Multimodal mechanisms of early mate-detection in the parasitoid wasp *Pimpla disparis* (Hymenoptera: Ichneumonidae)

by

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Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY

Spring 2015

Approval

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Degree: **Doctor of Philosophy (Biology)**

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Date Defended/Approved: April 20, 2015

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Abstract

Males of the hymenopteran parasitoid *Pimpla disparis* have been observed to aggregate on gypsy moth, *Lymantria dispar*, host pupae before the emergence of a female. This led me to test the hypothesis that males respond to chemical cues associated with parasitized pupae. Results of laboratory experiments suggest that females mark the host pupae they have parasitized and that males discern between such pupae and those not parasitized. Males continue to recognize parasitized pupae throughout the development of the parasitoid.

To investigate potential acoustic and vibratory cues that males may exploit to detect the presence and track the progress of a developing parasitoid (DePa; future mate) inside a host pupa, I analyzed DePa-derived cues by airborne sound and laser Doppler vibrometer recordings. Parameters (e.g., amplitude) of sound and vibratory cues change significantly over time and thus could 'inform' a visiting adult male about the stage of DePa's development.

To test the hypothesis that male *P. disparis* memorize and revisit the location(s) of parasitized host pupae as a strategy to attain mates, we color-coded *P. disparis* males in a field survey and recorded their behaviour. We learned that they revisit parasitized moth pupae on consecutive days, and arrest on those pupae with a near-emergence parasitoid. These results are supported by laboratory experiments, revealing that males memorize both the macro- and micro-locations of parasitized host pupae.

DePa's quiescence a few days before emergence could be a cue for a visiting male that the emergence of a mate will soon take place but it would not help the male to precisely predict the time of emergence. In contrast, oral fluid produced by emerging adult parasitoids may be indicative of the emergence process. I tested the hypothesis that semiochemicals associated with DePa's emergence arrest males on a parasitized host pupa. I found that these semiochemicals emanate from oral fluid secreted by parasitoids while chewing their way out of a host. Attraction of males to oral fluid semiochemicals from males and females indicates that mate-seeking males co-opt chemicals involved in the eclosion process as a mate finding cue, taking a 50% chance that the prospective mate is a female.

Keywords: *Pimpla disparis*; Ichneumonidae; Early mate detection; Learning and memorization; Sound and vibrations; Emergence semiochemicals

To my dear parents for everything...vă mulţumesc.

To my little wonders Felix and Elisa,

with all my heart...vă ador.

Acknowledgements

I would like to express my gratitude to everyone who guided me throughout this project. My journey would not have been possible without the involvement and support of some remarkable people whom I was fortunate to know over the years.

I would like to address my sincere thanks to Dr. Gerhard Gries for his dedicated supervision, the unconditional support and encouragement. He offered me the opportunity to pursue my education goals, and he guided and helped me throughout the whole process. His wisdom, incommensurable experience and knowledge are exemplary and guided my through the years.

I would also like to express my thanks to Regine Gries. Her experience, hard work and valuable technical skills have inspired me over the years. I would like to thank her for all the help in the laboratory, the technical assistance and advice, as well as for her kind and understanding character.

I gratefully acknowledge my supervisory committee members, Dr. Carl Lowenberger and Dr. Peter Belton, for their support, guidance and constructive comments during the conceptual and final stages of the project. I would also like to thank Dr. Silvia Dorn for having agreed to be External Examiner of my thesis defence, Dr. David Gillespie for having agreed to be Internal Examiner of my thesis defence, and Dr. Ronald Ydenberg for having agreed to chair the defence.

I also extend my thanks and appreciation to Dr. Paul Schaefer whose field observations initiated this project, for his valuable help with the field work and constructive comments during the preparation of manuscripts.

I have had the opportunity of working with so many remarkable laboratory members. I thank Dr. Stephen Takács for his acoustics expertise, insightful discussions, advice and sense of humor and encouragement along the way. I am also thankful for the friendship, assistance and interesting conversations with the old and new members of the lab. Especially, I would like to thank Kelly Ablard, Bekka Brodie, Eloise Rowland,

Samantha Vibert, Veronika Lambinet, Catherine Scott, Michael Hrabar, Sean McCann, Sebastian Ibarra and Dr. Grigori Khaskin.

My research was greatly helped by the contributions of several research assistants who worked with me. I thank them for all the energy and time they dedicated to this project. Specifically, I would like to thank Shari Ikoma, Jessika Iwanski, Lara Monk and Matthew Drake. Special thanks go to Michael Hrabar and Cesar Inducil for their valuable contributions to the development and testing of research ideas, their problem solving abilities and initiatives, technical skills, hard work, and rigorous data collection, and last but not least, for their interest in parasitoids and their fine sense of humor.

I thank Pilar Cepeda for her assistance with insect colony maintenance and bioassays and for enjoyable conversations over the years.

I would like to thank the staff of the Department of Biological Sciences at SFU for their dedication and for always being ready to help. Specifically, I thank Marlene Nguyen, David Carmean, Amelia Shu, Debbie Sandher and Barbara Sherman.

I extend my thanks to Ian Berkovitz, Marie Loughin and Dr. Tom Loughin for statistical consultation and advice, and Robert Birtch and Stephen DeMuth for graphical illustrations.

I would like to thank my parents, my husband, and family friends that helped me complete this journey through moral support and encouragement which have been decisive. I thank my daughter and my little son for inspiring me with enthusiasm and motivation; I thank them for their patience; they grew up during the course of this project.

My research was supported by a CGS-D Canada Graduate Scholarship from the Natural Sciences and Engineering Research Council of Canada (NSERC), Graduate Fellowships from Simon Fraser University, and by an NSERC-Discovery Grant and an NSERC - Industrial Research Chair to G.G. with Contech Enterprises Inc., SC Johnson Canada, and Global Forest Science as industrial sponsors.

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Glossary

Term	Definition[1]
Acoustic	Relating to sound
Arrest	Here, referred to as a behaviour where a male remains in the same microlocation (e.g., on or near the host pupa)
Arrestment	A behavioral response of a male induced by chemical and/or mechanical stimuli and characterized by a motionless state
Arrhenotoky	A form of parthenogenesis dominant amongst the Hymenoptera in which males develop from unfertilized eggs and females from fertilized ones (Quicke, 1997)
Behavior	The ways in which an organism adjusts to, and interacts with, its environment (Matthews & Matthews, 1978)
Cue	A feature of the world, animate or inanimate, that can be used by an animal as a guide to future action (Hasson, 1994; Maynard Smith & Harper, 2003)
Developing parasitoid	Parasitoid in the larval, pupal or pharate state that has not yet emerged from its host
Early mate detection	The strategy of males seeking virgin females at their site of emergence
Fitness	Reproductive success
Gregarious parasitoid	A parasitoid that lays multiple eggs in a single host developing in multiple adult parasitoids
Haplodiploid	Organisms in which the sex of an individual is determined by whether it possesses a haploid or diploid genetic complement (Quicke, 1997)
Idiobiont	A life history strategy in which the host does not develop after it is parasitized (Quicke, 1997)
Kairomone	Chemical signal that mediates interspecific interaction, benefiting only the receiver
Protandry	The early emergence of male parasitoids
Quasi-gregarious parasitoid	A parasitoid that lays a single egg in a host, but the hosts are clustered
Semiochemicals	Any chemicals that carry information about the emitter to a receiver (Quicke, 1997)

[1] References are included in the Introduction (Chapter 1) under the “References” section.

Sex pheromone	Chemical signal that mediates intraspecific communication between prospective mates, with both the signaler and receiver benefiting
Sexual kairomone	A kairomone used by the benefiting organism for sexual purposes (Ruther et al., 2002)
Sexual selection	Here referred to as intrasexual selection; competition between males for access to mates
Signal	An act or structure that alters the behavior of another organism which evolved because of that effect, and which is effective because the receiver's response has also evolved (Maynard Smith & Harper, 2003)
Solitary parasitoid	A parasitoid that lays a single egg in a host
Sonogram	Representation of sound as a distribution of intensity components and corresponding frequencies
Sound	The product of particle vibrations and the propagation as longitudinal waves of those vibrations through a medium
Sound intensity level	The sound acoustic power per unit of area relative to a fixed reference (e.g., the lowest sound intensity perceived by the human ear, $I_0 = 10^{-12}$ W/m^2)
Strategy	In the context of mating behavior, it represents a series of adaptations and tactics by which mating is achieved
Synomone	Chemical signal that mediate interspecific interaction, benefiting both the signaler and the receiver
Vibration	Mechanical oscillations about an equilibrium position in a solid medium

Introductory image: *Pimpla disparis*

Figure A. ***Pimpla disparis*** **males arresting on a wax moth pupa containing a developing parasitoid (Photo by Sean McCann)**

Chapter 1.

Introduction

1.1. General background

1.1.1. The adaptive significance of mate finding behavior in insects

Insects probably display the most impressive diversity of mating and social behaviors among animals (Choe & Crespi, 1997). The mating system of insects represents the behavioral, morphological and physiological mechanisms by which gamete union is accomplished (Emlen & Oring, 1977; Davies, 1991; Brown et al., 1997). Mating systems in insects are shaped by the reproductive biology of both males and females, the intensity of intrasexual competition and the distribution of partners in time and space (Emlen & Oring, 1977; Thornhill & Alcock, 1983; Godfray, 1994; Shuster & Wade, 2003; Metzger et al., 2010). The reproductive system of insects includes locating mates, choice of mates, selection of oviposition sites, and the factors affecting the fitness of larvae (Bailey & Ridsdill-Smith, 1991). Among these behaviors, mate finding is a first crucial step in shaping insect mating systems (Metzger et al., 2010).

The reproductive success of insects typically relies on an efficient strategy to locate mates. Males adopt strategies that maximize their genetic contributions to future generations through specific behavior in the context of reproduction (Thornhill, 1979). Generally, males search for females because a male's fitness increases with each female he inseminates, whereas a female's entire complement of eggs can usually be fertilized by sperm from a single or few males (Thornhill & Alcock, 1983). The fitness of a male is also affected by numerous biological and behavioral factors linked to his reproductive strategy such as production and management of sperm, capacity to acquire mates, and investment in offspring (Thornhill & Alcock, 1983; Roitberg et al., 2001;

Damiens & Boivin, 2005). The reproductive success of a male is generally limited to the number of females he mates with, whereas the reproductive success of a female is limited by the number and quality of her eggs, and thus the offspring she produces, as both offspring number and quality represent the currency of fitness (Snook & Pizzari, 2012). Females generally invest more in offspring than males, while males compete for mating partners (Ayasse et al., 2001). Males invest little in individual spermatozoa and rather increase their fitness by maximizing the number of sperm produced and the number of matings (Damiens & Boivin, 2005).

In most insect species, male fitness represents the quantity of offspring that their mate produces, but in hymenopteran parasitoids which are characterized by arrhenotokous parthenogenesis, male fitness is determined only by the daughters produced by their mates (Godfray, 1994; Quicke, 1997; Damiens & Boivin, 2005). There is selective pressure on males to increase their access to females as early as possible. Early emergence is one mechanism by which male parasitoids maximize their access to females and increase their fitness. Early emerging males mating with virgins are assumed to fertilize a greater number of eggs than males mating with previously mated females (Baughman, 1991). Conceivably, males mating with virgins ensure a greater fitness gain if sperm competition does not occur.

In addition to emergence patterns, male mate-searching behavior is affected by female and male distribution patterns (Alcock et al., 1978; Paxton, 2005). In parasitoids, the mating systems and distributions of females and males are shaped by the spatial distribution of hosts (Godfray, 1994; Ayasse et al., 2001). The distribution of hosts determines the distribution of oviposition sites for females, and consequently the emergence and dispersion sites of adult male and female parasitoids, and the mate finding strategies that males may adopt. In gregarious and quasi-gregarious parasitoids, mating commonly occurs at the emergence site (Godfray, 1994), but in solitary parasitoids where males and females emerge spatially separated, males often must seek emergence, oviposition or feeding sites of females (Godfray & Cook, 1997).

Lastly, the mate finding strategy that males adopt is determined by the distribution of receptive females in space and time, and by the reproductive life history of

females that affect the strength of sexual selection (Emlen & Oring, 1977; Thornhill & Alcock, 1983; Shuster & Wade, 2003; Paxton, 2005).

1.1.2. Overview of sensory mechanisms underlying mate finding behavior in parasitoids

Behavior of insects is mediated by a large number of external and internal stimuli (Kennedy, 1978; Harris & Foster, 1995), and there are hardly any examples which demonstrate that an insect uses only a single sensory modality to find a resource (Prokopy, 1986). When the resources are mates, insects typically engage in species-specific communication systems that rely on chemical, acoustic, vibratory or visual stimuli that could act independently or in combination. The main function of intraspecific communication is species and sex recognition (Matthews & Matthews, 1979) with consequences for reproduction and fitness.

Regardless of the communication modality, females and males exhibit sex-specific differences and adaptations that enhance reproductive success (Bailey, 1991). For example, if males and females use chemical signals during sexual communication, usually females call and males respond. If males emit pheromones, these pheromones often play a role at close range and during courtship, or as aphrodisiacs that induce female receptivity. In communication systems that are based on visual signals or displays, typically the males attract females. Decorative colors and patterns on wings of male butterflies make these males primary visual communicators, even though they may also use hairpencil-derived pheromones that inhibit wing movement and flight of females and mediate courtship behavior and mating. By carrying out their daily food-foraging flights, butterfly males visually attract receptive females who may approach them for mating (Rutowski, 1980). Similarly, nocturnal firefly males use light signals that reveal their position and attract females, whereas diurnal fireflies rely upon pheromones rather than ‘flashing lights’ for sexual communication (De Cock & Matthysen, 2005). In insects that emit sound signals for mate attraction, usually the males call and the females search, possibly dueting when the prospective mates approach each other. Cricket males acoustically call females but also produce close-range pheromones that induce receptivity in females (Otte & Cade, 1976; Bell, 1980). Bushcrickets produce

advertisement calls that have both air- and substrate-borne components, the latter travelling through plant stems and likely functioning in mate location (Keuper & Kühne, 1983; Hill, 2008). Cicada males produce long-range calling songs and short-range courtship songs, and females respond with audible wing-flicks, acoustically dueting with males before they make physical contact (Jérôme & Thierry, 2004). Males and females of various planthoppers perform duets via plant vibrations, with males searching for stationary females (Hill, 2008).

In these select but representative examples, the mate-finding challenge of males is affected by biological and physiological factors. Differences in signaling strategies by males and females relate to reproductive investment (Thornhill, 1979; Bailey, 1991). As stated before (subchapter 1.1.1), females invest more by producing costly eggs that are much larger than sperm. Females may need to mate with a limited number of males but await the arrival of males, whereas males are expected to search and compete for females. If males invest directly in developing eggs, males may become the choosy sex and females are expected to compete for males, a form of sex role reversal (Gwynne, 1985; Thornhill & Gwynne, 1986; Bailey, 1991).

Insect parasitoids must also address several challenges that largely control their fitness, particularly the challenges of finding mating and oviposition sites (Colazza et al., 2013). To reproduce successfully, most parasitoids need to solve problems such as where to find and search for mates, how to recognize them, and then how to behave in a manner that mating will ensue (Godfray, 1994; Godfray & Cook, 1997; Hardy et al., 2005). Like other insects during mate searching, parasitoids might integrate multimodal sensory information.

There are various mate finding strategies that parasitoid males pursue to successfully reproduce. Mainly, they search for emerged mates, await the emergence of prospective mates at emergence sites, or they combine both strategies. Each strategy is advantageous under specific conditions that are determined by the host habitat and host distribution, and the parasitoids' biology. Both movement and habitat preference of host insects determine the spatial and temporal distribution of parasitoid pupation sites, and thus the sites of emergence of parasitoid adults (Ayasse et al., 2001). In such systems,

the host patches exploited by parasitoid females should facilitate mate encounters. This concept is supported by observation that male parasitoids court females in host foraging or emergence patches (Godfray & Cook 1997; Hardy et al., 2005; Metzger et al., 2010). Male and female parasitoids that develop in the same host or host patch, with high levels of inbreeding, may not need to search extensively for mates, whereas solitary parasitoids whose hosts are widely spread, commonly engage in mate searches (Hardy et al., 2005). In gregarious and quasi-gregarious parasitoids, males most likely remain at the site where siblings or other conspecifics are expected to emerge. In solitary species, the rendezvous may occur in parts of the habitat where males can expect to find females, such as female emergence, oviposition or feeding sites (Godfray & Cook, 1997).

Next, I will highlight strategies that male parasitoids pursue and the sensory information that mediates mate finding.

Acquiring olfactory information

Detection of emerged females

Males of parasitoid wasps commonly search for and locate emerged mates primarily via sex pheromones (Godfray, 1994; Quicke, 1997), although in some species males enhance their mate-finding success by co-opting chemical cues associated with the host (Ruther, 2013).

a. Mate detection via sex pheromones

Sex pheromones are thought to be critically important during the mate finding process of parasitoid wasps (Godfray, 1994; Ruther et al., 2007). The sex pheromones of less than 25 species of parasitoid wasps have been identified (reviewed in Quicke, 1997; Kainoh, 1999; Ayasse et al., 2001; Keeling et al., 2004; Danci et al., 2006; Ruther et al., 2007; Ruther, 2013; Stökl et al., 2014). Depending on the mating system, volatile sex pheromones can be released by either sex of parasitoid wasp, but in most species sex pheromones are released by females (Quicke, 1997). Female-produced sex pheromones are either highly volatile and attractive to males over a long distance, or

they are low-volatile compounds such as cuticular lipids that mediate species recognition and courtship behavior at close range (Keeling et al., 2004; Ruther et al., 2007 and citations therein; Ruther, 2013; Stökl et al., 2014).

There is evidence for female-produced, volatile sex-attractant pheromones in a few ichneumonid species, including the gregarious larval endoparasitoid *Campoletis sonorensis* (Cameron) (Vinson, 1972), the pupal endoparasitoid *Ichneumon (= Pterocormus) promissorius* Erichson (Jewett & Carpenter, 1999), the pupal ectoparasitoid *Diapetimorpha introita* (Cresson) (Jewett & Carpenter, 1998), and the pupal endoparasitoid *Itoplectis naranyae* (Ashmead) (Itadani & Ueno, 2014). At least in some species, newly-emerged females most strongly attract males, whereas older females are less attractive.

Sex pheromone components produced by ichneumonid females have been (tentatively) identified only in the pupal endoparasitoid *Itoplectis conquisitor* (Say) (Robacker & Hendry, 1977), the larval endoparasitoid *Syndipnus rubiginosus* Walley (Eller et al., 1984), the larval parasitoid *Eriborus terebrans* (Gravenhorst) (Shu & Jones, 1993), and the larval-pupal ectoparasitoid *Roptocerus xylophagorum* (Ratzeburg) where the pheromone may be substrate-borne (Sullivan, 2002). In general, these are single- or multi-component pheromones, with components of low or high polarity.

Substrate-borne, female-produced sex pheromones could serve important roles in mediating mate location, especially in small cursorial parasitoids with limited dispersal capability (Godfray, 1994), or in species still capable of directed flight but commonly moving by walking on substrates and occasionally jumping in an apparently unpredictable manner. Females of *Aphelinus asychis* Walker, solitary parasitoids of aphids (Fauvergue et al., 1995), of *Trichogramma brassicae* Bezdenko, gregarious egg parasitoids of pyralid moths (Pompanon et al., 1997), of *Ascogaster reticulatus* Watanabe, solitary egg-larval parasitoids of tortricid moths (Kamano et al., 1989), of *Glyptapanteles flavicoxis* (Marsh) (Danci et al., 2006), gregarious larval parasitoids, and of *Metaphycus luteolus* (Timberlake), gregarious parasitoids of soft-bodied scales (Kapranas et al., 2013), all deposit “chemical footprints” or trail sex pheromones that males rely on to locate conspecific females once they have departed from their site of

emergence. This short-range communication system is representative of many gregarious species. If males remain at the emergence site, then females would not benefit from the production of long-range sex pheromones that attract males because pheromone biosyntheses would presumably incur metabolic costs while gaining little advantage (Salerno et al., 2012). Males remaining at the site of emergence may lose mating opportunities elsewhere but will potentially benefit by increasing their inclusive fitness by ensuring that their sisters are fertilized (Godfray, 1994).

Once potential mating partners have encountered each other, contact sex pheromones comprising less-volatile components such as methylated or unsaturated hydrocarbons on the females' cuticle, mediate communication, enable mate recognition and elicit stereotypic male courtship behavior before mating ensues (Steiner et al., 2006; Ruther et al., 2011; Ablard et al., 2012; Kühbandner et al., 2012; Ruther, 2013).

b. Mate detection via non-pheromonal semiochemicals (e.g., sexual kairomones) combined (or not) with sex pheromones

Parasitoids must acquire and integrate the information provided by a large variety of cues to make adaptive decisions for reproduction (van Alphen & Bernstein, 2008).

Non-pheromonal semiochemical cues evidently contribute to mate finding in parasitoid wasps. These cues may originate from the host itself (e.g., host pheromones, faeces, or lipid footprints) or may emanate from sources present in the host habitat (e.g., plants being attacked by the hosts) (Ruther, 2013). Such semiochemical cues may play roles in sexual communication systems of parasitoids because these cues could enhance the responsiveness of males to female sex pheromones, affect the release or production of sex pheromones, or serve directly as mate-finding cues (Ruther et al., 2002; Benelli & Canale, 2013).

Males can locate female emergence or oviposition sites (Godfray & Cook, 1997) by exploiting semiochemical cues associated with hosts. When female parasitoids produce low-volatile pheromones that induce courtship but not long-range attraction of males, males must rely on "infochemicals" other than pheromones for long-range orientation toward females (Steiner et al., 2007). These infochemicals may be the same host-associated cues that female parasitoids use to locate hosts (Steiner et al., 2007).

Host-specialist and -generalist parasitoids might also be expected to exploit different kinds of cues (Itadani & Ueno, 2014).

Females of the braconid wasp *Psyttalia concolor* (Szépligeti), endoparasitoids of tephritid fruit flies, locate host maggots by responding to kairomonal semiochemicals emanating from infested fruit. These kairomones appear to be key olfactory cues not only attracting *P. concolor* females to high-density host patches, but also guiding virgin males towards tephritid-infested fruits on which they may find newly emerged virgin females, thus raising a male's chance of locating more receptive females during his lifespan and increasing his fitness (Benelli & Canale, 2013). Because these kairomones function mainly in the context of mate location, they have been termed sexual kairomones (Ruther et al., 2002), although they may provide other benefits such as guiding parasitoids towards sugary liquids exuding from infested fruits.

Males of the ichenumonid wasp *Itoplectis naranyae* (Ashmead) search for female emergence sites and are likely to respond not only to female sex pheromones but also to host and habitat cues (Ueno & Tanaka, 1994; Itadani & Ueno, 2014).

As parasitoid females exploit both kairomones and synomones in their search for hosts, parasitoid males exploiting the same cues as do host-foraging females might increase their chance of encountering receptive females (Ruther, 2013). Males of the parasitoid *Venturia canescens* (Gravenhorst) respond both to female olfactory cues which elicit take off and orientation of males toward females, and to co-occurring kairomone cues that host-foraging females exploit (Metzger et al., 2010). These cues in combination afforded a significant increase in mate-finding efficiency by males.

Detection of prospective mates prior to their emergence

The reproductive success of parasitoid males depends on their ability to locate and inseminate receptive females (Ruther et al., 2007). Searching for emergence sites of females will be favored if females mate only once and are immediately receptive after emergence (Godfray, 1994; Itadani & Ueno, 2014).

Some parasitoid males search for female emergence sites, compete with other males at these sites, and await the emergence of conspecific females, as shown in species such as *Nasonia vitripennis* (Walker) (King et al., 1969), *Lariophagus distinguendus* (Forster) (Steiner et al., 2005), *Spalangia endius* Walker (King, 2006), *Megarhyssa* spp. and *Rhyssa* spp. (Matthews et al., 1979; Crankshaw & Matthews, 1981; Eggleton, 1991), and *Lytarmes maculipennis* (Smith) (Eggleton, 1990). Males of bethylid wasps in the genus *Goniozus* exemplify an extreme permutation of this strategy. They chew their way into female cocoons and mate with the female even before she emerges (Gordh, 1976). In the above examples, male parasitoids recognize chemical cues from host pupae with incipient emergence of prospective mates.

Some female parasitoids produce a sex pheromone during pupal development before emergence. This phenomenon has been reported in the braconid *Apanteles glomeratus* (L.) (Tagawa, 1977) and in the pteromalids *Nasonia vitripennis* (King et al., 1969) and *Anisopteromalus calandrae* (Howard) (Yoshida, 1978). Pre-emergence pheromone release by these females may increase their chance of being mated before departing from their emergence site in search for hosts. Still in the pupal stage, females of *A. calandrae,* larval parasitoids of the azuky bean weevil, *Callosobruchus chinensis* (L.), secrete a sex pheromone which triggers wing vibration in males and promotes mating as soon as the female emerges from an infested bean (Yoshida, 1978).

Mixed strategy

A mixed strategy, in which males detect females before or after they have emerged, has been revealed in the bethylid *Cephalonomia tarsalis* (Ashmead) (Collatz et al., 2009). Here, males mate with females either at the site of emergence following attraction to sex pheromone emanating from the females' cocoons, or males mate with females that have dispersed from the natal patch, following attraction to the females' sex pheromone or to kairomones associated with host faeces (Collatz et al., 2009). In this parasitoid, the same kairomones that guide a female to a potential host for oviposition, also guide a male to a potential mate encounter site, and serve as sexual kairomones (Ruther et al., 2002; Collatz & Steidle, 2008).

Females of *Lariophagus distinguendus*, polyphagous ectoparasitoids of the granary weevil *Sitophilus granarius* (L.), do not produce long-range pheromones (Ruther et al., 2000). In this parasitoid, male courtship behavior is mediated by a female-produced contact sex pheromone. Distinctively different from other parasitoids, the same sex pheromone is present in the pupal stage of both males and females, but the pheromone titer of males decreases shortly after their emergence (Steiner et al., 2005, 2006). In addition, females can be located by a male after they have left the emergence site to search for hosts. Both males and females are innately attracted to semiochemicals in faeces from their host (Ruther & Steidle, 2000; Steiner et al., 2007). By orientating towards the same volatiles that are exploited by host-foraging females, males may be guided to patches of potentially high female density and consequently increase their chance of mating.

Acquiring acoustic information

Pheromonal signals prevail during sexual communication in parasitoids (Quicke, 1997, 2014; Ayasse et al., 2001) but sound and vibrations add to the complexity of information conveyed (Vet et al., 2002). Parasitoids detect mechanosensory cues and signals during host foraging and courtship displays, respectively. Once a parasitoid male has located a female, both acoustic signals and close-range contact pheromones affect the outcome of courtship (van den Assem, 1986). Courtship is considered a mechanism for species and mate recognition, preventing hybridization (Sivinski, 1988). Courtship proceeds in a well-defined sequence of behaviors, including substrate antennation, trail following, wing fanning, and mounting of the female (Keeling et al., 2004). Typically, the courting male wing fans and thus generates acoustic signals with different functions. Wing fanning signals (*i*) mediate orientation of males to females in the ichneumonid *Campoletis sonorensis* (Cameron) (Vinson, 1972), (*ii*) enhance the females' activity in the braconids *Diachasmimorpha longicaudata* (Ashmead) (Sivinski & Webb, 1989) and *Diachasmimorpha krausii* (Fullaway) (Rungrojwanich & Walter, 2000), and (*iii*) induce sexual receptivity in females of the pteromalid *Nasonia vitripennis* (Miller & Tsao, 1974; van den Assem & Putters, 1980) and of the braconids *Cotesia rubecula* (Marshall) (Field & Keller, 1993) and *Cotesia marginiventris* (Cresson) (Joyce *et al.*, 2008). In the braconid wasp *Glyptapanteles flavicoxis*, females deposit sex pheromone

on substrate that elicits attraction and wing fanning in conspecific males which, in turn, induces sound and visual reply signals from females that help males orient toward them (Danci et al., 2010).

Acquiring visual information

Visual stimuli control innate responses in communicating insects (De Marco & Menzel, 2008). During mate searching, visual stimuli are essential and may become effective after primarily olfactory stimuli have mediated the process of resource location. This process can be viewed hierarchically at different levels, such as the habitat, patch and the resource itself (Prokopy, 1986). At each level, olfactory and visual stimuli can be assessed in a different manner. At the habitat level, the response to olfactory (or visual) stimuli may prevail, at the patch level the response to visual (or olfactory) stimuli may dominate, and at the resource level olfactory (or visual) stimuli may be preeminent, although olfactory and visual resource stimuli might also act simultaneously (Markl, 1974; Prokopy, 1986). Vision is common in insects, gives the most accurate directional information, and provides a range of cues of both color and shape for identity (Bailey, 1991). That the integration of visual and chemical stimuli is fundamental for mate location and courtship was well demonstrated in the braconid wasp *Psyttalia concolor.* Here, males respond particularly well when visual and chemical cues of females were presented simultaneously (Canale et al., 2013).

In parasitoids, the visual sense has been explored mostly in the context of associative learning of sensory information.

Learning of sensory information

Olfactory and visual stimuli release many types of innate behavioral responses by insects, such as orientation towards mates, appropriate oviposition sites and food sources (Kanzaki, 1996). Behavioral strategies that insects employ must be efficient and adaptive to circumstances which may change every moment (Kanzaki, 1996). If circumstances change, then insects must improve their responses through learning. Learning has been defined as the acquisition of neuronal representations of new information that influences relevant decisions and behaviors (Dukas, 2008, 2009). In

relation to visual and olfactory information, parasitoids can learn such new sensory information. Predominantly females learn chemical and visual cues, and spatial information associated with their hosts (Lewis et al., 2002; van Nouhuys & Kaartinen, 2008; Hoedjes et al., 2011). Females, in particular, can exploit detailed visual information especially during foraging for a food or host site, and they learn to discriminate between these sites based on visual characteristics (e.g., color, shape) (Wäckers & Lewis, 1999; Vet et al., 2002). Chromatic and achromatic plant cues are important mostly for parasitoids of endophytic pupal hosts because pupae do not feed and thus do not invoke plant volatile emissions from fed-on plant tissue (Wang & Yang, 2008) that would otherwise become attractive to parasitoids. Like female parasitoids, male parasitoids are capable of associative learning, but this trait has been explored in only a few species with reference to mate finding behavior.

Associative learning by male parasitoid wasps was first demonstrated with male *Nasonia vitripennis* which can learn to associate color with mates (Baeder & King, 2004). In the context of food location behavior and associated chemical cues, studies have shown that males of the parasitoid wasp *Pimpla alboannulatus* Uchida and *Pimpla luctuosa* Smith learn to associate odors with a specific food source (Iizuka & Takasu, 1999; Sato & Takasu, 2000).

A plausible explanation for the learning ability of male parasitoids is that any olfactory cues that they sense and learn during mating may help them later to find mates (Ruther, 2013). Studies with the aphid parasitoid *Aphidius ervi* Haliday have demonstrated that males indeed can learn olfactory cues in the mating environment. Males can learn to associate, and to respond with sexual display, to artificial vanilla odor provided they have sensed the vanilla odor during a mate encounter (Villagra et al., 2005). Moreover, the males' olfactory sensation in a given host habitat during mating can shape their preference for specific olfactory host habitat cues (Villagra et al., 2008).

When males of the ichneumonid wasp *Itoplectis conquisitor* were trained to respond to cups containing female pheromone-treated cotton, they then also responded to cups without pheromone, suggesting that the males had learned to associate the presence of the cup with the presence of pheromone in the cup (Robacker et al., 1976).

Finally, males of the braconid *Alabagrus texanus* (Cresson) patrol their habitats for the presence of female pheromones using spatial and temporal memory to avoid recently visited sites that are less likely to 'yield' virgin females (Goh & Morse, 2010).

In conclusion, parasitoid males can adjust their complex and often multimodal mate finding behavior in accordance with foraging conditions. Mate-seeking males may exploit and learn olfactory, visual and mechanical cues that are either directly associated with prospective mates or their respective habitats.

1.2. Life history of *Pimpla disparis*

Pimpla (*Coccygomimus*) *disparis* Viereck (Hymenoptera: Ichneumonidae: Pimplinae) is a polyphagous Palearctic species, parasitizing lepidopterous pupae including pupae of the gypsy moth, *Lymantria dispar* (L.) (Erebidae) and of other species in 14 lepidopteran families (Leonard, 1981; Schaefer et al., 1989). The greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), is an overwintering host that proved to be a good candidate for laboratory rearing of *P. disparis* (Weseloh & Anderson, 1982). *Pimpla disparis* was introduced to the United States as a biological control agent of gypsy moth during the mid 1970s and early 1980s (Weseloh & Anderson, 1982; Coulson et al., 1986; Schaefer et al., 1989). This parasitoid is holometabolous, multivoltine and solitary, attacking pre-pupae and pupae (1-9 d of age) (Fuester et al., 1989). Successfully parasitized host pre-pupae do not transform to the pupal stage (Fuester et al., 1989; Schaefer et al., 1989; Fuester & Taylor, 1993). The *P. disparis* larva develops within the host (pre)pupa. As an idiobiont parasitoid, it paralyses its host, with the host ceasing development after parasitism (Godfray, 2007).

Pimpla disparis has a haplodiploid sex determination with daughters arising from fertilized eggs and sons from unfertilized eggs, a phenomenon known as arrhenotoky (Godfray, 1994; Quicke, 2014). Haplodiploid sex determination means that females can determine the sex of their offspring by choosing whether to oviposit a fertilized or unfertilized egg. If superparasitism occurs in *P. disparis*, the elimination of supernumerary parasitoids is most likely the rule (Fuester et al., 1989), and if multiparasitism occurs, when in competition with native ichneumonids such as *Itoplectis*

conquisitor, the first larva to colonize a multiparasitized host is the most likely to survive to adulthood (Moser et al., 2008). Most (80-92%) *P. disparis* progeny emerging from laboratory-reared *L. dispar* host pupae are daughters (Fuester et al., 1989).Females of *P. disparis* are typically monandrous and may produce up to 199 progeny during a lifetime (Metterhouse, 1981). Females have a pre-oviposition period of 6-9 days, the entire life cycle from egg to adult takes 25-32 days, and adults live on average 20-28 days (Schaefer et al., 1989). Under laboratory conditions (25 ^{o}C, 40-60% RH, 16:8 (L:D) photoperiod) tracking 404 *L. dispar* host pupae, the average developmental time of *P. disparis* from egg to adult was 19.31 days (SEM $\pm$ 0.17)(Fuester & Taylor, 1993).

Field and laboratory studies with *P. disparis* have focused mainly on aspects pertinent to developmental strategies, behavior in relation to host characteristics, and host searching by females. More specifically, research has addressed habitat preference and overwintering potential of released parasitoids in biological control programs (Weseloh & Anderson, 1982). Other studies have investigated (*i*) the suitability, optimal age and gender of potential hosts for parasitism (Fuester et al., 1989; Schaefer et al., 1989; Fuester &Taylor, 1993) (*ii*) host attack behavior and host preference (Schaupp et al., 1992), (*iii*) stinging behavior and its impact on host fate (Fuester et al., 1997a), (*iv*) the level of parasitism and factors affecting parasitism (e.g, host density, habitat, or latitude) (Fuester et al., 1997b), (*v*) the effect of photoperiod and temperature on development and diapause (Yasuhara et al., 1998), and (*vi*) the occurrence of superparasitism (Fuester et al., 1989) and competition with other native ichneumonids in the form of multiparasitism and destructive host feeding (Moser et al., 2008).

1.3. Research motivation and questions

Parasitoids are used as biological control agents of insect pests. This applied aspect necessitates research to explore parasitoid and host insect interactions. Parasitoids have evolved diverse host-searching and host-attacking strategies (Wajnberg et al., 2008). Parasitoids are fascinating organisms and model insects for the development and testing of theory on foraging behavior and behavioral ecology including mating systems and sexual selection. *Pimpla disparis*, in particular, invokes a plethora of questions relevant to the fields of behavioral and evolutionary ecology.

Both the type of female mating system and the nature of male-male competition are key factors in determining the mate-locating strategy of males. In monandrous species, the first male to reach a virgin female has a great competitive advantage (Alcock et al., 1978). In general, competition occurs whenever the use of a resource by one animal makes it harder for others to obtain that resource. In sexual selection that resource is mates (Andersson, 1994; Andersson & Iwasa, 1996). One mechanism of sexual selection is scrambling to find a mate before rivals do. Males may achieve this by early emergence, rapid maturation, becoming reproductively active before females (protandry), and/or by rapid location of potential mates, well aided by sensitive sensory organs (Andersson & Iwasa, 1996). Males might also seek emerging or recently emerged females, simply by waiting at a site from which virgin females are likely to emerge (Alcock et al., 1978).

Males of parasitic Hymenoptera, including *P. disparis* (Doutt, 1964; Fuester & Taylor, 1993), commonly have a shorter developmental time than females. The shorter developmental time enables males to be present when virgin females become available which may mate only once or only shortly after emergence (Quicke, 2014). Indeed, laboratory-reared *P. disparis* males usually emerge 3–4 days before females (A Danci, personal observation), and wild males have been observed to aggregate on parasitized gypsy moth pupae prior to the emergence of a potential mate (P.W. Schaefer & S. Takács, personal observations). The underlying mechanisms of this type of mate location and the adaptive significance of this rather unusual behavior are completely unknown. The males' response to parasitized host pupae at this late stage of parasitism suggests that males sense signals or cues derived from the developing parasitoid (DePa) inside a host pupa, the decaying host pupa, or both. Males can be expected to have evolved complex mate detection and assessment abilities, because during their search for parasitized host pupae they may encounter pupae in all stages of parasitism. Typically, detection of host parasitism is the task of gravid females that seek suitable (unparasitized) host pupae. Female parasitoid wasps may integrate complex information to enhance the probability, reliability and accuracy of locating and assessing the suitability of host pupae (Wang & Yang, 2008). The same concept applies to mate-seeking *P. disparis* males that need to find and assess host pupae housing a prospective mate. Most immediate questions are: (1) how do males locate parasitized

host pupae; (2) how early (at what stage of DePa's development) do they sense parasitism; and (3) what are the cues that enable them to detect DePa?

As part of an efficient reproductive strategy and to increase potential mating opportunities, *P. disparis* males should be able (*i*) to monitor the development of DePa in a specific host pupa and (*ii*) to track DePa's development in multiple host pupae. This would be particularly important if males cannot determine the sex of DePa inside a host pupa and if other males have found the same parasitized pupa(e). If males sense parasitism of host pupae at an early stage, then the essential follow-up questions are: (4) can males learn and return to the location of parasitized host pupae, and (5) how do males sense the time of emergence of a prospective mate?

These intriguing questions are addressed in four research chapters of my thesis.

1.4. Overview of thesis chapters

My thesis is organized into six chapters, including this introductory chapter (Chapter 1), four research chapters (Chapters 2 - 5), and a concluding chapter (Chapter 6) summarizing the major findings and pointing out new directions for future research. My thesis follows an article-style format with the research chapters based on publications in refereed journals. Each research chapter is presented in the style and format prescribed by the journal that has published the manuscript and comprises an abstract, introduction, materials and methods, results, discussion, acknowledgments and a reference list. Figures and tables are at the end of each chapter. In the following section, I briefly outline the content of each research chapter.

In Chapter 2, I demonstrate that female *P. disparis* (chemically) mark host pupae and distinguish between parasitized and unparasitized pupae at early stages of parasitism. I show that males too distinguish between host pupae that were parasitized and those that were not, likely in response to the marker pheromone deposited by the ovipositing female. Aging parasitized host pupae remain attractive to males throughout DePa's development.

In Chapter 3, I investigate the acoustic cues that males exploit to detect the presence, and track the developmental progress, of a future mate inside a host pupal case. I gently stimulate host pupae with a paintbrush on days 1–23 post parasitism and analyze responses of DePas with airborne sound and laser Doppler vibrometer recordings. The results show that the parameters of sound and vibratory cues (amplitude, dominant frequency, upper limit of frequency band) change significantly over time and thus could 'inform' a visiting adult male about the stage of development of DePa. The visiting males also produce sound and vibratory cues that may inform DePa about their presence.

In Chapter 4, I test the hypothesis that male *P. disparis* identify, memorize, and revisit the location(s) of parasitized host pupae as a strategy to attain mates. In the field, we color-code males and observe their visits to parasitized host pupae on consecutive days. In the laboratory, we allow males to memorize both the location of corrugated-cardboard-cylinder "trees" holding parasitized host pupae and the micro-location of such pupae on these trees, and then assess the males' memory the following day. The combined results from field and laboratory studies indicate that male *P. disparis* learn the location of future mates and use spatial memory to integrate the information regarding the specific location of each parasitized host pupa. Finding a mate is a mandatory task for males to reproduce, whereas female *P. disparis* are haplodiploid and capable of reproducing without mating, which could explain why they do not seem to engage in active pheromonal signaling.

In Chapter 5, I test the hypothesis that the DePa inside the host pupal case produces a pheromone that attracts and arrests mate-seeking males, and that the pheromone is most effective during DePa's emergence from the host pupa. In laboratory experiments, I test the behavioral response of virgin males to experimentally opened host pupal cases containing a pupal DePa, or a recently eclosed parasitoid. The data indicate that the presence of DePas consistently arrests males, likely due to semiochemicals released during emergence, and that the arrestment cue emanates from oral fluid secreted by both female and male parasitoids while they chew their way out of a host pupal case.

In Chapter 6, I summarize significant findings and propose future research directions.

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1.6. Connecting Statement

As Dr. Paul Schaefer and Dr. Stephen Takács observed in the field, males of the parasitoid wasp *Pimpla disparis* aggregate on parasitized pupae of the gypsy moth, *Lymantria dispar*, prior to the emergence of a potential mate. The underlying mechanisms of this behavior were not known. Males may aggregate in response to (*i*) signals or cues associated with DePa inside a host pupa or (*ii*) marker pheromone deposited by ovipositing female parasitoids. In Chapter 2, I tested the hypothesis that ovipositing female *P. disparis* deposit marker pheromone that allows females and males to recognize parasitized host pupae.

Chapter 2.

Early detection of prospective mates by males of the parasitoid wasp *Pimpla disparis* Viereck (Hymenoptera: Ichneumonidae)[1]

2.1. Abstract

In some insect species, the presence of a mate at the time of eclosion appears to facilitate rapid mating, with positive fitness consequences for one or both mates. Field observations that males of the hymenopteran parasitoid *Pimpla disparis* Viereck aggregated on a gypsy moth, *Lymantria dispar* (L), host pupa before the emergence of a female led us to hypothesize that these males responded to chemical cues associated with parasitized host pupae. Results of laboratory experiments with wax moth, *Galleria mellonella* (L), host pupae suggest that female *P. disparis* chemically mark the host pupae they have parasitized and that males discern between such pupae and those not parasitized. As males continue to recognize parasitized host pupae throughout the development of the parasitoid, they could exploit not only the females' marker pheromone but possibly also semiochemical, visual or vibratory cues from the developing parasitoid inside the host pupa, the decaying host, or both. Irrespective, these cues could help males locate parasitized host pupae and time the emergence of a prospective mate.

[1] Danci, A., Inducil, C., Schaefer, P.W., and Gries, G. 2011. Environmental Entomology, 40(2): 405-411. http://dx.doi.org/10.1603/EN10237

2.2. Introduction

Reproductive success of insects typically relies on an effective strategy to attract or locate mates. Generally, males search for females because a male's fitness increases with each female he inseminates, whereas a female's entire complement of eggs can usually be fertilized by sperm from a single or few males (Thornhill and Alcock 1983).

The earliest possible detection of a prospective mate (in short "early mate detection") is a strategy that helps ensure the presence of potential mates at the time of their eclosion and facilitates rapid mating. This phenomenon has been documented in species of various taxa. For examples, larvae of the codling moth, *Cydia pomonella* (L), cocoon in aggregations from which protandrous males eclose and respond to sex pheromone disseminating from mature female pupae even before adult females eclose (Duthie et al. 2003; and references cited therein). Adult males of *Heliconius* spp. butterflies search for female pupae, inspect them regularly, and compete for positions on pupae and for access to a female as she ecloses (Deinert et al. 1994). Similarly unusual strategies have been reported in some hymenopteran parasitoids. Male *Nasonia vitripennis* (Walker) (Fam. Pteromalidae) discern between parasitized and non-parasitized *Calliphora* host puparia, compete for positions on them, and await the emergence of females (King et al. 1969). Males of the larval parasitoid *Goniozus galliola* Fouts (Fam. Bethylidae) chew their way into the cocoon of a female and copulate with her even before she emerges (Gordh 1976). Males of *Lariophagus distinguendus* (Forster) (Fam. Pteromalidae) strongly compete for access to monandrous females. They are attracted to infested grain and await the female's emergence, but might be distracted by preemergent males that mimic the female's pheromone (Steiner et al. 2005). In contrast, males of *Spalangia endius* Walker (Fam. Pteromalidae) apparently cannot discern between hosts containing a preemergent female or male, but can differentiate between hosts from which a female or a male had recently emerged (King 2006). Finally, males of *Megarhyssa* spp.(Fam. Ichneumonidae), parasitoid wasps that parasitize wood-boring horntail larvae in dead trees, show a high fidelity to particular host trees, patrol them daily, and form conspicuous aggregations on the bark of those trees where new adult wasp are about to emerge (Matthews et al. 1979, Crankshaw and Matthews 1981).

Males of the ichneumonid wasp *Pimpla disparis* also appear to engage in an early mate detection strategy. This solitary, polyphagous parasitoid wasp parasitizes moth pupae, including gypsy moth, *Lymantria dispar* (L.) (Schaefer et al. 1989). Field observations revealed that male *P. disparis* aggregate on parasitized gypsy moth pupae before emergence of a potential mate.

The males' attraction to a host pupa at this late stage of parasitism suggests that they respond to a signal derived from the developing parasitoid inside the host pupa, the decaying host, or both.

During their search for parasitized host pupae, males may encounter pupae at all stages of parasitism. In early stages, the signal would be more likely derived from the ovipositing female than the newly hatched parasitoid larva. Many female parasitoids mark the host after oviposition, thus deterring other females from attacking the same host (Van Lenteren 1981, Hofsvang 1990, Anderson 2002). Such marker pheromones may be deposited inside the host (Bai and Mackauer 1990), on its integument (McBrien and Mackauer 1991), or nearby (Van Lenteren 1981).

If female *P. disparis* were to pheromone mark parasitized host pupae to deter superparasitism, conspecific males could exploit the pheromone to find parasitized host pupae, and thus prospective mates. However, if the pheromone were to persist for only 3 d, as shown for several parasitoid wasps (Waage and Greathead 1986), then only those males that detect a host pupa within 3 d of parasitism would be able to exploit the pheromone as a cue for parasitized host pupae. This seems inefficient for a mate-location strategy. Thus, we predict that parasitism of host pupae remains apparent throughout the development of the parasitoid.

Male *P. disparis* would benefit from a marker signal that reveals not only whether a host is parasitized but also the mating status of the female that parasitized it. Females are haplodiploid and can control the sex of their offspring. While virgin females produce only sons, mated females can produce sons or daughters. By fertilizing an egg, they produce a daughter and by not fertilizing it they produce a son. If females were to produce a marker pheromone that serves as a persistent attractant for their daughters'

future mate, then one would expect mated females to mark host pupae containing a daughter differently than host pupae containing a son.

We tested the hypotheses that (1) female *P. disparis* pheromone mark host pupae and discern between parasitized and unparasitized pupae, (2) males exploit the pheromone as a cue for both the presence and sex of a developing parasitoid (future mate) inside host pupae, and (3) parasitism of host pupae remains apparent throughout the development of the parasitoid.

2.3. Materials and methods

2.3.1. Experimental insects

Pimpla disparis were field collected in northeast Maryland in the summer of 2002 to start a laboratory colony which was reared on pupae of laboratory host wax moth, *Galleria mellonella* (L.). Pupae were exposed for 24-72 h to female *P. disparis,* isolated in Petri dishes and kept at 20-25º C, 40-60% RH, and a photoperiod of 16:8 (L:D) h. Emergent parasitoids were transferred to mesh cages (46 × 46 × 46 cm) and provisioned with cotton wicks (1 × 5 cm; Richmond Dental, Charlotte, North Carolina) imbued in water and honey *ad libitum*. Those to be tested in bioassays were isolated according to age and sex (based on the presence of an ovipositor in females) and kept in separate cages. Voucher specimens of both species have been deposited in the museum of the Department of Biological Sciences at Simon Fraser University, BC, Canada.

2.3.2. General experimental design

Two-choice experiments 1-10 employed a Plexiglas cage (42 × 32 × 32 cm) fitted with two brown corrugated cardboards (Shippers Supply Inc., Delta, BC) (each 15.5 × 15.5 cm) as surrogate tree trunks, each holding one to three host pupae secured by a clear elastic cord (1-mm diameter, stretch magic bead and jewelry cord, Pepperell Crafts, Pepperell, MA) (Fig. 2.1). One day before bioassays, randomly assigned treatment boards were exposed to parasitism of the pupa by a virgin or mated female. Control boards were kept under the same conditions but were not exposed to insects.

Unless stated otherwise, in each bioassay replicate one 5- to 7-d-old female or two 5- to 7-d-old males were introduced into the cage and their behavior, including selection of oviposition sites, number of visits, and time spent on a pupa or the cardboard holding it, was recorded for 2 h (experiments 1-4) or 10 min (experiments 5-11).

Experiments 1-4: Do mated or virgin females discriminate between parasitized and unparasitized host pupae when selecting oviposition sites?

Experiments 1-4 (*N* = 31-33 each) tested the choice of oviposition site by experienced females that had brief (<3 h) exposure to one or two unparasitized host pupae the day preceding the experiment. An oviposition was deemed successful when a female engaged in intense searching, rigorously antennated a pupa, bent her abdomen ventrally, and inserted and withdrew her ovipositor within a minute. Parasitism (defined here as complete development of a parasitoid wasp inside the host pupa) was strongly correlated with such a short oviposition time, whereas prolonged oviposition typically resulted in physical damage of the host pupa and desiccation of the developing parasitoid. Experiments tested whether virgin or mated females select as oviposition site a pupa that was either not parasitized or parasitized by a virgin female (experiments 1-2) or by a mated female (experiments 3-4). In each replicate, one female was released into the cage, and her choice of oviposition site and her behavior was recorded.

Experiments 5-7: Do males discern between parasitized and unparasitized host pupae?

Experiments 5 and 6 (*N* = 10 each) tested whether males spent more time on a pupa or on the cardboard holding it, that was either not parasitized or parasitized by a virgin female (experiments 5) or by a mated female (experiments 6). Experiment 7 (*N* = 10) tested whether males spent more time on a pupa (and the cardboard holding it) that was parasitized either by a virgin or by a mated female. In each replicate, two males were released into the cage and the time they spent on boards and pupae was recorded. Two males, instead of one, were tested to enhance the probability of data collection.

Experiments 8 and 9: Do males discern between host pupae housing a developing male or female parasitoid?

Experiment 8 (*N* = 38) tested one pupa that was parasitized by a mated female and contained either a female or male parasitoid versus a pupa that was parasitized by a virgin female and contained a male parasitoid. Experiment 9 (*N* = 72) tested two pupae 1 d postparasitism that were parasitized by a mated female and contained either a developing female or male parasitoid. For each replicate, two males were released into the cage and the time they spent on a pupa, was recorded. After each replicate, pupae were isolated to determine the sex of the emergent parasitoid.

Experiment 10: Do males pheromone mark parasitized host pupae?

In experiment 10 (*N*=10), test stimuli consisted of two 1-d-old pupae each parasitized by a virgin female. The treatment pupa, but not the control pupa, had been exposed to virgin males for 1 h before each test, allowing them to contact the pupa and potentially deposit pheromone. For each bioassay replicate, two virgin males were released into the cage, and their number of visits and time on a pupa, and on the board holding it, were recorded.

Experiment 11: Are males capable of recognizing parasitized host pupae throughout parasitoid development?

Cohorts of 20 host pupae each were parasitized by mated females within the same day. At days 3, 5, 7, 10, 13, 15, 19, and 20 postparasitism, one pupa of a cohort was selected and bioassayed versus a host pupa only one day postparasitism. In each bioassay, two virgin males were released into the cage and their number of visits and time spent on each of the two pupae were recorded. Each pair of pupae was bioassayed in 2-3 replicates, each replicate testing the response of different males. Thereafter, the pupae were isolated to determine the sex of the emergent parasitoid. The total number of replicates that tested the response of males to paired pupae one day postparasitism versus either 3, 5, 7, 10, 13, 15, 19 or 20 d postparasitism was 9, 10, 10, 10, 9, 12, 7, and 4, respectively.

2.3.3. Statistical analyses

In two-choice experiments 1-4, the number of females responding to test stimuli were analysed with the χ^2 goodness-of-fit test. In two-choice experiments 5-10, the number of visits by males, and the time they spent on pupae and on cardboard, were analyzed with a Wilcoxon paired-sample test. In experiment 11, the effects of time postparasitism of host pupae on both the males' time spent on pupae and their number of visits to pupae were analyzed by analysis of variance (ANOVA) applying a linear regression to data points, each point describing the difference in response to paired treatment and control stimuli. All data were analyzed with Statistical Software JMP 7.0.2 (SAS Institute 2007), with $\alpha = 0.05$.

2.4. Results

In experiments 1 and 2 (Fig. 2.2), virgin and mated females selected as an oviposition site unparasitized host pupae more often than host pupae parasitized by a virgin female (Experiment 1: $\chi^2 = 4.1724$, $P = 0.0411$; Experiment 2: $\chi^2 = 5.7619$, $P = 0.0164$; Fig. 2.2). In experiments 3 and 4, both virgin and mated females failed to select as an oviposition site unparasitized host pupae more often than host pupae parasitized by a mated female (Experiment 3: $\chi^2 = 2.4615$, $P = 0.1167$; Experiment 4: $\chi^2 = 2.2857$, $P = 0.1306$; Fig. 2.2).

In experiments 5 and 6, males spent more time on a pupa parasitized by a virgin or a mated female than on unparasitized pupae (Experiment 5, left: $W = -22.5$, $P = 0.0039$; Experiment 6, left: $W = -22.5$, $P = 0.0039$; Fig. 2.3). Also, males spent more time on the cardboard holding a pupa parasitized by a virgin or a mated female than on the cardboard holding an unparasitized pupa (Experiment 5, right: $W = -27.5$, $P = 0.002$; Experiment 6, left: $W = -22.5$, $P = 0.0039$; Fig. 2.3). In experiment 7, when males were given a choice between a pupa parasitized by a virgin or by a mated female, they remained longer on the latter and longer on the cardboard holding it (time on pupa: $W = 21.5$, $P = 0.0078$; time on cardboard: $W = 22.5$, $P = 0.0215$; Fig. 2.3).

In experiment 8, 21 paired host pupae contained a male and no parasitoid (failed oviposition by mated female), 10 pairs contained a male and no parasitoid (failed oviposition by virgin female), and seven pairs contained a female and no parasitoid (failed oviposition by virgin female) (Fig. 2.4). In these seven pairs, males spent more time on a host pupa with a developing female than on a pupa with failed oviposition (W = -10.5, P = 0.0313; Fig. 2.4). There was not a single pair of pupae that contained a female and a male parasitoid. As this also applied to the 72 pairs of pupae in experiment 9, we could not analyze whether males discern between host pupae housing a developing male or female parasitoid.

In experiment 10 (Fig. 2.5) prior contact of parasitized pupae by males had no effect on the number of visits, and the time spent, by other males on such pupae or on control pupae, or the cardboards holding these pupae (visits on pupae: W = -5, P = 0.125; visits on cardboard: W = 0.5, P = 0.9883; time spent on pupae: W = -7.5, P = 0.0625; time spent on cardboard: W = 2.5, P = 0.8457; Fig. 2.5).

In experiment 11, time postparasitism of host pupae had no significant effect on the males' time spent on pupae (F ratio = 2.4964, P = 0.1184) or their number of visits to pupae (F ratio = 1.8591, P = 0.1769) (Fig. 2.6).

2.5. Discussion

Our data indicate that 1) virgin and mated female *P. disparis* mark, possibly chemically, the host pupae they have parasitized and select as an oviposition site unparasitized host pupae more often than host pupae parasitized by a virgin female, 2) males discern between host pupae that were not parasitized and those that were parasitized by a virgin or a mated female, 3) males spent more time on or near pupae parasitized by a mated female than they spent on or near pupae parasitized by a virgin female, and 4) males continue to recognize parasitized host pupae throughout the development of the parasitoid.

Females of *P. disparis* did not exhibit any obvious host marking behavior, but they likely deploy a marking pheromone. This would explain why females reject

potential host pupae 1 d postparasitism by a virgin female (Fig. 2.2). Many parasitic wasps are known to pheromone mark the hosts they have parasitized (Nufio and Papaj 2001, Hoffmeister and Roitberg 2002). The marker pheromone helps avoid self and superparasitism that would otherwise result in competition among larval offspring over limited resources (Stelinski et al. 2007), with potentially fatal outcomes for some or all competitors. Avoidance of self or superparasitism is essential in solitary parasitoid wasps that exploit a host which can support the development of only one parasitoid larva (Rogers 1975). Female parasitoids mark their parasitized hosts by external and/or internal chemical signals. They detect an external marker during antennation of prospective hosts and an internal marker when they probe them with their ovipositor. In the ichneumonid wasp *Nemeritis canescens* (Gravenhorst) the external marker is so specific that a female can discern between hosts she herself or another female has marked (Hubbard et al. 1987). The internal marker could deter further oviposition directly, or it could paralyze the host and induce changes in hemolymph composition that are detectable by other females. Other chemical changes could stem from the hatching and developing parasitoid larva (Hofsvang 1990). The parasitoid *N. vitripennis* induces persistent changes in the host's hemolymph that deter superparasitism (King and Rafai 1970).

Males clearly discerned between host pupae that were parasitized and those that were not (Fig. 2.3; experiments 5 and 6), likely in response to a marker pheromone. They also spent more time on or near host pupae parasitized by a mated female than they spent on or near host pupae parasitized by a virgin female (Fig. 2.3; experiment 7), suggesting that mated females deposit more or different marker pheromone than do virgin females. If so, the "intended" receivers of the females' marker signal are not just conspecific females. Females would benefit from a signal that informs them about parasitism of host pupae, but not from a signal that conveys the mating status of the signaler that parasitized the host pupa. Males, in contrast, would benefit from a marker signal that reveals the mating status of the female. Unlike virgin females, mated females can produce daughter wasps that could become prospective mates for males, if males were to revisit parasitized host pupae, track the development of daughter wasps, and time their emergence. This seems to be so, based on preliminary field observations.

If females indeed produce marker pheromones that serve as long-term attractants for their daughters' future mate, then one might argue that mated females may mark host pupae containing a daughter differently than a host pupa containing a son. We attempted to test this in experiment 9 by offering males a choice between two host pupae, each parasitized by a mated female, expecting males to respond more strongly to a pupa containing a female parasitoid than to pupa containing a male parasitoid, with the sex of parasitoids determined at the time of their emergence. However, in the 60 replicates we tested in experiment 9, not a single pupal pair contained a female and a male parasitoid, which would have allowed us to gauge the males' potential preference.

Males may or may not find host pupae immediately following parasitism. Similar number of visits and amount of time spent by males on pupae 1 d and 3-20 d postparasitism (Fig. 2.6) indicate that aging host pupae remain attractive to males. This implies that the female marker pheromone is persistent or that the developing parasitoid contributes over time to the attractiveness of host pupae. Other parasitoid wasps deploy marker pheromones that persist from a few hours up to 7 d (Bosque and Rabinovich 1979, Sugimoto et al. 1986, Hofsvang 1990, Stelinski et al. 2007). As the entire development of *P. disparis* in host pupae takes circa 3 wk, a marker pheromone would have to persist for that long. Yet, no such persistent marker pheromone has been reported. Instead, the females' pheromone may persist for some time but overlap with the onset of attractive and arrestant cues derived from the developing parasitoid inside host pupae. In *N. vitripennis*, males appear to respond to a short-lived marker pheromone deployed by females, but continue to respond to parasitized puparia of all ages, most strongly to those that have been parasitized the longest or from which the parasitoid has emerged (King 2006), likely because of parasitoid pheromone still adhering to host puparia.

If male *P. disparis* were to respond to semiochemical signals or cues originating from developing parasitoids inside host pupae, they would require repeated visits to host pupae to track the development of a prospective mate and time her emergence. Repeated visits, in turn, would necessitate that males memorize the location of parasitized pupae or that they pheromone mark them for ease of relocation. However,

equal number of visits by males, and equal amount of time they spent on host pupae with or without prior male contact (Fig. 2.5), indicate that males do not pheromone mark parasitized host pupae, and apparently rely on memory to relocate them. Such memorization skills have been demonstrated for females of the solitary parasitoid wasp *Hyposoter horticola* (Gravenhorst) that use visual landmarks to learn the position of multiple potential hosts, and up to several weeks monitor their development until they become susceptible to parasitism (van Nouhuys and Kaartinen, 2008). That male *P. disparis* do not pheromone mark parasitized host pupae may be adaptive in that such pheromone may attract competing males or other parasitoids to host pupae.

It will now be intriguing to identify the semiochemical signals or cues that allow female and male *P. disparis* to recognize parasitized host pupae, and to determine the time period within which they are effective.

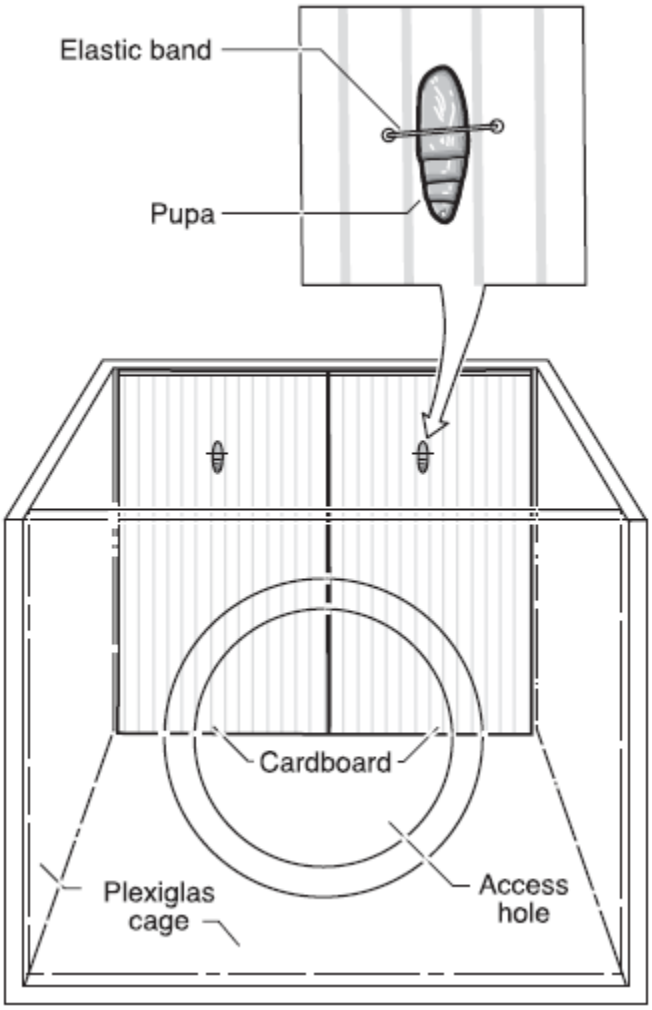

Figure 2.1. Design of two-choice experiments 1-11.
Note: 1) Plexiglas cage (42 × 32 × 32 cm) with access hole, 2) two separate pieces of brown corrugated cardboard (each 15.5 × 15.5 cm), 3) *G. mellonella* host pupae, and 4) clear elastic cord to secure pupa to cardboard.

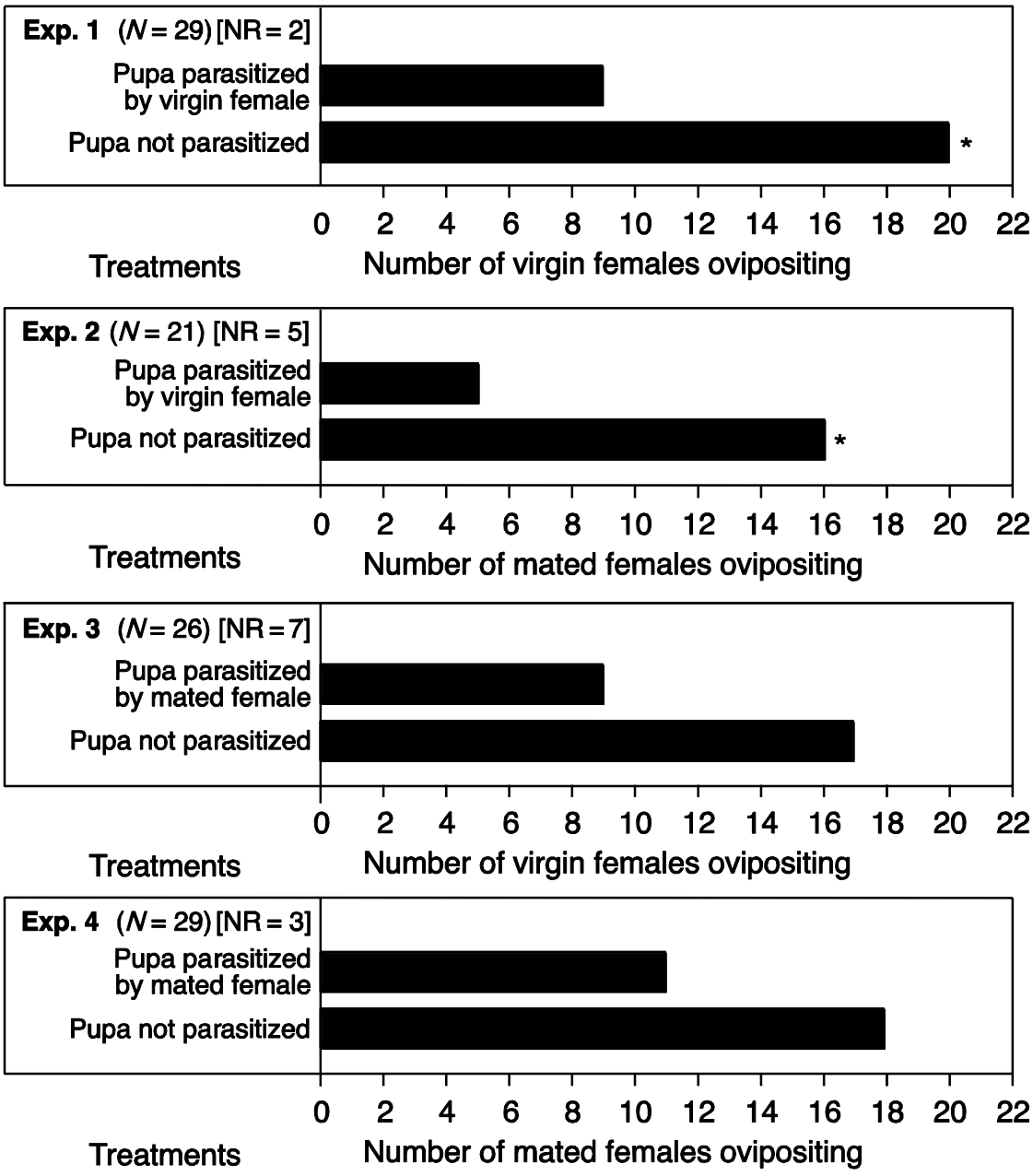

Figure 2.2. Number of virgin or mated female *P. disparis* selecting for oviposition *G. mellonella* host pupae that were either parasitized by virgin females or not parasitized (Experiments 1, 2), or parasitized by mated females or not parasitized (Experiments 3, 4).

Note: In each experiment, an asterisk (*) indicates a significant preference for a particular stimulus; $\chi 2$ test; *P < 0.05. Non responding females are reported in square brackets.

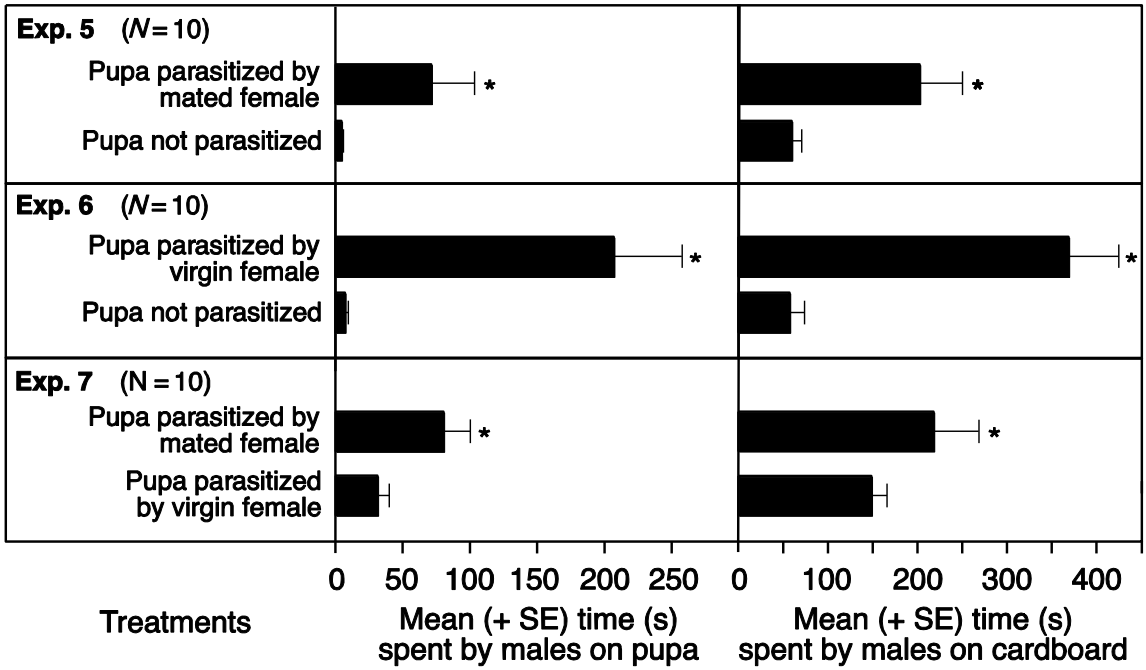

Figure 2.3. Mean (+ SE) combined time (sec) spent by two male *P. disparis* on *G. mellonella* host pupae (central column), or the cardboard carrying them (right column), that were not parasitized or parasitized by mated females (Experiment 5), not parasitized or parasitized by virgin females (Experiment 6), or parasitized by a virgin or a mated female (Experiment 7).

Note: For each pair of bars, an asterisk (*) indicates a significant preference for a particular stimulus; Wilcoxon paired-sample test; $P < 0.05$. There were no nonresponding insects in experiments 5-7.

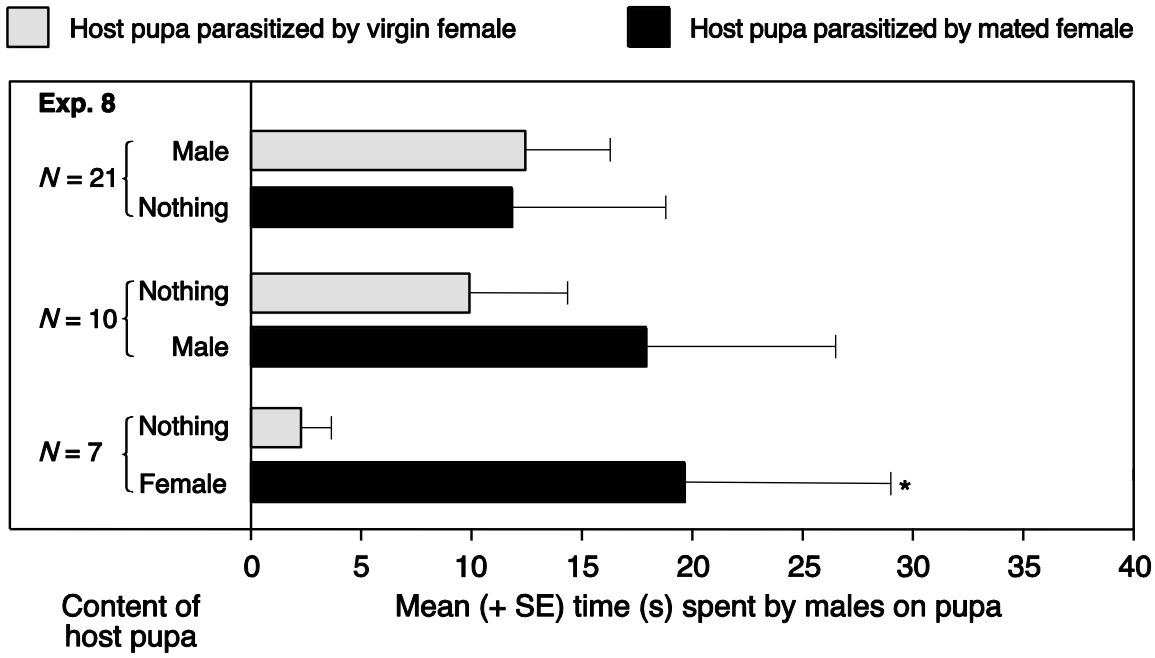

Figure 2.4. Mean (+ SE) combined time (sec) spent by two male *P. disparis* on *G. mellonella* host pupae that were parasitized by a virgin or a mated female and that contained a developing male or female parasitoid or neither.

Note: For each pair of bars, an asterisk (*) indicates a significant preference for a particular stimulus; Wilcoxon paired-sample test; $P < 0.05$.

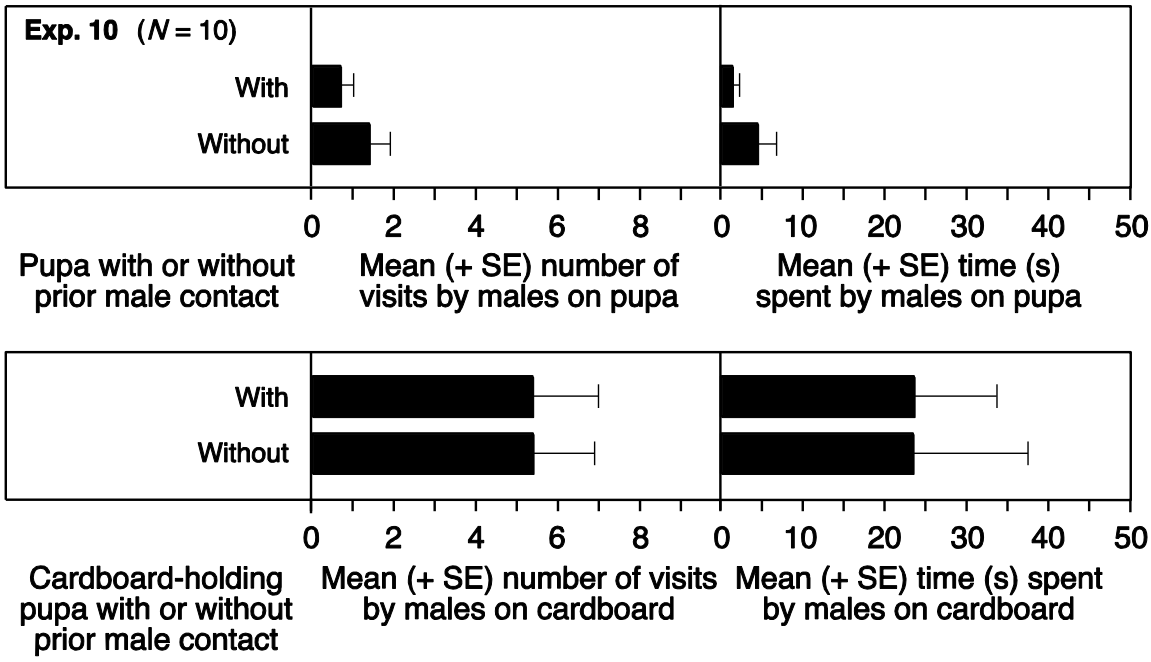

Figure 2.5. Mean (+SE) combined number of visits (central column), and mean combined time spent (right column) by two male *P. disparis* on parasitized *G. mellonella* host pupae, or the cardboard holding them, with or without prior contact by other males.

Note: For each pair of bars, there was no preference for a test stimulus; Wilcoxon paired-sample test; $P > 0.05$.

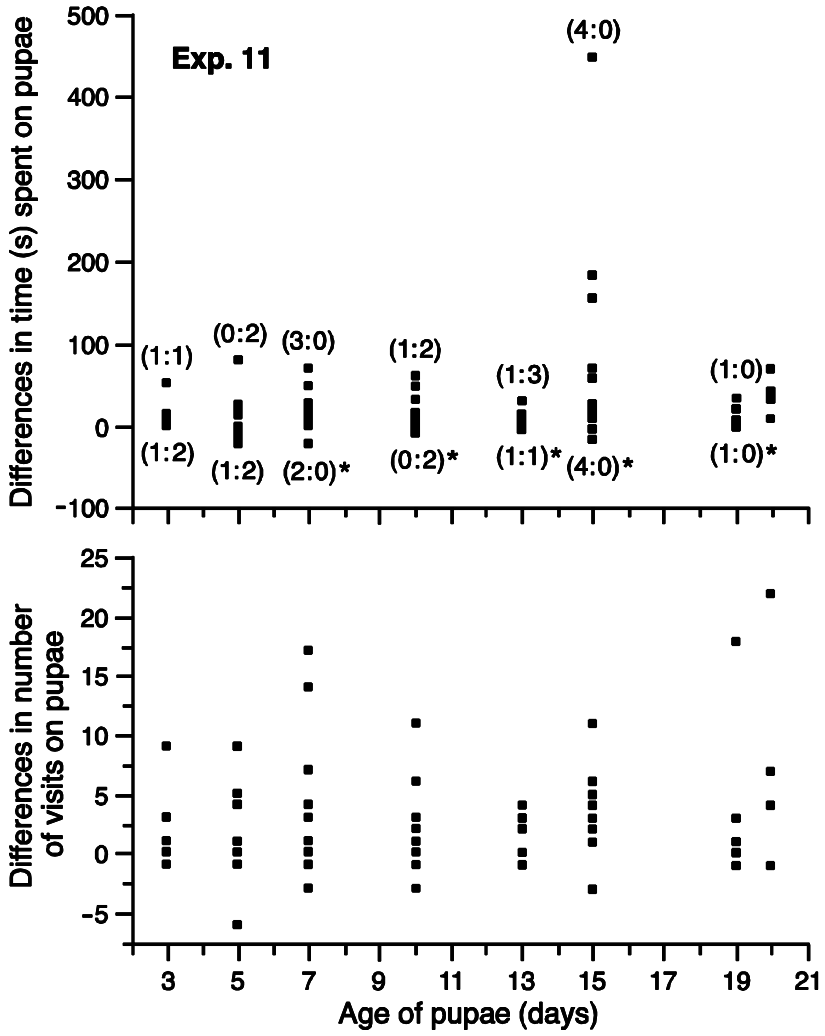

Figure 2.6. Comparison of combined time spent (parameter 1, top) and combined number of visits (parameter 2, bottom) by two male *P. disparis* on *G. mellonella* host pupae that were 1 d or 3–20 d postparasitism. There was no significant effect of time postparasitism of host pupae on either parameter; ANOVA, P > 0.05.

Note: 1) positive values indicate a preference for pupae 3–20 d postparasitism; 2) parentheses indicate the sex ratio (male:female) of parasitoids emerging from bioassay host pupae 3–20 d postparasitism (upper row) and 1 d postparasitism (lower row); 3) parentheses with an asterisk (*) indicate one host pupa in that group without a parasitoid emerging from it.

2.6. Acknowledgements

We thank Lara Monk for assistance with experiments, Bob Birtch for graphical illustrations, Sharon Hope for word processing, Ian Bercovitz for statistical advice, and one anonymous reviewer for constructive comments. This research was supported, in part, by a Natural Sciences and Engineering Research Council of Canada (NSERC) – Canada Graduate Scholarship to A. D., and by an NSERC Discovery Grant and NSERC – Industrial Research Chair to G.G., with Contech Enterprises, SC Johnson Canada, and Global Forest Science as industrial sponsors. Experimental insects were maintained in SFU's Global Forest Quarantine Facility, construction of which was completed through financial support from Global Forest Science (GF-18-2000-SFU-12).

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2.8. Connecting Statement

In Chapter 2, I brought evidence that female *P. disparis* mark the host pupae they have parasitized, presumably chemically, and that males exploit the same cues to discriminate between such pupae and those not parasitized. I also obtained behavioral evidence that males continue to recognize parasitized host pupae throughout DePa's development. Because the female marker is not likely to persist throughout DePa's development, it was conceivable that males rely on mechanical cues associated with movement of DePa. Moreover, I noticed that DePa can spin inside the pupa, producing both vibrations of the host pupa and audible sounds. Thus, in Chapter 3, I tested the hypothesis that males exploit DePa-derived acoustic and vibratory cues to detect the presence and track the developmental progress of DePa (a future mate) inside a host pupal case.

Chapter 3.

Mechanism of mate detection in parasitoid wasps: sound and vibratory cues change with developmental progress of future mates inside host pupal cases[2]

3.1. Abstract

Insects including parasitoid wasps use sound and vibratory signals in the context of sexual communication, mate recognition, courtship and mating. Males of the the parasitoid wasp *Pimpla disparis* Viereck (Hymenoptera: Ichneumonidae) detect insect host pupae parasitized by a conspecific female, learn their location, visit them repeatedly and remain on or near them when the prospective mate nears emergence. In the present study, the sound and vibratory cues that males exploit to detect the presence and track the developmental progress of a future mate inside a host pupal case are investigated. Responses are acquired from developing parasitoids (Depas) by airborne sound and laser Doppler vibrometer recordings, after gently stimulating each of 20 wax moth host pupae with a paintbrush on days 1-23 post parasitism. Sound and vibratory cues produced by DePa are detectable from day 7 onward and relate mostly to its spinning movements within the pupa. Parameters of sound and vibratory cues (amplitude, dominant frequency, upper limit of frequency band) change significantly over time and thus could 'inform' a visiting adult male about the stage of development of DePa. Adult males antennating a parasitized pupa, and flying around it also induce vibrations, which in turn may inform DePa about the presence of a male. There is no

[2] Danci, A., Inducil, C., Takács, S., Schaefer, P. W., and Gries, G. 2014. Physiological Entomology, 39(4): 292-303. DOI: 10.1111/phen.12075

experimental evidence for true signaling and rapid information exchange between DePa and adult males.

Delaying reply signals may help DePa avoid attacks by illicit receivers of such signals, including female (hyper)parasitoids and invertebrate predators.

3.2. Introduction

Vibratory cues or communication signals are commonly used by insects and arachnids (Hill & Shadley, 2001; Greenfield, 2002; Cŏkl & Virant-Doberlet, 2003; Drosopoulos & Claridge, 2006) and play roles in various contexts, including predator–prey interactions, resource competition, brood care, social interactions and sexual communication (Cocroft, 2001; Barth, 2002; Cocroft & Rodriguez, 2005; Casas & Magal, 2006; Cocroft *et al*., 2006; Hill, 2009; Vibert *et al.*, 2014).

Vibratory signals during sexual communication (e.g., mate location, mate recognition, courtship, mating) are known to occur in diverse insect taxa (Čokl & Virant-Doberlet, 2003; Virant-Doberlet & Čokl, 2004). Insects that use air-borne sound signals during courtship may also use the vibratory components of these signals as an additional channel. For example, substrate vibrations of a singing male bush cricket (Tettigoniidae) help to locate the signaller (Kalmring *et al.*, 1997). Some species of true bugs (Hemiptera) and of crickets and katydids (Orthoptera) emit both acoustic and vibratory signals (Gogala, 1985; Stölting *et al.*, 2002), each with a distinct frequency content and temporal pattern, produced by a specific mechanism, and serving in a particular context. Most insects communicating with any modality of mechanical signals use substrate (not air) as the optimal signal transmission channel (Cocroft & Rodriguez, 2005). For example, females of the Southern green stink bug, *Nezara viridula* (L.) produce plant vibrations that males exploit to locate mates (Čokl *et al.*, 1999; Virant-Doberlet *et al*., 2006).

In parasitoids, pheromonal communication prevails during mate finding, courtship and mating behavior (Vinson, 1972; Eller *et al.*, 1984; Quicke, 1997; Ayasse *et al.*, 2001). Once males have located a female, they court her using pheromonal and

acoustic signals (van den Assem, 1986). Male courtship songs can also cause substrate vibrations (Field & Keller, 1993; Vet *et al.*, 2002) and may induce receptivity in females (van den Assem & Putter, 1980).

The astounding ability of parasitoids to interpret vibratory cues has been most intensely studied in the context of host-parasitoid interactions. Parasitoids could use the same sensory ability to locate prospective mates instead of hosts. Foraging female parasitoids exploit substrate-transmitted vibrations from concealed endophytic hosts to locate them (Vet & van Alphen, 1985; Meyhöfer *et al.*, 1997; Meyhöfer & Casas, 1999; Vet *et al.*, 2002). Females of the pupal parasitoid *Pimpla turionellae* (L.), for example, use vibratory sounding cues (echolocation in solid substrate) to locate their mainly endophytic hosts (Fischer *et al.*, 2001). Vibratory sounding (Henaut & Guerdoux, 1982; Henaut, 1990; Wäckers *et al.*, 1998; Broad & Quicke, 2000) is a type of information gathering during which a female parasitoid induces substrate vibrations and then "reads" the resonance of the reverberating substrate for its relative solidity, thus allowing her to scan the substrate for hidden hosts. Similar to echolocation, vibratory sounding is considered as a form of 'self communication' in which the parasitoid is concurrently the sender and receiver, and locates a host independent of its stimuli (Vet *et al.*, 2002). This is effective for pupal parasitoids, because host pupae neither feed nor move and are often well concealed (Vet *et al.*, 1995). Male parasitoids searching for a prospective mate in host pupae could possibly employ the same tactic.

Parasitoids are capable of homing in on sounds or substrate vibrations produced by a potential host (Meyhöfer & Casas, 1999), and exploit these vibrations to determine a host's current activity and stage of development (Meyhöfer *et al.*, 1997). Females of the parasitoid *Tetrastichus planipennisi* Yang, for example, respond to movement and feeding vibrations associated with larvae of the emerald ash borer *Agrilus planipennis* Fairmaire and parasitize only those larvae that are actively feeding (Ulyshen *et al.*, 2010, 2011). Conceivably, movement and feeding vibrations of ash borer larvae may help *T. planipennisi* females not only locate hosts, but also assess their size and quality. Yet, even though large and small larvae produce vibrations of different amplitude, they are not correlated with preferential parasitism by *T. planipennisi* females (Ulyshen *et al.*,

2011). Still, male parasitoids searching for a prospective mate inside host pupae could possibly assess her stage of development based on the amplitude of her vibrations.

There is also counterespionage in that prospective host larvae detect and respond to vibrations from host-seeking parasitoids (Casas, 1989; Bacher *et al.*, 1994, 1996; Meyhöfer *et al.*, 1997; Meyhöfer & Casas, 1999; Low, 2012). Analogously, a prospective mate inside a host pupa could detect the presence of an adult male and signal her presence. However, the very same signals could be exploited by hyperparasitoids, providing a strong selective force on parasitoid larvae or pupae to keep quiet and inconspicuous.

Typically, parasitoid females rather than males respond to cues from host pupae (Meyhöfer & Casas, 1999; Broad & Quicke, 2000; Fischer *et al.*, 2001; Wang & Yang, 2008), although males of *Pimpla disparis* are an exception to this rule. The 'response motivation' of males, however, is mate location rather than oviposition. *Pimpla disparis* males memorize the location of parasitized host pupae (Danci *et al.*, 2013), visit them frequently and arrest on them when a prospective mate nears emergence, responding to semiochemicals associated with the emergence process (Hrabar *et al.*, 2012). These observations imply that males are capable of tracking the stage of a developing parasitoid (DePa) inside the host pupa. That males can recognize parasitized and unparasitized host pupae at early stages of parasitism (Danci *et al.*, 2011) appears to be based primarily on semiochemicals deposited by females during oviposition in host pupae. That males physically shake a parasitized host pupa (S. Takács, unpublished observation) suggests that they may also detect vibratory and/or acoustic cues associated with DePa. Video footage (see Supporting information, Video S1) of a 14-day-old DePa inside a partially opened host pupa reveals spinning movements of DePa that generate vibrations and sound that could be 'read' by a visiting male. Conceivably, the visiting male may even 'communicate' with DePa and obtain information encoded in DePa's reply signals.

To understand mate location and detection behavior of *P. disparis* further, the present study investigates whether DePa of *P. disparis* produce sound and vibrations inside host pupae that correlate with their developmental stage and thus reveals its time

of emergence to visiting males. The study also investigates whether visiting males, in turn, produce cues to inform DePa on their presence.

3.3. Materials and methods

3.3.1. Experimental insects

Parasitoid wasps *P. disparis* were field collected in northeast Maryland and reared on pupae of the wax moth *Galleria mellonella* (L.) in the Global Forest Quarantine Facility at Simon Fraser University (Danci *et al.*, 2011). Adult wasps were kept in mesh cages (46 × 46 × 46 cm^3) and provisioned them with water and moist honey *ad libitum*. Wax moth pupae were exposed to female *P. disparis* for 24-36 h, and the parasitized pupae were placed in Petri dishes maintained under an LD 16 : 8 h photocycle at 20-25 °C and 40–60% relative humidity. Newly-eclosed males were transferred to Plexiglas cages (10 × 10 × 7 cm^3) until they were 5-7 days old and ready to be tested in experiments.

3.3.2. Parasitism of wax moth pupae

A cohort of 50 wax moth pupae was exposed to female *P. disparis* for parasitism on the same day under the same conditions. Pupae were secured ventral-side up with elastic thread (diameter 0.5 mm) on pieces (5 × 3.5 cm^2) of corrugated cardboard (Shippers Supply, Canada). They were exposed to 1–2-week-old mated female *P. disparis* and the pupae were removed as soon as a female had oviposited. Of 50 parasitized host pupae, 20 were randomly selected for recordings and the remainder were allocated for dissections (one pupa every day) to track the developmental stage of parasitoids.

3.3.3. Vibration and sound recordings of parasitized host pupae

Laser recordings

Vibrations associated with DePa were recorded on the first day post parasitism until the adult parasitoid emerged. Every day, parasitized pupae were randomly selected from the cohort of 20 (see above), the cardboard holding a pupa was mounted on a Plexiglas holder (Fig. 3.1a) and the beam of a laser Doppler vibrometer (LDV; Polytec OFV-2500 with OFV-534 sensor head; Polytec International, Tustin, California) was pointed on the ventrum of the pupa between the forewings just below the forelegs. To obtain standardized recordings the procedure was as follows: after an initial 10.92 s of data acquisition (control recording), the pupa was gently stimulated with a paintbrush mimicking antennation of a visiting male, and the potential 'response' vibrations from DePa were then recorded for two 10.92-s intervals. Control pupae that were not parasitized were subjected to the same protocol.

For data analyses, representative recordings were selected for: (i) day 9 (±1) with DePa males and females being in the late larval stage, (ii) day 12 (±1) with DePa males and females being in the early pupal stage, (iii) day 16 (±1) with DePa males being in the late pupal stage and DePa females in the middle pupal stage, and (iv) day 19 (±1) with DePa females being in the late pupal stage. Vibrations caused by DePa were recorded using Polytec Vibrometer software, (version 4.7) in the time domain, with a sampling frequency of 12 kHz. To be able to compare laser vibrometer recordings and sounds recordings (see below), laser vibrometer files were converted from wave to log format compatible with the Joint Time-Frequency Analyzer (JTFA) developed with Labview-Graphical Programming for Instrumentation, version 7.0 (National Instruments Corporation, Austin, Texas), and then amplitude, dominant frequency, upper-limit frequency band, and duration of each vibration were measured. Displacement was calculated by multiplying the peak-to-peak amplitude (top to bottom range of the largest waveform) by the sensitivity factor of the laser vibrometer (VD-6: 10 mm s^{-1} V^{-1}) specified in the parameter settings of each vibrometer recording.

Sound recordings

After recording the vibrations, sound recordings (0-24 kHz) were also obtained from each pupa (Fig. 3.1b) which was paintbrush-stimulated as described above. Recordings proceeded in a soundproof room, using an AKG CK 61-ULS condenser microphone (AKG Acoustics, U.S. Nashville, Tennessee). The signal-to-noise ratio was improved by pre-amplifying (SC-2040 amplifier; National Instruments Corporation) the sound prior to digitizing with a PCI-MIO-16XE-10 data acquisition card (National Instruments Corporation) in an Intel Pentium 2.54 GHz computer at a sampling rate of 44.150 kHz. Sound was recorded continuously for approximately 5 min, and recordings were analyzed for waveform, frequency and time-frequency sound intensity (sonogram) using JTFA. In any recording, the peak-to-peak amplitude of the dominant frequency was determined by locating the largest waveform in the waveform window of JTFA, which provides V as the only recording unit. To determine sound intensity level, we measured the peak-to-peak (top to bottom) range of the largest waveform and applied the conversion formula: $20 \times \log_{10}$ (peak-to-peak voltage).

3.3.4. Video and vibration recordings of parasitized host pupae visited by a *Pimpla disparis* male

To determine potential interaction and information exchange between a male *P. disparis* and DePa, vibrations (see above) were recorded when a male *P. disparis* contacted or antennated a host pupa 14-16 days post parasitism. The cardboard holding the pupa was mounted in a Plexiglas cage ($10 \times 10 \times 7$ cm^3) (Fig. 3.1c), the laser beam aimed through a hole (diameter 0.5 cm) in the cardboard onto the dorsal surface of the pupa, and then a 5–7-day-old virgin male was introduced into the cage (Fig. 3.1c). Any vibrations caused by the male or by DePa were recorded using Polytec Vibrometer software in the time domain at a sampling frequency of 12 kHz. Concurrent video recordings (Canon Powershot S5IS and Canon FF 100; Canon Inc., Japan) allowed the behavior of the male, or the developing parasitoid within the host pupa, to be correlated with vibratory cues or signals. Each replicate employed a different male or a parasitized host pupa. An unparasitized wax moth pupa, affixed to cardboard and mounted on one lateral side of the Plexiglas cage, served as a control stimulus for the virgin male.

3.3.5. Statistical analyses

Temporal and spectral data of laser and sound recordings [duration, time interval between events, displacement (mm), sound intensity level (dB), dominant frequency (Hz) and upper limit of the frequency band (Hz)] were subjected to analysis of variance (ANOVA) and an all pairs, Tukey's honestly significant difference multiple comparison test ($\alpha = 0.05$) using JMP (SAS Institute Inc., Cary, North Carolina). To determine whether the type of stimulus (paintbrush or visiting male) that induced DePa spinning (clockwise or counter-clockwise rotations about its longitudinal axis) affected vibratory or acoustic characteristics of spinnings, three spins of either stimulus type were randomly selected, and vibratory or acoustic parameters (mean displacement and mean upper limit of the frequency band) were compared. To standardize duration, only data of the first 0.5 s of each recording were considered, taking into account that short spins lasted 0.48 s on average. Changes in sound and vibration data associated with DePas were analyzed over the course of their growth and transformation using repeated-measures ANOVA. Parameter estimates were analyzed in JMP using the Fit model platform and the multivariate analysis of variance (Manova) technique, including in the model such effects as time and gender interaction.

3.4. Results

3.4.1. Development of DePas in host pupae

From 20 parasitized pupae subjected to repeated recordings, 11 male and six female parasitoids emerged; parasitoids failed to emerge from three apparently parasitized pupae. The total developmental time of females was 4-5 days longer than that of males (Fig. 3.2a).

After paintbrush stimulation, six male and four female DePa responded consistently over the course of the experiment, typically performing long spins that generated vibrations and sound. Because vibrations and sound were recorded in sequence, and DePas were more responsive after the first paintbrush stimulation for vibration recordings, more vibratory data were collected than sound data.

3.4.2. Analyses of vibration recordings

Waveform, frequency and time-frequency sound intensity (sonogram) of paintbrush-induced response vibrations are depicted in Fig. 3.3(a). The duration of these vibrations did not change significantly over the course of development for DePa males (MANOVA, F-test = 6.2322, $P = 0.0836$) or DePa females (MANOVA, F-test = 58.1418, $P = 0.1651$) but did change significantly for both data sets combined (MANOVA, F-test = 17.6575, $P = 0.0207$) (Fig. 3.3b). There was no time and gender interaction (MANOVA, F-test = 1.0921, $P = 0.4720$).

The amplitude of vibrations (measured as displacement) increased significantly over time for DePa males (MANOVA, F-test = 9.7743, $P < 0.0001$) and DePa females (MANOVA, F-test = 0.7927, $P < 0.0289$) (Fig. 3.3b). There was significant time and gender interaction (MANOVA, F-test = 0.3675, $P < 0.0129$), with females generating larger displacements than males.

The dominant frequency increased significantly over time for DePa females (MANOVA, F-test = 10.2079, $P < 0.0001$) but not for DePa males (MANOVA, F-test = 24.1082, $P = 0.0591$) (Fig. 3.3b). There was no significant time and gender interaction (MANOVA, F-test = 0.3751, $P = 0.2026$).

The upper limit of the frequency band decreased significantly over time for DePa males (MANOVA, F-test = 33.9187, $P < 0.0001$) and DePa females (F-test = 2.1316, $P = 0.0005$) (Fig. 3.3b). There was significant time and gender interaction (MANOVA, F-test = 0.3787, $P = 0.0113$).

3.4.3. Analyses of sound recordings

The waveform, frequency and time-frequency sound intensity (sonogram) of sounds from wax moth host pupae after stimulation using a paintbrush on day 18 post parasitism by a female *P. disparis* are depicted in Fig. 3.4(a).

Sound intensity level increased significantly over time for DePa males (MANOVA, F-test = 13.1212, $P < 0.0001$) and DePa females (MANOVA, F-test = 5.8255, $P < 0.0001$) (Fig. 3.4b).

There was significant time and gender interaction for sound intensity level (MANOVA, F-test = 0.1301, $P = 0.0001$).

The upper limit of the frequency band increased significantly over time for DePa females (MANOVA, F-test = 0.6861, $P = 0.0258$) but not DePa males (MANOVA, F-test = 0.1616, $P = 0.0783$) (Fig. 3.4b). The dominant frequency of sound increased significantly over time for DePa males (MANOVA, F-test = 4.1927, $P < 0.0001$) and DePa females (MANOVA, F-test = 50.3305, $P < 0.0001$) (Fig. 3.4b). There was no significant time and gender interaction for the upper limit of frequency band (MANOVA, F-test = 0.0422, $P = 0.3631$) and for the dominant frequency (MANOVA, F-test = 0.1263, $P = 0.0543$).

As expected, the change in spectral sound characteristics translates into markedly different sound produced by DePa over time. The supplemental sound files containing sound recordings from the same DePa on days 8, 14 and 18 post parasitism (Fig. 3.2b) reveal changes in the sound pitch as a result of an increase in dominant frequency.

3.4.4. DePa vibrations in the presence of males

Behavior of males

When on or near a parasitized host pupa, males antennated it with their antennal tips, brushed it with their most distal antennomeres, groomed, arrested (occasionally > 2 h) and buzzed their wings. Both the mean upper limit of the frequency band and the displacement of vibrations caused by antennation differed significantly between the six males that were tested (ANOVA, $P_1 = 0.0006$ and $P_2 < 0.0001$, respectively) (Table 3.1). Single-pulse vibrations of parasitized pupae caused by buzzing males had a mean duration of 0.25 $\pm$ 0.05 s, a mean displacement of 0.38 $\pm$ 0.04 mm and a mean upper limit of the frequency band of 2.7 $\pm$ 0.62 kHz.

Response of DePa to the behavior of males

In the presence of a male, DePas (16-day-old) engaged in short (0.417 ± 0.024 s) spins, less frequent long spins (> 1 s) (Fig. 3.5a) and stereotypic knocks to the host pupal case (Fig. 3.5b). These knocks were detectable only with the laser vibrometer and did not generate sounds audible to human hearing as DePa spins or male buzzes did.

The mean duration of short spins did not differ significantly among DePas (ANOVA, P_6 = 0.2092) (Table 3.1) but the mean upper limit of the frequency band (ANOVA, P_7 = 0.0017) (Table 3.1) and the mean displacement (ANOVA, P_8 = 0.0043) (Table 3.1) did. The mean displacement of long spins (ANOVA, P_9 = 0.5767) (Table 3.1) and the upper limit of the frequency band (ANOVA, P_{10} = 0.7161) (Table 3.1) did not differ significantly among DePas. Both the mean displacement and mean upper limit of the frequency band of long spins differed between DePas (ANOVA, P_{11} = 0.0302; P_{12} = 0.02) (Table 3.2) but differences could not be attributed to the type of stimulus (paintbrush or male) that induced the spins. Similarly, within the first 0.5 s of long spins that were paintbrush-induced and the first 0.5 s of short spins induced by a male, both the mean peak amplitude and mean upper limit of the frequency band differed between DePas (ANOVA, P_{13} = 0.0013, P_{14} = 0.0079) (Table 3.2) but differences again could not be attributed to the type of stimulus (paintbrush or male) that induced the spins.

3.5. Discussion

The data support the conclusion that: (i) sound and vibratory cues associated with DePa inside a host pupa change over time; (ii) these changes may inform an adult male during repeat visits about the developmental progress of a future mate; (iii) males themselves produce sound and vibratory cues that may inform DePa about the presence of a male; and (iv) true communication or rapid information exchange between DePa and adult males likely does not take place.

Although *P. disparis* males seem to respond primarily to semiochemical cues when they detect host pupae at early stages post parasitism (Danci *et al.*, 2011), they could exploit sound and vibratory cues associated with DePa over the course of its

growth, metamorphosis and maturation to adulthood. As early as 7 days post parasitism, DePa produces distinct sounds and vibrations when stimulated with a paintbrush. Over time, irrespective of gender, the displacement of DePa vibrations increases (Fig. 3.3b), whereas the upper limit of the frequency band decreases (Fig. 3.3b). Similarly, the intensity level and dominant frequency of sound increase (Fig. 3.4b), resulting in a noticeably higher sound pitch. All changes in sound and vibratory characteristics are related to DePa increasing in size, assuming different forms (larva, prepupa, pupa) and sclerotization of the integument (Fig. 3.2b). The quiescence of the DePa a few days before its emergence, as well as the semiochemicals associated with the emergence process (Hrabar *et al.*, 2012), could then become indicators of DePa's imminent emergence, thus arresting mate-seeking males.

The subtle differences in vibratory cues provided by male and female DePa (Figs. 3.3b and 3.4b) could conceivably allow mate-seeking adult males to discriminate between DePa's sex, although laboratory and field observations of adult males on parasitized host pupae suggest that males fail to do so. Moreover, chemical secretion associated with emerging male or female parasitoids equally arrest males (Hrabar *et al.*, 2012).

A prerequisite of communication between adult insects, or immature and mature insects in the present study, is that partners both 'talk and listen'. Thus, whether *P. disparis* males produce signals or cues that could be noticed by DePa was carefully studied. In general, there is ample evidence that courting male parasitoid wasps fan wings, resulting in low-amplitude sound and substrate vibrations that serve as communication signals for females (e.g., Leonard & Ringo, 1978; Van den Assem & Putters,1980; Sivinski & Webb, 1989; Field & Keller, 1993; Danci *et al.*, 2010). Indeed, these courtship vibrations are critical to mating success in the parasitoids *Cotesia marginiventris* (Cresson) (Joyce *et al.*, 2008) and *Cotesia rubecula* (Marshall) (Field & Keller, 1993). Males of *P. disparis* antennate parasitized host pupae, causing vibrations that are detectable by the laser vibrometer (Fig. 3.6a) and likely also by DePa. Males also fly around parasitized host pupae with buzzing sounds that generate vibrations of these pupae (Fig. 3.6b). DePa appears to sense these vibrations because, subsequent

to a ‘buzzing event’, it stops knocking (see below), only to resume this behavior 8 s later (Fig. 3.7).

In the presence of an adult male, or sometimes spontaneously, DePas produce spins (Fig. 3.5a) and knocks (Fig. 3.5b). Because these knocks are rather stereotypic, they are first mistaken for recording artefacts. However, inter-recording variation in displacement, the upper limit of the frequency band and the time interval between knocks lead to the conclusion that DePas indeed produce these knocks. They result in subtle vibrations of the host pupa that a visiting adult male may detect when the legs or antennae make physical contact with the pupa. Subgenual receptors in legs of female *P. turionellae* pupal parasitoids exploit the resonance of self-produced vibrations as a means to locate concealed hosts (Wäckers *et al.*, 1998; Otten *et al.*, 2002) and antennal mechanoreceptors enable green stinkbugs to detect substrate (plant) vibrations (Jeram & Pabst, 1996). Moreover, substrate-borne vibrations such as these knocks (Fig. 3.5b) cause low-intensity, short-range sounds that antennae-borne sound receptors may receive, as suggested or shown for leafhoppers (Howse & Claridge, 1970), bees (Kirchner, 1994) and planthoppers (Romani *et al.*, 2009). Such receptors may also occur on antennae of *P. disparis* males that conspicuously tremble their antennae slightly above parasitized host pupae.

Whether DePa and adult males truly signal (communicate) is difficult to determine. Signaling implies intent to send information to a receiver, which then alters the receiver’s behavior and increases the signaller’s fitness (Bradbury & Vehrencamp, 1998). The spinning of DePa certainly has a behavior-modifying effect on males. They memorize the location of host pupae housing DePa, return to them repeatedly, and then await the emergence of a potential future mate (Danci *et al.*, 2013), thus likely enhancing their mating opportunities. A DePa female would also benefit from signaling her presence to a male. Although adult female *P. disparis* are able to produce male offspring without (Godfray, 1990, 1994; Quicke, 1997; Normark, 2003; Heimpel & de Boer, 2008; Danci *et al.*, 2013), they share more genes with their daughters than with their sons; however, the adult female must attract or arrest a male and mate to produce daughters. Despite these communication-linked benefits to both DePa and adult males, there might be severe constraints on the evolution of a communication system with rapid

signal exchange. On the part of the DePa, these constraints are likely trade-offs between the benefits of securing a future mate and the risks of being attacked by parasitoid females and invertebrate predators. The difference between the communication signals of a 'prospective' mate and the cues of either a foraging female parasitoid that would sting and feed on a DePa, or a hyperparasitoid that would hyperparasitize, may be too subtle to risk a prompt response by DePa. Its future benefits of survival may simply outweigh the benefits of a potential mate. If delayed replies from the DePa are the norm, this would explain why DePa rarely respond to playback recordings of male wing buzzing sounds (data not shown). It would also explain why males sometimes arrest on a host pupa for a long time, thus likely increasing their chance of detecting the presence of a DePa.

In conclusion, evidence is provided that movements of the DePa of *P. disparis* inside a host pupal case generate sounds and vibrations that may allow mate-seeking males to detect the presence of DePa. Changes in sound and vibration characteristics over the course of development of DePa may further allow males to approximate the time at which DePa will become a mature adult and emerge. Adult males antennating a parasitized pupa, and flying around it, also induced vibrations that may inform DePa about the presence of a male. There is no experimental evidence for rapid information exchange between DePa and adult males. Delaying reply signals may help DePa avoid attacks by illicit receivers of such signals including female (hyper) parasitoids and invertebrate predators.

Table 3.1. Analytical and descriptive statistics of parameters associated with behavioral activities of a *Pimpla disparis* pupa inside a wax moth host pupal case and adult males visiting such pupae.

Males			
Activity	**Parameters**	**Males (M) sampled (mean ± SE)**	**Statistics**
Antennation	Upper limit frequency band (kHz)	M1: 5.7 ± 0.3; M2: 3.9 ± 0.2 M3: 3.7 ± 0.4; M4: 4.6 ± 0.3 M5: 4.3 ± 0.2; M6: 5.5 ± 0.09	ANOVA; F_1 ratio = 5.34; P_1 = 0.0006
	Displacement (mm)	M1: 1.32 ± 0.23; M2: 0.58 ± 0.07 M3: 0.44 ± 0.04; M4: 0.3 ± 0.04 M5: 0.29 ± 0.02; M6: 0.38 ± 0.04	ANOVA; F_2 ratio =19.05; P_2 < 0.0001

Males			
Activity	**Parameters**	**Males (M) sampled (mean ± SE)**	**Statistics**
Buzzing[a]	Duration (s)	9 total buzzes from 6 males	Mean ± SE 0.25 ± 0.05
	Displacement (mm)	10 total buzzes from 6 males	Mean ± SE 0.38 ± 0.04
	Upper limit frequency band (kHz)	10 total buzzes from 6 males	Mean ± SE 2.7 ± 0.6
Pupae			
Activity	**Parameters**	**Pupae (Pu) sampled (mean ± SE)**	**Statistics**
Knocks	Time (s) between knocks	Pu1: 1.44 ± 0.03; Pu2: 1.52 ± 0.12 Pu3: 1.12 ± 0.12; Pu4: 2.37 ± 0.12 Pu5: 1.08 ± 0.05; Pu6: 1.61 ± 0.04	ANOVA; F_3 ratio = 7.6576; P_3 < 0.0001
	Upper limit frequency band (kHz)	Pu1: 3.04 ± 0.32; Pu2: 1.90 ± 0.069 Pu3: 2.52 ± 0.34; Pu4: 2.39 ± 0.14 Pu5: 4.21 ± 0.28; Pu6: 4.18 ± 0.12	ANOVA; F_4 ratio = 16.68; P_4 < 0.0001
	Displacement (mm)	Pu1: 0.77 ± 0.069; Pu2: 0.26 ± 0.02 Pu3: 1.06 ± 0.16; Pu4: 0.44 ± 0.008 Pu5: 1.06 ± 0.07; Pu6: 1.00 ± 0.02	ANOVA; F_5 ratio = 22.64; P_5 < 0.0001
Short spins[b]	Duration (s)	Pu1: 0.33 ± 0.03; Pu2: 0.42 ± 0.03 Pu3: 0.46 ± 0.07; Pu4: 0.37 ± 0.02	ANOVA; F_6 ratio = 1.65; P_6 = 0.2092
	Displacement (mm)	Pu1: 0.015 ± 0.001; Pu2: 0.023 ± 0.001 Pu3: 0.021 ± 0.0005; Pu4: 0.025 ± 0.001	ANOVA; F_7 ratio = 6.017; P_7 = 0.0043
	Upper limit frequency band (kHz)	Pu1: 1.15 ± 0.04; Pu2: 2.81 ± 0.62 Pu3: 2.50 ± 0.36; Pu4: 2.76 ± 0.07	ANOVA; F_8 ratio = 6.72; P_8 = 0.0026
Long spins[c]	Displacement (mm)	Pu1: 0.022 ± 0.002; Pu2: 0.022 ± 0.001 Pu3: 0.025 ± 0.001	ANOVA; F_9 ratio = 0.55; P_9 = 0.5767
	Upper limit frequency band (kHz)	Pu1: 2.58 ± 0.27; Pu2: 2.83 ± 0.24 Pu3: 2.88 ± 0.15	ANOVA; F_{10} ratio = 0.33; P_{10} = 0.7161

[a] Wing beat sound of flying male.

[b] <1s.

[c] >1s.

ANOVA, analysis of variance.

Table 3.2. Statistical comparisons of parameters associated with long spins (> 0.5 s) or short spins (< 0.5 s) of a *Pimpla disparis* parasitoid (Pa) inside a wax moth host pupa 14–16 days post parasitism.

Parameters	All pairs, Tukey's honestly significant difference multiple comparison test[a]				Analysis of variance
Comparison of parasitoid (Pa) long spins induced by paintbrush or male					
Displacement (mm)	Pa1: paintbrush	0.0203	—	B	F_{11} ratio = 2.67
	Pa1: male	0.022	A	B	P_{11} = 0.0302
	Pa2: paintbrush	0.03	A	—	
	Pa2: male	0.022	A	B	
	Pa3: paintbrush	0.026	A	B	
	Pa3: male	0.024	A	B	
Upper limit frequency band (kHz)	Pa1: paintbrush	2.21	A	B	F_{12} ratio = 2.92
	Pa1: male	2.58	A	B	P_{12} = 0.02
	Pa2: paintbrush	1.76	—	B	
	Pa2: male	2.83	A	—	
	Pa3: paintbrush	2.17	A	B	
	Pa3: male	2.88	A	B	
Comparison of parasitoid (Pa) long spins paintbrush-induced and short spins male-induced (only first 0.5 s of all spins used for analyses)					
Displacement (mm)	Pa1: paintbrush	0.006	—	B	F_{13} ratio = 6.12
	Pa1: male	0.018	A	B	P_{13} = 0.0013
	Pa2: paintbrush	0.023	A	—	
	Pa2: male	0.011	—	B	
	Pa3: paintbrush	0.009	—	B	
	Pa3: male	0.013	A	B	
Upper limit frequency band (kHz)	Pa1: paintbrush	2.67	A	—	F_{14} Ratio = 4.31
	Pa1: male	2.61	A	B	P_{14} = 0.0079
	Pa2: paintbrush	1.18	—	B	
	Pa2: male	1.46	A	B	
	Pa3: paintbrush	2.37	A	B	
	Pa3: male	2.84	A	—	

[a]Data associated with different uppercase letters are significantly different.

Spins were induced by painbrush stimulation of the host pupa or by the presence of an adult *P. disparis* male.

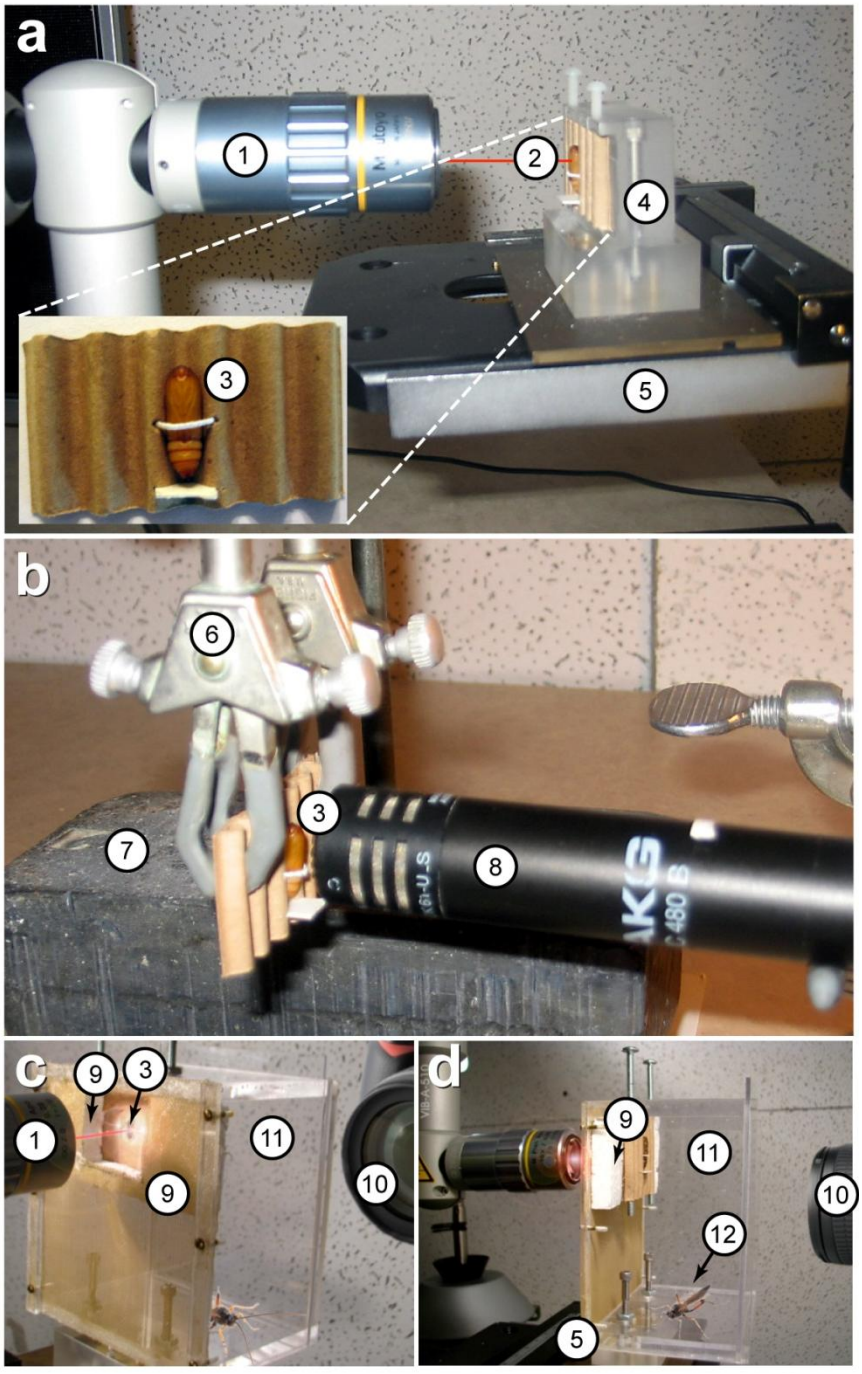
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Figure 3.1. **Experimental design for recording (a) vibrations and (b) sound from wax moth host pupae after paintbrush stimulation on days 1-23 post parasitism by a female *Pimpla disparis*; (c, d) front and back view of a modified design for video and vibration recordings of wax moth host pupae in the presence of, or in physical contact with, a male *P. disparis* at days 14–16 post parasitism by a female *P. disparis*. 1, laser Doppler vibrometer; 2, laser beam; 3, parasitized host pupa loosly mounted on a piece of corrugated cardboard; 4, Plexiglas holder; 5, metal plate; 6, holding clamps; 7, block of lead; 8, sonic microphone; 9, styrofoam; 10, lens of video camera; 11, Plexiglas cage; 12, male *P. disparis.***

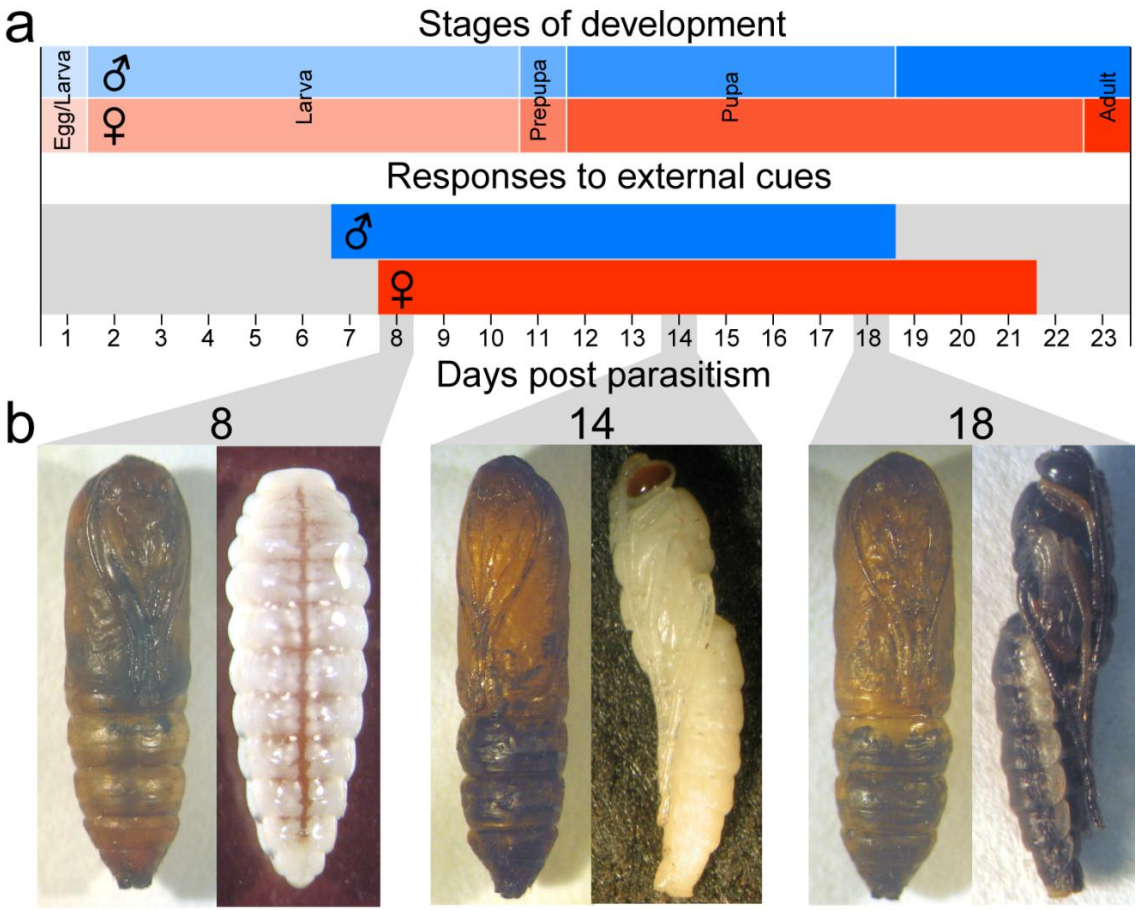

Figure 3.2. **(a) Time periods associated with developmental stages (egg, larva, pre-pupa, pupa and adult) of the parasitoid wasp *Pimpla disparis,* and periods of detectable sounds and vibrations related to movements of *P. disparis* inside a host pupal case; (b) Stages of *P. disparis* development on days 8, 14 and 18 post parasitism of the host pupa.**

Note: The Supporting information (Audio S1-S3) demonstrates the sounds associated with spinning motions of these stages.

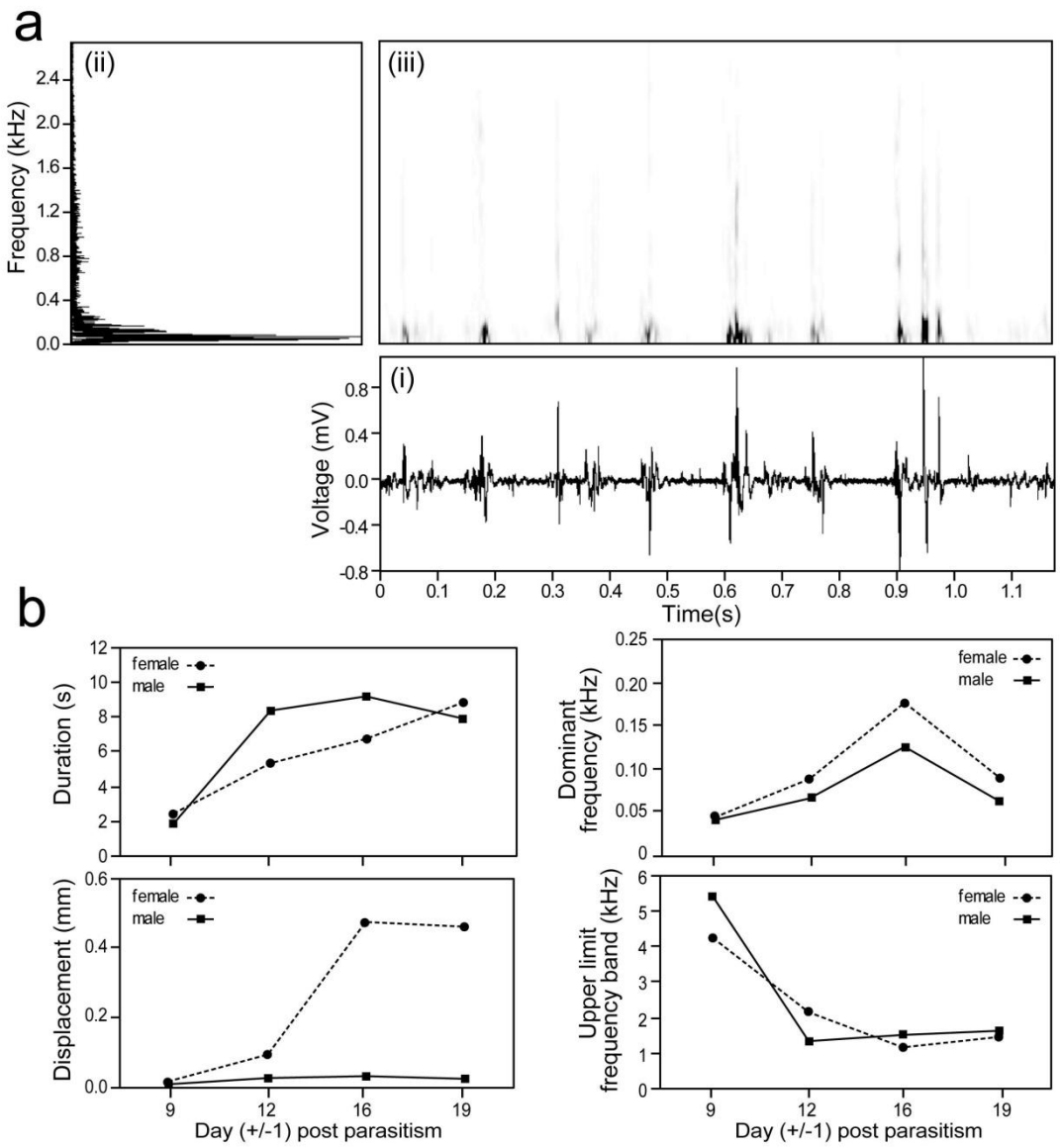

Figure 3.3. **(a) Representative recording of (*i*) waveform, (*ii*) frequency and (*iii*) time-frequency sound intensity (sonogram) of vibrations from wax moth host pupae after paintbrush stimulation on day 18 post parasitism by a female *Pimpla disparis*; (b) changes over time of vibration parameters (duration, displacement, dominant frequency, upper limit of the frequency band) produced by a developing male or female *P. disparis* inside a wax moth host pupa.**

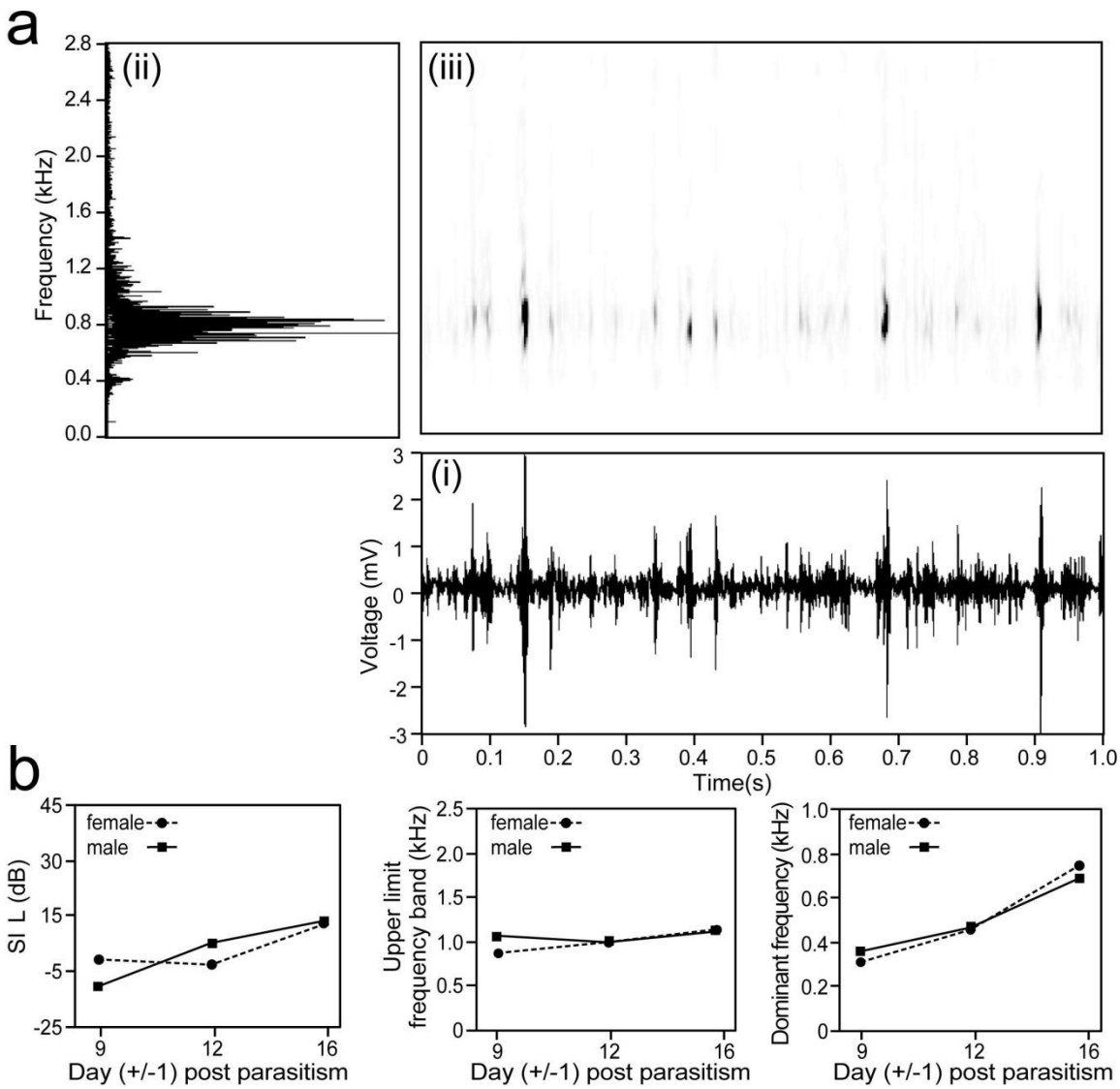

Figure 3.4. **(a) Representative recording of (*i*) waveform, (*ii*) frequency and (*iii*) time-frequency sound intensity (sonogram) of sounds from wax moth host pupae after paintbrush stimulation on day 18 post parasitism by a female *Pimpla disparis*; (b) changes over time of sound parameters [sound intensity level (SIL), dominant frequency, upper limit of the frequency band] produced by a developing male or female *Pimpla disparis* inside a wax moth host pupa.**

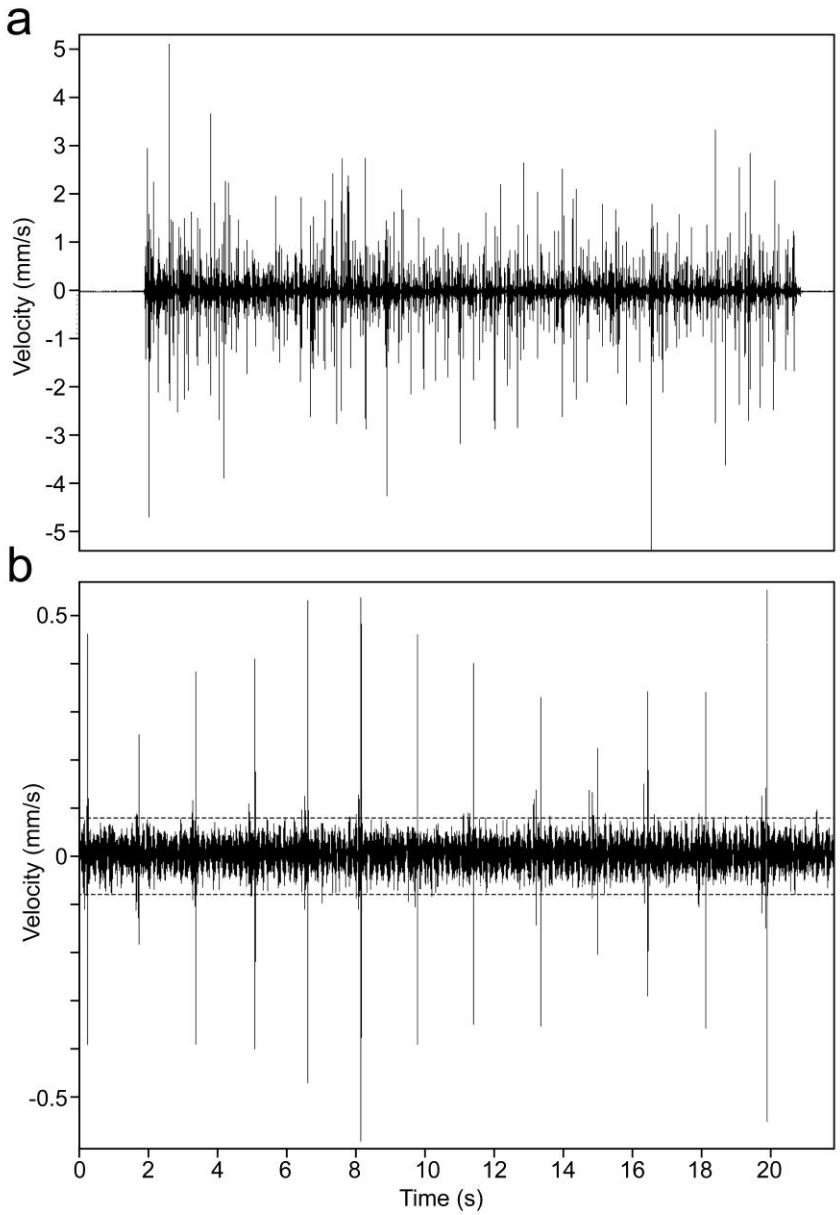

Figure 3.5. Representative recordings of vibrations associated with (a) long spins and (b) 'knocks' by a developing *Pimpla disparis* parasitoid inside a wax moth host pupa 16 days post parasitism of the pupa by a female *P. disparis.*

Note: The area between dotted lines represents the ambient noise level, as determined from recordings in the absence of vibrations.

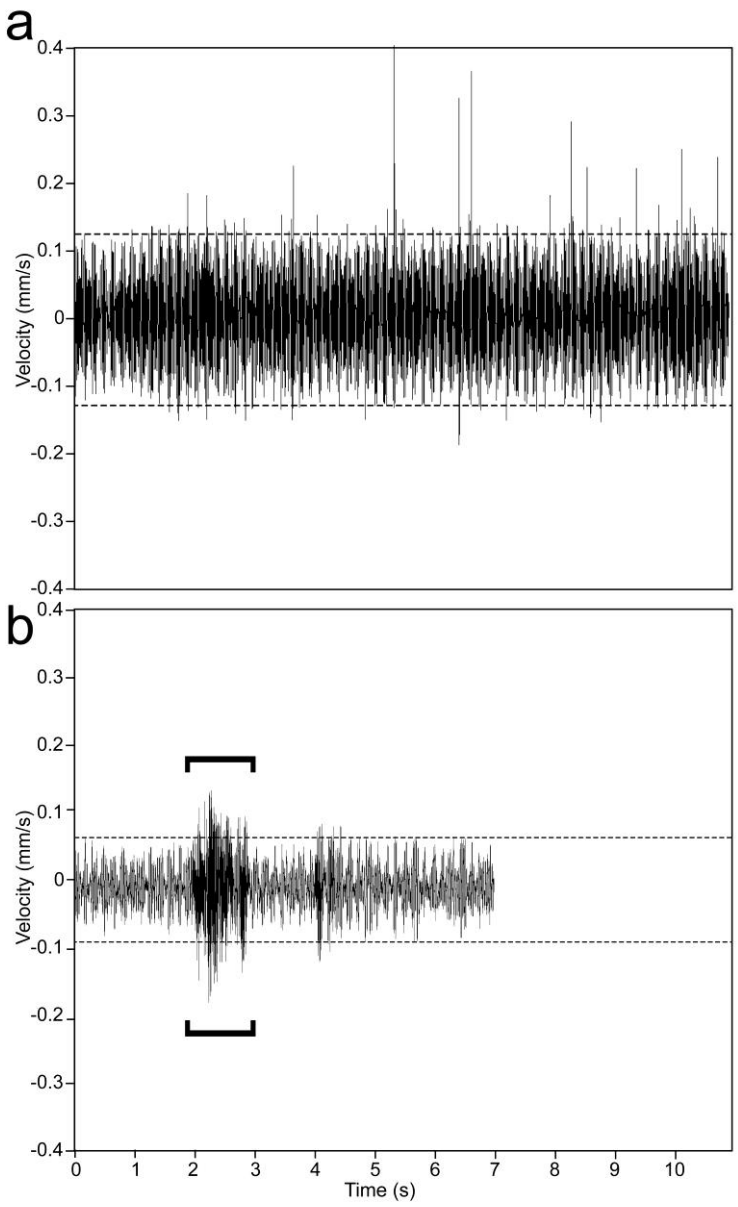

Figure 3.6. Representative recordings of vibrations caused when a male *Pimpla disparis* (a) antennated or (b) flew around a wax moth host pupa 16 days post parasitism of the pupa by a female *P. disparis.*

Note: The brackets in (b) indicate host pupal vibrations caused by the wing buzzing sound; the area between dotted lines represents the ambient noise level, as determined from recordings in the absence of vibrations.

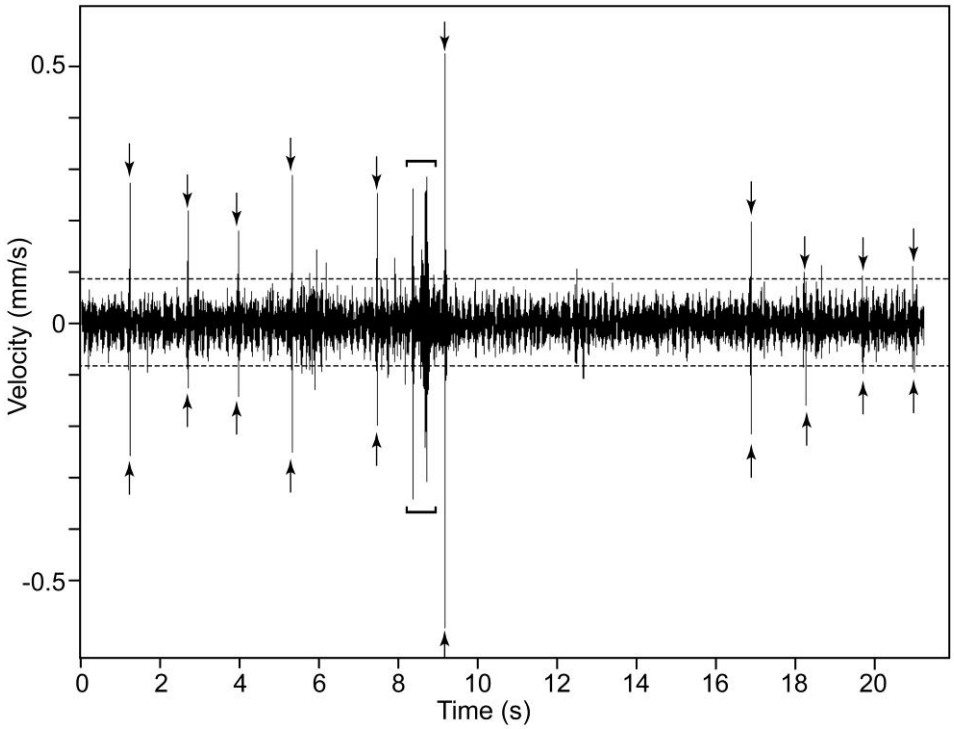

Figure 3.7. Recording of vibrations caused by (*i*) 'knocks' (demarked by arrows) of a developing *Pimpla disparis* parasitoid inside a wax moth host pupa (Fig. 3.5) and (*ii*) wing buzzing sound (demarked by brackets) of a male *P. disparis* flying near the same host pupa.

Note the cessation of knocks subsequent to the wing buzzing sound vibrations, and resumption of knocks 8 s later; the area between dotted lines represents the ambient noise level, as determined from recordings in the absence of vibrations.

3.6. Acknowledgments

We thank Pilar Cepeda for rearing wax moths, Matthew Drake for research assistance, Stephen DeMuth for graphical illustrations, Ian Bercovitz for statistical advice and three anonymous reviewers for constructive comments. This research was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC)-CGS to AD and by an NSERC – Discovery Grant and an NSERC – Industrial Research Chair to GG, with Contech Enterprises Inc. and Global Forest Science as industrial sponsors.

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3.8. Connecting Statement

In Chapter 3, I made vibration and sound recordings from parasitized host pupae throughout DePa's development. I showed that DePa-derived cues change significantly over time and thus could 'inform' a visiting adult male about the stage of DePa's development. It was not likely that males would remain for days on the same parasitized pupa to await the emergence of the prospective mate. Rather, it seemed plausible that males frequently re-visit a parasitized pupa to track DePa's development and gauge the time of emergence. In Chapter 4, I tested the hypothesis that *P. disparis* males memorize, and revisit, the location(s) of parasitized host pupae as part of a strategy to attain mates.

Chapter 4.

Learning provides mating opportunities for males of a parasitoid wasp[3]

4.1. Abstract

The ability of insects to learn locations of future resources has rarely been studied. Here, we show that males of the solitary parasitoid wasp *Pimpla disparis* Viereck (Hymenoptera: Ichneumonidae) learn locations of future mates. Male *P. disparis* reportedly arrest on parasitized pupae of wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), and gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Erebidae), when mate emergence is imminent. We tested the hypothesis that male *P. disparis* identify, memorize, and revisit the location(s) of parasitized host pupae as a strategy to attain mates. We colour-coded *P. disparis* males in the field and noticed that they revisit parasitized moth pupae on consecutive days, and arrest on those pupae with a near-emergence *P. disparis* parasitoid. In a laboratory experiment with two large corrugated cardboard cylinders (CCCs) as surrogate trees, each CCC bearing two parasitized moth pupae with a near-emergence *P. disparis* parasitoid or two pupae not parasitized, males on day 1 of the experiment visited parasitized pupae more often than pupae not parasitized. On day 2, when each CCC had been replaced and now carried pupae that were not parasitized, males returned to the same CCC, or the same micro-location on that CCC, which on day 1 had carried parasitized pupae. Field and laboratory data combined indicate that male *P. disparis* learn the location of future mates. With female *P. disparis* being haplodiploid and capable of reproducing without mating experience, the onus to find a mate is on males.

[3] Danci, A., Hrabar, M., Ikoma, S., Schaefer, P. W., and Gries, G. 2013. Entomologia Experimentalis et Applicata, 149(3): 229–240. DOI: 10.1111/eea.12129

They accomplish this by detecting parasitized pupae, learning their location, revisiting them frequently, and then arresting on them when the prospective mate nears emergence, taking a 50% chance that it is indeed a female.

4.2. Introduction

Learning (the acquisition of neuronal representations of new information) and memorization (the retention of newly acquired information over time) (Dukas, 2008) are not restricted to higher animals. They also occur in invertebrates like nematodes, snails, and insects (Smid & Vet, 2006). Learning plays an integral role in the decisions made by insects of many taxa (Papaj & Lewis, 1993). Insects rely on learning for all major life activities, including feeding, predator avoidance, aggression, social interactions, and mating (Dukas, 2008).

Social bees and ants use memorized visual cues to navigate between their nest and resources within a landscape (Zeil et al., 1996; Collett et al., 2003; van Nouhuys & Kaartinen, 2008). Honey bees, *Apis mellifera* L., learn the exact spatial location of their hive (Capaldi et al., 2000) and of profitable flower patches (Lehrer, 1993; Collett & Collett, 2002). Bumble bees rely on spatial learning to restrict visits to rewarding flowers within a patch (Burns & Thomson, 2005). Indeed, female parasitoid wasps too have the ability to learn and to form memory (Hoedjes et al., 2011). They repeatedly visit their nest while building and provisioning it, relying on their memory of visual cues to do so (e.g., Tinbergen, 1972).

The ability of an insect to learn the spatial location of future or potential resources, including mates, as opposed to resources it has already used, has seldom been reported. In one instance, the cleptoparasite *Argochrysis armilla* Bohart scouts for ground-nesting wasp females as they excavate and provision their burrows, returning later to oviposit into the burrows while wasp females are away foraging and unable to protect their brood (Rosenheim, 1987). Evidently, *A. armilla* is able to locate resources while they are conspicuous but unavailable, memorize their location, and to exploit them at a later time when they have become rewarding. Similarly, females of the parasitoid wasp *Hyposoter horticola* (Gravenhorst), which is an endoparasitoid of the Glanville

fritillary butterfly, *Melitaea cinxia* (L.), learn the location of host egg clusters, monitor their development, and parasitize 'during the single several-hour period when the host has developed into a larva but has not yet broken out of its egg shell', the only time parasitism will be successful (van Nouhuys & Ehrnsten, 2004; van Nouhuys & Kaartinen, 2008).

The spatial and temporal distributions of parasitoid wasps and their hosts are important determinants of parasitoid mating strategies (e.g., Godfray & Cook, 1997). When hosts are dispersed and parasitoids are solitary, males typically seek females near feeding or oviposition sites, whereas gregarious parasitoids, or parasitoids of gregarious hosts, often mate at shared pupation sites (Godfray, 1994; Godfray &Cook, 1997). The males' strategy of seeking virgin females at their site of emergence (henceforth 'early mate-detection') is adaptive because (1) females often do not re-mate (Alcock et al., 1978; Thornhill & Alcock, 1983) and (2) the presence of mates prior to or during eclosion facilitates rapid mating (Danci et al., 2011). Early mate-detection has been documented in species of various taxa (reviewed in Danci et al., 2011), including hymenopteran parasitoid wasps such as *Nasonia vitripennis* (Walker) (King et al., 1969), *Spalangia endius* Walker (King, 2006), *Lariophagus distinguendus* (Forster) (Steiner et al., 2005), and species of *Megarhyssa* and *Rhyssella* wasps (Matthews et al., 1979; Crankshaw & Matthews, 1981; Eggleton, 1991; Godfray & Cook, 1997).

Males of the solitary and polyphagous parasitoid wasp *Pimpla disparis* Viereck (Hymenoptera: Ichneumonidae) also engage in early mate-detection. Females parasitize host pupae in 14 families of the Lepidoptera (Schaefer et al., 1989), including gypsy moth pupae, *Lymantria dispar* (L.), in the Erebidae family (Leonard, 1981). Protandrous male *P. disparis* mate multiple times during their lifetime, but previous mating experience does not appear to affect their mate-seeking and mating behavior (A Danci, pers. obs.). Males usually emerge 3-4 days before females (A Danci, pers. obs.) and have been observed to gather on parasitized gypsy moth pupae in tree bark crevices (PW Schaefer, pers. comm.) and to arrest in response to a semiochemical cue associated with the emergence of a prospective mate from the host pupa (Hrabar et al., 2012). As emerging females do not engage in active long-range signaling (Hrabar et al., 2012), the males' ability to time their presence at parasitized host pupae prior to or during

emergence of a prospective mate is intriguing. A simple strategy for these males would be to stay for days on the same parasitized pupa, and to await the emergence of the prospective mate. For *P. disparis*, waiting seems a risky strategy, considering that males would take a 50% chance that the prospective mate is indeed a female (Hrabar et al., 2012), and that they would miss other mating opportunities elsewhere. Alternatively, if males were capable of identifying and memorizing the location of many parasitized pupae, they could repeatedly visit them, track their development, and more likely be present at the time of parasitoid emergence.

In a field survey and laboratory experiment, we tested the hypothesis that male *P. disparis* identify, memorize, and revisit the location(s) of parasitized host pupae as a strategy to attain mates.

4.3. Materials and methods

4.3.1. Experimental insects

Pimpla disparis emerged from gypsy moth pupae that we had field collected near the town of North East, MD, USA. We reared the wasps on pupae of gypsy moth (supplied by the USDA Forest Service, Northeastern Research Station, Hamden, CT, USA) and wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), in the Global Forest Insect Quarantine Facility of Simon Fraser University (Danci et al., 2011). We exposed host pupae to adult female *P. disparis* for 24 h, and isolated them in Petri dishes kept at 20–25 ºC, 40–60% r.h., and a L16: D8 photoperiod. We transferred emergent parasitoids to mesh cages (46 × 46 × 46 cm) and provisioned them with a cotton wick (1 × 5 cm; Richmond Dental, Charlotte, NC, USA) imbued with water and 20% honey (wt/vol), on which they could feed *ad libitum*. We isolated those to be tested in experiments according to age and sex and housed them in separate cages.

4.3.2. Tagging males for a field survey and laboratory experiment

To track the response of individual males in a field survey and laboratory experiment, we attached a coloured number tag (Bee queen number tags and glue;

Wienold Imkereibedarf, Lauterbach, Germany) to laboratory-reared virgin males. Each tag was reduced to a triangular shape to fit onto the wasps' notum. For the field survey, we also paint-marked (Sharpie acrylic paint markers, Michaels Art Supplies, Burnaby, BC, Canada) wild males (Figure 4.1b) that we had captured in the field. The one wild male that we had paint-marked at the time of his emergence from a field-collected gypsy moth pupa was a virgin at the time of his release. The mating status of all other paint-marked wild males was unknown.

4.3.3. Field observations of male *Pimpla disparis* in patches of parasitized host pupae

Finding a field site with at least a moderate population density of gypsy moth and *P. disparis* proved challenging due to re-occurring fungal epizootics that generally kept gypsy moth populations in check for the last decade. In Skowhegan, Maine (USA), in early July 2012, we located a row of 12 Lombardy poplar *Populus nigra* var. *italica* Muenchh (Salicaceae) trees that was heavily infested with gypsy moth larvae. We chose a tree near the center of the row (44º46'28.5"N, 69º37'09.5"W) on which to observe *P. disparis* mate-searching behavior. On the trunk of this tree, we selected two patches of 600 cm^2 each (Figure 4.1a) flanked by clusters of gypsy moth pupae for direct observations and video-surveillance of male *P. disparis* activities. As parasitism of gypsy moth pupae in or near both patches could not be ascertained in the field, and some pupae appeared to be diseased or damaged by predation, we supplemented both patches with field-collected gypsy moth (pre)pupae, and with laboratory-reared gypsy and wax moth pupae 17–21 days post parasitism by female *P. disparis*. As *P. disparis* is a generalist pupal parasitoid (Schaefer et al., 1989), presenting parasitized host pupae of two species in the same microhabitat was not likely to alter the behavior of male wasps. We randomly affixed the pupae to tree bark with hot glue (gun model AC-760; Michaels Art Supplies, Bangor, ME, USA), heating the glue just enough to 'tack', hand testing the temperature immediately prior to attaching the pupae. We separated pupae by at least 10 cm and assigned numbers by affixing a masking tape label next to them (Figure 4.1a).

We collected data by direct observations supported by video recordings (patch 1: Sony HDR-CX210; patch 2: Sony HDR-XR550; Sony of Canada, Toronto, ON, Canada) for review and confirmation of specific male wasp behavior. We released the number/color-tagged or paint-marked males (see above) onto the bark of a tree (44º46'28.5"N, 69º37'09.7"W) adjacent to the survey tree, and on each of 6 days recorded the males' visits to patches and host pupae therein, as well as the males' antennation of, and arrestment on or near, host pupae. Arrestment responses of males (being motionless for at least 5 s) usually occurred after antennation.

4.3.4. Do *Pimpla disparis* males memorize the location of host pupae?

In this laboratory experiment we tested the hypothesis that *P. disparis* males memorize the macro-location of corrugated cardboard cylinders (CCC) bearing parasitized host pupae and the micro-location of these pupae on such CCCs. We carried out the experiment in a still-air tunnel (1.10 × 1.10 × 2.40 m; no airflow) housing two brown CCCs (0.24 m diameter, 0.93 m high, spaced 1.1 m apart; Shippers Supply, Delta, BC, Canada) which served as surrogate tree trunks, and two sets of three black cylinders (each 1.0 × 0.11 m diameter) with or without white stripes which provided visually contrasting 'landmark' cues (Figure 4.1) for the macro-location of each trunk. In each experimental replicate (n = 10), we affixed with clear elastic cord (1 mm diameter; Pepperell Crafts, Pepperell, MA, USA) two parasitized wax moth pupae with a near-emergence *P. disparis* parasitoid to the randomly assigned treatment CCC, and we affixed two unparasitized wax moth pupae to the control CCC (Figure 4.1b). Then we released four laboratory-reared, number/colour-tagged (see above), 4- to 7-day-old virgin male *P. disparis* into the tunnel where we kept them with water and honey *ad libitum* for 2 days under a L16:D8 photoperiod, with ceiling-mounted light sources consisting of tubes of fluorescent 'daylight' (GE Ecolux Starcoat F32T8/SPX50/ECO, 5000K; General Electric, Fairfield, CT, USA) and tubes of 'broad-spectrum grow light' (F32T8/PL; Standard Products, Saint-Laurent, QC, Canada). Each replicate deployed a new set of CCCs and males. Instead of a single male, we released four males in each replicate to enhance the probability of data collection, and to address field observations that some males visited patches in small groups.

During the photophase of day 1, we allowed males to contact moth pupae, and to learn and memorize their location. During 3-4 h of observations, we recorded how often males hovered in front of, and landed upon, moth pupae, and how much time they spent antennating or arresting on or near them. At the end of the day-1 photophase, we gently covered resting males with a black cup (5.8 cm diameter $\times$ 2 cm high), removed both CCCs from the tunnel, and turned on the airflow for 3 min to exhaust semiochemicals. Then we placed two new CCCs each carrying two unparasitized wax moth pupae in the micro-locations of day 1 into the tunnel at day-1 macro-locations. Thereafter, we gently uncovered males under red light for the ensuing scotophase.

For 2 h from the onset of the day-2 photophase, we recorded (1) which pupae males contacted first; (2) how often males hovered in front of and landed upon or near ($<$1 cm) pupae; and (3) how much time males spent arrested on or near pupae or antennating them. We kept day-1 pupae in separate cages to confirm parasitism of treatment pupae, and to determine the sex of emerging parasitoids.

4.3.5. Statistical analyses

Data of the field survey on male *P. disparis* visits to parasitized host pupae do not warrant statistical analyses other than descriptive statistics. Data of the laboratory experiment were analyzed using a strip plot experimental design (Milliken & Johnson, 2009) with position (two on each CCC) and treatment (moth pupae parasitized or not) as the 'strips'. As a male's behavior in each replicate was not independent, he was considered a subsample of a replicate, and a replicate was considered the experimental unit. Thus, the total number of times males hovered in front of, and/or landed upon moth pupae, as well as the total time males spent arrested on or near moth pupae, or antennating them, were determined for each replicate by summing over the four colour-marked males during the 3-4 h of observations on day 1 and the 2 h of observations on day 2. Even though the four males were considered an experimental unit, colour-coding each male allowed us on day 2 to track individual visits and to determine the proportions of males returning to a specific CCC or the location of a pupa.

Do male *Pimpla disparis* recognize the location of parasitized host pupae on day 1 and exhibit a similar response on day 2?

We characterized qualitatively and quantitatively the behavioral responses of males to parasitized and unparasitized host pupae on day 1 and day 2, and we compared these responses between both days. We summed the activity over all four males within a replicate for each response criterion. The total number of times that males hovered in front of, and landed on or near, moth pupae were both considered count variables and were analyzed with Proc GLIMMIX of SAS® version 9.3 (SAS Institute, Cary, NC, USA) using generalized linear mixed models assuming a Poisson distribution with a log link function. The position of a pupa on a CCC (lower left or upper right), its status (parasitized or not), and day of observation (1 or 2) were included in the model as fixed effects, along with all corresponding interactions. Replicate, day*replicate interaction, and position*status*replicate interaction were included in the model as random effects.

The total time males spent arresting on or near moth pupae, or antennating them, is a continuous variable with many zero values (for unparasitized pupae), and some low and extremely high values; therefore, a natural logarithmic transformation was used to normalize total time and to control variability. Ln(total time) was subjected to a general linear mixed model analysis of variance. Fixed and random effects included in the models for total number of hoverings and total number of landings were also included in the model for this analysis using Proc MIXED in SAS. Because the inclusion of random effects leads to models with more complicated covariance structures, the classical degrees of freedom are biased, and consequently all analyses were performed using Kenward-Rogers degrees of freedom (Schaalje et al., 2001). All statistical tests were run using $\alpha = 0.05$. Pairwise comparisons carried out between levels of significant interactions were performed using t-tests.

Do male *Pimpla disparis* visit their favoured CCC of day 1 first on day 2?

Favoured CCC was defined as the CCC any male *P. disparis* on day 1 visited most often (definition 1), or for the longest time (definition 2), regardless of whether the CCC carried pupae that were parasitized or not (see Results). For each of the two definitions, we processed the data in four steps. We determined: (1) the favoured CCC on day 1 for each male; (2) the number of males returning first to their favoured CCC on day 2, keeping track whether a male just landed on that CCC, or on or near a pupa of that CCC; (3) the total number of males that visited either CCC on day 2, excluding from data analyses those males that did not visit any CCC on day 1 or 2, or that had no favoured CCC on day 1; and (4) the proportion of males returning first to their favoured CCC on day 2 expressed as the number of males returning divided by the total number of males.

The response variable of interest is the proportion of males returning first to their favoured CCC on day 2, for each definition of favoured CCC (see above). We used Proc GLIMMIX (SAS) for data analyses, the logit function as the link function, and we assumed a binomial distribution. Replicate was included as a random effect. Each treatment or control CCC had a 50% theoretical chance of being visited first by a male on day 2. We constructed the 95% confidence limits for the proportion of males returning first to their favoured CCC on day 2, concluding that a higher proportion returned to their favoured CCC than could be explained by random chance if the lower 95% confidence limit exceeds 0.5.

Do male *Pimpla disparis* visit their favoured host pupa of day 1 first on day 2?

For any given male *P. disparis* the favoured host pupa (parasitized or not) was defined as the host pupa he visited on day 1 most often (definition 1), or for the longest time (definition 2). For each of the two definitions, we processed and analyzed data as described above. Each of the two treatment and the two control pupae had a 25% theoretical chance of being visited first by a male on day 2. We constructed the 95%

confidence limits for the proportion of males returning first to their favoured pupa on day 2, concluding that a higher proportion returned to their favoured pupa than could be explained by random chance if the lower 95% confidence limit exceeds 0.25.

4.4. Results

4.4.1. Field observations of male *Pimpla disparis* in patches of parasitized host pupae

Of the four field-collected and four laboratory-reared male *P. disparis* we observed in the field, all visited pupae in patches 1 and 2 on each of two consecutive days (Figure 4.2). Moreover, three field-collected and three laboratory-reared males visited pupae on each of at least four consecutive days, with an estimated 60 ± 10 visits per day in patch 1, and 41 ± 9 visits per day in patch 2, over all males (Figure 4.2). Some field-collected and laboratory-reared males may have differed in their prior experience or mating status but clearly responded the same way.

Males visiting a patch, mostly hovered in front of pupae, possibly contacting them through brief antennation, but they also landed and arrested on or near pupae, and antennated them. For analyses, we counted as one visit when a male visited a patch even if he contacted, or arrested on, more than one pupa. During most days, on average over all males, the same male visited patch 1 for the following number of times: Day (D) 1: 3.87 ± 1.54 (mean ± SE); D2: 12.62 ± 4.64; D3: 6.87± 3.00; D4: 9.62 ± 2.71; D5: 7.87 ± 2.23; D6: 4.25 ± 1.37. The mean number of visits by the same male on patch 2 was: D1: 1.00 ± 0.50; D2: 4.87 ± 1.81; D3: 6.62 ± 2.57; D4: 8.75 ± 2.61; D5: 6.50 ± 2.01; D6: 3.37 ± 1.06.

At least three female *P. disparis* emerged from parasitized pupae (Table 4.1), including the 5J-labelled pupa of patch 2 which is shown in Figure 4.1c. The 5J pupa induced arrestment of the yellow-, pink-, and green-coded male. Only one male fully developed in a host pupa but did not emerge during the recordings (Table 4.1). Visiting males showed interest in pupae that contained a female or male parasitoid, supporting

the concept that they are unable to discern between a prospective mate and a male (Hrabar et al., 2012).

Visits by males to patches 1 and 2 commenced early in the morning (around 06:30 hours) and continued to early afternoon (around 13:00 hours) (Figure 4.2). Visits appeared rather stereotypic, following a specific search pattern between and within trees and patches. Wild green-, yellow-, and pink-coded males appeared to often search as a group, possibly because they were marked and released on the same day. Males stayed on leaf-bearing branches higher up in trees overnight and descended during the early morning just prior to dawn. Their search zone encompassed most, if not all, of the poplar row (30 m long $\times$ 3 m wide). Between visits to patches, males tended to leave the tree. When they returned on the same day, they often approached the tree and patches from different directions, rendering a response to long-range semiochemical cues unlikely. Patch visitations typically declined during the afternoon (data not shown).

4.4.2. Do *Pimpla disparis* males memorize the location of host pupae?

The status of moth pupae (parasitized or not) on days 1 and 2 (n = 10) had a significant effect on all three response criteria of males: (1) number of hoverings in front of pupae; (2) number of landings on or near pupae; and (3) time spent on or near pupae (Table 4.2, Figure 4.3). The effect of pupal status differed between days 1 and 2 for response criteria 1 and 2, but not 3 (status*day; Table 4.2). There was a significant effect for day on response criterion 3, but not on response criteria 1 and 2 (Table 4.2). The position of moth pupae on CCCs did not have a significant effect on any response criteria of males, nor did it have a significant effect when adjusted by status, day, or status*day (Table 4.2).

On day 1, males hovered in front of, and landed on or near parasitized pupae on treatment CCCs significantly more often than on unparasitized pupae on control CCCs (Figure 4.3A and B, Table 4.3). Results were similar on day 2 even though parasitized pupae on treatment CCCs had been replaced with unparasitized pupae; for both response criteria of males, results did not differ on days 1 and 2 (Figure 4.3A and B, Table 4.3). Males hovered significantly more often in front of control unparasitized pupae

on day 1 than on day 2 (Figure 4.3A, Table 4.3). Summed over days 1 and 2, the estimated mean time males spent on parasitized moth pupae was longer than the estimated mean time they spent on unparasitized pupae (Figure 4.3C, Table 4.3). Moreover, the estimated mean time males spent arrested on, or antennated, moth pupae (parasitized or not) was greater on day 1 (48.5 ± 0.39 s) than on day 2 (14.5 ± 0.39 s) (Figure 4.3C, Table 4.3).

Each of the two parasitized pupae tested on day 1 yielded a female *P. disparis* in replicates 1, 2, 5, 7, 8, 9, and 10, and a male *P. disparis* in replicates 3, 4, and 6. Whether pupae housed a developing male or female parasitoid had no significant effect on the males' number of visits (t-test: t = 0.235, d.f. = 8, P = 0.82) or the time they spent on a pupa (t = -0.379, d.f. = 8, P = 0.71). Of the 31 males that visited a particular CCC most often on day 1 (favoured CCC based on number of visits), 27 males chose the CCC which carried parasitized pupae. Of 24 males that spent most time on a particular CCC on day 1 (favoured CCC based on time spent), all chose the CCC which carried parasitized pupae. The proportion of males returning on day 2 to the CCC they had visited most often on day 1 was significantly higher than would be expected based on chance, as indicated by the confidence limit exceeding 0.5 (dotted line in Figure 4.4A).

Of the 27 males that visited one particular pupa most often on day 1, 25 chose a parasitized pupa (favoured pupa based on frequency of visits). Of the 23 males that spent most time on one particular pupa on day 1, all chose a parasitized pupa (favoured pupa based on time spent). The proportion of males returning on day 2 to their favoured pupa from day 1 was significantly higher than would be expected based on chance, as indicated by the lower confidence limit exceeding 0.25 (dotted line in Figure 4.4B).

4.5. Discussion

Our data support the hypothesis that male *P. disparis* learn, memorize, and revisit the location of host pupae containing prospective mates. In field surveys, males visited patches of parasitized host pupae repeatedly on the same day and on consecutive days (Figure 4.2), arresting on those pupae where the emergence of a prospective mate was imminent or had recently taken place, likely due to residual

semiochemicals (Hrabar et al., 2012). In the laboratory experiment, males learned and memorized both the macro-location of the CCC that had borne parasitized host pupae and the micro-locations of pupae on that CCC (Figure 4.3, Tables 4.2 and 4.3). With female *P. disparis* being haplodiploid and capable of reproducing with or without mating experience (Godfray, 1994; Quicke, 1997; Normark, 2003; Heimpel & de Boer, 2008), and with females not actively signaling for mates (Hrabar et al., 2012), the onus to find a mate and reproduce is on males. Males accomplish this by detecting host pupae that are parasitized (Danci et al., 2011), learning and memorizing their location (this study), tracking the development of prospective mates inside host pupae (A Danci, C Inducil, S Takács, PW Schaefer & G Gries, unpubl.), repeatedly visiting host pupae with prospective mates near emergence (this study), and then arresting when the emergence is imminent or underway (Hrabar et al., 2012).

The major advantage of learning over innate behavior is that it allows individuals to adjust to the specific conditions they experience at a certain place and time (Dukas, 2006). Not surprisingly then, the ability to learn has been documented in many insect species (Papaj & Lewis, 1993; Dukas, 2005, 2008; Collette, 2008) including parasitoid wasps (Vet et al., 1995; Tamo et al., 2006). Commonly though, mostly females engage in learning. Female parasitoids, for example, learn chemical cues that originate from their host(s) or the food source of their host(s) (Turlings et al., 1993; Vet et al., 1995; Iizuka & Takasu, 1998; Smid, 2006; Smid et al., 2007; Hoedjes et al., 2011). Learning of such semiochemical cues has been demonstrated in more than 30 parasitic wasp species (Steidle & van Loon, 2003). Female parasitoid wasps also learn colours, shapes, patterns, and spatial information (Wardle, 1990; Wardle & Borden, 1990; Wäckers & Lewis, 1999; van Nouhuys & Ehrnsten, 2004; van Nouhuys & Kaartinen, 2008; Hoedjes et al., 2011). Male parasitoids too can learn chemical and visual cues. Males of the parasitoid wasp *Pimpla alboannulatus* Uchida seek and feed on non-host food and learn to associate its odour with the resource (Sato & Takasu, 2000). Males of *Pimpla luctuosa* Smith appear to learn honey odor while feeding (Iizuka & Takasu, 1999; Sato & Takasu, 2000). Moreover, males of the parasitoid wasp *N. vitripennis* learn to associate colour with mates, but their ability to do so depends upon the type of colour and the type of reward (Baeder & King, 2004). Here, we present evidence that male *P. disparis* learn and memorize the macro- and micro-location of prospective mates.

Male *P. disparis* face the challenge to track evolving cues from future or potential mates. Semiochemical, visual, and vibratory cues associated with parasitized host pupae change as prospective mates develop inside them (Danci et al., 2011; A Danci, C Inducil, S Takács, PW Schaefer & G Gries, unpubl.). For example, parameters of sound and vibratory cues (amplitude, dominant frequency, upper limit of frequency band) that are related to rapid spinning of prospective mates change significantly over time and thus could 'inform' a visiting adult male about their stage of development. Male *P. disparis* apparently register these changes because their arrestment response intensifies as the developing parasitoid nears emergence from a host pupa (Danci et al., 2011). Even though male *P. disparis* recognize the multimodal cues associated with parasitized host pupae, and may be able to gauge the developmental status of a parasitoid inside a host pupa, this information becomes useful only if updated over time, necessitating that *P. disparis* use spatial memory to help them pinpoint the specific location of each parasitized host pupae for information update. Males likely use spatial memory to accomplish the task. This would explain why males on day 2 of the laboratory experiment visited first the CCC which on day 1 had borne parasitized pupae, even though on day 2 all pupae were not parasitized and lacked any semiochemical or vibratory cue associated with parasitism. It would also explain why males on day 2 returned first to the micro-location of their favoured day-1 pupa (which nearly exclusively was parasitized), and on the 'new' pupa in the very same location performed a stereotypical behavioral sequence of antennation and arrestment as if the pupa was parasitized. Males apparently relied on their day-1 spatial memory to return to the macro- and micro-location of host pupae that contained prospective mates. Possession of a spatial memory does not rule out that males on day 1 recognized parasitism of these pupae based on specific semiochemical, vibratory, or visual cues associated with them.

Memory is not a unique and stable unit of information storage but rather a dynamic process (Menzel, 2010). In species ranging from snails and insects to mammals, memory can be short- and long-term (Smid et al., 2007). Formation of long-term memory usually requires repeated and well-interspaced learning events, and is achieved by synthesis of specific proteins, while short-term memory requires only a single learning experience and is independent of protein synthesis (Smid et al., 2007).

Memory dynamics are likely linked to the ecology of a species (Menzel, 2001). Bumble bees use spatial memory when they repeatedly return to the same foraging area, remember and revisit rewarding plants, and sometimes repeat the same sequence of plant visits on each foraging trip over several days (Burns & Thomson, 2005). Specifics of the environment and spatial distribution of flowers determine the scale at which bumblebees use their spatial memory (Thomson et al., 1982; Burns & Thomson, 2005). In mate-seeking male *P. disparis*, the location of trees carrying parasitized host pupae, and the abundance and distribution of host pupae on those trees, could all affect memory dynamics. During day 1 of the laboratory experiment, males visited parasitized pupae on average 10.7 $\pm$ 2.28 times and spent on average 1 343.68 $\pm$ 408.59 s on them. This experience apparently sufficed for memory to form, because males on day 2 readily returned to the location of these pupae even though now they were not parasitized. In the field survey during each of 6 days males revisited patches 1 and 2 many times (Figure 4.2). These repeated visits over 6 days likely facilitated formation and reinforcement of short- or long-term spatial memory.

Landmarks, or the arrangement of landmarks, could play a role in the spatial memory of mate-seeking male *P. disparis.* Although we have not tested this experimentally, we can infer that males in our laboratory experiment relied on the visually distinct landmarks (black cylinders with or without white stripes) in an otherwise homogeneous environment to form a spatial memory of those CCCs that bore parasitized host pupae. The fundamental importance of landmarks to demarcate essential resources was first demonstrated by Tinbergen's (1972) pioneering study on female digger wasp, *Philanthus triangulum* Fabricius, that memorize the arrangement of landmarks to relocate their nest hole. After having contacted a parasitized pupa, male *P. disparis* exhibit circuiting flights around CCCs and their surrounding landmarks, reminiscent of the highly structured 'learning flights' of wasps and bees when they memorize the location of their nest using visual landmarks (Zeil, 1993).

Learning by male insects is commonly linked to reproductive strategies. For example, males of the braconid wasp *Alabagrus texanus* (Cresson) appear to learn spatial and temporal information that facilitates mate search. They integrate knowledge as to where a virgin female has recently emerged and are attracted to them by sex

pheromone to adjust short-term search patterns and avoid recently visited sites that are less likely to yield contact with a virgin female (Goh & Morse, 2010). Male halictine bees patrol for females among food plants and repeatedly return to 'profitable' plants, relating their position to local landmarks (Barrows, 1976). Male mason bees, *Hoplitis anthocopoides* Schenck, compete for and defend territories for mate encounter, learn their location, and return to them in the morning (Eickwort, 1977). Similarly, males of paper wasp, *Polistes* spp., maintain mate-encounter territories, depart at night and return each morning (Polak, 1993), obviously having learned their location.

In conclusion, we show that male *P. disparis* learn, and frequently return to, macro- and micro-locations of host pupae that contain prospective mates, thus affording opportunities to mate as soon as females emerge.

Table 4.1. Species and identification tag of moth pupae (GM, gypsy moth; WM, wax moth), date of parasitism by female *Pimpla disparis* in the laboratory, and status of *P. disparis* in host pupae in patch 1 or 2 (Figure 4.1.a) during field observation of mate-searching male *P. disparis* in Skowhegan (Maine, USA) during 22–30 July 2012

Pupae	Day of parasitism (in July)	Status of parasitoid in host pupa during 22–30 July
Patch 1		
GM; 3D	3	Failed to develop
WM; 5K	5	Male fully developed but not yet emerged
WM; 3C	3	Gender unknown, emerged on 24 July
GM; 13A	13	Late instar close to pupation
Patch 2		
WM; 5J	5	Female emerged on 27 July (at 12:20 hours)
WM; 10I	10	Female fully developed but not yet emerged
WM; 10E	10	Female emerged on 30 July (at 11:00 hours)
WM; 5F	5	Gender unknown, emerged on 23 July (pupal case empty on 24 July)
WM; 5G	5	Dead parasitoid in host pupal case (crack noted on 28 July)
WM; 10H	10	Female pupa attacked by spider on 29–30 July

Table 4.2. **General linear mixed model analysis of the number of times male *Pimpla disparis* hovered in front of, or landed on or near, moth pupae, and the time males spent on or near pupae, as affected by the factors (1) position of wax moth pupa (upper or lower level; see Figure 4.1.c) on corrugated cardboard cylinders, (2) status of moth pupae (parasitized or not); and (3) day of observations (1 or 2), or their interactions**

Factor	Responses of males on days 1 and 2								
	No. hoverings			No. landings			Time (s) spent on pupa		
	d.f.	F	P	d.f.	F	P	d.f.	F	P
Position of pupa	1,32.67	0.82	0.38	1,27.17	0.01	0.90	1,27	0.16	0.69
Status of pupa	1,33.03	53.61	<0.0001	1,27.7	40.45	<0.0001	1,27	76.41	<0.0001
Position*status	1,32.26	0.75	0.39	1,27.13	2.21	0.15	1,27	0.14	0.71
Day of observation	1,8.82	2.53	0.15	1,9.88	0.06	0.82	1,36	11.46	0.0017
Position*day	1,72	2.49	0.12	1,72	0.16	0.69	1,36	1.23	0.27
Status*day	1,72	44.57	<0.0001	1,72	12.69	0.0007	1,36	2.15	0.15
Position*status*day	1,72	0.06	0.80	1,72	0.07	0.79	1,36	0.05	0.82

Table 4.3. **Analysis of the number of times male *Pimpla disparis* hovered in front of, or landed on or near, wax moth pupae in pairwise comparisons (t-tests) of interactions between the status of moth pupae [parasitized (P) or not parasitized (NP)] on treatment cylinders (TC) or on control cylinders (CC) and observation day (1 or 2)**

				Responses of males					
				No. of hoverings			No. of landings		
Status	Day	Status	Day	d.f.	t	P	d.f.	t	P
NP on CC	1	NP on CC	2	12.82	3.20	0.0071	20.88	1.47	0.16
NP on CC	1	P on TC	1	32.7	-3.92	0.0004	34.39	-3.51	0.0013
NP on CC	2	NP on TC	2	72	-8.72	<0.0001	72	-6.60	<0.0001
P on TC	1	NP on TC	2	8.124	-0.34	0.74	8.286	-1.36	0.21

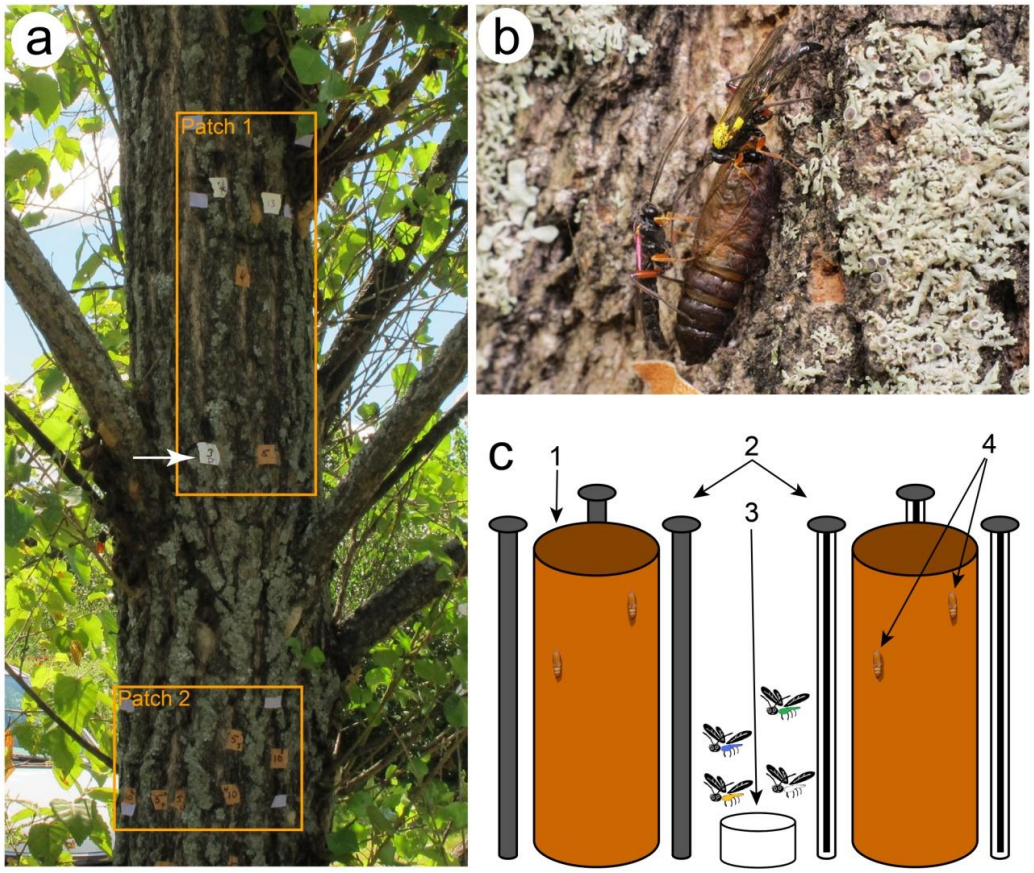

Figure 4.1. **(a) Location of patches 1 (15 × 40 cm) and 2 (20 × 30 cm) on a poplar tree in Skowhegan (Maine, USA) that were surveyed for visits of male *Pimpla disparis* during 22–30 July 2012. Identity of pupae was assigned by affixing a label (see white arrow) next to them. (b) Two male *P. disparis* that had previously been captured and colour-coded in the field now awaiting the emergence of a prospective mate. (c) Experimental design for testing whether male *P. disparis* memorize the macro-location of corrugated cardboard cylinders (CCCs) bearing parasitized host pupae and the micro-location of parasitized pupae on those cylinders; 1 = CCCs (24 × 46 cm) serving as surrogate tree trunks; 2 = sets of black cylinders (each 100 × 11 cm) with or without white stripes providing visually contrasting 'landmark' cues for the macro-location of each CCC; 3 = four 4- to 7-day-old colour-coded male *P. disparis* released in the center of the still-air tunnel for each experimental replicate; 4 = two wax moth pupae parasitized (17–20 days post parasitism by female *P. disparis*) or not.**

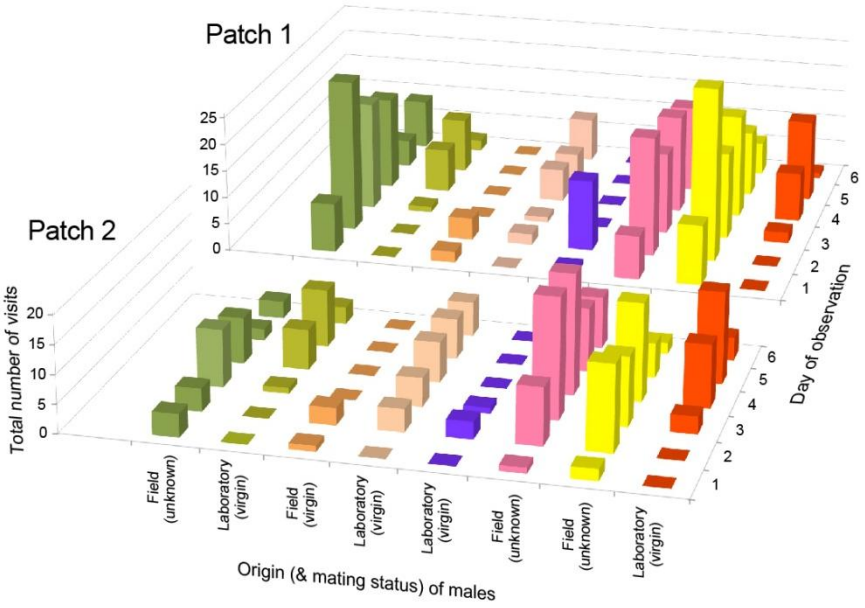

Figure 4.2. Total number of visits of eight individually marked *Pimpla disparis* males in each of two patches (Figure 4.1.a) on a poplar tree in Skowhegan (Maine, USA) on each of six consecutive days (25–30 July 2012).

Note: Males had been laboratory-reared or field-collected, and they were virgins or had unknown mating status. Each patch contained various numbers of gypsy or wax moth pupae parasitized by female *P. disparis* (Table 4.1).

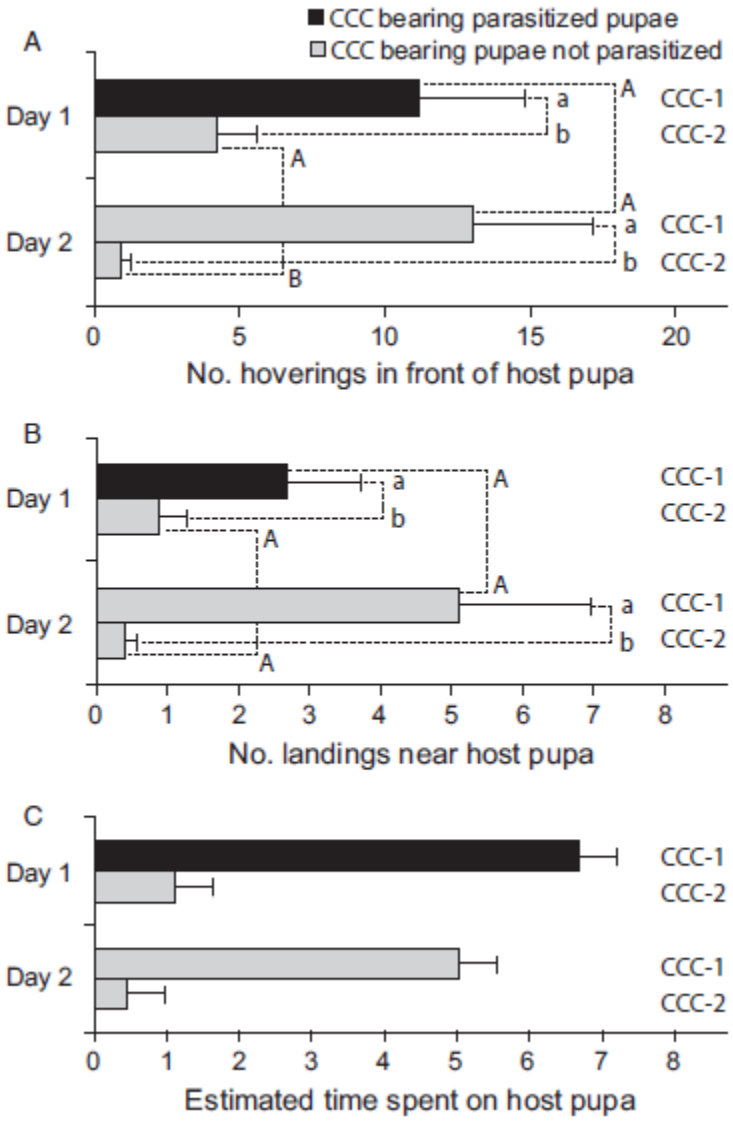

Figure 4.3. Mean (+ SE) number of times *Pimpla disparis* males (A) hovered in front of wax moth pupae affixed to corrugated cardboard cylinders (CCC) as surrogate tree trunks (Figure 4.1.c), (B) landed on or near them, and (C) the time (s) males spent arrested on or near pupae that were not parasitized (light bars) or parasitized (17–20 days post parasitism by female *P. disparis*) on days 1 and 2 of the experiment.

Note: Different lower or upper case letters on bars connected by dotted lines denote statistically significant differences (pairwise comparisons, t-test: $P < 0.05$). For detailed results of statistical analyses see Tables 4.2 and 4.3. For the criterion 'time spent on pupa' we could not assign letters to bars because for 'pupa status*day' $P > 0.05$ (see Table 4.2).

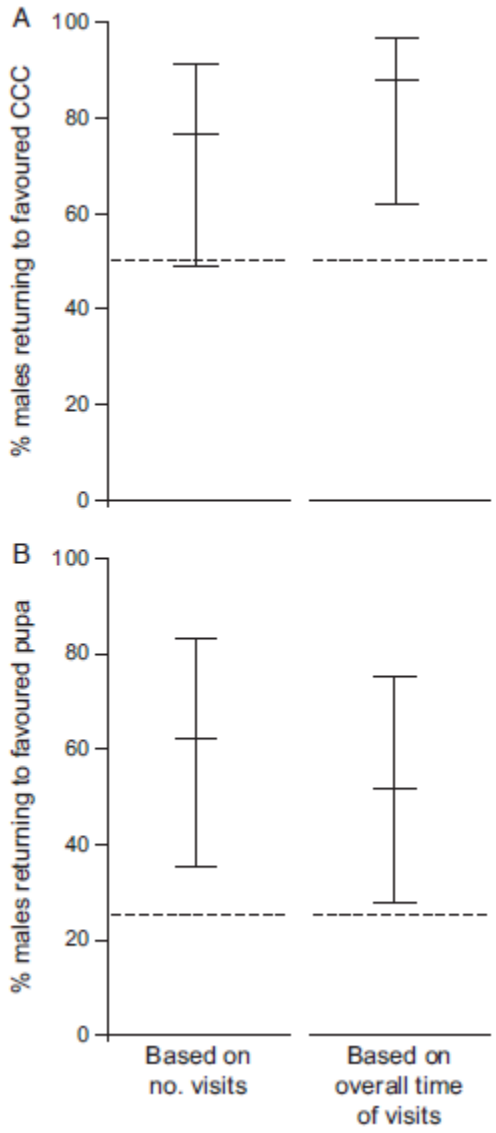

Figure 4.4. Estimated mean percentage (+ 95% confidence intervals) of male *Pimpla disparis* returning on day 2 of the experiment first to (A) the favoured corrugated cardboard cylinder (CCC) as surrogate tree trunk, and (B) the favoured host pupa which on day 1 males had visited the most often or for the longest time.

Note: Confidence limits are asymmetrical because estimates were back-transformed from the log scale. Each of the two CCCs, and each of the four pupae, had a 50 and 25% theoretical chance, respectively, of being visited first by a male on day 2, as indicated by the dotted lines.

4.6. Acknowledgements

We thank Pilar Cepeda for rearing wax moths, Ronald Hozmer for allowing us to use trees, electricity, and other supply material for the field survey on his property (Skowhegan, ME, USA), Stephen DeMuth for graphical illustrations, Marie Loughin and Tom Loughin for advising on and helping with statistical analyses, and two anonymous reviewers for constructive comments. This research was supported by SFU Graduate Fellowships and a Natural Sciences and Engineering Research Council of Canada (NSERC) - Canada Graduate Scholarship to AD, and by a NSERC–Discovery Grant and a NSERC–Industrial Research Chair to GG, with Contech Enterprises and Global Forest Science (GF-18-2007-226, GF-18-2007-227) as sponsors.

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4.8. Connecting Statement

In Chapter 4, I demonstrated in field surveys that *P. disparis* males learn the location of parasitized host pupae, revisit them on consecutive days, and arrest on them when the emergence of DePa is imminent. These results were supported by laboratory experiments, revealing that males memorize the macro- and micro-locations of parasitized host pupae. As it became evident in recordings of sound and vibratory cues produced by DePas over the course of their development (Chapter 3), DePas are quiescent a few days before emergence. This phenomenon could be a cue for a visiting male that the emergence of a mate will soon take place but it would not help the male to precisely predict the time of emergence. In contrast, the oral fluid produced by adult parasitoids while chewing their way out of a host pupa may signal an ongoing emergence. In Chapter 5, I tested the hypothesis that semiochemicals associated with DePa's emergence arrest males on a parasitized host pupa.

Chapter 5.

In the nick of time: Males of the parasitoid wasp *Pimpla disparis* respond to semiochemicals from emerging mates[4]

5.1. Abstract

Males of the parasitoid wasp *Pimpla disparis* Viereck (Hymenoptera: Ichneumonidae) aggregate on parasitized gypsy moth, *Lymantria dispar* (L.), host pupae when the emergence of a prospective mate is imminent or under way. We tested the hypotheses that the developing parasitoid (DePa) inside the host pupal case produces a pheromone that attracts and arrests mate-seeking males, and that the pheromone is most effective during the emergence of the parasitoid from the host. Results obtained in two-choice laboratory experiments, with 4-7-d-old virgin males, indicate that (1) DePa-derived semiochemicals arrest males, (2) the opening of a host pupal case strongly arrests males, and (3) the arrestment cue emanates from oral fluid secreted by both female and male parasitoids while they chew their way out of a host pupal case. This phenomenon implies that emerging females, which are haplodiploid and can reproduce without mating, do not engage in active pheromone signaling to attract males, and that mate-seeking males co-opt chemicals involved in eclosion as a mate-finding cue, taking a 50% chance that the prospective mate is a female.

[4] Hrabar, M., Danci, A., Schaefer, P. W., and Gries, G. 2012. Journal of Chemical Ecology, 38(3): 253–261. DOI: 10.1007/s10886-012-0079-9

5.2. Introduction

Ever since parasitic Hymenoptera (Parasitica) diverged from their ancestral phytophagy, their evolution has been driven, in part, by the natural history of their hosts. This host link spurred an adaptive radiation that resulted in the adoption of diverse mating systems (Quicke, 1997), ranging from staking out a common emergence site where brothers compete for access to sisters (Somjee et al., 2011), to lekking, where countless “bachelors” swarm around a nuptial site attracting females into the melee (Quicke, 1997).

The spatial and temporal distributions of parasitoids and their hosts are important determinants of parasitoid mating strategies (Alcock, 1978). When hosts are dispersed and parasitoids solitary, males typically seek females near feeding or oviposition sites, whereas gregarious parasitoids, or parasitoids of gregarious hosts, often mate at shared pupation sites (Godfray, 1994). Whether males focus their mate searching at sites of female emergence, feeding, or oviposition depends, to a large extent, on when and where receptive females are most abundant (Hölldobler, 1983; Thornhill and Alcock, 1983; Godfray, 1994).

The ichneumonid wasp *Pimpla disparis* Viereck is a primary, solitary endoparasitoid of lepidopteran pupae (Fuester et al. 1989; Schaefer et al., 1989; Fuester and Taylor 1993). One host is pupae of gypsy moth, *Lymantria dispar*, which are commonly found in bark crevices or under leaves of hardwood trees (Leonard, 1981).

Male *P. disparis* engage in an early mate detection strategy (Danci et al., 2011), whereby they locate parasitized gypsy moth, each containing a single conspecific developing parasitoid (DePa), periodically revisit these pupae, and then aggregate and arrest around them just prior to emergence of the prospective mate (Fig. 5.1a). This behavior implies that there exists some form of signaling or communication between DePas and adult males searching for them. The signal could be a pheromone, considering that pheromone-mediated communication is common within adult parasitic Hymenoptera (Hölldobler, 1984; Quicke, 1997; Ayasse et al., 2001; Metzger et al., 2010), and that both contact (Steiner et al., 2006; Ruther et al., 2011) and airborne female pheromones have been identified (e.g., Syvertsen et al., 1995, and references

therein; DeLury et al., 1999; Krokos et al., 2001; Collatz et al., 2009; Nichols et al., 2010). If the *P. disparis* signal were a pheromone, males would be best able to pinpoint the emergence of DePa if the pheromone changed in effectiveness over time. In order to emerge from the host pupal case, the adult parasitoid chews an opening while producing an oral fluid which seems to soften the pupal case (personal observation), as has previously been suggested for other ichneumonid species (M.G. Fitton, cited in Quicke, 1997). The timing and brief production of this oral fluid make it an ideal candidate source for the "emergence pheromone".

We tested the hypotheses that (1) the pre-emergence DePa produces a pheromone that attracts or arrests males; (2) the pheromone is most effective just prior to, or during, the emergence of a parasitoid; and (3) the oral fluid of the emerging parasitoid contains the emergence pheromone.

5.3. Materials and methods

5.3.1. Experimental insects

Pimpla disparis were field collected near the town of North East, Maryland, USA, and reared on pupae of the laboratory host wax moth, *Galleria mellonella,* in the Global Forest Insect Quarantine Facility of Simon Fraser University (Danci et al., 2011). Host pupae were exposed for 24–72 h to female *P. disparis* adults*,* isolated in Petri dishes, and kept at 20–25 ºC, 40–60% RH, and a 16L:8D photoperiod. Emerged parasitoids were transferred to mesh cages (46 × 46 × 46 cm) and provisioned with cotton wicks (1 × 5 cm; Richmond Dental, Charlotte, NC, USA), imbued with water and honey, on which to feed *ad libitum*. Those to be tested in bioassays were isolated according to age and sex (based on the presence of an ovipositor in females) and housed in separate cages.

5.3.2. General bioassay design

Two-choice experiments were conducted in clear Plexiglas cages (9 × 6 × 9 cm), lined on the inner right and left sides with single-face corrugated cardboard (Shippers Supply Inc., Delta, B.C., Canada) which served as surrogate tree trunks (Fig. 5.1b).

Unless otherwise stated, bioassay host pupae were frozen ("freeze-killed") on dry ice for 15 min, thawed for 15-20 min, and then attached with elastic thread (1 mm diam., stretch magic bead and jewellery cord, Pepperell Crafts, MA, USA) to small cardboard squares (3 × 3 cm) (Fig. 5.1.c) randomly assigned to the left or right side of the cage. For each replicate, a 4–7-d-old virgin male was introduced into the center of the cage and the time it spent on, or immediately adjacent to, host pupae recorded over 15 min. Each male was bioassayed only once for each set of test stimuli. In experiments testing candidate pheromone sources (see below), cages were washed, and new cardboard was affixed to the sides between replicates, to prevent cross contamination.

5.3.3. Acquisition and bioassay of candidate pheromone sources

We predicted the presence of at least trace amounts of pheromone on DePa or on the host pupal case. Therefore, moth pupae, 17 d post parasitism, were opened, the DePa removed and immersed in 3 ml of hexane in a 20-ml vial for 10 min. (without agitation) at room temperature. Intact host pupal cases were immersed in separate hexane-containing vials 2 min. and agitated gently. This change in protocol for pupal case washes was prompted by concerns that 10-min immersions may soften the pupal case and allow solvent to diffuse to the DePa inside. Gentle agitation was applied to maximize extraction of chemicals from the pupal case surface. The supernatant of several washes was combined and evaporated under a stream of nitrogen, such that 10 µl equalled 1 host pupa, or 1 DePa, equivalent. For bioassays, 10-µl aliquots of washes, or of solvent controls, were uniformly applied to freeze-killed unparasitized host pupae.

5.3.4. Acquisition of headspace volatiles

Groups of 20, 16-d-old parasitized pupae were placed into each of three linearly interconnected Pyrex® glass tubes (2 × 12 cm). Charcoal-filtered air was drawn at 0.5-1 $L.min^{-1}$ through the chambers and a Pyrex® glass tube (4 mm ID × 100mm), containing 250 mg of Porapak-Q (50–80 mesh, Waters Associates, Inc.; Milford, MA, USA) held in place with glass wool. At 24-h intervals, aerations were stopped briefly and volatiles eluted from the Porapak-Q with 2 ml of redistilled pentane, after which aerations were resumed. Extracts were concentrated, as needed, under a stream of nitrogen.

5.3.5. Acquisition of oral secretions

Oral secretions of emerging male or female parasitoids were obtained by (*i*) gently grasping their thorax between the thumb and forefinger, (*ii*) wicking the resulting droplet (~0.2 µl) of oral fluid (Fig. 5.1e) into a micro-capillary tube (1 × 0.58 mm; Fig. 5.1f; Davies and Madden, 1985), and (*iii*) by dispensing the oral fluid into hexane by blowing lightly through the capillary tube.

5.3.6. Specific bioassay experiments

Hypothesis 1: Pre-emergence DePa produces pheromone that attracts or arrests males

Experiment 1 (Table 5.1) tested whether males arrest for longer periods on or near a freeze-killed pupa 17 d post-parasitism, than on a pupa that is not parasitized. Freeze-killed, rather than live, pupae were bioassayed to exclude sound and motion of DePa as potentially confounding factors affecting the responses of males. Following bioassays, the treatment pupae were opened to confirm the presence of a DePa, and to note the developmental stage. As males recognized parasitized pupae in experiment 1, experiments 2–4 (Table 5.1) tested whether pheromone was present on, and can be washed by solvent off, the surface of host pupal cases 17 d post-parasitism **(**experiment 2), and the integument of ~17-d-old male or female DePa (experiments 3 and 4). Finally, experiments 5-7 (Table 5.1) tested whether the putative pheromone disseminated from host pupae prior to, as well as during, the emergence of the parasitoid.

Hypothesis 2: Pheromone is most effective just prior to or during the emergence of a parasitoid

Experiment 8 (Table 5.1) tested whether opening of the host pupal case resulted in a release of pheromone, thus giving mate-seeking males a timing signal for emerging parasitoids. In each replicate, the bioassay male was given a choice between a host pupa either containing a pupal DePa or an eclosed 1–3-d pre-emergence adult male or female parasitoid, and a control unparasitized host pupa of similar age. During the first

15 min. of each bioassay, both pupae were kept intact. The male was then removed, and retained for ~30 sec. under a 30-ml Solo plastic cup, while the treated pupa was either left intact or received a 2-mm incision at the cephalic end to simulate the initial cut a parasitoid makes to initiate emergence (Fig. 5.1d; supplemental video S2). During the second 15 min of each bioassay, the male's preference for treatment or control pupae was again recorded.

Hypothesis 3: Oral secretion contains the emergence pheromone

Experiments 9 and 10 (Table 5.1) tested whether oral fluid, secreted by male and female adult parasitoids while chewing an opening in the host pupal case, contained the pheromone that attracts and arrests males. For each replicate, the oral secretion was collected from an emerging parasitoid by wicking the liquid into a capillary tube and then immediately dotting it onto a live unparasitized moth pupa that was bioassayed against a live untreated moth pupa.

5.3.7. Statistical analyses

In the two-choice experiments 1–7 and 9–10, the times that males spent on or near the treatment or control host pupal cases were compared using a Wilcoxon signed-rank test. In experiment 8, all possible variables (intact or incised host pupal case; sex of DePa; pupal DePa or eclosed parasitoid inside host pupal case) were subjected to a 3-way analysis of variance (ANOVA). Differences between means for each variable were analyzed by Tukey tests. All data were analyzed with the Statistical Software JMP 7.0.2 (SAS Institute 2009), with $\alpha = 0.05$.

5.4. Results

Hypothesis 1: Pre-emergence DePa produces pheromone that attracts or arrests males

In experiment 1, males spent more time on or near freeze-killed parasitized pupae than on freeze-killed unparasitized pupae ($W = 86$; $P = 0.001$; Fig. 5.2). In

experiments 2–4, males spent more time on unparasitized host pupae treated with hexane washes of (*i*) parasitized host pupal cases (experiment 2), (*ii*) female DePa pupae (experiment 3), or (*iii*) male DePa pupae (experiment 4) than on host pupae treated with the equivalent amount of hexane (Exp. 2: $W = –39$, $P < 0.001$; exp. 3: $W = –56$, $P < 0.001$; exp 4: $W = –174$, $P < 0.001$; Fig. 5.3). In experiment 5, males did not discriminate between unparasitized pupae treated with headspace volatile extract of host pupae containing a pre-emergence DePa (17–18 d post parasitism) or treated with the equivalent amount of pentane ($W = –31$, $P = 0.1$; Fig. 5.4). In experiments 6 and 7, males spent more time on unparasitized host pupae, treated with headspace volatile extract of host pupae containing an emerging adult male (19–20 d post parasitism) or adult female (20–22 d post parasitism) parasitoid, than on unparasitized host pupae treated with the equivalent amount of pentane (Exp. 6: $W = –102$, $P < 0.001$; exp. 7: $W = –44$, $P < 0.007$; Fig. 5.4).

Hypothesis 2: Pheromone is most effective during the emergence of a parasitoid

In experiment 8, pupae incised in the second period arrested males longer than intact pupae during the first period, irrespective of the sex and developmental stage of the DePa inside (Fig. 5.5, Table 5.2). Developmental stage, but not sex, of DePa affected arrestment times of bioassay males. Female parasitoids already eclosed within the incised host pupae elicited longer arrestment by males than did female DePa in the pupal stage. This was not the case for eclosed males or male pupal DePa. When parasitized host pupae were kept intact, males spent equal amounts of time on pupae during the first and the second 15-min bioassay periods.

Hypothesis 3: Oral secretion contains the emergence pheromone

In experiments 9 and 10, males spent more time on live unparasitized moth pupae, treated with oral secretions of emerging female (experiment 9) or male (experiment 10) parasitoids, than on live unparasitized moth pupae not treated (Exp. 9: $W = –28$; $P = 0.002$; Exp. 10; $W = –39$, $P < 0.001$; Fig. 5.6).

5.5. Discussion

Our data support the hypothesis that detection of pre-emergence and emerging mates in *P. disparis* is mediated by semiochemicals. Attraction and arrestment of males in response to (*i*) freeze-killed, parasitized host pupae, which could "signal" only with semiochemicals, (*ii*) solvent washes of parasitized host pupae or isolated DePas, and (*iii*) headspace volatiles of parasitized live host pupae with emerging parasitoids, all implicate DePa-derived semiochemicals as a means by which males pinpoint emergence of prospective mates. Experimental opening of host pupal cases containing a pupal DePa, or a recently eclosed parasitoid, induced strong arrestment of males, likely due to a burst of semiochemicals release. Parasitoids chewing their way out of the host pupal case (Fig. 5.1d; supplemental video S2) release these semiochemicals with the oral fluid, making them a true "emergence cue".

To implicate semiochemicals as the single modality males exploit in order to pinpoint the time of emergence of prospective mates, we needed to exclude visual and vibratory cues. A DePa inside a host pupal case engages in spontaneous spins, which cause vibrations that increase in magnitude but decrease in frequency as DePas mature (Danci et al., unpubl.), thus potentially providing information to a visiting male about the stage of development. As males arrested in response to freeze-killed host pupae and DePas that could no longer spin, they could not have relied on vibrational cues. Moreover, when males responded to body washes of DePas, and to headspace volatiles of host pupae with emerging parasitoids, they could not have relied on visual cues such as discoloration of the host pupal case. Collectively, these data implicate DePa-derived semiochemicals as the source of information that alerts males to the incipient or ongoing emergence of a prospective mate.

Changes over time in the composition and/or abundance of DePa semiochemicals could carry critical information for mate-seeking males. Although we do not know anything about the identity of the chemical(s), and hence cannot discuss qualitative changes, the strong responses of males to freshly incised host pupal cases containing a pupal DePa or an eclosed adult, but not to intact host pupal cases, points to

semiochemical concentration as critical information used by males to gauge the emergence time of a prospective mate.

While investigating the source of the “emergence semiochemical”, we noticed that mate-seeking males pay particular attention to the incision line that parasitoids chew into their host pupal case to open and emerge from. Moreover, macro-videography of this chewing revealed oral fluid (Supplemental video) that appeared to soften the pupal case. These observations made us hypothesize that the semiochemicals are present in the oral fluid released during eclosion. Collecting oral fluids from emerging male and female parasitoids (Fig. 5.1, e, f), and immediately testing them in bioassays, revealed that they induced strong arrestment, wing fanning, antennation, and even copulatory attempts, by males. Thus, these semiochemicals may originate from mandibular glands, which have been implicated or shown to be the source of sex pheromones in some ichneumonid wasps (Davies and Madden, 1985) and sphecid wasps (Ayasse et al., 2001). For example, males of the European beewolf, *Philanthus triangulum* Fabricius, secrete sex pheromone from their mandibular gland that attracts females into their territory, which they cordon off to secure a harem (Herzner et al., 2007).

Insects utilize both signals and cues in their search for prospective mates (Metzger et al., 2010). A cue refers to any kind of sensory information present in the environment (Ruxton and Schaefer, 2011), whereas a signal implies intent on the part of the sender (Wilson, 1975) and requires an active process. Pheromone-based sexual communication with “intentional” signaling is well documented in hymenopteran parasitoids (Syvertsen et al., 1995, and references therein; Quicke, 1997), but may not exist in *P. disparis*. The communication systems of some solitary wasp species seem inefficient, in that males are attracted as strongly to emerging males as they are to emerging females (e.g., Heatwole, et al., 1962; Robacker and Hendry, 1977; Davies and Madden, 1985; Quicke, 1997; Ayasse et al., 2001; Steiner et al., 2005; King, 2006; Ruther and Steiner, 2008). Interpretations of this rather peculiar phenomenon, in general, include sensory limitations on the part of the males (Thornhill and Alcock, 1983; O’Neill, 2001), and mimicry of female pheromone by (juvenile) males (Eliyahu et al., 2009). The latter phenomenon has been implicated in some mating systems as playing a role in reducing male aggression (Peschke, 1987; Cremer, et al., 2002), stealing nuptial

gifts (Eliyahu et al., 2009), and in post-copulatory mate guarding (Field and Keller, 1993). In the *P. disparis* mating system, female mimicry has no obvious selective advantage. As female *P. disparis* do not respond to hexane extract of oral secretion (data not shown), our original hypothesis that the oral secretion contains an aggregation pheromone, *sensu* Borden (1985), that attracts males and females and thus facilitates outbreeding and genetic diversity, was not supported. An alternative explanation is that many ichneumon females may not pheromone signal at all, and that males, instead, respond to "pre-existing" information associated with prospective mates (Godfray and Cook, 1997; Ruther and Steiner, 2008), such as metabolites present on the integument (Howard, 1993). This explanation is applicable to *P. disparis.* Males respond to semiochemicals in oral fluids of both male and female conspecifics (Fig. 5.6), suggesting that emerging females indeed do not signal with long-range sex pheromone.

The metabolites in oral fluids of *P. disparis* may serve a primary function of softening the host pupal case to facilitate the parasitoid's emergence; secondarily, they appear to have been co-opted by males to assist them in locating emerging females which, once fully emerged, do not seem to engage in long-range attraction of searching males, although they do seem to possess a sex-specific cuticular (contact) pheromone that elicits courtship behavior in males (M.H., personal obs.). As these oral fluids are secreted only during emergence, and dissipate thereafter, they are a highly reliable indicator of parasitoid emergence. The strategy of males, of responding to these secretions, may represent a trade-off between a high probability of encountering an emerging conspecific, and a 50% chance of it being a male instead of a mate.

With the emerging female not actively signaling and revealing her location, the onus to find a mate and reproduce is on males. Females, in contrast, are haplodiploid, and thus are capable of reproducing with or without mating experience (Quicke, 1997; Normark, 2003; Heimpel and de Boer, 2008). Such female parasitoids have reduced evolutionary pressure to allocate resources toward pheromone production and signaling, which may attract predators and, instead, may allocate more resources toward oviposition (Godfray and Cook, 1997; Ayasse et al., 2001; Steiner and Ruther, 2009). This would explain why males ensure their reproductive success by co-opting chemicals

already present in oral secretions of emerging females (and males) as a mate-finding cue.

Table 5.1. Stimuli tested in Experiments 1-10

Exp.#	*N*	Stimuli tested	
		Stimulus 1	**Stimulus 2**
1	19	Freeze-killed wax moth pupa (F-KWMP) 17 days post parasitism	F-KWMP not parasitized
2	15	F-KWMP not parasitized, treated with hexane wash of WMP 17 days post parasitism[a]	F-KWMP not parasitized, treated with hexane (10 µl)
3	21	F-KWMP not parasitized, treated with hexane wash of female *P. disparis* pupa[a]	F-KWMP not parasitized, treated with hexane (10 µl)
4	31	F-KWMP not parasitized, treated with hexane wash of male *P. disparis* pupa[a]	F-KWMP not parasitized, treated with hexane (10 µl)
5	22	F-KWMP not parasitized, treated with headspace volatile extract of WMP 17-18 days post parasitism[b,c]	F-KWMP not parasitized, treated with pentane (10 µl)
6	20	F-KWMP not parasitized, treated with headspace volatile extract of WMP 19-20 days post parasitism[b,d]	F-KWMP not parasitized treated with pentane (10 µl)
7	13	F-KWMP not parasitized, treated with headspace volatile extract of WMP 20-22 days post parasitism[b,e]	F-KWMP not parasitized, treated with pentane (10 µl)
8	22 (T1)[f]	0-15 min *Intact* live WMP containing male *P. disparis* pupa (w) 15-30 min *Intact* live WMP containing male *P. disparis* pupa (x)	0-15 min Intact live WMP not parasitized 15-30 min Intact live WMP not parasitized

Exp.#	*N*	Stimuli tested	
		Stimulus 1	**Stimulus 2**
	22 (T1)	0-15 min *Intact* live WMP containing male *P. disparis* pupa (y) 15-30 min *Incised* live WMP containing male *P. disparis* pupa (z)	0-15 min Intact live WMP not parasitized 15-30 min Intact live WMP not parasitized
	23 (T2)	0-15 min *Intact* live WMP containing eclosed adult male *P. disparis* (w) 15-30 min *Intact* live WMP containing eclosed adult male *P. disparis* (x)	0-15 min Intact live WMP not parasitized 15-30 min Intact live WMP not parasitized
	23 (T2)	0-15 min *Intact* live WMP containing eclosed adult male *P. disparis* (y) 15-30 min *Incised* live WMP containing eclosed adult male *P. disparis* (z)	0-15 min Intact live WMP not parasitized 15-30 min Intact live WMP not parasitized
	40 (T3)	0-15 min *Intact* live WMP containing female *P. disparis* pupa (w) 15-30 min *Intact* live WMP containing female *P. disparis* pupa (x)	0-15 min Intact live WMP not parasitized 15-30 min Intact live WMP not parasitized
	39 (T3)	0-15 min *Intact* live WMP containing female *P. disparis* pupa (y) 15-30 min *Incised* live WMP containing female *P. disparis* pupa (z)	0-15 min Intact live WMP not parasitized 15-30 min Intact live WMP not parasitized

Exp.#	*N*	Stimuli tested	
		Stimulus 1	**Stimulus 2**
	20 (T4)	0-15 min *Intact* live WMP containing eclosed adult female *P. disparis* (w) 15-30 min *Intact* live WMP containing eclosed adult female *P. disparis* (x)	0-15 min Intact live WMP not parasitized 15-30 min Intact live WMP not parasitized
	21 (T4)	0-15 min *Intact* live WMP containing eclosed adult female *P. disparis* (y) 15-30 min *Incised* live WMP containing eclosed adult female *P. disparis* (z)	0-15 min Intact live WMP not parasitized 15-30 min Intact live WMP not parasitized
9	10	Oral secretion[g] of emerging female *P. disparis* applied to live WMP not parasitized	Intact live WMP not parasitized
10	12	Oral secretion[g] of emerging male *P. disparis* applied to live WMP not parasitized	Intact live WMP not parasitized

[a] Aliquots of 0.5 body-wash equivalents of host pupa, or *P. disparis* pupa (chemicals washed off 0.5 host pupa, or developing parasitoid, respectively) were bioassayed in each replicate

[b] Aliquots of 70 insect-hour equivalents (volatiles released by 70 parasitized wax moth pupae over one hour) were bioassayed in each replicate

[c] No *P. disparis* were yet emerged

[d] Some male *P. disparis* were emerging

[e] Some female *P. disparis* were emerging

[f] T Treatment

[g] One-insect-equivalent of oral secretion of emerging male or female *P. disparis* was bioassayed in each replicate

Table 5.2. Three-factor ANOVA comparing effects of, and interactions (×) between, parasitoid sex, developmental stage (pupal vs eclosed), and host pupal status (intact vs incised)

Effects tested	*F* Ratio	*P* Value
Experimental design 1: Parasitized pupae (intact/intact)		
parasitoid sex	0.51	0.48
parasitoid developmental stage	0.33	0.57
pupal status	2.1	0.15
parasitoid sex × parasitoid developmental stage	0.0020	0.96
parasitoid sex × pupal status	0.010	0.92
parasitoid developmental stage × pupal status	0.080	0.78
parasitoid sex × parasitoid developmental stage × pupal status	0.50	0.48
Experimental design 2: Parasitized pupae (intact/incised)		
parasitoid sex	0.58	0.45
parasitoid developmental stage	0.98	0.32
pupal status	110	<0.001
parasitoid sex × parasitoid developmental stage	0.048	0.83
parasitoid sex × pupal status	0.016	0.9
parasitoid developmental stage × pupal status	15	<0.001
parasitoid sex × parasitoid developmental stage × pupal status	2.0	0.16

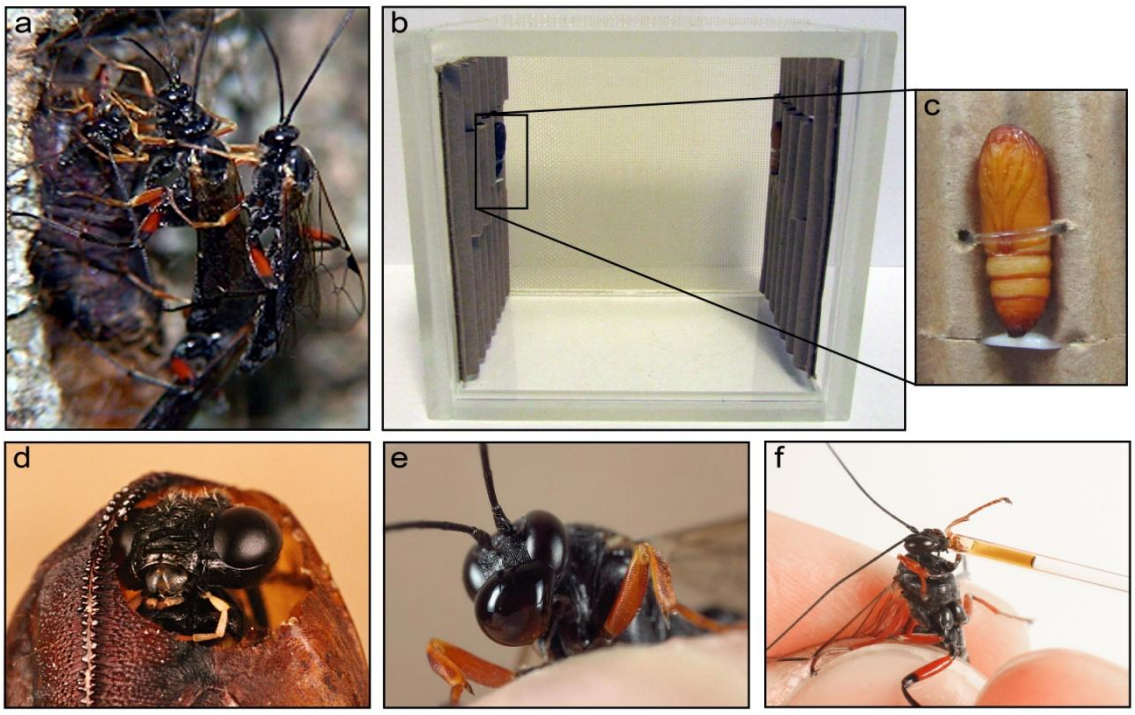

Figure 5.1. **a) Photograph of several male *Pimpla disparis* awaiting the emergence of a mate from a gypsy moth, *Lymantria dispar*, host pupa; b) experimental setup deployed in two-choice experiments 1-10, comprising a clear Plexiglas cage with mesh back, and sides lined with single-face corrugated cardboard; c) insert: close-up of wax moth, *Galleria mellonella*, host pupa affixed to cardboard with elastic thread, and supported at the base with a small cardboard plate; photographs of *P. disparis* d) emerging from a host pupal case, e) secreting oral fluid, and f) releasing fluid into a micro-capillary tube.**

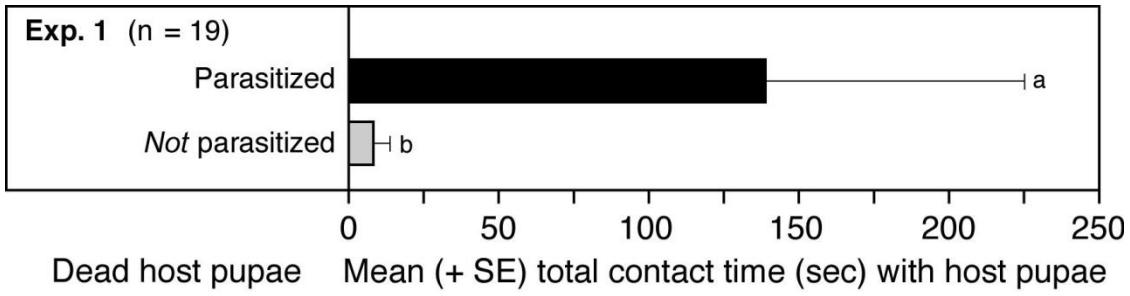

Figure 5.2. Mean (+ SE) total time (sec.) spent in experiment 1 (see Table 5.1) by 4–7-d-old virgin male *Pimpla disparis,* in a 15-min duration bioassay, at or near freeze-killed *Galleria mellonella* pupae, either 17 d post parasitism by female *P. disparis*, or not parasitized.

Note: A different letter on bars indicates a difference in response to test stimuli; Wilcoxon paired-sample test; $P < 0.05$

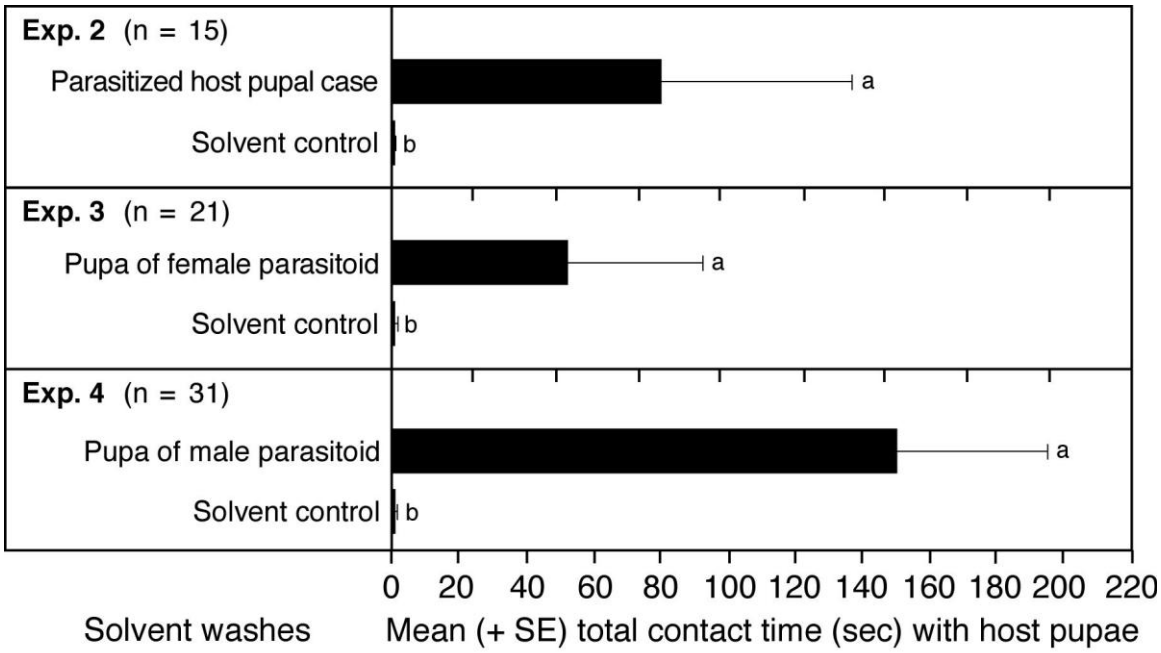

Figure 5.3. Mean (+ SE) total time (sec.) spent in experiments 2–4 (see Table 5.1) by 4–7-d-old virgin male *Pimpla disparis,* in a 15-min duration bioassay, at or near freeze-killed wax moth, *Galleria mellonella,* pupae treated with a 1 insect-equivalent wash of (*i*) intact *G. mellonella* pupae 17 d post parasitism (Exp. 2), (*ii*) 17-d-old female developing parasitoid (DePa) (Exp. 3), or (*iii*) 17-d-old male DePa (Exp. 4).

Note: In each experiment, different letters on bars indicate a difference in the response to test stimuli; Wilcoxon paired-sample test; $P < 0.05$

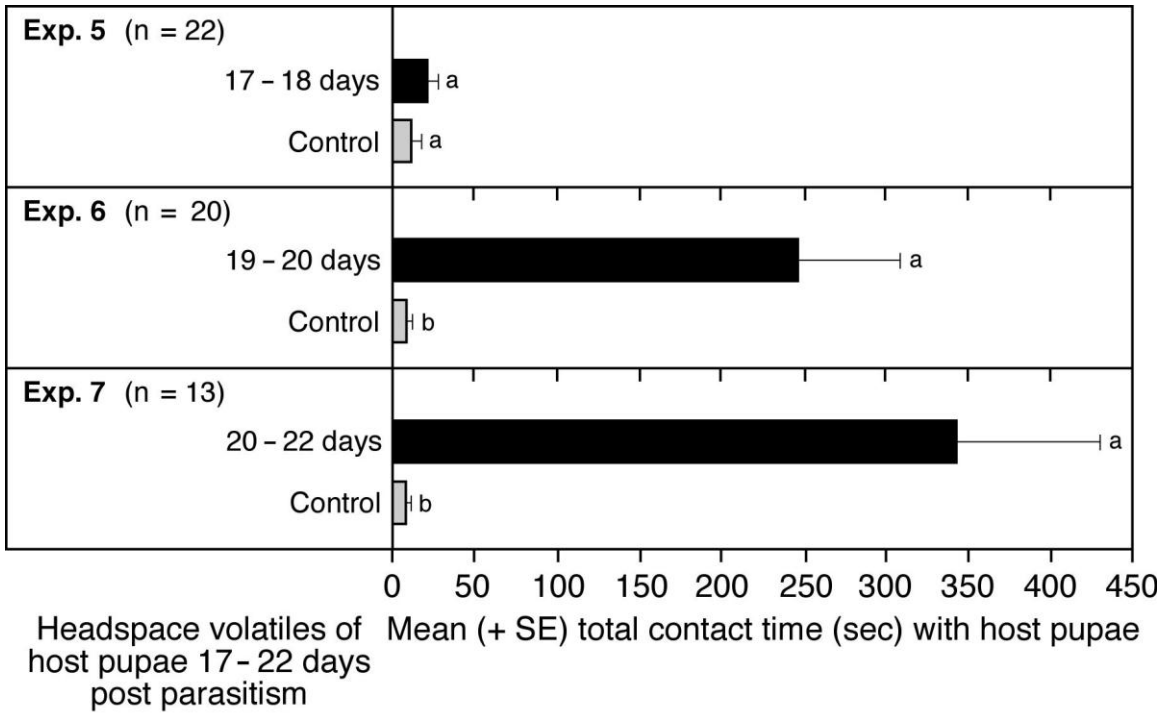

Figure 5.4. Mean (+ SE) total time (sec.) spent in experiments 5–7 (see Table 5.1) by 4–7-d-old virgin male *Pimpla disparis,* in a 15-min duration bioassay, at or near freeze-killed wax moth, *Galleria mellonella,* pupae treated with headspace volatile extract of live host pupae (*i*) 17–18 d post parasitism (with no parasitoids emerging yet) (Exp. 5), (*ii*) 19–20 days post parasitism (with some males emerging) (Exp. 6), or (*iii*) 20–22 days post parasitism (with some females emerging) (Exp. 7).

Note: In each experiment, different letters on bars indicate a difference in response to test stimuli; Wilcoxon paired-sample test; $P < 0.05$

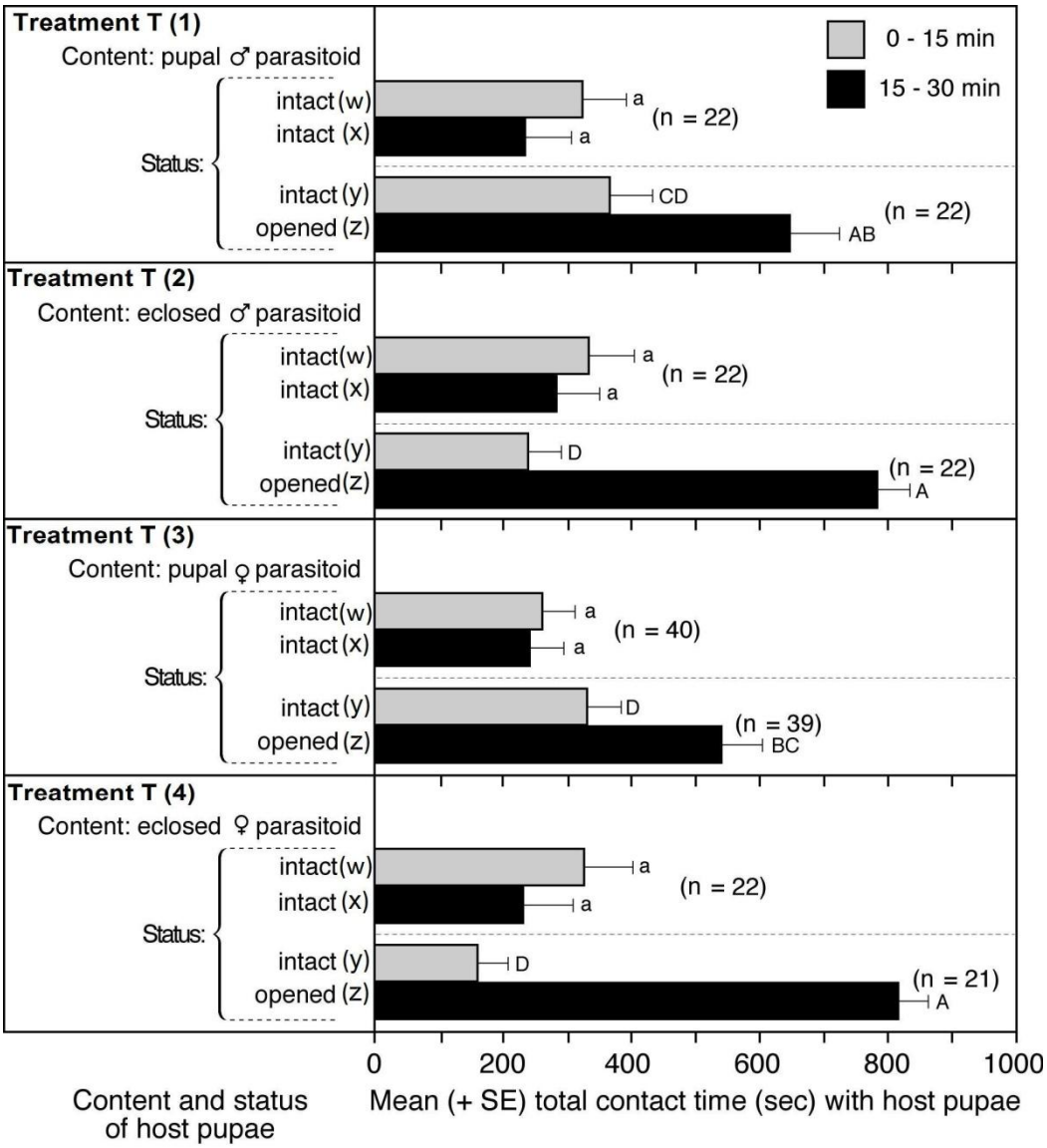

Figure 5.5. Mean (+ SE) total time (sec.) spent in experiment 8 (see Table 5.1) by male *P. disparis* in two consecutive bioassays, each of 15 min duration, at or near wax moth, *G. mellonella,* pupae 17–22 d post parasitism, that were kept intact or incised, and contained a male or female *P. disparis* pupa or a male or female eclosed adult. Paired intact/intact host pupae data were analyzed and assigned a lower case letter; paired intact/incised host pupae data were assigned an upper case letter. Within and between treatments 1-4, all bars with the same lower case letter superscript or the same upper case letter superscript do not differ. All data were analyzed by 3-factor ANOVA (see Table 5.2 for details).

Note: (1) more explanations of test stimuli w, x, y, and z are provided under experiment 8 in Table 5.1; (2) in treatments 2 and 4, “eclosed” refers to parasitoids that have eclosed within, but not yet emerged from, the host pupal case.

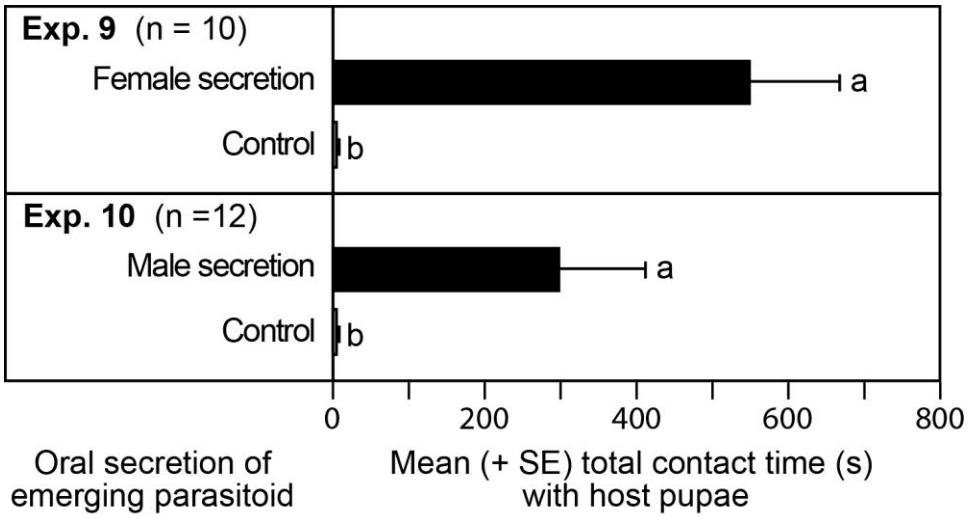

Figure 5.6. Mean (+ SE) time (sec.) spent in experiments 9-10 by 4–7-d-old virgin male *Pimpla disparis,* in a 15-min duration bioassay, at or near live wax moth, *Galleria mellonella,* pupae that were either treated with an oral secretion from an emerging female (Exp. 9) or male (Exp. 10) conspecific. The control consisted of a live untreated *G. mellonella* pupa.

Note: In each experiment, different letters on bars indicate a difference in response to test stimuli; Wilcoxon paired-sample test; $P < 0.05$

5.6. Acknowledgements

We thank Sean McCann and Stephen Takács for photographs and for technical support and advice regarding macro photography and video setup, Stevo DeMuth for graphical illustrations; Ian Bercovitz for statistical advice, Pilar Cepeda for assistance with insect rearing; Jessika Iwanski for assistance in bioassays and in organizing the quarantine facility, and two anonymous referees for meticulous reviews and constructive comments. Funding was provided by a Natural Sciences and Engineering Research Council of Canada (NSERC) – Discovery Grant and by an NSERC – Industrial Research Chair to G. G., with Contech Enterprises, SC Johnson Canada, and Global Forest Science as sponsors.

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Chapter 6.

Conclusions and Future Work

6.1. Conclusions

Life histories of parasitoids are incredibly diverse and often even “weird”, and in their complexity and intricacy are probably unequalled in the animal world (Vet et al., 2002). Parasitoids are not only important biological control agents of insect pests, they are also perfect model insects for answering fundamental questions in behavioral and evolutionary ecology. The ichneumonid parasitoid *Pimpla disparis* offered particularly intriguing phenomena for study, and thus became the target species for my thesis research.

In my thesis, I aimed to disentangle the intricate sexual communication system of *P. disparis*. I approached this study through field surveys and experimental manipulation, using state-of-the-art technology to answer the challenging question as to how males locate and communicate with prospective mates. This question was particularly intriguing because females remain hidden in host pupae just before they emerge and then immediately mate with a male awaiting her emergence. While female parasitoids are well known for their host location and selection ability (Vinson, 1984), nothing was known about how *P. disparis* males discern between host pupae that do, or do not, contain a prospective mate, and how males track her development and time of emergence.

Pheromone marking of host larvae or pupae is common in parasitic wasps. It reduces the incidence of super- and possibly multi-parasitism and furthers the survival of a female’s progeny. While the marker pheromone deters other females from re-attacking the same host it could also be a cue for *P. disparis* males that the host pupa contains a

future mate. In Chapter 2, I demonstrate that *P. disparis* males do indeed discriminate between marked (parasitized) and unmarked host pupae at early stages of parasitism, likely in response to the female marker pheromone.

Host-foraging parasitoid females home in on sounds or substrate vibrations produced by their hosts during various types of activities (Meyhöfer & Casas, 1999), or they detect concealed hosts through self-produced vibrations (Wäckers et al., 1998). Some parasitoid males and females communicate via mechanosensory signals during courtship (Vet et al., 2002). In Chapter 3, I provide evidence that mechanosensory signals or cues even play a role in the context of mate location. Mate-seeking males of *P. disparis* respond to mechanosensory cues from future mates that are still concealed inside host pupae. I show that the developing parasitoid (DePa), which is essentially the future mate, produces vibrations while spinning inside the host pupal case. I further show that physical parameters of these vibratory cues change in accordance with DePa's developmental progress. These changes could possibly inform a visiting male about DePa's stage of development and allow the male to gauge the time of emergence of his future mate.

Parasitoid females commonly learn in the context of host forging. They learn semiochemical cues associated with their hosts or plants damaged by their hosts. They also learn visual cues associated with their hosts such as colours, shapes and patterns, or the spatial information of host distribution and density. The ability of an insect to learn the spatial location of future or potential resources, including mates, as opposed to resources it has already used, has been reported in only a few species (Rosenheim, 1987; van Nouhuys & Ehrnsten, 2004; van Nouhuys & Kaartinen, 2008). In Chapter 4, I demonstrate that parasitoid males also possess this cognitive trait. Males of *P. disparis* are capable of detecting, learning and memorizing the location of future resources, here, host pupae housing a prospective mate.

In parasitoids, mate finding is primarily mediated by sex pheromones (Godfray, 1994; Quicke, 1997) which are often released by females and comprise components of different volatility that are effective at long or short range. Pheromonal communication typically takes place after the female has emerged, but in some parasitoids the males

detect pheromones from potential mates even before they emerge. In Chapter 5, I provide evidence for the presence of semiochemical cues that inform mate-seeking *P. disparis* males about the incipient or ongoing emergence of a prospective mate from a host pupa. The response of males to oral fluid semiochemicals from males and females indicates that mate-seeking males co-opt chemicals involved in the eclosion process as a mate finding cue, taking a 50% chance that the prospective mate is a female.

As female *P. disparis* do not appear to engage in active long-range signaling, meeting a female at her emergence site seems to be the only chance for a male to gain reproductive fitness. Males may encounter host pupae of various stages post-parasitism and appear to recognize parazitized pupae throughout DePa's development. To locate future mates, males seem to rely on different sensory modalities in a hierarchical manner. At the habitat level, males may respond to chemical and visual cues to locate patches of host pupae. At the patch level, males again may rely on chemical and visual cues to locate individual host pupae. To assess the status of host pupae, males apparently interpret sound and vibratory cues, and possibly sense a chemical cue (marking pheromone?) deposited by the parasitizing female. The latter option would likely be contigent upon recent parasitism of the host pupae. To monitor the development of future mates inside host pupae, males then memorize their locations, revisit them frequently, and track changes in sound and vibratory cues. Finally, they sense the emergence of a prospective mate based on chemical cues associated with the emergence process.

This strategy appears to be effective in a population with a stable sex ratio. However, when host pupae are scarce or when very few males are present to locate (parasitized) pupae, females would rarely be found and mated, and thus would rarely be able to produce daughters with whom they share more genes than with their sons. It is plausible, but would have to be tested experimentally, that in such a context an alternative mate-finding or mate-attracting strategy prevails, in which the emergent female might engage in active long-range pheromonal signaling.

6.2. Future Work

My research has answered important questions, but has generated many more questions. I will highlight some of these in the next couple of paragraphs.

I have shown that *P. disparis* females mark, possibly chemically, the host pupae they have parasitized, and that males discriminate between such pupae and those not parasitized. I have also shown that males recognize parasitized host pupae throughout the development of the parasitoid. If the marking is mediated by a marking pheromone, it would now be intriguing to identify this pheromone and to assess the time over which the pheromone has signal characteristics to both females and males.

I have demonstrated that DePas generate both vibratory and sound cues and that these cues change in accordance with DePa's development. The receptors that sense these cues are not known. Locating them (on legs or antennae?), describing their morphology, and studying the modes of signal reception and transduction would greatly further our understanding of sensory receptors in *P. disparis,* and in parasitoids in general.

I have shown that males co-opt oral fluid semiochemicals from males and females to pinpoint the time of emergence of a prospective mate. These fluids may originate from mandibular glands, as has been shown in other hymenopteran wasps. It would be interesting to dissect adult *P. disparis* for the presence of such glands, and to determine the glands' ultra-structure through histological methods. It would be equally interesting to identify the semiochemicals in gland extracts. This approach would require sophisticated analytical techniques including, but not limited to, coupled gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry. Preliminary data indicate that key semiochemicals may be present in only trace quantities and are not be easily identified (AD, unpublished data).

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Appendix A

Supplementary material

Additional supporting information can be found in the online version of the articles under the DOI reference, or the files can be accessed from the SFU Library website.

Chapter 3

Supporting information [Danci, A., Inducil, C., Takács, S., Schaefer, P. W., and Gries, G. (2014) Physiological Entomology, 39(4): 292–303. DOI: 10.1111/phen.12075]

Audio S1. Sound recording of a *Pimpla disparis* larva 8 days post parasitism of the host pupa.

Filename: audioS1.mp3

Audio S2. Sound recording of a *Pimpla disparis* pupa 14 days post parasitism of the host pupa.

File name: audioS2.mp3

Audio S3. Sound recording of a *Pimpla disparis* pupa 18 days post parasitism of the host pupa.

File name: audioS3.mp3

Video S1. Video of spinning 14-day-old *Pimpla disparis* pupa inside host pupa.

File name: videoS1.mp4

Chapter 5

Supporting information [Hrabar, M., Danci, A., Schaefer, P. W., and Gries, G. (2012) Journal of Chemical Ecology, 38(3): 253–261. DOI: 10.1007/s10886-012-0079-9]

Video S2. Video of adult *Pimpla disparis* chewing its way out of the host pupal case (Video by Michael Hrabar).

File name: videoS2.mp4