EFFECT OF PHYSIOLOGICAL AND BEHAVIOURAL CHARACTERISTICS OF PARASITOIDS ON HOST SPECIFICITY TESTING OUTCOMES AND THE BIOLOGICAL CONTROL OF *PAROPSIS CHARYBDIS*

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To Barbara Barratt and Kath Dickinson, outstanding scientists, inspiring mentors and patient friends - without your enthusiastic and supportive introduction to scientific research I wouldn't have had the determination or desire to keep going when the road got rough. Things have rarely gone as planned, but thanks to you I know there are many exciting research opportunities awaiting me, and some amazing scientists that I can look forward to working with in the future.

Abstract of thesis submitted in partial fulfilment of the requirements for the Degree of Ph.D.

Effect of physiological and behavioural characteristics of parasitoids on host specificity testing outcomes and the biological control of *Paropsis charybdis*

By Tara J. Murray

An established host-parasitoid-hyperparasitoid system was used to investigate how the physiological and behavioural characteristics of parasitoids influence the outcomes of laboratory-based host specificity tests. The characteristics of the two pteromalid egg parasitoids, *Enoggera nassaui* (Girault) and *Neopolycystus insectifurax* Girault, were assessed and interpreted in regard to the particular host specificity testing methods used and the control of the eucalypt defoliating beetle *Paropsis charybdis* Stål (Chrysomelidae) in New Zealand.

The physiology of *N. insectifurax* was examined to determine how to increase production of female parasitoids that were physiologically capable and motivated to parasitise *P. charybdis* eggs in laboratory trials. *Neopolycystus insectifurax* were found to be more synovigenic than *E. nassaui*. Provisioning them with honey and host stimuli for three days, and allowing females to parasitise hosts in isolation (i.e. in the absence of competition) was an effective means of achieving these goals.

No-choice tests were conducted in Petri dish arenas with the four paropsine beetles established in New Zealand. All four were found to be within the physiological host ranges of *E. nassaui* and *N. insectifurax*, but their quality as hosts, as indicated by the percent parasitised and offspring sex ratios, varied. The results of paired choice tests between three of the four species agreed with those of no-choice tests in most instances. However, the host *Trachymela catenata* (Chapuis), which was parasitised at very low levels by *E. nassaui* in no-choice tests, was not accepted by that species in paired choice tests. A much stronger preference by *N. insectifurax* for *P. charybdis* over *T. catenata* was recorded in the paired choice test than expected considering the latter was parasitised at a high level in the no-choice test. The presence of the target host in paired choice tests reduced acceptance of lower ranked hosts. Both no-choice and choice tests failed to predict that eggs of the

acacia feeding beetle *Dicranosterna semipunctata* (Chapuis) would not be within the ecological host range of *E. nassaui* and *N. insectifurax*.

Behavioural observations were made of interspecific competition between *E. nassaui* and *N. insectifurax* for access to *P. charybdis* eggs. Two very different oviposition strategies were identified. *Neopolycystus insectifurax* were characterised by taking possession of, and aggressively guarding host eggs during and after oviposition. They also appeared to selectively oviposit into host eggs already parasitised by *E. nassaui,* but did not emerge from significantly more multi-parasitised hosts than *E. nassaui*. *Enoggera nassaui* did not engage in contests and fled when approached by *N. insectifurax*. Although often prohibited from ovipositing by *N. insectifurax, E. nassaui* were able to locate and begin ovipositing more quickly, and did not remain to guard eggs after oviposition. It is hypothesised that although *N. insectifurax* have a competitive advantage in a Petri dish arena, *E. nassaui* may be able to locate and parasitise more host eggs in the field in New Zealand, where competition for hosts in is relatively low.

The biology of the newly established encyrtid *Baeoanusia albifunicle* Girault was assessed. It was confirmed to be a direct obligate hyperparasitoid able to exploit *E. nassaui* but not *N. insectifurax*. Field and database surveys found that all three parasitoids have become established in many climatically different parts of New Zealand. Physiological characteristics were identified that may allow *B. albifunicle* to reduced effective parasitism of *P. charybdis* by *E. nassaui* to below 10%. However, the fact that hyperparasitism still prevents *P. charybdis* larvae from emerging, and that *B. albifunicle* does not attack *N. insectifurax,* may preclude any significant impact on the biological control of *P. charybdis*.

Overall, parasitoid ovigeny and behavioural interactions with other parasitoids were recognised as key characteristics having the potential to influence host acceptance in the laboratory and the successful biological control of *P. charybdis* in the field. It is recommended that such characteristics be considered in the design and implementation of host specificity tests and might best be assessed by conducting behavioural observations during parasitoid colony maintenance and the earliest stages of host specificity testing.

Keywords: biological control, host specificity testing, no-choice test, choice test, parasitoid, hyperparasitoid, parasitoid behaviour, *Eucalyptus*, *Acacia*, *Paropsis charybdis*, *Enoggera nassaui*, *Neopolycystus insectifurax*, *Baeoanusia albifunicle*

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CHAPTER 1: INTRODUCTION

1.1 CLASSICAL BIOLOGICAL CONTROL

Classical biological control is a pest management tactic that aims to re-establish the link between exotic pests and their natural enemies (Waage & Greathead 1988). This usually entails the transfer of one or more beneficial organism from a pest's country of origin to the country it has invaded. These biological control agents (BCAs) are intended to become permanently established in the receiving country. Classical biological control in this form began in 1888 with the introduction of vedalia beetle (*Rodolia cardinalis* (Mulsant)) against cottony cushion scale (*Icerya purchasi* Maskell) in California. Successful biological control results in the suppression of the target pest below a level considered economically damaging (Louda et al. 1997). Early successes with entomophagous BCAs are reviewed by DeBach (1964). Cameron et al. (1993) summarises all introductions of BCAs into New Zealand up to 1987, with evidence for many cases of pest suppression.

Classical biological control is currently one of the more acceptable methods for weed and arthropod pest management. It is widely perceived as being environmentally benign or 'friendly'. This results from an upsurge in the awareness of risks associated with broad spectrum chemical pesticides, particularly since the publication of 'Silent Spring' (Carson 1963), in which the extent of environmental damage being caused by pesticides was brought to the attention of the North American public. In addition to direct toxicity, disadvantages of pesticides include re-infestation from unsprayed areas, pest resurgence, and pesticide resistance. Proponents of classical biological control highlight its ability to provide a self-replicating, self-spreading, sustainable and targeted alternative to chemical pest control. Despite these positive attributes, some of the characteristics responsible for successful establishment and effective pest suppression by BCAs also make them potentially dangerous invaders (McEvoy 1996). In recent decades much of the discussion around classical BCAs has focused on the risks they pose to non-target organisms and ecosystems. In this time the already vast literature on biological control, including several peer-reviewed international journal series devoted to the subject (e.g. Biological Control, BioControl, Biocontrol Science and Technology), has been augmented by a number of conferences, special issues, international workshops and symposia on these issues (e.g.

Withers et al. 1999; Follett & Duan 2000; Van Driesche & Reardon 2004). Concerns around the lack of pre- and post-introduction assessment of BCAs were being raised in New Zealand as early as the mid 1980s (e.g. Roberts 1986). There is now widespread agreement that these risks are real and must be reduced or ideally eliminated by utilising only the most host-specific agents. This is in stark contrast to earlier attitudes that considered a wide host range to be beneficial (Cameron et al. 1993). Current practices in most developed countries generally preclude introductions of, for example, generalist vertebrate herbivores or predators, as BCAs. However, still regarded favourably are certain phytophagous insects to control weeds and entomophagous insects (parasitoids and predators) to control arthropod pests. The latter are the subjects of this study.

1.2 Evaluating the Risks of Biological Control Agents

Entomophagous BCAs can pose risks to non-target native and introduced beneficial insects on a number of levels. These include direct trophic interactions, direct interference and also indirect interactions via an intermediate species such as a shared natural enemy or shared host. The latter two are reviewed by Secord & Kareiva (1996). Direct trophic interactions have been easier to assess and are the basis of most pre-release risk assessment in biological control programs. These interactions will be the focus of this study.

Publications on the negative impacts of biological control have increased significantly since the early 1990s (Bennett 1993; Duan & Messing 1997; Louda et al. 1997; Van Driesche & Hoddle 1997; Boettner et al. 2000; Follett & Duan 2000; Louda et al. 2003; Babendreier et al. 2005). Although generalist predatory vertebrates have certainly caused significant non-target harm (see Simberloff & Stiling 1996 for review) direct evidence for and against non-target effects by insect BCAs, such as extinction or displacement of native fauna, is lacking, and there has been considerable debate on the issue (e.g. Ehler & Hall 1982; Funasaki et al. 1988; Howarth 1991; Simberloff & Stiling 1996). Legislators have responded to the concerns raised by implementing guidelines and regulations around the introduction of BCAs. How the required data should be collected and interpreted to assess the risks and benefits of an agent before its introduction has not been clearly identified. Generally, the host specificity of an agent is used as a measure of risk. In countries where comprehensive risk assessments are required, host specificity is estimated by a

combination of published literature and laboratory tests, as well as field observations and experiments in the agent's native range (Van Driesche & Reardon 2004; Babendreier et al. 2005).

Potential non-target impacts thought to have occurred in New Zealand include the displacement of native parasitoids by *Trigonospila brevifacies* (Hardy), and the attack of native weevils and a beneficial introduced control agent *Rhinocyllus conicus* (Froelich) by *Microctonus aethiopoides* Loan (Barratt et al. 1997; Munro & Henderson 2002). These agents were introduced before the implementation of stringent pre-release assessments. The Entomology Division of the Department of Scientific and Industrial Research (DSIR, replaced by the Crown Research Institutes in 1992) drew up its own set of criteria to minimise risks associated with introducing BCAs in the early 1980s (Roberts 1986). Formal recognition of such risks has been included in the recent environmental legislative reform that culminated in the Hazardous Substances and New Organisms (HSNO) Act 1996, implemented with respect to new organisms in 1998. The Environmental Risk Management Authority (ERMA) was established to assess and make decisions upon any application to introduce a new organism. Under the Act a cautionary approach is taken to protect native species, introduced beneficial organisms, ecosystem processes and the health, safety and economic wellbeing of New Zealand people (see Harrison et al. 2005 for review). Other countries such as Australia, Canada, Mexico, South Africa, the USA and members of the EU have also implemented risk-adverse legislation around the introduction of BCAs, but their regulatory processes are generally less streamlined and less stringent compared to the New Zealand model (Sheppard et al. 2003; Bigler et al. 2005). States within the USA, for example, each have their own regulatory framework governed by an assortment of different agencies, and in most countries regulation around the introduction of BCAs is based on historical legal acts derived for purposes other than biological control.

In New Zealand it must be demonstrated that a candidate BCA poses minimal risks before it can be imported into quarantine, then, before it can be released into the environment, evidence must be provided showing that specific native and beneficial organisms will not be harmed. The key tool for assessing risk is host specificity testing, initially developed for phytophagous insects (see Zwolfer & Harris 1971 for review). Historically, this involved

conducting no-choice tests to ensure particular commercially-valued plants (i.e. other crops) were not within an agent's host range (McEvoy 1996). The development of the centrifugal phylogenetic method (Wapshere 1974) transformed laboratory-based host specificity testing. Under this method agents are exposed to a sequence of successively more distantly related plants and a selection of relevant unrelated cultivated plants to delimit their *host range*. This relies upon the fact that phytophagous insects use secondary chemicals produced by plants to locate and identify their hosts, and these compounds are more likely to be similar between more closely related plants. Currently, host specificity tests based on this method are considered to provide reliable but conservative predictions of the likely host range of phytophagous agents (Lopez-Vaamonde & Moore 1998). There is a movement, however, to modernise the method considering the major scientific advances in recent years (Briese 2005). Methods initially developed for phytophagous BCAs are still relied upon to assess the risks posed by entomophagous BCAs such as parasitoids. Although much of the theory is applicable, these methods are not entirely transferable (Goldson & Phillips 1990). In particular, insect pests lack any analogy to the taxonomically shared secondary chemicals that have historically underpinned the selection of non-target species to be assessed. This may explain why phylogenetic relatedness is less likely to predict the host range of a parasitoid (Haye et al. 2005). Furthermore, compared to plants, it is intrinsically more difficult to work with and maintain multiple insect species in the laboratory because they are mobile, have specific feeding and ecological requirements, and their behaviour is more likely to be influenced by confinement. This limits the range of tests, non-target organisms and replication that can be achieved in biological control programs.

1.3 Host Specificity Testing of Parasitoids

McEvoy (1996) described host selection as a hierarchical sequence of opportunities and constraints. Exploitation of a host by a parasitoid is determined by the ability of the adult female to locate, accept and oviposit into that host within a given environment. This process is thought to be driven by chemical cues derived from the plant-host-complex. These include volatile cues and contact kairomones associated with the host, the plant on which the host is found, and produced by interactions between the plant and the feeding host, host frass and host eggs or larvae (Turlings et al. 1990; Mattiacci et al. 1995; Withers & Browne 2004). Laboratory tests are generally accepted to overestimate parasitoid host

ranges by preventing parasitoids from exhibiting their full repertoire of host finding behaviours. In the natural environment, the processes of locating the host habitat and the host within it can filter out numerous potential host species (Goldson & Phillips 1990; Hill 1999), but in the confinement of the laboratory this filtering process is largely prevented.

Globally, a variety of host specificity test designs have been employed, such as 'black–box' no-choice, sequential choice, paired choice and multiple choice (see van Lenteren et al. 2006a for review). One of the most common debates about host specificity testing is whether to use choice or no-choice tests. As pointed out by Barratt et al. (1999), this depends on the intent of the test. No-choice tests maximise the likelihood of attack and thereby determine the widest range of hosts the parasitoid could attack and/or successfully develop on. Choice tests provide information on host preferences, especially in the presence of the target (Barratt et al. 1999; Withers & Browne 2004). Some studies have used only no-choice tests (e.g. Lopez-Vaamonde & Moore 1998) and others only choice tests (e.g. Fuester et al. 2004). A growing number of studies now combine both, especially in New Zealand (Field & Darby 1991; Goldson et al. 1992; Barratt et al. 1997; Porter 2000; Froud & Stevens 2004; Withers & Browne 2004; Zilahi-Balogh 2004). This increases the information available with which to estimate ecological host ranges. However, detailed comparisons of the predictive value of each test type have rarely been made and how the results of different methods should be weighted and interpreted are not well defined. Ideally, a number of tests might be used, but this is often unachievable as the process of doing so is costly and constrained by time, resources and the practicality of working with live insects. Increased efficiency and accuracy is required to assess parasitoid host ranges in the laboratory and make predictions concerning the extent of any non-target impacts that are likely to occur post-release.

1.4 IMPROVING HOST SPECIFICITY TESTING

Significant advances have been made in determining which arthropod pests are suitable for biological control, which agents pose fewer risks, and what physiological and physical factors might need to be controlled during host specificity testing (see Babendreier et al. 2005 and Briese 2005 for reviews). Laboratory-based host specificity tests like those described in section 1.3 have become commonplace (e.g. Field & Darby 1991; Neale et al.

1995; Lopez-Vaamonde & Moore 1998; Sands & Coombs 1999; Duan & Messing 2000; Morehead & Feener 2000; Porter 2000; Mansfield & Mills 2002; Fuester et al. 2004). No single test is expected to predict the ecological host range of all candidate BCAs and there have been numerous reviews and discussions concerning which of the various methods to use, guidelines to follow, and other factors to take into consideration (Zwolfer & Harris 1971; Pschorn-Walcher 1977; Goldson & Phillips 1990; McEvoy 1996; Withers et al. 1999; Van Driesche & Reardon 2004; Babendreier et al. 2005; Briese 2005). Although these reviews make many valid points and have stimulated much needed international discussion around host specificity testing, there is still relatively little experimental evidence on which to base the choice and design of testing methods. The interpretations of results obtained using different methods are even less well evaluated. The problem remains therefore, that it is not known how effectively the tests and the interpretation of their results,, predict host ranges, let alone the extent and implications of any non-target attack.

Developing an in-depth understanding of the biology and behaviour of both target and agent could play a key role in selecting and accurately interpreting the most appropriate set of tests for a given candidate BCA. Post-release evaluations and experimental case studies employing retrospective host-specificity testing should help to validate the decisions made. A number of studies of this type have been conducted in recent years (e.g. Barratt et al. 1997; Duan & Messing 2000; Benson et al. 2003; Louda et al. 2003; Van Driesche et al. 2003; Haye et al. 2005; Morrison & Porter 2005; Barron 2007). Each of these has provided valuable insight regarding the accuracy of host specificity tests and factors of importance in their implementation and interpretation. For example, Benson et al. (2003) reported that parasitism of *Pieris virginiensis* Edwards by *Cotesia glomerata* (L.) represented a false positive result because that non-target host occupies a habitat not naturally searched by the parasitoid in the north-eastern United States. Barratt (2004) explained how initial host specificity testing of *M. aethiopoides* may have failed to predict attack of *R. conicus* in New Zealand because of the unsuitable physiological state of the hosts used in the tests.

Retrospective host specificity testing involves comparing the realised host ranges of BCAs that were introduced with little or no pre-release testing, to host ranges predicted by post-release laboratory tests. Such studies may provide a means of calibrating laboratory tests so

they can more accurately estimate ecological host ranges and predict non-target impacts (Van Driesche & Murray 2004a; Briese 2005). In turn biological control practitioners should be better equipped to interpret the results of laboratory-based host specificity tests and conduct risk-benefit analyses with greater confidence. However, comparing predicted and realised host ranges is only the first step towards improvement. The cause of disparities must be determined and laboratory tests and the interpretation of their results modified accordingly. Babendreier et al. (2005) reviewed multiple factors that influence the outcomes of host specificity tests. Identifying how physiological and behavioural characteristics influence, or are influenced by, these factors, will complement this knowledge and increase the accuracy with which ecological host ranges of parasitoids can be predicted. The range of cues and stimuli that affect parasitoid behaviour is vast and only partly understood. It is also extremely difficult to reproduce natural conditions within a laboratory environment. Behavioural observations during host specificity tests will improve the understanding of the mechanism of host selection. They can identify risks such as host mortality as a result of probing (i.e. inserting the ovipositor into the host) and provide information on the relative acceptability of the host. This knowledge is important both with regard to selecting the most specific and effective agents, and preventing the rejection of suitable agents (Zwolfer & Harris 1971; Mansfield & Mills 2002). It could help avoid future omissions, testing errors, and misinterpretations of results that have been blamed for some of the most recent unexpected non-target impacts (see Briese 2005). Viewing biological control in the context of the evolutionary constraints surrounding the host-parasitoid interaction and developing a greater understanding of the characteristics of agents that provide safe and effective control compared to those that do not, may increase control efficiency and minimise risks to non-targets in the long term.

Biological control as a science has evolved rapidly over the last 100 years. At one time the aim was simply to find and import a natural enemy to suppress a pest. Subsequently, the criteria and efficiency with which targets and agents are selected, reared and released have been improved. Internationally, an awareness of the potential risks associated with the importation of BCAs has been recognised, and legislation implemented around assessing these risks. At present the ability to use existing test procedures to accurately assess risks and benefits in a timely and cost effective manner is limited by the constraints imposed by the laboratory environment and a lack of understanding of what drives the success of

specific BCAs. Retrospective host specificity testing, that includes behavioural observations with both successful and unsuccessful agents, is one means of verifying and improving the predictive ability of host specificity testing methods currently relied upon.

1.5 Study System in Which to Examine Host-Specificity Testing

In this section eucalypt production forestry in New Zealand and the seven insect species used in this study are introduced. These include four herbivorous beetles, two primary parasitoids and a hyperparasitoid, representative specimens of which have been deposited in the National Forest Insect Collection, FRNZ, held at Scion, Rotorua. The system is suitable for assessing the ability of laboratory testing methods for use in pre-release host specificity assessments because: 1) most of the species have been established in New Zealand for some time and are well studied; 2) one parasitoid has provided effective control of the target pests while the other appears to be less effective; 3) the system includes several non-target hosts and there is evidence that at least one of these is not within the realised host range of the parasitoids. The system provides a unique opportunity to assess the consequences of self-introductions that have occurred subsequent to the implementation of a successful biological control program, a situation that has not been assessed before. In this case the second primary parasitoid and the hyperparasitoid represent such introductions.

Eucalypt forestry and Australian paropsine beetles in New Zealand

As a long term crop, spread widely over variable terrain, and from which a blemish-free product is not required, plantation forestry in New Zealand is ideally suited to classical biological control. Most of the predominantly northern hemisphere softwood timber species that make up 97% of New Zealand plantation forests were introduced in the absence of their native insect associations, and have few pests as a result (Nuttall & Alma 1986; Sheridan 1989). In contrast, native Australian tree species suffer significant insect damage when grown in New Zealand (Bain 1977a; Richardson & Meakins 1986; Barrett 1998; Withers 2001). The geographical proximity, prevailing weather patterns, climatic similarity, and volume of trade and travel between New Zealand and Australia provide regular colonising opportunities for Australian insects (Ridley et al. 2000; Withers 2001). Over half of all New Zealand's insect forestry pests originate from Australia and specialise

on *Eucalyptus* L'Herit (Myrtaceae), which makes up just 2% of the industry (Withers 2001; MAF 2008). Historically, desirable fast-growing coppicing eucalypts have failed to excel in production forestry primarily because of their susceptibility to the defoliating beetle *Paropsis charybdis* Stål (Anon. 1976; Thomson 1977; Nicholas & Hay 1990). *Eucalyptus nitens* (Deane et Maiden) Maiden, highly regarded for short fibre pulp, is particularly suited to plantation forestry in the New Zealand environment but severe defoliation by *P. charybdis* effectively prohibited its production on a commercial scale for many years (Baker & de Lautour 1962; Anon. 1976; Miller et al. 1992).

Eucalypt feeders are usually highly specific because of the secondary plant chemicals produced by their host plants (Steven 1973; Ohmart 1991). This makes them ideal targets for biological control. In both Australia and New Zealand the primary eucalypt defoliators are paropsine beetles (Coleoptera: Chrysomelidae) (Kelly & Reid 1999). Most are uncommon in Australia and regulated by a range of natural enemies, especially tachinid and hymenopteran parasitoids that inflict high levels of primary parasitism and hyperparasitism (Cumpston 1939; Edwards & Suckling 1980; Tanton & Epila 1984; Selman 1985; Naumann 1991; Tribe 2000). Four paropsine species are established in New Zealand and 13 others have been intercepted since 1955 (Murphy 2005). As the main eucalypt defoliator in New Zealand, *P. charybdis* has been extensively studied (e. g. Clark 1930; Styles 1970; Steven 1973; McGregor 1984; Murphy 1998). It is uncommon in Australia, occurring in the ACT and Tasmania, and was discovered in New Zealand in the Port Hills, Canterbury in 1916 (Thomson 1922; Clark 1930; Styles 1970). It is now found wherever eucalypts are grown in New Zealand (Dugdale 1965; Styles 1970; White 1973). Oviposition is largely restricted to the sub-genus *Symphyomyrtus*, section Maidenaria (de Little 1979; Bain & Kay 1989).

Trachymela sloanei (Blackburn), *T. catenata* (Chapuis) and *Dicranosterna semipunctata* (Chapuis) have caused less defoliation of commercially planted trees in New Zealand than *P. charybdis* so far, but as its relatives they are perceived as having the potential to become pests. *Trachymela sloanei*, detected in Auckland in 1976, is established in much of the North Island and possibly the Marlborough Sounds in the South Island (Steven & Mulvay 1977; Bain 2001b, a, 2002). It is native to New South Wales, Victoria and the ACT, and

has caused extensive damage since establishing in California (Paine et al. 2000). In New Zealand it shows a preference for *E. nitens* and has occasionally been responsible for severe defoliation (Walsh 1998). It may be responsible for the majority of defoliation in the Gisborne region (pers. ob.). Adult beetles consume expanding leaf shoots in addition to flush foliage, thereby preventing re-foliation (Millar et al. 2000; Paine et al. 2000). *Trachymela catenata* is not known to have caused significant damage in New Zealand. Its range is restricted to Gisborne and northern Hawke's Bay where it was detected in 1992 (Barrett 1998). A thesis by D.P. Barrett (1998) on the biology and ecology of *T. catenata* in New Zealand appears to be the only literature on the species. *Dicranosterna semipunctata* was discovered in Auckland in 1996 and occurs from Northland to the Bay of Plenty where it is a minor pest on *Acacia melanoxlyon* R. Br. (Walsh 1998; Appleton 2001b; Nicholas & Brown 2002). Two hymenopteran egg parasitoids (*Enoggera polita* Girault and *Neopolycystus* sp.), related to those considered in this study, have been identified exploiting *D. semipunctata* in New South Wales (Appleton 2001a, b; Nicholas & Brown 2002).

Parasitoids of paropsine beetles in New Zealand

Parasitoids play a significant role in the natural regulation of paropsines in Australia (Greaves 1966; Tanton & Epila 1984) and as such were identified as a practical means of *P. charybdis* control in New Zealand where small blocks of eucalypts were scattered across large areas and managed by multiple groups (Clark 1930). Classical biological control programs for *P. charybdis* began in the 1930s. Most candidate species considered were not released because of hyperparasitism or failure to rear on *P. charybdis* in quarantine (Bain & Kay 1989; Kay 1990). Four agents, *Froggattimyia tillyardi* Malloch, *Neopolycystus* sp., *Enoggera nassaui* (Girault) and *Cleobora mellyi* Mulsant have been introduced and the latter two have established (Murray et al. 2008).

Enoggera nassaui (Hymenoptera: Pteromalidae) were introduced from Western Australia, an area where *P. charybdis* does not occur, in 1987. The parasitoid dispersed quickly throughout New Zealand and in many regions suppressed *P. charybdis* populations such that additional chemical control was not required (Kay 1990). It is a polyphagous solitary egg parasitoid, having been reared from 21 species from five paropsine genera and one

psyllid (Naumann 1991; Mo & Farrow 1993; Nahrung & Murphy 2002). In New Zealand *P. charybdis* is *E. nassaui's* only known field host, and it has occasionally been induced to parasitise *T. catenata* and *D. semipunctata* in the laboratory (Murphy & Kay 2004; Murphy 2005). In the laboratory, 100% parasitism of *P. charybdis* is common, but in the field parasitism averages between 0 and 50% (Bain & Kay 1989; Jones & Withers 2003). High winter mortality, as a result of poor climate matching, has been proposed to explain historically low levels (< 20%) of *P. charybdis* parasitism by *E. nassaui* in early spring in the cool central regions of the North Island (Murphy & Kay 2000). Consequently, a Tasmanian strain of *E. nassaui* was introduced to the Bay of Plenty region in 2000 in an attempt to improve spring control.

A second solitary egg parasitoid, *Neopolycystus* sp., incorrectly identified as *Neopolycystus insectifurax* Girault (Pteromalidae), was released in 1987 but did not establish (Kay 1990; Berry 2003). An apparently self-introduced confirmed population of *N. insectifurax* (Berry 2003) has since established in the Bay of Plenty and regions to the north of this (Murphy 2002). *Neopolycystus insectifurax* is polyphagous attacking at least 10 species of *Paropsis* and *Chrysophtharta* and many other 'unidentified' species in Australia (Cumpston 1939; Tanton & Epila 1984; Bouček 1988; Mo & Farrow 1993; Tribe 2000). *Paropsis charybdis* is the only host from which it has been reared in New Zealand and parasitism is generally low (Jones & Withers 2003). Its limited success is thought to result from poor synchrony with *P. charybdis* oviposition peaks and adaptation to temperatures higher than are normally experienced in most of New Zealand (Bain & Kay 1989; Tribe & Cillié 2000).

The third parasitoid investigated in this study is *Baeoanusia albifunicle* Girault (Encyrtidae). This hyperparasitoid was first reared from *P. charybdis* eggs parasitised by *E. nassaui* in the Bay of Plenty in late 2001 and it is not thought to have dispersed far from this location (Murphy 2002). It is an uncommon, yet widely distributed native of Australia, where it has been reared from parasitised eggs of five species of *Paropsis* and *Chrysophtharta* (Cumpston 1939; Tanton & Khan 1978; Tribe 2000). High levels of hyperparasitism have made it difficult to locate *E. nassaui* in the Bay of Plenty since *B. albifunicle* was detected. Hyperparasitism is expected to impede the annual population growth of *E. nassaui*, further inhibiting *P. charybdis* control in early spring (Murphy

2002). In Australia, *N. insectifurax* tends to dominate when it occurs in sympatry with *E. nassaui* and *B. albifunicle* (Cumpston 1939). If *N. insectifurax* is immune to hyperparasitoid attack, as it appears to be, it could assume some of the regulatory role against *P. charybdis* expected to be lost by *E. nassaui* in New Zealand (Tribe & Cillié 2000; Murphy 2002; Jones & Withers 2003).

1.6 THESIS GOALS

Hymenopteran primary parasitoids are often recognised as a particularly effective and relatively safe group from which to select BCAs. Although there is legislation in place in New Zealand that requires extensive pre-release host specificity testing of candidate BCAs there is little empirical evidence regarding the ability of the various laboratory-based testing methods to accurately predict post-release host ranges. Retrospective analysis of established parasitoids has been identified as a useful means of testing and improving this accuracy. In this study the established pest/parasitoid/hyperparasitoid system described above is exploited to investigate how and why particular behavioural and physiological characteristics define the effectiveness of a BCA.

Two established egg parasitoids of the eucalypt defoliator *P. charybdis* are compared. Previous studies have indicated that despite their similarities the two species have some distinctly different behavioural characteristics (S. Mansfield unpub.) and that *E. nassaui* is a more effective BCA than *N. insectifurax* in the field (Jones & Withers 2003). The presence of several non-target hosts, including at least one that is thought to be outside of the ecological host range of *E. nassaui,* provides the opportunity to compare the outcomes and predictive abilities of choice and no-choice host specificity tests. The behavioural and physiological characteristics of the two parasitoid species are considered in terms of the consequences for their rearing and treatment in quarantine prior to host specificity testing. How their characteristics influence the outcomes of host specificity tests are discussed with regard to the appropriateness of testing methods, test conditions and the correct interpretation of test results. The interactions between the two primary parasitoids and a hyperparasitoid are also explored to highlight the degree to which behavioural characteristics and ecological interactions influence realised host ranges and pest suppression when pest management is based on classical biological control.

Goal 1: Compare the physiological and behavioural characteristics of two established egg parasitoids that differ in their effectiveness as BCAs of a forestry pest and discuss how such characteristics may influence the outcomes of different host specificity tests.

Goal 2: Compare the host ranges predicted by choice and no-choice tests in the laboratory to the realised host ranges of two parasitoids of a forestry pest in New Zealand.

Goal 3: Investigate the general biology of a self-introduced hyperparasitoid of an effective biological control agent and assess how its interactions with two established primary parasitoids and their physiological and behavioural characteristics may influence the biological control of a forestry pest in New Zealand.

Based on these broad objectives a number of experiments have been devised, the specific objectives of which are given in the following chapters. In chapter 2, some physiological and behavioural characteristics of the two primary parasitoids are identified and compared, primarily to facilitate the production of suitable insects for experiments in the subsequent chapters. In chapter 3, no-choice tests are used to determine the physiological host ranges of the two parasitoids and to assess the sex ratio of their progeny when reared on different hosts. In chapter 4, paired choice tests are conducted and the results compared to those of no-choice tests in chapter 3 to assess the value of the information gained from the different test types. Host preferences and the accuracy with which choice and no-choice test results reflect the realised host ranges of the two species are assessed. Following from observations made in chapter 2 and earlier in chapter 4 that the two species will compete for access to hosts the effect of parasitoid density on the acceptance of less preferred hosts is investigated. This is expanded in chapter 5 where their host acceptance behaviour and the nature of competitive interactions between the two species are assessed in detail. These interactions are discussed with regard to the different behavioural and physiological characteristics of the species and their possible consequences for biological control of the target pest. Finally in chapter 6 the dynamic nature of biological control as a method of pest management is considered in a study of the hyperparasitoid that arrived subsequent to successful suppression of *P. charybdis*. The biology of the hyperparasitoid, its ability to exploit the two primary parasitoids, and the overlap in their distributions are determined and discussed in terms of the future control of the target pest.

CHAPTER 2: INSECT CULTURES & PREPARING PARASITOIDS FOR EXPERIMENTAL TRIALS

2.1 INTRODUCTION

Seven laboratory colonies were required for the experiments conducted in this study (Fig. 2.1). Although rearing methods have been established for *P. charybdis* and *E. nassaui*, new and improved techniques were required for the remaining species. Insect rearing is a science in itself as all species have unique requirements, and there is an abundance of detailed literature on the subject (e.g. King & Leppla 1984; Singh & Moore 1985). Many species, for instance, will not mate and/or oviposit when caged. Such problems can often be mitigated by providing substrates that allow communication between individuals, or which provide them with particular physical, tactile or chemicals cues. Natural light, adequate space and airflow, appropriate insect densities (see section 4.3) and adequate nutrition may also have important roles in stimulating mating and egg laying and other normal behaviour in caged environments. Fortunately, methods that have been used successfully for one species can often be adapted for other closely related species.

In addition to producing sufficient numbers of insects for experimental work, methods to produce individuals of a quality comparable to that found under natural conditions must be determined. In particular, parasitoids capable of parasitising the target host at similar levels as in nature, and hosts that display normal behavioural and physiological defences against parasitism, must be produced.

In section 2.2 methods used to maintain insect cultures are described. Section 2.3 expands on the particular difficulties encountered producing *N. insectifurax* females for use in the experiments described in chapters 3, 4 and 5. Rearing techniques and behavioural observations made during rearing directly affected experimental protocols used throughout the study. Details of these protocols are presented in section 2.4.

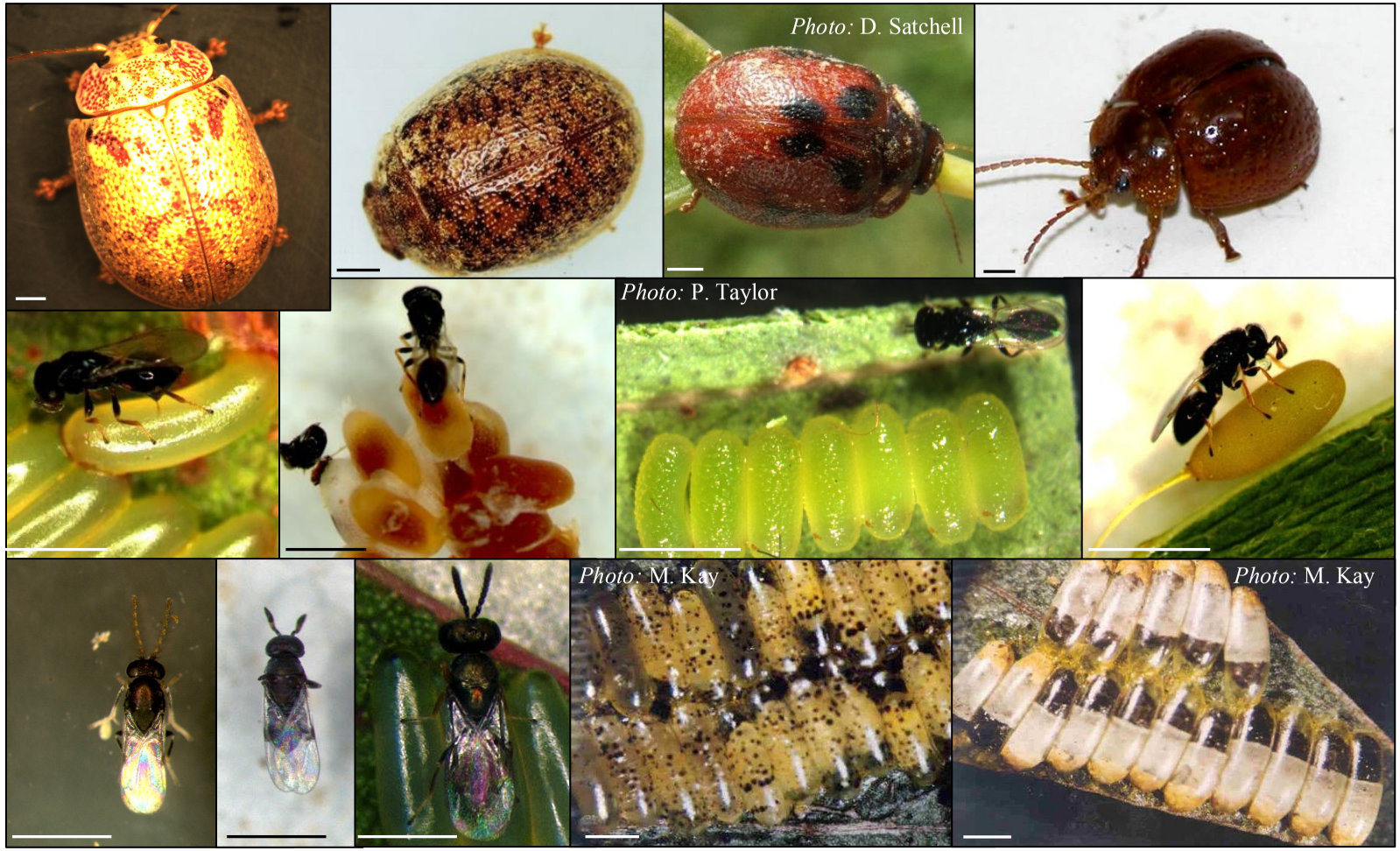

Figure 2.1: Top from left – *P. charybdis*, *T. sloanei*, *T. catenata*, *D. semipunctata*, scale bars = 1 cm. **Middle -** *E. nassaui* on eggs of: (from left) *P. charybdis*, *T. sloanei*, *T. catenata*, *D. semipunctata,* scale bars = 1 mm. **Bottom from left –** male *B. albifunicle*, female *B. albifunicle*, *N. insectifurax*, *P. charybdis* eggs parasitised by: *E. nassaui*, *N. insectifurax, s*cale bars = 1 mm. Photos by author except where indicated otherwise.

2.2 INSECT CULTURES

All seven insect colonies were established from field-collected insects (Table 2.1). Most were supplemented throughout the study with additional field-collected adults (*P. charybdis*, *D. semipunctata*), or parasitised eggs (*E. nassaui*, *N. insectifurax*, *B. albifunicle*), primarily between November and April each year.

Table 2.1: Life stage, location, and primary collection dates of field collected insects used to establish and maintain seven laboratory colonies during this study. A = Adult, P = parasitised *P. charybdis* eggs. See Appendix 1 for geographical coordinates.

Species	Life stage	Source location & collection date
P. charybdis	A	Rotorua, Kapenga, Nov. 2005, July 2006
T. sloanei & *T. catenata*	A	Gisborne, Mar. 2006
D. semipunctata	A	Kerikeri, Jan. 2005 / 2006, Pirongia Dec. 2007
E. nassaui	P	Southland, Feb. / Mar. 2006, Kapenga Dec. 2006
N. insectifurax	P	Wairakei & Waihi, Mar. 2006, Kapenga, Feb. 2007
B. albifunicle	P	Wairakei, Feb. 2006, Waihi, Dec. 2006, Rotoiti, Feb. 2007

Paropsis charybdis

Paropsis charybdis were maintained in an environmentally controlled room (22 ± 2 ^{o}C, 65% r.h., 14L:10D). Fifty to 200 adults were contained in two perspex cages (1.0 m tall x 0.7 m x 0.7 m) with removable front panels for access. Adults were fed new growth *E. nitens* foliage. Foliage was collected weekly from Kapenga plantation (Appendix 1) 7 km south of Rotorua adjacent to State Highway 30 and kept fresh by placing the cut stems into a jar of tap water . Eggs laid on the foliage were collected three times per week by plucking off the leaf tip to which they were adhered. These eggs were stored at 4 ^{o}C until required for parasitoid rearing or experiments.

Trachymela sloanei & Trachymela catenata

Trachymela sloanei and *T. catenata* were maintained on *E. nitens* as described for *P. charybdis*. Foliage was renewed, and eggs harvested, twice weekly and stored at 4^{o}C until required for experiments. As *T. sloanei* deposit their eggs in bark crevices, rather than on leaf blades, artificial oviposition sites were created. The method of Millar et al. (2000) was followed by pinning stacks of 2 cm^2 pieces of 1 mm cork sheet to pieces of foam that were

then placed among the stems and leaves of the *E. nitens* foliage. Females laid their eggs between the cork layers. Individual cork pieces bearing eggs were harvested three times a week. As beetles were not easily obtainable from the field some eggs were allowed to hatch and complete development to ensure reproductive adults were added frequently to each colony.

Dicranosterna semipunctata

Dicranosterna semipunctata were maintained from November to March in 2005, 2006 and 2007. Adults were contained in ventilated perspex cages (1.0 m tall x 0.7 m x 0.7 m) and fed fresh new-growth *A. melanoxylon* foliage, collected locally every 2-3 days. Individual eggs were harvested three times per week by plucking off the leaf to which they were adhered, and stored at 4 ^{o}C until required. Initially, *D. semipunctata* were maintained in the environment controlled room housing the *P. charybdis* colony. However, insufficient natural light disrupted egg laying behaviour, resulting in an inconsistent supply of eggs for use in experiments. Beetles were relocated to a workshop bench with abundant natural light and maintained under ambient conditions.

Enoggera nassaui* & *Neopolycystus insectifurax

Parasitoids were maintained in an environmentally controlled quarantine facility (22 ± 2 ^{o}C, 65% r.h., 14L:10D) and provisioned with pure honey, renewed weekly, as a carbohydrate source. Initially, both species were reared in large Petri dishes (90 mm diameter) in which ten 2 to 3-day-old adult wasps were presented with seven 1 to 7-day-old *P. charybdis* egg batches (stored at 4 ^{o}C since collection) three times a week. After 24 h, adults were removed and the eggs left to develop. Any *P. charybdis* larvae that emerged from unparasitised eggs were removed to prevent cannibalism of adjacent eggs. Parasitoids that emerged in each dish remained together with access to food and mates until they were required for experiments or colony maintenance.

A method was sought partway through this study to increase female parasitoid numbers. Females capable of oviposition were required for all experiments, but the two species showed no sexual dimorphism, making female identification difficult. The *N. insectifurax*

colony exhibited a male-biased sex ratio and the proportion of females produced by *E. nassaui* was also lower than desired. This is a common problem in crowded parasitoid colonies (Wylie 1976; Waage 1982, 1986; Jervis 2005). Most hymenopteran parasitoids are able to choose the sex of their offspring at oviposition by laying fertilised (female) or unfertilised (male) eggs (Waage 1986). There is substantial evidence that female eggs are preferentially allocated to higher-quality hosts (Charnov et al. 1981) (see also chapter 3). On seven occasions in October 2007, up to 20 solitary parasitoids of each species were presented with a *P. charybdis* egg batch in a 55 mm Petri dish, in parallel with a group of parasitoids presented with multiple egg batches for colony maintenance, as described in the previous paragraph. The '*individuals'* received egg batches of the same age as their counterparts in the main '*colony'* and were exposed to them for the same 24 h period under identical environmental conditions. Upon emergence, 20 parasitoids of each species were collected from a *colony* rearing dish and from several *individual* rearing dishes (< 20 individuals were present per dish). Parasitoids were frozen and dissected to determine their sex. The average yield of female progeny produced by each species under the two methods was compared using a non-parametric Wilcoxon ranked-sums test (SAS version 9.1). A significantly higher female sex ratio was obtained from both *E. nassaui* (z = -2.3168, P = 0.0204) and *N. insectifurax* (z = -2.5252, P = 0.0127) using the *individual* rearing method (Fig. 2.2).

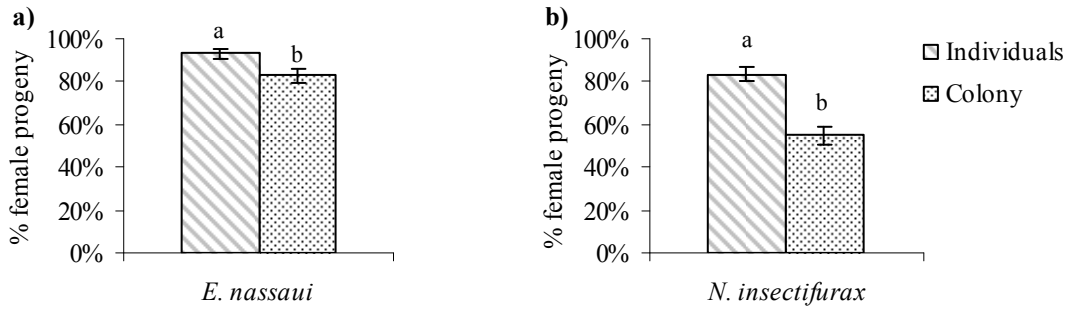

Figure 2.2: Yield ($\bar{x}$ ± SE) of female progeny following 24 h exposures of *P. charybdis* eggs to solitary (individuals) and groups (colony) of **a)** *E. nassaui* and **b)** *N. insectifurax*, in the laboratory (22 °C, 65 % r.h. 14L:10D). Means with different letters above are significantly different at $P < 0.05$.

The *individual* method was subsequently adopted for rearing both species to maximise the availability of females. Individual wasps were presented with host eggs three times per

week to produce cohorts of equal-aged adults for the 'host age' and 'wasp age' experiments in section 2.3, and for all experiments reported on in chapters 4 and 5. The success of this method is thought to be a consequence of reducing direct physical competition between females. This will be discussed in detail in chapter 5.

Baeoanusia albifunicle

Hyperparasitoids were maintained in 65 mm Petri dishes in a controlled climate cabinet (Custom made, Scion) (22 ± 2 ^{o}C; 65% r.h., 14L:10D). Twice weekly, honey and three to five groups of three *P. charybdis* egg batches, exposed to *E. nassaui* females for the preceding 24 h, were presented to groups of five 3-5 day-old *B. albifunicle* females. Wasps were removed after 48 h and the eggs left to develop. Hyperparasitoid emergence occurred after c. 14 days. Newly-emerged adults were supplied with honey, replenished twice weekly, on 2 cm^2 pieces of paper towel.

2.3 MOTIVATING *NEOPOLYCYSTUS INSECTIFURAX* TO OVIPOSIT

2.3.1 Introduction

The following study was an essential prerequisite to investigating the direct behavioural interactions between *E. nassaui* and *N. insectifurax* in the laboratory. *Paropsis charybdis* is uncommon in Australia (Styles 1970; Edwards & Suckling 1980) and there are no published accounts of it being parasitised there by *N. insectifurax*, and few (e.g. Nahrung & Murphy 2002) by *E. nassaui*. Both species do reproduce on *P. charybdis* in New Zealand, but field parasitism by *N. insectifurax* is relative low compared to *E. nassaui* (Jones & Withers 2003). This was thought, in part, to be a result of poor synchrony with the oviposition peaks of *P. charybdis* in December and February (Tribe & Cillié 2000). It may also indicate that *P. charybdis* is not a natural host of *N. insectifurax*, or is a low-ranked host. The same could be said for *E. nassaui*, yet it appears highly motivated to oviposit into *P. charybdis* eggs in New Zealand and under a range of laboratory conditions. *Enoggera nassaui* frequently parasitise all available hosts within 24 h in the laboratory, while *N. insectifurax* require up to 72 h to consistently achieve similar rates (pers. ob.). Observations of solitary females of each species in the laboratory have found that *E. nassaui* quickly locate and begin parasitising hosts. *Neopolycystus insectifurax* take

considerably longer to show interest in host eggs and tend to aggressively guard them in the presence of conspecifics, rather than commence oviposition (S. Mansfield unpub.).

Regardless of the rank of *P. charybdis* as a host, *N. insectifurax* may simply take longer than *E. nassaui* to assess and accept eggs of any species. Factors such as eggload, (Minkenberg et al. 1992) previous host experience, and environmental conditions (van Alphen & Visser 1990; Wang et al. 1997), are well known determinants of the rate at which low-ranked hosts become acceptable. The experiments below were designed to determine if factors easily manipulated in the laboratory could be used to maximise the motivational state of *N. insectifurax* so that it might begin assessing *P. charybdis* eggs as quickly as *E. nassaui*. Similarly motivated individuals of each species were required so as to observe (chapter 5) how the two species interact when both make contact with one host and therefore compete for that host as an oviposition resource.

2.3.2 Ovigeny

Differences in the process of oogenesis must be taken into account when comparing the behaviour of parasitoid species. The rate at which eggs develop in the ovaries can affect a parasitoid's motivational state at any given time, as it determines the number of mature eggs present (eggload) and therefore its capacity to parasitise a host (Withers & Browne 2004). Parasitoid ovigeny is thought to represent a continuum from pro-ovigenic, i.e. having a full complement of mature eggs at emergence, to synovigenic, where eggs continue to mature over the wasp's life (Jervis et al. 2001). In the latter case, a nutritional input or host stimulus is often required before maturation occurs (Rosenheim & Rosen 1992). Both species considered here host-feed (Clausen 1962), and this may provide such a nutritional input. Evidence suggests that *E. nassaui* is close to the pro-ovigenic end of this scale as it is capable of parasitising host eggs 24 h after emerging (S. Mansfield unpub.). One undescribed species of *Neopolycystus* in Australia is known to be pro-ovigenic, having the capacity to mate and commence oviposition within minutes of adult emergence (Appleton 2001b). If in contrast, *N. insectifurax* tend towards synovigeny, this may explain why individuals of the same age as *E. nassaui* are relatively less motivated to parasitise *P. charybdis* eggs. Determining the process of oogenesis exhibited by *N. insectifurax* may help resolve what stimuli are required before it becomes motivated to parasitise hosts.

Objectives

To determine if *N. insectifurax* tend toward pro-ovigeny or synovigeny, and whether the presence of food or host stimuli can enhance the rate at which eggload increases.

Methods

Thirty solitary *N. insectifurax* adults (0-4 h old) were exposed in Petri dishes to one of five treatments (six wasps per treatment): water only (W), honey + host remains (HR) (leaf tip bearing empty *P. charybdis* egg shells, see Fig. 2.3), water + live hosts (WH), honey (HY), honey + live hosts (HH). This was repeated six times for 12, 24, 48, 72 and 96 h in environmentally controlled cabinets (22 ^{o}C, 65% r.h., 14L:10D), giving a total of 36 replicates per time/treatment combination. Water and honey were provided on paper towel placed in each dish and renewed daily. *Live hosts* refers to *P. charybdis* egg batches < 24 h old that were replaced every 24 h in the 48, 72 and 96 h treatments. Wasps were frozen immediately after each experiment. Wasp sex and number of mature eggs in the ovaries were determined by making a slide preparation of the abdomen that was viewed (100 x mag.) using a binocular microscope (Axioskop2, Zeiss, Germany).

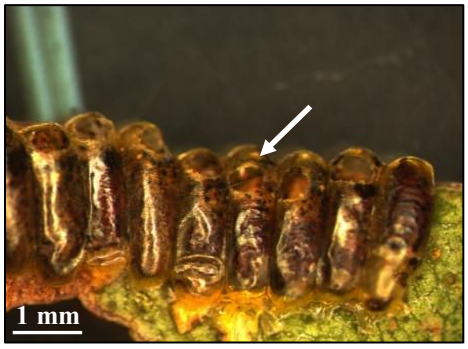

Figure 2.3: An example of 'host remains'. Parasitised *P. charybdis* eggs on an *Eucalyptus* leaf tip from which adult parasitoids have previously emerged. The mottled black and orange colouration of the egg shells and exit holes (indicated with arrow) made during parasitoid emergence specify that the pictured eggs were parasitised by *E. nassaui*.

Progeny that emerged from host eggs following the HH and WH treatments were counted and included in the eggload count of each parent wasp. These procedures were repeated over 12, 24 and 48 h with *E. nassaui* females for the HY and W treatments (15 replicates each). Average eggload of *N. insectifurax* females submitted to each time/treatment combination was initially compared using a GLM ANOVA. Mean eggload of *N. insectifurax* under each of the three honey treatments between 24 h and 96 h, of *E. nassaui* on honey and on water between 24 h and 48 h, and of *N. insectifurax* vs. *E. nassaui* reared on water only and honey only, were each compared using Generalised Linear Model ANOVA with Poisson distribution (Proc GENMOD, SAS 9.1, SAS Institute, 1999).

Results

As wasp sex could only be verified upon completion of each experiment, the total number of females ultimately exposed to each time/treatment combination ranged from 7-24 (Table 2.2). There were significant differences in the mean eggload of females exposed to each treatment ($F = 317.95$, df = 5, $P < 0.001$), each time ($F = 352.32$, df = 4, $P < 0.001$) and treatment by time ($F = 64.44$, df = 16, $P < 0.001$). No eggs were found in the ovaries of any females at 12 h. The interaction between treatment and time resulted from a steady increase in eggload with time for all three treatments that included honey, but not for water only or water + hosts (Fig. 2.4). Significantly fewer eggs were found in the ovaries of females in the latter two treatments (W and WH), most of which died within 24-48 h of the longer experiments. There were significant differences in the eggload of females submitted to each of the three honey treatments ($F = 10.81$, df = 2, $P < 0.001$) and all times ($F = 163.7$, df = 3, $P < 0.001$). Honey-fed females with access to live hosts had the highest eggload (HH, $\chi^2 = 6.45$, $P = 0.0111$) and these increased steadily with time (Fig. 2.4). Mean eggload of females with access to honey and host remains (HR) was greater than that of females with access to honey alone (HY, $\chi^2 = 20.47$, $P < 0.001$). Under both these treatments eggload increased with time and began to stabilise after 72 h (Fig. 2.4).

Table 2.2: Total number of females out of 36 parasitoids exposed for each time/treatment combination.

Time (h)	W	HR	WH	HY	HH
12	16	20	16	21	17
24	23	22	22	20	24
48	17	17	24	20	20
72	15	20	12	21	19
96	10	7	14	8	8

Eggload of *E. nassaui* increased with time ($F = 75.95$, df = 2, $P < 0.001$); however, there was a time-treatment interaction ($F = 86.72$, df = 2, $P < 0.001$). This was explained by a steady increase in eggload over time in the presence of honey (Fig. 2.5a) while eggload only increased in the presence of water up to 24 h and then decreased at 48 h as most females died (Fig. 2.5b). The eggload of *E. nassaui* fed honey or water was significantly higher after 24 h than *N. insectifurax*. After 48 h a significant species by time interaction

indicated there was no difference in eggload between the species on either honey or water ($F = 8.43$, df = 1, $P = 0.0037$).

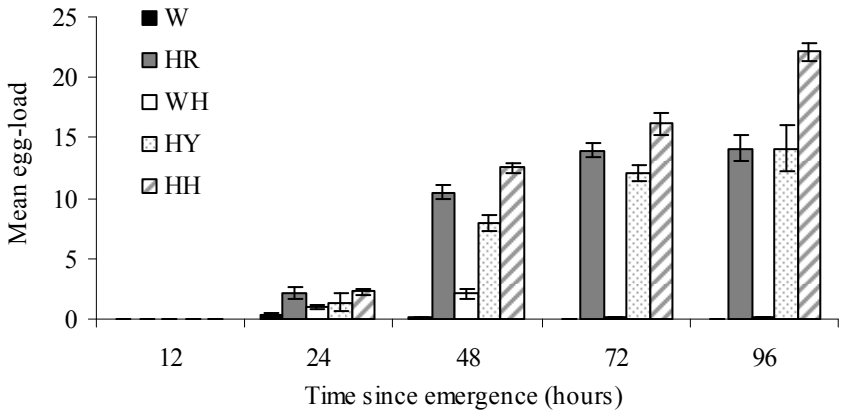

Figure 2.4: Eggload ($\bar{x}$ ± SE) of *N. insectifurax* exposed to five different treatments for 12, 24, 48, 72 or 96 h (22 °C, 65% r.h., 14L:10D) immediately following emergence. W = water only, HR = honey + host remains, WH = water + live hosts, HY= honey only, HH = honey + live hosts.

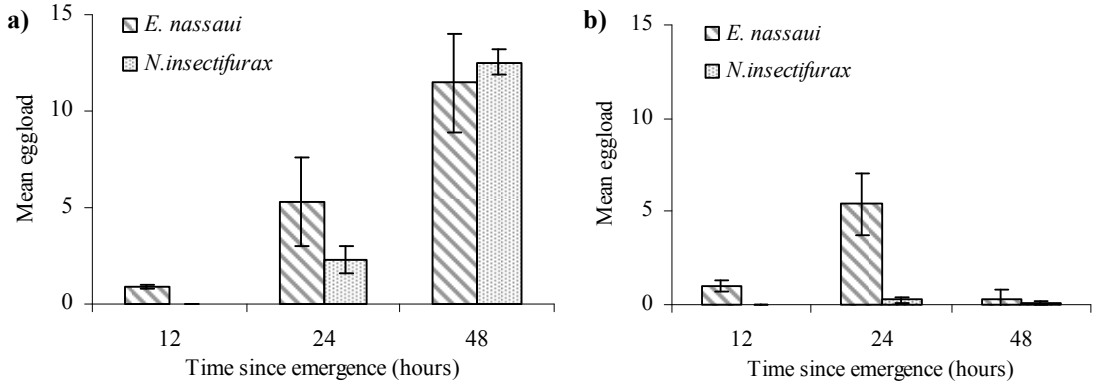

Figure 2.5: Eggload ($\bar{x}$ ± SE) of *E. nassaui* and *N. insectifurax* fed **a)** honey only or **b)** water only, for the first 12, 24 and 48 h after emergence (22 °C, 65% r.h., 14L:10D).

Discussion

Enoggera nassaui is not strictly pro-ovigenic but has a shorter pre-oviposition period than the relatively more synovigenic *N. insectifurax*. This difference in oogenesis may cause the two species to experience very different motivational states towards hosts, especially low-ranked hosts during the first 48 h post-emergence. For example, *E. nassaui* are capable of parasitism within 24 h of emergence, but because of their slower rate of egg maturation, *N. insectifurax* may not be physiologically capable of parasitism for 48 h. If they are, eggload

may still be very low. The motivation of *N. insectifurax* to accept a low-ranked host during this time may be reduced because fitness losses could be increased by wasting limited eggs on poor quality hosts (Heimpel & Rosenheim 1998). Two-day-old *E. nassaui*, with their much higher eggload, may be time-limited rather than egg-limited, and therefore motivated to accept even low-ranked hosts to avoid incurring fitness costs by dieing with eggs in their ovaries (Godfray 1994).

Although eggload does not determine the motivational state of a parasitoid on its own, it can provide clues as to when and under what conditions the parasitoid is most likely to accept hosts. In this study, the eggload of *N. insectifurax* was highest when females were provided with honey and live hosts for 96 h. Nutritional input from honey and host feeding has often been shown to have a positive effect on eggload and parasitism rates (Wylie 1976; Rosenheim & Rosen 1992; Ferreira de Almeida et al. 2002; Giron et al. 2004). All else being equal, a fed and mated parasitoid with a high eggload is more likely to locate and accept a natural host than is an identical wasp with a low eggload. For example, *Aphytis linganensis* Compere individuals have significantly reduced chances of finding hosts in 30 minute experiments when eggload is low (Rosenheim & Rosen 1991). If there is a degree of physiological and behavioural plasticity, chemical stimuli associated with the host, rather than just its nutritive value if host feeding occurs, may also increase eggload and improve a parasitoid's motivational state (Rosenheim & Rosen 1991; Mangel & Heimpel 1998; Jervis et al. 2001). However, the presence of live hosts during colony maintenance allows oviposition experience, and may lead to reproductive senescence if all eggs are laid. It is generally desirable therefore to use naïve parasitoids in host specificity tests, because experience with one host species can affect the acceptance of the same or other species during subsequent encounters (Withers & Browne 2004; see also section 4.2). In the absence of live hosts, the eggload of *N. insectifurax* still increased for up to 72 h, especially when host remains were present. This reflects the situation in previous laboratory colonies, where almost 100% parasitism was achieved by leaving *N. insectifurax* with host eggs for three days. It is not clear if the presence of the host remains, or the leaf (host habitat cue) to which they are adhered provides the stimulus that increases the rate of oogenesis. It was not considered necessary to distinguish the effects for the purpose of this study.

2.3.3 Parasitoid age

As parasitoids age they become increasingly time-limited if mature eggs still remain in the ovaries. This limitation can affect the range of host species or instars the parasitoid will accept (Völkl & Mackauer 1990; Weisser 1994; Riddick 2003; Withers & Browne 2004). Fecundity may also vary with age depending on whether the parasitoid is pro-ovigenic or synovigenic as discussed in the previous section, or is suffering from host deprivation (Minkenberg et al. 1992; Withers & Browne 2004). Determining if female age affects the motivation of *N. insectifurax* to accept *P. charybdis* eggs may aid in the selection of individuals that are likely to display oviposition behaviour as will be required to conduct observational experiments in chapter 5.

Objectives

To compare the levels of parasitism achieved by *N. insectifurax* females of different ages, and to determine at what age *N. insectifurax* is capable of achieving parasitism levels equal to those of three-day-old *E. nassaui*.

Methods

Groups of colony-reared *N. insectifurax* and *E. nassaui* adults were provisioned with honey and host remains and held in an environmentally controlled room (22 ^{o}C, 65% r.h., 14L:10D) for three, six or eight days following emergence. One-hundred and twenty wasps (30 x 3, 6 and 8-day-old *N. insectifurax* = N3, N6, N8, and 30 x 3-day-old *E. nassaui* = E3) were placed in individual Petri dishes and supplied with honey for 2 h. Each was then presented with a batch of 8-12 *P. charybdis* eggs < 48 h old, that had been viewed under a microscope (Stemi SV6, Zeiss, Germany, 10 x mag.) to ensure minimal embryonic development had occurred (Fig. 2.7a). Wasps were removed and frozen after 4 h and dissected to determine their sex. Egg batches were incubated separately (22 ^{o}C, 65% r.h., 14L:10D) and the number of individual eggs yielding parasitoid progeny was recorded. All *P. charybdis* larvae that hatched were removed to prevent cannibalism of adjacent eggs. Paired Wilcoxon ranked-sums tests were used to compare the mean proportion of eggs parasitised by wasps of each age. *P*-values were adjusted with a sequential Bonferroni procedure (R Development Core Team, 2008) to control for increased type-I-error resulting form multiple comparisons.

Results

Ninety-two to 100% of females of all ages parasitised some of the host eggs provided. Three-day-old *E. nassaui* parasitised a significantly higher proportion of individual eggs (Fig. 2.6) compared to three, six and eight-day-old *N. insectifurax* (N3 z = -3.7142, P = 0.0025; N6 z = -3.4557, P = 00044; N8 z = -4.4200, P = 0.0006). There were no significant differences between the mean proportion of eggs parasitised by *N. insectifurax* of any of the different ages tested.

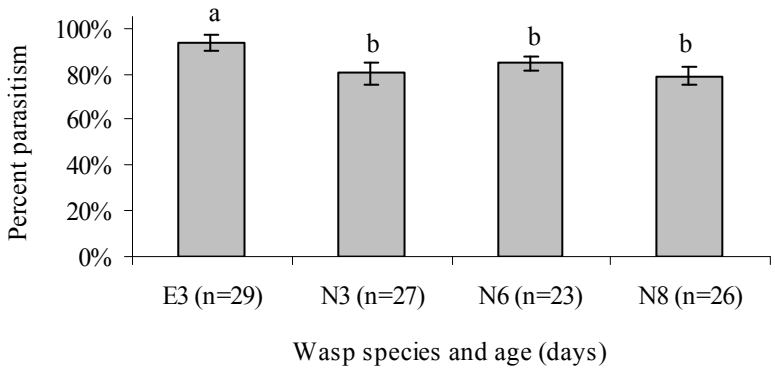

Figure 2.6: Proportion ($\bar{x}$ ± SE) of eggs parasitised by 3-day-old *E. nassaui* (E3) and *N. insectifurax* of three (N3), six (N6) and eight (N8) days of age during a 4 h laboratory experiment (22 ^{o}C, 65% r.h.). Means with different letters above are significantly different at $P < 0.01$.

Discussion

The highest level of parasitism achieved by *N. insectifurax* of any age was still significantly lower than that of 3-day-old *E. nassaui*. There is no reason, therefore, to consider using the two species at different ages. Doing so would only add unwanted complexity to the rearing process and potentially create other physiological and behavioural differences between the species. If the behaviour of equal-aged *E. nassaui* and *N. insectifurax* is to be compared in chapter 5, then this age must be chosen to ensure both species are in a similar physiological state with regard to their motivation to oviposit. Assuming oogenesis occurs on a continuum from complete pro-ovigeny to synovigeny (Jervis et al. 2001), and that ovigeny of *E. nassaui* and *N. insectifurax* occur at different points on that continuum, then parasitoids must be allowed to age sufficiently so that both species are able and motivated to oviposit, but not so long that processes such as oosorption begin to influence motivation.

As a strongly synovigenic species (section 2.3.2.), the willingness of naïve *N. insectifurax* to oviposit should increase with age, as a function of increasing eggload, host deprivation and time limitation (e.g. Withers & Browne 2004). However, there was no significant increase in parasitism by six or eight-day-old *N. insectifurax* compared to three-day-olds in this study. Because *E. nassaui* is able to survive slightly longer in the laboratory in the absence of food compared to *N. insectifurax* is thought to have the capacity to resorb eggs (S. Mansfield unpub.). If egg resorption does occur, motivation of older *E. nassaui* may decrease, especially if sufficient food is not available. Considering the risk of resorption by *E. nassaui*, and given that no significant increase in parasitism was achieved by allowing *N. insectifurax* to age more than three days, 3-day-old individuals of both species will be used in comparative experiments in chapter 5. Using parasitoids at the youngest age possible in the maintenance of colonies also reduces the time required to rear successive parasitoid generations, thereby allowing more efficient use of limited resources.

2.3.4 Host age

In a review of physiological interactions between parasitoids and their hosts, Strand (1986) noted that most parasitoids are adapted to develop on a specific host stage (i.e. egg, larva, pupa or adult), indicating that the developmental state of the host is critical in determining host suitability. Egg parasitoids used for biological control, primarily *Trichogramma* species (Hymenoptera: Trichogrammatidae), have often been shown to find late-stage host eggs, that contain well developed embryos, less acceptable than younger host eggs (e.g. Reznik & Umarova 1990; Makee 2005). In a series of 30 minute laboratory experiment, S. Mansfield (unpublished data) observed very few attempts by solitary *N. insectifurax* to oviposit into undeveloped *P. charybdis* eggs (Fig. 2.7a). On one occasion, however, a batch of well developed eggs (Fig. 2.7d) was accidentally supplied, and the female immediately responded by beginning to parasitise them. The lack of motivation to attack young eggs in the previous experiments might indicate, therefore, that host eggs need to develop substantially before they are suitable for oviposition. This was supported by the observation that, at 22 ^{o}C, parasitoids in the laboratory colony required up to three days to parasitise freshly laid host eggs, which, if not parasitised, would hatch out *P. charybdis* larvae after 4-5 days at that temperature.

Objectives

To determine the levels of parasitism achieved by 3-day-old *N. insectifurax* when presented with *P. charybdis* eggs of different ages, and to compare these to parasitism levels achieved by 3-day-old *E. nassaui* under the same conditions.

Methods

Two hundred batches of 5-13 colony-reared *P. charybdis* eggs < 48 h old were viewed under a microscope (Stemi SV6, Zeiss, Germany, 10 x mag.). Those showing minimal signs of embryonic development (Fig. 2.7a) were divided into four groups. One group was immediately relocated to 4 ^{o}C to suspend development. The remaining three groups were maintained at 22 ^{o}C for 24, 48 and 72 h respectively before also being relocated to 4 ^{o}C. After a further 24 h, all four groups were returned to 22 ^{o}C. Eggs were reassessed to ensure batches within each group shared the same visible signs of development (Fig. 2.7a-d). These groups were regarded to represent < 1, 1, 2 and 3 days of development. The most well developed group was further divided to represent 2.5 and 3 days of development as the prolegs of pharate 1st instar larvae were more clearly visible in the latter group.

At the time the groups of egg batches were returned to 22 ^{o}C, 168 3-day-old *N. insectifurax* and 30 *E. nassaui* adults were placed into individual Petri dishes provisioned with honey. All wasps had been reared in groups at 22 ^{o}C and provided with honey since emergence. After 2 h, an egg batch from one of the five groups was presented to each *N. insectifurax* adult, and < 1-day-old batches were presented to *E. nassaui* adults. After a further 4 h, wasps were recaptured, frozen and dissected to confirm their sex. Host eggs were incubated at 22 ^{o}C and any *P. charybdis* larvae that hatched were removed to prevent cannibalism of adjacent eggs. Percent parasitism was recorded after 4-5 days when parasitised eggs changed in colour. The mean proportion of eggs parasitised was compared between all age groups using a non-parametric Kruskal-Wallis ANOVA (SAS version 9.1). Paired Wilcoxon ranked-sums tests were then used to assess which means differed from one another. *P*-values were adjusted using a sequential Bonferroni procedure to control for an increased chance of type-I-error resulting from multiple comparisons.

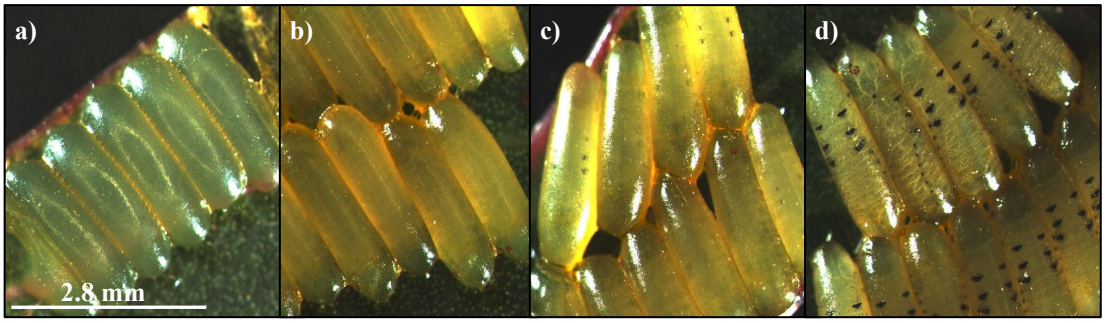

Figure 2.7: Visible stages of *P. charybdis* egg development at 22 °C: **a)** < 1-day-old, clear, no visible structures, blue/green; **b)** 1-day-old, opaque, anterior/posterior orientation visible, blue/green to yellow; **c)** 2-days-old, prolegs, segmentation and movement perceptible, yellow; **d)** 3-days-old, pharate 1st instar larvae well developed, prolegs, segmentation, hairs and spiracles visible, larvae hatch within 24 h.

Results

A total of 58 replicates were discarded across all host ages as verification of wasp sex indicated they had been exposed to males. Over 93% of females provided with hosts 2-days-old or younger successfully parasitised some eggs, whereas only 50% and 6.25% successfully parasitised any 2.5 and 3-day-old eggs respectively. Host age had a significant effect on parasitism (H = 60.23, df =5, P < 0.0001). The mean proportion of eggs parasitised by *N. insectifurax* decreased with increasing host age from 91.4% of < 1-day-old to just 1.27% of 3-day-old eggs (Fig. 2.8). Significantly fewer 2.5 and 3-day-old hosts were parasitised compared to hosts 2-days-old or younger eggs (z = -3.34, P = 0.0014). When presented with < 1 and 1-day-old eggs, parasitism by *N. insectifurax* was better or equal to that of *E. nassaui* on < 1-day-old eggs, but not significantly different.

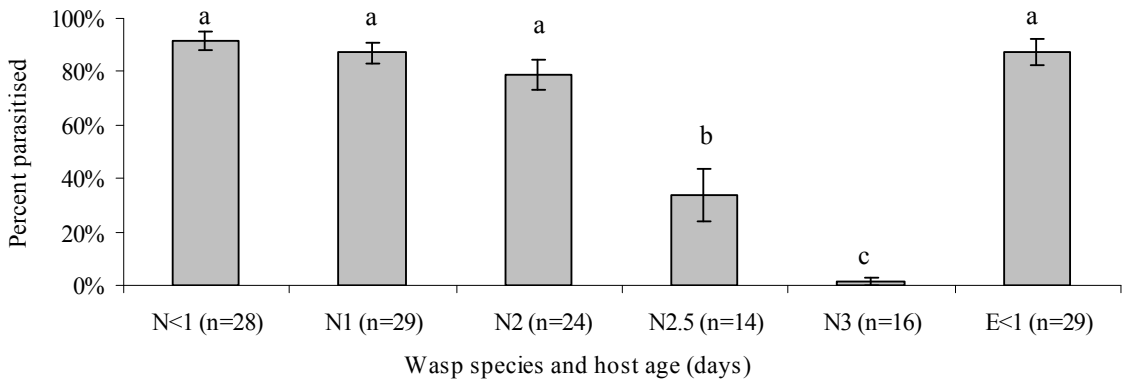

Figure 2.8: Percent parasitism ($\bar{x}$ ± SE) of *P. charybdis* eggs exposed for 4 h at 22 °C to < 1-day-old *E. nassaui* (E < 1) and < 1, 1, 2, 2.5 and 3-day-old *N. insectifurax* (N < 1, N1, N2, N2.5 and N3). Means with different letters above are significantly different at P < 0.05.

Discussion

Many larval parasitoids selectively attack older host instars but whether this preference is driven by the age or size of the hosts is difficult to determine (e.g. Joyce et al. 2002; Jervis 2005). The effects of host age on acceptance by egg parasitoids have mostly been studied with regard to optimising the use of *Trichogramma* species for biological control. Preferences for younger hosts have often been recorded (Reznik & Umarova 1990; Makee 2005) but several species also show preferences for particular host species as a function of their relative size (e.g. Mansfield & Mills 2002). For idiobiont parasitoids, a host represents a fixed parcel of resources that determines the eventual size of its progeny and in turn their fecundity (Rosenheim & Rosen 1992). Therefore, selecting larger host eggs theoretically increases the fitness of a parasitoid. In New Zealand, *P. charybdis* eggs, that do not change in size as they age, are probably the only hosts encountered by many *E. nassaui* and *N. insectifurax*. Consequently, individual parasitoids probably encounter hosts of very uniform size and host age may be a more important indicator of quality.

Chorion thickness, and the ratio of chorion thickness to egg volume, have been shown to preclude parasitism of some species by *Trichogramma platneri* Nagarkatti, and limit the acceptability of others (Mansfield & Mills 2002). Chorion ultrastructure of paropsine eggs has also been cited as a potential inhibitor of parasitism (Murphy 2005) but it is not known if structure or thickness-to-volume ratios change as these eggs age. Such changes could potentially influence host acceptance as a function of host age. Both *E. nassaui* and *N. insectifurax* appear to arrest host embryo development, and feed on the undifferentiated egg contents rather than embryo tissue. It is likely, therefore, that a reduction in the volume of the host egg that is not occupied by the host embryo, rather than chorion characteristics, reduces the acceptability of older host eggs for oviposition.

Larval parasitoids have to cope with the immune response of their host (Strand 1986 and references therein). This response often results in an increased rate of encapsulation with host age (Salt 1968). Insect eggs, in contrast, have no cellular defences (Salt 1968). Host age effects on the survival and development of egg parasitoids are therefore more likely to be a function of digestibility or the time available for parasitoid development. Host eggs develop from a single cell to a pharate 1st instar larva before hatching. Egg parasitoids are

generally understood to disrupt host embryogenesis either as a direct result of larval feeding and movement, or by the injection or secretion of substances by the adult parasitoid or larva, respectively, that arrest host development or cause necrosis (Salt 1968; Strand 1986). If feeding disrupts *P. charybdis* embryogenesis, the period of time that the host is susceptible to parasitism by *N. insectifurax* may be relatively short. This period would be limited by the time required by the parasitoid to hatch and begin feeding, and its ability to digest any host tissue already formed. An opportunity for the movement of *N. insectifurax* larvae to disrupt host development has been observed. At 22 ^{o}C, parasitoid larvae hatch within 24-48 h, and they are very mobile within the host egg for at least the next 48 h until they occupy almost its entire volume. There is also opportunity for adult *N. insectifurax* to inject development-arresting or necrosis causing factors into host eggs as oviposition is preceded by a period of probing (see chapter 5). During probing, the ovipositor is inserted into the egg and moved around. After oviposition there is a change in the structure of the host egg contents radiating out from the parasitoid egg (see Fig. 5.6c). Although the nature of this change has not been assessed it could potentially be associated with necrosis factors.

Regardless of which of the above strategies a parasitoid employs, at a certain point in the host's development a parasitoid will be unable to arrest that development or will have insufficient time to hatch and physically disrupt host embryogenesis. Tribe (2000) reported that parasitism of *Trachymela tincticollis* (Blackburn) by *Neopolycystus* sp., in Western Australia, decreased with host age from 24 h to 72 h and was unsuccessful at 96 h. Similarly, in this study *N. insectifurax* parasitised almost all available hosts that had been allowed to develop for up to two days, but successful parasitism of older eggs decreased markedly. Some 66% of 2.5-day-old eggs failed to yield parasitoid progeny. Only two 3-day-old host eggs were successfully parasitised and *P. charybdis* larvae hatched from 142 (91%). As direct observations were not made, it is not clear if eggs from which parasitoids did not emerge were rejected by the adult female parasitoids, or were accepted and parasitoid larvae failed to complete their development because of factors such as those described in the previous paragraph. The results do suggest, however, that at between two and three days (22 ^{o}C) host embryogenesis has progressed to a point beyond which larval feeding, movement, or chemically mediated factors have no effect. At this point, host eggs are either rejected or are no longer susceptible to parasitism that does occur.

The ability of parasitoid females to assess the developmental state of a host may depend on both physical and chemical cues. *Enoggera nassaui* and *N. insectifurax* assess the external surface of host eggs by walking up and down the length of the egg and antennating (see chapter 5) before ovipositing. This type of behaviour has been shown to be associated with the detection of host kairomones for host recognition, and with assessing egg shape and volume (Schmidt & Smith 1885). The latter could possibly vary with developmental state. Both parasitoids also probe with the ovipositor, especially before ovipositing in the first egg of a batch. This may provide further chemical and physical information to assess the developmental state of the host.

2.3.5 Optimum temperature

In observations that will be made in chapter 5 to compare the oviposition behaviour of *N. insectifurax* to that of *E. nassaui*, temperature may influence the motivation of *N. insectifurax* to parasitise *P. charybdis* eggs. Increasing temperature may increase parasitoid activity or accelerate the parasitoid's egg maturation rate (Minkenberg et al. 1992; Rosenheim & Rosen 1992; Wang et al. 1997). These changes might increase host encounter rates (Rosenheim & Rosen 1992) and therefore the likelihood of *N. insectifurax* displaying oviposition behaviour. As the observations in chapter 5 are to be of short duration (30 min.) the temperature at which they are conducted may not alter the motivational state of the wasps but temperature effects on egg maturation prior to the observations could influence the state of the parasitoids going into the experiments. If so, temperature could potentially be manipulated during colony maintenance to maximise the chance that oviposition behaviour is exhibited when required for experiments.

Objectives

To assess the effect of temperature on parasitism and sex allocation by *N. insectifurax* on *P. charybdis* in the laboratory.

Methods

A single cohort of newly-emerged (< 2 h old) *N. insectifurax* was split into three equal groups. Each group was maintained in a temperature-controlled cabinet at 18, 22 or 27 ^{o}C

(65% r.h., 14L:10D) and provisioned with honey and host remains. Three days after emergence, 20 parasitoids from each temperature regime were separated into individual Petri dishes and provided with honey and a single batch of *P. charybdis* eggs (< 48 h old) for 24 h. Each wasp was subsequently frozen and its sex verified by preparing a slide of the abdomen to view the genitalia. Egg batches that had been exposed to male parasitoids were discarded and those exposed to females were incubated (22 ^{o}C) until any parasitoid progeny emerged. All *P. charybdis* larvae that hatched were removed to prevent cannibalism of unhatched or parasitised host eggs.

This entire procedure was repeated on six occasions until > 30 host egg batches had been exposed to a female parasitoid at each temperature. An equal number of egg batches, and individual eggs when possible, were allocated to each temperature on each occasion. Following the first three repeats of the experiment, parasitoid progeny that emerged from three egg batches per temperature were frozen and dissected to determine sex ratios. Only batches that were completely parasitised, and from which no progeny escaped or died, were used. Mean percent parasitism and percent of eggs that collapsed (i.e. no *E. nassaui* adults or *P. charybdis* larvae emerged) were compared between temperatures using a non-parametric Kruskal-Wallis ANOVA. Wilcoxon ranked-sums tests, with *P*-values adjusted using a sequential Bonferroni procedure, were conducted to determine at which temperatures means were significantly different from each other.

Results

All but one female at each temperature oviposited in at least some of the host eggs provided (Table 2.3). As temperature increased, there was a non-significant trend for parasitism to increase (H = 4.835, df = 2, P = 0.089) and a corresponding decrease in the proportion of host eggs that collapsed. Significantly more host eggs collapsed at 18 ^{o}C than 27 ^{o}C (z = -2.4333, P = 0.0261). The sex ratios of parasitoid progeny produced under all three temperatures were almost identical (H = 1.3472, df = 2, P = 0.5099).

Table 2.3: Proportion ($\bar{x}$) of individual host eggs from which parasitoid progeny emerged, and that collapsed, following 24 h exposure to female *N. insectifurax* reared and tested at three different temperatures. The proportion ($\bar{x}$) of female progeny that emerged from a subset of parasitised host batches are indicated. Means (within columns) with different letters beside are significantly different at $P < 0.05$.

Temperature	Females tested	Females ovipositing	$\bar{x}$ Eggs parasitised (%)	$\bar{x}$ Eggs collapsed (%)	$\bar{x}$ Female progeny (%)
18°C	39	38	78.21 [a]	21.60 [a]	86.38 [a]
22°C	41	40	80.46 [a]	16.96 [ab]	86.06 [a]
27°C	37	36	90.55 [a]	6.46 [b]	84.50 [a]

Discussion

A direct link between parasitism rates and eggload has been shown for some hymenopteran parasitoids, as has a link between egg maturation and temperature (Rosenheim & Rosen 1991; Minkenberg et al. 1992). In this study, there was a trend for increased parasitism by *N. insectifurax* when rearing temperature was increased from 18-27 °C, but this was not significant. Ferreira de Almeida et al. (2002) conducted similar experiments to those described here but with the larval parasitoid *Tachinaephagus zealandicus* Ashmead (Hymenoptera: Encyrtidae). Although no significant difference was found in the number of hosts killed between 20 and 27 °C, fewer attacks were made, and less progeny produced, at the higher temperature of 29 °C. In the present study, in contrast, there was a slight reduction in the number of *N. insectifurax* progeny yielded at lower temperatures. This reduction might indicate that the parasitoids move more slowly and have a lower host-encounter rate at lower temperatures. However, if we assume that all eggs that collapsed did so because of failed parasitism attempts or rejection after probing, the declining trend disappears. Tribe (2000) reported a 20% increase in the number of *T. tincticollis* eggs that collapsed following probing by hyperparasitoids that did not oviposit. Probing can cause fluid to leak from a host resulting in desiccation. Host feeding can have a similar effect, and some parasitoids, but not *N. insectifurax*, use separate hosts for feeding and oviposition. If the collapse of host eggs did result from probing this would suggest that rather than parasitoids having a reduced rate of host encounter at lower temperatures, there may be an increase in probing without oviposition, or a lower rate of parasitoid survival when parasitism does occur. The former may indicate that wasps reared at 18°C require slightly more nutrients or time to mature their eggs than those reared at 27 °C.

The slight upwards trend in successful parasitism with increasing temperature provides some evidence that the upper temperature threshold for oviposition by *N. insectifurax* is above 27 ^{o}C. This may indicate that the *N. insectifurax* population originated from an area of Australia with a warmer climate than is experienced in most parts of New Zealand. Tribe (2000) hypothesised that a high optimum temperature may have caused the failure of the original introduction of *Neopolycystus* sp., from Perth, to establish in New Zealand. He made a similar conclusion regarding the failure of another egg parasitoid, *Procheiloneurus* sp., to establish in South Africa, noting it showed a progressive bias towards a male sex ratio at temperatures below 27 ^{o}C. No such bias was recorded here for *N. insectifurax*.

2.3.6 Conclusions

The purpose of this series of experiments was to develop a rearing method for *E. nassaui* and *N. insectifurax* that would maximise the expression of oviposition behaviour when required, without significantly increasing the complexity of the rearing process. Although *E. nassaui* and *N. insectifurax* are unlikely to experience the exact same physiological state at any given time, it was desirable to rear them under identical conditions to avoid introducing unknown and potentially confounding effects. Based on the evidence presented above, it was decided that both species would be reared using the individual method (section 2.2), with access to honey and host remains to stimulate egg maturation (section 2.3.2) at 22 ^{o}C (section 2.3.5). During all experiments, 3-day-old wasps (section 2.3.3) and *P. charybdis* egg batches < 48 h old (2.3.4) would be used.

2.4 Experimental & Analytical Protocols

The following protocols were developed based on the results presented in sections 2.2 and 2.3 and observations made during insect rearing to take into account technical limitations. These protocols were used in all experiments unless otherwise stated.

2.4.1 Experimental procedures

Host eggs refer to batches of *P. charybdis* eggs that were presented to parasitoids on a 1-2 cm piece of the *E. nitens* foliage on which they had been laid. Eggs were collected from the colony Monday, Wednesday and Friday and used in experiments on the same day or

stored overnight at 4°C. To ensure eggs were no more than 48 h old at the beginning of each experiment, each batch was viewed under a microscope (10 x mag) to confirm minimal embryonic development had occurred. When eggs of other host species were used in experiments the same procedures were followed.

Colony reared *E. nassaui*, *N. insectifurax*, and *B. albifunicle* adults emerged in the presence of conspecifics from approximately five other parasitised *P. charybdis* egg batches. These individuals remained together to mate and feed on honey in their respective colonies before being used in experiments at the appropriate age. The wasps used in experiments were either **naïve**, having no experience with fresh host eggs or fresh leaf material, or were **pre-tested**. Pre-testing was conducted as a means of identifying female parasitoids as *E. nassaui* and *N. insectifurax* show no obvious sexual dimorphism. This pre-testing method could potentially influence the acceptance threshold for different host species, or the perceived availability of preferred hosts (Rosenheim & Rosen 1992). These issues are discussed in detail in section 4.2. In the absence of pre-testing, the number of females in a given experiment would be unknown. This would increase the number of replicates and therefore the number of host eggs, wasps, and time required to complete an experiment. This would have statistical implications given that equal replication of all treatments on any given day could not be guaranteed. Pre-testing was conducted 2-4 h before experiments. Individual parasitoids were presented with a *P. charybdis* egg batch in a Petri dish until they raised the abdomen to insert the ovipositor. At this point, the parasitoid was immediately removed from the egg batch before it could oviposit. Similar methods have been used by Tribe (2000) to identify female parasitoids and by Wylie (1976) to stimulate host attack. *Baeoanusia albifunicle* (chapter 6) were not pre-tested because males and females could easily be distinguished by the morphology of the antennae (Fig. 2.1).

Regardless of whether pre-testing occurred, individual parasitoids were held in isolation for a period of 2-4 h before most experiments. Each was placed in a Petri dish and supplied with honey and **host remains**. The latter consisted of a *P. charybdis* egg batch (adhered to piece of *E. nitens* foliage) from which parasitoid adults had previously emerged (Fig. 2.3).

Enoggera nassaui and *N. insectifurax* were only presented with the remains of eggs from which members of their own species had previously emerged.

Upon completion of experiments in which pre-testing was not employed, **wasp sex** was verified by dissection. This was achieved by freezing the wasps for 48 h before removing the abdomen with fine-tipped forceps under a dissection microscope (Stemi V6, Zeiss, Germany). The abdomen was placed on a microscope slide with a drop of tap water and flattened under a coverslip. Males and females were identified based on their genitalia as viewed under a binocular microscope (Axioskop2, Zeiss, Germany) at 100x magnification. This method was also used to determine the number of mature eggs (**eggload**) in the ovaries of female wasps.

2.4.2 Data analysis & statistical procedures

In most experiments, data for *E. nassaui* and *N. insectifurax* were analysed separately, although non-statistical comparisons of the two species are made in the text. The majority of data collected was **percentage data** with unbalanced replication, and therefore violates the assumptions of ANOVA. Although percentage data can be made to fit the assumptions using the arcsine square-root transformation this approach was rejected because: 1) transformation of percentage data does not always make the data fit the assumptions of ANOVA; 2) it was deemed valuable to use the same tests wherever appropriate, for simplicity and for comparability between experiments. Percentage data was analysed using non-parametric Kruskal-Wallis ANOVA (Proc NPAR1WAY, SAS 9.1, SAS Institute, 1999) to test for overall differences between treatment means. This test is robust enough to cope with unbalanced replication. Multiple Wilcoxon ranked-sum two-sample tests (Proc NPAR1WAY Wilcoxon, SAS 9.1) were then used determine which particular pairs of means differed from one another. When $n < 40$ the t approximation to the Wilcoxon statistic was used. P-values were adjusted using the sequential Bonferroni procedure (R Core Development Team, 2008) to preserve 95% confidence when making multiple comparisons of the same data.

Count data was assessed in several ways. In chapter 3, a General Linear Model ANOVA (Proc GLM, SAS 9.1), able to cope with unbalanced replication, was used to compare

female tibia length. Proc GENMOD (SAS, 9.1) was used to fit generalised linear models to count data with non-linear response distributions (eggload, chapter 2; number of eggs parasitised, chapter 4; days alive, chapter 6) by defining an alternative response distribution and an appropriate link function (distribution = binomial, link = logit; distribution = Poisson, link = log). When significant overall treatment effects were found, chi-square tests on the differences of least square means were used to evaluate which treatment means were different from one another. When replication was low, Fisher's exact test (Proc freq exact, SAS, 9.1) was considered a more appropriate test of no association than a standard chi-square test. This test was used in chapter 4 to assess the number of eggs parasitised in no-choice and choice tests and the number of females that probed each host species first in choice tests, and in chapter 5 to assess the number of 'first ovipositions' representing multiparasitism by each parasitoid species.

Behavioural data (chapter 5) was recorded in real time using *The Observer* (Noldus Information Technology, version 5.0). Details of data collection procedures are presented in chapter 5, appendix 3 and appendix 5. Count, percentage and duration data for each behavioural state recorded were organised using *The Observer* software, and means were compared using non-parametric procedures in SAS 9.1 as described above.

2.5 SUMMARY

The techniques developed to rear *P. charybdis*, *T. sloanei*, *T. catenata*, *D. semipunctata*, *E. nassaui*, *N. insectifurax* and *B. albifunicle* are given in section 2.2. A method to increase the proportion of female progeny of *E. nassaui* and *N. insectifurax* is defined.

In section 2.3 ovigeny, parasitoid age, host age and temperature are assessed with regard to their effects on the willingness of *N. insectifurax* to oviposit into *P. charybdis* eggs.

- *Neopolycystus insectifurax* appears to be more synovigenic than *E. nassaui* and female eggload is increased significantly by supplying nutrients (honey) and host stimuli (remains of host eggs attached to *E. nitens* foliage).

- Parasitism does not increase significantly if *N. insectifurax* is allowed to age more than three days before having access to host eggs, and remains slightly lower than parasitism by 3-day-old *E. nassaui.*
- Host age has a significant effect on successful parasitism, with eggs more than 2-days-old proving to be unsuitable hosts in most cases.
- Parasitism and sex ratio do not differ significantly when *N. insectifurax* are reared at 18, 22 and 27 ^{o}C, but more host eggs collapse at the lower temperature.

Results are used to define a method of rearing parasitoids that are physiologically able and highly motivated to parasitise *P. charybdis* eggs as required for experiments in chapter 5. Experimental protocols are developed based on these findings and knowledge acquired during the establishment and maintenance of the insect cultures. The methods of *pre-testing* and *verifying wasp sex* are described. Details are given on the type, and choice, of statistical procedures used to analyse percentage data and count data.

CHAPTER 3: PHYSIOLOGICAL & ECOLOGICAL HOST RANGES

3.1 INTRODUCTION

The recognition that host specificity testing as a key component of biological control programs has gathered momentum since the early 1980s. A number of case studies and reviews have illustrated the actual and potential risks to which non-target organisms are exposed when exotic BCAs are introduced (Howarth 1991; Simberloff & Stiling 1996; Van Driesche & Hoddle 1997; Boettner et al. 2000; Louda et al. 2003). Most documented non-target impacts involve agents introduced in the 19th and early 20th centuries, when little or no pre-release risk assessment was carried out (Simberloff & Stiling 1996). The intrinsic value of non-crop plants, native flora and insect fauna was rarely considered at that time. A wide host range was even considered beneficial, allowing a control agent to reproduce on alternative species when the target pest became scarce (Cameron et al. 1993). The selection of less 'risky' BCAs has improved significantly as the understanding of population dynamics and the ecological effects of introducing organisms to novel environments has developed. For example, organisms such as generalist vertebrate predators are no longer considered for importation as classical BCAs. Endo-parasitoids, which develop within the body of a single host, are perceived to pose less risk because the intimacy of the host-parasitoid relationship results in a high degree of oligophagy (Vet & Dicke 1992). Groups characterised by this level of specificity are now favoured as BCAs, both to reduce the risk of non-target impacts, and to provide more effective pest control (Onstad & McManus 1996).

Host specificity tests for parasitoids are based on methods developed for phytophagous insects (Van Driesche & Murray 2004a). However, because there are fundamental differences in the way parasitoids locate, recognise, and accept their hosts compared to phytophagous insects, these methodologies are not necessarily suitable. Early tests for parasitoids examined the acceptance and suitability of a few closely related species in no-choice tests. These tests are now considered as only the starting point of much more comprehensive risk assessments that will be discussed further in chapter 4.

Determining the physiological host range of a candidate BCA is the first step in assessing its degree of specificity and therefore what risk, if any, it may pose to non-target organisms in the receiving country. The physiological host range of a parasitoid refers to the complement of species on which it can complete its development, and is generally assessed in the laboratory and by consulting the available literature (Sands & Van Driesche 2004; Van Driesche & Murray 2004a). Once physiological host range is established it can form the basis on which to estimate ecological host range. The latter refers to the current and evolving set of species on which the parasitoid will complete its lifecycle when released into the environment in the receiving country (Onstad & McManus 1996) and is determined by its ability to: 1) locate the host's habitat; 2) locate the host within the habitat; 3) accept the host for oviposition; 4) develop on the host (Doutt 1959; Van Driesche & Murray 2004b). The realised host range in the new environment is generally expected to be narrower than the laboratory defined physiological host range. However, because of the dynamic nature of the host-parasitoid relationship, a narrower realised host range should not simply be assumed. Exactly which species will be exploited, when, and to what extent, is a combination of their physiological suitability and the biotic and abiotic context of the new environment (Onstad & McManus 1996; Strand & Obrycki 1996).

Under natural conditions in New Zealand, *E. nassaui* and *N. insectifurax* are only known to parasitise *P. charybdis*, one of four paropsine species established in the country. The host range of *N. insectifurax* has not been assessed in the laboratory. Previous laboratory studies of *E. nassaui* have reported *D. semipunctata* is a suitable host, *T. catenata* is not accepted, and *T. sloanei* is accepted for oviposition only occasionally (Barrett 1998; Murphy & Kay 2004; Murphy 2005). In this chapter, a no-choice test, designed to maximise host acceptance, was used to assess the physiological host ranges of *E. nassaui* and *N. insectifurax*. The willingness of *E. nassaui* and *N. insectifurax* to oviposit into each of the species was determined (section 3.2) and the ability of each accepted species to support their complete development was confirmed (section 3.3). In section 3.4 field collected *D. semipunctata* eggs were assessed to confirm if that species was within the ecological host range of *E. nassaui* in New Zealand. This provided a basis upon which to assess the predictive ability of host specificity testing methods in chapter 4.

3.2 ACCEPTANCE OF HOSTS FOR OVIPOSITION

For parasitoids, ecological host range is driven by the ability of the immature parasitoid to complete its development in a host species located and accepted by the parent female. Assessing host location behaviour in quarantine during host specificity testing is extremely difficult because of containment regulations and limited space. Host acceptance is therefore the most informative level of behaviour that can be easily studied. Host acceptance is generally believed to provide a highly conservative estimate of potential host range upon which to base a more comprehensive host specificity testing program as part of an ecological risk assessment. Prior to assessing the ability of the immature stages of *E. nassaui* and *N. insectifurax* to develop in the eggs of each of the four paropsine species that are currently established in New Zealand (Fig. 2.1) it was necessary to determine which of them the adult parasitoids would accept for oviposition in the laboratory.

Objective

To determine which paropsine species of the four established in New Zealand are accepted for oviposition by *E. nassaui* and *N. insectifurax* in the laboratory.

Methods

Solitary, naïve, 2-day-old *E. nassaui* and *N. insectifurax* adults were presented with either two *D. semipunctata* eggs or one batch (< 8 eggs) of *P. charybdis*, *T. sloanei* or *T. catenata* eggs. As evidence of host acceptance only was required, rather than parasitism levels, small egg batches were used. Similarly, only two individually laid *D. semipunctata* eggs were used per replicate. Conserving eggs in this was made available more eggs of each species for use in other experiments and for maintaining parasitoid colonies. Parasitoid behaviour was observed under a binocular dissection microscope (Stemi SV6, Zeiss, Germany) and those seen inserting their ovipositor into a host egg were left with them for 24-48 h. These host eggs were subsequently placed on a glass slide in a drop of water and pressed flat under a coverslip. Slide preparations were examined under a binocular microscope (100 x mag., Axioskop2, Zeiss, Germany) to determine the presence of parasitoid eggs.

Results

Both *E. nassaui* and *N. insectifurax* were observed probing eggs of all four paropsine species. Slide preparations of probed eggs confirmed that some individuals of both parasitoid species also oviposited into the eggs of all four species (Fig. 3.1.).

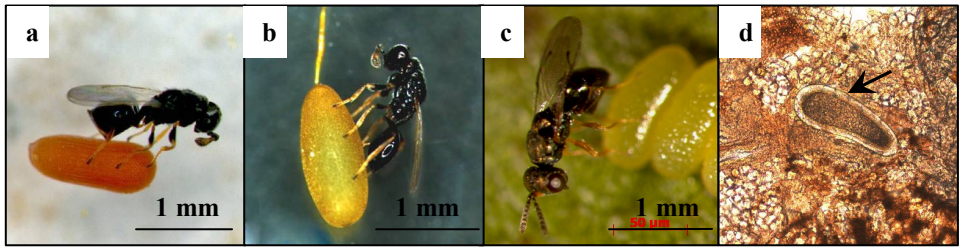

Figure 3.1: *E. nassaui* probing egg of **a)** *T. sloanei* and **b)** *D. semipunctata* in the laboratory. **c)** *N. insectifurax* probing egg of *T. catenata*. **d)** Slide preparation (200 x mag.) of *T. sloanei* egg into which *N. insectifurax* has oviposited (parasitoid egg indicated with arrow) in the laboratory (22 ^{o}C, 65% r.h.).

Discussion

Both *E. nassaui* and *N. insectifurax* have previously been shown to parasitise eggs of a number of paropsine species in the laboratory (Naumann 1991; Tribe 2000). It was not unexpected, therefore, that all four species tested in this study would be accepted. Polyphagy is relatively high among egg parasitoids, compared to parasitoids of other host stages. Some *Trichogramma* species have exceptionally wide host ranges encompassing up to 150 species from seven orders (Clausen 1962; Curl & Burbutis 1978). Strand (1986) proposed that egg parasitoids do not have to develop specialised host relationships because they are not exposed to the host's cellular defences. Janzen (1975) further suggested that the limited nature of eggs in time and space may prevent them supporting specialist parasitoids. As multiple paropsine species will oviposit on the same eucalypt species in Australia (Selman 1994) eggs of several closely related species may well be encountered by a searching parasitoid on any given tree. Polyphagy may therefore confer significant fitness gains.

3.3 Suitability of Hosts for Parasitoid Development

In section 3.2 all four paropsine species established in New Zealand were accepted for oviposition by *E. nassaui* and *N. insectifurax* in the laboratory. The second step to assess

the physiological host ranges of the two parasitoid species was to determine if their immature stages were able to complete their development within the eggs of each accepted species. Physiological host range in conjunction with data on parasitism levels and progeny sex ratios, is useful for estimating the ecological host ranges of candidate BCAs, and for assessing whether they pose direct risks to non-target species.

Objectives

To assess the ability of *E. nassaui* and *N. insectifurax* to complete development in eggs of four paropsine species into which they are known to oviposit in the laboratory, and to compare relative levels of parasitism and offspring sex ratios on each host species.

Methods

Three-day-old *E. nassaui* and *N. insectifurax* adults were placed individually into 55 mm Petri dishes. Each parasitoid was provisioned with honey and a single *D. semipunctata* egg or a batch of *P. charybdis*, *T. sloanei* or *T. catenata* eggs. All host species were exposed to *E. nassaui* for 24 h. *Dicranosterna semipunctata* eggs were exposed to *N. insectifurax* for 24 h and the three other species were exposed to *N. insectifurax* for 48 h. The 48 h duration was used because *N. insectifurax* had been observed to be very slow to initiate parasitism (S. Mansfield pers. comm.). The 24 h duration was used for all hosts with *E. nassaui*, and for *D. semipunctata* with *N. insectifurax*, as there was a high risk that eggs would become desiccated and collapse as a result of excessive probing if exposed for longer periods. Each wasp was frozen after exposure and dissected to verify its sex. Host eggs found to have been exposed to males were discarded. Host eggs exposed to females were incubated for up to 21 days (22 ^{o}C, 70% r.h, 14L:10D). Beetle larvae that hatched were removed to prevent cannibalism of adjacent eggs. Emergent parasitoid progeny were dissected to verify their sex, and the hind-tibia-length (h.t.l.) of female progeny was recorded.

As host species were neither simultaneously, nor consistently, available, and parasitoid sex could not be determined prior to their use in this experiment, full replication of all parasitoid-host combinations tested in this study could not be achieved at any one time. Replicates of all host-parasitoid combinations were each accumulated over 5-7 days

between November 2006 and May 2007, with the aim to achieve 50 replicates (i.e. egg batches exposed to confirmed females) per combination. A much larger number of *T. catenata* batches were exposed to *E. nassaui* as only 11 progeny were reared from the first 50 replicates, and more were deemed necessary to accurately determine sex ratio. Although accumulating data through time to achieve sufficient replication is statistically problematic it is a common reality in studies involving live insects (e.g. Mansfield & Mills 2002, 2004). Every effort was made to ensure consistency of experimental conditions (e.g. temperature, humidity, photoperiod, host age, wasp age) to minimise any 'day effect'. This included running 3-44 replicates of one or more parasitoid-host combination each day.

Mean percent parasitism could not be compared between host species because the individual number of eggs per batch could not be determined for *T. sloanei*, because of the spatial arrangement of their eggs within batches. Also, *D. semipunctata* eggs are not naturally arranged in batches, therefore only one egg was presented to each female, and only binary data (parasitised vs. not parasitised) could be recorded. Instead, the mean proportion of *E. nassaui* and *N. insectifurax* that parasitised hosts on each *test day* were compared (separately for each parasitoid species) using a non-parametric Kruskal Wallis ANOVA. As no 'day effect' was found (*E. nassaui* H= 0.8261, df = 5, P = 0.9753; *N. insectifurax* H = 1.0433, df = 5, P = 0.9590) Wilcoxon two-sample ranked-sums tests were used to compare the mean proportion of females per day that parasitised hosts of each species. As test were run between each pair of host species, P-values were adjusted using the sequential Bonferroni procedure to control for increased type-I-error resulting from multiple comparisons. Mean percent female progeny that emerged per day, from each host species parasitised, were compared as above, using Wilcoxon two-sample ranked-sums test with P-values adjusted using the sequential Bonferroni procedure. Average h.t.l. of female progeny was compared between host species, as an estimate of relative size, using a General Linear Model ANOVA (because of the unbalanced replication) with Tukey's Studentised Range Test for mean separation.

Result

Eggs of all four paropsine species supported the complete development of *E. nassaui* and *N. insectifurax*. The proportion of egg batches parasitised, and from which adult offspring

emerged, varied significantly for both parasitoid species by host species, as did offspring sex ratios and size of female offspring (Table 3.1). *Paropsis charybdis* and *D. semipunctata* were readily accepted by both parasitoid species. *Trachymela sloanei* batches were only parasitised by a small proportion of each parasitoid species. *Trachymela catenata* were accepted by *N. insectifurax* at high levels comparable to *D. semipunctata* but were only rarely (9/142 batches) accepted by *E. nassaui.*

Table 3.1: Number and proportion of egg batches of four host species in which *E. nassaui* and *N. insectifurax* completed development (22 °C). Proportion ($\bar{x}$) of eggs parasitised within batches is shown for the three species for which individual eggs could be counted. The proportion of female offspring and their hind-tibia length (h.t.l., $\bar{x}$ ± SE) is indicated for each parasitoid-host combination. Means with different letters beside (within parasitoid species) are significantly different at $P < 0.05$.

Parasitoid-host combination	Batches exposed to adult females				Dissected progeny		
	Total	Parasitised	% batches	% eggs	Total	% female	h.t.l. (µm)
Enoggera nassaui							
P. charybdis	102	102	100.0[a]	86.1	473	88.3[b]	47.8 ± 2.10[a]
D. semipunctata	91	71	78.0[b]	67.6	73	98.6[a]	44.4 ± 1.75[b]
T. sloanei	72	12	16.7[c]	-	26	88.5[ab]	33.0 ± 2.65[c]
T. catenata	142	9	6.3[c]	2.8	17	100.0[a]	40.0 ± 2.50[d]
Neopolycystus insectifurax							
P. charybdis	99	96	97.0[a]	82.8	729	62.6[a]	46.6 ± 2.91[a]
D. semipunctata	58	39	67.2[b]	73.8	39	17.9[b]	43.1 ± 1.21[b]
T. sloanei	53	3	5.7[c]	-	4	0.0[b]	-
T. catenata	49	34	69.4[b]	63.4	150	4.7[b]	42.1 ± 1.07[b]

As more than 900 parasitoid progeny emerged from *P. charybdis* egg batches, only 58% of *E. nassaui*, and 90% of *N. insectifurax*, were dissected to assess sex ratios. All progeny that emerged from the other host species were dissected, although a small proportion (~ 3%) escaped. At least 88% of *E. nassaui* progeny reared from every host species were female. A significantly higher proportion of females were reared from *T. catenata* and *D. semipunctata* compared to *T. sloanei* and *P. charybdis* (Table 3.1). *Neopolycystus insectifurax* showed a male-biased sex ratio on all hosts except *P. charybdis*. All four *N. insectifurax* progeny reared from *T. sloanei* eggs were male.

Tibia length of *E. nassaui* reared from each species declined as follows: *P. charybdis* > *D. semipunctata* > *T. catenata* > *T. sloanei* (Table 3.1, Tukey's HSD, $P < 0.05$). Mean h.t.l of *E. nassaui* was marginally longer than *N. insectifurax* on *P. charybdis* and *D. semipunctata*. Female *N. insectifurax* reared from *P. charybdis* were significantly lager than those reared from *D. semipunctata* or *T. catenata* (Tukey's HSD, $P < 0.05$), but the difference in mean h.t.l. between females reared from *D. semipunctata* and *T. catenata* was not significant.

Discussion

Although there is no evidence from the field that *D. semipunctata*, *T. sloanei* and *T. catenata* are exploited by *E. nassaui* or *N. insectifurax*, all three supported their complete development in the laboratory. High variability in relative parasitism levels and sex ratios on the different hosts suggests these hosts are neither physiologically equivalent nor perceived by ovipositing females to be of equal quality. Based on parasitism levels alone it would appear that *P. charybdis* and *D. semipunctata* are high quality hosts for both species, *T. sloanei* is a poor quality host, and *T. catenata* is a high quality host for *N. insectifurax*, but a poor quality host for *E. nassaui*. However, the sex ratios of offspring that were reared from each host species do not completely reflect this interpretation.

While successful parasitism may indicate the physiological suitability of a host, the proportion of progeny that are female is often cited as a measure of host preference or of quality as perceived by the ovipositing female (Ode & Strand 1995; Mansfield & Mills 2004). Parasitoid wasps select the sex of their offspring by controlling insemination during oviposition, such that fertilised eggs become females and unfertilised eggs become males. Females typically account for 65% or more of the adult progeny of parasitoid wasps that control sex in this way (Wylie 1976). Waage (1986) reviewed the adaptive patterns of both progeny and sex allocation by parasitoids. One factor strongly influencing sex allocation was mating structure. In the laboratory, *E. nassaui* and *N. insectifurax* males eclose first and mate with females that subsequently emerge from the same egg batch (pers. ob.). This strategy is also used by some gregarious *Trichogramma* and solitary scelionid egg parasitoids which, like *E. nassaui* and *N. insectifurax,* parasitise batches of eggs (Waage 1982; Waage & Ng 1984). The scelionids usually allocate a male to the first or second host

egg, followed by a number of females, then another male and series of females until the whole batch is parasitised (Waage 1982). This pattern has been explained by the theory of *local mate competition* (LMC) (Hamilton 1967) under which a parent female should produce only enough male offspring to fertilise all her daughters. The strategy may provide fitness gains when host patches are sparse, when they are defended by other females, or if female density is low and mating occurs near the emergence site (Jervis 2005). If mating structure alone drives sex allocation by *E. nassaui* and *N. insectifurax*, sex ratios on each host species should have been similar in these laboratory tests, with the exception of *D. semipunctata* because their eggs were presented individually rather than in batches (see pg. 49). This may explain the results for *E. nassaui* (88-100% female progeny on all host species), but not *N. insectifurax*, as the latter allocated considerably different proportions of female progeny (0-62%) to the host species assessed.

Parasitoid sex ratios have also been explained by the theory of *conditional sex allocation* (Charnov et al. 1981). Under this theory, perceived host quality leads to the preferential oviposition of one sex. Host size is often regarded as an indicator of host quality (see section 2.3.5) because of a positive correlation between host size and parasitoid fitness (Charnov et al. 1981; Jones 1982; Waage & Ng 1984; Rosenheim & Rosen 1992; Ode & Strand 1995; Mansfield & Mills 2002). Jones (1982) suggested that where there is a preference for large hosts, female progeny will be allocated to the very largest available. Tribe (2000) observed that the variable size of an unidentified *Neopolycystus* species in Western Australia was determined by the size of the host eggs from which they emerged. In this study, eggs ranged from largest to smallest in the order *P. charybdis* > *D. semipunctata* > *T. catenata* > *T. sloanei* (Appendix 2, Fig. 2.1). This ranking matches that of the average h.t.l. measured for both *E. nassaui* and *N. insectifurax,* confirming that there is a positive correlation between host size and parasitoid size. For the species assessed here, there is usually little variability in within-batch egg size (Appendix 2.). If, however, small eggs are present in batches they are still parasitised and produce relatively smaller, but apparently functional, wasps. As the experiments conducted here consisted of no-choice tests with naïve wasps, host quality as a function of host size could only be based on a preference for an absolute size, or perceived relative to the size of the rearing host (in this case *P. charybdis*) experienced at eclosion (e.g. Jones 1982; Hare 1996; Joyce et al. 2002).

Because *E. nassaui* progeny emerged from few *T. sloanei* (16.7%) and *T. catenata* (6.3%) batches it is likely their eggs are poor quality hosts. This may result from the small size of their eggs relative to *P. charybdis*, but no size-dependent reduction in host quality was reflected in offspring sex ratios. Females accounted for 88.5% and 100% of progeny that emerged from *T. sloanei* and *T. catenata* eggs respectively. As differential mortality between the sexes is a possibility, and only a small number of progeny eclosed to be dissected, the accuracy with which sex ratio can be estimated is limited. A few exploratory egg dissections indicated that a higher proportion of *T. sloanei* eggs may have been parasitised and failed to develop. It is possible, therefore, that eggs of these two species were *perceived* to be high quality hosts and readily parasitised, but were not particularly suitable for parasitoid development. Interestingly, *E. reticulata* produce approximately equal numbers of both sexes on *T. sloanei* in the laboratory, but parasitise only 0-20% of available eggs (Millar et al. 2000). Here, a large proportion of *N. insectifurax* (69.4%) parasitised *T. catenata* batches yet the majority of their offspring were male. *Neopolycystus insectifurax* may therefore have perceived *T. catenata* as a poor quality host but continued to oviposit in the absence of an alternative. *Trachymela sloanei* appears to be a poor host both physiologically and as perceived by *N. insectifurax*.

Parasitism of *D. semipunctata* by *E. nassaui* and *N. insectifurax* was high, yet > 98% of the progeny of the former, and < 18% of the latter, were female. This disparity raises the question as to whether the two parasitoid species have a different sequence of sex allocation or a different perception of host quality. The eggs of *D. semipunctata* occur singly, rather than in batches, and were presented so in this study. They are also deposited on the leaves of *Acacia*, rather than *Eucalyptus*, and are therefore unlikely to be within either wasp species' ecological host range (see section 3.4). Based on the LMC theory, Waage (1982) predicted, and found some evidence for, a slightly female-biased sex ratio close to 0.5 for parasitoids exploiting single eggs. Neither *E. nassaui*, nor *N. insectifurax* fit this prediction. One possible explanation is that they are both adapted to hosts that lay eggs in batches rather than individually, and have developed different sequences of sex allocation that are not adjusted to account for batch size. *Neopolycystus insectifurax* could follow a 'male first' rule as described by Waage (1982) and *E. nassaui* a 'female first' rule. Alternatively, if batch size *is* assessed in addition to egg size (and both species do appear to assess the entire surface of an egg batch when first encountered), single eggs may be

perceived as a low quality resource by *N. insectifurax*, and sex allocation adjusted accordingly. *Enoggera nassaui* were observed to be very eager to parasitise *D. semipunctata* (section 4.2) and batch size may be of little importance to them as they have different post-oviposition behaviour (see chapter 5). Assessing the sequence of sex allocation by each species, combined with a measure of sex ratios produced when artificial batches of multiple *D. semipunctata* eggs are created, would help to determine what drives the differential response of *E. nassaui* and *N. insectifurax* to this species.

In addition to size, the actual and perceived quality of hosts may be influenced by other physical characteristics. As noted earlier, chorion structure has been implicated as a physical defence against parasitism and an important factor in host recognition and acceptance (Mansfield & Mills 2002). Unlike the smooth thick chorion of readily accepted *P. charybdis* and *D. semipunctata* eggs, the chorion of *T. catenata* is dimpled and covered with a sticky coating. It also appears to be structurally weaker, breaking easily when pressed flat onto a slide under a coverslip. *Neopolycystus insectifurax* were unperturbed by this coating. They appeared to have some difficultly gripping the egg surface while inserting the ovipositor, but successful oviposition occurred nevertheless. *Enoggera nassaui* were reluctant to parasitise *T. catenata* eggs. Wasps quickly moved away from these batches after drumming on their surface with the antennae. The sticky coating may explain this rejection either as a result of physical interference with oviposition, as suggested by Murphy (2005), or some chemical signature that limits recognition of the eggs as potential hosts. Internal chemical factors could account for the poor survival of *E. nassaui* on *T. catenata*. Biochemical substances injected to halt host development can be incompatible with some hosts, resulting in failed parasitism attempts (Luhring et al. 2000).

Trachymela sloanei eggs are also dimpled, with a relatively thin chorion. They are unlikely to be encountered by *E. nassaui* and *N. insectifurax* in nature, as they are deposited under eucalypt bark (Steven & Mulvay 1977). Some eggs in *T. sloanei* batches are inaccessible to parasitoids because they are deposited in a jumbled mass in crevices. It has been observed in colony upkeep that beetle larvae hatching from partially parasitised batches begin eating adjacent eggs, killing any developing parasitoids. Exploiting batches that can only be partially parasitised may therefore risk high offspring mortality.

The data collected in this study indicate the physiological host ranges of *E. nassaui* and *N. insectifurax* in New Zealand. Although the mechanisms are not clear, some host species were undoubtedly preferred over others. The question arises as to how the parasitoids would respond when given a choice between species. Preference testing is often the next step in ecological risk assessments for candidate BCAs and will be explored in chapter 4. The parasitism levels and sex ratios observed in this section will be useful to interpret the ability of choice and no-choice experiments to predict parasitoids' host ranges. Of particular interest is the ability to detect low levels of parasitism, such as those of *E. nassaui* on *T. catenata* and *N. insectifurax* on *T. sloanei*, as this will influence what constitutes sufficient replication and whether testing methods are appropriate to the specific biology of the parasitoid. Understanding parasitoid behaviour is essential to correctly interpret the biological implications of low parasitism levels. These issues will be addressed by comparing the results of the no-choice experiments carried out here with choice and no-choice experiments conducted in chapter 4, and by a detailed investigation of the behaviour of the two parasitoid species in chapter 5.

3.4 *DICRANOSTERNA SEMIPUNCTATA* AS A FIELD HOST OF *ENOGGERA NASSAUI*

Other than *P. charybdis*, *D. semipunctata* is the only paropsine established in New Zealand that *E. nassaui* had previously accepted in the laboratory. This result is considered a 'false-positive' because parasitised eggs have not been found in the field, despite the host's abundance and direct releases of *E. nassaui* into the host's *Acacia* habitat (Murphy & Kay 2004). A false-positive result refers to a laboratory-predicted host range (physiological host range) that is larger than the realised host range (ecological host range). The dangers of false-negative results, i.e., predicting a narrower than realised host range, receive significant attention (Howarth 1991; Boettner et al. 2000), but false-positives are rarely discussed. Because of the conservative approach taken in New Zealand to the risk assessment of BCAs, certain host specificity tests are expected to overestimate host ranges (Goldson et al. 1992). The ability to interpret whether physiological suitable hosts represent ecological false-positives is essential, as suitable BCAs could otherwise be rejected. This could result in continued losses due to uncontrolled pest damage or, more often, the use of non-specific chemical controls in the apparent absence of a biological control alternative. In this study, it was considered important to correctly identify *D. semipunctata* as a physiologically suitable host that is outside of *E. nassaui*'s ecological

range, allowing the accuracy with which laboratory experiments can predict ecological host ranges to be assessed in chapter 4.

Objective

To confirm that parasitism of *D. semipunctata* eggs by *E. nassaui* in the laboratory represents a false-positive result.

Methods

Field-collections of *D. semipunctata* eggs were made in March, November and December of 2007. Eggs were collected from *A. melanoxylon* at two Bay of Plenty (BP) and two Waikato (WO) sites (Table 3.2). *Enoggera nassaui* is well established in these regions (Murray et al. 2008), and each site was adjacent to eucalypt trees suitable for *P. charybdis* oviposition. Unfortunately eucalypt foliage was inaccessible, and *P. charybdis* eggs could not be collected to confirm the presence of *E. nassaui* in these specific sites at this time. Field-collected *D. semipunctata* eggs were incubated (22 ^{o}C, 70% r.h. 14L:10D) and assessed daily for emergence of *D. semipunctata* larvae or parasitoids. Eggs that failed to hatch within 14 days were assessed for visible signs of parasitism before being recorded as 'collapsed' eggs.

Results

A total of 992 *D. semipunctata* eggs were collected. Larvae hatched from approximately 80% of these (*Athenree* data not included) while the remaining proportion collapsed with no visible signs of parasitism (Table 3.2).

Table 3.2: Number of field-collected *D. semipunctata* eggs that were parasitised, that collapsed, or from which *D. semipunctata* larvae hatched in the laboratory (22 °C, 70% r.h. 14L:10D). The proportions of Athenree eggs that collapsed and hatched were not distinguished. WO = Waikato region, BP = Bay of Plenty region (see Appendix 1 for geographical coordinates).

Date	Site (region)	Eggs collected	Parasitised	Collapsed	Hatched
19/03/07	Athenree (BP)	118	0	-	-
15/11/07	Athenree (BP)	102	0	-	-
26/11/07	Rotorua (BP)	62	0	15	47
28/11/07	Tamahere (WO)	100	0	16	84
01/12/07	Pirongia (WO)	212	0	36	176
05/12/07	Pirongia (WO)	125	0	31	94
14/12/07	Pirongia (WO)	273	0	52	221
Total		**992**	**0**	**150**	**622**

Discussion

In small cage host specificity tests, the first two stages of host selection (location of host habitat and location of host within habitat) are effectively bypassed. This can lead to an overestimate of host range (Curl & Burbutis 1978; Goldson et al. 1992; Sands 1993). As a consequence, physiological host range determined in the laboratory is not expected to predict ecological host range with 100% accuracy. In nature, only a fraction of host species in which development is possible will be parasitised. This is because in addition to overcoming any physiological barriers the parasitoid must be seasonally, geographically, and ecologically coincident with the host in the new environment (Doutt 1959). No-choice tests conducted in section 3.3 indicate that if *D. semipunctata* occurred in *E. nassaui's* eucalypt habitat, or if *E. nassaui* searched *Acacia* foliage, it could be utilised as a host. As no *E. nassaui* emerged from field-collected *D. semipunctata* eggs it is probable that its parasitism in the laboratory is a false-positive result. This concurs with the results of Murphy & Kay (2004), who found no parasitism of 668 *D. semipunctata* eggs collected from a stand of *A. melanoxylon* into which *E. nassaui* had been intentionally released.

Only a few studies have been conducted worldwide to assess the accuracy with which laboratory tests can predict field host ranges of introduced parasitoid BCAs (Louda et al. 2003). In several, laboratory predictions have been indicative of realised host ranges (e.g.

Barratt et al. 1997) while in others the realised range has been narrower (Van Driesche et al. 2003; Froud & Stevens 2004), or wider (Goldson et al. 1992; Louda et al. 1997). Briese (2005) suggested that wider than predicted host ranges have probably resulted from the omission of species that should have been tested, or misinterpretation of results. Unnatural laboratory conditions have also been used to explain disparities between predicted and realised ranges. Confinement, in particular, can inhibit or prevent normal host location and acceptance behaviour (Froud & Stevens 2004). In a review of larval parasitoids of the leaf beetle *Lilioceris lilii* (Scopoli), false-positive results occurred frequently in small Petri dish test arenas (Casagrande & Kenis 2004). The parasitoids exhibited wider host ranges, weaker preference for the target host, and higher efficacy on the target than occurred in the native environment. Despite their limitations, small-arena, no-choice tests do have a role in predicting BCA host ranges. Withers and Browne (2004) advocated their use in the initial stages of host specificity testing programs as a highly conservative starting point from which to assess risks posed by candidate BCAs. Mansfield & Mills (2002) proposed that physiological host ranges determined in the laboratory using such methods may be sufficient to predict risks posed by polyphagous parasitoids to non-target hosts that coincide temporally and spatially with the target host.

Most paropsine beetles are specific to *Eucalyptus*. Eucalypt leaves contain very high levels of secondary plant compounds, such as oils, tannins, phenols and surface waxes (Ohmart & Edwards 1991; Selman 1994). Many of these are regarded to provide some defence against insect herbivory, to which paropsine beetles have adapted and overcome. Parasitoids of herbivores that have such specialised relationships with their host plant are likely to be highly attracted to that plant's volatile emissions and herbivore-induced plant volatile emissions (Vet & Dicke 1992). Indeed, Paine *et al.* (2004) reported that the attraction of the egg parasitoid *Avetianella longoi* Siscaro (Hymenoptera: Encyrtidae) to the eucalypt habitat of its target host was so strong that no formal host range testing was deemed necessary before its introduction to California, where there are no native eucalypts or other myrtaceous plants. Parasitoids may also search very particular microhabitats within the host habitat. Curl & Burbutis (1978) found that although *Trichogramma nubilale* Ertle & Davis is almost exclusively host specific to the European corn borer in the field, it could successfully parasitise eggs of 17 out of 21 other lepidopteran species tested in the laboratory. They determined this was because of an adaptive behaviour and

ecological coincidence with the host-plant, within which the parasitoid only searches a specific region for its host. Similarly, Tribe (2000) suggested that the failure of *E. nassaui* and *Neopolycystus* spp. to establish in South Africa resulted from the fact that the available host, *T. tincticollis*, lays its eggs in bark crevices, rather than on leaf blades where the wasps search. These examples illustrate the likely importance of the physical nature of the host-plant, and its volatile emissions, to parasitoid host location and acceptance behaviour. These concepts will be discussed further in chapter 4.

It seems reasonable to conclude that *D. semipunctata* eggs are not utilised by *E. nassaui* in the field because of the spatial asynchrony between the location of the eggs and the habitat searched by the wasp. Although beyond the scope of this thesis, it would be interesting to assess how large a test arena would need to be before *E. nassaui* would fail to parasitise *D. semipunctata* eggs on *A. melanoxylon*. It would also be informative to monitor parasitism of sentinel *P. charybdis* eggs placed in the foliage of *A. melanoxylon* adjacent to *E. nitens*.

Recognising false-positive and false-negative results, or ideally, preventing their occurrence, is fundamental to accurately predicting parasitoid host ranges from laboratory host specificity tests. The ability to correctly interpret host ranges predicted in the laboratory, is possibly more useful than trying to emulate natural conditions so that laboratory host ranges exactly match those seen in the field, as has been the aim in the past few decades. Field observations and ecological theory predict that physiologically suitable hosts that do not overlap with a natural enemy in time or space will remain free of attack. Laboratory host ranges must therefore be interpreted in light of the behavioural ecology of the host and the parasitoid (e.g. Haye et al. 2005). Behavioural ecology includes how hosts are located and accepted and how the conditions experienced during host specificity tests influence parasitoid behaviour. To this end, the host ranges of *E. nassaui* and *N. insectifurax* predicted using two common types of host specificity tests are compared in chapter 4, while the behavioural ecology of the two species is investigated in chapter 5.

3.5 SUMMARY

Eggs of *P. charybdis*, *T. sloanei*, *T. catenata* and *D. semipunctata* are accepted for oviposition and support the complete development of *E. nassaui* and *N. insectifurax* in the laboratory. Parasitism of the four host species by *E. nassaui* ranges from 100 – 6.3% in the order *P. charybdis* > *D. semipunctata* > *T. sloanei* > *T. catenata*, and by *N. insectifurax* from 97 - 5.7% in the order *P. charybdis* > *T. catenata* > *D. semipunctata* > *T. sloanei.* The size of female progeny of both parasitoid species varies significantly, ranging from largest to smallest in the order *P. charybdis* > *D. semipunctata* > *T. catenata* > *T. sloanei*.

Parasitism levels, sex allocation, and host size data, provide different explanations for the possible rank order preferences of the four hosts tested. The roles of mating structure and perceived host quality on host acceptance and sex allocation are discussed with reference to the theories of *local mate competition* and *conditional sex allocation*. The possible influences of physical egg characteristics, other than size, are also considered.

No evidence is found to support the possibility that *D. semipunctata* is within the ecological host range of *E. nassaui* in New Zealand. Although this species is clearly within the physiological host range of *E. nassaui* and *N. insectifurax*, it most likely escapes parasitism in the field because it oviposits on *A. melanoxylon* rather than eucalypt foliage.

CHAPTER 4: CHOICE VS. NO-CHOICE TESTS AND THE EFFECT OF PARASITOID DENSITY

4.1 INTRODUCTION

In recent decades substantial advances have been made in the selection of appropriate target pests and candidate BCAs for classical biological control (Hoelmer & Kirk 2005; Kuhlmann et al. 2006). Despite extensive discussion of the risks of introducing exotic BCAs and the implementation of legislation to minimise these risks (see chapter 1), specific methods for their assessment are still lacking. Risk assessment is primarily based on the host specificity of the BCA. This specificity is usually estimated by conducting host range tests in the laboratory, which can take many different forms. Zwolfer and Harris (1971) reviewed early host range testing methods and their shortcomings, pointing out particular considerations that needed to be met. Sheppard (1999) reviewed methods used for weed BCAs since the time the centrifugal phylogenetic approach for selecting potential non-targets was devised by Wapshere (1974). This review noted that a standard set of procedures had not been adopted, and provided a guide for choosing appropriate procedures in view of the agent's biological characteristics and any information on its specificity.

The development of host range testing methods for entomophagous BCAs lags behind that of weed BCAs because of a relative lack of concern for non-target insects. Many researchers have independently devised their own host specificity testing procedures for individual biological control programs (e.g. Barratt et al. 1997). Goldson and Phillips (1990) provided one of the first lists of 'considerations' for the assessment of entomophagous BCAs. These included understanding the phenology, behaviour, population dynamics and ecology of the agent, its target, and potential non-targets. A multi-phase screening process for assessing host specificity was suggested that incorporated the available literature, determining host range and relative suitability of hosts in the laboratory, and the likely coincidence of the target and non-targets in the receiving country. We now know of many specific factors that must be considered in host range tests (e.g. Barratt et al. 1999; Withers et al. 1999; Withers & Browne 2004; van Lenteren et al. 2006b).

The uptake of the suggestions by Goldson & Phillips (1990) and others can be seen in the application of host specificity testing for entomophagous BCAs in New Zealand today, where a cautionary approach to importing BCAs must be taken by law. Risk analysis often begins by estimating host specificity from the available literature, the agent's country of origin, and other countries into which it has been imported. A series of laboratory tests to determine physiological host range and to estimate the levels of non-target attack that could be expected usually follow. Although one set of tests can not adequately assess risks posed by all entomophagous BCAs, it would be beneficial for scientists and regulators to have guiding protocols for choosing tests and to standardise their implementation and interpretation. To set such protocols it is necessary to assess the ability of laboratory tests to predict field host ranges. In this chapter, the aim is to compare choice to no-choice tests, which are the two main test categories commonly applied in biological control programs.

The roles and values of choice versus no-choice host specificity tests have been debated on several occasions (Sheppard 1999; Withers & Mansfield 2005). Sheppard (1999) noted that no-choice tests were the most commonly used for weed BCAs. No-choice tests are popular as they are highly conservative, offering a means to estimate the widest possible host range a BCA may express (Goldson & Phillips 1990; Withers & Browne 2004). However, some researchers argue that choice tests provide more realistic predictions of which physiologically suitable non-target hosts will be utilised in the field, where the full set of host finding behaviours, rather than just acceptance behaviours, can be expressed. Others consider choice tests misleading as BCAs, especially very small and relatively immobile ones, are unlikely to encounter more than one host at a time under natural conditions (e.g. Field & Darby 1991; Hill 1999; Mansfield & Mills 2004). Without information on the availability of more or less preferred hosts, these agents have no opportunity to choose between them. Instead, they accept or reject each host encountered based on stimuli present at the time, previous experience, and physiological state (Edwards 1999; Barratt 2004). Many researchers also consider choice tests to be problematic because cues associated with species X may influence the response of the agent to species Y, resulting in false positives or false negatives. Others advocate choice tests to avoid false negative results. Fuester et al. (2004), for example, used choice tests that include the target to avoid misinterpreting the failure of un-motivated parasitoids to oviposit as rejection of non-target hosts. Berndt et al. (2007) used a sequential no-choice design with a *target – non-target* -

target sequence when faced with a similar problem while testing the braconid *Cotesia urabae* Austin & Allen.

In an extensive review of methods used to assess non-target effects, Babendreier et al. (2005) considered the use of choice versus no-choice tests for parasitoid BCAs. Of 26 biological control programs that included some form of host specificity testing they found that 23 included no-choice tests, 11 choice tests, and 8 combined both. They concluded that no-choice tests have often provided good estimates of non-target effects and found little evidence that choice tests overestimate parasitoid host ranges as has been argued for weed BCAs. The also noted that there have been few comparisons of different methods used among studies and that it is timely to do so.

Implementing the ideal set of host specificity tests is limited by the availability of hosts and parasitoids and also by time and resources. Compromises must be made with regard to which species to assess, and how. Understanding what factors can differentially influence the outcomes of choice and no-choice tests will help researchers choose tests that can provide the most informative results under the circumstances. Of particular importance is how to interpret results from a number of tests in light of the physical test conditions and the physiological state of the insects. In the case of *E. nassaui* and *N. insectifurax*, parasitoid density was regarded as one factor that might significantly influence test outcomes, as competitive behaviour (investigated in detail in chapter 5) had been observed during colony maintenance. In section 4.2 the results of paired choice tests are compared to the no-choice tests conducted in chapter 3. In section 4.3 the effect of parasitoid density on the acceptance of less-preferred hosts is investigated.

4.2 Comparing Choice vs. No-Choice Host Specificity Tests

A variety of laboratory tests have been used to assess parasitoid host ranges (van Lenteren et al. 2006a). These include no-choice, sequential choice, choice-minus-target, paired choice and multiple choice. No-choice tests are often employed to assess the physiological host range of a candidate BCA and to select a smaller number of 'at risk' non-target hosts to subject to further analysis such as choice tests. As few studies have directly compared the predictive ability of no-choice and choice tests, it is not clear if the information gained

from one or the other, or a combination of the two, is more ecologically relevant (Withers & Mansfield 2005). This has consequences for the efficient use of insects and other limited resources during the risk assessment of candidate BCAs. Choice tests can be particularly difficult to conduct as large numbers of the appropriate stages of target and non-target hosts are required simultaneously. In the paired choice test, the target and one non-target host are presented together to determine if the agent will show a preference for the target host if it occurs in sympatry with the non-target. Because of the physical limitations imposed by the quarantine environment, host range tests are usually conducted in small arenas, such as Petri dishes. There is evidence to suggest that both confinement, and the presence of the target host, can influence the behaviour of the candidate BCA being tested (Sands 1993; Withers & Browne 2004). Incomplete understanding of these influences can make the results of no-choice and paired choice tests difficult to interpret.

Objectives

To determine the host preferences of *E. nassaui* and *N. insectifurax* when given the choice between pairs of physiologically suitable hosts, and to compare the predictive accuracy of choice tests to those of no-choice tests conducted in chapter 3.

Methods

Motivated 3-day-old female wasps were identified by pre-testing (see section 2.4) 2 h prior to experiments. For each paired choice test (Table 4.1), each solitary wasp was presented with equal-sized egg batches of two host species in 55 mm Petri dishes for 1-6 h (22 ^{o}C, 65% r.h.). Shorter durations were used for *E. nassaui* because this species had previously been observed to parasitise whole egg batches in < 1 h (chapters 2 and 3), which could mask host preferences if both batches presented in these choice tests were fully exploited. *Paropsis charybdis* and *T. catenata* eggs were presented on the *E. nitens* leaves on which they had been laid. Individual eggs were removed with a scalpel to reduce batch size to 4 or 8 eggs (see Table 4.1). As the eggs of *D. semipunctata* are laid individually, fewer eggs were available for use in these experiments so batch size was reduced to four eggs when *P. charybdis* and *T. catenata* were paired with this species. To construct comparable artificial *D. semipunctata* batches, groups of four indivuidual eggs were glued side by side with chicken egg-white onto the surface of *A. melanoxylon* leaves. Ten control replicates were

included alongside four of the paired choice tests when sufficient eggs were available (see Table 4.1). These were used primarily to assess if individual parasitoids had sufficient time and motivation, during the test period, to parasitise all available hosts. Each control replicate consisted of two batches of either four or eight *P. charybdis* eggs, matching the test replicates. For all tests with *E. nassaui*, and for the *P. charybdis* vs. *D. semipunctata* test with *N. insectifurax,* observations were made to record which host was probed first. Observations were made at five minute intervals during the *P. charybdis* vs. *D. semipunctata* tests with *E. nassaui* to determine after what time the second host accepted was probed.

Table 4.1: Duration of exposure, number of replicates, and number of eggs per batch exposed to *E. nassaui* or *N. insectifurax* in paired choice tests with three physiologically suitable host species (22 ^{o}C, 65% r.h.).

Host pair compared	Test date	Duration (h)	Replicates	Eggs per batch
Enoggera nassaui				
P. charybdis vs. *D. semipunctata**	17-11-08	1	30	4
P. charybdis vs. *T. catenata*	5-05-08	2	30	8
*D. semipunctata vs. T. catenata**	30-01-09	1	30	4
Neopolycystus insectifurax				
*P. charybdis vs. D. semipunctata**	03-02-09	6	30	4
P. charybdis vs. *T. catenata**	26-03-08	6	33	8
T. catenata vs. *D. semipunctata*	05-04-08	6	29	4

* Run in conjunction with 10 positive control replicates of 2 x *P. charybdis* egg batches.

Following all experiments, wasps were removed and egg batches incubated (22 ^{o}C, 65% r.h.). Beetle larvae that hatched were removed to prevent cannibalism of adjacent eggs. The proportion of eggs per batch from which parasitoid progeny emerged was recorded as percent parasitism. Mean parasitism was compared between each species pair (by parasitoid) using Wilcoxon two-sample ranked-sums tests. The Fisher's exact test was used to compare the total proportion of female *E. nassaui* and *N. insectifurax* that parasitised each host species in choice and no-choice tests, and also the proportion that probed each species first when order was recorded. A one-way ANOVA was used to compare mean time lag before *E. nassaui* females probed the second host species, when they did so.

Results

When given the choice between *P. charybdis* and *D. semipunctata* eggs 29/30 *E. nassaui* were observed antennating and probing both. In 18/30 replicates *P. charybdis* was assessed in this way first, this was not significantly more often than *D. semipunctata* (Fisher's exact test, $P = 0.1964$). Subsequent assessment of *D. semipunctata* occurred on average between 30 and 35 min into the test but not all were parasitised. This lag was significantly longer ($F = 6.23$, df = 1, $P = 0.0189$) than when *D. semipunctata* was assessed first (12/30 replicates) in which case *P. charybdis* were assessed 20 to 25 min into the test on average. The same average time lag was observed before the second *P. charybdis* batch was probed in the control replicates. In total, 93.3% of *E. nassaui* females parasitised *P. charybdis* ($\bar{x}$ = 89.2% of eggs), and 76.7% parasitised *D. semipunctata* ($\bar{x}$ = 40.8% of eggs, Table 4.2). *Enoggera nassaui* chose to oviposit exclusively in *P. charybdis* over *T. catenata* and in *D. semipunctata* over *T. catenata* (Table 4.2). Four females in the former test, and five in the latter, briefly contacted *T. catenata* eggs first, tapping them with the antennae. This was significantly less than first contacted *P. charybdis* (Fisher's exact test, $P < 0.0001$) and *D. semipunctata* (Fisher's exact test, $P < 0.0001$). In the latter test, one female probed the *T. catenata* eggs but did not oviposit, while 28/30 probed *D. semipunctata* eggs and 27 oviposited. There was no significant difference in the proportion of *E. nassaui* females that parasitised each host species in choice compared to no-choice tests (Table 4.2).

Neopolycystus insectifurax showed a preference for *P. charybdis* over *D. semipunctata* ($z = 591$, $P < 0.001$, Table 4.2). Significantly more females (25/29) accepted *P. charybdis* first (Fisher's exact test, $P < 0.0001$). Eleven eventually parasitised *D. semipunctata* eggs but only one did so without also parasitising *P. charybdis.* A preference was also shown for *P. charybdis* over *T. catenata* ($z = 626$, $P < 0.0001$). Only 3/33 females parasitised *T. catenata* and only one of these did not also parasitise *P. charybdis*. Significantly more *N. insectifurax* females parasitised *D. semipunctata* and *T. catenata* eggs in no-choice compared to choice tests, regardless of which species they were paired with (Table 4.2). When given the choice between *D. semipunctata* and *T. catenata*, 48.3% of females failed to parasitise either host, but 20.7% parasitised both hosts. As observations were not made it is not known which host was accepted first in the latter case. Overall, *D. semipunctata* was parasitised in 10% more replicates than *T. catenata*, but this difference was not significant ($z = 834$, $P < 0.3515$). *Neopolycystus insectifurax* consistently parasitised only one of the

two 8-egg *P. charybdis* control batches for the *P. charybdis* vs. *T. catenata* test, remaining in contact with the parasitised batch throughout the test. In contrast, 70% of control females parasitised both 4-egg batches in the *P. charybdis* vs. *D. semipunctata* test.

Table 4.2: Proportion of *E. nassaui* (*n* = 30) and *N. insectifurax* (*n* = 29-33) that parasitised *P. charybdis* (P), *D. semipunctata* (D) and *T. catenata* (Tc) eggs in paired choice tests (**C**, this chapter) compared to no-choice tests (**NC**, chapter 3). The proportion that parasitised both host species (**Both**), and mean proportions of individual eggs parasitised in each choice test ($\bar{x}$ **Parasitism**) are also shown. Host 1 and 2 refer to the host species as indicated in parentheses in column one. $P \leq 0.05$ indicates significant differences between values (C vs. NC = Fisher's exact test; $\bar{x}$ parasitism Host 1 vs. Host 2 = Wilcoxon ranked-sums test).

	Host 1			**Host 2**			**Both**	$\bar{x}$ **Parasitism**		
	NC	**C**	***P***	**NC**	**C**	***P***	**C**	**Host 1**	**Host 2**	***P***
Enoggera nassaui										
P (1) – D (2)	100.0	93.3	0.0503	78.0	76.7	1.0000	76.7	89.2	40.8	< 0.001
P (1) – Tc (2)	100.0	100.0	-	6.3	0.0	0.3625	0.0	97.9	0.0	< 0.001
D (1) – Tc (2)	78.0	86.7	0.4300	6.3	0.0	0.3625	0.0	65.8	0.0	< 0.001
Neopolycystus insectifurax										
P (1) – D (2)	97.0	93.3	0.3298	67.2	36.7	0.0073	33.3	83.3	23.3	< 0.001
P (1) – Tc (2)	97.0	90.9	0.1648	69.4	9.1	< 0.001	6.1	66.2	2.8	< 0.001
Tc (1) – D (2)	69.4	31.0	0.0019	67.2	41.4	0.0367	20.7	30.2	33.6	0.7029

Discussion

In this discussion no-choice and choice tests are compared in the light of the results presented above. Possible interpretations of agreements and disparities between the two testing methods are discussed with regard to what these might indicate about the host-parasitoid relationships investigated in this study. This is followed by an overview of some specific factors that can influence the outcome or interpretation of no-choice and choice tests, and their implications for host specificity testing and risk assessment of BCAs.

Choice vs. no-choice tests

Both *E. nassaui* and *N. insectifurax* have been shown to exhibit clear host preferences in choice tests. These preferences generally agree with the results of no-choice tests. Exclusive preferences were shown for hosts that sustained very high levels of parasitism in

no-choice tests when paired with hosts that sustained very low levels (*E. nassaui* on *P. charybdis* (100%) vs. *T. catenata* (6.3%), and *D. semipunctata* (78%) vs. *T. catenata* (6.3%)). Clear and statistically significant preferences were also apparent for hosts that sustained 20-30% higher parasitism in no-choice tests than the hosts they were paired with in choice tests (e.g. both parasitoids on *P. charybdis* vs. *D. semipunctata,* and *N. insectifurax* on *P. charybdis* vs. *T. catenata*). These results, especially those for *E. nassaui* on *P. charybdis* vs. *D. semipunctata,* are similar to those reported by Porter (2000). In that study, minor parasitism of native non-target fire ants relative to introduced target fire ants in no-choice tests, corresponded to a three to fourfold preference for the target in paired choice tests.

Parasitism of *T. catenata* by *N. insectifurax* was high (69.4%) on no-choice tests, and only 30% less that that of *P. charybdis.* In choice tests however, *T. catenata* was rarely accepted. The no-choice result suggests *T. catenata* is very acceptable and likely to be utilised in the field, but the choice result indicates a strong preference for *P. charybdis* that may exclude *T. catenata* from the ecological host range of *N. insectifurax*. The disparity between the two tests warns against relying on only one testing method. Haines et al. (2003) reported on the failure of historic choice tests, in the absence of no-choice tests, to reveal the acceptability of *Chamaecytisys proliferus* (L.) as a non-target host of the broom seed beetle *Bruchidius villosus* (F.). When these choice tests were later repeated, the non-target host was accepted at low levels, similar to those for *T. catenata* by *N. insectifurax* in this study. Although these results may still have been interpreted to indicate only minor risk of non-target parasitism, substantial attack of *C. proliferus* has since been observed in the field. Haines et al. (2003) suggested that choice tests that include the target are not, on their own, a robust means of observing acceptance of lower ranked hosts of phytophagous insects. These observations show that non-target impacts cannot be assumed to be insignificant on the grounds that a strong preference is shown for the target in choice tests.

Choice tests were not able to detect any preference by *N. insectifurax* between *D. semipunctata* and *T. catenata*, which suffered very similar levels of parasitism in no-choice tests (67.2% vs. 69.4%). Detecting a preference was made more difficult by the fact that observations were not made to determine which species was accepted first when both were

parasitised. No-choice results indicated both were highly acceptable, while the choice test indicated both were low ranked. Furthermore, progeny from no-choice tests showed a strongly male-biased sex ratio. This suggests that both species are low ranked yet physiologically suitable hosts that become acceptable, at least for the production of male offspring, when *N. insectifurax* is deprived of more preferred hosts as occurred in the 48 h no-choice test. This highlights the importance of conducting experiments of an appropriate duration. In this case, both choice and no-choice tests were probably too long, and direct observations during the experiment would have provided more informative results.

Low levels of parasitism in choice and no-choice tests

In no-choice tests, 100% of *E. nassaui* females parasitised *P. charybdis* and 6.3% parasitised *T. catenata* (section 3.3). In the paired choice test, however, only *P. charybdis* was parasitised. If only choice tests had been conducted *T. catenata* would not have been placed within the physiological host range of *E. nassaui*. Indeed, previous sequential and paired choice tests failed to detect parasitism of this host (Barrett 1998), although replication was also very low. Limited replication is a common problem in host specificity tests and it is likely that parasitism of *T. catenata* was only observed in this study as a result of the high replication used.

The failure of the choice test to predict parasitism of *T. catenata* may seem inconsequential given that only 6.3% parasitism occurred in the no-choice test. The biological implications of low levels of non-target attack are poorly understood, but their interpretation may become critical in future risk assessments. On one hand, minor non-target attack may be outweighed by the benefits of controlling the pest (e.g. Porter 2000). Alternatively, failure to detect low levels of attack could have serious negative implications, especially if the non-target is a highly valued BCA, a native beneficial, or a threatened, restricted or poorly known native species. Moderate non-target parasitism can sometimes be sustained with barely detectable impacts on abundance, but at other times relatively low levels can translate into substantial impacts (Barlow et al. 2004). This is probably determined by density dependent responses and the life-history of the agent, non-target and target hosts (e.g., phenological synchrony). In New Zealand, the BCA *M. aethiopoides*, introduced in 1982 against *Sitona discoideus* Gyllenhal, parasitises several native weevils and the

introduced BCA *R. conicus* (Barratt et al. 1997). This is one of the few biological control programs for which extensive post release evaluation that included the non-target organisms has occurred. Although significant impacts were not confirmed in this case, most programs do not even evaluate the consequences of 'minor' non-target attack as it is difficult to quantify its impact in the field. What appears to be the first attempt to do so was made by Barlow et al. (2004). Using a discrete-time Ricker model, it was estimated that when two native weevil genera experienced 15% field parasitism by *M. aethiopoides*, 8% population suppression occurred. The weevil species sustained relatively high levels of parasitism in no-choice laboratory tests (47-88%) but low levels (< 5%) in some field locations (Barratt et al. 1997). A similar model estimated a 5% suppression of abundance of New Zealand's endemic red admiral *Bassaris gonerilla* (F.) by the cabbage butterfly parasitoid *Pteromalus puparum* (L.) (Barron 2007). Neither of the cited studies attempted to directly compare field impacts with parasitism levels in the laboratory. In this study no-choice parasitism of *T. catenata* by *E. nassaui* was much lower than that observed in the laboratory on the non-target hosts attacked by *M. aethiopoides*. This may indicate a chance for spill-over parasitism of *T. catenata*, but significant population impacts are unlikely.

Choice tests & false negative results

Parasitism of *T. catenata* is not known to occur under natural conditions in New Zealand. However, as is the case for many non-targets, *T. catenata* is uncommon, has a restricted distribution, and few eggs have been located in the field for assessment. It is not clear, therefore, if the *P. charybdis* vs. *T. catenata* choice test accurately predicted that *T. catenata* will not be represented in the realised host range of *N. insectifurax*, or if the presence of *P. charybdis* interfered with the natural acceptance of *T. catenata*. Volatile chemical cues and contact kairomones associated with the target host or its substrate (host-plant complex) have been shown to influence the response of some parasitoids to other species in choice tests, resulting in false negative or false positive results (see Withers & Browne 2004). Parasitism of *Irenimus aequalis* (Broun) by *Microctonus hyperodae* Loan, for example, was found to be considerably lower in the presence of the target host (Goldson et al. 1992). The unknown affects of mixing chemical signals causes some practitioners to disapprove of choice tests or at least advocate caution in their interpretation. Gilbert & Morrison (1997), for example, opted for sequential choice tests to asses for non-target attack by phorid flies while avoiding 'inappropriate attacks' that might

arise from the build up of odours from multiple ant species if they were presented to the flies simultaneously in closed containers.

False negative results may occur in choice tests if a preference is developed for one species through experience with its associated chemical cues. There are numerous examples of experience-induced changes in responsiveness to target and non-target hosts (Turlings et al. 1993; Cortesero & Monge 1994; Kitt & Keller 1998; Withers & Browne 2004). Cameron and Walker (1997) found that several non-target species attracted oviposition attempts by *Cotesia plutellae* Kurdjumov in no-choice tests, but subsequent wind tunnel experiments indicated that the parasitoid responses were probably elicited by cues associated with the host plant, cabbage. There are also a number of cases where rearing host and previous experience have been shown not to alter host acceptance or preferences (Sands & Coombs 1999; Duan & Messing 2000; Porter 2000). The probability that experience-induced preferences will occur in host specificity tests is usually minimised by using parasitoids that have had no oviposition experience. A problem may still arise, however, if the rearing host is included in the choice test, as parasitoids can gain experience with that host at eclosion. The host preferences of some *Trichogramma* spp. for example can be affected by both rearing host and previous oviposition experience (Bjorksten & Hoffmann 1998). In this study the target host *P. charybdis* was, by necessity, also the rearing host as it was the only species that could be maintained in large numbers in an egg producing state throughout the study. To determine if this influenced parasitoid responses in choice tests would require that the tests be repeated with parasitoids reared on *D. semipunctata* or *T. catenata*. This was not possible because of limited egg availability. For *E. nassaui* at least, the similarity between parasitism levels on non-target hosts in choice and no-choice tests would suggest that rearing host did not significantly influence host acceptance.

Choice tests & false positive results

Chemical cues associated with a preferred host can also elicit false positive non-target attack (Field & Darby 1991; Withers & Browne 2004). During choice tests under confined conditions, parasitoids may be stimulated to parasitise hosts they would not normally accept, because of direct contact with the target host or the presence of chemical cues from

the target host causing a state of central excitation (Withers & Mansfield 2005). In this study, the presence of the target (*P. charybdis*) did not stimulate attack by *E. nassaui* or *N. insectifurax* on the less preferred host *T. catenata*. In fact there was no parasitism by *E. nassaui* and a significant reduction by *N. insectifurax*. These results provide strong evidence that *T. catenata* is actively rejected by both species in the presence of *P. charybdis*. It is difficult to determine if *T. catenata* would become acceptable to *N. insectifurax* after all available *P. charybdis* were parasitised. It was evident from control replicates and colony observations that *N. insectifurax* tends to remain on a host batch long after oviposition is complete (see also chapter 5). Failure to accept a substantial proportion of *T. catenata* may therefore reflect the search behaviour of *N. insectifurax,* and no-choice test results may be more representative of its likely response to this host if encountered in the natural environment, even after an oviposition experience with *P. charybdis*. In contrast, both no-choice and choice results indicate that although *D. semipunctata* is a lower ranked host of *E. nassaui* than *P. charybdis* it is likely to be accepted if encountered in the environment. In choice tests *E. nassaui* tended to probe (and presumably parasitise) *P. charybdis* first, but as they quickly parasitise whole egg batches and resume host searching almost immediately afterwards, they may be willing to accept slightly less suitable hosts when they are encountered, regardless of previous experience. This possibility is investigated further in chapter 5.

False positive results could ultimately lead to the rejection of suitable BCAs. *Cotesia rubecula,* for example, would probably be rejected as a BCA in North America by today's host specificity standards because it parasitises the native pierid butterfly *Pieris napi oleracea* Harris in the laboratory (Van Driesche et al. 2003). Laboratory parasitism of *T. catenata* and *D. semipunctata* by *N. insectifurax* may represent false positive results, although there is insufficient field data to confirm this. There is evidence however (section 3.3) (Murphy & Kay 2004) that parasitism of *D. semipunctata* by *E. nassaui* in choice and no-choice laboratory tests is a false positive result.

Eggs of *D. semipunctata* elicit a very strong response from *E. nassaui*. The parasitoid is quickly arrested and begins antennating and probing the eggs with rapid movements. Why these eggs are so readily accepted is not clear, although they are similar in size to *P.*

charybdis eggs and larger than those of the two lower ranked *Trachymela* species. Parasitoid survival may be lower on *D. semipunctata* eggs. Despite the fact that most *D. semipunctata* eggs were probed in the choice test with *P. charybdis*, *E. nassaui* emerged from just 40%. It seemed that *E. nassaui* spent more time probing *D. semipunctata* eggs than *P. charybdis* eggs before ovipositing, but this was never quantified. Excessive probing can cause eggs to desiccate and die (Tribe 2000) regardless of whether parasitism has occurred. This may explain why *E. nassaui* emerged from such a low proportion of *D. semipunctata* eggs even though some eggs in most batches were parasitised.

Dicranosterna semipunctata feeds on *A. melanoxylon* in New Zealand. In Australia this plant grows as part of a mixed understory beneath a *Eucalyptus* canopy (Nicholas & Brown 2002). In New Zealand, it is mainly grown alone or alongside eucalypts in farm-forestry plantations. There is no evidence to date that *E. nassaui* searches *A. melanoxylon* for hosts, even when in close proximity to eucalypts (e.g. Murphy & Kay 2004). Parasitism of *D. semipunctata* in the laboratory probably results from the inability of the parasitoids to express normal host location behaviours under confined conditions. Long and short-range host location behaviours filter out some physiologically suitable hosts from a parasitoid's ecological host range (Goldson & Phillips 1990). Habitat separation has been identified as the filter that protects the native butterfly *P. virginiensis* from the cabbage white butterfly BCAs *C. rubecula* and *C. glomerata* in North America (Benson et al. 2003). Some species, such as the oligophagous *C. plutellae*, appear to be attracted more to their hosts' habitat than the host itself (Cameron & Walker 1997). In a novel experiment, Morehead & Feener (2000) directly inserted eggs of *Apocephalus paraponerae* Borgmeier (Diptera: Phoridae) into the bodies of ant species outside its realised host range. The survival of these parasitoid's showed the extent to which long and short range host location cues, rather than direct physiological interactions alone, can limit the ecological host range of this parasitoid. Host-plant and habitat preferences are now recognised as factors that can help to identify potential non-target species that do not need to be included in host specificity tests (Kitt & Keller 1998; Orr et al. 2000).

4.3 Effect of Parasitoid Density in Choice and No-Choice Tests

As noted in the previous chapters, host specificity testing is often constrained by time and resources. Experimental design may suffer if space for cages is limited or parasitoids and non-target hosts for tests and controls are few in number. Most researchers can not afford the time required to conduct exploratory investigations, such as those detailed in chapter 2, to determine the best conditions under which to assess a parasitoid's host range. Instead, less-than-ideal methods are frequently employed to utilise limited numbers of insects when and where they are available. In many cases, non-target species are difficult to obtain and maintain in the laboratory. For these reasons, multiple parasitoids are commonly exposed to groups of hosts in specificity tests (Field & Darby 1991; Neale et al. 1995; Barratt et al. 1997; Kitt & Keller 1998; Duan & Messing 2000; Morehead & Feener 2000; Porter 2000; Babendreier et al. 2003). Goldson et al. (1992), for example, found it more economical to expose three parasitoids to 50 hosts in choice tests when native non-target weevil species and space for cages were limited. Such practices can serve to increase the number of parasitoids tested while conserving limited numbers of hosts.

Multiple parasitoids have also been used to ensure the presence of at least one female when the BCA being tested is sexually monomorphic, as is the case for both *E. nassaui* and *N. insectifurax*. For this reason, using multiple parasitoids per test arena was considered in the initial stages of this study as a means of achieving balanced test designs, in which equal numbers of replicates per treatment could be conducted simultaneously. However, both *E. nassaui* and *N. insectifurax* are known to compete for access to *P. charybdis* eggs, and *N. insectifurax* are suspected to defend host eggs (S. Mansfield Unpub.). Furthermore, in section 2.2 the presence of multiple females was shown to affect sex allocation. As a result of these observations parasitoid density was recognised as a factor that had the potential to influence the outcome of host specificity tests. It was decided that multiple parasitoids should not be used in the host range tests and the other experiments conducted in chapters 2, 3, 4 and 6. To qualify this decision, the effect of defensive behaviour on the results of host specificity tests is investigated in the following section, and also in chapter 5.

Objectives

To assess the effect of parasitoid density on acceptance of non-target hosts by *E. nassaui* and *N. insectifurax* in choice and no-choice tests, and to determine if parasitoid density could therefore be manipulated to improve the predictive accuracy of such tests.

Methods

For each of ten replicates with each parasitoid species (one per day), nine 55 mm Petri dishes were set up containing either one, two or four (three dishes for each density) pre-tested (section 2.2) female parasitoids. A batch of eight *P. charybdis* eggs, eight *T. catenata* eggs, or a batch of each, was added to each dish. Eggs were exposed to the parasitoids for 2 h (22 ^{o}C) before being incubated (22 ^{o}C, 70% r.h. 14L:10D). After several days, parasitised and unparasitised eggs could be distinguished and the latter were speared with a pin to prevent beetle larva hatching and consuming adjacent eggs. The number of eggs from which parasitoid progeny ultimately emerged at each density, and in choice vs. no-choice tests, were compared using Generalised Linear Model ANOVA with binomial distribution (Proc GENMOD, SAS 9.1).

Results

Enoggera nassaui successfully parasitised almost all *P. charybdis* eggs in choice and no-choice tests whereas no *T. catenata* eggs were parasitised (Fig. 4.1a). Significantly more *P. charybdis* (F = 5.8, df = 1, P = 0.0195) were parasitised in the no-choice test ($\bar{x}$ = 78.3% ± 0.08) compared to the choice test ($\bar{x}$ = 75.7% ± 0.16). There was no significant difference in the parasitism of *P. charybdis* as a function of parasitoid density (*E. nassaui*: F = 1.36, df = 2, P = 0.2645; *N. insectifurax*: F = 0.27, df = 2, P = 0.7667). *Trachymela catenata* were parasitised by *N. insectifurax* in both choice and no-choice experiments but parasitism of *P. charybdis* was higher (Fig. 4.1b). Parasitism of *P. charybdis* by *N. insectifurax* did not differ significantly between choice and no-choice tests (F = 0.93, df = 1, P = 0.34). More *T. catenata* eggs were parasitised in no-choice compared to choice tests, but this difference was marginally not significant at the 5% level (F = 3.49, df = 1, P = 0.0670). There was also a non-significant trend for increased parasitism of *T. catenata* at higher parasitoid densities (F = 2.66, df = 2, P = 0.0790, Fig. 4.1b) under both choice and

no-choice conditions. More *P. charybdis* and *T. catenata* eggs collapsed when exposed to *N. insectifurax* compared to *E. nassaui*, regardless of parasitoid density or test design.

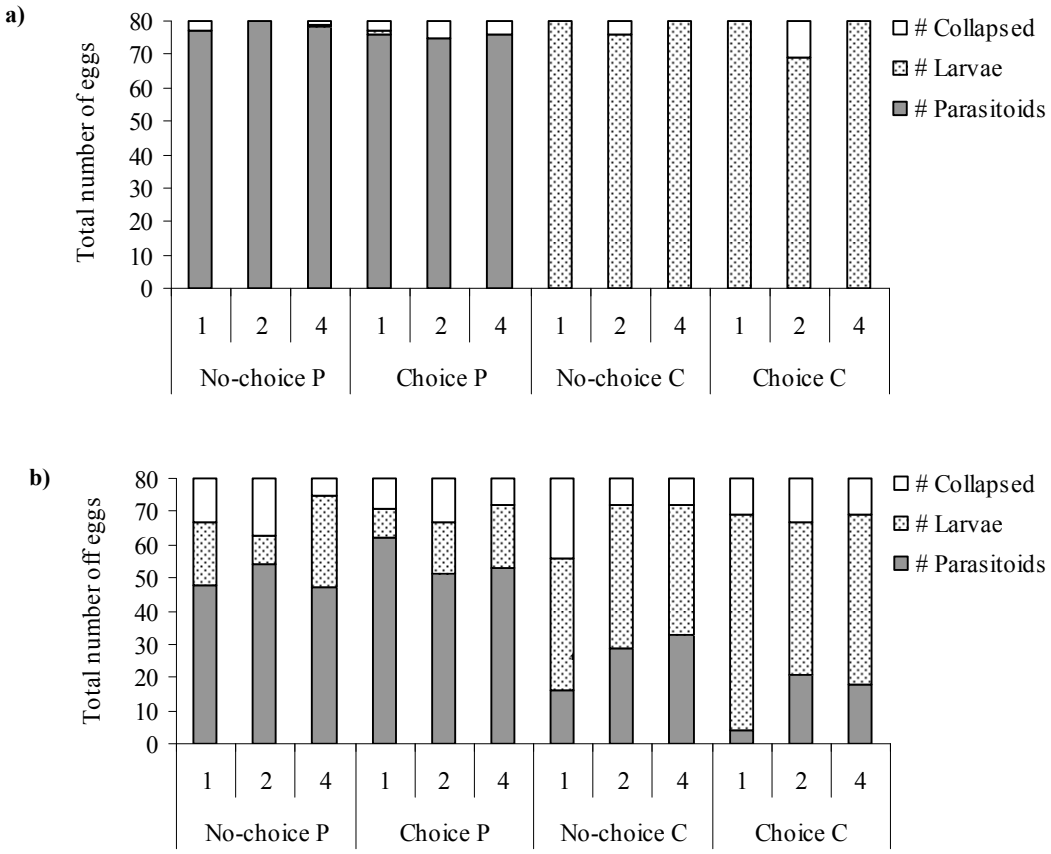

Figure 4.1: Total number of emergent parasitoids, emergent beetle larvae and collapsed eggs following exposure of *P. charybdis* (P) and *T. catenata* (C) egg batches to **a)** 1, 2 or 4 *E. nassaui* and **b)** 1, 2 or 4 *N. insectifurax* for 2 h in no-choice and choice tests (22 ^{o}C, 65% r.h.).

Discussion

When host specificity tests are conducted for biological control programs, parasitoids and non-target hosts are often very limited in number, resulting in low replication. In a review of published parasitoid host specificity tests Babendreier et al. (2005) reported that more than half the cited studies included only 1-10 replicates per treatment. Low replication with non-target hosts is often regarded as better than no replication, but this can limit the power of any statistical analyses conducted (Hoffmeister et al. 2006). Multiple parasitoids are sometimes used in host specificity tests to increase the number of parasitoids exposed to non-target hosts in these situations. Doing so may be justified if previous research has shown parasitoid density has no effect on parasitism (Barratt et al. 1997; Babendreier et al.

2003), or when cage size or host placement and density is manipulated to reduce interactions between individual parasitoids (Neale et al. 1995). More often, the effects of host density might be assessed (e.g. Ferreira de Almeida et al. 2002) but the role of parasitoid density is rarely considered.

As discussed in section 4.2, the presences of volatiles and contact kairomones associated with a target host and its substrate have the potential to stimulate parasitoids to oviposit into hosts they would not normally accept. Therefore, false positive results may occur in choice tests that include the target. Increasing parasitoid density in choice tests may further increase the acceptance of non-targets, as individual parasitoids may be prevented from utilising the target host. Parasitism by *E. nassaui* of the non-target host *T. catenata* was not significantly influenced by parasitoid density, regardless of the presence of the target *P. charybdis*. This result agrees with previous no-choice (section 3.3) and choice (section 4.2) tests in which *T. catenata* was rarely accepted in the absence of other hosts, and not at all when *P. charybdis* was present. This in turn strengthens the evidence that *T. catenata* was actively rejected as a less preferred host, and that chemical cues associated with the target host do not cause *E. nassaui* to accept less preferred hosts in confinement, even when competition for the target host is high.

Aggressive behaviour of *E. nassaui* and *N. insectifurax* observed during colony maintenance suggested that when multiple parasitoids are present in small test arenas some individuals may directly prevent others from ovipositing. There was no compelling evidence that this occurred in this study. There was a slight decrease in parasitism of *P. charybdis* at a density of four *N. insectifurax* compared to two, but the difference was not significant. Parasitism of *T. catenata* actually increased with parasitoid density. This might indicate that, as less preferred hosts, *T. catenata* eggs are not defended. Unlike *N. insectifurax*, parasitism by *E. nassaui* appeared to be limited by host availability, with almost all *P. charybdis* eggs parasitised even with a density of one female parasitoid per test arena. As *E. nassaui* parasitise host eggs very quickly they may have had time to parasitise all available host eggs even if a significant proportion of time was spent defending them from other females. Increasing numbers of host eggs available may have increased the chance of seeing a direct competition effect. Observing the parasitoids

throughout the experiment would also have provided an opportunity to detect such an effect, and this became a significant focus of chapter 5.

The presence of multiple parasitoids could reduce effective parasitism without direct interaction between individuals if they have a mechanism to avoid superparasitism. Such mechanisms are common among solitary parasitoids (van Alphen & Visser 1990). Effective parasitism could also be reduced as a result of host egg mortality if superparasitism is not avoided, or if rejection occurs only after the host egg is probed. *Enoggera nassaui* readily assess *P. charybdis* and *D. semipunctata* by probing, and under confined conditions will often return to and probe previously parasitised eggs. When superparasitism occurs, only one parasitoid egg will survive (see section 5.3). If many females oviposit into one host there may be insufficient resources within that host for any parasitoid larvae to develop, and the host egg will eventually collapse (Tribe 2000). Even in the absence of oviposition, multiple probing events, or associated host feeding, may cause the host egg to lose fluid and collapse. As a result, even if more parasitoid eggs are laid at higher parasitoid densities the totally proportion of host eggs from which adult parasitoids ultimately emerge may decrease. There was no evidence of this occurring with *E. nassaui* as < 5/80 eggs collapsed at any density. This was despite the fact that, at a density of one, parasitoids were able to parasitise all eight *P. charybdis* eggs in the 2 h experiments. This suggest up to four oviposition attempts may be made per host egg at a density of four parasitoids. *Paropsis charybdis* eggs have been observed to collapse after having been exposed to large groups (≈ 12-20) of *E. nassaui* for > 24 h during colony maintenance. Experiments of a longer duration (> 2 h) may therefore have shown an increase in the proportion of collapsed host eggs.

Contrary to expectations, increasing parasitoid density was not found to strongly influence the acceptance of less preferred hosts. Aggressive defence of host eggs by individual parasitoids and avoidance of superparasitism may explain why the total proportion of eggs parasitised did not increase significantly in the presence of multiple parasitoids. The results however, did not provide direct evidence for this, and did not indicate why parasitism did not decrease. The experiment may have been more informative if direct observations had been made to quantify the relative handling times of the different hosts and explore the

consequences of any direct interactions between parasitoids. These factors may be important if experiments are conducted for inappropriate durations. Parasitism of acceptable hosts may be prevented in tests of short duration, and parasitism of less preferred hosts could be increased in tests of long duration. The outcomes of direct interactions between parasitoid species and avoidance of superparasitism and/or multiparasitism will be directly assessed in chapter 5.

4.4 SUMMARY

In section 4.2 the host preferences of *E. nassaui* and *N. insectifurax* are assessed in paired choice tests, and parasitism of each host species is compared to the results of no-choice tests from chapter 3. Both parasitoid species show a strong preference for *P. charybdis* over *T. catenata*. Choice test results for *E. nassaui* closely match no-choice results but fail to show that *T. catenata* is within its physiological host range. *Neopolycystus insectifurax* shows a much stronger preference for *P. charybdis* over *T. catenata* in choice tests than expected considering the no-choice test results. As in the no-choice test, it is difficult to detect any preference of *N. insectifurax* between *D. semipunctata* and *T. catenata* in choice tests. The acceptance of *T. catenata* appears to be reduced by the presence of any other suitable host species. There is no evidence that either parasitoid species is stimulated to accept non-target hosts because of the presence of the target host. Overall, comparing choice and no-choice test results provides substantially more information than either test alone. In Petri dish arenas, neither choice nor no-choice tests are able to accurately predict that *D. semipunctata* is not within the ecological host range of *E. nassaui*, as was shown in chapter 3 (section 3.3).

In section 4.3, parasitoid density is considered as a factor that may influence the outcome of choice and no-choice host specificity tests and the accuracy with which those tests are able to predict field host ranges. Parasitoid density has no significant effect on parasitism of *P. charybdis* or non-target hosts in choice and no-choice tests. There is a slight, but non-significant, increase in parasitism of the non-target host *T. catenata* by *N. insectifurax* at higher parasitoid densities. The mechanisms by which parasitoid density could affect host acceptance and effective parasitism are discussed with particular focus on the influence of host defence and the avoidance of superparasitism.

CHAPTER 5: COMPETITION BETWEEN SPECIES & CONTRASTING BEHAVIOURAL STRATEGIES

5.1 INTRODUCTION

In order to make parasitoid host range testing meaningful, it is necessary to understand the multitrophic system in which the parasitoid forages. In addition to the physical and chemical environment this includes parasitoid behaviour, learning, and interactions between species. Parasitoids are difficult to observe under natural conditions but there is an extensive literature on behaviour from laboratory studies. Prior to the 1980s there was substantial research into host handling times and host recognition mechanisms. More recently, the role of plant volatiles, herbivore-induced plant volatiles, and contact kairomones, in host-habitat location, host recognition, and the ability of parasitoids to detect and learn chemical cues, have been at the forefront of behavioural studies (Cave et al. 1987; Turlings et al. 1990; Vet & Dicke 1992; Turlings et al. 1993). The information gained in these areas is extremely valuable in biological control. Understanding such processes when selecting potential BCAs, has improved control efficiency and reduced risks to non-targets. However, with the shift in focus towards chemical ecology there has been less time for general biological and ecological observations, which are equally important to effective biological control.

When time and resources are limited, the early stages of host specificity studies for parasitoids may rely on a 'black-box' approach. Potential non-targets are exposed to the agent, and parasitoids that subsequently emerge are recorded (i.e.% parasitism). Occasionally, studies include an observational component such as recording which host is accepted first during choice tests (e.g. Mansfield & Mills 2004). Measures of host preference such as the ratio of host acceptance to host contacts (van Dijken et al. 1986) and comparisons of handling times for target and non-target hosts might also be included (Mansfield & Mills 2004). Behavioural observations made before and during the initial stages of host specificity testing are particular useful (Duan & Messing 1997; Gilbert & Morrison 1997; Mansfield & Mills 2004). Such observations can provide information necessary for maintaining viable insect colonies and identify factors that need to be controlled for during host specificity tests (e.g. chapter 2). They may also indicate if

probing occurs in the absence of oviposition which is important if probing alone can kill non-targets.

The role of competition between parasitoids must also be considered in biological control programs. A common shortcoming of early programs was a lack of information on the biology of the agents being assessed. This limits the understanding of how multiple agents might interact to influence control efficacy, and hinders the identification of characteristics that are actually responsible for a program's success or failure (DeBach & Rosen 1991). Multiple BCAs of a target pest may compete directly or indirectly via agonistic interspecific interactions, adversely affecting their ability to regulate the target (Batchelor et al. 2005). Globally, there has been considerable debate on (see Pschorn-Walcher 1977 for review), and a shift away from, importing multiple BCAs, once favoured as a means of achieving additive control. Currently, importing one or a few highly effective agents is preferred, and the incentive to minimise introductions is strengthened by the fact that each imported agent poses potential non-target risks that are costly to assess.

Both *E. nassaui* and *N. insectifurax* can parasitise a high proportion of *P. charybdis* eggs in the laboratory (chapter 3). In the field however, *E. nassaui* parasitises a much higher proportion of the pest population (Jones & Withers 2003). During laboratory rearing, the two species were observed to exhibit agonistic behaviour towards conspecifics in the presence of hosts. Subsequently, parasitoid density at the time of oviposition was found to have a significant influence on sex allocation behaviour, especially for *N. insectifurax* (chapter 2). Furthermore, in choice and no-choice tests (chapter 4) *N. insectifurax* were seen to remain in contact with any host batch they had parasitised, even when a second batch of equally suitable hosts was available. These observations concur with the unpublished results of Dr. S. Mansfield, who quantified the oviposition behaviour of solitary females and conspecific female pairs of *E. nassaui* and *N. insectifurax*.

This study compares the behavioural characteristics of *E. nassaui* and *N. insectifurax* when they are forced to compete with each other for access to hosts. It assesses whether the differences noted during interspecific competition are apparent during interspecific competition, and whether this can help to explain why *N. insectifurax* is a less effective

BCA for *P. charybdis* in New Zealand. In section 5.2, the oviposition behaviour and direct interactions of the two competing species will be quantified. Indirect interactions, such as parasitoids' abilities to detect parasitised hosts, and their responses to these, will be quantified in section 5.3. Observing interactions between extremely small, fast-moving insects such as these parasitoids is difficult. It requires simultaneously recording the subject displaying an action, the action itself, the subject being acted upon, and a time factor, while looking down a microscope. Although video technology has been successfully used in some studies (e.g. Merfield et al. 2004), cameras capable of tracking very small insects are rarely obtainable and reviewing video footage adds a substantial amount of time to data collection. For this study, *The Observer* software (Noldus Information Technology, Wageningen, The Netherlands) was selected as a recording method. It is designed specifically for the collection, analysis and presentation of behavioural data, and unlike other methods has the capacity to record and store data in real time. It can later sort and analyse this data or export it to other programs for additional statistical analyses, thereby avoiding repeated data entry. As a configuration (coding scheme to record events) is established before the observation takes place, and time-keeping is automated, this software provides an objective means of recording large amounts of data accurately and quickly. This recording method has been used in several similar behaviour studies (e.g. Field & Calbert 1998; De Vis et al. 2003)

5.2 Direct Interspecific Interactions

In chapter 2 it was found that the proportion of female offspring produced by *E. nassaui* and *N. insectifurax* in laboratory colonies could be improved by exposing host egg batches to solitary parasitoids, rather than to groups of parasitoids. The improvement was thought to result from a reduction in direct competition, as females were observed competing with their conspecifics for access to the hosts. In a previous study (S. Mansfield unpub.) conspecific *E. nassaui* were found to have pushing contests to win possession of host eggs. Conspecific *N. insectifurax* were even more aggressive, frequently biting off their opponent's legs or antennae in addition to pushing. Such behavioural attributes associated with resource defence may have consequences in terms of the risks posed by, and efficacy of, parasitoids introduced as BCAs when multiple agents are present. As these two species now occur in sympatry in New Zealand understanding the direct interactions between them

should assist in predicting the impact of their co-occurrence on the future biological control of *P. charybdis*.

Objectives

To determine if *E. nassaui* and *N. insectifurax* will compete for access to host eggs and whether they display different behavioural characteristics that can explain why *E. nassaui* is a more effective control agent in New Zealand.

Methods

A configuration was created (Appendix 3) using *The Observer* 5.0 (Noldus Information Technology, Wageningen, The Netherlands) and loaded on to a handheld PC. This configuration denoted the observed subjects (*E. nassaui* and *N. insectifurax*), a number of independent variables, and defined all behaviours expected (based on earlier exploratory observations of parasitoid pairs) to be observed during the following experiment. Following protocols developed in section 2.3 to maximise the likelihood that parasitoids would be physiologically motivated to oviposit, 3-day-old females of each species were identified by pre-testing. The confirmed females were held in individual Petri dishes with honey and host remains for approximatly 2 h until observations began. Each observation was conducted for 30 mins using a dissection microscope (Stemi V6, Zeiss, Germany, 10 x mag.). Before observations, independent variables were recorded and an 'egg map' was drawn by hand so that individual eggs could be marked if parasitised and their fate tracked. Two female parasitoids, one of each species, were then placed together in a Petri dish containing a batch of 7-13 fresh *P. charybdis* eggs. From this moment on, all parasitoid actions and interactions were directly observed, and recorded with *The Observer* via the handheld PC. Each individual parasitoid could exhibit only one behavioural state at any time, hence the beginning of one state automatically signalled the end of the previous state. An observation was discontinued if no parasitoid made contact with the host eggs within five minutes, or if only one made contact with the eggs and no patasitoid interactions occurred within ten minutes. To avoid observer fatigue, only one consecutive hour of observations was made at any time, and no more than three hours of observations were conducted on a single day. Observations were all conducted between 1200-1700 h.

Following each observation, unparasitised eggs were pricked with a pin to ensure *P. charybdis* larvae did not emerge to feed on neighbouring parasitised eggs and prevent parasitoid emergence. Eggs were incubated (22 ^{o}C, 70% r.h., 14L:10D) and the number and species of the parasitoids that emerged were recorded for each batch. Reference to the marked egg maps allowed the winners of any multiple-parasitism events to be determined. Latency, duration, proportion of observed time, and number of event occurrences were calculated for each behavioural 'state' by *The Observer*. Species means were compared using non-parametric two-sample Wilcoxon signed-rank tests (SAS Institute, 1999).

Results

Twenty-eight observations were accumulated over 37 days (Fig. 5.1). Ambient temperature ranged from 22-27 ^{o}C. As no significant difference in parasitism was detected between these temperatures in section 2.3.6 this variable was not included in subsequent analysis.

All 28 *N. insectifurax* oviposited, parasitising 117 hosts (Table 5.1). Only 15/28 *E. nassaui* oviposited. 13/50 *E. nassaui* ovipositions occurred while they had sole possession of the hosts, before *N. insectifurax* located them (Fig. 5.2). Two were achieved when *N. insectifurax* failed to detect *E. nassaui* as it approached from behind. The remaining 35 (70.0%) occurred when the two species 'shared' possession of the eggs. In contrast, 94 (80.3%) of *N. insectifurax* ovipositions occurred when they had sole possession of host eggs and only 23 (19.7%) occurred when the host was shared with *E. nassaui* (Fig. 5.3). Eighteen eggs were multiparasitised in eleven observations. Of these *E. nassaui* emerged from five and *N. insectifurax* from thirteen, but in all cases only one parasitoid emerged. *Neopolycystus insectifurax* superparasitised five eggs and *E. nassaui* none.

There was no significant difference between *E. nassaui* and *N. insectifurax* in the average latency from the beginning of the observation to display of the host assessment behaviours *DrumW, Drill* and *Hfeed* (see appendix 3 for full description of individual behavioural states). Latency to oviposition was significantly shorter for *E. nassaui* (z = 245, P = 0.0185, Fig. 5.3). When *N. insectifurax* located the hosts first (n = 14/28) they maintained possession throughout ten observations, prohibiting *E. nassaui* from ovipositing (Table 5.1). In the remaining four observations *E. nassaui* also gained access to the eggs,

ovipositing in the presence of *N. insectifurax*. Fourteen *E. nassaui* located host eggs first. Eleven subsequently lost possession to *N. insectifurax*, and three were able to remain with the hosts and continue ovipositing when joined by *N. insectifurax*. No *E. nassaui* maintained exclusive possession of host eggs throughout any observation.

Table 5.1: Number of observations and proportion of observed time during which 28 *E. nassaui* and *N. insectifurax* spent in possession of, or ovipositing into, host eggs during 30 min observations. The proportion of on-host time (time in contact with host eggs) is divided into time spent conducting oviposition behaviours (*Contact, DrumW, Drill, Hfeed, Ovip*) and defensive behaviours (*Aware*, *Patrol, Bite, Chase, Flap*). $P < 0.05$ indicates significant differences between mean values.

		E. nassaui	***N. insectifurax***	***P***
Possession	First to locate host eggs	14	14	-
	Stole from other species	0	11	-
	Lost to other species	11	0	-
	Maintained possession throughout	0	10	-
	Joined opponent †	4	3	-
Oviposition	Number of ovipositing ♀'s	15	28	-
	Number of ovipositions	50	117	-
	Ovipositions per ♀($\bar{x}$ ± SE)	1.8 ± 2.3	4.2 ± 1.7	0.0003
	Ovipositions per ovipositing ♀($\bar{x}$ ± SE)	3.3 ± 0.6	4.2 ± 0.3	0.1814
	Oviposition duration (s) ($\bar{x}$ ± SE)	27.4 ± 3.0	38.2 ± 4.4	0.1647
Time spent on-host as % of total observed time	Total on-host time ($\bar{x}$ ± SE)	28.7 ± 0.05	90.7 ± 0.02	< 0.001
	Oviposition behaviours ($\bar{x}$ ± SE)	92.1 ± 0.03	55.8 ± 0.02	< 0.001
	Defensive behaviours ($\bar{x}$ ± SE)	0.0 ± 0.00	30.4 ± 0.02	< 0.001
	Other (e.g. groom/rest) ($\bar{x}$ ± SE)	27.4 ± 0.03	38.2 ± 0.02	< 0.001

† Joined opponent = instances in which an individual located host eggs already occupied by their opponent, mounted the host eggs in view of the opponent and oviposited at least once.

There were 297 interactions between *E. nassaui* and *N. insectifurax* (see table 5.2), all instigated and won (parasitoid remained in contact with host eggs after the interaction while the 'loser' fled) by *N. insectifurax*. Of these, 275 involved direct attacks (*chase*, *bite*) on *E. nassaui* by *N. insectifurax* and the remainder involved a display where *N. insectifurax* orientated itself towards *E. nassaui* and flapped its wings (*flap*). *Enoggera nassaui* responded by running away (*flee*), but failed to respond to *flap* in five instances. Two additional defensive displays were exhibited by *N. insectifurax* when in possession of host eggs. The first, *patrol,* involved walking or running around the perimeter of the egg batch with head and antennae erect, looking around the arena or towards *E. nassaui* rather than at the host eggs. The second, *aware,* involved standing motionless on the host eggs

with head and antennae erect, looking around the arena or towards *E. nassaui* if nearby. On average, these two behaviours were displayed for 25.0% of each observation and occurred regardless of whether *E. nassaui* was approaching the host eggs or not.

The two parasitoid species differed significantly (Table 5.2) in the mean number of occurrences and mean duration of all behaviours, except resting on the host (*Hrest*). *Enoggera nassaui* displayed all off-host behaviours for longer, and on average spent 71.4% of observed time away from host eggs. They made significantly more individual contacts with host eggs than *N. insectifurax* during each observation ($z = 1160.5$, $P < 0.001$, Table 5.2) but were immediately chased away by *N. insectifurax* following approximately 2/3 of these. When able to remain in contact with host eggs (on-host), *E. nassaui* displayed behaviours associated with oviposition (*Contact, DrumW, Drill, Hfeed, Ovip*) for $\bar{x} =$ 92.1% of the time (Table 5.1). In contrast, while *N. insectifurax* spent an average of 90.7% of observed time in contact with host eggs, oviposition behaviours were displayed for only $\bar{x} =$ 55.8% of this. Remaining on-host time was primarily allocated to defensive behaviours (30.4%) and grooming (*Hgroom*, 13.2%). Overall, however, *N. insectifurax* did spend more time per observation conducting host assessment behaviours, grooming while in contact with the host (*Hgroom*), and displaying defensive behaviours. Following an oviposition, *N. insectifurax* showed a 61% probability of displaying defensive behaviours and a 35% probability of resuming host assessment while *E. nassaui* showed a 96% probability of resuming host assessment (Appendix 4).

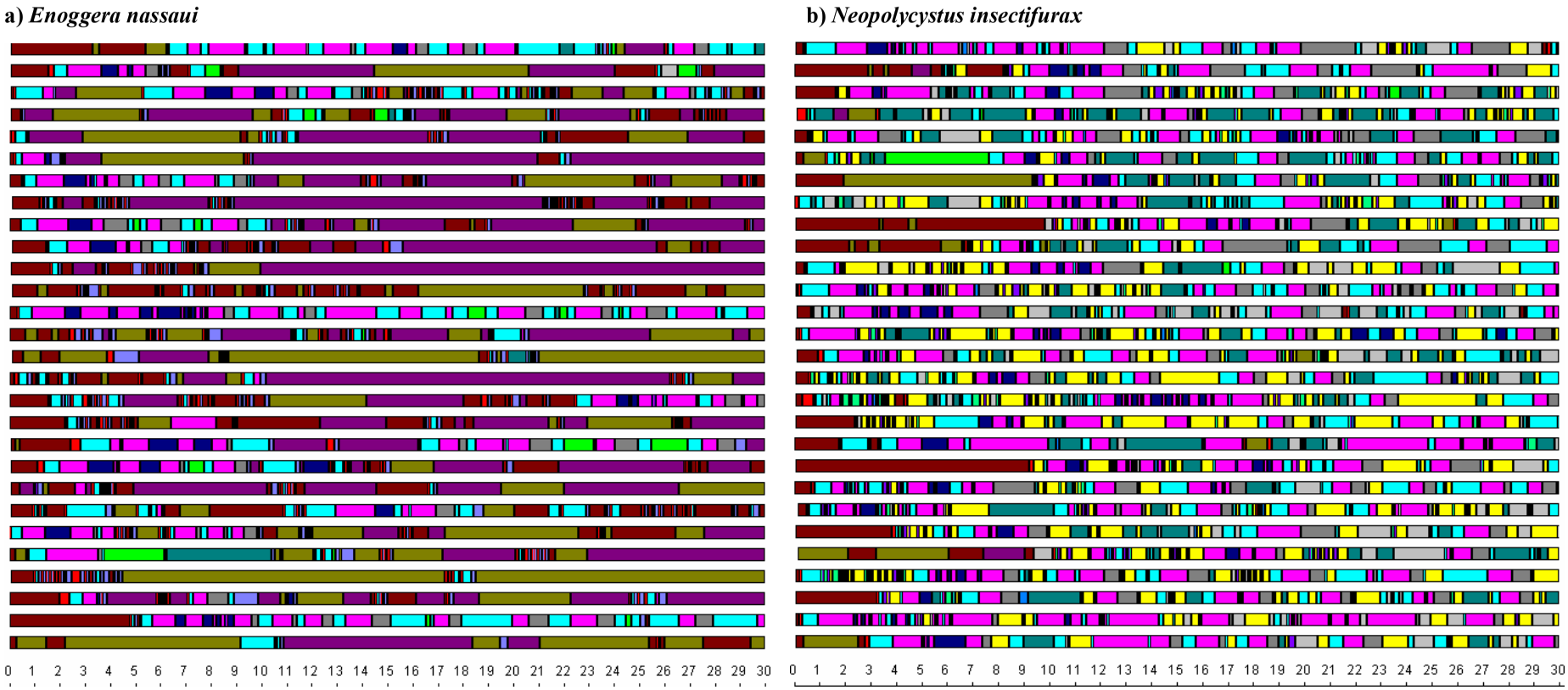

Figure 5.1: Time spent by **a)** *E. nassaui* and **b)** *N. insectifurax* exhibiting different behavioural states during 30 min observations. Each horizontal bar represents one observation (n = 28) from 0 to 30 minutes left to right. Each colour codes for one of 16 behavioural states. For example, purple, which dominates (a), indicates the period of time *E. nassaui* was *resting*, while the yellow prominent in (b) indicates instances when *N. insectifurax* was *patrolling* around the host eggs. A full key to colour codes is given in Appendix 3. The duration of each behaviour is shown by the length of bar coloured for that state. In general, it can be seen that in (a) only a few behaviours were exhibited, each for a long duration, whereas in (b) behaviours occurred for shorter durations and there were more state changes. Each observation included one wasp of each species, hence adjacent bars in (a) and (b) represent the actions of, and interactions between, one *E. nassaui* and one *N. insectifurax* observed simultaneously.

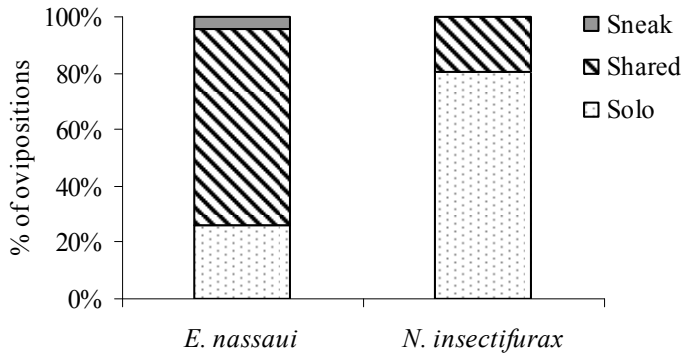

Figure 5.2: Proportion of ovipositions by *E. nassaui* (n = 50) and *N. insectifurax* (n = 117) when in sole possession of host eggs (solo), when possession was shared by both species (shared), and when the opposing species was in possession of the host eggs (sneak).

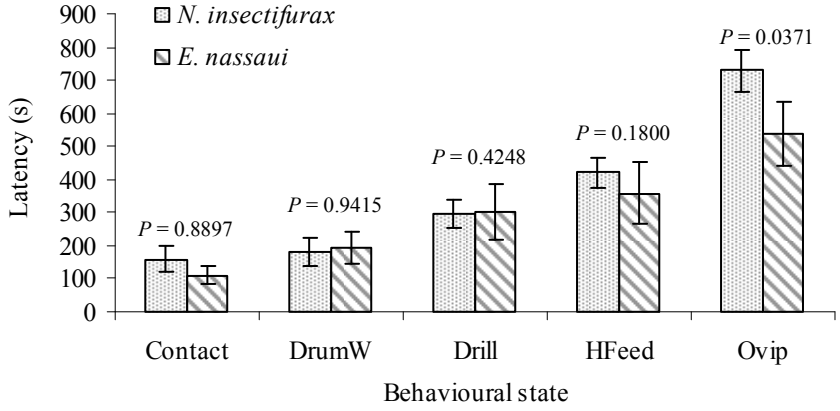

Figure 5.3: Latency ($\bar{x}$ ± SE) from the beginning of observation to the first occurrence of behavioural states associated with oviposition for *E. nassaui* and *N. insectifurax*.

Table 5.2: Number of times ($\bar{x}$ ± SE) and duration ($\bar{x}$) for which each behavioural state was displayed by *E. nassaui* and *N. insectifurax*. The first 12 states (*Contact-Hgroom*) occurred when parasitoids were in contact with the host (on-host) and the remainder occurred while not in contact with the host (off-host). Behaviours associated with oviposition are shaded in pale grey, defensive behaviours in dark grey and other behaviours are unshaded. $P < 0.05$ indicates species means that are significantly different.

	Number of occurrences ($\bar{x}$ ± SE)			Duration ($\bar{x}$) (mins & seconds)		
State	***E. nassaui***	***N. insectif.***	***P-value***	***E. nassaui***	***N. insectif.***	***P-value***
Contact	10.3 ± 1.1	1.4 ± 0.1	< 0.0001	52	10	< 0.0001
DrumW	7.1 ± 0.9	13.8 ± 0.7	< 0.0001	2 m 46 s	4 m 21 s	0.0008
Drill	4.0 ± 1.0	11.6 ± 0.8	< 0.0001	2 m 49 s	7 m 5 s	< 0.0001
Hfeed	1.4 ± 0.3	3.3 ± 0.3	0.0002	38 s	57 s	0.0117
Ovip	1.8 ± 0.4	4.2 ± 0.3	0.0003	48 s	2 m 35 s	< 0.0001
Aware	0.6 ± 0.2	14.2 ± 1.1	< 0.0001	1 s	1 m59 s	< 0.0001
Patrol	0.0 ± 0.0	5.9 ± 0.7	< 0.0001	0 s	5 m 11 s	< 0.0001
Flap	0.0 ± 0.0	0.8 ± 0.2	< 0.0001	0 s	6 s	< 0.0001
Bite	0.0 ± 0.0	4.9 ± 0.6	< 0.0001	0 s	31 s	< 0.0001
Chase	0.0 ± 0.0	4.9 ± 0.7	< 0.0001	0 s	29 s	< 0.0001
Hrest	0.0 ± 0.0	0.2 ± 0.1	0.0437	22 s	11 s	0.0523
Hgroom	0.3 ± 0.1	7.0 ± 0.8	< 0.0001	14 s	3 m 32s	< 0.0001
Flee	10.4 ± 1.1	0.0 ± 0.0	< 0.0001	24 s	0 s	< 0.0001
OffWalk	8.0 ± 0.9	1.6 ± 0.2	< 0.0001	4 m 25 s	1 m 56 s	0.0003
Groom	3.4 ± 0.5	0.7 ± 0.2	< 0.0001	6 m 21 s	45 s	< 0.0001
Rest	3.6 ± 0.5	0.1 ± 0.1	< 0.0001	9 m 12 s	6 s	< 0.0001

Discussion

Direct and indirect competition between female parasitoids of the same guild is common (Pschorn-Walcher 1977). Host-guarding and direct agonistic interactions have been observed in many families (Cave et al. 1987; Mills 1991; Field & Keller 1999; Batchelor et al. 2005). Understanding these interactions is important if a species-complex approach to biological control is taken, so that compatible agents are selected (Pschorn-Walcher 1977). The most effective agents tend to exhibit superior host finding abilities and are more closely synchronised with their host but are inferior competitors. These 'r-selected' species should ideally be released first to demonstrate their control potential in the absence of competition, as additional agents may be unnecessary (but see Ehler 1979). Such counter-balanced competition, i.e. between good searchers with poor defence and poor searchers with strong fighting abilities, is common in forestry parasitoid complexes (Mills 1991).

Overall *N. insectifurax* are innately more aggressive than *E. nassaui*. They were characterised by taking possession of and defending host egg batches, and always responded to the presence of *E. nassaui*, instigating all interactions. Substantial time was spent patrolling around and guarding host batches ($\bar{x}$ = 23.9% observed time) even if *E. nassaui* was not approaching. This presumably aids the detection of opponents. Extended periods of brood guarding and defence, as exhibited by *N. insectifurax,* have rarely been observed in parasitoids (Hardy & Blackburn 1991; Goubault et al. 2007a; Nakamatsu et al. 2009). Guarding behaviour has been seen in ectoparasitic bethylids and chelonids (Hardy & Blackburn 1991; Batchelor et al. 2006). It may be beneficial if the probability of finding further unparasitised hosts is low (Hardy & Blackburn 1991), if egg production is costly (Nakamatsu et al. 2009), or if survivorship of the current brood will be low if they are not guarded (Goubault et al. 2007a). *Neopolycystus insectifurax* also groomed frequently while on the host batch. This form of grooming appeared to involve rubbing a substance from the host surface over the head and forelegs and may therefore be somehow involved in signaling host possession.

Enoggera nassaui took no interest in *N. insectifurax*, except to flee in response to attack. As a moderately r-selected species (relative to *N. insectifurax*), *E. nassaui* may maximise its fitness by being better adapted for host searching and distributing its progeny. In contrast, the competitive strength of *N. insectifurax* (relatively K-selected) may come at the expense of host searching. *Enoggera nassaui* were able to locate hosts and begin ovipositing more quickly than *N. insectifurax* and made repeated attempts ($\bar{x}$ = 10.4 in 30 min) to access the hosts despite losing contests. The ability of *E. nassaui* to quickly locate and begin parasitising hosts could reflect differences in eggload, as low eggload can reduce searching intensity (Rosenheim & Rosen 1991). However, every effort was made to minimise such a disparity by following the rearing procedures developed in chapter 2, so eggload should not have influenced searching in this instance. In chapter 4, *E. nassaui* were seen to resume searching shortly after parasitising an egg batch, often locating and parasitising the second batch. In contrast, laboratory reared *N. insectifurax* remained with hosts for some time (>24 h) post-parasitism. *Enoggera nassaui* have a shorter pre-oviposition period (section 2.3.2) and development time than *N. insectifurax*, completing development and eclosing two days earlier (9 vs. 11 days at 22^{o}C). This may increase the resource value (see below) of guarded hosts to *E. nassaui* because if they multiparasitise

them shortly after *N. insectifurax* their larvae may still eclose first, possibly winning possession of that host (see section 5.3). This may explain the persistence of *E. nassaui* in trying to access hosts after losing contests, and the post-ovipositional host guarding of *N. insectifurax*.

The eggs of *N. insectifurax* may also be at risk of hyperparasitism for longer than *E. nassaui's*. In Australia, where there is a larger and more complex guild of parasitoids associated with a diverse fauna of eucalypt-specific paropsine beetles (Cumpston 1939; Tanton & Epila 1984; Selman 1985; Kelly & Reid 1999), the forces of competition and hyperparasitism inevitably exert strong selection pressure on parasitoids. The different oviposition strategies demonstrated by *E. nassaui* and *N. insectifurax* may represent alternative adaptations to surviving in this highly competitive environment.

To maximise fitness-per-host, parasitoids face a trade-off between ovipositing, host searching and host defence (Waage 1986). Depositing many eggs in one place, risks' losing them all if that patch is found by a predator, is superparasitised or hyperparasitised. Parasitoids travelling between patches, risk predation or injury during flight, and may fail to locate new hosts. Injury or death sustained during host defence reduces any chance of future reproductive success. Evolutionary game theory predicts individuals will avoid direct contests that may result in injury unless resources are scarce. It is not surprising; therefore, that female-female competition in the Parasitica rarely escalates beyond brief, ritualised interactions with minimal physical contact (Batchelor et al. 2005). Contest outcomes are generally determined by physical asymmetries like body size, or resource value asymmetries such as ownership, egg- vs. time-limitation, batch size, rate of encounter with unparasitised hosts, and rate of encounter with competitors (Hardy & Blackburn 1991; Field & Calbert 1998; Goubault et al. 2007b; Nakamatsu et al. 2009). The dominance of *N. insectifurax* in this study probably resulted from its larger size (section 3.3) making it too risky for *E. nassaui* to fight. As a smaller parasitoid, *E. nassaui* has less chance of winning, so avoiding interactions and directing energy into host searching may increase its fitness. Conversely, a larger parasitoid like *N. insectifurax,* able to defend its brood with relative ease, risks more by travelling between patches and benefits from monopolising any hosts it does find.

When parasitoids meet on a host patch, an owner-intruder situation is commonly formed (Field & Calbert 1999). The first to arrive takes possession of the hosts becoming the 'owner'. A resource-value asymmetry is created when the owner begins ovipositing and an 'intruder' subsequently arrives, because the host is more valuable to the female that has already invested some progeny (Mills 1991; Goubault et al. 2007a). Mills (1991) found that intraspecific interactions between parasitoids of the ash bark beetle were generally won by 'owners', while interspecific interactions showed a clear hierarchy of dominance between species. In contests between conspecific *Trissolcus basalis* (Wollaston) (Scelionidae), ownership is a stronger predictor of contest winning than is size (Field & Calbert 1998, 1999). Asymmetries and ritualised displays usually prevent escalated fighting between females (e.g. Goubault et al. 2007b). One might expect conspecific females to be more physically and competitively 'symmetrical' than females of different species. If so, interactions between the latter should escalate to physical combat less often. This seems to be the case with *E. nassaui* and *N. insectifurax*. When conspecific pairs compete, contests are often violent, with owners winning interactions and intruders failing to oviposit (S. Mansfield unpub.). In contrast, however, Batchelor et al. (2005) observed that fatal contests between bethylids were more likely to occur during interspecific competition than conspecific competition for their host, the coffee berry borer.

Co-exploitation by *N. insectifurax* and *E. nassaui* occurred on several occasions favouring oviposition by *E. nassaui.* Co-exploited egg batches were not larger than batches defended by *N. insectifurax*, nor had their owners arrived more recently or laid fewer eggs. As such, there was no apparent reason for these batches to hold less value for *N. insectifurax* and not be worth defending. Unlike examples of co-exploitation by other parasitoids (e.g. Field & Calbert 1998), *N. insectifurax* became very agitated and usually ceased to oviposit in the presence of *E. nassaui.* Furthermore, only one example of possible co-exploitation of a *P. charybdis* egg batch has been recorded in the field, despite several years of intensive egg collection (Jones & Withers 2003; Murray et al. 2009). It is suggested therefore that true co-exploitation did not occur in this study, and these events might be better regarded as cases of *E. nassaui* winning interactions. There is no obvious explanation for the behaviour of *N. insectifurax* in these instances which is a possibly an artefact of confinement or rearing conditions over multiple generations.

Host feeding secures nutrients for oocyte production so may be considered as a trade-off between ovipositing now and ovipositing in the future (Ferreira de Almeida et al. 2002; Giron et al. 2004). *Neopolycystus insectifurax* was expected to exhibit longer feeding bouts than *E. nassaui* as it is larger, more synovigenic (chapter 2) and directed considerable energy into defending hosts, yet the opposite occurred. *Neopolycystus insectifurax* did, however, feed more frequently, so a higher proportion of their time was dedicated to securing nutrients overall. To sustain intensive host searching, *E. nassaui* may also have very high energy demands. As *E. nassaui* were frequently chased off hosts, they may have fed as long as possible to satisfy this requirement whenever the opportunity arose.

This study predicts direct interactions will occur between *E. nassaui* and *N. insectifurax* in the field, but this remains unevaluated. Their co-existence dynamics will depend on their direct interactions but also on host density, physiological differences (chapter 2), distribution overlap (chapter 6) and indirect interactions. The latter potentially include conspecific superparasitism, multiparasitism and hyperparasitism, all of which can affect brood survival. Multiparasitism was observed here for the first time, with both species emerging on some occasions. This is investigated in more detail in the following section.

5.3 MULTIPARASITISM BY *E. NASSAUI* AND *N. INSECTIFURAX*

In the previous section, *N. insectifurax* was able to out-compete *E. nassaui* during direct competition for access to hosts in the laboratory. Whether this occurs in the field is less clear because multiple parasitoids may only rarely arrive on a host batch at the same time. Individual parasitoids that locate the same host batch at different times may still compete if both oviposit into it. This is superparasitism if the two ovipositing parasitoids are conspecifics, and multiparasitism if they are different species. Only one batch of eggs containing both *E. nassaui* and *N. insectifurax* has ever been collected from the field (Jones & Withers 2003; Withers *pers. com*.), so it has been assumed that if multiparasitism occurs one species must consistently out-compete the other. In section 5.2, both species responded to and oviposited into hosts already parasitised by a female of the other species (their 'opponent'). Multiparasitism is generally considered maladaptive for solitary parasitoids because only one offspring can usually complete development per host. However, there are instances in which it can be adaptive (van Alphen & Visser 1990). In section 5.2 it was

observed that when parasitised and unparasitised eggs were present within a batch some *N. insectifurax* appeared to actively select to oviposit into parasitised hosts first. To assess if this was the case, an experiment in which adults of each species had access to parasitised eggs without coming into direct contact with an opponent was necessary.

Objectives

To determine if *E. nassaui* and *N. insectifurax* are able to distinguish between parasitised and unparasitised host eggs and whether this influences their decision to oviposit. To determine whether species, or the order in which the species oviposit, is more likely to influence the outcome of multiparasitism.

Methods

Twenty observations were accumulated following the protocols and configuration described in section 5.2. 'Modifiers' were added to this configuration so that any egg submitted to antennating, drilling, host-feeding or oviposition could be identified as unparasitised, self-parasitised or parasitised by the opposing species (Appendix 5). The behavioural states *aware*, *patrol*, *bite*, *chase* and *flap* were grouped into a single state termed *defensive*, and the behaviour '*jab'* was added to the configuration (Appendix 5). Hosts (batches of 10 *P. charybdis* eggs) were exposed to each parasitoid of the pair sequentially, not simultaneously as in section 5.2, so each observation consisted of two parts. In Part I, the actions of a solitary *E. nassaui* were recorded for 30 min or until 5/10 eggs had been observed to be parasitised. After parasitising five eggs, *E. nassaui* was removed and immediately replaced with a solitary *N. insectifurax*, the behaviour of which was recorded for the next 30 min (Part II). The procedure was repeated with hosts being exposed to *N. insectifurax* in Part I followed by *E. nassaui* in Part II. As *N. insectifurax* often took longer than 30 min to parasitise five eggs in Part I, they were observed until the required ovipositions occurred, but their actions were only recorded for the first 30 min. A total of 20 observations were made alternating *E. nassaui* and *N. insectifurax* as the first individual to have access to the hosts. Ovipositions were recorded on a hand-drawn egg map to track the fate of each parasitoid egg. Exposed egg batches were subsequently incubated (22 ^{o}C, 70% r.h.) and assessed daily for signs of parasitoid competition within multiparasitised and superparasitised eggs. The species of each parasitoid that emerged

was recorded. Mean latency to, duration of, proportion of observed time exhibited, and number of occurrences were calculated for each behavioural state and compared between species and between host states (parasitised or unparasitised) within species. Mean parasitism, multiparasitism, and proportion of multiparasitism events won in Part II, were also compared between species. Comparisons were all made by way of non-parametric two-sample Wilcoxon signed-rank tests.

Results

In Part II, *E. nassaui* oviposited in significantly more eggs (67/100) than *N. insectifurax* (46/100) (z = 135.5, P = 0.0167, Table 5.3). Two eggs were superparasitised by *E. nassaui*. Multiparasitism accounted for 73.9% and 47.7% of ovipositions by *N. insectifurax* and *E. nassaui* respectively. As such, there was a significant difference in the number of ovipositions representing parasitism vs. multiparasitism by *N. insectifurax* (14 vs. 32 eggs, z = 65.5, P = 0.0069) but not *E. nassaui* (33 vs. 32 eggs, z = 108.5, P = 0.8183). Also in Part II, 9/10 *N. insectifurax* and 7/10 *E. nassaui* oviposited into a parasitised egg first. Overall 85.0% and 55.0% of the first two eggs parasitised by *N. insectifurax* and *E. nassaui* represented multiparasitism, this difference was significant (Fisher's exact test $P = 5.547^{-6}$). More *N. insectifurax* (57.8%) than *E. nassaui* (42.2%) emerged from multiparasitised hosts. Both species emerged from a higher proportion of multiparasitised hosts if their parent was the second individual to oviposit (Fig. 5.4). Visual assessments of multiparasitised hosts failed to find evidence that > 1 parasitoid larva eclosed per host.

Table 5.3: Number of ovipositions by *E. nassaui* and *N. insectifurax* into *P. charybdis* eggs in Part II.

	E. nassaui	***N. insectifurax***
Total eggs parasitised	67/100	46/100
Parasitised	33/50	14/50
Multiparasitised	32/50	32/50
Superparasitised	2/100	0/100
Oviposited into unparasitised host first	3/10	1/10
Oviposition into parasitised host first	7/10	9/10
Offspring from multiparasitised eggs	27/64	37/64

There was no significant difference between species in the mean latency from the beginning of an observation to the first oviposition (z = 375, P = 0.3564, Fig. 5.5). Neither species showed a significant difference in latency on parasitised vs. unparasitised eggs (*E. nassaui* P = 0.7783; *N. insectifurax* P = 0.2518). *Neopolycystus insectifurax* did appear to begin ovipositing into parasitised eggs more quickly than unparasitised eggs ($\bar{x}$ = 9 m 57 s vs. 17 m 42 s, Fig. 5.5) and more quickly than *E. nassaui* overall ($\bar{x}$ = 9 m 02 s vs. 10 m 14 s, z = -0.7181, P = 0.2407), but these differences were not significant. *Enoggera nassaui* were quicker to begin ovipositing into *unparasitised* eggs compared to *N. insectifurax* (z = 58, P = 0.0145). Latency to oviposition was shorter for *E. nassaui* when given access to the hosts first rather than second (P = 0.0345), but slightly longer for *N. insectifurax* (P = 0.1434).

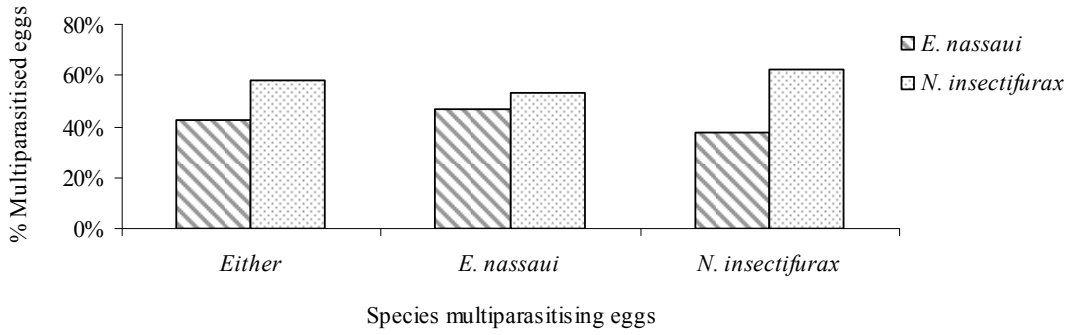

Figure 5.4: Total proportion of multiparasitised eggs from which *E. nassaui* or *N. insectifurax* emerged depending on which species was responsible for the multiparasitism (i.e. oviposited second).

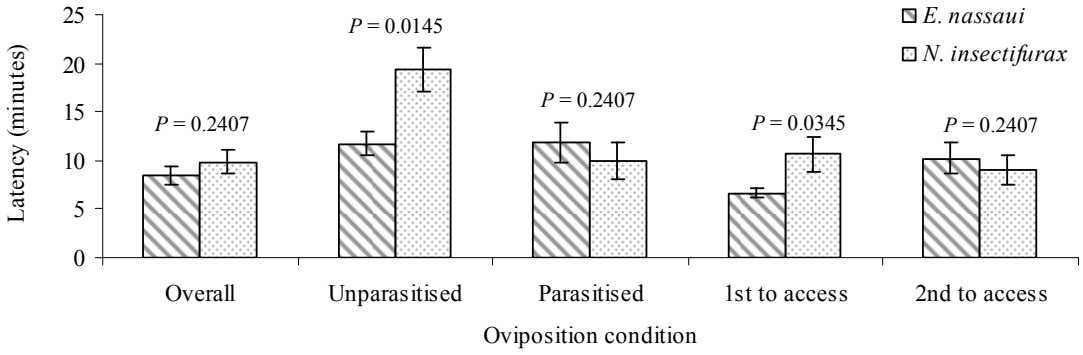

Figure 5.5: Latency ($\bar{x}$ ± SE) to first oviposition in Part II observations into: any host (= overall), an unparasitised host, or a parasitised host. Latency to first oviposition into any host when *E. nassaui* and *N. insectifurax* were first vs. second to have access to the hosts (n = 10 each species, 18-24 ^{o}C).

Overall, there was no significant difference in the time taken by *E. nassaui* to assess hosts (*DrumW, Drill, Hfeed*) when eggs parasitised by *N. insectifurax* were present (Part II), compared to when only unparasitised hosts were present (Part I, Table 5.4). However, significantly more time was spent antennating (*DrumW*) and drilling into (*Drill*) the individual eggs that were parasitised by *N. insectifurax*. These results together indicate that unparasitised eggs in the presence of parasitised eggs in Part II were accepted with less assessment than were unparasitised eggs alone in Part I, a comparison not directly made in Table 5.4. The mean time spent by *E. nassaui* females ovipositing into unparasitised and parasitised eggs was not significantly different (Table 5.4). In contrast, *N. insectifurax* spent significantly less time assessing and ovipositing into hosts parasitised by *E. nassaui* than into unparasitised hosts (Table 5.4), and oviposition duration was significantly longer when hosts were already parasitised ($\bar{x}$ = 61.6 s vs. 37.6 s, z = 67, p = 0.0414). *Neopolycystus insectifurax* spent significantly more time (z = -2.3812, P = 0.0139) displaying defensive behaviours when eggs parasitised by *E. nassaui* were present ($\bar{x}$ = 20.5% of observed time) than when they were not ($\bar{x}$ =11.8% of observed time).

Table 5.4: Time ($\bar{x}$ ± SE) spent by *E. nassaui* and *N. insectifurax* displaying oviposition related behaviours on unparasitised and parasitised eggs in Part II observations, and displaying oviposition and defensive behaviours in Part I, when only unparasitised hosts were present, compared to Part II, when parasitised hosts were also present. $P < 0.05$ indicates means are significantly different.

	Unparasitised	**Parasitised**	***P***	**Part I**	**Part II**	***P***
E. nassaui						
DrumW	2 m 19 s ± 24 s	4 m 05 s ± 38 s	0.0298	3 m 35 s ± 23 s	3 m 12 s ± 25 s	0.1549
Drill	3 m 44 s ± 44 s	7 m 25 s ± 1 m 28 s	0.0257	6 m 36 s ± 32 s	5 m 34 s ± 53 s	0.1023
Hfeed	2m 35 s ± 13 s	1 m 04 s ± 14 s	0.0283	1 m 06 s ± 11 s	44 s ± 10 s	0.0526
Ovip	1 m 50 s ± 20 s	1 m 21 s ± 17 s	0.1602	2 m 50 s ± 1 m 36 s	1 m 36 s ± 13 s	0.0046
N. insectifurax						
DrumW	2 m 57 s ± 19 s	1 m 55 s ± 20 s	0.0307	4 m 53 s ± 53 s	2 m 26 s ± 15 s	0.0010
Drill	5 m 11 s ± 47 s	3 m 05 s ± 50 s	0.0345	7 m 50 s ± 40 s	4 m 18 s ± 38 s	0.0030
Hfeed	59 s ± 15 s	13 s ± 06 s	0.0147	1 m 21 s ± 13 s	36 s ± 09 s	0.0085
Ovip	3 m 02 s ± 29 s	42 s ± 11 s	0.0012	3 m 06 s ± 53 s	1 m 52 s ± 22 s	0.1356
Defend	-	-		3 m 04 s ± 39 s	6 m 10 s ± 43 s	0.0139

Discussion

Up to 200 species of hymenopteran parasitoids, and most families, have been shown to discriminate between parasitised and unparasitised hosts (Nufio & Papaj 2001). Some never or rarely super- or multiparasitise hosts (e.g. Potting et al. 1997; Gauthier & Monge 1999) while the majority reject parasitised hosts at least after experiencing unparasitised hosts (Potting et al. 1997). Hosts of most solitary parasitoids can only sustain a single parasitoid offspring. As superparasitism and multiparasitism can delay or prevent offspring development (Potting et al. 1997; Ardeh et al. 2005) their avoidance is generally considered adaptive (van Lenteren 1981). The ability of solitary parasitoids to discriminate can theoretically increase their efficiency as BCAs by reducing wastage of eggs, hosts, and time, and the level of competition and therefore mortality suffered by the offspring (van Lenteren 1981; van Alphen & Visser 1990; Nufio & Papaj 2001; Ardeh et al. 2005).

Superparasitism was very low in this study, suggesting both *E. nassaui* and *N. insectifurax* are able to recognise self-parasitised eggs. *Neopolycystus insectifurax* can also distinguish between self-parasitism and hosts parasitised by *E. nassaui*. They appeared to selectively multiparasitise hosts with ≈ 74% of all ovipositions per female and 85% of the first two ovipositions being into parasitised hosts. Host guarding behaviour (Fig. 5.6a) was also exhibited twice as often in the presence of parasitised hosts. Although assessment by *E. nassaui* of, and oviposition into, parasitised compared to unparasitised hosts did take significantly longer, almost identical proportions of ovipositions were made into each. This indicates that *E. nassaui* does not distinguish between unparasitised hosts and those parasitised by *N. insectifurax* when accepting a host for oviposition. Although the ability to detect and avoid *intraspecific* superparasitism is common among solitary parasitoids the ability to discriminate against *interspecific* parasitism is rare (van Lenteren 1981; Agboka et al. 2002; Ardeh et al. 2005). Like many parasitoids therefore, *E. nassaui* may only have the ability to avoid self-superparasitism and may treat all other hosts equally.

Whether an individual will super- or multiparasitise can be influenced by egg load, experience, and time since the initial parasitism (van Randan & Roitberg 1996; Agboka et al. 2002; Ardeh et al. 2005). Discrimination can be based on external factors such as marking pheromones, or internal factors such as physiological changes in the egg contents

or the presence of parasitoid larvae (Hardy & Blackburn 1991; Nufio & Papaj 2001). Marking pheromones could be costly as they may also alert hyperparasitoids to the presence of hosts. Both *E. nassaui* and *N. insectifurax* rigorously assess the external host surface before drilling, and appear to assess the internal environment with the ovipositor. No obvious marking behaviour, such as dragging the tip of the abdomen across the host after oviposition, was observed. If neither species applies marking pheromones, the arrestment of *N. insectifurax* on parasitised hosts may reflect their ability to detect any recently parasitised hosts rather than exactly who they were parasitised by (e.g. Field & Keller 1999). The wound created during oviposition, or a parasitism-induced change in the host contents detected in the fluid or volatiles oozing from that wound, may be the signal that it has been parasitised (Field & Keller 1999).

The preference of *N. insectifurax* to multiparasitise, compared to the apparent disregard shown by *E. nassaui*, may indicate a similar scenario to that seen with *Eretmocerus mundus* Mercet and *E. eremicus* Rose & Zolnerowich (Aphelinidae). The former does not discriminate between unparasitised hosts and those parasitised by *E. eremicus*, but is a strong larval competitor, able to win interactions within the host. Larvae of *E. eremicus* are not competitive, and adults actively avoid multiparasitism (Ardeh et al. 2005). Similarly, when there is little delay between ovipositions, *Telenomus busseolae* Gahan (Scelionidae) always out competes *T. isis* Polaszek and *Campoletis chlorideae* Uchida usually out competes *Eriborus argenteopilosus* (Cameron) (both Ichenumonidae) when they multiparasitise eggs of their respective noctuid hosts, regardless of which oviposits first (Agboka et al. 2002; Bajpai et al. 2006). When longer delays are induced, however (24 h and 18 h respectively), the first to oviposit often out-competes the other. This is usually attributed to the fact that the first larva to eclose, or that which develops faster, gains a competitive advantage over its opponent. Based on developmental speed, *E. nassaui* should be more willing to multiparasitise than *N. insectifurax*, which could explain their persistence in returning to occupied hosts (section 5.2) and their lack of discrimination. *Neopolycystus insectifurax* could selectively oviposit into parasitised eggs first to minimise the delay between larval eclosions, thereby maximising the chances of their slower developing offspring. It is difficult to estimate how successful this apparently risky strategy would be given they probably face competition from multiple other parasitoid species in the natural Australian environment (e.g. Tanton & Epila 1984). *Neopolycystus*

insectifurax does appear to have inferior searching abilities and test-females had been withheld from hosts for three days which could potentially induce a perception of host scarcity. The willingness of *N. insectifurax* to multiparasitise may therefore represent a trade-off between doing so and resuming host searching in the host-limited environment.

Unlike the examples discussed above, neither *E. nassaui* nor *N. insectifurax* consistently won multiparasitism. Individuals had a greater chance of winning when they were the second, rather than the first, to oviposit. The actual mechanism by which contests between these species are won within multiparasitised hosts is not clear. Larvae of both are highly mobile and can be seen 'swimming' within the host within 24 h and 48 h of oviposition respectively. Although both species eggs were often visible immediately after multiparasitism, on no occasions were multiple larvae subsequently observed. The outcome of multiparasitism may therefore be determined very early, possibly before larval eclosion. Ovicide has been observed in ectoparasitic bethylidae, which consume the eggs of their opponents (Hardy & Blackburn 1991), and the ectoparasitoid *Bracon hebetor* Say, which will puncture eggs of opponents with their ovipositor (Strand & Godfray 1989). The latter was observed of *N. insectifurax* on several occasions after their 30 min observations period (Fig. 5.6b), and may have some role in this species' dominance. During recorded observation periods physical ovicide was not seen, but there was opportunity for a chemically mediated form of ovicide. Substances can be injected during oviposition, or secreted by eggs and larvae of some parasitoids to paralyse hosts or stop host development (Salt 1968; Strand 1986). Potentially, such substances could also inhibit the development of any other parasitoid's eggs present in the host. *Enoggera nassaui* and *N. insectifurax* do appear to stop the development of *P. charybdis*. A distinct change in host composition can be seen emanating out from *N. insectifurax* eggs in particular within minutes of oviposition (Fig. 5.6c). By the following day the entire host contents appears to be 'broken down'.

Multiparasitism may be adaptive when hosts are scarce and competition between individual parasitoids is high (van Alphen & Visser 1990; Potting et al. 1997). The pay-off of multiparasitism is probably higher for *E. nassaui* when competing against *N. insectifurax*, due to its faster development time. *Neopolycystus insectifurax* may counteract this disparity in development time by attempting physical or chemical ovicide of *E.*

nassaui eggs. Because *N. insectifurax* are at risk of mortality from superparasitism, multiparasitism and hyperparasitism for longer, adult females may also obtain a fitness gain from brood guarding. As neither species consistently out-competed the other in this study, it would be interesting to see if either rejects parasitised hosts after first being given experience with unparasitised hosts, or if a greater delay between ovipositions were induced. Theoretically, hosts should become less acceptable for multiparasitism as the parasitoid larva inside develops and consumes more of the limited resource, and guarding duration by *N. insectifurax* should reflect this.

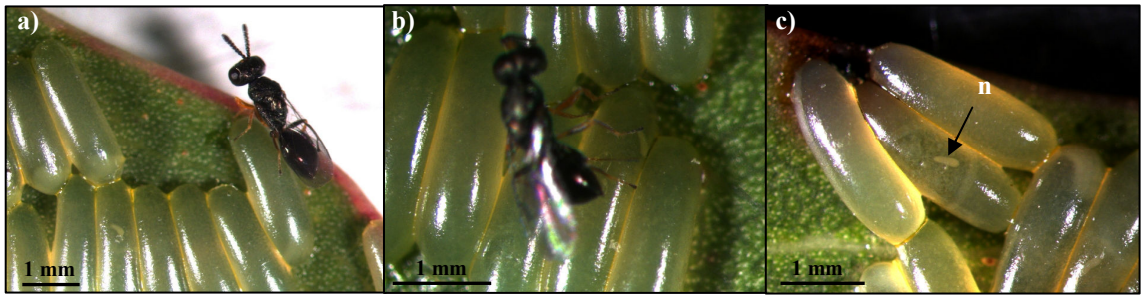

Figure 5.6: a) Guarding behaviour (*aware*) of *N. insectifurax*, **b)** *N. insectifurax* 'jabbing' egg of another parasitoid with the ovipositor, **c)** *P. charybdis* egg contents breaking down around *N. insectifurax* egg (n).

In this chapter it has been shown that *N. insectifurax* is more competitive that *E. nassaui* in the laboratory during direct competition between adult females. *Neopolycystus insectifurax* also appears to be slightly more competitive when multiparasitism occurs. However, in the natural environment there is likely to be little direct interaction between individuals and greater delays between ovipositions when multiparasitism occurs. The competitive advantage held by *N. insectifurax* may therefore be reduced by the potentially superior host searching abilities and faster development of *E. nassaui*. However, the detection in 2001 of a hyperparasitoid that attacks *E. nassaui* in New Zealand, now adds another level of competition to this system. The ability of this hyperparasitoid to affect *N. insectifurax* and the geographical overlap between populations of all three species is explored in the next chapter, and the potential implications for the biological control of *P. charybdis* in New Zealand are discussed.

5.4 SUMMARY

Enoggera nassaui and *N. insectifurax* show different behavioural strategies when forced to compete to oviposit into the same host in the laboratory. *Neopolycystus insectifurax* are characterised by taking possession of host egg batches and aggressively guarding them. *Enoggera nassaui* flee when approached by *N. insectifurax* and do not engage in contests. Compared to *N. insectifurax*, *E. nassaui* are able to locate and begin assessing hosts more quickly. *Neopolycystus insectifurax* prohibit *E. nassaui* from spending much time in contact with the hosts, but when contact is made *E. nassaui* spend most of their time conducting oviposition behaviours. *Neopolycystus insectifurax*m in contrast, spend considerable time actively guarding the hosts, and less time ovipositing.

Both *E. nassaui* and *N. insectifurax* multiparasitise host eggs when presented with mixed batches of parasitised and unparasitised hosts. *Enoggera nassaui* do not appear to discriminate between unparasitised hosts and hosts parasitised by *N. insectifurax*. *Neopolycystus insectifurax* do discriminate, and often choose to oviposit into eggs parasitised by *E. nassaui* before ovipositing into unparasitised hosts. There is some evidence that *N. insectifurax* might commit physical and/or chemical ovicide of *E. nassaui* eggs. Neither *E. nassaui* nor *N. insectifurax* consistently win multiparasitism, but both win more often when they are the second to oviposit into the host.

CHAPTER 6: BIOLOGY OF *B. ALBIFUNICLE &* ITS POTENTIAL IMPACT ON THE BIOLOGICAL CONTROL OF *P. CHARYBDIS*

6.1 INTRODUCTION

Unintentional introductions of exotic natural enemies of established pests are often only detected by chance and are rarely investigated in detail. These arrivals have the potential to both provide and compromise the biological control of pests through top-down processes such as parasitism, hyperparasitism and predation (Rosenheim 1998; Sullivan & Völkl 1999; Withers 2001). The self-introductions of *N. insectifurax* and *B. albifunicle* to New Zealand, both first detected in the Bay of Plenty region in 2001 (Murphy 2002; Berry 2003), may exemplify both possibilities. These two discoveries provided a unique opportunity to study the consequences of successive parasitoid incursions in respect to their positive and negative impacts on an established biological control program. They also add another dimension to the question of what characteristics improve or compromise the effectiveness of *E. nassaui* and *N. insectifurax* as control agents of *P. charybdis* in New Zealand.

The ecological role of obligate hyperparasitoids and their ability to disrupt biological control programs through top-down constraints on primary parasitoid population growth have been reviewed by Rosenheim (1998) and Sullivan & Völkl (1999). Both cite some models that predict disruption will occur, and others that predict it will not, or will even improve control by stabilising fluctuating herbivore and primary parasitoid densities. Experimental studies that provide evidence of hyperparasitism reducing pest regulation by primary parasitoids in some instances and having no significant impact in others are also acknowledged. Hyperparasitoids can certainly jeopardise the successful establishment of BCAs by limiting the numbers that can be reared in quarantine for release and screening for hyperparasitoids prior to the importation of new agents is now considered essential (Lopez-Vaamonde & Moore 1998; Berry & Mansfield 2006). Several early importations of BCAs for *P. charybdis* were unsuccessful because of to hyperparasitism (Bain & Kay 1989). This is not surprising considering that hyperparasitoids are integral in the regulation of parasitoid populations that attack defoliating, sap-sucking and wood boring insects in Australian eucalypt forests (Greaves 1966; de Little 1982; Tanton & Epila 1984; Selman

1985). The extent of this regulation is apparent from the high levels of parasitism achieved in their absence when primary parasitoids have been used as BCAs of paropsine beetles in South Africa and New Zealand (Bain & Kay 1989; Tribe & Cillié 2000).

The family Encyrtidae, to which *B. albifunicle* belongs, consists almost entirely of internal parasites of insects and arachnids and they are commonly used as BCAs (Noyes 1988). Most are solitary primary parasitoids, but gregarious, polyembryonic, and hyperparasitic species are not uncommon. *Baeoanusia albifunicle* is one of three species in its genus and is widely distributed in Australia (Cumpston 1939; Tribe 2000; Schmidt & Noyes 2003). Its biology has not been studied in detail. Tribe (2000) described it as an obligate hyperparasitoid of *Enoggera* spp., noting the relatively large size of the larval head and mandibles, and a female-biased sex ratio. In New Zealand *E. nassaui* and *N. insectifurax* are the only primary parasitoids of *P. charybdis* eggs and hence the only host species potentially available to *B. albifunicle*. Historically *E. nassaui* achieved up to 90% parasitism of *P. charybdis* in the North Island (Murphy & Kay 2000) but since 2002 has suffered high levels of hyperparasitism in the Bay of Plenty (Jones & Withers 2003) and become scarce in some areas where the hyperparasitoid is present. Its scarcity has lead to speculation that *E. nassaui* is being suppressed by *B. albifunicle*, disrupting the control of *P. charybdis* (Jones & Withers 2003; Berry & Mansfield 2006). Indeed, after 15 years of effective suppression by *E. nassaui* in the Bay of Plenty, damage by *P. charybdis* began to increase soon after the detection of *B. albifunicle* (B. Poole, pers. com.), although no quantitative evidence for this has been presented. Rosenheim (1998) warns that observations of high hyperparasitism alone are not reliable indications of a significant impact on the efficacy of primary parasitoids, giving examples of BCAs that perform poorly, and others that remain economically successful in the presence of hyperparasitoids. Even so, as species in the genus *Neopolycystus* are thought not to be hyperparasitised by *B. albifunicle* (Tribe 2000; Tribe & Cillié 2000) the self-introduced *N. insectifurax* could potentially compensate for a hyperparasitoid-driven reduction in *P. charybdis* control by *E. nassaui.* There is currently no quantitative evidence that such a decline, or substitution, is occurring (Jones & Withers 2003).

In chapters 2, 3 and 4 *E. nassaui* was shown to parasitise *P. charybdis* at higher levels and more readily that *N. insectifurax*. Field monitoring has indicated similar trends (Jones & Withers 2003; Murray et al. 2008). The evidence suggests that, at best, *N. insectifurax* may complement the actions of *E. nassaui* by extending control later into the summer. In chapter 5 it was shown that host guarding may reduce parasitism rates for *N. insectifurax* relative to *E. nassaui*. In this chapter the possibility that the behavioural and physiological advantages held by *E. nassaui* may become obsolete in the presence of the newly arrived hyperparasitoid, and that the aggressive nature of *N. insectifurax* may allow it to become a relatively more effective control agent of *P. charybdis,* is explored.

The basic biology, ecology and behaviour of the hyperparasitoid are assessed here to assist in predicting the extent to which it could disrupt the biological control of *P. charybdis*. Adult longevity, fecundity and sex ratio are determined and compared to *E. nassaui* (section 6.2, 6.3). Experiments are conducted to confirm *B. albifunicle* is an obligate hyperparasitoid (section 6.4) and whether it is host specific to *E. nassaui* or primary parasitoids within *P. charybdis* eggs (section 6.5). In section 6.6 the timing (in relation to primary parasitism) and location (inside or outside the primary parasitoid body) of hyperparasitoid oviposition is determined. In the event that *B. albifunicle* has the ability to reduce *E. nassaui* populations, the impact on *P. charybdis* control will be strongly influenced by the geographical overlap between the hyperparasitoid and the two primary parasitoid species. To this end the New Zealand distributions, in particular the southern limits, of the three species are examined (section 6.7).

6.2 ADULT LONGEVITY

As *B. albifunicle* has rarely been associated with *E. nassaui* in its native Australian habitat it is not clear whether the lifecycle of the hyperparasitoid is closely synchronised with those of this primary parasitoid in New Zealand. The longevity of *B. albifunicle* relative to that of *E. nassaui* will influence the amount of time the hyperparasitoid has to locate and parasitise this host in the field. This may affect the degree to which the *E. nassaui* population can be reduced by the hyperparasitoid annually and therefore the level of disruption to the control of *P. charybdis*.

Objective

To determine the adult longevity of laboratory reared *B. albifunicle* for comparison to available data on the longevity of its known New Zealand host *E. nassaui*.

Methods

Eighty female and 51 male hyperparasitoids were separated into individual Petri dishes (65 mm diameter) upon emergence, and placed in growth cabinets (22 ^{o}C, 70% r.h., 14L:10D). Each wasp was exposed to one of four treatments: honey, honey diluted with water (honey-water, 1:9), water, or no treatment. Honey, honey-water and water were provided on 2 cm^2 pieces of paper towel and 'no treatment' consisted of a clean piece of paper towel. All treatments were refreshed daily and dead wasps recorded. Longevity (days alive) was compared between and within the sexes and between treatments using Generalized Linear Models with Poisson distribution (Proc GENMOD, SAS 9.1).

Results

Both sexes survived longer on honey and honey-water than on water or no treatment (F = 242.2, df = 3, $P < 0.0001$, Table 6.1). No significant difference was detected in mean longevity between males and females ($F = 0.03$, df = 1, $P = 0.8654$), despite the fact that the maximum longevity of females on honey and honey-water was 33 and 30 days longer than males respectively. Longevity did not differ significantly between females maintained on honey and honey-water ($\chi^2 = 0.59$, df = 1, $P = 0.444$), but it was significantly greater on honey for males and for males and females combined.

Table 6.1: Longevity ($\bar{x}$ ± SE and maximum) (days) of adult *B. albifunicle* females, males, and both sexes combined, when reared since emergence on each of four treatments in the laboratory (22 ^{o}C, 75% r.h., 14L:10D). Means, within columns, with different letters beside are significantly different at $P < 0.05$.

	Female		Male		Female + Male	
Treatment	**Mean** ± SE	**Max**	**Mean** ± SE	**Max**	**Mean** ± SE	**Max**
Honey	56.7 ± 6.5^{a}	102.5	55.4 ± 3.0^{a}	69.5	55.4 ± 3.0^{a}	102.5
Honey-water	51.9 ± 5.3^{a}	74.5	33.7 ± 2.7^{b}	44.5	33.7 ± 2.7^{b}	74.5
Water	1.9 ± 0.1^{b}	2.5	0.8 ± 0.1^{c}	1.5	2.2 ± 0.1^{c}	2.5
No treatment	1.4 ± 0.1^{b}	1.5	1.6 ± 1.3^{c}	2.5	1.6 ± 0.1^{c}	2.5

Discussion

Most parasitoids require food as adults to sustain longevity and reproduction (Ferreira de Almeida et al. 2002). This may be in the form of nectar, honeydew, pollen or protein from host feeding (Giron et al. 2004; Jervis et al. 2008). Food provides energy for host searching, assessment, oviposition and oocyte production in species that are not pro-ovigenic. An abundance of food has been shown to promote longevity and female fecundity (Giron et al. 2004; Jervis et al. 2008) so it was not unexpected that *B. albifunicle* had increased longevity when provided with dilute or pure honey. Of more interest was the combined longevity of male and female *B. albifunicle* relative to *E. nassaui*. When supplied with honey, average longevity of *B. albifunicle* (54.41 ± 2.98 days) was longer than previously determined for *E. nassaui* (42.00 ± 6.06 days) using the same methods as outlined above (S. Mansfield unpub.). This is substantially longer than a parasitoid of this size is likely to survive in the natural environment (Cumpston 1939; Mansfield & Mills 2002). Although *B. albifunicle* can live almost as long on honey-water as on honey, Mansfield found the survival of *E. nassaui* on honey-water ($\bar{x}$ = 8 days) was significantly shorter. *Enoggera nassaui* would therefore be at greater risk of starvation under natural conditions, where available carbohydrate sources may be less calorific than pure honey. This suggests *B. albifunicle* may have a physiological advantage over *E. nassaui* that could allow it to exploit the primary parasitoid very effectively even if their lifecycles are not well synchronised in the New Zealand environment.

6.3 Fecundity, Offspring Sex Ratio & Percent Parasitism

Lifetime fecundity and intrinsic rate of population increase are generally lower for hyperparasitoids than primary parasitoids (Sullivan & Völkl 1999). Also, observing high levels of hyperparasitism in the field does not necessarily equate to significant impacts on the primary parasitoid population (Sullivan & Völkl 1999), or on biological control because the host of the primary parasitoid is still killed (Tanton & Epila 1984). However, by comparing the fecundity, sex ratio and parasitism levels achieved by *B. albifunicle* and *E. nassaui* under optimum laboratory conditions useful information may be obtained on the potential of *B. albifunicle* to impact the *E. nassaui* population. This may, in turn, improve the accuracy with which predictions can be made about the ability of *B. albifunicle* to disrupt the biological control of *P. charybdis*.

Objectives

To determine the fecundity of laboratory reared *B. albifunicle* and the sex ratio of their progeny for comparison to available data on *E. nassaui,* and to determine levels of hyperparasitism attained on *E. nassaui* in the laboratory.

Methods

Ten female *B. albifunicle* were placed in a growth cabinet (22 ^{o}C, 70% r.h., 14L:10D) in separate Petri dishes (65 mm diameter). Each dish was provisioned with honey and a *P. charybdis* egg batch of 5-31 ($\bar{x}$ = 15) eggs parasitised 24 h earlier by *E. nassaui*. Egg batches were replaced daily until all ten *B. albifunicle* had died. Egg batches removed each day were returned to the growth cabinet in separate Petri dishes and the number of *E. nassaui*, *B. albifunicle* and *P. charybdis* that emerged were recorded, as were the number of eggs from which no insects emerged. The sex of *B. albifunicle* adults can be determined based on the morphology of the antennae and progeny sex ratios were calculated for each parent female in this way. Parasitism (%) and the number of progeny per female were compared (non-statistically) to data collected for *E. nassaui* in an earlier study (S. Mansfield unpub.) which followed the same methods outlined above for *B. albifunicle*. Only the first 14 days of data were used for this comparison as this was the duration of the *E. nassaui* study.

Results

On average *B. albifunicle* oviposited on 19 consecutive days and survived an additional five days after oviposition ceased (Table 6.2). Mean lifetime fecundity was 127.2 progeny per female with a maximum of 182. Over a 14-day period, *B. albifunicle* produced an average of 103.8 progeny per female, slightly less than previously recorded for *E. nassaui* (123.1). A total of 69.9% of *B. albifunicle* progeny were female with up to 8♀:1♂ emerging per egg batch. The sex ratio of hyperparasitoid progeny over the lifetime of a parent female ranged from 1.2 to 3.9 females to 1.0 males ($\bar{x}$ = 2.6 ± 0.3), with an average of 2.4, and maximum of 6.0 males per batch. Three of the ten females produced only male offspring during the last five to seven days of their reproductive lives.

Table 6.2 Number of days that *B. albifunicle* females (n = 10) survived (total longevity) and continued to lay eggs (repro. longevity) when provided with a fresh batch of host eggs each day (22 °C, 70% r.h., 14L:10D). Also shown are the number of host eggs provided to the females from which emerged either nothing, *P. charybdis*, *E. nassaui* or *B. albifunicle* (total) and the number of *B. albifunicle* of each sex.

	Longevity (days)		No.	Emergence				*B. albifunicle*	
	Total	Repro.	Eggs	None	*P. charybdis*	*E. nassaui*	*B. albifunicle*	Female	Male
Min.	14.0	13.0	223.0	107.0	0.0	3.0	87.0	62.0	19.0
Max.	32.0	27.0	421.0	198.0	21.0	67.0	182.0	124.0	74.0
Mean	24.2	18.8	304.7	144.2	9.2	24.1	127.2	88.9	38.3

Baeoanusia albifunicle successfully hyperparasitised 41.8% of all *P. charybdis* eggs (Fig. 6.1) while *E. nassaui* emerged from an additional 7.9%. Assuming *B. albifunicle* is an obligate hyperparasitoid and *E. nassaui* can parasitise 75.2% of *P. charybdis* eggs in the laboratory (S. Mansfield unpub.), *B. albifunicle* successfully hyperparasitised only 55.7% of available hosts. Between 8.9% and 16.0% of eggs parasitised by *E. nassaui* escaped hyperparasitism, depending on whether all eggs that collapsed (i.e. no primary parasitoids, secondary parasitoids or *P. charybdis* emerged) were assumed to have been parasitised or not. The number of *P. charybdis* eggs that collapsed was substantially higher than when Mansfield exposed *P. charybdis* eggs to *E. nassaui* alone for the same duration (Fig. 6.1).

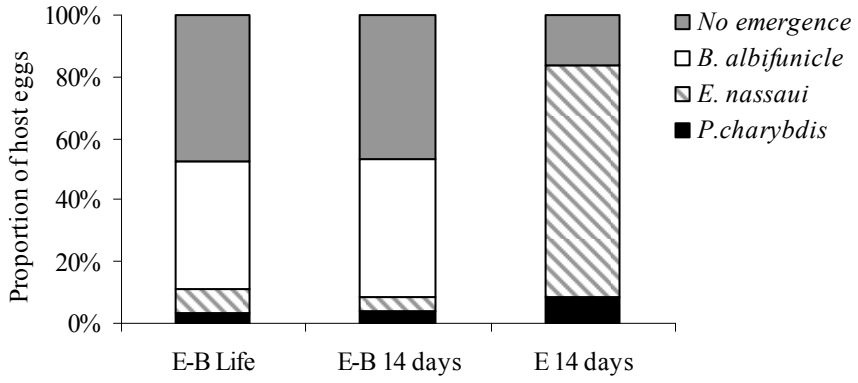

Figure 6.1: Proportion of *P. charybdis* eggs from which emerged *P. charybdis*, *E. nassaui*, *B. albifunicle* or nothing (no emergence) following exposure in the laboratory (22 °C, 70% r.h., 14L:10D) to either *E. nassaui* for 1 h followed by *B. albifunicle* for 24 h (E-B life, n = 10; E-B 14, n = 10) or to *E. nassaui* only for 24 h (E 14, n = 10, reproduced from S. Mansfield unpub.). 'E-B life' includes all data collected until the 10 wasps had died while 'E-B 14' and 'E 14' include only data collected over the first 14 days of survival.

Discussion

Based on the study of aphid hyperparasitoids, Sullivan & Völkl (1999) noted that secondary parasitoids have a relatively low fecundity compared to primary parasitoids. However, fecundity varies greatly even for individual hyperparasitoid species on different hosts. The average lifetime fecundity of *B. albifunicle* (127 eggs) for example, is similar to that of *Alloxysta pleuralis* (Cameron) (113), substantially higher than *Asaphes vulgarus* Walker on *Lysiphlebus cardui* Marshall via *Aphiis fabae* Scopoli (51) and substantially lower than the same hyperparasitoid on *Aphidius uzbekistanicus* Luzhetski via *Sitobion avenae* (F.) (1143).

Like its host (section 3.3), *B. albifunicle* probably has the ability to choose the sex of its offspring. In this study *B. albifunicle* progeny had a female-biased sex ratio (69.9% female) comparable to that recorded by Tribe (2000) (68.5% female) on the source population of *E. nassaui* prior to its introduction to New Zealand. This is lower than *E. nassaui* on *P. charybdis* (88.3% females, section 3.3). Sex allocation by the aphid hyperparasitoid *Dendrocerus carpenteri* (Curtis) has been studied in detail (Chow & Mackauer 1996) and is strongly correlated to host quality as a function of aphid size. As individual *P. charybdis* eggs within batches are almost identical in size, host size is unlikely to drive sex allocation by *B. albifunicle*. They may instead use a standard pattern of allocation. As discussed in section 3.3, some parasitoids deposit one male egg then allocate females to most remaining hosts so that only enough males are produced to fertilise their sisters. On average, *B. albifunicle* allocated 2.4 males per egg batch, and only female progeny emerged from 13 batches. There is no evidence therefore that *B. albifunicle* follows the 'one male then all females' strategy. Several females produced only male progeny near the end of their reproductive lives, after 81 to 124 female progeny had already been produced, suggesting that stored sperm was used up before all eggs were laid.

High host egg mortality, as illustrated by eggs collapsing rather than *P. charybdis* or parasitoids emerging, could indicate that *E. nassaui* is not a particularly suitable host for *B. albifunicle*. However, it is more likely that collapsed eggs became desiccated because of excessive probing. Tribe (2000) found evidence for a direct effect of probing on unparasitised *Trachymela tincticollis* eggs in the form of a 20% increase in mortality

following exposure to a hyperparasitoid. Considering the confined laboratory conditions and 24 h experimental period, it is quite possible that *B. albifunicle* probed the limited number of eggs exposed to them multiple times. If so, 55.7% hyperparasitism of *E. nassaui* in the laboratory may be an underestimate. This level of hyperparasitism is similar to that of *E. nassaui* by *Neblatticida* sp. nr. *lotae* (Girault) (Encyrtidae) which ranged from 46.7-86.2% on three different beetle hosts in the laboratory (Tribe 2000). This species was also found to hyperparasitise 27% of *E. reticulata* Naumann via *T. tincticollis* in the field. In contrast, *B. albifunicle* hyperparasitises 64.8% of *E. nassaui* via *Paropsis geographica* Baly and *Chrysophtharta amoena* (Clark) in the laboratory, but has only been found in 2.6% of field collected eggs. Tribe concluded however that this low level resulted from eggs being collected when they were less than four days old, and that sufficient time had not passed for hyperparasitism to occur (see also section 6.6).

In this study, *B. albifunicle* produced fewer female offspring and successfully parasitised a smaller proportion of hosts than did its own host, *E. nassaui*. These differences agree with the understanding that lifetime fecundity and intrinsic rate of increase are generally lower for hyperparasitoids than primary parasitoids (Sullivan & Völkl 1999). This does not necessarily preclude a hyperparasitoid from having an impact on a primary parasitoid population. In fact, data suggest that *B. albifunicle* has a strong potential to reduce the effective parasitism of *P. charybdis* by *E. nassaui* to below 10%. There was evidence for a reduction of this scale in at least one Bay of Plenty site between January and March 2003 (Jones & Withers 2003). The subsequent effect on the control of *P. charybdis* is difficult to determine. Although hyperparasitism may be detrimental to the overall success of *E. nassaui*, it still prevents *P. charybdis* hatching. One could predict therefore that in the presence of *B. albifunicle* the *P. charybdis* population would initially decrease, but may show a resurgence in subsequent seasons, due to a gradual decline of *E. nassaui*. Only two seasons of primary and hyperparasitoid abundance data have been collected in the field since the detection of *N. insectifurax* and *B. albifunicle*. Several more years may be required before any hyperparasitoid-driven reduction in *E. nassaui*, resulting in an increase in *P. charybdis* survival, would become apparent. Furthermore, as *N. insectifurax* is now also parasitising substantial numbers of *P. charybdis* at certain times of year, this could potentially conceal a reduction in *P. charybdis* control by *E. nassaui* (Jones & Withers 2003). The ultimate impact of *B. albifunicle* on *P. charybdis* control will depend on

whether it is restricted to *E. nassaui* as its host. Its impact may be greater if it is a facultative hyperparasitoid able to develop on *P. charybdis* in the absence of a primary parasitoid, or negligible if *N. insectifurax* is immune to hyperparasitism and can substitute for *E. nassaui*. These two possibilities are addressed in the following two sections.

6.4 Obligate or Functional Hyperparasitism

Obligate hyperparasitoids are secondary parasitoids that can only develop in or on a primary parasitoid host, while facultative hyperparasitoids are able to develop as primary or secondary parasitoids in both parasitised and unparasitised hosts (Sullivan & Völkl 1999). Obligate hyperparasitism requires a more specialised relationship with the primary host. As only two primary parasitoids of *P. charybdis* exist in New Zealand for *B. albifunicle* to exploit, the hyperparasitoid's success will require a high degree of ecological synchrony with at least one of them. Tribe (2000) concluded that *B. albifunicle* is an obligate hyperparasitoid in south-western Australia. However, Murphy (2002) described that *B. albifunicle* oviposits into unparasitised *P. charybdis* eggs but does not develop until these are subsequently parasitised by a primary parasitoid. Even if *B. albifunicle* is an obligate hyperparasitoid this oviposition strategy could provide the opportunity for it to evolve into a facultative hyperparasitoid. Considering the uncertain taxonomy of hymenopteran parasitoids and the unknown origin of the New Zealand *B. albifunicle* population, it is possible that the organism present here is not the same as that studied by Tribe. Its mode of hyperparasitism, therefore, requires confirmation.

Objective

To confirm that *B. albifunicle* is an obligate hyperparasitoid.

Methods

Forty *P. charybdis* egg batches were placed in separate Petri dishes provisioned with honey. Ten batches each were presented to *B. albifunicle*, *B. albifunicle* then *E. nassaui*, *E. nassaui*, or *E. nassaui* then *B. albifunicle*. These were exposed to *E. nassaui* for 2 h and *B. albifunicle* for 6 h (22 ^{o}C, 70% r.h.). Treatments were synchronised so that all exposures to *B. albifunicle* occurred simultaneously. The number of *P. charybdis* larvae, primary

parasitoids and hyperparasitoids that emerged after a period of incubation were recorded. Several exploratory dissections of additional parasitised and unparasitised eggs probed by *B. albifunicle* were made to record the presence and location of hyperparasitoid eggs.

Results

Host eggs exposed only to *B. albifunicle* produced only *P. charybdis* larvae while those exposed to *B. albifunicle* followed by *E. nassaui* or to *E. nassaui* alone produced primary parasitoids (Fig. 6.2). Of those eggs presented to *B. albifunicle* after *E. nassaui*, 91.8% were hyperparasitised, and *E. nassaui* emerged from an additional 2.7%. The highest level of egg mortality (no emergence) occurred in the presence of *B. albifunicle* alone (8.4%). Exploratory dissections of probed host eggs found no evidence that *B. albifunicle* oviposited in the absence of a primary parasitoid egg or larva. Hyperparasitoid eggs were usually found within the primary parasitoid egg or larva, although a few were found outside, but close to the primary parasitoid (see Fig 6.5b, 6.5c & 6.5d pg. 117).

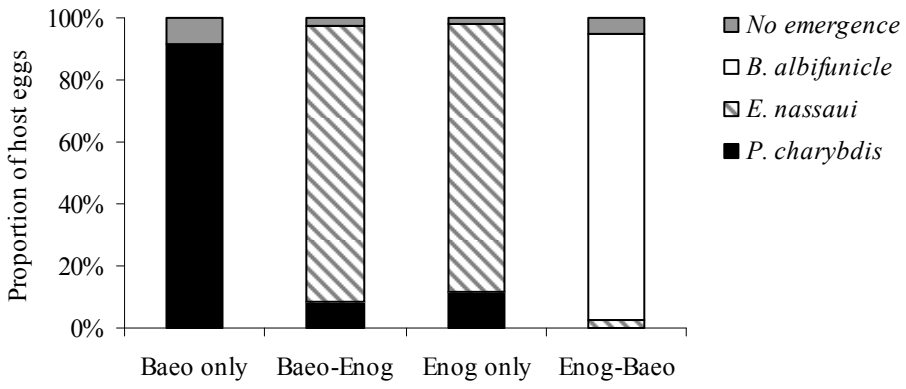

Figure 6.2: Proportion of *P. charybdis* eggs from which *P. charybdis*, *E. nassaui*, *B. albifunicle* or nothing (no emergence) emerged following exposure in the laboratory to (from left to right) *B. albifunicle* only, *B. albifunicle* then *E. nassaui*, *E. nassaui* only or *E. nassaui* then *B. albifunicle* (22 ^{o}C, 70% r.h.).

Discussion

If *B. albifunicle* were a facultative hyperparasitoid it could significantly reduce *E. nassaui* numbers causing local extinction, but would also have the ability to act as a BCA of *P. charybdis* itself. The failure of hyperparasitoids to emerge from *P. charybdis* eggs that were not already parasitised by *E. nassaui* indicates that *B. albifunicle* has not established a

primary relationship with *P. charybdis*. *Baeoanusia albifunicle* achieved a very high level of hyperparasitism (> 90%) in the laboratory relative to previous laboratory (section 6.3) and field observations (Jones & Withers 2003). The former may be because eggs were exposed to *B. albifunicle* for a shorter duration (6 h vs. 24 h) resulting in fewer *P. charybdis* eggs being probed multiple times and becoming desiccated as discussed in section 6.3. Higher levels of hyperparasitism in the laboratory, than previously recorded in the field, agree with the findings of Tribe (2000). These levels do not necessarily confirm *B. albifunicle* will have a significant impact on *E. nassaui* under natural conditions (Rosenheim 1998), but suggest it has the potential to do so. As an obligate hyperparasitoid its population size should be closely linked to that of *E. nassaui*, particularly if unable to utilise *P. charybdis* eggs parasitised by *N. insectifurax*. The latter possibility is explored in the following section.

6.5 PHYSIOLOGICAL HOST RANGE

Obligate endo-hyperparasitoids are usually host specific, either to primary parasitoids within a genus or to a particular herbivore host (Sullivan & Völkl 1999). For example, only a few hyperparasitoids of aphids attack a broad range of unrelated aphids or primary parasitoids (Sullivan & Völkl 1999). For *B. albifunicle*, at this point in time in New Zealand, host specificity would only imply it does not hyperparasitise *N. insectifurax* or paropsines other than *P. charybdis*. As an obligate hyperparasitoid (section 6.4) *B. albifunicle* probably arrived in New Zealand with it primary parasitoid host. Because *N. insectifurax* was detected at the same time in the same region, it is the obvious candidate. There is no evidence that *N. insectifurax* is utilised in New Zealand (Jones & Withers 2003), but one example of attack on *Neopolycystus* sp. has recently been recorded in Australia (Nahrung & Duffy 2008). Host specificity to the genus *Enoggera*, as suggested by Tribe (2000), would indicate there is potential for *N. insectifurax* to compensate for any decline in the control of *P. charybdis* by *E. nassaui*. Restriction to primary parasitoids attacking *P. charybdis* could allow *E. nassaui* to find refuge in the eggs of the three other paropsine species present in New Zealand, of which *D. semipunctata* is the most common.

Objectives

To determine if *N. insectifurax* is within the physiological host range of *B. albifunicle*, and if *B. albifunicle* is able to detect and successfully parasitise *E. nassaui* in the eggs of the established paropsine beetle, *D. semipunctata*.

Methods

Eighty *P. charybdis* egg batches were exposed to either a single *E. nassaui* adult for 1 h (*n* = 20), a single *N. insectifurax* adult for 24 h (*n* = 40) or no primary parasitoid (*n* = 20) (22 ^{o}C, 65% r.h.). Parasitoids were dissected to confirm their sex and egg batches that had been exposed to confirmed females were incubated in a growth cabinet for 24 h (22 ^{o}C, 65% r.h.). Each batch was then exposed to a solitary hyperparasitoid female for 2 h before being dissected to record the presence of primary and secondary parasitoid eggs.

In a second experiment, 40 *P. charybdis* egg batches of 8-26 eggs were exposed to either a single 2-day-old *E. nassaui* (*n* = 20) or 6-day-old *N. insectifurax* female (*n* = 20) for 24 h (females confirmed by dissection). Each was subsequently exposed to a solitary *B. albifunicle* female for 24 h (22 ^{o}C, 65% r.h.). Eggs were incubated (22 ^{o}C, 65% r.h.) and the number of 1^{o} and 2^{o} parasitoids that emerged per batch was recorded.

In a third experiment, 20 individual *D. semipunctata* eggs and 20 *P. charybdis* egg batches (2-9 eggs) were each presented to a solitary *E. nassaui* female for 2 h (confirmed by dissection), then a solitary *B. albifunicle* female for 24 h (22 ^{o}C, 65% r.h.). Eggs were incubated and the number of parasitoids and hyperparasitoids that emerged was recorded.

Results

In the first experiment, a total of 20 *P. charybdis* egg batches parasitised by *E. nassaui*, 19 by *N. insectifurax* and 20 unparasitised, were ultimately exposed to *B. albifunicle*. Hyperparasitoid eggs were dissected from 95% of those parasitised by *E. nassaui.* No hyperparasitoid eggs were found within unparasitised *P. charybdis* eggs or those parasitised by *N. insectifurax*. However, during pilot study dissections used to practice the

dissection technique, *B. albifunicle* was found to have oviposited in eggs parasitised by *N. insectifurax* on two occasions. Their eggs were not deposited directly into the *N. insectifurax* larvae, as they were into *E. nassaui* larvae (see Fig. 6.5b & 6.5c pg. 117), but rather were floating freely in the *P. charybdis* host medium (Fig. 6.5f). Furthermore, whereas only one to three hyperparasitoid eggs were found in *E. nassaui* larvae, up to 12 were deposited next to the *N. insectifurax* larvae.

In the second experiment, 20 *P. charybdis* egg batches and 77% of all individual eggs were parasitised by *E. nassaui*. Hyperparasitoids emerged from 92% of these (Fig. 6.3). *Neopolycystus insectifurax* parasitised 60% of the *P. charybdis* eggs and none of these were successfully hyperparasitised. Only 30% of *D. semipunctata* eggs were parasitised by *E. nassaui* in the third experiment, therefore only six eggs were effectively available for hyperparasitism. *Baeoanusia albifunicle* successfully oviposited and developed in four (67%) of these (Fig 6.4).

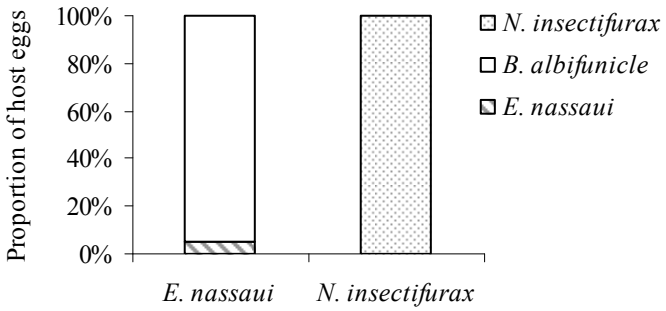

Figure 6.3: Proportion of *P. charybdis* eggs from which *B. albifunicle*, *E. nassaui* and *N. insectifurax* emerged following exposure to *B. albifunicle* after either *E. nassaui* (*n* = 20 batches, 304 eggs) or *N. insectifurax* (*n* = 20 batches, 314 eggs).

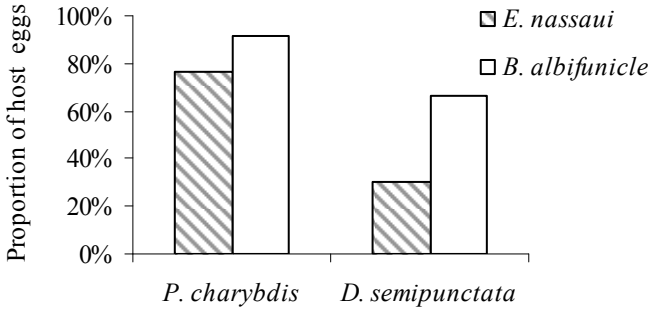

Figure 6.4: Total proportion of *D. semipunctata* (*n* = 20 eggs) and *P. charybdis* (*n* = 20 batches, 108 eggs) parasitised by *E. nassaui,* and proportion of those eggs then hyperparasitised by *B. albifunicle*.

Discussion

Low levels of hyperparasitism by *B. albifunicle* have been recorded on *E. nassaui* and *E. reticulata* Naumann via *C. amoena*, *C. decolorata* (Chapuis), *P. geographica* and *P. atomaria* Oliver in Australia (Tribe 2000). It is not therefore host specific at the level of

the herbivore hosts. This is confirmed in this study with the successful hyperparasitism of *E. nassaui* via *D. semipunctata* in the laboratory. The arrangement and location of some of the fore-mentioned hosts' eggs are quite different, suggesting that *B. albifunicle* is able to search a variety of host habitats and respond to non-specific host cues. It may be searching for a particular polyphagous primary parasitoid or be broadly polyphagous itself. *Baeoanusia albifunicle* has recently been strongly associated with *Neopolycystus* sp. in South-East Queensland (Nahrung & Duffy 2008) but this relationship has not been confirmed experimentally. With this exception, *B. albifunicle* has only been reared in the field from species in the genus *Enoggera*, and has failed to exploit *Procheiloneurus* sp., and *Neopolycystus* sp. (Western Australia) (Tribe 2000) and now *N. insectifurax* in the laboratory. Evidence suggests therefore that it is not widely polyphagous and may be specific to the genus *Enoggera*.

As an obligate hyperparasitoid with only one host available in New Zealand, *B. albifunicle* must exhibit strong temporal synchrony with *E. nassaui* to survive. As it has successfully established and increased in abundance (Jones & Withers 2003) this is presumably the case. It therefore has strong potential to significantly impact *P. charybdis* control by *E. nassaui*. In section 6.3 *B. albifunicle* was able to reduce effective parasitism by *E. nassaui* to below 10%. As *N. insectifurax* does not appear to be exploited by the hyperparasitoid it has some capacity to compensate for this. No endo-hyperparasitoids have been reared from natural populations of *N. insectifurax* in Australia, although few have been sampled. Tribe (2000) recorded the emergence of the ecto-hyperparasitoid *Signiphora* sp., (Signiphoridae) and dissected the eggs of *Neblatticida* sp. (Encyrtidae) from larvae of an unidentified species of *Neopolycystus* in south west Australia. No endoparasitic species, including *B. albifunicle*, have been induced to successfully hyperparasitise *N. insectifurax* in the laboratory. Tribe suggested the parasitoid's fat bodies may be too large for the hyperparasitoid larvae to ingest. Hyperparasitoids also have the ability to reduce primary parasitism indirectly by influencing parasitoid foraging behaviour (Rosenheim 1998). For example, primary parasitoids may abandon incompletely exploited host patches if interrupted, or to spread risk if they detect a high level of hyperparasitism in the patch. This is not favourable for *E. nassaui* in New Zealand as any unparasitised eggs in a batch of *P. charybdis* are likely to hatch and the larvae will consume or damage the adjacent parasitised eggs (pers. ob.). Directly defending parasitised eggs may prevent

hyperparasitism and be less costly than moving long distances to new host patches. The aggressive guarding behaviour seen of *N. insectifurax* in chapter 5 suggested this species has evolved in a highly competitive environment. Its behaviour could be driven by competition with other primary parasitoids or be a defence against top-down regulation by hyperparasitoids. Hyperparasitoids in turn have developed means of avoiding aggressive primary parasitoids such as avoiding direct interactions and jumping away if they come under attack (Hübner & Völkl 1996). The latter response is possibly used by *B. albifunicle* as it has powerful hind legs that it uses to jump considerable distances (pers. ob.). Because of the risks involved in travelling between patches, it may be beneficial when hyperparasitism is high for females to guard hosts they have parasitised, rather than search for more host patches, Under this scenario, optimum guarding duration will depend on the period of time that the primary parasitoid larvae are vulnerable to hyperparasitism. This is considered in the following section.

6.6 Timing and Location of Oviposition

Baeoanusia albifunicle is a direct endo-hyperparasitoid as it only attacks parasitised hosts (section 6.4). This contradicts the initial description of its biology given when it was first detected in New Zealand (Murphy 2002). It is not clear if the hyperparasitoid oviposits directly into *E. nassaui* larvae, or if the hyperparasitoid eggs are deposited into the *P. charybdis* egg and enter the primary parasitoid only after hatching. The former is more likely based on *P. charybdis* dissections already made in section 6.5. Hyperparasitoid eggs were almost always found inside *E. nassaui* larvae, but on the two occasions that eggs were found in *P. charybdis* eggs parasitised by *N. insectifurax* they were external to the parasitoid larvae. Ovipositing outside the primary parasitoid's body may expose the hyperparasitoid egg to cytotoxins produced by the primary parasitoid (Strand & Vinson 1984) in order to paralyse or digest the herbivore embryo. Successful hyperparasitism may therefore be limited by the ability of *B. albifunicle* to either: locate and reach the primary parasitoid larva with its ovipositor when that larva is still small; or to pierce the larva's integument and overcome any active defence mechanisms when the larva is well developed. Consequently, vulnerability to hyperparasitism is not constant through time (Strand & Vinson 1984). The duration of this vulnerability may affect a hyperparasitoid's ability to impact a primary parasitoid population by limiting successful hyperparasitism in the field.

Objectives

To confirm that *B. albifunicle* eggs survive only if deposited directly into *E. nassaui* eggs and to establish the duration for which *E. nassaui* a vulnerable to hyperparasitism.

Methods

Fifty *P. charybdis* egg batches were each exposed to a solitary *E. nassaui* female for 2 h followed by a solitary *B. albifunicle* female for 2 h after either 2, 4, 6, 12 or 24 h (10 batches each) had elapsed. Egg batches were incubated (22 ^{o}C, 70% r.h., 14L:10D) and parasitoids that subsequently emerged were recorded. In a second experiment, 80 *P. charybdis* egg batches were each exposed to a solitary *E. nassaui* female for 1 h followed by a solitary *B. albifunicle* female for 2 h after an interval of either 30, 1, 24, 12, 18, 15, 17 or 16 h (in that order, 20 batches each). Each interval between exposure to primary and secondary parasitoids was conducted on a separate day. This was necessary because intervals were adjusted as the experiment progressed to delimit the minimum interval required for successful hyperparasitism. Following exposure, egg batches were stored at < 4 ^{o}C for up to 24 h before being dissected by dissolving the hard external coating from around the egg batch with bleach and pressing the softened eggs flat onto a microscope slide under a coverslip. The number of *E. nassaui* and *B. albifunicle* eggs, and the location of the latter within or outside of *E. nassaui*, were recorded for each egg batch by viewing the slide preparations under a microscope at 100 - 200 x magnification. The length of *E. nassaui* eggs dissected out immediately following the 15, 16 and 17 h intervals was measured. *E. nassaui* egg size and percent hyperparasitism were compared between intervals in a series of non-parametric Wilcoxon ranked-sums tests with *P*-values adjusted using a sequential Bonfferoni procedure to preserve 95% confidence.

Results

Of the 50 *P. charybdis* egg batches exposed to *B. albifunicle* 2 - 24 h after exposure to *E. nassaui* all but four were at least partially parasitised by *E. nassaui*. This gave an average of 7.3 primary parasitoids available for hyperparasitism per replicate for each interval treatment. No hyperparasitoids emerged from eggs exposed to *B. albifunicle* less than 12 h after primary parasitism (Table 6.3). A single hyperparasitoid emerged following the 12 h interval and after the 24 h interval 27.4% of individual *E. nassaui* (11.6% of *P. charybdis*

eggs) were hyperparasitised (Table 6.3).

Table 6.3: Total number of *P. charybdis* egg batches and individual eggs successfully exposed to *B. albifunicle* 2-24 h after exposure to *E. nassaui* (22 °C, 70% r.h., 14L10D). The proportion of *P. charybdis* eggs parasitised by *E. nassaui* and therefore available for hyperparasitism is shown as is the proportion of those eggs from which *E. nassaui* and *B. albifunicle* subsequently emerged.

Time since 1° parasitism	# Batches	# Eggs	% Parasitism of *P. charybdis*	% Emergence	
				E. nassaui	*B. albifunicle*
2 h	9	151	41.8	100.0	0.0
4 h	9	150	50.4	100.0	0.0
6 h	10	151	48.3	100.0	0.0
12 h	10	150	49.3	98.7	1.3
24 h	8	151	36.4	71.7	28.3

In the second experiment, egg dissections showed that *B. albifunicle* eggs initially have a tail-like structure, but this is not visible on hyperparasitoid eggs located within primary parasitoid eggs and larvae (Fig. 6.5a). There was no evidence of hyperparasitism at any interval less than 16 h since primary parasitism (Table 6.4). After 16 h, 5% of egg batches and 1% of all primary parasitoids within them, were hyperparasitised. The proportion of primary parasitoids hyperparasitised increased steadily up to the 24 h interval (62%) then declined slightly at 30 h. The proportion of primary parasitoids superparasitised by *B. albifunicle* (i.e. > 1 hyperparasitoid egg present) followed a similar pattern. After 16 h and 17 h intervals all hyperparasitoid eggs were located inside *E. nassaui* eggs (Fig. 6.5b) or larvae (Fig. 6.5c-e). Significantly more hyperparasitoid eggs were present after a 24 h interval, and 25% of primary parasitoids contained more than one hyperparasitoid egg (Fig. 6.5d). Almost half of the hyperparasitoid eggs at this interval were outside of the primary parasitoid hosts and there was some evidence, in the form of a burst larval integument (Fig 6.5d), that they had been squeezed out of the primary parasitoid during the dissection process.

There was a significant increase in the length of *E. nassaui* eggs from 15 to 16 h (z = -4.9612, $P < 0.001$) and 16 to 17 h (z = -2.3670, $P < 0.001$) (Fig. 6.6). However, there was no significant difference in the length of 17 h eggs that were hyperparasitised compared to those that were not hyperparasitised (z = 770.5, P = 0.3205).

Table 6.4: Number of 1° (*E. nassaui*) and 2° (*B. albifunicle*) parasitoids dissected from *P. charybdis* eggs that had been exposed to *B. albifunicle* 1-30 h after *E. nassaui*. The proportion of 1° parasitoids hyperparasitised, and super-hyperparasitised (i.e. > one 2° parasitoid egg present) are shown along with the proportion of 2° parasitoid eggs found inside and outside of the 1° parasitoid eggs and larvae.

	P. charybdis	Parasitoid eggs		Hyperparasitism			
Interval	**eggs**	**1°**	**2°**	**% of 1°**	**% Super.**	**% Inside**	**% Outside**
1 h	157	121	0	0.0	-	-	-
12 h	106	110	0	0.0	-	-	-
15 h	79	56	0	0.0	-	-	-
16 h	66	56	1	1.8	0.0	100.0	0.0
17 h	77	72	24	26.4	6.9	100.0	0.0
18 h	81	66	32	33.3	9.0	87.5	12.5
24 h	78	71	128	62.0	25.4	50.8	49.2
30 h	101	109	58	39.4	10.1	93.1	6.9

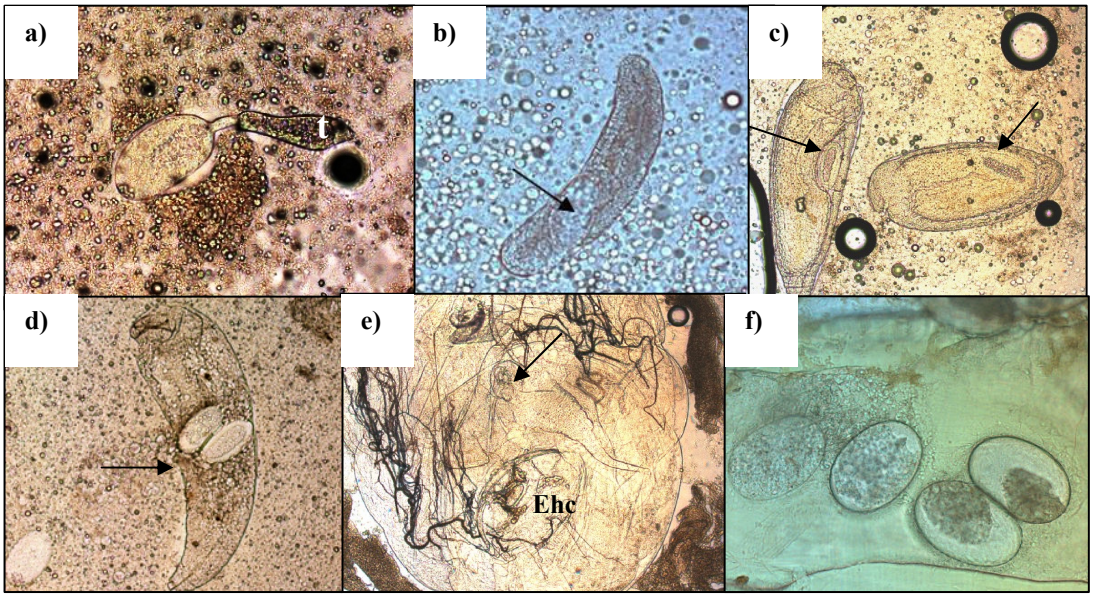

Figure 6.5: Eggs of *B. albifunicle* (indicated with arrows): **a)** immediately after oviposition into *P. charybdis* showing 'tail'(t); **b)** within *E. nassaui* egg; **c)** within early instar *E. nassaui* larvae. **d)** Breach (indicated with arrow) in primary parasitoid integument through which one *B. albifunicle* egg has possibly been squeezed out during slide preparation. **e)** Early larval instar of *B. albifunicle* (indicated with arrow) within a well developed *E. nassaui* larva (Ehc = *E. nassaui* head capsule). **f)** Four *B. albifunicle* eggs floating freely within a *P. charybdis* egg that has been parasitised by *N. insectifurax* (*N. insectifurax* not visible).

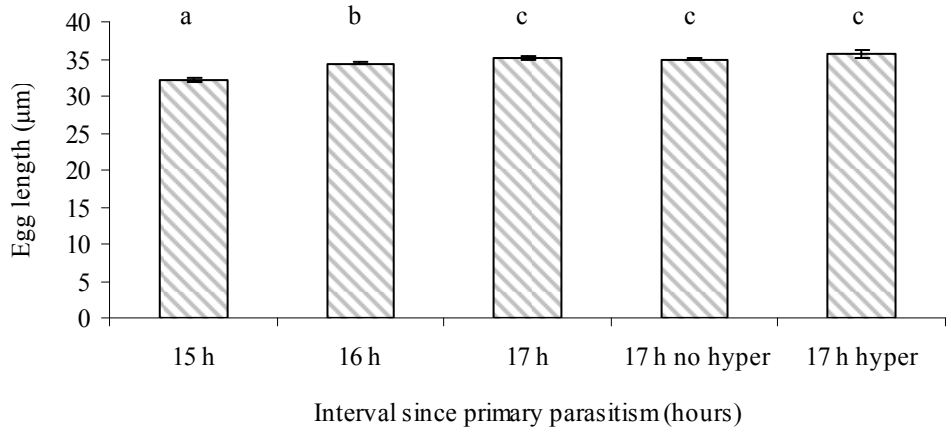

Figure 6.6: Length ($\bar{x}$ ± SE) of *E. nassaui* eggs dissected from *P. charybdis* eggs 15, 16 and 17 h after oviposition, and length of those *E. nassaui* eggs dissected 17 h after oviposition that were hyperparasitised (17 h hyper) compared to those that were not hyperparasitised (17 h no hyper) (22 ^{o}C, 70% r. h., 14L:10D).

Discussion

Upon its initial detection in New Zealand Murphy (2002) stated that *B. albifunicle* oviposited into unparasitised *P. charybdis* eggs and developed only if those eggs were subsequently parasitised by *E. nassaui*. The larvae of *E. nassaui* were said to be consumed by the hyperparasitoids and *P. charybdis* eggs exploited by only *B. albifunicle* would develop normally. No evidence was given as to how this was determined for *B. albifunicle* but the strategy is known to occur among other parasitoids. *Perilampus tasmanicus* Cameron (Pteromalidae) crawls to and enters the body of *P. atomaria* as a planidium, then ceases to develop further until the host is parasitised by a primary parasitoid such as *Eadya paropsidis* Huddleston & Short (Braconidae). In Australia, Tribe (2000) determined *B. albifunicle* was an obligate hyperparasitoid by presenting paropsine eggs to the primary and secondary parasitoids simultaneously, so the order in which oviposition occurred was not ascertained. Tribe suggested that *B. albifunicle* oviposited after the primary parasitoid as hyperparasitism was extremely low in 0-4 day old field-collected paropsine eggs, but older eggs were not collected to test this theory.

The evidence presented here and in section 6.4 confirms that *B. albifunicle* usually only oviposit into parasitised paropsine eggs. Furthermore, the hyperparasitoid probably attempts to oviposit directly into the primary parasitoid host. Although *B. albifunicle* eggs have a tail-like structure, which could *potentially* indicate motility, most eggs were

deposited directly into *E. nassaui* unless super-hyperparasitism occurred. In the latter case some hyperparasitoid eggs were found outside the *E. nassaui* egg or larva but it was unclear whether these had been deposited where they lay, were squeezed out because the larvae could not accommodate them, or were squeezed during slide preparation. The 'tail' of *B. albifunicle* eggs could instead be a yoke source, but although the stalk that attached it to the rest of the egg was present, the tail itself was never visible on eggs located inside *E. nassaui* eggs and larvae.

Enoggera nassaui does not appear to by susceptible to hyperparasitism by *B. albifunicle* for the first 12-16 h after oviposition at 22 ^{o}C. This may result from the primary parasitoid eggs being too small to detect, to reach, or to accommodate the hyperparasitoid egg. Indeed, *E. nassaui* eggs 15 and 16 h old were significantly smaller than eggs 17 h old or older. Strand and Vinson (1984) found that only 3rd instar larvae of *Telenomus heliothidis* Ashmead were large enough to be hyperparasitised by the facultative hyperparasitoid *Trichogramma pretiosum* Riley. Eggs were sometimes laid outside but next to 1st instar larvae, but these failed to develop. It was suggested this was the result of a cytolytic toxin associated with the development of the primary parasitoid. *Trichogramma pretiosum* failed to hyperparasitise older *T. heliothidis* larvae. Similarly, in this study hyperparasitism by *B. albifunicle* decreased when the time elapsed since primary parasitism was >24 h. This could result from the larval integument becoming too strong to penetrate, there may be insufficient time for the hyperparasitoid to develop before the primary parasitoid pupates, or older larvae may have an immune response to which the hyperparasitoid is susceptible. Overall *E. nassaui* appears to be vulnerable to hyperparasitism by *B. albifunicle* for only a short duration. To have a significant impact of the *E. nassaui* population *B. albifunicle* must therefore be well synchronised with its host temporally, and exhibit high host finding efficacy.

Primary parasitoids of paropsines in Australia come under significant pressure from hyperparasitoids (Greaves 1966) and many have evolved mechanisms to avoid hyperparasitism (section 6.5). In chapter 5 *E. nassaui* was observed to oviposit deep into *P. charybdis* eggs while *N. insectifurax* usually oviposited near the host's upper surface. This may represent an adaptation by *E. nassaui* to avoid attack from smaller hyperparasitoid species. As *N. insectifurax* guards its brood, no such adaptation would be necessary.

6.7 Overlap in Distribution Between Primary & Secondary Parasitoids

The hyperparasitoid *B. albifunicle* has the potential to cause a significant reduction in the population of *E. nassaui* in New Zealand (section 6.3) (Jones & Withers 2003). This in turn could severely impede the biological control of *P. charybdis*. The recently established primary parasitoid *N. insectifurax* appears to have behavioural and physiological characteristics that make it impervious to hyperparasitism by *B. albifunicle* (section 5.2, 6.5). *Neopolycystus insectifurax* may therefore be able to substitute for a hyperparasitoid-driven decline in *E. nassaui*. If so, the degree to which *B. albifunicle* will affect the biological control of *P. charybdis* in New Zealand will depend on the geographical overlap between it and the two primary parasitoids.

Objective

To determine the geographical distributions of, and overlap between, *B. albifunicle*, *N. insectifurax* and *E. nassaui* in New Zealand.

Methods

A list of all known distribution records for *E. nassaui*, *N. insectifurax* and *B. albifunicle* was compiled from the records of the Forest Health Database, Scion. Field surveys were conducted between December 2007 and January 2008 in the Northland and Gisborne regions and in at least one site from each Crosby region (as defined by Crosby et al. (1998)) in the South Island. These represented the first directed surveys for *N. insectifurax* and *B. albifunicle* in the South Island. Surveyed sites included eucalypts on roadsides, public parks and reserves, and permission was sought to access private farm forestry and plantation forestry land. Lower foliage (< 2 m above ground) was assessed for signs of *P. charybdis* damage before being thoroughly searched for egg batches. When foliage was not accessible from the ground but *P. charybdis* damage was apparent, pole-pruners were used to gather foliage from up to 10 m high. All live egg batches collected were maintained in Petri dishes until *P. charybdis* larvae or parasitoids emerged and could be identified to species. Remains of egg batches were also collected and assessed under a microscope to determine if they had been parasitised by *E. nassaui* or *N. insectifurax* based on markings on the egg shells. This did not allow detection of *B. albifunicle* as the colouration of hyperparasitised eggs is indistinguishable from that of eggs parasitised by *E. nassaui* alone.

Results

Parasitised egg batches were collected from 21 sites representing 12 of 16 regions in the South Island (Fig. 6.7) as well as Kerikeri (ND) and Wairoa (GB). This field data, in conjunction with database records, shows that *E. nassaui* has now been recovered from 20 regions (Fig. 6.7a). Regions where *E. nassaui* has not been recorded represent those that have not yet been surveyed specifically for its presence (TK, RI, WI, WA, SI, FD) and three surveyed regions (HB, SC, MK) where *P. charybdis* egg batches could not be located during this study. Considering *E. nassaui* has been established for over 20 years and is present in regions neighbouring these particular locations it is unlikely to be absent from them.

Both *N. insectifurax* and *B. albifunicle* were recovered from the South Island for the first time during this study. The hyperparasitoid, previously recorded only from three North Island regions (BP, TO, CL) was located in ND, GB and six South Island locations as far south as Roxburgh (MB, NC, MC, OL, CO) (Fig. 6.7b). In addition to previous records from BP and CL, the presence of *N. insectifurax* was confirmed for the first time in ND, GB, NC, MB, and KA (Fig. 6.7c).

Discussion

Biological control of *P. charybdis* in New Zealand may vary between regions because of the presence and absence of different natural enemies. In particular, it may be reduced where *B. albifunicle* is present but *N. insectifurax* is not. This is because *N. insectifurax* has been shown here to be immune to hyperparasitoid attack, and therefore has the potential to substitute for a hyperparasitoid-driven decline in control provided by *E. nassaui*. When *E. nassaui*, *Neopolycystus* sp. and *B. albifunicle* occur in sympatry in south-western Australia, *Neopolycystus* sp. tends to predominate over *E. nassaui* (Cumpston 1939). Like *N. insectifurax* in New Zealand, it is more abundant in January and February (Tribe 2000; Tribe & Cillié 2000; Jones & Withers 2003). The two primary parasitoids have been estimated to parasitise more than 50% of *P. geographica* and *C. amoena* eggs in south-western Australia (Tribe 2000). Less than 2% of eggs exposed in the field for < 4 days were hyperparasitised by *B. albifunicle* but Tribe concluded that the majority of hyperparasitism would occur when eggs were older than this.

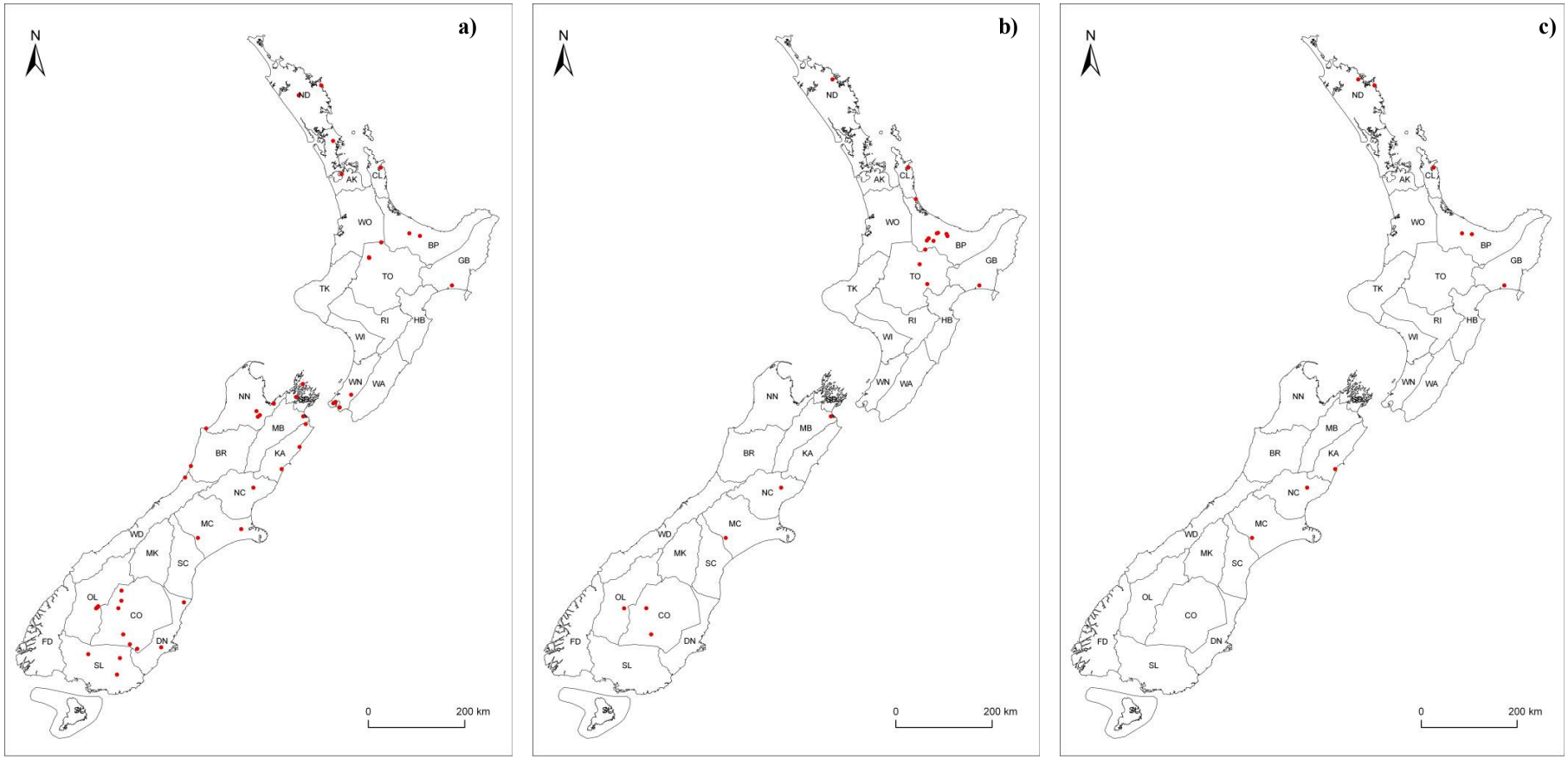

Figure 6.7: Known distribution of: **a)** *E. nassaui*; **b)** *B. albifunicle*; **c)** *N. insectifurax* in New Zealand as of January 2008.

In New Zealand, all three parasitoid species have established in Northland (ND), with a warm wet climate, the dry central South Island with hot summers and cold winters, and in the cool central North Island. It is unlikely therefore that their distributions are primarily climate-limited in New Zealand. Although a complete data set is not yet available, both *N. insectifurax* and *B. albifunicle* are expected to be established wherever their hosts are present with the exception of Southland. Extensive collections of *P. charybdis* eggs have been made in Southland in the last three years, but to date neither *N. insectifurax* or *B. albifunicle* have been detected. This could be climate related as *N. insectifurax* has a higher temperature threshold than *E. nassaui* in the laboratory (S. Mansfield unpub.) but as they appear to be established in Central Otago (CO), they may simply have not yet reached Southland (SL). Taupo (TO), Marlborogh (MB), Central Otago and Otago Lakes (OL) are the only regions where *B. albifunicle* has currently been found in the absence of *N. insectifurax*. Biological control of *P. charybdis* in these areas may therefore be at risk. However, as *N. insectifurax* is established in regions adjacent to, and most importantly south of, Taupo and Marlborough, the parasitoid probably is present in those regions also. The biological control of *P. charybdis* is therefore not expected to be affected in most of New Zealand with the possible exception of regions south of Central Otago, where it is only a minor pest.

6.8 SUMMARY

In the laboratory *B. albifunicle* has a greater longevity that *E. nassaui*. It is slightly less fecund and produces 20% fewer female offspring. High host-egg mortality suggests excessive probing by *B. albifunicle* which may indicate that *E. nassaui* is either not the most suitable host for its development or that the duration of this experiment was too long. Overall, *B. albifunicle* hyperparasitises over 55% of *E. nassaui* in the laboratory. This effectively reduces emergence of *E. nassaui* to 10-20% of *P. charybdis* eggs depending on whether host eggs that become desiccated and collapse are considered to be hyperparasitised or not.

Baeoanusia albifunicle is confirmed to be an obligate hyperparasitoid with only one primary parasitoid host, *E. nassaui*, in New Zealand. It is able to detect and parasitise this host in the eggs of the *Acacia*-feeding paropsine beetle *D. semipunctata*. *Enoggera nassaui*

is vulnerable to hyperparasitism for a limited duration and successful hyperparasitism almost certainly requires *B. albifunicle* to oviposit directly into *E. nassaui* eggs or larvae. Immature primary parasitoids that have developed for < 12-16 h and >24 h at 22 ^{o}C are less susceptible to hyperparasitism than 16-24 h old parasitoids. *B. albifunicle* oviposits into both the eggs and larvae of *E. nassaui* but very small eggs are either not located or are too small to accommodate hyperparasitoid eggs.

Baeoanusia albifunicle has physiological characteristics that may allow it to successfully exploit *E. nassaui* even if its lifecycle is not completely synchronised with this host in the New Zealand environment. Its inability to hyperparasitise *N. insectifurax* may preclude any significant impact on the biological control of *P. charybdis*. Both *N. insectifurax* and *B. albifunicle* have established in regions of New Zealand from Northland to Mid Canterbury. The Central Otago and Otago Lakes regions in the South Island are the only areas in which *B. albifunicle* is present and *N. insectifurax* is not likely to have established yet. It is possible that the biological control of *P. charybdis* may be threatened in these areas and to the south. However, *P. charybdis* is only a minor pest in these regions as they experience particularly cool winter conditions relative to much of the rest of the country.

CHAPTER 7: GENERAL DISCUSSION

Over the past two decades identifying the risks associated with introducing exotic organisms (BCAs) for the purpose of suppressing pests has been at the forefront of biological control discussion. There have been calls for more accurate, standardised methods for assessing these risks but little direction given as to how to achieve this. Members of the scientific community have suggested methods of 'best practice' based on their awareness of factors known to influence host specificity tests (e.g. Goldson & Phillips 1990; Withers et al. 1999; Barratt 2004). Empirical evidence concerning how and why particular factors influence test outcomes is still largely lacking.

This study investigated the links between physiological and behavioural characteristics of parasitoid BCAs as well as how these can influence the outcomes and interpretation of host specificity tests. The role of interspecific interactions between BCAs and with a hyperparasitoid, and host-parasitoid spatial synchrony were also considered with respect to the likely ecological host ranges of two primary parasitoids and their impact on the control of the forestry pest *P. charybdis*. This kind of behavioural-ecological approach has been advocated in the past as a means of studying host-parasitoid dynamics and predicting the effectiveness of candidate BCAs (e.g. Luck 1990). Here, in addition, this approach was taken to assess the appropriateness and interpretation of host specificity tests that are the basis upon which risk assessments of candidate BCAs are made.

Several physiological and behavioural characteristics of the established BCAs, *E. nassaui* and *N. insectifurax*, were identified and are discussed in section 7.1 along with their potential to influence the outcomes of laboratory choice and no-choice tests. In section 7.2 the appropriateness of choice and no-choice tests for predicting host ranges and how these can be interpreted in light of known physiological and behavioural characteristics of the candidate BCAs is discussed. The biology of the recently established hyperparasitoid *B. albifunicle* was investigated and its distribution relative to *E. nassaui* and *N. insectifurax* was determined. The influence of the hyperparasitoid on the future control of *P. charybdis* in New Zealand is considered in section 7.3 in light of the physiological and behavioural characteristics of all three parasitoids.

7.1 Influence of Physiological & Behavioural Characteristics on Host Specificity Testing

Designing the most predictive host specificity tests possible within the constraints of a quarantine laboratory environment and correctly interpreting them is essential for accurate host range evaluation. Doing so requires a good understanding of the biology and behaviour of the host, potential non-target hosts and the candidate BCA itself. Observations made during parasitoid colony maintenance can provide such information. These observations can be used to adjust the conditions under which parasitoids are maintained before and during host specificity tests to maximise the effectiveness and accuracy of those tests (e.g. Zilahi-Balogh 2004).

7.1.1 Physiological characteristics

Physiological characteristics of *E. nassaui* and *N. insectifurax* investigated in chapter 2 go some way to explaining the disparities in their behaviour observed in chapter 5. In turn physiological and behavioural characteristics are useful for correctly interpreting the results of choice and no-choice tests in chapters 3 and 4. Of particular importance was the finding that *E. nassaui* and *N. insectifurax* exhibit different degrees of synovigeny (section 2.3). Natural selection theory predicts parasitoid ovigeny characteristics will be adjusted to match expected host encounter rates (Jervis et al. 2001). This requires a degree of physiological and behavioural flexibility. As a result ovigeny can potentially be influenced by laboratory conditions and therefore affect the outcomes of host specificity tests. In this laboratory study, a slow rate of egg maturation by *N. insectifurax* was found to translate into lower eggload relative to *E. nassaui* over the first few days following emergence. Motivation to oviposit was therefore relatively low during this time, which effectively increased the pre-oviposition period of *N. insectifurax* compared to *E. nassaui*. Failing to recognise a low motivational state could potentially lead to false negative results being obtained from no-choice host specificity tests designed for a more motivated parasitoid. This was counteracted here by running no-choice tests of long (24-48 h) duration. Low motivation could also result in choice tests wrongly predicting strong preferences for the target host compared to non-target species. Such effects were avoided in this study by provisioning *N. insectifurax* with honey and host stimuli and allowing them to age for 72 h before choice tests and behavioural experiments. Consequently, the strong preference

shown by *N. insectifurax* in choice tests for *P. charybdis* over *D. semipunctata* and in particular *T. catenata,* compared to those that would have been expected based on no-choice results, can be attributed with more confidence to actual preferences.

Progeny sex ratios also differed between *E. nassaui* and *N. insectifurax* colonies. Parasitoid sex ratios are usually strongly female biased (Wylie 1976) yet only 55% of *N. insectifurax* progeny reared from *P. charybdis* were female. Like most hymenopteran parasitoids *E. nassaui* and *N. insectifurax* determine the sex of individual offspring by laying fertilised (= female) or unfertilised (= male) eggs. There is an extensive literature indicating that female offspring are allocated preferentially to higher quality hosts (e.g. Charnov et al. 1981; Jones 1982; Waage & Ng 1984). Sex ratio can therefore often be used as an indicator of host quality in host specificity tests. The low female sex ratio of *N. insectifurax* made it difficult to obtain females for host specificity testing and could have potentially produced misleading results from host specificity tests concerning host quality and acceptability. It also restricted the study of oviposition behaviour, and prohibited direct comparison of *N. insectifurax* behaviour to that of the more highly motivated *E. nassaui*. Investigating this disparity brought to light important behavioural characteristics of the two parasitoid species (see section 7.1.2). This allowed the development of a more effective method of rearing parasitoids for host specificity testing, and signalled the potential for particular behavioural characteristics to influence the outcomes of these tests.

Male-biased sex ratios are a common problem in parasitoid colonies (Waage 1986). As *N. insectifurax* were reared in large groups in which they showed aggression towards one another it was hypothesised that offspring sex ratio was adjusted in response to competition. High adult parasitoid densities may indicate a reduced chance of offspring survival because of the potential for superparasitism. Alternatively, the presence of parasitoid eggs already in a host may signal a depleted resource and therefore a host of lower quality. Allocating female offspring to hosts under these conditions may reduce reproductive fitness. By presenting *P. charybdis* eggs to solitary *N. insectifurax* females, competition was eliminated and the proportion of female progeny rose to 84%.

Offspring sex ratios from non-target hosts can be informative in the interpretation of choice and no-choice tests because they can provide information on relative host quality. In no-choice tests in chapter 3, for example, *N. insectifurax* allocated few female progeny to solitary eggs of *D. semipunctata*. In preceding and subsequent chapters, *N. insectifurax* was found to be strongly synovigenic, had strong competitive abilities at the expense of host searching and exhibited post-oviposition host-guarding. These characteristics suggest host-batch size is probably an important indicator of host quality for *N. insectifurax* because it invests substantial time and energy into guarding any host it accepts. Therefore, *N. insectifurax* may have allocated female offspring only occasionally to solitary *D. semipunctata* eggs because doing so provides minimal fitness gain.

7.1.2 Behavioural characteristics

As noted in the previous section, *E. nassaui* and *N. insectifurax* differed in their oviposition behaviour but also in their responses to, and interactions with, other parasitoids. *Neopolycystus insectifurax* was characterised by aggressively defending hosts (section 5.2). This behaviour allowed *N. insectifurax* to produce more offspring than *E. nassaui* when competing for hosts in the laboratory. However, host-guarding appeared to occur at the expense of host searching ability. *Enoggera nassaui* were quick to abandon hosts when approached by the larger, aggressive *N. insectifurax*, even if they had commenced oviposition. It was hypothesised that *E. nassaui* could afford not to defend their brood because of their shorter pre-oviposition period, and faster egg maturation and development time. These physiological characteristics coupled with effective host searching and quicker host handling may allow *E. nassaui* to encounter and parasitise more hosts than *N. insectifurax* during their lifetime. This ability was not apparent in confined laboratory tests because *E. nassaui* were physically excluded by *N. insectifurax* from accessing host eggs and were unable to leave the test arena and search for unoccupied hosts.

The oviposition strategies described above may represent two solutions to the problem of optimising oviposition success in a highly competitive environment. Paropsine beetles in Australia are extremely diverse (Selman 1985) and have a similarly diverse suite of natural enemies (e.g. Tanton & Khan 1978; de Little 1982; Tanton & Epila 1984; Tribe 2000).

Many have a wide host range within the genus *Eucalyptus* so the eggs of multiple species may be present on the leaves of a single plant. Unsurprisingly, many paropsine egg parasitoids are polyphagous and competition for hosts may be intense within the trophic guild. From an evolutionary perspective, if competition for hosts is high and substantial time and energy must be invested to locate unparasitised hosts, then fitness gains will be made by either ensuring more hosts are encountered or that offspring survive to eclosion from any hosts that are encountered. Interestingly, not only did *N. insectifurax* exhibit aggression and host-guarding, a form of 'maternal care', but they also appeared to be able to recognise hosts parasitised by individuals other than themselves (section 5.3). Both *E. nassaui* and *N. insectifurax* avoided superparasitism, but in many instances *N. insectifurax* actively multi-parasitised eggs previously parasitised by *E. nassaui,* and there were indications that physical or chemical ovicide was committed. These parasitoids could therefore prove very useful for studying the evolutionary mechanisms behind aggression, maternal care, and the ability to discriminate between self-parasitism, conspecific parasitism and parasitism by another species.

The fourth trophic level may also have a substantial impact on oviposition strategies, i.e., rather than defending their brood from conspecifics and other competitors, *N. insectifurax* may have evolved their defensive strategy against hyperparasitism in Australia. In the comparatively simple New Zealand context, *N. insectifurax* is not exploited by the only established paropsine hyperparasitoid, *B. albifunicle* (section 6.5). Consequently, any fitness gains associated with aggressively defending host resources from hyperparasitoids are lost.

Host-guarding behaviour like that observed of *N. insectifurax* has the potential to strongly influence the outcomes of host specificity tests. In a recent review, Withers & Browne (2004) recommended that exposing parasitoids to non-target hosts in groups could increase their motivation to accept less preferred hosts and therefore help in the detection of the widest possible host range that a parasitoid could express. However, in this study the behaviour of parasitoids in groups was found to potentially reduce or prohibit parasitism, not because of host rejection or low motivation, but as a result of direct competition. This could lead to unparasitised non-target hosts being incorrectly interpreted as falling outside

a parasitoid's host range. In choice tests, hosts that would not normally be accepted might be parasitised if individuals were stimulated to oviposit by the presence of a preferred host, but were physically prevented from accessing that host. Directly observing parasitoids during these tests could prevent misinterpretation of such results. There was no compelling evidence that parasitoid density caused either outcome in this study, although parasitism of *T. catenata* by *N. insectifurax* did increase slightly with increased parasitoid density.

7.2 Appropriateness & Interpretation of Choice vs. No-Choice Tests

In this study, choice and no-choice test results agreed in most instances. No-choice tests showed the four paropsine species tested were within the physiological host ranges of *E. nassaui* and *N. insectifurax*. Choice tests produced the same results with one exception. *Trachymela catenata* was not accepted by *E. nassaui* when paired with the target host *P. charybdis* or with *D. semipunctata*. This result may indicate that either *E. nassaui* has a very strong preference for *P. charybdis* or that parasitism of *T. catenata* in no-choice tests is a false positive result. Parasitism of *T. catenata* was very low in the no-choice test (6.3%). Absence of attack on *T. catenata* in the presence of more preferred hosts provides some evidence that choice tests might fail to predict very low levels of non-target attack. The importance of this depends on the ecological implications of low attack rates and these are still poorly understood. Non-target attack does not necessarily translate into severe non-target impacts, nor do strong preferences for the target host necessarily preclude non-target attack in nature (Barlow et al. 2004). This reiterates that the risks to non-target organisms must be weighed against the benefits of pest suppression, although there are some instances (e.g. when the non-target is a threatened native species) where any non-target attack is unacceptable.

Parasitism of *T. catenata* by *N. insectifurax* also declined substantially in choice tests compared with no-choice tests. In light of the behavioural characteristics of *N. insectifurax* described in section 7.1.2, an explanation other than preference alone is possible. Host-guarding behaviour (linked to physiological characteristics as described in section 7.1) may have influenced the test results because *N. insectifurax* has a tendency to remain in contact with a host once accepted. Indeed parasitism of all hosts declined in choice compared with no-choice tests. The fact that parasitism of *D. semipunctata* did not decline to the same

degree as *T. catenata* when paired with *P. charybdis* indicates that guarding was only a contributing factor. Parasitism of *P. charybdis* declined only slightly compared with the no-choice test therefore *P. charybdis* is clearly a much preferred host. Choice tests may therefore provide reliable information on host preferences but not necessarily the strength of those preferences. That strength may significantly influence any impact on non-target hosts in nature, and additional studies may be required to predict this with confidence.

Both choice and no-choice tests failed to predict that *D. semipunctata* is not in the ecological host range of *E. nassaui* or *N. insectifurax*, as was confirmed in section 3.4. It seems reasonable to conclude that *D. semipunctata* escapes parasitism by these two species in New Zealand because it feeds and oviposits on *A. melanoxylon* rather than eucalypt. Although spatial separation is generally regarded to provide refuge for physiologically suitable non-targets hosts that do not share the habitat of the target host (e.g. Benson et al. 2003) several parasitoid BCAs introduced to New Zealand have expanded their habitat range beyond that of the target host. These include *M. aethiopoides* and *Diglyphus iseae* (Walker) that moved into subalpine habitats from pastoral and urban habitats respectively, and *T. brevifacies* from a horticultural habitat to native forests (Munro & Henderson 2002). There are many studies in which parasitoids have been shown to initially orient to their host's food plant and to only detect and orientate to the host itself over short distances (e.g. Kitt & Keller 1998). Parasitoids of herbivores that are specialist eucalypt feeders would be expected to search for hosts by orientating to volatile emission from eucalypts. They should therefore show stronger habitat fidelity than the aforementioned BCAs. Parasitism of *D. semipunctata* eggs that were presented on *A. melanoxylon* leaf tips in both choice and no-choice tests indicates that in the confines of a Petri dish neither *E. nassaui* nor *N. insectifurax* are able to respond in a normal way to stimuli that provide information about the search habitat. This suggests they are only using short range host acceptance cues. Acceptance or rejection of the hosts encountered will be strongly influenced by their physiological condition and any experience from before the test that has provided information on the availability of more preferred hosts. Inhibition of normal host selection behaviour in the laboratory that leads to false positive results continues to be an area of concern regarding the use of no-choice tests.

Biological control practitioners are also wary of choice tests for several reasons. Firstly, there is concern that the presence of the target host may stimulate attack on non-target hosts, causing false positive results (Vinson 1976). This study provided no evidence for such an effect, in fact the opposite was observed (section 4.2). The presence of *P. charybdis* reduced parasitism of *D. semipunctata* and *T. catenata* by *N. insectifurax* and completely excluded parasitism of *T. catenata* by *E. nassaui*, as noted above. False negatives resulting from a strong preference for the target or rearing host (often the same species) are of equal concern but there is little evidence that this occurs. The failure of *E. nassaui* to parasitise *T. catenata* in the presence of *P. charybdis* may be such a case. Both false negative and false positive results have the potential to cause a biological control program to fail. Understanding the physiology and behaviour of a candidate agent and observing its behaviour during host specificity tests may assist in identifying and correctly interpreting these false results.

Choice tests also present difficulties for statistical analysis (e.g. Hoffmeister et al. 2006). The simultaneous presentation of two or more host species violates the assumption of independence making standard ANOVA inappropriate (Roa 1992). Also, even if the same number of individuals of each species are present at the beginning of a test, as soon as one or the other is parasitised the relative proportions of each species available have changed, unless replaced. Addressing these issues was considered to be beyond the scope of this thesis, however, every attempt was made to avoid the use of inappropriate analysis methods. Non-parametric analyses and Generalized Linear Models were used to deal with unbalanced replication, data that were not normally distributed and percentage data. Parasitism of each host species in choice tests was compared to parasitism of the same host in no-choice tests as suggested by van Lenteren et al. (2006a). As the statistical power of non-parametric tests is sometimes considered low (Hoffmeister et al. 2006) the results they provided were used only to back up clearly observed effects.

In general, there are two lines of thought about how host specificity tests should be run. The first aims to maximise the likelihood of acceptance of non-targets so that the widest possible fundamental host range can be estimated (e.g. Withers & Browne 2004). The second aims to obtain a more accurate prediction of ecological host range by giving more

consideration to the entire host selection process including host habitat location, location of host within habitat, host acceptance and host suitability (e.g. Kitt & Keller 1998). Both approaches are valid with regard to implementing host specificity tests. The first is appropriately cautious and more achievable in the laboratory environment, but runs the risk of rejecting suitable agents. Attempting to replicate the natural environment is commendable but severely limited by the physical constraints of quarantine facilities. Instead it may be more efficient to gain a better understanding of the behaviour and physiology of a BCA and how it responds to particular conditions in the laboratory, and then incorporate this into the interpretation of test results. Haye et al. (2005) concluded that laboratory host specificity tests may only identify host suitability, and alone cannot predict actual impact on non-targets. Considering the current concerns over the validity of choice and no-choice test results the overall conclusion has to be one of proceeding with caution. This is the stance already taken in New Zealand under the HSNO Act, and it is common practice to use both choice and no-choice tests. Agreement between these tests allows predictions to be made with more confidence. Disparities should be regarded as signals that further investigation is required. In this study combining the results of choice and no-choice tests provided a greater understanding of how the parasitoids might respond to hosts in the field than either test could have provided alone.

7.3 BIOLOGICAL CONTROL OF *PAROPSIS CHARYBDIS* IN NEW ZEALAND

Biological control agents exist in a dynamic multi-trophic environment. They are introduced to play a role within that environment specifically because they are living organisms that have the ability to move and adapt to it. For this reason the successful introduction of a BCA does not represent an end point. BCAs introduced to New Zealand from Australia, in particular, may encounter new hosts, natural enemies and competitors from their native range that were not present when that agent was initially introduced. Since the successful establishment of *E. nassaui* in New Zealand in 1987, the biological control of *P. charybdis* has been limited primarily by the parasitoid's inability to tolerate the cool winter conditions experienced in some regions (Murphy & Kay 2000). The self-introduction and establishment of a direct competitor of *E. nassaui*, *N. insectifurax,* and a natural enemy, *B. albifunicle*, have recently changed this situation. Control of *P. charybdis* in the future will be strongly influenced by the interactions between these three species.

Interspecific competition between parasitoid BCAs of the same target pest can reduce the effectiveness of individual species, but the combined parasitism achieved by an aggregate is generally expected to be greater than any single species (e.g. Ehler 1979; Bajpai et al. 2006). The establishment of *N. insectifurax* in New Zealand was initially expected to add substantially to the control of *P. charybdis* by *E. nassaui.* However, there has been little evidence of this in the field (Jones & Withers 2003). As discussed in section 7.1.1, several factors have been identified that may explain why *N. insectifurax* is less effective than *E. nassaui*. In particular, relative to *N. insectifurax*, *E. nassaui* has a shorter pre-oviposition period because it is less strongly synovigenic (section 2.3), and develops more quickly in the host, resulting in a shorter generation time. It has strong colonising abilities, as proven by its rapid establishment and spread throughout the country upon introduction (Kay 1990), and is slightly more closely synchronised with *P. charybdis* oviposition peaks in New Zealand (Jones & Withers 2003). *Enoggera nassaui* also appears to be more adept at finding hosts (section 5.1). These characteristics are among the most frequently cited as being common to successful BCAs (see Pschorn-Walcher 1977 for review). The study also suggests that the aggressive pre- and post-oviposition defence of host eggs observed in chapter 5 may limit the success of *N. insectifurax* in New Zealand. As indicated earlier, this behaviour undoubtedly confers an advantage in the native range of the parasitoid where the presence of numerous parasitoids of paropsine eggs generates a highly competitive environment. This advantage was apparent in the laboratory when only one batch of hosts was made available to pairs of *N. insectifurax* and *E. nassaui* (section 5.2). However, in New Zealand where *E. nassaui* is the only direct competitor faced by *N. insectifurax* the propensity to host-guard at the expense of host-searching is probably disadvantageous. *Enoggera nassaui's* physiological characteristics coupled with the fact that it appears to resume host searching shortly after parasitising a batch of host eggs (sections 4.2 & 5.1) may result in a higher encounter rate with *P. charybdis* eggs and therefore increased parasitism relative to *N. insectifurax* over its lifetime. Maximising search efficiency and parasitising as many batches as possible may be particularly advantageous when hosts occur at high densities as *E. nassaui* may be able to increase in abundance relative to *N. insectifurax*. At extremely low densities superior host finding abilities may also confer an advantage to *E. nassaui*.

Despite these shortcomings, *N. insectifurax* is now well established in New Zealand and has spread to most regions of the country as documented in section 6.7. Its behaviour of actively attempting to multi-parasitise hosts already parasitised by *E. nassaui* (section 5.3) may have contributed to this. Although the regional abundance of *N. insectifurax* has not been assessed, this study suggests this species could play an increasingly important role in *P. charybdis* control because of the establishment of the hyperparasitoid *B. albifunicle*. Initially the arrival of *B. albifunicle* was expected to devastate the control of *P. charybdis*. Indeed, in section 6.3, *B. albifunicle* was found to have the capacity to reduce effective parasitism by *E. nassaui* to 10-20%. Similar estimates have been made from field surveys (Jones & Withers 2003). In this study it was confirmed (section 6.5) that *N. insectifurax* is not exploited by *B. albifunicle*. Therefore, in areas where *B. albifunicle* is present (section 6.7) *N. insectifurax* does have the potential to substitute for *E. nassaui*. However, *P. charybdis* control may still suffer to some degree. *Neopolycystus insectifurax* is thought to have higher temperature requirements than *E. nassaui* and as such *E. nassaui* remains the primary control agent active against the first spring generation of *P. charybdis* (Kay 1990; Jones & Withers 2003). A hyperparasitoid driven reduction in the numbers of *E. nassaui* going into the over-wintering population will increase the time taken for the *E. nassaui* population to build up in the spring to levels sufficient to suppress the population growth of and damage caused by the first of the two *P. charybdis* generations.

7.4 Limitations and Future Research

Although not necessarily ideal, the experimental designs used throughout this study reflect the usual set of difficulties experienced during laboratory/quarantine-based host specificity testing. For example, limited numbers of insects and the inability to distinguish male and female primary parasitoids had particular implications in regards to achieving equal and simultaneous replication of multiple experimental treatments. By necessity, parasitoids used in experiments were reared only on the target host, and in some cases were exposed to target host eggs before experiments to confirm they were female. As discussed in section 4.2, these experiences are not thought to have greatly influenced host acceptance in this study, but repeating choice tests with parasitoids reared on a different host, such as *D. semipunctata*, could be used to confirm this.

Although *D. semipunctata*, *T. catenata* and *T. sloanei* were regarded not to be within the ecological host ranges of *E. nassaui* and *N. insectifurax* there is limited field data to confirm this. *Trachymela sloanei* and *D. semipunctata* are unlikely to be exploited by these parasitoids because their eggs are not located on eucalypt leaf blades like *P. charybdis*. *Trachymela catenata* may be exploited, but the species is uncommon, with a restricted geographical range. The fact that no parasitoids were reared from field collected *D. semipunctata* eggs is a good indication that this species is not exploited. However, if parasitised *P. charybdis* eggs could have been found on *E. nitens* adjacent to *A. melanoxylon* stands were *D. semipunctata* were collected, this would have provided more conclusive evidence that these parasitoids do not search *A. melanoxylon* for hosts.

A key conclusion from this study was that behavioural observations can provide information to aid the interpretation of non-target host acceptance or rejection in host specificity tests. For example, observational data could be particularly useful in identifying when low or no parasitism of non-targets in the laboratory represents false negative or false positive results. In sections 3.3, 4.2 and 4.3, longer and more rigorous observations during no-choice and choice tests may have helped identify the conditions under which *T. catenata* was accepted or rejected by both parasitoid species. Whether low levels of parasitism in the laboratory translate into ecological impacts in the field is an aspect of host specificity testing that will require significant attention in the future.

The behavioural aspects of this study (section 5.2 & 5.3) indicated exciting opportunities for future work. The practical implications for host specificity testing and successful biological control resulting from the host guarding behaviour of *N. insectifurax* were discussed in section 5.2 and the preceding sections of this chapter. More generally, however, the assessment of the mechanisms by which parasitoids out-compete each other in instances of multiparasitism could be investigated using this system and there is also opportunity to explore the evolution of aggression and brood guarding.

Although the practical aspects of the biological control of *P. charybdis* were not initially a focus of this study, the information being collected was clearly relevant, and an extensive interpretation of the situation was attempted (see section 7.3). There are numerous

opportunities for future work on this continuing problem. For example, it would be particularly interesting to test the predictions made in this study regarding the future control of *P. charybdis* by measuring the relative abundances of the three parasitoid species in the field, now that all are well established. Comparing parasitoid species ratios to the level of *P. charybdis* control achieved in different regions throughout the spring and summer months would be a useful starting point.

7.5 Concluding Remarks

Post-release evaluations of introduced BCAs, including their non-target impacts, are still uncommon. This may change as more agents are introduced after having undergone extensive pre-release tests, especially if the legislation under which they are released requires it, as is now the case in Australia. Such studies as well as retrospective host specificity testing of BCAs that were introduced before pre-release testing was required, have the potential to provide empirical evidence as to how and why some BCAs are effective and do not have major non-target impacts, while others fail, or cause significant non-target harm. This thesis has added to a growing number of such studies.

As parasitoid host ranges can vary spatially and temporally and potentially adapt to changing conditions, accepting only completely monophagous parasitoids for biological control is probably unrealistic. The risks posed by BCAs must therefore be weighed against the benefits of their release. This idea is not new, neither is the understanding that choosing appropriate methods by which to assess host specificity is fundamental to this risk-benefit analysis. However, the major hurdles to overcome at present may be in choosing the conditions under which host specificity tests will be run and interpreting their results in light of those conditions. First and foremost, more time should be allocated to observing the behaviour of candidate BCAs so that behavioural and physiological characteristics can be factored in to test designs. This will improve the quality of data obtained from host specificity tests and make better use of limited insects, time and other resources. Secondly, no-choice host specificity tests conducted within the constraints of the quarantine environment are generally accepted to overestimate parasitoid host ranges. Rather than berate the use of this tool because of its inability to accurately predict host ranges, its value as a means of gathering data on parasitism rates and sex ratios and for

selecting physiologically suitable hosts for more comprehensive testing should be recognised. Thirdly, choice tests are not necessarily always more or less likely to reflect the conditions a parasitoid may encounter in the natural environment and therefore are not always more or less relevant to predicting their ecological host ranges. Choice tests should be recognised for the ability to provide data on host preferences that can be used in conjunction with the information gained from behavioural observations and no-choice tests to better understand the candidate BCA. Combining data from a number of sources and interpreting it with a good understanding of the physiology and behaviour of the parasitoid may result in the more accurate prediction of the likely behaviour of that parasitoid when released into a specific new environment. The following suggestions for improving host specificity testing of parasitoids are made based on combining data in such a way:

1) The use of completely naïve parasitoids is not necessarily achievable or appropriate. Because most parasitoids that are seriously considered for release are expected to be relatively host specific, they can usually only be reared in sufficient numbers for testing on the target host. They will therefore gain some experience of that host at eclosion. Host stimuli may also be important for inducing egg maturation and therefore motivation to assess and accept hosts encountered in host specificity tests. Exposure to host stimuli without allowing oviposition may ensure a high level of motivation and reduce the potential for false negative results.

2) Parasitoid density can directly affect a parasitoid's ability to parasitise a host, or influence host acceptance or sex allocation. Parasitoid density experienced by individual parasitoids in laboratory colonies and during host specificity tests should reflect this and be considered when interpreting results. Understanding whether BCAs will encounter competitors in the field, and how they may respond to them would also be useful for estimating control efficiency.

3) Choice and no-choice tests should be recognised for their ability to contribute different types of information that can be combined to better understand a BCA and therefore predict its likely ecological host range. If non-target attack is predicted by either test, the level of impact might be estimated based on information such as parasitism rates, time taken to accept the host, preference rankings, and sex allocation in choice compared to no-choice tests.

4) Biological control is a dynamic process and parasitoids interact within and between trophic levels. Competitive interactions should be assessed and agents should be screened for hyperparasitoids in quarantine before introduction as this may affect the efficiency with which they can suppress the host. Future risks of hyperparasitoid incursions should also be considered. Post release evaluations to assess non-target impacts provide an opportunity to screen for the arrival of competitors and hyperparasitoids into the system and this can provide useful information on the continued efficacy of pest control.

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APPENDICES

Appendix 1: Geographic co-ordinates for locations where insects were collected to establish and maintain insect cultures (section 2.2), to assess for field parasitism (section 3.4) and to determine species distributions (section 6.7). Region codes follow Crosby et al. (1998) These are the standard area codes used to record arthropod specimens' localities in New Zealand and are included in Fig. 6.7. E = *E. nassaui*, N = *N. insectifurax*, B = *B. albifunicle*, P = *P. charybdis*, D = *D. semipunctata*, Ts. = *T. sloanei*, Tc = *T. catenata*.

Species	East	North	Location	Region
B N D	2597650	6664025	Kerikeri	ND
E N	2631450	6651425	Kerikeri	ND
E	2585500	6631204	Kaikohe	ND
E	2584955	6630985	Knudsen, Kaikohe	ND
E	2656060	6535870	Dome Forest, Dibbles Block	AK
B	2754890	6480715	Whitianga	CL
B E N	2754615	6480655	Cooks Beach	CL
E	2673778	6466783	Papatoetoe	AK
B	2770330	6414990	Waihi Beach	BP
D	2769360	6411720	Athenree	BP
D	2717620	6372225	Tamahere	WO
D	2696295	6347435	Pirongia	WO
B	2816290	6344950	Rotoiti	BP
B E N	2813830	6343810	Kawerau A5B	BP
B E	2835960	6338220	Kawerau	BP
B P	2796937	6333421	Longmile Rd. Rotorua	BP
B E N	2796610	6333340	Rotorua	BP
B E N P	2793220	6328855	Kapenga	BP
B	2806875	6328135	Lake Tarawera	BP
E	2755801	6325217	Tokoroa	WO
B	2790050	6310160	Wairekei	TO
E	2730150	6294160	Pureora	TO
E	2731100	6293025	Maraeroa	TO
E	2730430	6292860	Maraeroa	TO
Ts	2948645	6270865	Fox St., Gisborne	GB
Tc	2928550	6269795	Gentle Annie Hill	GB
Ts	2932895	6267120	Ross Estate, Mauntuke	GB
B	2794015	6238420	Poronui	TO
B E N Ts	2901985	6235770	Waiatai Rd.	GB
E	2693345	6008365	Rimutaka Catchpool	WN
E	2579640	6003500	Maori Bay, Pelorous	SD
E	2661131	5993270	Onslow Rd.,Wellington	WN
E	2660810	5993105	Hutt & Onslow Rd., Wellington	WN
E	2656735	5990773	Karori Cemetery	WN
E	2656640	5990530	Karori Cemetery	WN
E	2532875	5990015	Grampians Walkway, Nelson	NN
E	2533440	5989815	Grampian Hill, Nelson	NN
E	2669720	5982035	Rimutaka Catchpool	WN

Appendix 1 continued

Species	East	North	Location	Region
E	2497625	5974275	Olivers Rd., Spooners Range	NN
E	2504625	5965865	Hiwipango	NN
B E	2594409	5963498	Blenheim	MB
E	2500400	5962600	Golden Downs	NN
E	2599494	5947422	Seddon	KA
E	2393800	5938227	Westport	NN
E	2586729	5899987	Dunluce	KA
E	2362146	5860181	Victoria Park, Greymouth	BR
E N	2549712	5853896	Oaro	KA
E	2350175	5836445	Hokitika	WD
B E N	2491160	5815282	Balmoral	NC
E	2466266	5729189	Lincoln	MC
B E N	2376550	5711050	Montalto	MC
E	2218781	5601447	Glenfolye Station	CO
E	2218525	5580390	Bendigo	CO
E	2347630	5577010	Airedale	DN
E	2168626	5566521	Queenstown Hill	OL
B E	2211985	5564980	Cromwell	CO
B E	2166077	5564621	Queenstown	OL
B E	2222214	5510509	Roxburgh	CO
E	2235876	5489743	Raes Junction	CO
E	2300820	5483230	Mt. Allan	DN
E	2251200	5480411	Southland	SL
E	2250974	5479926	Glen Dhu	CO
E	2149845	5469025	Dipton	SL
E	2215385	5461000	Osyter creek	SL

Appendix 2: Egg length (mm) of laboratory-reared paropsine species established in New Zealand.

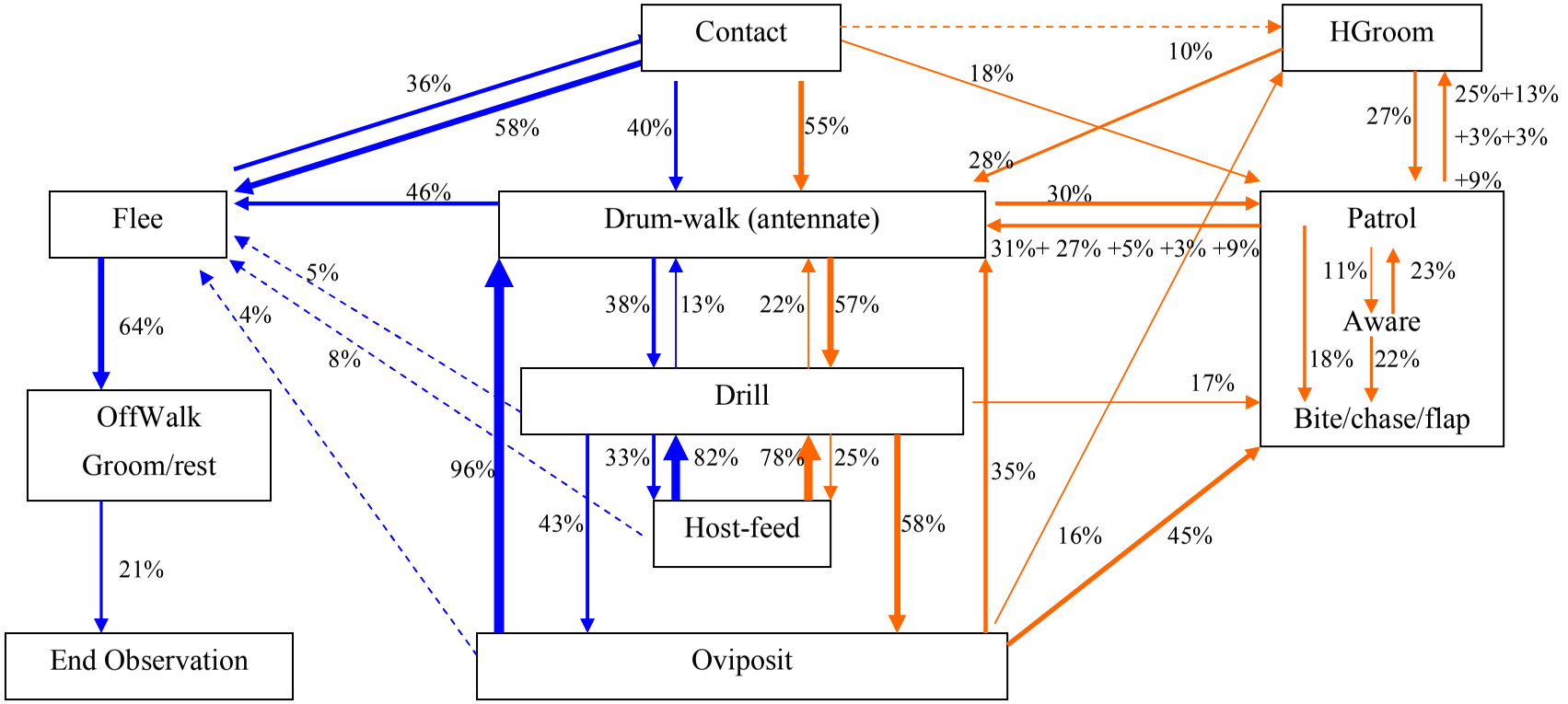
Rep	*P. charybdis*	*D. semipunctata*	*T. catenata*	*T. Sloanei*
1	2.92	2.25	1.92	-
2	2.83	2.25	1.92	-
3	2.83	2.25	1.83	-
4	2.75	2.25	1.83	-
5	2.75	2.08	1.83	-
6	2.83	2.42	2.00	-
7	2.83	2.33	1.92	-
8	2.92	2.58	1.83	-
9	2.83	2.25	2.00	-
10	2.92	2.33	2.00	-
11	2.92	2.17	1.83	-
12	2.92	2.25	1.92	-
13	2.92	2.67	1.67	-
14	2.75	2.17	1.75	-
15	2.50	2.33	1.83	-
16	2.92	2.42	1.83	-
17	2.83	2.25	1.75	-
18	2.92	2.33	1.75	-
19	2.67	2.17	1.83	-
20	2.75	2.25	1.83	-
21	2.83	2.42	1.75	-
22	2.75	2.33	1.67	-
23	2.83	2.42	2.08	-
24	2.83	2.42	1.75	-
25	3.00	2.25	2.08	-
26	2.83	2.25	1.92	-
27	2.75	2.33	2.00	-
28	2.83	2.25	1.83	-
29	2.92	2.17	1.83	-
30	2.92	2.17	1.75	-
31	2.75	2.25	1.92	-
32	2.83	2.33	1.92	-
33	2.92	2.25	1.83	-
34	2.83	2.17	1.92	-
35	2.83	2.42	1.92	-
36	3.00	2.42	1.83	-
37	2.92	2.17	1.92	-
38	2.92	2.25	1.83	-
39	2.92	2.17	1.92	-
40	2.83	2.25	1.83	-
10	2.75	2.50	1.92	-
42	2.83	2.33	1.92	-
43	2.75	2.25	2.08	-
44	2.92	2.08	1.75	-
45	2.75	2.25	1.92	-
46	2.75	2.33	1.92	-
47	2.67	2.42	1.92	-
48	2.75	2.17	1.83	-
49	2.83	2.33	1.92	-
50	2.75	2.50	1.92	-
Average ± SE	**2.83 ± 0.01**	**2.30 ± 0.02**	**1.87 ± 0.01**	**1.60**[†]

[†] *T. sloanei* eggs were not measured in the course of this study. Reported value from T. Withers *pers. com*.

Appendix 3: Configuration programmed into *The Observer* and used to record actions and interactions of *E. nassaui* and *N. insectifurax* in section 5.2. Independent variables were recorded before or after each observation. Behavioural states displayed by each subject were recorded in real time during observations. Colours used to depict behavioural states in Fig. 5.1 (pg. 83) are displayed beside each state description.

Parameter	Description	Value
Subjects	1 x pre-tested *Enoggera nassaui* female	Enog
	1 x pre-tested *Neopolycystus insectifurax* female	Neo
Settings		
Recording method	Actions recorded continuously or at set intervals	Continuous
Duration	Maximum time from start to finish of observation	30 minutes
Duration basis	Observed time or elapsed time	Observed time
Independent variables		
Batch size	Number of eggs in batch	6-15 eggs
Lab temp	Ambient room temperature at time of observation	0-30 ^{o}C
Enog ovip	*E. nassaui* oviposited on at least one occasion	Yes/no
Neo ovip	*N. insectifurax* oviposited on at least one occasion	Yes/no
Interaction	Subjects acted in response to one another on the egg batch on at least one occasion	Yes/no
Neo win	*N. insectifurax* gained/retained possession of egg batch following at least one interaction	Yes/no
Enog win	*E. nassaui* gained/retained possession of egg batch as above	Yes/no
Ownership change	The species in possession of the egg batch changed due to an interaction on at least one occasion	Yes/no
Behavioural state		
Contact	Walks or alights onto egg batch from elsewhere in the arena	
DrumW	Taps antennae (antennating) on egg batch while walking	
Drill	Inserts or moves ovipositor around within host egg, abdomen vertical	
Ovip	Oviposits in host egg, remains still with ovipositor inserted, abdomen horizontal	
Patrol	Walks/runs around perimeter of egg batch with antennae and head up	
Hfeed	Feeds on host egg contents	
Hgroom	Grooms while standing on egg batch	
Hrest	Remains motionless with head tucked towards body while on egg batch	
OffWalk	Walks/runs within the test arena without contacting the egg batch or leaf	
Groom	Grooms within the test arena without contacting the egg batch or leaf	
Rest	Remains motionless in the test arena without contacting the egg batch or leaf	
Aware	Remains still with antennae and head up, sometimes turning head side to side	
Flap	Flaps wings while facing other subject	
Bite	Bites other subject	
Chase	Moves rapidly and directly towards other subject	
Flee	Moves rapidly away from other subject in response to *flap*, *bite* or *chase*	

Appendix 4: Probability of transition, from the point of host contact, between each of the main behavioural states displayed by *E. nassaui* (blue) and *N. insectifurax* (orange) in 30 minute observations of 28 pairs (one of each species) of parasitoids. Most transitions with probabilities < 5% are not shown. Arrows are weighted to indicate the main behavioural pathways of each parasitoid species. Where multiple states are presented in individual boxes, probability values indicate the combined total of transition to all of those states (eg. *N. insectifurax* has a 30% probability of following *Drum-walk* with one of the states *Patrol*, *Aware*, *Bite*, *Chase* or *Flap*).



Appendix 5: Configuration programmed into *The Observer* and used to recording the actions of *E. nassaui* and *N. insectifurax* in section 5.3. *Modifiers* were used to record the state of the individual egg being acted upon at any given time by each subject.

Parameter	Description	Value
Subjects	1 x pre-tested *Enoggera nassaui* female	Enog
	1 x pre-tested *Neopolycystus insectifurax* female	Neo
Settings		
Recording method	Actions recorded continuously or at set intervals	Continuous
Duration	Maximum time from start to finish of observation	30 minutes
Duration basis	Observed time or elapsed time	Observed time
Modifiers		
Enog egg	Host egg previously parasitised by *E. nassaui*	
Neo egg	Host egg previously parasitised by *N. insectifurax*	
Fresh egg	Un-parasitised host egg	
Independent variables		
Lab temp	Ambient room temperature at time of observation	0-30 ^{o}C
Previous oviposition	Species that had oviposited into the observed egg batch before the individual currently being observed	*E. nassaui* *N. insectifurax* None
Multiple-parasitism	At least one instance of multiple-parasitism observed	Yes/no
Super-parasitism	At least one instance of super-parasitism observed	Yes/no
Behavioural state		
DrumW	Taps antennae (antennating) on egg batch while walking	
Drill	Inserts or moves ovipositor around within host egg, abdomen vertical	
Ovip	Oviposits in host egg, remains still with ovipositor inserted, abdomen horizontal	
Jab	Directs the ovipositor at or into a parasitoid egg within a host egg	
Hfeed	Feeds on host egg contents	
Hgroom	Grooms while standing on egg batch	
Hrest	Remains completely still with head tucked towards body while on egg batch	
Defensive	*Patrols* or stands *aware* as described in Appendix 3	
OffHost	Subject is in test arena but not in contact with host eggs	