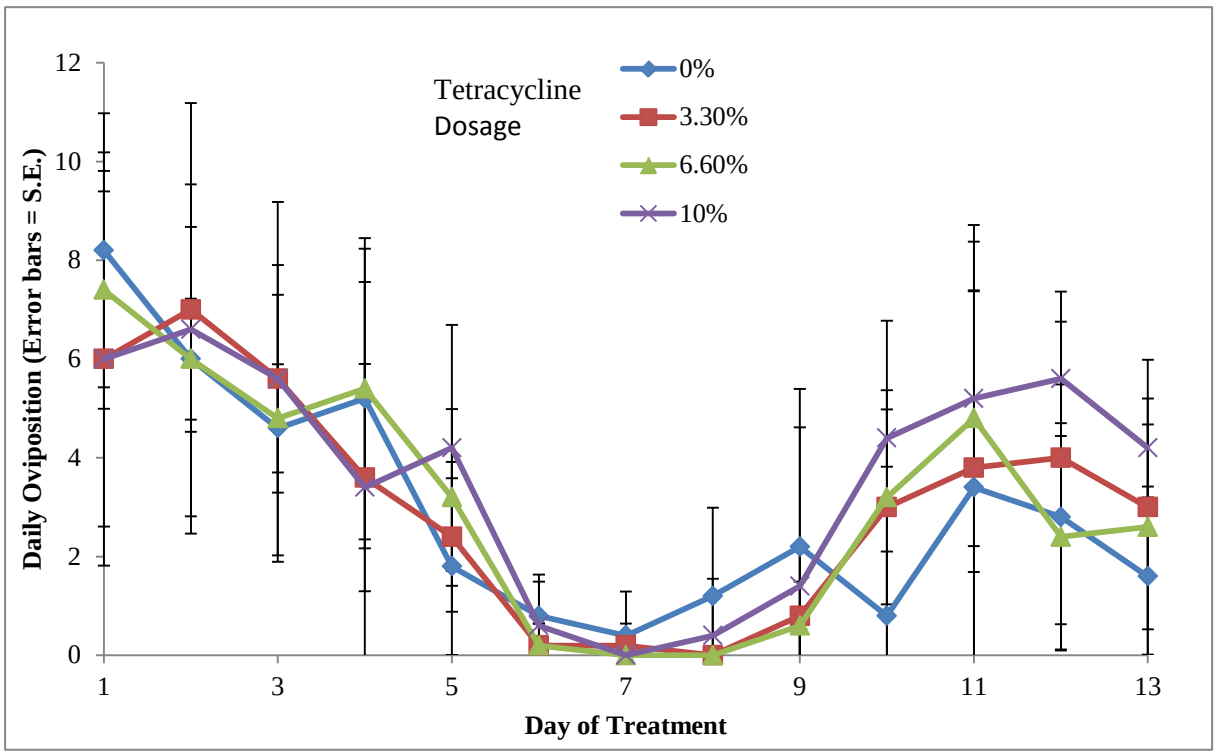

Figure 4.2 – The effects of Tetracyline dosages on oviposition of C. nigritus. Insects were given the same food and conditions for 5 days, followed by a syrup meal on days 6-8 containing various conecntrations of tetracycline antibiotic. Higher doses of tetracycline appear to demonstrate higher oviposition post treatment, especially prominent in the 10% group, which shows the highest oviposition rate (purple).

Table 4.1 – Descriptive statistics of C. nigritus post Tetracycline treatment.

	Tetracycline treatment group			
	0,0%	3,3%	6,6%	10,0%
Mean oviposition	54,24	20,93	81,04	45,80
Standard deviation	37,34	24,87	10,78	14,97
Total hatch	36	29	53	55
Total ovipostion	60	73	68	106

4.4.3 Effects on oviposition

Overall there was a significant difference between the pre-treatment period and post-treatment period (fig. 4.2) (*d.f. = 1, F = 72.86, P<0.001*), indicating an effect of Tetracycline treatment on these insects as a whole. Previous to Tetracycline treatment the difference between all groups was non-significant (*d.f. = 3, F = 0.73, P=0.538*). After the treatment period however, there was a significant effect of dosage type on oviposition (*d.f. = 3, F= 9.71, P<0.001*), indicating that the significant difference occurred only post treatment (total oviposition is represented in table 4.1).

4.4.4 Effects on egg viability

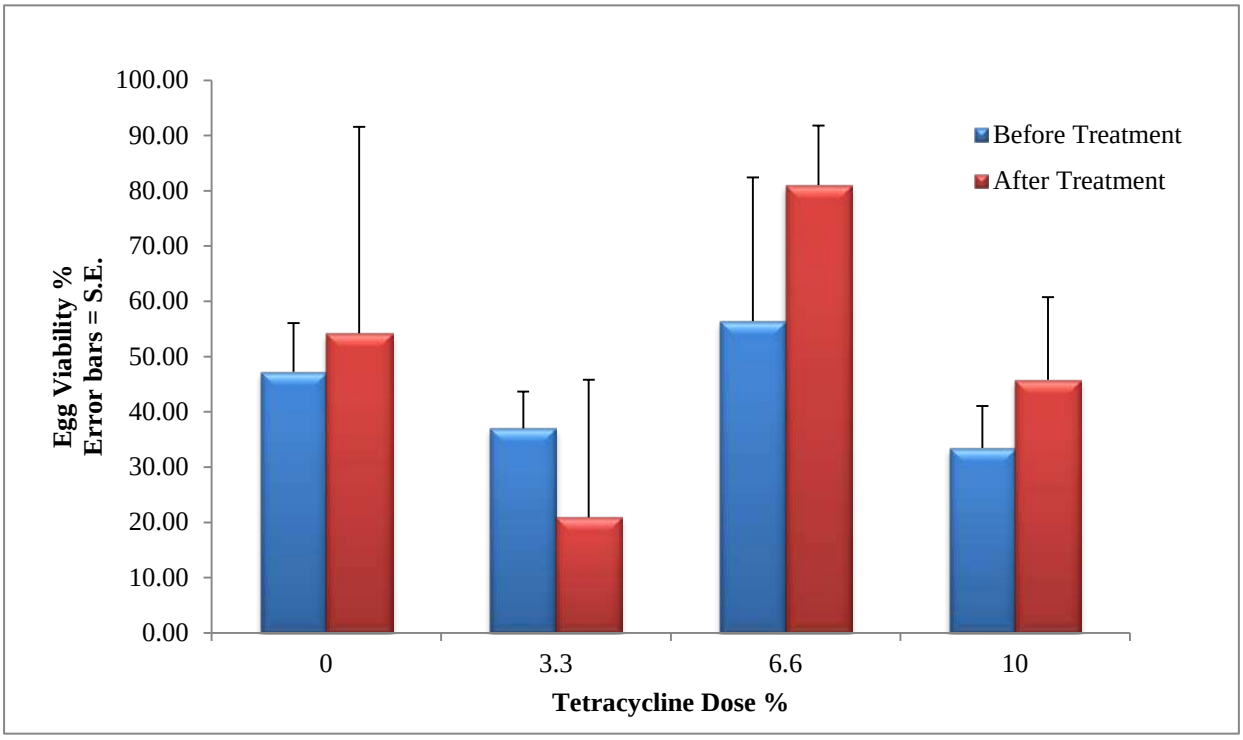

Figure 4.3 – The effects of Tetracycline on egg viability in C. nigritus. Tetracycline treaments see higher post – treatment viability.

Even though superficially it appears that post-treatment egg viability is higher (see fig.4.3), a Mann-Whitney test showed the difference between egg viability before and after treatment to

be non significant (W = 348.5, d.f. = 1, P = 0.098). Differences between treatment groups were shown to be non significant before and after treatment.

4.4.5 PCR Results

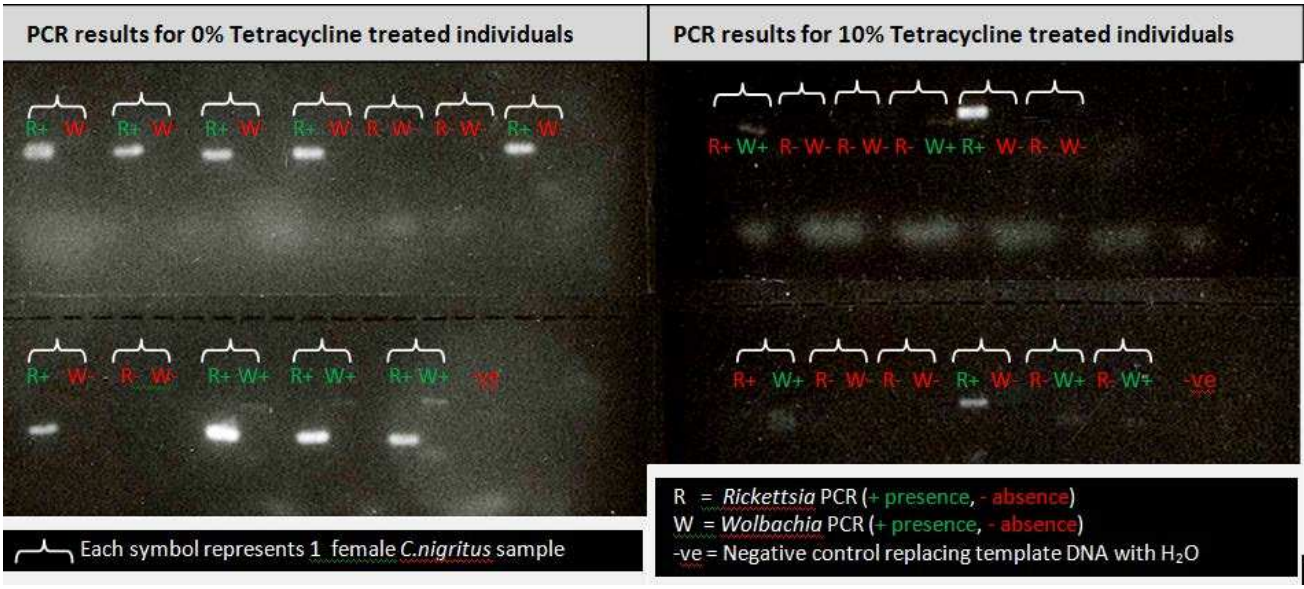

Figure 4.4 – PCR results comparing the effects of Tetracycline treatment of bacterial presence. The image shows electrophoresis gels, with DNA bands representing positive amplification of either Wolbachia or Rickettsia genes.

A qualitative difference can be noted between the two treatment groups (fig. 4.4), with Tetracycline treated insects having a lower bacterial prevalence in this case. A Chi-squared test showed a significant difference between bacterial prevalences between the 0 and 10% treatments (d.f. = 1, $X^2 = 3.909$, P = 0.048).

4.5 Discussion

Experiment 1 shows an unusual effect of Tetracycline, causing the reduction (and almost total inhibition) of oviposition in the individuals treated with 10% Tetracycline/syrup solutions. Although previous studies carried out on coccinellids had shown Tetracycline to be an

effective method of curing bacterial infection and increasing fecundity, these data show the inverse effect on egg-laying. This dramatic decrease in productivity seen in the beetles treated with antibiotics could be due to the elimination of beneficial and mutualistic male-killing bacteria, as has been noted in a number of other insects (Dobson *et al.* 2002, Hosokawa *et al.* 2009). Perhaps more likely considering other data in this chapter, is the direct negative effect of the antibiotic on the insects, either through acting as an antiphagant, or by directly affecting the reproductive system of the beetles. Antiphagous properties of antibiotics on insects have been noted before (Kermerrac & Mauleon 1989), and reducing the amount of food the insects ingest could also limit their fecundity (Ponsonby & Copland 2007). This supposition would also go some way to explaining the gradual return to normal oviposition observed once the experimental period had finished.

Experiment 2 demonstrates an effect of different Tetracycline dosages on oviposition, with the highest oviposition being encountered in the higher tetracyline treatments (see figs. 4.2 & table 4.1). The most unusual observation to be made here is that 10% Tetracycline in this experiment led to increased oviposition, contrary to the results found in Experiment 1. These two experiments were performed approximately two years apart, on the same lab culture, but yielded very different results. Factors that could lead to such a dramatic difference are difficult to determine. If antiphagant properties of the antibiotic/ damage to the reproductive system was the original cause of the oviposition decrease, then it is difficult to see why the same treatment would lead to an increase in oviposition in insects treated two years later.

This anomaly may highlight a potential problem with the way biocontrol agents are raised. Most mass rearing occurs in a stringently controlled environment, using only one or two prey species of food item. There is a strong tendency for insects to adapt quickly to these artificial conditions, as this lab environment offers a very strong persistant selection pressure. As a result, it is thought that insects that are mass-reared for long enough become quite different

from the wild populations which founded them (Miyatake 1998). All manner of life history traits can alter during a species' time in the laboratory, including dietary preference, larval development and survival (Bellutti 2011). The olfactory system is particularly susceptible to change during mass rearing (Bellutti 2011). Long term selection pressures in the lab can even lead to complete loss of gustatory response (Bernays *et al.* 2003).

It is certainly possible that gustatory response has changed during the rearing of *C. nigritus* in captivity. Beetles that would normally be polyphagous in the wild have been 'forced' into oligophagy, in this case feeding only on two species of scale insect. It is possible that this accounts for a change in gustatory response to the Tetracycline treatment. This change could also be explained by numerous bottlenecking events combined with artificial selection for individuals with a particular food preference. Sinse gustatory sensitivity to a substrate can be altered by even a single base pair mutation (Inomata *et al.* 2004), this change in the population's taste response is possible within even a short time period. This kind of gustatory plasticity is also supported by the literature about fluctuating host preference detailed in Chapter one. A comparison of the dietary preferences and gustatory responses of wild type and long mass-reared insects could therefore be valuable in the future. This provides a new hypothesis on the plasticity of gustatory reception in predators. Gustatory plasticity could be measured by testing of dietary preference, or specific gustatory cues.

Irrespective of the unusual difference between these two experiments, the variation in oviposition seen between Tetracycline dosages in Experiment 2 suggests at least a partial elimination of *Rickettsia* and *Wolbachia*, especially in the higher dosage groups. Direct post-experiment analysis of the infection status of the different treatment groups was unavailable at this point in the project. However, subsequent comparison of the offspring of the 0% and 10% groups was made seven months later. The results of diagonstic PCR (shown in figure 4.4) give a qualitative insight into the effect of Tetracycline on endosymbionts in *C. nigritus*.

This provides evidence that the 10% tetracyline treatment given to one population had reduced the prevalence and/or titre of bacterial infections significantly (*P = 0.048*) (*Rickettsia* infected individuals were especially numerous in the untreated population, containing nine infected individuals compared to only two in the Tetracycline treated group). The significant difference in oviposition between the 0% and 10% groups post-treatment would also suggest at least partial elimination of male-killing bacteria by Tetracycline.

Effects of endosymbionts of oviposition have been noted in other insect families (Zchori-Fein *et al.* 2001), but most studies focus on overall fecundity as a measure of effects on bionomics. Whilst the proportion of viable offspring did not appear to be affected by antibiotic curing (figs. 4.4 & 4.5), the total number of offpring produced by each treatment group was highest after 6.6 and 10% antibiotic treatments (fig.4.5). In terms of mass rearing it would appear that Tetracycline treatment is beneficial for productivity of *Chilocorus nigritus*, and might be considered as an inexpensive means of increased the numbers of individuals hatched in this and other coccinellids. The advised dosage would potentially lie somewhere between 6.6% and 10% based on the findings on overall fecundity. This kind of treatment should also be tested on a far greater number of individuals, and a number of wild and lab cultures of *Chilocorus nigritus* in order to test for cases where Tetracycline inhibits oviposition. It might for instance be advisable to perform Tetracycline treatment only on long term lab cultures.

Whether the subsequent curing effect of this treatment has wider implications for the bionomics of the species should also be determined in order to optimise field efficacy. Some of the other areas of interest relevant to biocontrol would be pesticide resistance, pathogen resistance, functional response, dietary preference, pheromone production and olfactory gustatory responses; all of which can be influenced by endosymbiont infections.

The increases in productivity seen with antiobiotic treatment could be outweighed by the negative consequences of eradicating endosymbionts. *C. nigritus* currently appears to suffer from very few pathogenic fungi and limited parasitism (Ponsonby 2009), and shows some evidence of pesticide resistance and tolerance (Hattingh & Tate 1995). Eradicating useful traits like these would evidently be detrimental; so the question of whether these traits are associated with endosymbiotic infection remains a valuable topic for further research. These results do however suggest that the influence of endosymbiont infections in biocontrol systems could be more than previously thought; thus the effects on bionomics of predators in general is worthy of further investigation.

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Chapter 5 – The effect of endosymbionts on functional response of *C. nigritus*.

5.1 Abstract

Male killing endosymbionts have been shown to affect the functional response of biocontrol agents, altering one of the bionomic characteristics most relevant to pest suppression. This experiment aimed to determine whether genetic background or endosymbiont infection status altered the functional response or prey consumption of *Chilocorus nigritus*. Cage experiments were set up to monitor consumption of the scale insect *Aspidiotus nerii* at increasing prey densities. Two genetically distinct biotypes of the beetle were used (LS1 and ENT) as well a subline known to harbour a lower level of endosymbiont infection (LS1TC). Significant differences were observed in prey consumption between LS1 and ENT males, and females showed a higher level of prey consumption overall. Comparisons between LS1 and LS1TC strains showed for the first time that *C. nigritus* individuals harbouring endosymbionts have a higher rate of prey consumption than uninfected strains.

5.2 Introduction

Functional response is arguably the most essential bionomic characteristic belonging to a biocontrol agent, describing the ability of a predator to eat and suppress a pest population (Pervez & Omkar 2005). The functional response also describes the ability of a predator to suppress pests at different population densities. Broadly speaking there are three classifications of functional response often used to describe predator-prey interaction (fig.5.1). Type I functional response represents a linear relationships between host density and numbers of prey eaten. Type II refers to a decreasing curve, which often represents satiation of the predator at higher host densities. The third, and most preferable response for a biocontrol agent is type III, showing an upwards curve (i.e. the predatory insect eats more pest insects as prey density increases).

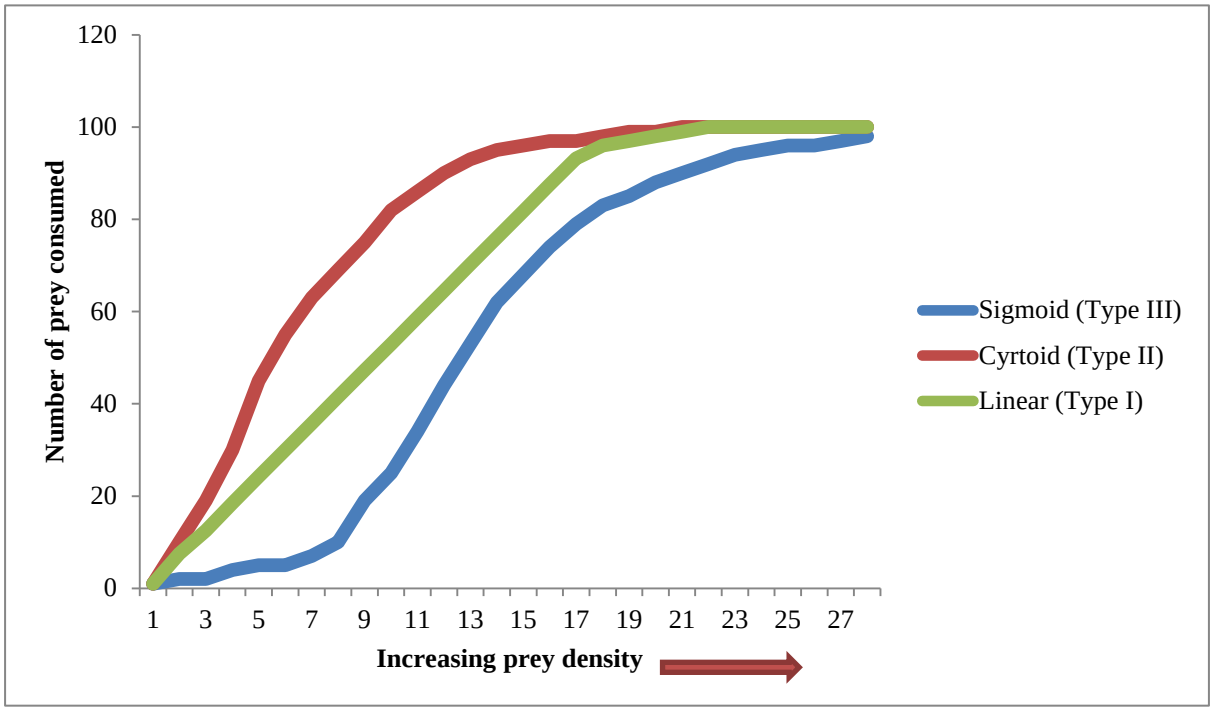

Figure 5.1 – The theoretical categories of functional response.

Separate specialised strains of an insect species are referred to as biotypes. Given the right environment, one biotype can become more effective as a biocontrol than another. A classic

example of this is seen in the ragwort flea beetle *Longitarsus jacobaeae* (Waterhouse), which was released as two biotypes in the U.S.A. (Szucs *et al.* 2011). The strain of *L.jacobaeae* which originated in Italy provided substantial control of Tansy Ragwort (*Senecio jacobaea* L.) along the Pacific Coast, mainly in areas where the climate was similar to that of the beetle's native Mediterranean climate. When Ragwort managed to colonise the colder, higher elevation regions of the Pacific North-West, pest suppression was provided by a different biotype, an Italian-Swiss hybrid. This example shows how adaptations can occur below the species level, leading to different bionomics between populations. In this case these adaptations were thought to be related to temperature tolerances and different functional reponses.

There is some direct evidence of functional response and feeding behaviour varying between insect biotypes. A well studied example is the whitefly *Bemisia tabaci* (Gennadius), a species complex containing over twenty biotypes (Perring 2001). The most voracious biotypes are *B. tabaci* B (identified by Brown *et al.* 1995), and *B. tabaci* Q (Horowitz *et al.* 2003). However, there is considerable variation across the known biotypes, in terms of functional response and a whole number of other bionomic characteristics (Perring 2001). In a classical and inundative biocontrol context, it has been suggested that introducing a combination of several biotypes can be the best way to provide successful biocontrol in a variety of agronomical situations (Tauber & Tauber 1993). This practise effectively increases the number of genotypes and phenotypes in the founder population therefore improving the chances of at least one genotype/phenotype establishing a new population and providing successful control. Using a guild of predator biotypes could potentially provide a tool with which to improve glasshouse scale control.

There is also evidence to suggest that endosymbiont infection can alter functional response. *Wolbachia* infection in parasitoid *Trichogramma brassicae* (Bezdenko) specifically increases host handling time, and thus alters the functional response, reducing the biocontrol efficacy of the

infected strain (Farrokhi *et al.* 2010). These effects have yet to be established in coccinellids, but could have implications for mass rearing projects, as well as release programs; potentially causing a reduced feeding capacity in infected individuals. There is also growing evidence showing that the olfactory responses of insects can be altered by these bacteria, in some cases enhancing responses to food items (Peng & Wang 2009, Peng et al. 2008). Handling time and olfactory response are hypothetically linked to one another, and thus there is potential for endosymbionts to affect functional response through this mechanism.

Data on the feeding responses of *Chilocorus nigritus* are limited, but recently the effects of temperature regimes on feeding rate have been identified. When fed on *Abgrallaspis cyanophylli*, *C. nigritus'* daily prey consumption peaks at cyclic temperatures, but at constant temperatures of 30°C individual ladybirds consume the equivalent of seven individuals per day (Ponsonby & Copland 2000). This rate may vary dependent on prey species, as other authors have observed different daily rates on other hosts (Jalali & Singh 1989). As mentioned in Chapter one, this variation may be in some part due to the different relative sizes of different host species; thus one adult *Abgrallaspis cyanophylli* may be equivalent in nutritional value to several adults of a smaller species.

Responses of aphidophagous coccinellids to prey density is commonly a type II curvilinear increase in prey consumption, observed in a number of aphid predators (Gharari *et al.* 2003, Omkar & Pervez 2005, Jafari & Goldasteh 2009, Khan 2010, Saleh *et al.* 2010). Type II responses have also been noted (Mandour et al. 2006) as well as type III responses to arachnid prey items (Sohrabi & Shishehbor 2007). While type II functional responses appear to be the predominant trait in aphidophagous species, coccidophagous species remain considerably under-researched, perhaps due to the relative difficulty of rearing and controlling the density of scale insects. It has been suggested that coccidophagous ladybirds consume prey in a more efficient manner than aphidophagous species, resulting in a lesser degree of satiation (Mills

1982, Dixon 2002, Magro *et al.* 2002). One might expect insects with this trait to display a type III sigmoid response to prey density, but functional response data on scale insect prey are notably absent in the literature, with the exception of some data on psyllids (Da Silva *et al.* 1992). This study therefore aimed to categorize the response of *Chilocorus nigritus* to density of prey insect *Aspidiotus nerii*. In addition three lineages of *C. nigritus* were used to test for differences between biotypes and endosymbiont infection types.

5.3 Methodology

5.3.1 Insect cultures

Chilocorus nigritus individuals from three different lines were used for comparison. This included the previously mentioned LS1 and ENT strains, as well as a sub-culture derived from the LS1 lab culture (named LS1TC). The LS1TC lineage (LS1+Tetracycline) had been previously treated with 10% tetracycline treatment in order to eliminate male killing bacteria (see Chapter four). The elimination of *Wolbachia* and *Rickettsia* bacteria was only partial after that initial treatment, and thus two subsequent antibiotic feeds were given to this culture for a period of three days each time. During this period all scale insect food was removed. Beetles had been maintained and reared at 26°C on overlapping generations of uniparental *Aspidiotus nerii*.

5.3.2 Prey consumption methodology

Cumulatively over a period of five days, a total of thirty individuals from each line were used (six per day) and placed onto a potato tuber inoculated with different densities of uniparental *Aspidiotus nerii*. Scale insect cultures were approximately six weeks old, containing adult females only. Ladybirds were placed individually in custom made vented cages which allowed

individuals to be restricted to a specific area of the potato tuber's surface. The containers consisted of 17 mm lengths of 50 mm outer diameter (40 mm inner diameter) plexigass tubing with 10 mm x 5 mm of neoprene foam glued around the base being used to create a tight seal between the container and the potato itself (see. fig.5.2). Mesh was welded on top for ventilation. This was secured to the potato surface using several elastic bands. After inoculation for up to a week with scale crawlers, prey densities on tubers ranged from 41-2760 individuals per cage.

Figure 5.2 – Design of insect cages for Chilocorus on Aspidiotus nerii. (Left) Scale insect cage. (Right) Cage attached to infested tuber

Total number of scale insects under each container was counted and one adult beetle was placed inside, before securing the cage to the tuber surface. Tubers were separated into three densities, low (0-200 scale insects), medium (201-1000), and high (1000+) per cage. Each day, two beetles from each biotype were placed onto each of the three densities i.e., six beetles per day for a period of five days, totalling 30 replicates for each biotype.

Cages and tubers were placed into large sealed plastic tubs containing an open container of saturated ammonium nitrate solution, which maintained humidity at 66% rh. This was in turn maintained at 26°C on a 12:12 light regime. Total number of scale insect individuals consumed was measured for each ladybird after 24 hours. Evidence of predation was usually in the form of a 'chewed' or removed scale cover.

5.3.3 Data analysis

Correlations between daily prey consumption and prey density were made via Minitab 16 in the form of a linear regressions using density as the independent variable. Other functions tested with this data set included logit and polynomial models, but a negative linear correlation provided the best fit for this data set. Comparisons were also made between male and female functional response, and between all three biotypes/infection types via a balanced ANOVA (after testing for normal distribution via an Anderson-Darling test for normality, $P<0.005$). For analytical purposes, densities were also sub-divided into broad categories as follows; 1-100, 101-200, 201-300, 301-400, 401-1000 and 1000+ scales per cage, and differences between daily consumption were tested again at these densities with ANOVA and Student's t-tests for differences between two groups.

5.4 Results

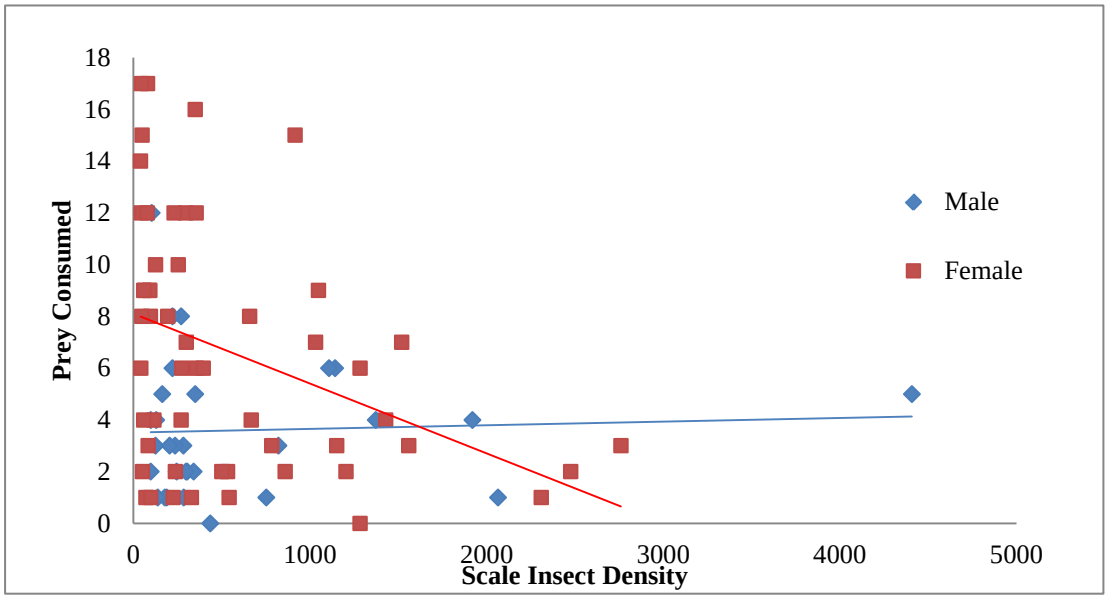

Figure 5.3 – General functional response of Chilocorus nigritus (n=84). Male and female functional responses are shown. The spread of data shows that female prey consumption is generally higher than in males,

but shows a decrease in prey consumption at higher prey densities. Conversely, males display a stable low level of predation.

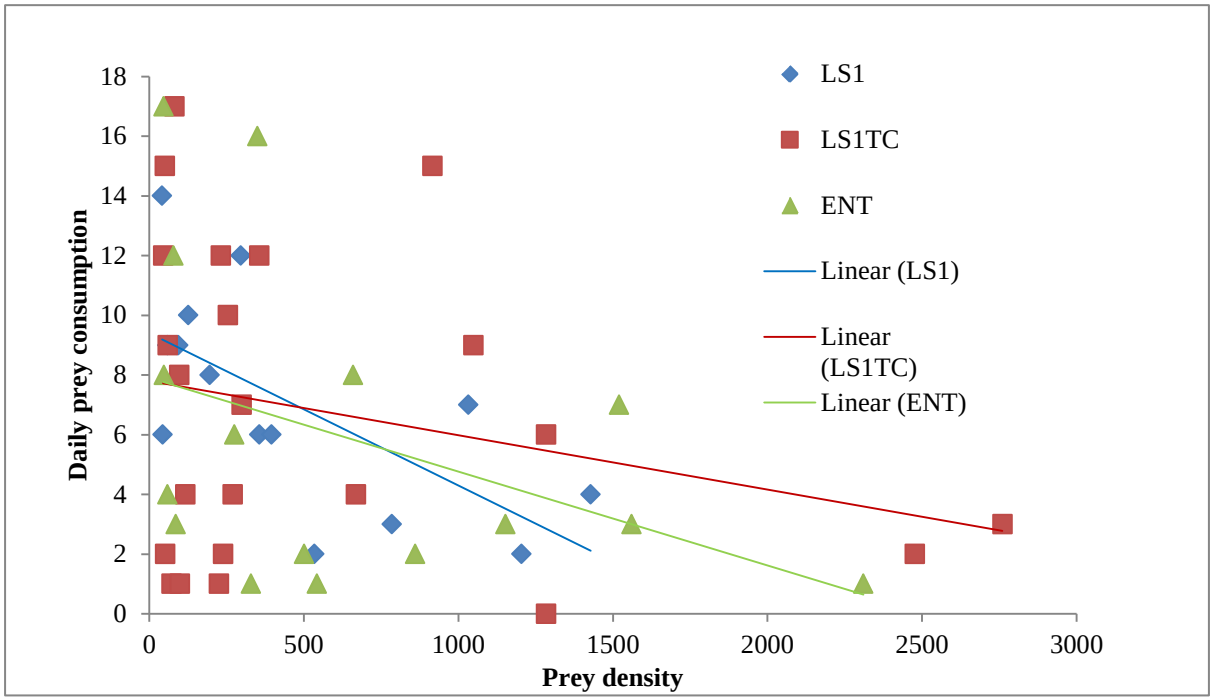

Figure 5.4 – Functional responses of different C. nigritus biotypes and infection types (females only, n= 54). All groups can be seen to decline in voracity at the highest prey densities, but displaying a large amount of variation at any particular density.

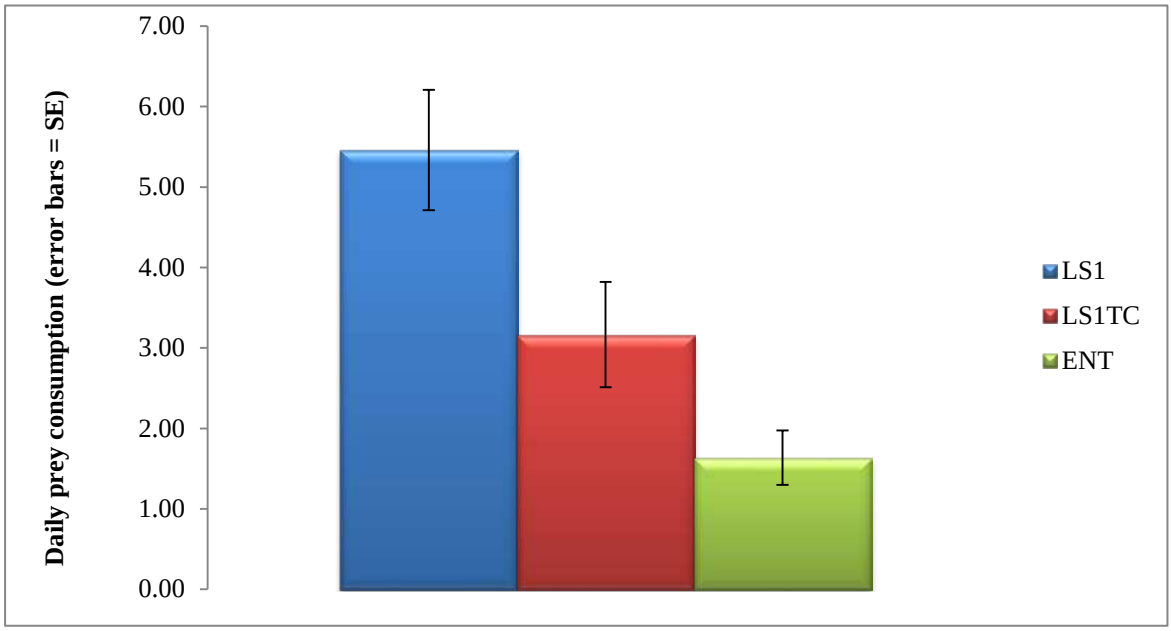

Figure 5.5 – Mean daily consumption of Aspidiotus nerii by male Chilocorus nigritus lines. Differences can be noted between strain of Chilocorus nigritus (ENT and LS1), and between infection types (LS1 and LS1TC). This is represented by non overlapping error bars which give some indication of the significant difference between variances of these groups.

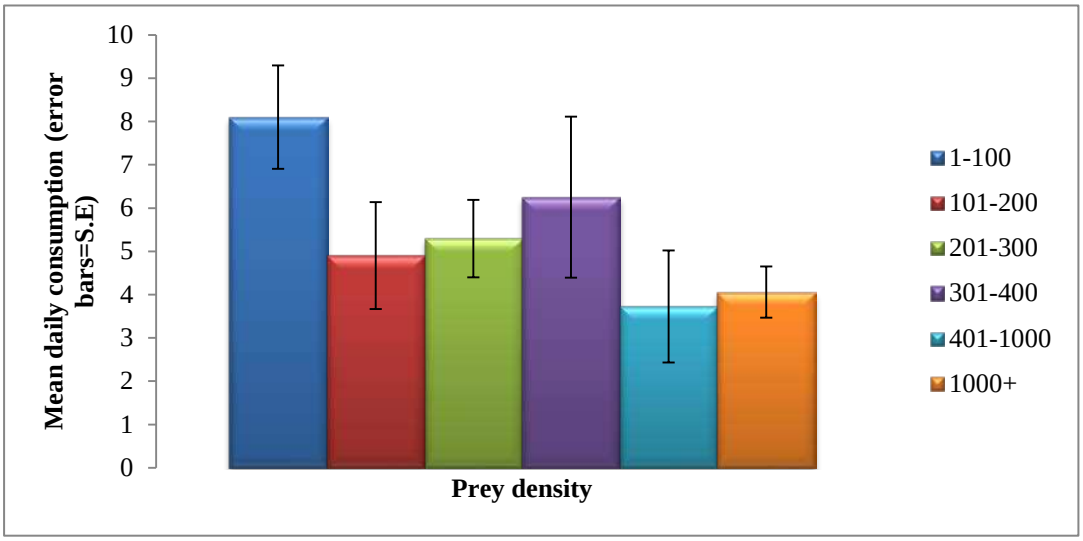

Figure 5.6 – Mean prey consumption of Chilocorus nigritus at different host densities. Higher densities of prey show reduced prey consumption by the predator.

Table 5.1 – Descriptive statistics of Chilocorus nigritus daily consumption rates.

	Mean daily consumption (no. of individual scale insects consumed)	**Standard error of the mean**	**Standard deviation**	**N**
Females	6.611	0.644	4.732	54
Males	3.6	0.481	2.634	30
LS1	7.133	0.910	3.523	28
LS1TC	6.78	1.09	5.2	29
ENT	5.88	1.30	5.19	27

Pooled data from female beetles showed a significant negative correlation between prey density and prey consumption (*F = 7.94, P = 0.006*), also demonstrated graphically in figure 5.3. The decrease in prey consumption at higher densities was also noted when data were sub-divided into categories (see fig.5.6). Prey consumption at densities higher than 400 scale per cage were significantly lower than prey consumption at *A.nerii* densities between 1-100 (400-1000, *d.f. = 1*,*T = 2.29*, *P = 0.033*; 1000+, *d.f. = 1,T = 0.304, P = 0.005*).

When means from both male and females were combined, no significant differences were observed between daily consumption of the three lines of *C. nigritus* (shown in figure 5.4 and figure 5.5) (*d.f. = 2, F = 1.39, P = 0.255*). Similarly, the differences between daily consumption in the three lines were non-significant when tested in just females (*d.f. = 2, F = 0.1, P = 0.909*). However, when only males were tested there was a significant difference between the mean daily consumption in the three biotypes/lines (*d.f. = 2, F = 10.0, P = 0.001*) (see fig.5.5).

Significant differences were noted between male and female beetles (fig 5.3), with females displaying a higher level of prey consumption (*T = -4.95, P < 0.001*) (see table 5.1). Comparison of prey consumption between biotypes LS1 and ENT (represented in fig. 5.4) also resulted in a significant difference when only males were considered (*d.f. = 1, T = 4.36, P*

= *0.001*). Comparisons of infected (LS1) and uninfected beetles (LS1TC) also showed a significant difference in mean prey consumption (*d.f. = 1,T = 3.10, P = 0.009*) (fig.5.5).

5.5 Discussion

Contrary to what might be expected in a coccinellid species, there appeared to be a significant decrease in number of prey consumed at higher densities (figs.5.3 & 5.6). This was especially the case with females, which exhibited a negative linear response to increased host density. In this case, R-squared values for all potential trendline functions were low, but the negative linear function had the highest value of fit (r-squared = *0.139*) and the correlation was deemed significant (*P = 0.006*). This sudden drop in prey consumption may be due to some unexpected factors such as tritrophic effects.

For example, potato plants contain several steroidal glycoalkaloids which contribute to their bitterness and are considered to play a part in pest resistance (Fragoyiannis *et al.* 2001). Whilst the tubers used in this project are an 'un-natural' host for *Aspidiotus nerii*, high levels of phytophagy by scale insects could still stimulate particularly high levels of these glycoalkaloids as a non-specific defence mechanism. Scale insects in particular have been shown to sequester toxic and antiphagant plant compounds as a defence mechanism (Loaiza *et al.* 2007). If sequestered in scale insects, glycoalkaloids could easily inhibit *C. nigritus* ability to consume its prey, and this could explain why at high prey density there is significantly lower prey consumption. This warrants further investigation as comparative quantitative analysis of these plant compounds via GC/MS could provide insight as to whether these compounds are indeed produced in higher quantities when tubers are being attacked. Extracts made from *A.nerii* individuals could also be tested to determine whether plant compounds are sequestered in the insect tissues, and to what extent.

Unlike the females, males showed no significant decrease or effect of density on daily consumption (*F= 0.06, P = 0.802*). It may be that the densities measured in this study recorded represent the upper asymptote of the male functional response, and thus the predicted increase in prey consumption accompanying increases in prey abundance occurs at much lower densities. Thus the data show that males were satiated at much lower prey densities than females, with a mean of 3.6 insects consumed daily, compared to 6.61 in females (see table 5.1). This difference in male and female consumption has been shown several times in predatory coccinellids (Mandour *et al.* 2006, Khan 2010), and has been demonstrated previously in *C. nigritus* (Ponsonby & Copland, 2000 and Omkar & Pervez 2003). The ecological reason for this still remains untested, but could be explained by the high reproductive pressure put on females during oviposition. This reproductive pressure would require higher nutritional input.

Differences observed between ENT and LS1 support the idea that geographically separate biotypes can have different functional responses (at least in the males of the population). This re-enforces findings of other studies which have observed similar differences between biotypes (Perring 2001). More importantly, this highlights intraspecific variation as a variable which might have been neglected in biological control programs. Selecting the most effective biotype (in this case LS1) for the correct agronomic situation might make a considerable difference to the pest suppression they can provide. Additionally, Tauber & Tauber's suggestions (1993) of using a guild of predators from different biotypes seem to be worth consideration in inundative control programs.

Comparisons between LS1 and LS1TC lines also showed a significant difference in daily consumption, demonstrating for the first time that *C. nigritus* strains harbouring endosymbionts can have a higher rate of prey consumption than those that have been treated

to eliminate infection. Potentially, the reason for high prey consumption in infected individuals is to provide additional nutrition for the endosymbionts. This effect that has been observed before in *Aedes* mosquitoes, which have increased metabolic rate and rely on the host for certain amino acids (Evans *et al.* 2009). This is also the first evidence of a strong 'beneficial' effect of endosymbionts in this species (in terms of biocontrol efficacy), where previous experiments have identified deleterious traits associated with infection, such as lower oviposition (Chapter four). Maintenance of infection within the population may therefore be an important consideration for rearing in insectaries. The mechanism behind this variation in voracity could be linked to numerous aspects of the predator's biology such as the olfactory and gustatory responses, visual prey location and ability to digest prey; areas which would benefit from further research.

Males of this species may contribute less to the level of pest suppression, both because of the lower level of daily consumption and the smaller number of males found in the female biased populations. However, these data provide evidence that both lineage and infection status influence the ability of male *C. nigritus* to suppress scale insects. This would especially be the case in ageing insectary cultures, which have been shown to become more male biased with age (Ponsonby 2009). While these differences have only been shown when feeding on *Aspidiotus nerii*, they could potentially explain some of the ambiguities in the literature about prey preference from populations around the world (detailed in Chapter one). It will therefore be important to test if these differences also occur on other prey items.

The findings of this study suggest that high variability in the feeding characteristics could occur in biocontrol agents in general, whether due to different endosymbiont infections, or different genetic background. The importance of these factors in the rearing and release of BCAs is potentially large, as many notable species are known to harbour endosymbionts (Enigl *et al.* 2005, Farrokhi *et al.* 2010, Floate *et al.* 2006) and have high levels of genetic

variation (Szucs *et al.* 2011). Testing for intraspecific variation and endosymbiont infection in economically important predators other than *C. nigritus* could therefore be a valuable venture.

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Chapter 6 – The chemical ecology of *Chilocorus nigritus*.

6.1 Abstract

This study presents preliminary results on the analysis of the volatile and non-volatile compounds present in *Chilocorus nigritus*. GC/MS was used to analyse airborne volatile compounds collected from three biotypes of the beetle to determine any intraspecific variation in the array of compounds. To investigate the presence of non-volatile compounds which could act as contact pheromones, Solid Phase Micro Extraction (SPME) swab samples were collected from insect cuticles and analysed using GC/MS. Direct extraction of reflex blood was also analysed directly by GC/MS in order to look for defence compounds often associated with ladybirds. Samples obtained from headspace collection and from cuticular swabs showed no compounds of interest to pheromone research. However, direct extraction of haemolymph in acetone identified an alkaloid compound similar to (+-) hippodamine. Although the biological function of this compound in *Chilocorus nigritus* has yet to be established, examples of the function of hippodamine in similar species suggest it could serve as an alarm pheromone or as an attractant kairomone.

6.2 Introduction

Biological control agents such as *C. nigritus* are costly to produce and success rates are sometimes erratic, even when conditions are apparently favourable (Ponsonby, 2009). The reasons for such failure are often unclear and much has yet to be discovered of the complex relationships that biocontrol agents have with their environments. A crucial step to enhance our understanding of the behaviour and reproduction of *C. nigritus* is to explore its chemical ecology. So far, the majority of research has been focused on pheromones in pest species themselves (Flint & Doane 1996). However, less work has been done on an alternative, predator-focused research, and the complex interactions between the prey, the predator species and the host plant in which these interactions take place.

Volatile compounds produced by either the host plant or the prey insect can play a role as attractant kairomones for beneficial predators. Kairomone interaction has been suggested in *C. nigritus*, which increases its searching behaviour in the presence of biparental *Aspidiotus nerii*, one of *C. nigritus*' most suitable and essential prey items (Boothe & Ponsonby 2006). It has also been suggested that this attraction could be due to gustatory or olfactory cues. One potential cue is *A.nerii*'s female sex pheromone identified by Einhorn *et al.* (2006), which has already demonstrated its potential use as an attractant for the parasitoids *Aphytis chilensis* (Howard) and *Aphytis melinus* (DeBach) and has been implicated in stimulating oviposition behaviour in the latter species (Lo Pinto *et al.* 2002 and Millar & Hare 1993). Kairomonal attraction has also been observed in similar coccinellid species such as *Coccinella septempunctata.* (L.) Ninkovic *et al.* (2001) found that *C. septempunctata* responded with increased searching behaviour where the aphid species *Rhopalosiphum padi* (L.) was present (or had recently been present) on barley. This particular ladybird species uses methyl salicylate (a plant produced compound) as one if its olfactory cues for prey location (Zhu & Park 2004), thus plant compounds and tri-trophic interactions are also important.

Conspecific interactions between the ladybirds themselves are also of interest in biocontrol. The beneficial ladybirds *C. septempunctata* and *Adalia bipunctata* (Al Abassi, *et al.* 1998) are attracted to 2-isopropyl-3-methoxy pyrazine produced by conspecifics (previously thought only to act as an alarm pheromone). This compound could in theory be used to retain valuable ladybird predators in particular areas, avoiding problems due to predator dispersal. Any such compound would be a great asset where *C. nigritus* is used, as this particular ladybird species will disperse when the density of the prey is low (Ponsonby & Copland 2007). There are a number of other examples of aggregation pheromones in coccinellids, which are becoming of great interest to control of pests (Brown *et al.* 2006, Verheggen *et al.* 2007).

It is also important not to underplay the potential effects of contact pheromones in the communication of this species, namely the non volatile compounds found on the cuticle of the insects. An example is the pheromone (Z)-pentacos-12-ene found in *Cheilomenes sexmaculata* (Fabricius) larvae (Klewer *et al.* 2007). This alkaloid causes adult female ladybirds to avoid laying sites already inhabited by members of the same species, and may therefore be of great significance in the population distribution and dynamics of these predators (Klewer *et al.* 2007).

With the production of any communication chemical there can be variations between individuals from the same species. Reflex bleeding is also another common communication mechanism in coccinellids, whereby the ladybird exudes a filtered form of haemolymph containing a number of alkaloids. The primary role of alkaloids is defence against predation, as alkaloids often act as anti-phagants to deter potential predators (Eisner *et al.* 2002). Intraspecific variation in volumes of reflex fluid produced and the quantity of alkaloids produced have been noted, and may be a result of underlying genetic variation (Holloway *et al.* 1991, Kajita *et al.* 2010). These defence substances can be present across all life stages

including eggs and larvae (Kajita *et al.* 2010) and any absence or deficiencies in alkaloid production could be detrimental to the insect's ability to defend itself. Thus variation within a population may lead to variable fitness in its defensive capacity.

In addition to genetic variability, variation in endosymbiont infection may be able to affect pheromone synthesis and production within a species. Experiments in *Drosophila melanogaster* show that removal of *Wolbachia* reduces mate recognition and discrimination by up to 50% (Koukou *et al.* 2006). This is thought to be in some part due to *Wolbachia* found in the antennal lobe, which may have adapted to alter the behaviour and/or pheromone production of the host. In *Drosophila paulistorum* cuticular hydrocarbons are thought to vary intraspecifically, partially as a result of this behavioural modification by *Wolbachia* (Kim *et al.* 2004, Chao *et al.* 2010). These bacterial species can also inhibit or enhance olfactory responses to cues such as the semiochemical produced by their food source (Peng & Wang 2009, Peng *et al.* 2008). Effects of previously identified endosymbiont infections on *C. nigritus* (Chapter three) are therefore of great interest in terms of the possible impact on somatic pheromone glands and the olfactory system.

The aim of this study was to conduct a preliminary study of volatile and non-volatile compounds produced by *C. nigritus* and *A. nerii,* and to determine any underlying variation between populations of *C. nigritus* biotypes, and further investigate the predator's chemical ecology.

6.3 Methodology

Table 6.1. Experimental groups (prey-predator-host plant) selected for the extraction of volatiles, including control groups.

Group	Description
1-CONT	Control – Insect chamber run empty for the whole period.
2-TUBERS	Potato tubers only.
3-DAMAGE	Tubers mechanically damaged with entomology pin.
4-UPSCAL	Uniparental *A.nerii* on tubers (25 tubers)
5-BPSCAL	Biparental *A.nerii* on tubers (25 tubers)
6-ORIG*	Long term captive *C. nigritus* from Wyebugs ltd. (LS1)
7-ORICUR*	Long term captive *C. nigritus* from Wyebugs ltd. previously treated with tetracycline hydroxide to cure bacterial infection. (LS1TC)
8-INDIAN*	Recently acquired wild caught *C. nigritus* from Bangalore, India. (IND)
9-ORIMAL*	Wyebugs ltd. strain *C. nigritus* males. (LS1)
10-ORIFE *	Wyebugs ltd. strain *C. nigritus* females. (LS1)
11-ORISY	Wyebugs ltd. mixed sex *C. nigritus* feeding only on syrup. (LS1)

* Indicates that the group was being fed on 25 tubers infested with biparental *A.nerii.*

6.3.1 Insect Cultures

Potato tubers (cv Desiree) were used as the host plant to rear *A. nerii*, and also to determine any potential plant related attractants. Uniparental *A.nerii* was also used in conjunction with biparental *A.nerii* for comparison. Intraspecific variation in the compounds produced by *C. nigritus* was tested for by comparing insects from different biotypes and infection types. In addition to direct GC/MS analysis of the volatiles, Solid Phase Micro Extraction (SPME) was used to collect non-volatiles and cuticular samples from the insects; these samples were also analysed using GC/MS. The insects used for the pheromone extractions were of specific ages where reproductive pheromones were likely to be produced. *C. nigritus* populations were at least 8 weeks old and contained approximately 30 beetles. *A. nerii* cultures were 7 weeks old.

6.3.2 Collection of volatiles

The apparatus shown in Figure 6.1 was set up and run empty for 1 week after acid washing in HCl and multiple rinses of distilled H_2O. The collection medium used was Poropak Q™, of which approximately 500 mg were packed into each tube with glass wool. Tubes were then cleaned of unwanted volatiles using dichloromethane, and dried under slow flowing nitrogen. They were then heat treated at 210-220°C, under nitrogen flow, for 2.5 hours. Tubes were stored at room temperature and sealed with aluminium foil. Table 6.1 shows the experimental groups selected and a brief description of the characteristics of each group. Each of the groups was run through the extraction process for 12 days at room temperature at a flow rate of 1-2 L/min. Extraction tubes were then sealed again with aluminium foil.

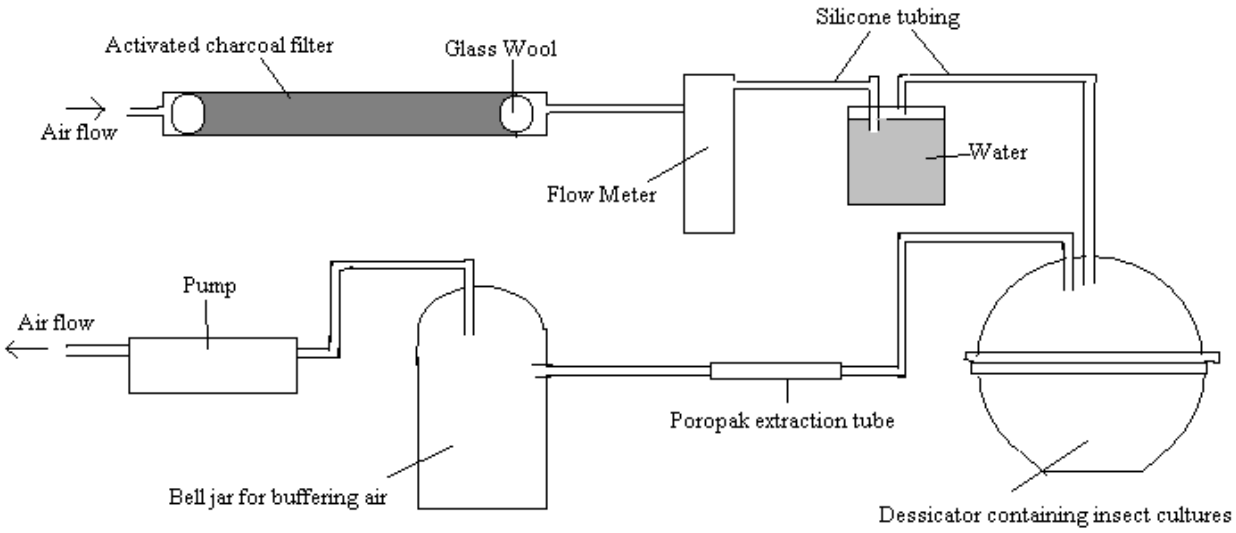

Figure 6.1 – Pheromone collection apparatus. Air was passed through the system, over the Poropak adsorbant in order to collect volatile compounds present in the headspace.

6.3.3 Volatile Analysis

Volatiles adsorbed onto the Poropak were eluted with pesticide residue grade hexane. Samples were then analysed on a Hewlett Packard 5890 GC (using a non-polar DB-5 30m x 0.25 mm x 0.25 μm film thickness) and Carlo Erba QMD1000 Mass spectrometer (70eV). The temperature program used was 50˚C for 5 minutes then ramped up at 5˚C/minutes up to 250˚C, then raised to 300˚C at 20˚C/minutes. The injection port was held at 250˚C and 1μl of each eluted sample was injected. The chromatogram results are presented in Figure 6.2. For comparative purposes, the same samples were also analysed on a Fisons Instruments MD800 and GC8000 containing a DB-5 (30m x 0.25 mm x 0.25 μm film thickness) at 70eV. All temperature parameters were the same as the experiments run on the HP and Carlo Erba equipment. Similar results were obtained with the Fisons Instruments apparatus.

6.3.4 Solid Phase Micro-Extraction (SPME)

SPME was used in two ways to explore the presence of contact (non-volatile) pheromones on the insects. On the first method, SPME fibres were used on the cuticles of *C. nigritus*; 100μm polydimethylsiloxane (PDMS) fibres from Sigma-Aldrich® were rubbed for 30 seconds across the elytra (protective wing case) of a single insect. The second method involved using PDMS fibres for direct sampling of beetle haemolymph (tissue fluid). Haemolymph samples were prepared as follows; male, female and mixed sex *C. nigritus* (the latter consisting of 4 individuals, 2 male and 2 female) were agitated to produce reflux bleeding (haemolymph). 1μl of haemolymph from each beetle was mixed with 200 μl of acetone; dichloromethane was initially used, but was subsequently discarded in favour of acetone due to solubility problems. PDMS fibres were loaded with the acetone solution and then injected into the GC port.

GC/MS analyses of the samples collected by both methods were performed on a Hewlett Packard 5890 GC (using a non-polar DB-5 30m x 0.25 mm x 0.25 μm film thickness) and Carlo Erba QMD1000 Mass spectrometer (70eV). Each fibre was heat treated in the GC port at 250˚C to desorb any contaminants prior to cuticle/haemolymph sampling. The temperature program used was 50˚C for 5 minutes then ramped up at 5˚C/minutes up to 250˚C, then raised to 300˚C at 20˚C/minutes. The GC port was held at 250˚C.

6.4 Results

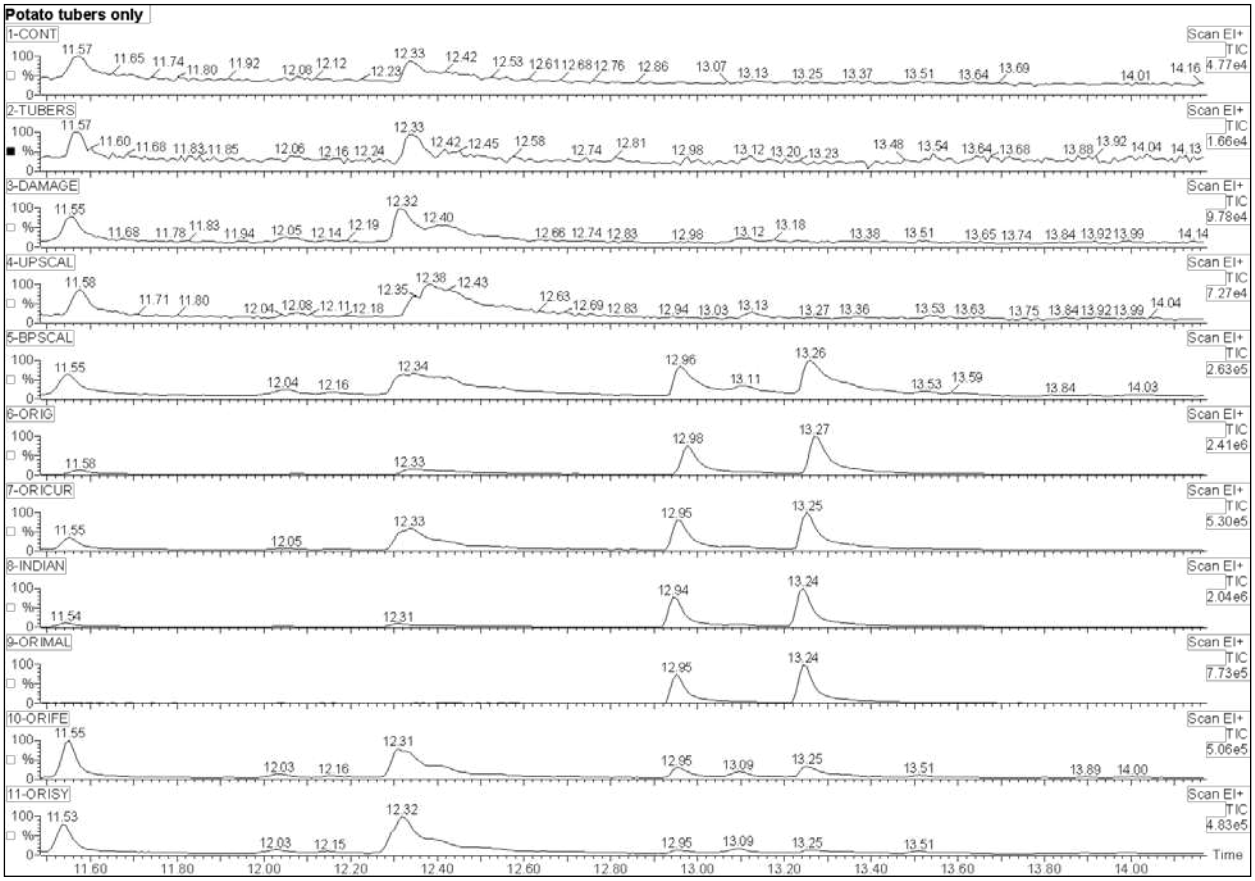

Figure 6.2. Chromatograms for all the sample groups. This figure shows volatile headspace components were the same across all groups.

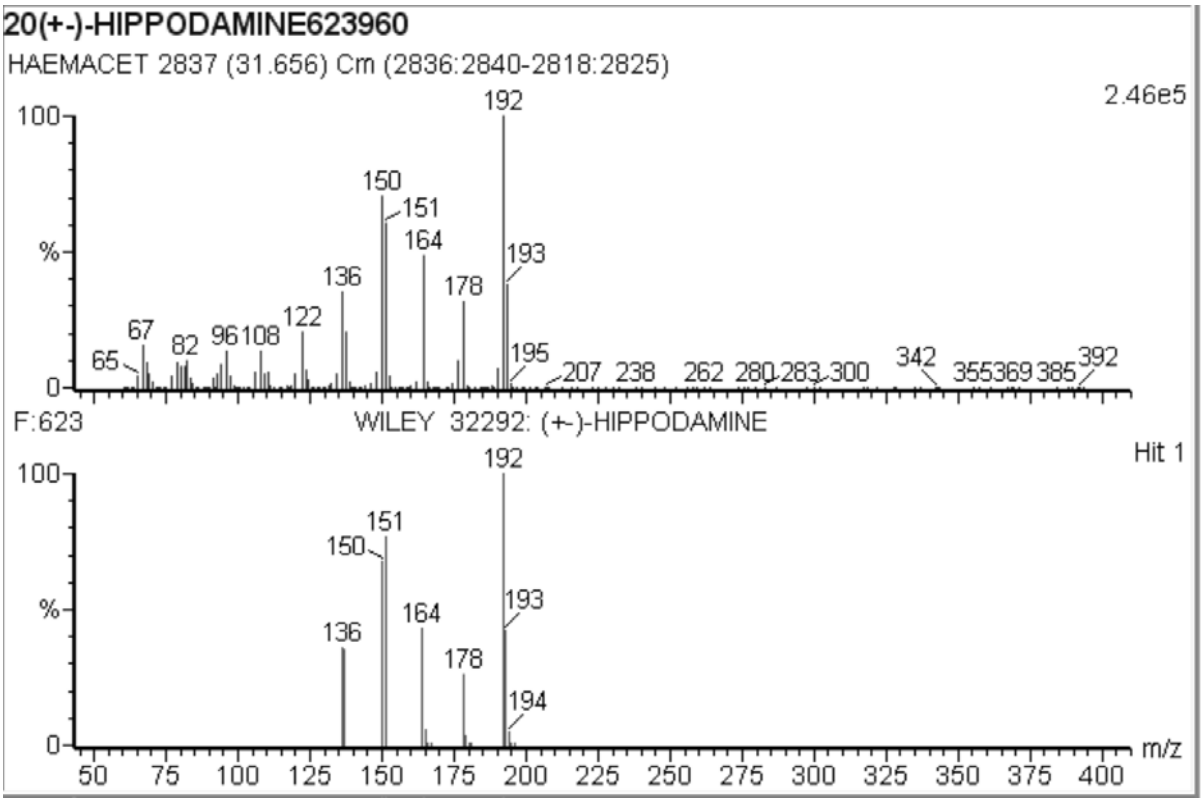

Figure 6.3. Mass spectra of hippodamine from mass spectrometry library (bottom) and unknown compound from C. nigritus haemolymph (top). Hippodamine provided the top match to the unknown spectrum, indicating high similarity to the library compound.

6.5 Discussion

6.5.1 Volatile analysis

GC/MS results for all the samples analysed are shown in Figure 6.2. Most of the compounds extracted on Poropak appeared to be common to all samples including control. This suggests that there were few volatile compounds produced in any significant quantity by any of the insect (or plant) samples. However, there was one exception, 2- diethylbenzene (Rf 12.95, 13.25), which was found in all samples except the controls and uniparental scale group; 1,2-Diethylbenzene has also been reported in a similar pest, *Hyadaphis tataricae* (Aizenberg)(Hedin *et al.* 1999). Thus, 1,2-diethylbenzene may be of interest in terms of its biological function in

the *C. nigritus*/*A.nerii* relationship. However, this result must be treated with caution, since degradation of Poropak adsorbent produces a similar compound, 1,2- diacetylbenzene (Sturaro *et al.* 1991); further research will be needed to ascertain whether the compound is in fact an insect pheromone or an artifact due to the sample collection method. No differences between the compounds produced by different biotypes and infection types were detected.

6.5.2 Solid Phase Micro Extraction (SPME) analysis

GC/MS analysis of the SPME samples obtained from the elytra of *C. nigritus* showed no differences in the compounds identified with respect to those found in control samples. This suggests the absence of cuticular hydrocarbons used as pheromones amongst *C. nigritus* individuals. However, GC/MS analysis of the haemolymph extracts suggests the presence of a compound similar to (+-) hippodamine (see fig. 6.3). Hippodamine has been reported in similar ladybirds such as *Hippodamia convergens* (Guerin-Meneville)(Tursch *et al.* 1974), and *Chauliognathus pulchellus* (Macleay) (Moore & Brown 1978). In the case of *Hippodamia convergens,* hippodamine is thought to act as an alarm pheromone. Moreover, two other members of the *Chilocorus* genus species, *C. renipustulatus* (Scriba) and *C. Cacti* (L.), are known to produce similar alkaloids, nominally Chilocorine A, B, C and D (Laurent *et al.* 2002, Huang *et al.* 1998, Shi *et al.* 1995 & McCormick *et al.* 1994). In addition to its alarm pheromone activity, hippodamine can act as an attractant kairomone in *Dinocampus coccinellae* (Schrank)(a ladybird parasitoid), promoting oviposition (Al Abassi *et al.* 2001).

The semi quantitative nature of GC/MS provides a limited comparison for differences between hippodamine production between individuals. However, it would be beneficial to quantify any intraspecific variation in the amount of reflex blood produced, and the concentration of alkaloids in the fluid. This could be achieved in the future by methods similar to those used by Holloway *et al.* (1991); a study which identified significant amounts of

variation in the both the volume and the concentration of coccinelline found in the reflex blood of *Coccinella septempunctata*. Such variations in *C. nigritus* could be responsible for further bionomic variation in this species (see previous chapters). This could especially be the case under the influence of endosymbionts, which have already shown to either parasitize (see Chapter four) or enhance (see Chapter five) the resources of the beetle.

Establishing the structure and biological function of this alkaloid compound will be the next step to enhance our understanding of the behaviour and reproduction of *C. nigritus*.. The fact that the compound is present in the haemolymph may have an effect when trying to maintain healthy populations of ladybirds for use as biocontrol agents, since their haemolymph may attract harmful parasitoids (Al Abassi *et al.* 2001). Additionally the role of this alkaloid as a defence chemical or alarm pheromone can alter behaviours such as oviposition (Klewer *et al.* 2007) and both intraspecific and interspecific cannibalism (Kajita *et al.* 2010), thus having a dramatic influence on the insect's use as a biocontrol agent.

The precise biological function of the compound found in *C. nigritus* has yet to be established, but could be ascertained through electrophysiological recordings of antennal responses, as well as behavioural assays such as olfactometry. Further analysis of synthesised hippodamine isomers, using the already published synthetic route (Newton *et al.* 2008) will be needed to ascertain the exact nature of this hippodamine compound. A comparative study of the compound found in *C. nigritus* with those found in *C. renipustulatus* and *C. cacti* for example, could be useful in the full identification of the *C. nigritus* alkaloid. The experiment established that there were no detectable differences in volatile production between the various biotypes, but did provide an insight into these novel aspects of the chemical ecology of the species. The findings provide the potential for a new avenue of research into the defence chemicals produced, and variation within these compounds, which may have a dramatic effect on the predator's ecology.

6.6 References

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Chapter 7 – The efficacy of *Chilocorus nigritus* biotypes and infection types in ornamental glasshouses.

7.1 Abstract

Despite success in wild field releases, the performance of *Chilocorus nigritus* has been inconsistent in ornamental glasshouse situations. Endosymbionts and genetic variation have been shown to cause significantly variability in the prey consumption and fecundity of *Chilocorus nigritus*. It has been argued that variability allows a biocontrol agent to be flexible in a variety of agronomic situations, such as those encountered in ornamental glasshouses. Two biotypes (LS1 and ENT) and one uninfected sub-line of the LS1 biotype (LS1TC) were released in the Palm House and Temperate House at Royal Botanical Gardens, Kew, in order to test the efficacy of a guild of biotypes on scale insect control.

Scale insects were monitored on twenty plants in the two glasshouses over a period of eighteen months from April 2009 until October 2010. Larval *C. nigritus* from the three strains were released in April 2010. Reduction in three species of scale insect was observed during the period of introduction, including *Abgrallaspis cyanophylii*, *Pinnaspis buxi* and *Diaspis boisduvallii*. *Steinernema feltiae* combined with fungal entomopathogen *Verticillium lecanii* also showed a significant amount of scale suppression on *D.boisduvalii.* No significant effects of beetle introductions were noted on soft scale species, indicating a strong preference for the Diaspididae. This experiment showed a promising level of pest suppression, and further highlights the need for prey preference data in *C. nigritus*.

7.2 Introduction

Botanical glasshouses usually house a large number of rare plant species from various world locations, which despite improvements to plant import legislation (Forestry Commission of Great Britain 2009) are often imported alongside a number of insect pests (Reed 2011). As a result, glasshouses can have great difficulty in preventing and controlling new invasive pests, often due to conflicts between issues of plant protection and public safety. Botanical glasshouses are often frequented by the public, and in addition, usually house a number of animals, both vertebrate and invertebrate. As such, strong applications of insecticides are not advised, and glasshouses tend to use mild 'over the counter' pesticides which are often ineffective. As a result there is a great demand for effective biological control wherever possible.

In the United Kingdom, *C. nigritus* is used as a scale insect predator in glasshouse environments. Despite the relatively controlled nature of a glasshouse, the efficacy of *Chilocorus nigritus* as a means of controlling scale insect pests has been found to be somewhat variable (Ponsonby, 1995). Even under climatically similar conditions, establishment and pest suppression ability of *C. nigritus* can vary significantly (Ponsonby 1995). This variability is potentially due to abiotic factors such as pesticide usage, as well as numerous biotic elements (host plant, type and abundance of prey items, predation, competition etc.). Ornamental glasshouses provide a particularly heterogeneous environment, with many host plants, prey types and microclimates, and thus require an adaptable biocontrol agent, capable of dealing with changing circumstances. It has been suggested that biocontrol introductions should consist of a variety of biotypes/phenotypes in order for the predator population to be adaptable to a variety of agronomic situations (Tauber & Tauber 1993). The homogeneous gene pool highlighted in lab cultures (Chapter two) means that captive bred *C. nigritus* are likely to have fewer potential phenotypes, and as a population are potentially less capable of

dealing with these changing circumstances. This homogeneity could be one of the factors limiting the success of this important scale predator. However, whilst genetic variation within a population remains low there is still significant variability between populations, highlighted by barcoding data (Chapter two). Thus there is potential to introduce several different genotypes to provide a wider range of bionomic characteristics.

In addition to genetic differences, different endosymbiont infection types have been observed depending on the individual and population of *C. nigritus* (Chapter three). Differing infection type therefore provides an additional source of variation within the population, and another range of potential phenotypes (e.g. tetracycline treated individuals display higher oviposition). It has been suggested that the manipulation of male killing bacteria in inundative and classical biocontrol is a useful and undervalued resource, and as such has recently gained a lot of attention (Floate *et al.* 2006, Zindel *et al.* 2011, Ahantarig & Kittayapong 2011). Despite the interest in this area of research, endosymbionts are largely disregarded in the assessment and risk assessment of biocontrol programs (Zindel *et al.* 2011).

Thus far in the current thesis it has been demonstrated that both endosymbiont infection and genetics of *C. nigritus* vary geographically, and also that endosymbiont infection may have significant effects on host fecundity and functional response; characteristics essential to successful pest suppression. Ultimately this project aims to establish how these factors could hinder/assist the application of this species as a biocontrol agent. Once insects had been sourced from several localities as well as being treated in order to create a line of insects with low endosymbiont infection, there was an opportunity to test these different insects in a field environment. This chapter details the release of two *C. nigritus* cultures (ENT and LS1) as well as one uninfected sub-line derived from LS1 (named LS1TC) in glasshouses at Royal Botanical Gardens, Kew, London, over the field seasons of 2009 and 2010. Additionally, larval *C. nigritus* were tested for their ability to establish and efficacy in controlling scale populations.

Recent studies have suggested that larvae can consume all life stages of scale insect (Ponsonby & Copland 2000), yet there have been no field experiments to test larval *C. nigritus* in glasshouses since then. As such, the different biotypes were released as 1st , 2nd and 3rd instars. The aim was to monitor and compare the establishment of the three larval 'biotypes', and also determine whether combining biotypes such as these would provide adequate control of scale pests.

7.3 Methodology

7.3.1 Scale insect monitoring

Initially several visits were made in Spring 2008 to the Temperate House and Palm House in Royal Botanical Gardens (RBG), Kew, London. A thorough inspection and consultation with glasshouse staff led to the selection of ten plants in each glasshouse suitable for the study of scale insect populations, and release of biocontrol agents. Criteria for suitable plants included size, accessibility, and either the presence of a substantial scale insect population, or a recent history of infestation.

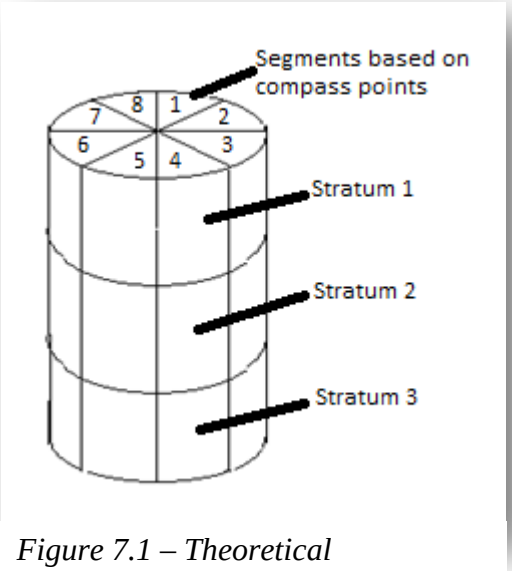

Figure 7.1 – Theoretical partitioning of plant strata

No control plants were available for this study due to the nature of heterogeneous plant collections. Whilst making assumptions about population change of insects without a control group could be precarious, it is considered to be the only way to assess the influence of classical biocontrol agents, and has been used to do so in a number of cases (Debach & Rosen

1991). Thus it was decided to be an unfavourable but necessary concession in the experimental design.

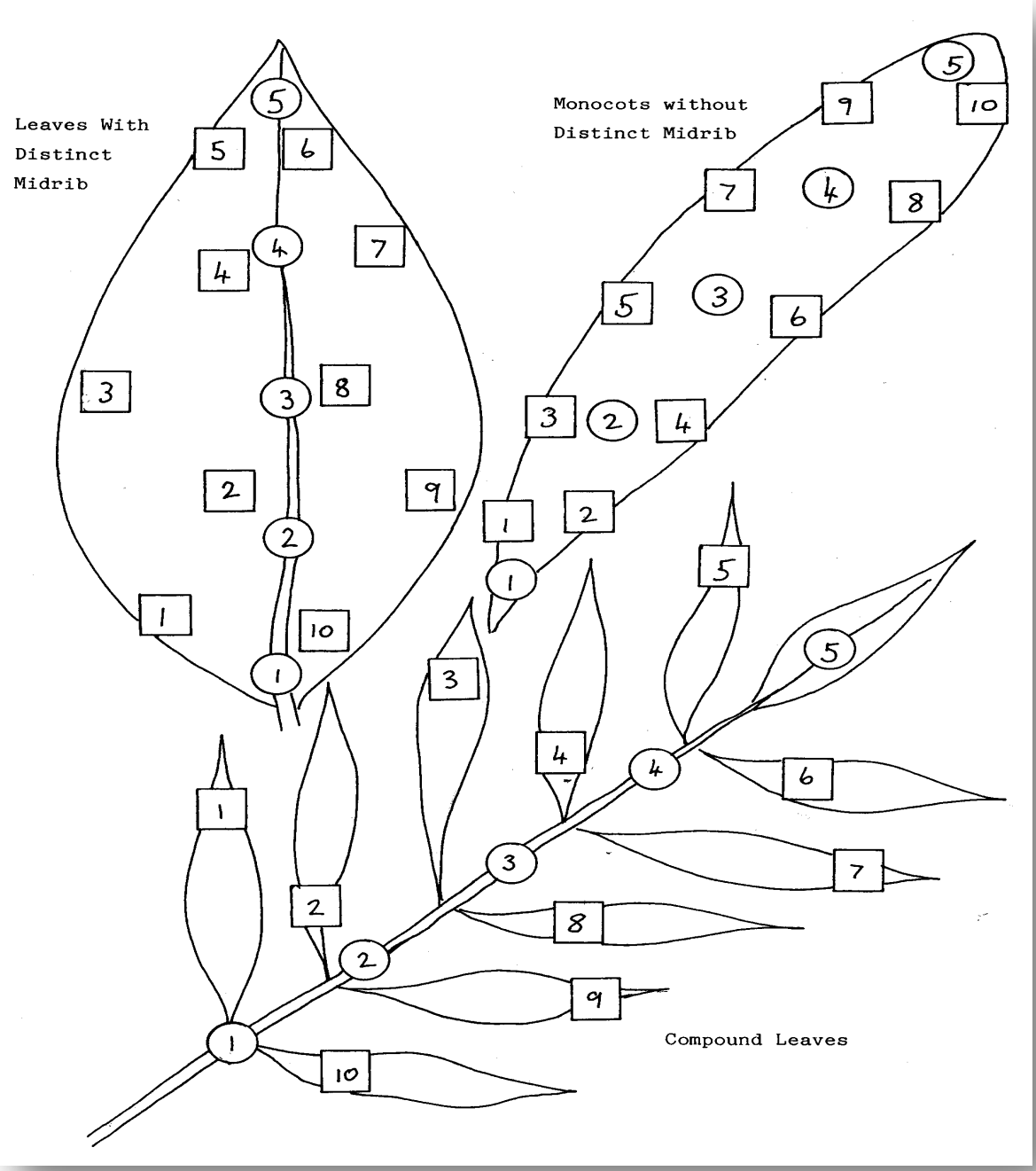

Figure 7.2 –Site selection points on different leaf morphologies (Ponsonby 1995)

Scale insect abundance was then measured monthly from April 2009 through to October 2010 on all twenty plants. This was performed by a method of random stratified sampling detailed in Ponsonby 1995. This method provided a uniform method for quantifying and comparing scale insects of plants of varying size and morphology. The height of each plant was divided into three strata of approximately 80cm (delineated by using masking tape at 80cm intervals up the accessible parts of the plant). Smaller plants sometimes filled only one or two of these strata. Each stratum was further sub-divided into three additional sub-strata, and 8 segments based on compass points. These theoretical segment divisions (shown in figure 7.1) provided the basis for the random stratified sampling method.

Within each major stratum, ten positions on the plant were picked by using sets of random numbers to select first a sub-stratum (numbered 1-3), followed by a positional segment based on compass direction (numbered 1-8) (see fig 7.1). These ten positions were also selected on alternating side of the leaf. In order to select randomly on individual leaves, a further random number was used to select for position on the leaf (fig.7.2). Once a position was selected, total numbers of adult scale (A), intermediate stages (I) and crawlers (C) were recorded on the stem, midrib, interveinal tissues and lateral veins where applicable (table 9.1 see appendix). A standard area of the leaf was examined by counting all scale insects within the area of a standard entomology eyeglass (24mm diameter). Temperature and humidity were measured using a combination of data loggers (TinyTag Talk data logger, Gemini Ltd., West Sussex, UK), and temperature probes already in use by RBG, Kew. Pesticide usage and other biocontrol releases were also recorded as a matter of course by glasshouse staff.

7.3.2 Biocontrol release program

An augmentative release program using the three strains/infection types of *C. nigritus* (ENT, LS1 and LS1TC) began in March 2010, and continued through until September of 2010. 1st, 2nd and 3rd instar *C. nigritus* were placed strategically on plants where scale insect populations were high and had remained prevalent over the period of monitoring (see table.7.1). Larvae were introduced directly onto areas of highest scale insect density on filter paper, or directly applied with a paintbrush. The release schedule occurred every month directly after scale insects had been counted on the host plant. During the release program (and before fresh larvae had been released) recordings were made of any signs of establishment from previous releases. This included looking for larvae, pupae/exuviae, and adults.

At the end of the study, scale insect samples were taken and after clearing in potassium hydroxide, were run through increasing concentrations of ethanol to dehydrate the samples. Insects were then stained with acid fuchsin and mounted on slides with Canada balsam. Scale insects were identified to at least the level of genus and to species level where possible.

Table 7.1 - Host plant selection at Kew Gardens

Glasshouse	Location	Host Plant	Scale insect	Number of *C.nigritus* released per month
Temperate House	1	*Encephalartos arenarius*	*Saissetia sp.*	None
Temperate House	2	*Encephalartos trispinosus*	*Saissetia sp.*	**45 individuals (e.g. 15 x LS1, ENT and LS1TC)**
Temperate House	3	*Strelitzia reginae*	*Unidentified white diaspid*	None
Temperate House	4	*Agyrodendron peralatum*	*Saissetia sp.*	None
Temperate House	5	*Cordyline fruticosa 1*	*Saissetia coffeae*	**45 individuals (e.g. 15 x LS1, ENT and LS1TC)**
Temperate House	6	*Cordyline fruticosa 2*	*Saissetia coffeae*	**45 individuals (e.g. 15 x LS1, ENT and LS1TC)**
Temperate House	7	*Cordyline fruticosa 3*	*Saissetia coffeae*	None
Temperate House	8	*Unknown low lying fern*	*Saissetia sp.*	None
Temperate House	9	*Syzgium paniculatum*	*Saissetia sp.*	None
Temperate House	10	*Leucodendron argenteum*	*Abgrallaspis sp.*	**90 individuals (e.g. 30 x LS1, ENT and LS1TC)**
Palm House	11	*Pandanus sp.*	*Diaspis biosduvallii*	**90 individuals (e.g. 30 x LS1, ENT and LS1TC)**
Palm House	12	*Pandanus vandermeeschi*	*Diaspis biosduvallii*	**90 individuals (e.g. 30 x LS1, ENT and LS1TC)**
Palm House	13	*Rhapis excelsa 1*	*Pinnaspis sp.*	**45 individuals (e.g. 15 x LS1, ENT and LS1TC)**
Palm House	14	*Rhapis excelsa 2*	*Pinnaspis sp.*	**45 individuals (e.g. 15 x LS1, ENT and LS1TC)**
Palm House	15	*Clerodendrum nutans 1*	*Saissetia coffeae*	**15 individuals (e.g. 5 x LS1, ENT and LS1TC)**
Palm House	16	*Clerodendrum nutans 2*	*Saissetia coffeae*	**15 individuals (e.g. 5 x LS1, ENT and LS1TC)**
Palm House	17	*Barringtonia racemosa*	*Saissetia sp.*	None
Palm House	18	*Oxyanthus pyriformis*	*Coccus hesperidum*	None
Palm House	19	*Tambourissa purpurea*	*Saissetia sp.*	None
Palm House	20	*Dictyosperma album*	*Diaspis biosduvallii*	None

7.3.3 Data analysis

Due to the sedentary nature and low dispersal ability of scale insects, their spatial distribution on host plants tends to be clustered (Kim & McPheron 1993). When using random sampling methods this leads to high levels of variation, and results do not display a normal distribution. Clustered distribution was confirmed on all populations of scale found at Kew Gardens by performing a linear regression of mean scale counts vs. standard deviations for each plant and each stratum within that plant. All populations showed a significant linear correlation between mean and standard deviation ($P<0.001$-$P<0.0000000$), confirming the assumption that scale insect are distributed contagiously.

As a result of the non-normal distribution, graphs were created using the log (x+1) transformation of the means at each sampling time. Untransformed data were treated with non-parametric statistics. Comparisons between the April-September periods of 2009 and 2010 (before and after *C. nigritus*' introduction) were made using the Kruskal-Wallis test for differences in distributions. This method makes no assumption of independence between observations. This was performed both on the total combined number of individuals found on the plant, and on numbers located on individual strata and from particular life stages. In addition to biological interactions, effects of temperature and humidity were also tested by regressing monthly climatic means against mean scale numbers observed. All statistical analyses were performed in Minitab 16.

7.4 Results

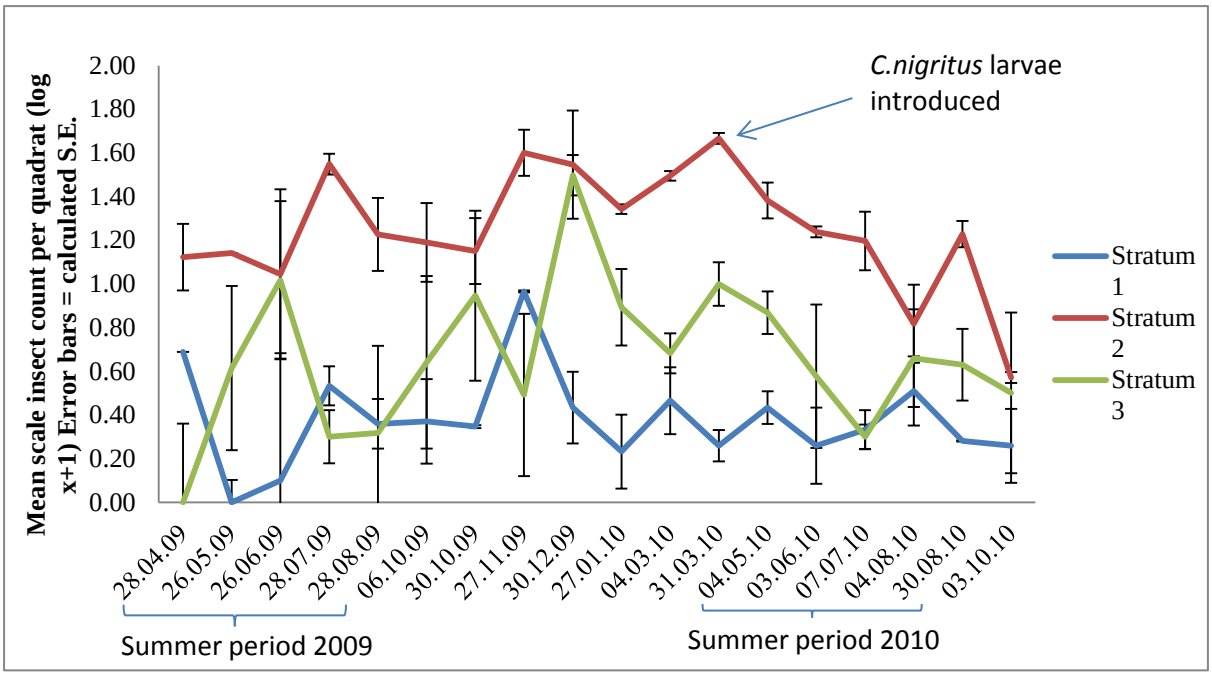

Figure 7.4 – Abgrallaspis sp. monitored on Leucadendron argenteum (Temperate House, Royal Botanical Gardens, Kew). Scale population appears to decrease after introduction of predators.

7.4.1 *Leucadendron argenteum* (L.) (fig 7.5)

Figure 7.5 – Abgrallaspis sp. on Leucadendron argenteum (Temperate House) (second sampling stratum)

Comparison of 2009 and 2010 summer periods (April-September) showed a significant difference between the mean number of *Abgrallaspis sp.* found before and after *C. nigritus* larvae were introduced. *Abgrallaspis sp.* populations were significantly suppressed after the augmentative release had begun (fig.7.4), with significant reductions in the numbers of intermediate stages (*d.f. =1, H = 31.72, P<0.001*) and adults (*d.f. = 1, H = 7.79, P = 0.005*) found in the second stratum (where scale density was highest, fig.7.5). There was no significant correlation between the number of insects observed and mean temperature (*P=0.949*) or humidity (*P=0.089*).

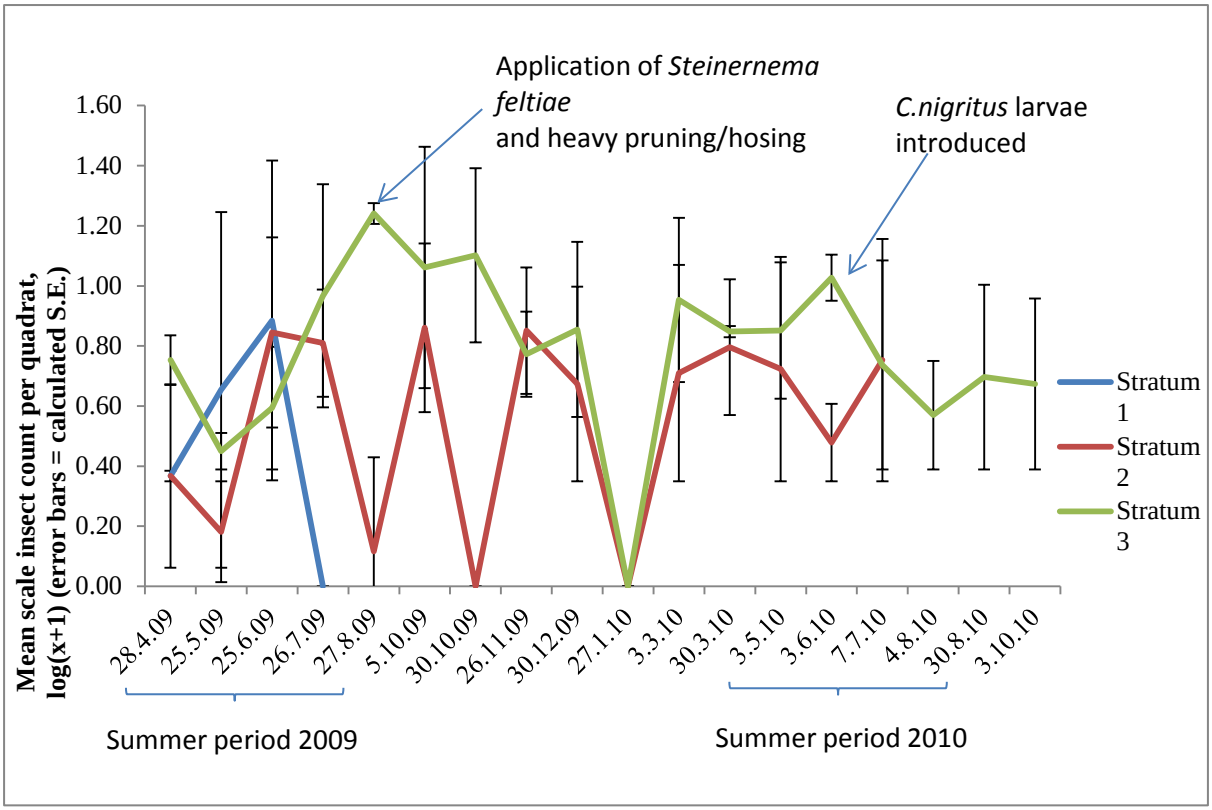

Figure 7.6 – Diaspis boisduvallii (Boisduval) *population monitored on Pandanus vandermeeschii (Palm House, Royal Botanical Gardens, Kew)*

7.4.2 *Pandanus vandermeeschii* (Balf) (Fig. 7.7)

After comparison of the summer periods of 2009 and 2010 there was a significant change in *Diaspis boisduvallii* (Signoret) population numbers in the intermediate stages ($H = 6.16$, $d.f. = 1$, $P = 0.013$). Other life stages showed no significant changes. Significant drops in scale population also occurred in the months following application of the nematode *Steinernema feltiae* (Filipjev) and heavy pruning carried out by glasshouse staff (fig.7.6). There was no significant correlation of scale insect population size with temperature ($P = 0.484$) or humidity ($P = 0.191$).

Figure 7.7 – Pandanus vandermeeschii infested with D.boisduvallii (Palm House)

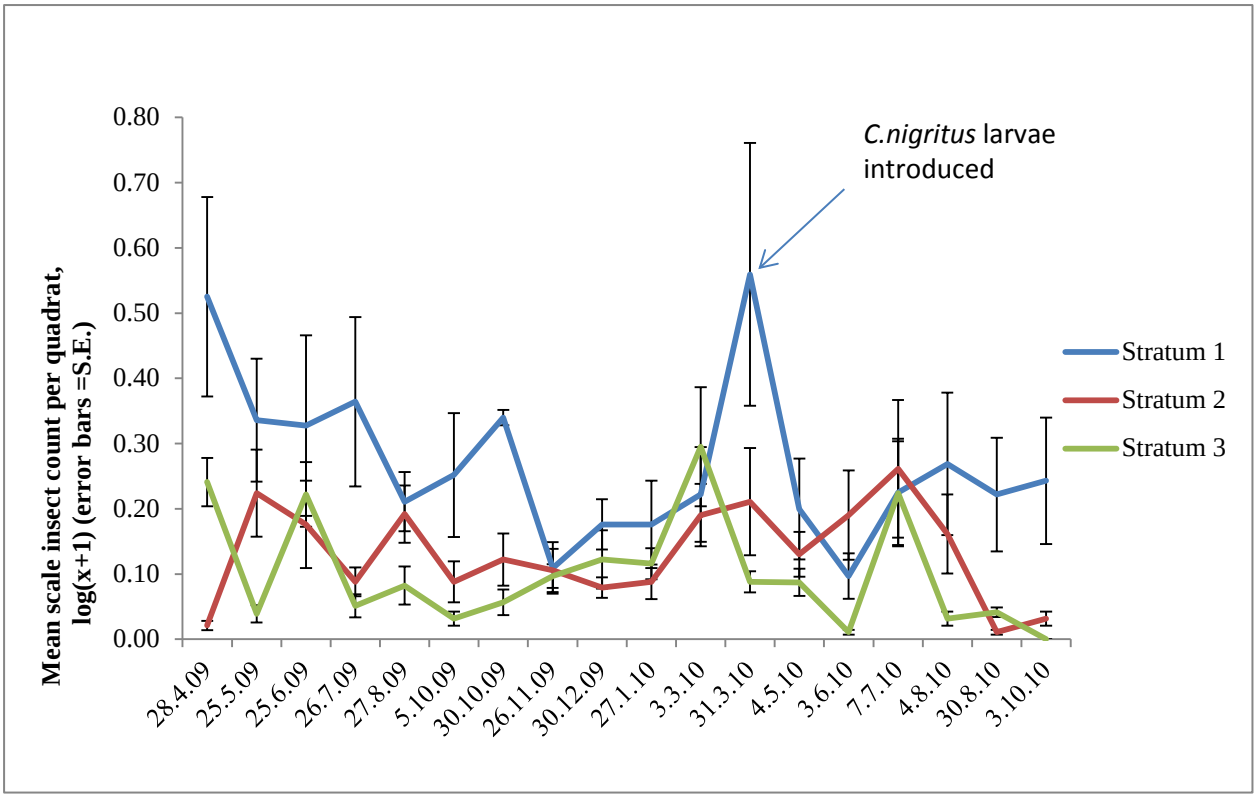

Figure 7.8 – Pinnaspis sp. Population monitored on Rhapis excelsa (1) (Palm House, Royal Botanical Gardens, Kew). Stratum 1 in particular appears to have lower numbers of scale post biocontrol introduction.

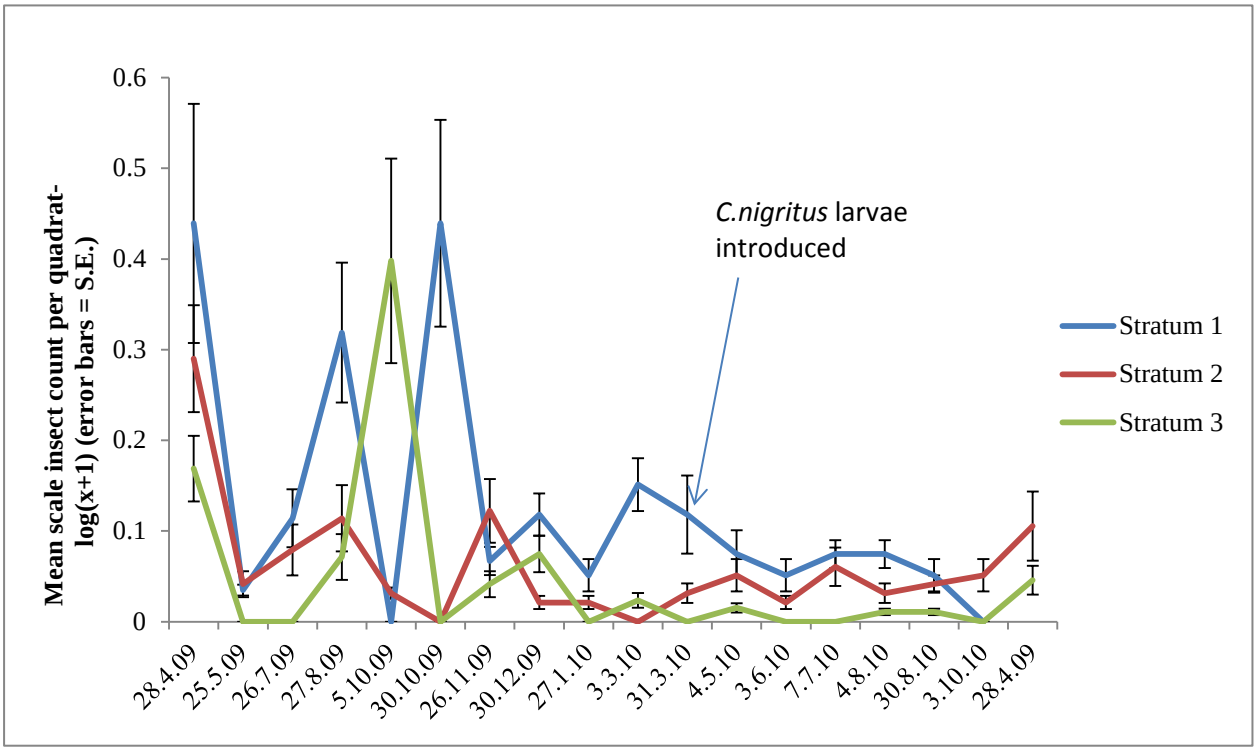

Figure 7.9 – Pinnaspis sp. population monitored on Rhapis excelsa (2) (Palm House, Royal Botanical Gardens, Kew). Scale populations appear to be smaller post-release.

7.4.3 *Rhapis excela* (Thunb.)

(Fig.7.10)

Figure 7.10 – Rhapis excelsa (Palm House)

Rhapis excelsa 1 - There was a significant difference observed between the summer periods of 2009 and 2010 (*d.f. = 1, H = 1.03, P = 0.039*), indicating a reduction in the mean number of intermediate *Pinnaspis buxi* (Bouche) instars (Fig 7.8, instars shown in fig. 7.11). Regressions between abiotic factors yielded no significant correlations with temperature (*P = 0.888*) or humidity (*P = 0.197*).

Figure 7.11 – Pinnaspis buxi on Rhapis excelsa (Palm House)

Rhapis excelsa 2 – No significant difference was seen between the two summer periods before and after release of larvae despite an apparent decline (fig. 7.9). The group closest to undergoing a significant change during this period was the adult imagos (*H = 3.78. d.f. = 1, P = 0.052*), but when strata were tested separately there were still no statistical significances. Additionally no significant correlations were observed with abiotic factors, temperature (*P = 0.855*) or humidity (*P = 0.110*).

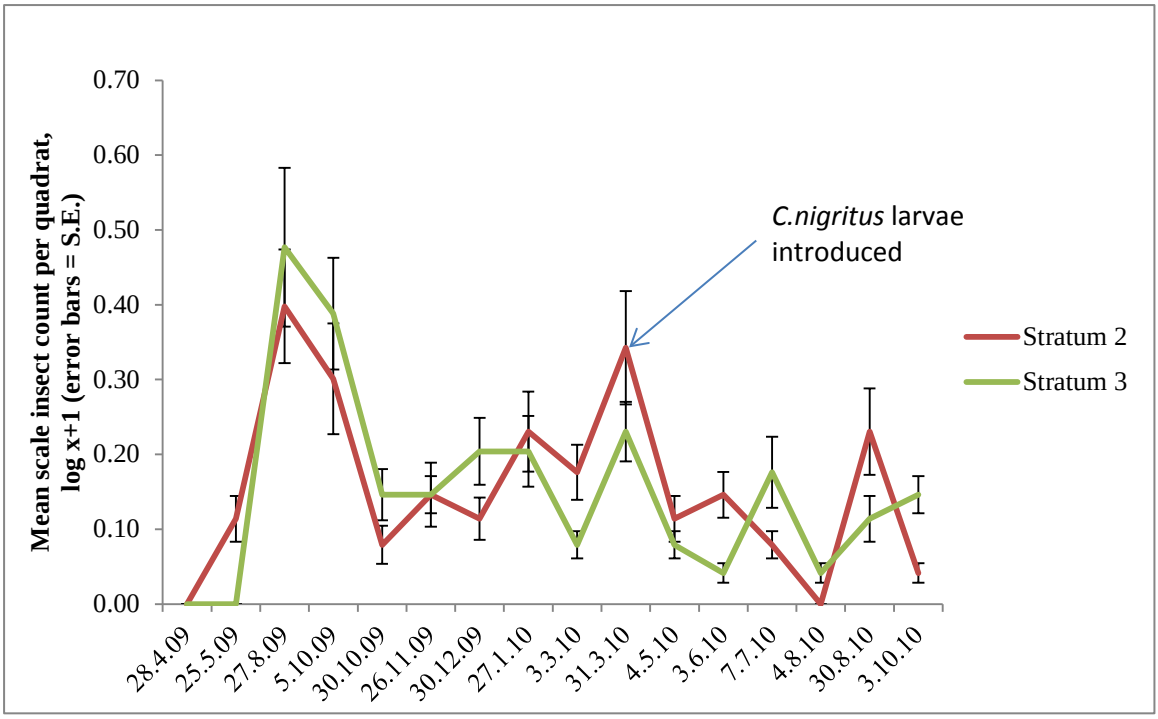

Figure 7.12 – Saissetia sp. monitored on Cordyline fruticosa (2) (Temperate House, Royal Botanical Gardens, Kew). A slight reduction in scale is observed immediately after release.

7.4.4 *Cordyline fruticosa* (L.) (Fig 7.14)

There was no significant different between numbers of *Saissetia sp.* (fig.7.12, scale insects are depicted in fig 7.13) before and after the inundative release. Three *C. nigritus* adults were observed on this plant on the 7th June 2010.

Figure 7.14 – Cordyline fruticosa

Figure 7.13 – Saissetia sp. on Cordyline fruticosa (Temperate House)

Date	Product used	Dilution	Quantity	Target pest
2009-01-14	Dynamec	50ml/100l	300l	Spider mite, chilli thrips
2009-01-22	Gyro	40ml/100l	150l	Whitefly, mealy, chilli thrips
2009-01-28	Dynamec	50ml/100l	200l	
2009-02-11	Gyro	40ml/100l	300l	
2009-02-25	Nemasys + Mycotal	250million + 500g	80l	Scale, mealy
2009-03-11	Nemasys	250million	80l	Scale, mealy
2009-03-18	Nemasys + Mycotal	250million + 500g	80l	Scale, mealy
2009-03-26	Nemolt + Chess	50ml/100l, 20g/100l	250l	Whitefly, Aphids
2009-04-07	Nemolt + Chess	100ml/100l, 20g/100l	200l	Whitefly, Aphids
2009-04-23	Nemasys + Mycotal	250million + 500g	80l	Scale, mealy
2009-04-30	Conserve + Calypso	7.5 ml/10l + 2.5ml/10l	100l	Mealy, Chilli thrips
2009-06-03	Nemasys + Mycotal	250million + 500g	80l	Scale, mealy
2009-10-22	Calypso + Conserve	2.5ml/10l + 10ml/10l	500l	Chilli thrips
2009-10-28	Calypso + Conserve	2.5ml/10l + 10ml/10l	600l	Chilli thrips

Date	Product used	Dilution	Quantity	Target pest
2010-03-17	Calypso + Conserve	2.5ml/10l + 10ml/10l	800l	Chilli Thrips, Mealy, Whitefly
2010-03-24	Dynamec	5ml/10l	800l	Chilli Thrips, Mealy, Whitefly
2010-03-30	Calypso + Conserve	2.5ml/10l + 10ml/10l	800l	Chilli Thrips, Mealy, Whitefly
2010-06-22	SB Invigorator	20ml/10l	1000l	Chilli thrips
2010-06-23	Nemasys & Mycotal		80l	Chilli thrips
2010-06-29	Mycotal		1000l	Chilli thrips
2010-07-06	Gazelle + SW7	25g/100l + 1ml/l (0.1%)	1000l	Chilli thrips
2010-07-13	Dynamec + SW7	5ml/10l + 1ml/l (0.1%)	1000l	Chilli thrips
2010-07-20	SB Invigorator	20ml/10l	1200l	Chilli Thrips, Mealy, Whitefly
2010-07-27	Gazelle + SB Invig	25g/100l + 20ml/10l	1300l	Chilli thrips
2010-08-03	Dynamec + SW7	5ml/10l + 1ml/l (0.1%)	1000l	Chilli thrips
2010-08-10	SB Invigorator	20ml/10l	1000l	Chilli thrips
2010-08-17	Gazelle + SW7	25g/100l + 20ml/10l	1300l	Chilli thrips
2010-08-24	Dynamec + SW7	5ml/10l + 1ml/l (0.1%)	1000l	Chilli thrips
2010-08-31	SB Invigorator	20ml/10l	1000l	Chilli thrips
2010-08-14	Mycotal		80l	Chilli thrips

Table 7.2 – Spray regimen in Palm House, Kew 2009 and 2010 (Temperate House data not available). No chemical sprays co-incided with significant scale insect declines, but Nemasys and Mycotal biological control applications did appear to have an effect on Pandanus vandermeeschii.

7.5 Discussion

A promising level of pest suppression was found after inundative release on three plants, including three species of scale insect (*Diapis biosduvallii, Abgrallaspis sp.* and *Pinnaspis buxi*, feeding on three host plants respectively, on *Pandanus vandermeeschii, Leucadendron argenteum* and *Rhapis excelsa*). *A.cyanophylli* had been used as a successful food item for the mass rearing of all beetles previous to release, and the same species of *Abgrallaspis* was located on the silver tree *Leucadendron argenteum* in the Temperate House. This population underwent a significant decrease in the months following release, perhaps unsurprisingly considering *C. nigritus'* known preference for this genus. The *Abgrallaspis* population had been increasing in numbers for the entire period of monitoring up until the biocontrol release. After release, the population decreased significantly every month for a period of four months, during which time two pupal cases were found on the plant, indicating larvae had completed development. Additionally, there was evidence of predation in the form of scale covers that had been eaten or overturned. This evidence shows a clear ability for *C. nigritus* populations to suppress *Abgrallaspis* in a field situation as well as previously demonstrated in captivity.

In captive circumstances the beetle will consume a mean of 510 *A.cyanophylli* in order to complete development (Ponsonby & Copland 2000). A total of 450 larvae were introduced during the field experiment, which could easily have eradicated the population found on this plant given the known voracity of *C. nigritus*. However, only partial elimination of the pest was encountered. Whilst this could still be considered a successful release in terms of pest suppression and plant health, there are still questions remaining as to why more larvae did not appear to develop on the plant. In this particular example, both scale insects and predators were highly exposed due to the morphology of the plant (see fig.7.5), and may have been particularly vulnerable to predators. Additionally, coccinellids are notorious for dispersing or avoiding oviposition once many larvae are present on a prey patch, in part due to the presence

of certain larval chemicals. This effect has mainly been studied in the context of adult females, which often avoid oviposition sites where conspecific larvae are already present (Oliver *et al.* 2006, Doumbia *et al.* 1998). Larvae have somewhat limited dispersal ability, but have adapted to avoid cannibalism from other ladybirds by avoiding pheromone tracks secreted by other larvae on the plant. Specifically, larval tracks reduce foraging behavior in some species, and thus reduce their ability to consume pests (Meisner *et al.* 2011). Dispersal of the larvae away from the main food patch or reduced feeding in relation to the threat of cannibalism could be the reason why few pupae were observed. The role of larval trails would be interesting to explore in relation to this behavior, especially as these pheromone tracks often consist of similar alkaloids to the hippodamine compound found in the reflex blood of *C. nigritus* (Burman *et al.* 2010, see also Chapter six).

Another diaspid, *Pinnaspis buxi,* was found on two adjacent *Rhapis excelsa* plants. Despite the potential for interaction between these two populations (e.g. migration of crawlers from one plant to the other), a reduction in scale numbers was observed in the summer of 2010 after larvae were introduced on the first plant (fig.7.8). This was not the case in the second plant, despite there being a decrease in numbers that was close to significant. This may be because this plant had lower numbers of individuals to begin with (Wilcoxon's matched pairs test showed the plants to have significantly different population sizes, (d.f. = 1, $P < 0.001$), and thus statistical differences were more difficult to identify. *Pinnaspis buxi* are a known host of *C. nigritus*, with 3rd and 4th instar larvae consuming 19 and 41 individuals per day respectively in a lab environment (Samways & Wilson 1988). These findings assert the hypothesis that *Pinnaspis* is a suitable prey item for both rearing and field situations. No pupal cases or other signs of long term establishment were noted in this case, although inspection of the entire plant was difficult to accomplish successfully.

A similar introduction of *C. nigritus* carried out in 1993 showed substantial control of all scale insect species in Plantasia Botanical Gardens (Swansea), but other contributory factors had made it unclear whether the ladybirds themselves had a significant effect in glasshouses at RBG, Kew (Ponsonby 1995). One of the main factors suggested as a contributor to population decline (and potentially confounding the results observed from the introduction of predators), was the use of chemical sprays and other control regimens carried out in the glasshouses. These factors are often necessary parts of glasshouse management, and are thus difficult prevent in the experimental setup. During this experiment it was requested that glasshouse staff refrain from spraying experimental plants where possible, but despite this there were some necessary applications made during both summer periods (table 7.2). However, many of these compounds were not targeted towards scale control, and no chemicals appeared to cause significant declines in scale population, perhaps due to inherent ability of scale insect covers to protect the insect from spray applications (Donahue & Brewer 1998) or pesticide resistance (Nel *et al.* 1978).

However, one spray treatment did appear to reduce scale insect numbers significantly; namely the introduction of *Steinernema feltiae* combined with fungal entomopathogen *Verticillium lecanii* (Viegas). This combination appeared to significantly decrease the population of *Diaspis biosduvallii* on *Pandanus vandermeeschii* directly after the initial inoculum (fig.7.6). This plant was highlighted as suffering a heavy infestation, and as such was also treated with heavy pruning and hosing to remove scale insect residues and mealybug infestations. These factors combined may have contributed to the lower population found in the following summer season, and thus it is difficult to attribute the reduction in scale insects specifically to predation by *C. nigritus*. *Steinernema feltiae* has been demonstrated as a successful method of control in numerous other insect groups, including whitefly (Cuthertson *et al.* 2003) and fungus gnats (Jagdale *et al.* 2004). *Verticillium lecanii* has also been demonstrated as an effective biocontrol of aphids (Ashouri *et al.* 2004) and thrips (Ahmadi *et al.* 2004). This study provides evidence that

S.feltiae and *V. lecanii* can provide substantial control of scale insects. It has been suggested that guilds of biocontrol agents can be the most successful way to suppress pests in a dynamic environment (Rott & Ponsonby 2000) and thus *S .feltiae* and *V .lecanii* could be a valuable addition to the guild of existing predators and parasitoids used in glasshouses for scale insect control.

This experiment also represents the first use of *C. nigritus* larvae as a method of scale suppression, rather than eggs and adults. There has been some ambiguity in the literature regarding which life stages the early instars are able to consume. It was originally suggested that larvae could only prey upon first instar crawlers (Samways & Wilson 1988), but recently it has become evident that larvae can also attack later life stages (Ponsonby & Copland 2000, Boothe, 2010). The release program in RBG, Kew has re-asserted the more recent findings, with significant effects observed in both intermediate instars and adult scale. Whether larval introduction is as effective as using solely adults is unknown, although this will likely vary depending on host plant and host insect. Larval stages have in some cases been shown to be more 'effective' at consuming diaspids than adult *C. nigritus* when considering only numbers eaten (Samways & Wilson 1988), but there are also many contrary examples (Jalalhi & Singh 1989). Ultimately conclusions on this should be made with the weight of scale insect consumed rather than the number (i.e. eating one adult scale insect may be the equivalent of eating many 1st instar crawlers, and thus the two are not comparable) (Ponsonby & Copland 2000).

Whilst diaspid prey were particularly abundant in both glasshouses, there were relatively low densities of soft scale present at any given time during the field trial. This was despite the presence of the more common glasshouse species, *Saissetia coffeae* and *Coccus hesperidum* (L.) in several localities. However, the data collected on *Cordyline fruticosa* (2) in the Temperate House suggest that larval introduction had no significant effect on the population of *S.coffeae.* Adult

ladybirds were observed on this plant (albeit not feeding) on one occasion after release. It is possible that these insects had dispersed from another area in the glasshouse, or could have been one of the adults introduced as part of the standard biocontrol regimen in the Temperate House. They appeared to provide very little control, at least at these low densities. This fits very well with the numerous records of preference for diaspid prey in the literature (Dixon 2000, Ponsonby & Copland 2007, Chazeau 1981), especially noted in South African individuals (Samways 1984). However, significant differences may be confounded by the low densities of *Saissetia* encountered. It could be advantageous to carry out field trials in other areas where soft scale species are more abundant. It would be particularly valuable to establish if any biotypes are effective on soft scale such as *Saissetia coffeae* (Walker) in the laboratory setting, as these results on soft scale seem to suggest a preference for diaspids.

The experiment introduced several biotypes and infection types of the ladybird. Very few signs of establishment were noted and therefore there were too few individuals available for an informative recapture. It was originally intended that patterns of establishment on certain host types could have been identified (i.e. do uninfected individuals establish better on a particular host?). Whilst these data are unavailable, this study highlights the need for prey preference and functional response data on these prey insects, particularly to compare the efficacy of different biotypes and infection types. If scale insect species obtained from Kew could be established successfully in the lab, differences in prey consumption and preference could be tested across the range of host insect genera, including *Pinnaspis*, *Abgrallaspis* and *Diaspis* and *Saissetia*. The biotypes most capable of dealing with a prey item (*Pinnaspis buxi* for example), might reflect the biotype that led to the suppression of *Pinnaspis* observed in the field study.

7.6 Conclusions

The potential for a guild of predators, parasites and pathogens to provide successful scale insect control is emphasized by this work, and if combined with greater phenotypic diversity within guild species, could cover a much broader range of agronomic situations and pest species. This study does show that introducing multiple biotypes into a glasshouse environment can be effective in providing scale insect control, but clarity on this issue needs to be informed by more rigorous testing of different scale insect prey in the lab. Collection of a wide range of prey species and more *C. nigritus* biotypes could provide even more different phenotypes of *C. nigritus* with which to test the species plasticity.

Importantly the data collected in this study also provide a wealth of information about the population dynamics of scale insects for future work.

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Chapter 8 – Research summary and implications

The research carried out provides new information about Chilocorus nigritus that will inform both future research and current rearing practice for this species, but can also inform research and applied use of other coccinellids. Most importantly, the work highlights a number of issues that can be applied much more widely to the field of biocontrol in general.

8.1 The significant of genetic variability.

The work detailed in Chapter two determined a surprising level of genetic variability between the different populations of *C. nigritus* studied (in accordance with Objective 1, Chapter one). The bionomic properties of different strains have been shown to vary, perhaps most notably in the level of prey consumption observed in LS1 strains compared to ENT (Chapter five). Endosymbiont infection type and prevalence also varied between the geographically isolated populations LS1, ENT, IND and RSA. This demonstrates for the first time that *Chilocorus nigritus* exists as several biotypes across its geographical range, and that these biotypes can have different characteristics and abilities to suppress pests.

Selection of the most efficient biotypes for a specific agronomic situation will now be possible given some further research. If these biotypes can be tested across a range of prey species and at a range of climatic conditions, the specific niche for each biotype can be ascertained. In a heterogeneous environment, with many plant and pest species, it may be beneficial to introduce a mixture of biotypes in order to 'cover all the bases'; e.g. some biotypes will be more capable of dealing with a particular plant/pest/climate than others, and *vice versa.* Conversely, it may be that some biotypes provide the optimal characteristics to deal with one very specific condition, such as the kind of monoculture of plants and pests found in a citrus plantation. The genetic variability encountered between these biotypes may therefore add a great deal of flexibility to the way we approach a particular pest problem.

Unlike the pattern observed between biotypes, genetic variability of *C. nigritus* within a population (and especially within an insectary population) is low. This highlights the

important issue of genetic bottlenecking in biocontrol, an occurrence that could potential limit the adaptability of *C. nigritus* to different situations (temperature, food type, host plant etc.). The genetic bottleneck encountered when a small number of founder individuals are collected is narrowed even further by keeping them in a very specific environment (in this case, selecting for individuals that survive well at 26°C, feeding on two host insects from one host plant, and being maintained with a strict 12:12 light regimen).

Genetic bottlenecks are often highlighted as a source of inbreeding depression, whereby and insect population starting from a small founder population will retain deleterious recessive alleles (Kirkpatrick & Jarne 2000). It has been argued that this kind of event is harmful to the insect population (Kirkpatrick & Jarne 2000), but in some cases the bottleneck actually 'purges' these deleterious genes, causing the inbred population to be 'healthier' than the population it came from (Facon *et al.* 2011). So in this sense it remains unclear what effect the bottlenecks have on the health of *C. nigritus*, a question which constitutes another viable area of research for future projects. However in terms of its functionality as a biocontrol agent, this bottlenecking undoubtedly reduces the adaptability of the beetle to changing climates and pest, and limits the potential for future evolution (Franks *et al.* 2011). This issue is something that should perhaps be considered more when rearing a generalist predator and bottleneck events are indeed receiving more attention in relation to modern classical biocontrol (Franks *et al.* 2010, 2011).

Regardless, this work shows clearly that treating a predator as a single species, and ignoring the underlying variability created by geographical isolation or otherwise, may be ignoring the inherent flexibility which can make the predator population more adaptable in different situations. Just as a particular species of biocontrol agent can be advised for a particular pest problem, so can a particular biotype.

8.2 The significant of chemical ecology

Olfactory cues are vital to so many aspects of an insect's life, including the way it interacts with plants, other species, and insects of the same species (Carde & Millar 2004). Chapter six details the first step in our understanding of pheromone communication in *C. nigritus*, identifying a potential alarm pheromone hippodamine, which could affect *Chilocorus nigritus* itself, and also its predators (Burman *et al.* 2010). Many steps are still needed to clarify the ecological importance of this compound. First of all, whether *C. nigritus* or other species respond to hippodamine could be established via electro-antennography, which directly assesses the responses of antennae to individual compounds. This will establish whether this compound is active as a pheromone in this species.

To be more certain of the behavioural function of hippodamine, behavioural experiments will also be required. Alkaloids are almost exclusively used for alarm pheromones in coccinellids, and thus determining whether *C. nigritus* behaves adversely to them is important, e.g. does the compound cause dispersal, reduced oviposition and feeding, etc. Additionally, complete identification of the compound and its isomeric form need to be established. Whilst some related insect species do share a common basic pheromone (Svensson *et al.* 2009), most pheromones compounds (including alkaloids) are species-specific (Slogett & Davis 2010). Therefore the chiral structure of the alkaloid produced by *C. nigritus* needs to be identified to clarify whether this is the case with this hippodamine compound. If we should wish to synthesise this compound for future experiments, a full identification will be essential.

Chemical ecology research could also help us to answer questions about prey preference, highlighted in the thesis. Previous studies have already identified that the presence of scale insects on a plant (even after insects are removed), causes increased searching behaviour (Boothe & Ponsonby 2006). So there is evidence to say that olfactory cues play a part in *C. nigritus* prey searching behaviour in addition to visual cues. Finding how the compounds eliciting a response differ between prey species and between host plants could be the key to

understanding prey preference in this species. This could also highlight any differences in prey preference between biotypes and infection types, so testing responses of the ladybirds to scale insect produced compounds could be fruitful.

8.3 The significance of endosymbionts.

The project has identified several novel endosymbionts present in *C. nigritus* populations (Objective 2; Chapter one). This opens up a number of avenues of research on their evolutionary history, and effects on the biocontrol system. Bionomic variability within the beetle populations appears to be closely linked to a beetle's infection status as well as its biotype, which may go some way to explaining the ambiguities noted in the literature about this species (Objective 3; Chapter one). Endosymbionts were first shown to be a source of bionomic variability in Chapter four, with infected individuals showing reduced reproductive output when compared to uninfected ladybirds. This suggests at least a partial parasitism by *Ricketssia* and *Wolbachia*, which are potentially utilising a significant amount of their host's nutritional resources; resources which would have otherwise been directed towards reproduction. This is the first observation of this kind in *C. nigritus*, and shows a strong interaction between endosymbiont and host.

These negative effects have been observed in almost all other coccinellid species harbouring these endosymbionts (Chapter one). It has already been suggested that ladybirds are particularly prone to these kinds of male killing bacteria due to their egg laying behaviour and sibling cannibalism (Hurst & Jiggins 2000). This pattern suggests a commonality within the family Coccinellidae, and also implies that ladybirds could be a successful group of biocontrol predators to test artificial curing on in the future. This is especially true of species of high economic, importance which could see significant increases in the number of individuals reared after bacterial elimination. Several notable species of interest to biocontrol programs remain untested for endosymbionts, including *Cryptolaemus montrouzieri* Mulsant, *Stethorus*

punctillum (Weise), *Chilocorus baileyi* and *Rhyzobius forestieri* (Pope), which would be good candidates for future screening projects.

In cases where parasitic effects are encountered, complete elimination of endosymbionts can be achieved given the correct curing protocol, and it should also remain in this state for a considerable amount of time (due to the low levels of horizontal transmission expected with these bacteria) (Vavre *et al.* 1999). However, levels of vertical and horizontal transmission remain unexplored in *C. nigritus*, and if examined could provide information about the persistence of antibiotic curing in the predator population. In other insects, mechanisms of horizontal transmission have been suggested via shared parasitism with other infected individuals (Vavre *et al.* 1999), and shared food resources (Sintupachee *et al.* 2006). These possibilities would be worth exploring in *C. nigritus* and other coccinellids.

On the whole, the consequences of these findings can inform biocontrol producers, whereby the number of insects produced could be significantly increased by using tetracycline treatment. However, whilst antibiotic curing is likely to increase the number of individuals produced in a mass rearing project, the resulting offspring are also likely to have a lower level of prey consumption (based on the observations made in Chapter five). This is an important consideration for biocontrol producers and complicated the issue somewhat, if only because it may render their 'product' more productive, but less effective. Whether the positive effects of increased fecundity outweigh the effects of reduced prey consumption is unknown, but could be determined in future research projects.

In a field situation, determining whether endosymbionts are beneficial or detrimental to *C. nigritus* may be a different case altogether. This is highlighted by Chapter seven and previous studies (Ponsonby 1995), which showed variable rates of success in suppression of different scale insect species on different host plants in ornamental glasshouses (Objective 4, Chapter one). In the laboratory, positive effects of endosymbionts on prey consumption and negative effects on fecundity were noted, but strictly in individuals fed on *A. nerii*. These

characteristics may vary between *C. nigritus*' numerous potential prey species, and even on different host plants, making the glasshouse environment more unpredictable. Chapter five suggests the potential for plants under attack from scale insects to produce toxic or antiphagant compounds, potentially affecting predator, prey and endosymbiont. There are also a number of examples of natural antibiotic effects of plant compounds on male killing endosymbionts. Therefore, with the sheer number of potential trophic interactions that can occur between plant, endosymbiont, pest and predator, making assertions based on only this project's finding on *A. nerii* would be unwise. In a generalist predator like *C. nigritus*, endosymbionts could have very different effects on the beetle depending on (a) which prey it was feeding on, (b) which host plant it was on, or even (c) the temperature the insects were maintained at (since temperature fluctuations are also known to affect the growth and reproduction of endosymbionts).

Clearly more research is needed in this area to establish which infection types are most successful in different agronomic situations. This project has shown that some individuals harbour infections whilst others do not, leading to a degree of reproductive variability and variability in prey consumption within a population. If the main goal is to produce a predator that is effective in ornamental glasshouses (a highly variable and heterogeneous environment), then this variability caused by endosymbionts may be considered an asset to the predator (rather than a limiting factor that simply reduces its reproductive ability). It could be beneficial to keep infected and uninfected individuals within a population, if only to provide a number of phenotypes capable of adapting to different situations. In the long term, predator diversity may be one of the keys to sustainable biocontrol. If this species is going to be used to its full potential, future research efforts should aim to establish the specific niches of *Chilocorus nigritus*' biotypes and infection types, and in which situations they are at their most effective.

8.4 My future work

Whilst I would aim to continue along the numerous avenues of research opened up by this project, I will mainly continue to work specifically on the chemical ecology of insects at the Swedish Agricultural University (SLU) in Alnarp, Sweden. I am currently developing additional skills in analysis of pheromones and plant volatiles in order to undertake new projects related to insect biodiversity in Sweden. In addition, I plan to use these new-found techniques and resources to explore the chemical ecology of *C. nigritus* further, including the use of electro-antennography to determine the response of the ladybird to the defence compound hippodamine (preliminarily identified in Chapter six, Burman *et al.* 2010). In doing so I would also hope to build up collaboration and communication between Canterbury Christ Church University and SLU, and answer some of the questions left by this thesis.

8.5 References

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9.0 Appendix

Host Plant: **Scale Species:** **Stratum:** **Date:**

q	*h*	*q*	*h*	*q*	*h*	*q*	*h*	*q*	*h*
6	2	6	1	5	3	8	1	8	3
6	2	1	2	6	1	4	2	8	3

			*Stem**				*Midrib***						*Lateral Vein***					*Interveinal Tissues****				
	No	**Score**	**Tn**	**A**	**I**	**C**	**Rn**	**Score**	**Tn**	**A**	**I**	**C**	**Rn**	**Tn**	**A**	**I**	**C**	**Rn**	**Tn**	**A**	**I**	**C**
↑	**1**						3						7					7				
↓	**2**						1						10					2				
↑	**3**						3						5					8				
↓	**4**						1						3					5				
↑	**5**						5						9					1				
↓	**6**						4						6					4				
↑	**7**						3						1					7				
↓	**8**						2						8					9				
↑	**9**						1						9					9				
↓	**10**						1						8					3				
	Mean																					

*No counted on 12mm of stem either side of (and including) the petiole
**No counted on 24mm length of mibrib/vein selected at random zone indicated
***No counted in a 24mm diameter circle of tissue selected at random zone indicated

*(**Score** 0 = none, 1 = scattered individuals, 2 = small colonies (10-50), 3 = large colonies (50-100), 4 = confluent infestation)*

Table 9.1 – Example data sheet used on a single stratum