Local Mate Competition, and Mechanisms, Functions and Fitness Consequences of Courtship and Mating Behaviour in the Parasitoid Wasp *Ooencyrtus kuvanae* (Hymenoptera: Encyrtidae)

by

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M.Sc., Oxford Brookes University, 2006
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Dissertation Submitted In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Department of Biological Sciences Faculty of Science

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Abstract

Males and females of the egg parasitoid wasp *Ooencyrtus kuvanae* (Howard) (Hymenoptera: Encyrtidae) emerge as sexually mature adults from gypsy moth, *Lymantria dispar* (L.), host egg masses. Sibling or non-sibling males compete intensely for mating opportunities with spatially clustered monandrous females who are briefly receptive. Mated females typically disperse prior to ovipositing, whereas males disperse in search of mates. My main objectives were to examine the occurrence of local mate competition (LMC), and to tease apart the mechanisms, functions, and fitness consequences of courtship and mating behaviour in *O. kuvanae*.

As predicted by LMC theory, with increasing numbers of foundresses on a host egg mass, the proportion of emerging males increases. Males exhibit one of two mating tactics, a mate-at-once (MAO) tactic, and a harem-gathering and -guarding (HGG) tactic. MAO males immediately mate any receptive female they encounter. HGG males mate the first receptive female they encounter, then transfer a unique pheromone-tag to females without prior male contact, and finally relocate and mate those females they themselves have tagged. Females do not incur direct fitness costs by mating with multiply-mated males.

Males are attracted to a close-range female sex pheromone comprising (5S)-methylheptacosane and (5R,17S)-dimethylheptacosane. Conversely, males are repelled by the blend of (5R)-methylheptacosane and (5R,17R)-dimethylheptacosane. This suggests that the stereochemistry of these two hydrocarbons may differ between males and females, and that it could be an underlying mechanism in mate recognition and mate assessment.

Immediately prior to copulation, males engage females in a brief pre-copulatory ritual, then mate, and thereafter execute a lengthier post-copulatory ritual. Both rituals entail physical interactions rather than pheromone transfer. Following the pre-copulatory ritual, females enter a receptive state that persists after copulation, whereby a female is susceptible to additional copulations by sneaker males, who compete with the first male to mate for post-copulatory ritual rites. The post-copulatory ritual accelerates the awakening of an in-trance female, who then never mates again. First male sperm precedence lies with the first male to engage a female in the post-copulatory ritual. Therefore, the ritual may represent a male adaptation to prevent sperm competition.

**Keywords:** Alternative reproductive tactics; Sex pheromone; Courtship ritual; Local mate competition; Sperm precedence; Kin discrimination
To Logan and Evan, my extraordinary nephews, who always remind me that the unknown marks the beginning of a journey, and that there is always adventure in searching for the answers.
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Lastly, I’d like to acknowledge those supportive and loving family and friends that passed while I was working on this degree; they will now cross the finish line with me in spirit. In memory of Geneva Ablard, Dorothy Turpen, Jane Gale, and Maureen Curran.
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# Glossary

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternative reproductive tactics (ARTs)</td>
<td>Behavioral traits and other traits used in mating. The tactics may be part of one or more strategies (i.e., different strategies may employ the same tactic, though they would do so somewhat differently(^1)).</td>
</tr>
<tr>
<td>Alternative strategy</td>
<td>Individuals are genetically polymorphic, carrying different alleles linked to different strategies. Each strategy results in equal fitness under frequency-dependent selection (Gross, 1996).</td>
</tr>
<tr>
<td>Enantiomer</td>
<td>One of a pair of molecular entities (e.g. one of two stereoisomers) which are mirror images of each other and non-superposable; also known as “optical isomers” (IUPAC, 1997).</td>
</tr>
<tr>
<td>Quasi-gregarious</td>
<td>Solitary species that develop in hosts that are aggregated (Damiens and Boivin, 2005).</td>
</tr>
<tr>
<td>Quiescent (see “trance”)</td>
<td>In a state or period of inactivity or dormancy.</td>
</tr>
<tr>
<td>Ritual</td>
<td>A series of actions or type of behaviour regularly and invariably followed (Oxford Dictionaries, 2012).</td>
</tr>
<tr>
<td>Solitary parasitoid</td>
<td>Only one larva per host completes development. (Mackauer and Chau 2001).</td>
</tr>
<tr>
<td>Stereoisomer</td>
<td>Isomers that possess identical constitution, but which differ in the arrangement of their atoms in space (IUPAC, 1997).</td>
</tr>
<tr>
<td>Strategy</td>
<td>The decision rules of an individual. Genetically-based - when there are alternative strategies, the population is genetically polymorphic, with individuals carrying different alleles linked to different strategies. Each strategy results in equal fitness under frequency-dependent selection (Gross, 1996; R. Ydenberg, pers. comm.).</td>
</tr>
<tr>
<td>Tactic</td>
<td>Behaviour resulting from the decision rule. A strategy may give rise to different behavioral tactics in different situations (R. Ydenberg, pers. comm).</td>
</tr>
<tr>
<td>Trance (see “quiescent”)</td>
<td>A […] state characterized by an absence of response to external stimuli […] (Oxford Dictionaries, 2012).</td>
</tr>
</tbody>
</table>

\(^1\) References listed in Introduction chapter under the “Literature cited” section.
Figure A. *Ooencyrtus kuvanae* male (L) and female (R) (Photo by Sean McCann)
1. Introduction

In the introductory chapter, I will introduce the main topics that my research focused on, provide a rationale for my choice of study specimen, and present an overview of some of the outstanding questions or desired research associated with these topics. I will then provide a general background to mating systems, mating tactics, mating stages (pair formation, pre-copulatory, copulatory, and post-copulatory behaviour) within a mating sequence, sex pheromones, local mate competition, and sperm competition in some species of the Hymenoptera, with emphasis placed on parasitoid wasps. Following this, I will provide a more thorough background on my study specimen, the parasitoid wasp *Ooencyrtus kuvanae*, discussing the biology of *O. kuvanae* within the context of my research. I will present a brief summary of each research chapter, highlighting the main hypotheses, methods, and some of the key results. Finally, I will briefly touch on the importance of my thesis and how my findings will contribute to our understanding of mating systems in parasitoid wasps.

1.1. Dissertation intent

My thesis explores mating behaviour, alternative reproductive tactics (ARTs), chemical communication, local mate competition, and sperm competition in a parasitoid wasp. Previous research on these topics often has been undertaken with an intra-disciplinary approach, and in some cases revealed only the mechanism, or the function, underlying the behaviour, and the fitness consequences associated with it (Allen et al., 1994; King and Fischer, 2005; Shuker et al., 2006; Johansson and Jones, 2007; Brockmann, 2008).

Consequently, I embarked on an interdisciplinary approach throughout my research to explore both the mechanisms and functions as well as the fitness consequences associated with these processes in an attempt to address outstanding
questions in the pertinent literature, conduct novel research, and provide new avenues for future work on mating system theory and local mate competition.

Parasitoid wasps are model organisms for research within these contexts. Their mating systems are diverse (Godfray, 1994) and the stages that make up a mating sequence can be teased apart experimentally (van den Assem, 1986). Sexual selection, which is associated with ARTs, is usually intense (Brockman, 2008), and hymenopterans are at the forefront of studies on chemical communication (Ayasse et al., 2001). There are high levels of local mate competition (Quicke, 1997), and there is evidence of sperm competition (Simmons, 2001).

For these reasons, my research contributes to mating system theory by adding to our understanding of the conditions that apply, the degree to which each sex controls stages of a mating sequence (e.g., pair formation, pre-copulation, copulation, and post-copulation), and the underlying mechanisms of each stage (Brown et al., 1997). Teasing apart stages of a mating sequence may also reveal the presence of male ARTs based on a theoretical model that predicts at least two ARTs can coexist when males intensely compete for monandrous females that are numerous, spatially clustered, and synchronously receptive for a short period of time (Shuster and Wade, 2003); these characteristics are common in many parasitoids (Godfray, 1994). Examination of ARTs can advance our understanding as to why particular phenotypes take the form they do (Taborsky et al., 2008).

Courtship and/or mating behaviour of hymenopterans are to some degree mediated by sex pheromones that consist of cuticular hydrocarbons (CHCs) (Ayasse et al., 2001). Thus far, sex pheromones have been identified in many species of insects, of which social hymenopterans exhibit some of the most diverse sex pheromones (Ayasse et al., 2001), including recognition pheromones and mate assessment pheromones (Johansson and Jones, 2007). Although there has been extensive research on these sex pheromones, there is still a need for more research on pheromones that trigger aspects of mating behaviour other than attraction (Ayasse et al., 2001). And even when pheromone components that mediate courtship and mating behaviour have been identified, research has not always tested synthetic components in bioassays (Ayasse et al., 2001). There is also a need for interdisciplinary research that combines exploration
of chemical and behavioural processes to further our understanding of pheromones that function in mate recognition and mate assessment, and the source of pheromones in parasitoids (Guerrieri et al., 2001).

Antennation is a mechanism underlying the transfer of close-range and contact sex pheromones in parasitoids during courtship and/or mating. This is because antennae can be the site of pheromone release and detection. Some pheromone glands have been identified in the antennae of male parasitoids (Guerrieri et al., 2001; Romani et al., 2008; Steiner et al., 2010); however, antennal glands of female parasitoids are known in very few species (Ayasse et al., 2001).

Research on sex allocation by female parasitoids and local mate competition (LMC) has been extensive. Unfortunately, the effect of local resource competition (LRC) among larvae is one of the most important confounding factors in experimental studies of LMC (Godfray, 1994). Research on a parasitoid such as O. kuvanae that meets all the assumptions of LMC, but whose larvae do not compete for local resources (and thus do not distort the sex ratio in favor of sons) could shed light on existing LMC theory.

Insects are ideal models for exploring adaptations to sperm competition because there are numerous behavioural, morphological and physiological mechanisms underlying the transfer, manipulation, removal, and utilization of sperm (Simmons, 2001). Research thus far on sperm competition in parasitoids has been focused primarily on behavioural traits, with less emphasis on morphological traits that function to reduce or avoid sperm competition, and the mechanisms mediating these traits. In addition, paternity testing has been primarily based on the use of allozyme markers and phenotypic markers; DNA microsatellite analysis has never been implemented for paternity testing in an egg parasitoid.

1.2. General background

Mating systems of animals rely on behavioral, morphological and physiological characteristics that will maximize reproductive success prior to, during and post copulation (Arnqvist and Rowe 2005). Since females are most often the limiting sex (since they often have greater parental investment) in mating systems, selective forces
that shape male sexual behavior can include female choice, spatial and temporal occurrence of females, and their reproductive biology (Thornhill and Alcock 1983; Godfray, 1994; Eberhard, 1996).

Female choice in parasitoids has been suggested to take the form of cryptic choice post-copulation, in the parasitoid wasp *Nasonia vitripennis* (Beukeboom, 1994). However, there is also evidence that *N. vitripennis* females choose a more sexually attractive mate prior to copulation (Blaul and Ruther, 2011). This is because ovipositing females can positively influence the attractiveness of their sons, by selecting hosts that are a good source of nutrients. Well nourished sons could be perceived as high-quality mates who are able to advertise their quality to females (Godfray, 1994), giving them a competitive advantage not only over siblings, but also non-siblings.

Consequently, competition among males can be intense even though a female-biased sex ratio, commonly seen in parasitoids, is thought to reduce competition among males for mating opportunities (Hamilton 1967; Taylor 1981; Abe et al., 2007). Intense competition between males can give rise to ARTs which are influenced by spatial, temporal, and reproductive parameters in those female parasitoids that mate only once in their lifetime, synchronously emerge as sexual adults, and mate at their emergence site (Quicke, 1997; Shuster and Wade, 2003). Male ARTs (not to be confused with alternative strategies, conditional strategies or mixed strategies; see Glossary) in insects are very common, and can take numerous morphological forms as seen in the fig wasp *Pseudidarnes minerva* (Cook et al., 1997), or behavioural forms as seen in the parasitic wasp *Cotesia rubecula* (Field and Keller, 1993), to obtain copulations (Brockmann, 2008).

Body size (Brockmann, 2008), speed (Byers et al., 2010), sexual experience (Clutton-Brock et al., 1989), high sensitivity to female pheromone for locating virgins (Carde and Baker, 1984), and/or the ability to successfully perform pre- and post-copulatory rituals ["ritual" meaning a series of established behaviours] (van den Assem, 1986), also influence the choice of ART as these traits are indicators of male endurance and quality which can convey overall health (Rantala et al., 2002), and thus may function as a passive mechanism by which a choosy female will mate with the quality male (Wiley and Poston, 1996).
Some female defense polygynous mating systems, whereby males compete for multiple mating opportunities and prevent rival males from gaining access to mates by guarding harems, or a series of individual females (Thornhill and Alcock, 1983), are highlighted by pre- and post-copulatory rituals. Mating sequences with both a pre- and post-copulatory stage are known to occur in some parasitoid wasps (Table 1.1). These rituals are known among a small number of parasitoids to be behaviourally similar in mechanism (Alzofon, 1984; van den Assem, 1986), but the underlying function(s) of these rituals are poorly understood (Kajita, 1986; Allen et al., 1994; Brown et al., 1997; Quicke 1997).

Pre-copulatory rituals of parasitoids in the Pteromalidae and Aphelinidae entail repetitive, stereotypic strikes of the female by the courting male (van den Assem and Povel, 1972; van den Assem, 1986; van den Assem et al., 1989) that place her in a "trance-like" (unmoving, unresponsive) receptive state (henceforth “trance”) prior to, and during copulation (Khasimuddin and Debach 1975; van den Assem, 1989). Pre-copulatory rituals in males can also take the form of mate guarding by (i) staying in contact with a female through physical mounting as in Aphytis melinus (Allen et al., 1994), (ii) non-physical contact by fighting off rival males prior to mating as in Melittobia spp. (van den Assem, 1986), or (iii) by guarding in absentia [e.g., female mimicry] as in Lariphagus distinguendus (Ruther and Steiner, 2008).

Although pre- and post-copulatory rituals can be similar behaviourally, post-copulatory rituals tend to take longer (Table 1.1), may entail non-stereotypic patterns, as in Spalangia endius (King and Fischer, 2005), and may be a male adaptation to reduce or prevent sperm competition (Brown et al., 1997; Simmons, 2001). By engaging in a post-copulatory ritual males gain the time to remove rival sperm, manipulate the female’s storage of sperm in favour of their own, and/or inhibit the female’s receptivity through effective guarding, as in A. melinus (Allen et al., 1994). Post-copulatory rituals can take the form of physical-contact mate guarding (Parker, 1974) as in the Aphelinidae (Vigiani and Battaglia, 1983), non-physical-contact mate guarding as in C. rubecula (Field and Keller, 1993), or guarding in absentia via sex pheromone that function to inhibit receptivity and curtail attractiveness, as suggested in S. endius (King and Fischer, 2005).
Sex pheromones are defined as odours produced by either males or females that are involved in the process of mate choice, with three different types of pheromone prevailing. Species-recognition pheromones function to distinguish individuals of different species and reproductive status; mate-recognition pheromones function in advertising sex, receptivity, and specific phases or coordination of sexual behaviour between the sexes, mate-assessment pheromones serve individuals of one sex to discriminate between individuals of the other sex (Johansson and Jones, 2007). Unlike species- and mate-recognition pheromones, mate-assessment pheromone must convey the identity and quality of the sender. Also, species-recognition, mate-recognition, and mate-assessment pheromones are not mutually exclusive.

Sex pheromones play a critical role in sexual communication among hymenopterans (Ayasse et al., 2001). In solitary and quasi-gregarious parasitoid species, where mating typically occurs at the emergence site, the emission of long-range sex pheromones is uncommon (Godfray, 1994). Instead, close-range pheromones mediate mate attraction as shown in the Braconidae (Syversten et al., 1995), Chalcididae (Mohamed and Coppel, 1987), Trichogrammatidae (Pompanon et al., 1997), Pteromalidae (van den Assem et al., 1980; Sullivan 2002), Ichneumonidae (Shu and Jones 1993), and Aphididae (McClure et al., 2007). Close-range sex pheromones can also facilitate mating stages within a mating sequence (van den Assem and Povel, 1972; Takahashi and Sugai, 1982; Godfray 1994; Ruther et al., 2000; Steiner et al., 2006), sexual inhibition, and recognition (Singer, 1998; Nichols et al., 2010).

Sex pheromones that function within a close range are mostly comprised of complex blends of CHCs. (Ayasse et al., 2001). Cuticular hydrocarbon (CHC) compounds can differ because of methylated branches with chiral centers that give rise to enantiomers (Mori, 2007). There is increasing evidence that many species can discriminate between enantiomers resulting in intra- and inter-specific communication as shown in the solitary bee Andrena wilkella (Tengo et al., 1990).

Intra- and inter-specific communication can also be achieved through quantitative or other qualitative differences in the CHC pheromone blend, or via differential release rates of the pheromone blend (Johansson and Jones, 2007). Unique body odour of individual specimens has been shown in badgers, shrews and deer (Cantoni et al., 1996;
Lawson et al., 2000; Buesching and Macdonald, 2004), and only implied for *Drosophila* fruit flies (Billeter et al., 2009). Unique body odour is likely to occur also in those hymenoptera which use recognition pheromones as part of their communication system, as shown in bees and ants where kin recognition relies on nest- and colony-specific pheromones (Vander Meer and Alonso, 2002; Ozaki et al., 2005; Herzner et al., 2006; van Wilgenburg et al., 2006; Sramkova and Ayasse, 2009).

Specimen-specific pheromone (unique body odor) might be expressed by genes analogous to those that regulate recognition pheromones across taxa or by phenotypic plasticity (Ghalambor et al., 2010). For example, *Desaturase 1* and *Desaturase 2* genes in insects have been shown to produce enzymes that govern the biosyntheses of CHCs and cause variation in CHC profiles (Coyne et al., 1999). The underlying intraspecific molecular mechanisms for CHC biosynthesis are not yet fully understood but they do play a major role in honeybees (Barchuk et al., 2007) and ants (Vander Meer et al., 1989). Moreover, insects may evolve different phenotypes by altering the genes they express. Phenotypes may differ in morphological, physiological or behavioral traits with simple or complex genetic bases (Takahashi et al., 2001).

Specimen, species, nest, colony, and kin recognition could mechanistically be based on the phenotype-matching model (Holmes and Sherman, 1982). This model demonstrates that animals with high capabilities to learn, such as parasitoids (Godfray, 1994), learn their CHC phenotype, or that of others, and then compare the unfamiliar type to their learned recognition template, as in the desert queen ant (Lahav et al., 1998).

Kin recognition in species that inbreed, such as many parasitoid wasps, has been thought to affect a female’s adjustment of offspring sex ratio; however, kin recognition, as an effect of relatedness, has not been demonstrated in parasitoid wasps (Reece et al., 2004; Shuker et al., 2004b; Martel et al., 2008). Local mate competition (LMC) is one of the most important factors affecting a female’s adjustment of offspring sex ratio (Hamilton, 1967; Cook et al., 1994). In order for LMC to exist, certain assumptions should be met within population structures. Firstly, the LMC theory is based on the premise that a female is able to adjust the sex ratio of her offspring (Shuker et al., 2005). This is possible due to the haplodiploid sex determination system.
which enables females to control whether they allow an egg to be fertilized and produce a diploid daughter, or not fertilized, and produce a haploid son. In these systems, mothers are more closely related to their daughters than to their sons, so are likely to favor the production of daughters, resulting in a female-biased sex ratio. Secondly, mating will frequently take place between siblings on discrete patches, and brothers will compete for mates (Nadel and Luck, 1992; Flanagan et al., 1998).

However, when females are ovipositing in the presence of other females on a discrete patch, the theory of LMC predicts that the optimal sex ratio will become more male-biased as the number of foundresses increases (Waage and Lane, 1984). This is based on the idea that when at least two mated females oviposit in the same patch, their sons will compete for mating opportunities not only among themselves but also among non-siblings. Adjusting the sex ratio to be more male-biased improves the chance of outcompeting rival males thereby securing more mating opportunities.

The prediction is based on Hamilton’s (1967) equation \[ r = \frac{(n-1)}{2n} \], but was later modified to incorporate haplodiploidy \[ r = \frac{(n-1)(2n-1)}{n(4n-1)} \] (Hamilton, 1979). It functions under the pretense that as the number of ovipositing foundresses (n) in a patch increases, the optimal sex ratio of males (r) will increase. For a number of parasitoid wasp species, notably \textit{N. vitripennis} (Werren, 1983; Frank, 1985; King and Skinner, 1991; Luck et al., 2001), experimental manipulations of foundress numbers have confirmed this prediction of LMC.

Relatedness among foundresses is another factor that is thought to affect optimal offspring sex ratio in structured populations. If female parasitoids can recognize kin, it has been suggested that siblings ovipositing on the same patch should produce a lower proportion of sons, thus further reducing competition among sons (Frank, 1985; Taylor and Crespi, 1994; Shuker et al., 2004a) and increasing their chances of mating within a narrow time constraint.

Narrow time constraints for males to mate with monandrous females can also select for male adaptations to sperm competition. Sperm competition takes place in a female when there is a temporal and spatial overlap of sperm from at least two males to fertilize ova (Simmons, 2001). There is evidence of sperm competition in \textit{L.}

8
distinguendus, whereby the first male to mate sires a greater proportion of the offspring (van den Assem et al. 1989), and also in N. vitripennis, where greater proportions of offspring are sired by the first male to mate, and in some cases, by the second male to mate (Holmes, 1974; Beukeboom, 1994).

Adaptations to reduce, or prevent sperm competition among insects can include specialized genitalia, longer durations in copula, many copulation bouts, and variations of post-copulatory behaviour as a form of mate guarding (Gwynne, 1984; Thornhill, 1984).

First-male sperm precedence can be achieved through specialized genitalia in insects, which can take the form of a long aedeagus that delivers sperm to a female storage organ such that this sperm is the first, and sometimes only sperm a female will use (Simmons, 2001). Last-male sperm precedence can be achieved by males with specialized aedeagi covered in structures, such as spines, that function to scoop sperm out from the first male to mate, as seen in the damselfly Calopteryx maculate (Waage, 1979).

Sperm precedence can also be achieved by males which deliver more sperm over a longer duration, or engage in repeated bouts of copulation that flush out the sperm of the first male to mate (Simmons, 2001). The latter behaviour would be advantageous only for males which mate with females that have an expandable or elastic spermatheca. An inelastic spermatheca can cause displacement of sperm when the spermatheca approaches its carrying capacity, as shown in the wasp Dahlbominus fuscipennis (Wilkes, 1966). Under these conditions, sperm from the first male to mate enters the spermatheca, and then travels to the spermathecal duct which enters into the vagina where fertilization takes place. Sperm from the second male to mate accumulates at the opening of the spermatheca with entry impeded (Wilkes, 1966). Thus, the first sperm to enter and fill the spermatheca is the first to leave through the spermathecal duct (Simmons, 2001), resulting in first-male sperm precedence.

Sperm precedence may also rely on effective pre- and post-copulatory rituals by males. Specifically, post-copulatory rituals as a form of mate guarding are selected for as the risk of sperm competition increases. This is evident in A. melinus males. The
proportion of offspring they sire correlates with the time they spend guarding a female against rival males (Allen et al., 1994). Post-copulatory rituals can also function in the acceptance and storage of sperm, and/or be a foundation for a female’s assessment of males (Thornhill and Alcock, 1983), but evidence of these particular functions are lacking in parasitoids.

1.3. Study specimen

My study specimen was the solitary egg parasitoid wasp *Ooencyrtus kuvanae* (Hymenoptera: Encyrtidae). It was introduced to the U.S.A. from Japan in 1908 as a biological control agent of the gypsy moth, *Lymantria dispar* (Brown, 1984). Currently, it is established throughout Europe (Tryapitsin, 1989; Zerova, 1989), Africa, and the Eastern United States (Brown, 1984) where it is still utilized as an effective biological control agent.

As a result, most research on *O. kuvanae* has been tailored toward its multivoltine life history patterns (Crossman, 1925; Dowden, 1961; Hofstetter and Raffa, 1997), host-foraging behaviour (Bellinger et al., 1988; Hofstetter and Rafa, 1998), female oviposition behaviour (Tadic and Bincev, 1959; Weseloh, 1972) and population dynamics (Schieferdecker, 1969; Odell et al., 1989).

The mating system of the 2-mm long *O. kuvanae* takes place on host gypsy moth egg masses, which measures 2-3 cm in diameter and comprises several hundred eggs. Eggs in the uppermost layer (15-20%) are parasitized by 2-3 foundresses (Crossman, 1925) that insert a single egg into each accessible host egg. Females can chose to fertilize an egg and produce a daughter, or not to fertilize it, and produce a son. Haploid sons derive their entire genome from their mother, whereas daughters are diploid and receive genes from both parents. Son and daughter wasps complete development inside the host egg and emerge as sexually mature adults after 3-4 weeks of development. Although the sex ratio in *O.kuvanae* is female-biased, a male-biased sex ratio may occur as it was inferred that parent females are greatly influenced by LMC (Cook et al., 1994), but empirical data are still lacking. After a male has secured a female by engaging in either the HGG or MAO tactic, a male engages a female in a brief pre-copulatory ritual,
then mates and performs a post-copulatory ritual, which lasts significantly longer (Alzofon, 1984). Alzofon (1984) observed that (i) during the pre-copulatory ritual the male uses his antennae to make contact with the female’s antennae, (ii) during the post-copulatory ritual the male uses his antennae, forelegs, and head to make contact with the female, and (iii) that the female contacts the male with her mouthparts. If a female does not engage in the post-copulatory ritual, she can mate repeatedly up to four times (Alzofon, 1984). Whether a female re-mates after having engaged in the post-copulatory ritual is unclear (Alzofon, 1984).

Mated females may disperse (Tadic and Benciv, 1959) after 24 hours seeking new hosts to parasitize (Hofstetter and Raffa, 1998); females can parasitize as many as 200 eggs in the field (see Brown, 1984). Unlike females, males typically remain on the host egg mass and wait for newly emerging females (Tadic and Benciv, 1959).

1.4. Chapter summaries

My thesis consists of seven chapters, including this brief introductory chapter, five research chapters and a concluding chapter summarizing major findings. The thesis is organized as an article-style thesis. Research chapters closely resemble manuscripts that have already been published (Chapters 3-5), or are submitted for review (Chapters 2 and 6). Each research chapter (manuscript) is presented in the format prescribed by the journal that has published, or may publish, the manuscript. It typically comprises an abstract, introduction, materials and methods, results, discussion and a reference list. In the following sections, I highlight the content of each research chapter and the concluding chapter.

In Chapter 2, I explore whether alternative reproductive tactics (ARTs) by *O. kuvanae* males coexist, as predicted by models with parameters that apply to *O. kuvanae* females which are spatially clustered and numerous. Using behavioural bioassays, high-speed cinematography and environmental scanning electron microscopy (ESEM) as experimental techniques, I demonstrate that male *O. kuvanae* exhibit two ARTs, a mate-at-once (MAO) tactic and a harem-gathering and -guarding (HGG) tactic. MAO males invariably mate any susceptible female immediately upon
encounter, whereas HGG males typically mate the first receptive female they encounter, then pheromone-tag those females without prior male contact, and mate with them at a later time.

In Chapter 3, I test the hypothesis that *O. kuvanae* females use a close-range sex pheromone that attracts males during courtship. Gas chromatographic - mass spectrometric analyses of male and female body surface extracts reveal two candidate pheromone components with one and two chiral centres, respectively, that allow for eight possible combinations of optical isomers. In a series of behavioural bioassays with blends of stereoselectively synthesized isomers, I show that a certain blend attracts males whereas another repels them. The results suggest that these isomers may function in both mate attraction and mate assessment.

In Chapter 4, I explore the mechanisms and functions of the pre- and post-copulatory rituals in the *O. kuvanae* mating sequence, using behavioural bioassays, high-speed cinematography, and gas chromatography - mass spectrometry of body surface extracts. I reveal that males engage females in a pre-copulatory ritual that consists of stereotypical physical interactions, and functions to put females into a trance-like quiescent state. Conversely, the post-copulatory ritual relies on non-stereotypical physical interactions by males that function as a form of mate guarding in that the male accelerates the awakening of in-trance females, who will then reject mating attempts by other males.

In Chapter 5, I explore whether local mate competition (LMC) occurs in *O. kuvanae* because life history traits of *O. kuvanae* meet the assumptions of LMC. I test this by manipulating the number of wasp foundresses on host gypsy moth egg masses. I show that with increasing numbers of wasp foundresses on an egg mass, the proportions of emerging sons increases. These results support the conclusion that LMC applies to the *O. kuvanae* mating system.

In Chapter 6, I investigate sperm competition between *O. kuvanae* males. I manipulate females to mate with two males, recording the following parameters: (i) the order in which males mate, (ii) the time they remain *in copula*, (iii) the number of post-copulatory rituals, and (iv) the duration of post-copulatory rituals. I show that the
microstructure of the males’ aedeagus is not conducive to removing sperm of a prior mate from the female, and that copulation duration and number of copulations do not affect paternity. By DNA microsatellite analyses of parents and daughters, I reveal that the second male to mate can sire offspring, but that there is strong first male sperm precedence in those males who begin the post-copulatory ritual first. These results suggest that the post-copulatory ritual is an adaptation to sperm competition.

In Chapter 7, I tie all the chapters together, summarize key findings and propose future research directions.

1.5. Research significance

My dissertation provides an example of interdisciplinary research that combines behavioural, chemical, and molecular processes to explore the mechanisms and functions associated with stages of a mating sequence, and their fitness consequences. This approach deepens our understanding of selective forces that may shape mating systems of other parasitoids, and it reveals intricacies of intra- and inter-sexual communication in the Insecta.

Moreover, I apply new experimental methods to study the mating system of parasitoid wasps. As a result, I have discovered novel behaviours and I could contribute to the interpretations of previously reported data. These new methods, or modifications thereof, may prove useful in future studies of parasitoid mating systems.

Finally, O. kuvanae afforded me the opportunity to study LMC for the first time in a quasi-gregarious parasitoid. I could explore its behaviour outside the context as a biological control agent, and contribute to the currently little knowledge on this species’ basic biology, and the genus as a whole. I have studied an insect mating and communication system, but many of my findings compare in mechanism and function to vertebrates.
Table 1.1. *Occurrence [Y = yes; N = no] and duration (s) of pre-copulatory (pre-cop.) and post-copulatory (post-cop.) behaviour in parasitoid wasps*

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Brood</th>
<th>Pre-cop. association &amp; duration (s)*b</th>
<th>Post-cop. association &amp; duration (s)b</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aphelinidae</strong></td>
<td>A. lingnanensis</td>
<td>G</td>
<td>Y = PC (3.30±1.37)</td>
<td>Y = PC (105.3±37.3)</td>
<td>Gordh &amp; Debach, 1978</td>
</tr>
<tr>
<td></td>
<td>A. maculicornis</td>
<td>S</td>
<td>Y = PC (ca. 4)</td>
<td>N</td>
<td>Khasimuddin &amp; Debach, 1975</td>
</tr>
<tr>
<td></td>
<td>A. melinus</td>
<td>G</td>
<td>Y = PC (5)</td>
<td>Y = PC (ca. 149)</td>
<td>Allen et al., 1994</td>
</tr>
<tr>
<td></td>
<td>A. longiclava</td>
<td>-</td>
<td>Y = PC (unknown)</td>
<td>Y = PC (130±0.42)</td>
<td>Viggiani &amp; Battaglia, 1983</td>
</tr>
<tr>
<td></td>
<td>C. noacki</td>
<td>-</td>
<td>Y = PC (9.50±4.99)</td>
<td>Y = PC (61±0.36)</td>
<td>Viggiani &amp; Battaglia, 1983</td>
</tr>
<tr>
<td></td>
<td>E. asterobemisiae</td>
<td>S</td>
<td>Y = PC (2.42±3.25)</td>
<td>Y = PC (1561±523)</td>
<td>Viggiani &amp; Battaglia, 1983</td>
</tr>
<tr>
<td></td>
<td>E. partenopea</td>
<td>S</td>
<td>Y = PC (unknown)</td>
<td>Y = PC (18.6±9.36)</td>
<td>Viggiani &amp; Battaglia, 1983</td>
</tr>
<tr>
<td></td>
<td>E. pergandiella</td>
<td>S</td>
<td>Y = PC (unknown)</td>
<td>N</td>
<td>Viggiani &amp; Battaglia, 1983</td>
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<tr>
<td></td>
<td>Prospaltella spp.</td>
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<td>Y = PC (unknown)</td>
<td>Y = PC (unknown)</td>
<td>Kajita, 1986</td>
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<td><strong>Bethylidae</strong></td>
<td>C. tarsalis</td>
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<td>Y = PC (ca.37.13)</td>
<td>N</td>
<td>Cheng et al., 2003</td>
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<td>A. ervi</td>
<td>S</td>
<td>Y = Non-PC (78±0.006)</td>
<td>N</td>
<td>Villagra et al., 2011</td>
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<td></td>
<td>B. hebetor</td>
<td>G</td>
<td>Y = Non-PC (unknown)</td>
<td>N</td>
<td>Antolin &amp; Strand, 1992</td>
</tr>
<tr>
<td></td>
<td>C. flavipes</td>
<td>G</td>
<td>Y = PC (unknown)</td>
<td>N</td>
<td>Kimani &amp; Overholt, 1995</td>
</tr>
<tr>
<td></td>
<td>C. rubecula</td>
<td>S</td>
<td>Y = Non-PC (ca. 3)</td>
<td>Y = Non-PC</td>
<td>Field &amp; Keller, 1993</td>
</tr>
<tr>
<td></td>
<td>P. concolor</td>
<td>S</td>
<td>Y = Non-PC (20.30±3.49)</td>
<td>N</td>
<td>Benelli et al., 2012</td>
</tr>
<tr>
<td><strong>Eulophidae</strong></td>
<td>Melittobia spp.</td>
<td>G</td>
<td>Y = Non-PC (unknown)</td>
<td>Y = PC (unknown)</td>
<td>van den Assem, 1986</td>
</tr>
<tr>
<td><strong>Pteromalidae</strong></td>
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<td>G</td>
<td>Y = PC (unknown)</td>
<td>N</td>
<td>van den Assem et al., 1980</td>
</tr>
<tr>
<td></td>
<td>L. distinguendus</td>
<td>S</td>
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<td>Y = PC (ca. 17)</td>
<td>van den Assem et al., 1989</td>
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<td>G</td>
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<td>Y = PC (15)</td>
<td>Jachmann &amp; van den Assem, 1993</td>
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<tr>
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<td>S. endius</td>
<td>S</td>
<td>Y = PC (6)</td>
<td>Y = PC (22)</td>
<td>King &amp; Fischer 2005</td>
</tr>
</tbody>
</table>

*Brood type: G = Gregarious; S = Solitary; - = unknown; *PC = Physical Contact
1.6. Literature cited


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2. **An alternative reproductive tactic: a parasitoid wasp gathers and guards a harem by pheromone-tagging virgins**

2.1. **Abstract**

Alternative reproductive tactics (ARTs) are the outcome of decisions to obtain copulations in reproductive competition. Mating tactics that male insects exhibit can be based on their competitive ability, or depend on conditions such as a competitive setting and the spatial and temporal distribution of receptive females. When females are clustered and numerous, two or more mating tactics can coexist. We predicted that this concept is applicable to the egg parasitoid wasp *Ooencyrtus kuvanae* (Hymenoptera: Encyrtidae), because wasps emerge *en masse* as sexually mature adults from gypsy moth, *Lymantria dispar*, egg masses. We reveal that male *O. kuvanae* exhibit two ARTs, a mate-at-once (MAO) tactic, and a harem-gathering and -guarding (HGG) tactic. MAO males invariably and immediately mate females they encounter. HGG males (i) typically mate the first receptive female they encounter, (ii) then find and assess other females, (iii) tag those without prior male contact, and finally (iv) return to, and mate with, all females they themselves have tagged. Females do not incur a direct fitness cost by mating with multiply-mated males. HGG males rely on their speed, unique tag pheromone, and on the females’ rejection of HGG males except the one that pheromone-tagged them. The tagging pheromone mediates mate recognition and assessment.

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2.2. Introduction

Alternative reproductive tactics (ARTs) are the expression of two or more ways by which competing males - or sometimes competing females - obtain copulations. ARTs occur frequently in insect mating systems with intense sexual selection (Taborsky et al., 2008; Roff, 2011), are often heritable, and may or may not result in equal fitness (Taborsky et al., 2008).

Similar to a conditional strategy (Gross 1996), ARTs are primarily condition- and status-dependent (Taborsky et al., 2008). When condition-dependent with such conditions as intense male-male competition over many receptive females within a discrete patch (Emlen and Oring, 1977; Alcock, 1978; Thornhill and Alcock, 1983; Shuster, 2010), or status-dependent when a male's competitive ability is greater than his rivals (Gross, 1996), male insects are expected to adjust their alternative male phenotype traits. These phenotype traits may be expressed as different mating tactics (Brockmann and Penn, 1992; Brockmann, 2002; Shuster and Wade, 2003). Such mating tactics include female defense polygyny whereby males prevent others from gaining access to a female (Emlen and Oring, 1977), scramble competition (Brockmann, 2008), or mate searching (Alcock and Buchmann, 2011).

In many parasitoid mating systems, males likely implement ARTs because females often (i) emerge protandrously from a discrete patch (spatially clustered), (ii) are receptive upon emergence, (iii) mate once in their lifetime, and (iv) outnumber males 2:1 (Godfray, 1994; Quicke, 1997; Godfray and Cook, 1997). Under these conditions, males have a small window of opportunity to mate a female; male ARTs may be associated with differences in a male’s ability to quickly locate receptive females. For example, when receptive females are spatially clustered and synchronously receptive, males choosing a harem gathering and guarding (HGG) tactic could increase the number of receptive females available to them because search time and distance to locate females are minimized (Shuster and Wade, 2003); if males choose to implement an HGG tactic under these conditions, they should be able to deliver sperm to many females (Roitberg et al., 2001; Boivin et al., 2005). Extensive spatial clustering of receptive females also increases female encounter rates for males, and therefore may select for another ART to persist. For example, less competitive males might decide to choose a mate-at-once
(MAO) tactic with any female they encounter; MAO males may have lower fitness than HGG males but may still be reproductively successful (Brown et al., 1997; Shuster and Wade, 2003), and overall more successful than if they tried to use the HGG tactic.

Male ARTs can also be affected by female choice (Brockmann, 2008). In a female-defense polygynous mating system, the females’ mate choice may help shape the males’ ART and be based on indirect criteria such as choosing the male that has secured most of the females (Thornhill and Alcock, 1983; Wiley and Poston, 1996).

Parasitoid males may secure females without aggression (van den Assem, 1996), even though aggression and weaponry tend to evolve when females are spatially clustered and asynchronously receptive, as in Muscidifurax spp. (van den Assem et al., 1980; Thornhill and Alcock, 1983). When parasitoid females are spatially clustered and synchronously receptive, weaponry is less conspicuous (Shuster and Wade, 2003) and competition may be less aggressive, taking the form of display threats as in Nasonia vitripennis Ashmead (King et al., 1969), contests of speed and agility as in Tetrastichus atricapillus Waterston (van den Assem et al., 1980), or in absentia mate guarding as in Spalangia endius Walker (King and Fischer, 2005).

Males guard mates in absentia through volatile or non-volatile short-range pheromones (Greenfield, 1981; Delisle et al., 2000; Ayasse et al., 2001). Such pheromones may be transferred primarily through antennation, a key behavior exhibited by parasitoids during courtship and/or mating. Antennae are sexually dimorphic (van Baaren et al., 1999), house sex pheromone glands (Isidoro and Bin, 1995; Bin et al., 1999; Steiner et al., 2010) or pheromone receptors (Quicke, 1997; van Baaren et al., 2007), and differ in the number, shape, and type of sensory sensilla (Chapman, 1982), contingent on their function in detection and release of pheromone.

Males compete for a positional advantage to release pheromones that (i) counteract attractants associated with receptive females (see Ayasse et al., 2001), (ii) convey discreet courtship messages understood only by females of their choice (Thornhill and Alcock, 1983), or (iii) quickly place females into a receptive state (Bin et al., 1999).
Life history traits of the egg parasitoid wasp *Ooencyrtus kuvanae* (Howard) (Hymenoptera: Encyrtidae) are conducive to the coexistence of several male ARTs (Thornhill and Alcock, 1983; Shuster and Wade, 2003; Gross, 1996). This quasi-gregarious, 2-mm wasp parasitizes eggs of gypsy moth, *Lymantria dispar dispar* L.. Female wasps insert a single egg into each accessible egg of a host egg mass that measures 2–3 cm across and contains several hundred eggs covered in setae (Brown, 1984). By allowing to fertilize an egg, female insects produce a daughter, and by not fertilizing it, they produce a son. Within 4 weeks, wasps complete development inside host eggs and emerge *en masse* as sexually mature adults that can live 4–6 weeks. Females emerge up to 1 d later, are immediately receptive to mating, and are twice as numerous as their brothers. However, a male-biased sex ratio can occur among non-siblings due to local mate competition (Somjee et al., 2011) and because males typically remain on the host egg mass as long as there are mating opportunities, whereas mated females disperse within 24 h, seeking new gypsy moth egg masses (Brown, 1984). Females mate only once in their lifetime shortly after emergence, whereas males mate multiply over their lifetime. Mate attraction is mediated by close-range pheromone components (Ablard et al., 2012). When a male encounters a single female, a stereotypic sequence of events ensues consisting of a brief (ca. 4 s) pre-copulatory ritual, copulation, and a post-copulatory ritual, which may last 15-67 s. Both rituals are mediated by tactile signalling (Ablard et al., 2011).

Our objectives were to explore the presence of ARTs in *O. kuvanae*. We hypothesized that (1) at least two male ARTs (HGG and MAO) coexist when males are competing over spatially clustered receptive females, (2) that one tactic would provide more mating opportunities than another tactic, (3) females indirectly choose males, (4) these tactics are condition- and status-dependent, and that (5) females do not incur a fitness cost as a result of these tactics.
2.3. Materials and methods

2.3.1. General protocol

A colony of *O. kuvanae* was started with specimens field collected near the town of North East, Maryland (39°36´N, 75°55´W) (USA). All insects were reared under a 16:8 h light:dark regime at 22-25 °C and 50-70% relative humidity (RH) (Hofstetter and Raffa, 1997) in the Global Forest Quarantine Facility of Simon Fraser University, on eggs of gypsy moth supplied by the U.S. Forest Service (Hamden, Connecticut, USA). Insects were contained in Plexiglass cages (40 × 40 × 30 cm) and provisioned with cotton wicks (1 × 10 cm; Richmond Dental, Charlotte, NC, USA) soaked in a 30% honey water solution every 2 days. Wasps were used in all experiments within 1 day after emergence to avoid adverse effects associated with aging (van den Assem, 1996). Every 21 days, 10 gypsy moth egg masses (each consisting of several hundred eggs) were introduced to be parasitized by female wasps. Fourteen days later, parasitized eggs were removed and 800 eggs were placed singly into translucent plastic cups (103.5 ml) secured with a lid. Emergent insects were provisioned with a honey water-soaked cotton wick (1 × 1.3 cm) and separated by sex and similar size under a microscope.

2.3.2. Laboratory experiments

Mating behavior of males in a non-competitive setting

To explore mating behavior by males in a non-competitive setting, a virgin male was introduced to a single sexually inexperienced female under a Petri dish lid (2.5 × 1.0 cm, unless stated otherwise) similar in size to a gypsy moth egg mass (experiment 1a; *n*=15). After mating a female, he was introduced within 1 min to another female under a new Petri dish lid; this procedure was repeated five times. The same male was then introduced to a group of five sexually inexperienced females (experiment 1b; *n*=15) under a Petri dish lid. Courtship and mating behavior of each male and female were recorded.
Mating behavior of males in a competitive setting

To investigate the ARTs males exhibit in a competitive setting (experiment 2), four sexually inexperienced females were introduced within 1 min to two sexually inexperienced males (n=10) under a Petri dish lid. This type of competitive setting is likely to occur on a gypsy moth egg mass and was designed to gather specific evidence for a HGG tactic. Consequently, replicates where both males exhibited the MAO tactic (n=3) were discarded. The insects were video-tracked [CK3900N video camera (Meiji Techno Co. LTD, Japan); Samsung DVD-RW/-R recorder; Insignia viewing screen 30.5 × 22.9 cm] until 15 min after the last female mated. Data of male mating behavior, latent period prior to and between courtship, and the males (n=20) that mated females (n=40) were recorded via focal sampling.

Prevalence of specific alternative reproductive tactics

To quantify the prevalence of the MAO and HGG tactic (experiment 3; n=15), four sexually inexperienced females were introduced to two sexually inexperienced males under a Petri dish lid, and the ART of each male was recorded.

Mechanisms of HGG tactic: Antennation between male and female, microstructure of antennomeres involved, and exploration of pheromone transfer

To examine the interactions between an HGG male and a female, the behavior was filmed with the Fastec imaging camera IN1000M2GB custom-fitted with a Canon macro lens (MP-E 65 mm f/2.8, 1-5×) and equipped with Fastec imaging software version 3.0.4. Footage was obtained at rates of 1000 frames/s, with a resolution of 1280 × 1024. In each replicate (n=7), a female and two males were placed on filter paper and retained in a glass Petri dish on scale to the field of view of the camera lens (12 mm). Frame-by-frame analysis of footage with MiDAS player software version 5.0.0.3 determined that the apical antennomere of the HGG male and female come in contact with each other (henceforth “tagged”).

The microstructure of male and female antennae was examined by means of focus-stacked imaging, and environmental scanning electron microscopy (ESEM) of freeze-killed, 1-day-old virgin females (tagged or not; n=7 each), and 1-day-old virgin
males (with or without tagging experience; \(n=7\) each). Focus-stacked images were obtained with a Pentax K-7 digital SLR camera (Pentax Imaging Company, Golden, CO) fitted with a 10× objective lens (Mitutoyo M Plan Apo, 0.28 numerical aperture, Mitutoyo, Kanagawa, Japan) and a StackShot™ Macro Rail (controlled rail adjustment: 0.01 mm, Cognisys, Kingsley, MI, U.S.A.). Image composites were obtained using Zerene Stacker software version 1.04 (Zerene Systems LLC, Richland, WA, U.S.A.).

ESEM images were obtained by mounting insects onto a peg (12.7 mm diam), covered with a conductive carbon adhesive tab, and imaging with the ESEM FEI Quanta FEG 4000 (FP Innovations, Hillsboro, OR, USA) at magnifications of 1500×, 2500×, and \(\geq 5000\times\) within a chamber kept at ambient temperature, using 1.5 Torr pressure, an accelerating voltage of 15 kV, and a Gaseous Secondary Electron Detector with a 1-mm aperture.

To test for pheromone transfer, experiments 4 and 5 (\(n = 22\) each) were designed to manipulate female antennae tagged by an HGG male. Two sexually inexperienced males and four virgin untagged females were placed on filter paper and covered with a Petri dish lid. Mating behavior was observed and all MAO males were discarded. In experiment 4, tagged females were removed, cold-anesthetized, and immobilized with an insect pin (size 0) inserted into the tip of their wings. The distal antennomeres A11 and A12 of these females were thinly coated (masked) with a solution of a non-toxic glue and water (van den Assem and Jachmann, 1982), which was applied with a closed-end glass filament (1 \(\times\) 0.58 \(\times\) 102 mm; A-M Systems Inc., Carlsborg, WA, USA). Untagged females were treated the same way except that their proximal (instead of distal) antennomeres A2 and A3 were masked. All such treated females were then allowed to warm up but kept isolated until they moved normally and the solution had dried. In each of 22 replicates, the tagged and an untagged female were then confined with the HGG male that had tagged the female. The males’ mate choice in this non-competitive setting was recorded. Experiment 5 was identical in design except that tagged females were not treated to mask the pheromone. We could not mask antennae of HGG males because it acutely impaired their ability to locate females.
Harem guarding function

To determine whether (pheromone) tagging functions as a form of *in-absentia* mate guarding in a competitive setting (experiment 6), two males were confined with three females in a Petri dish (30 mm diam). The males’ mating tactic was not determined as it would not have affected experimental results. One of the three females was virgin and not yet tagged, one was virgin but tagged, and one was tagged and mated with an HGG male not present in this experiment, and thus not receptive (*n*=20). The mated female was expected not to re-mate (Ablard et al., 2011) but to increase the sense of competition among the males. The males’ first choice of a female (tagged or not), the behavior of each of the two females in response to the males, and the time elapsed until each of the two females mated were recorded. Evasion behavior of females was recorded when they stayed >2 mm away from a male or when they quickly moved away when he approached within ~2 mm.

Harem gathering function

With evidence emerging (experiments 2 & 6) that tagged females are (nearly) assured mates for the male that tagged them, experiment 7 was designed to test whether the tactic of tagging females generates additional mating opportunities for HGG males in a competitive setting. Three females and two males (*n*=15) were placed in a Petri dish (30 mm diam). Female #1 was a virgin without tag; male #1 had no prior contact with any female; female #2 was a virgin but had been tagged by HGG male #2; female #3 had been mated with a male absent from this experiment. The mated female was expected not to re-mate but to increase the sense of competition among the males. The first mate choice by #1- and #2-males was recorded simultaneously by several observers, each tracking a male or female throughout the bioassay.

Fitness cost to females mated with multiply-mated males

Experiment 8 tested whether males with varying degree of mating experience adversely affect the females’ reproductive output. To test this, a 1-day-old, sexually inexperienced male and female were placed on filter paper (9 cm diam., Whatman©) under a Petri dish lid and allowed to mate (*n*=13). Post-mating, the male was removed and within 5 min introduced to another 1-day-old, sexually inexperienced female confined under another Petri dish lid. This protocol was repeated five times, resulting in
females which copulated with males that had mated 0-5 times before. Each female was placed separately in a Petri dish (14 × 2.5 cm), kept in a controlled environment (16:8 light:dark regime; 22-25°C; 50-70% RH), and provisioned with honey water-soaked cotton wicks and 100 L. dispar eggs to parasitize. Two weeks later, each female was removed, and the parasitized eggs were kept until offspring emerged. The total number of progeny and of daughters each female had produced was recorded. Whether males’ ejaculate contains nutrients was not tested; this is not evident in other parasitoids (Godfray, 1994). Females not fed within 3 days perish, suggesting females do not likely receive sustaining nutrients from a male’s ejaculate which could affect their reproductive output in this context.

2.3.3. Data analyses

In experiments 1a and 1b, a binomial distribution was used to determine whether males employ alternative tactics to obtain copulations in a non-competitive setting. In experiments 2 and 4-7, a χ²-test was run to determine whether (i) in a competitive setting females tagged by an HGG male mated the HGG male that had tagged them (experiment 2), (ii) HGG males mated the females they had tagged with or without the pheromone being masked (experiments 4, 5), (iii) males rejected as mates females tagged by a HGG male (experiment 6), and (iv) whether HGG males were more successful than MAO males in mating untagged females (experiment 7). Descriptive statistics of normally-distributed data were used to determine the frequency of ARTs (experiment 3). A paired t test was used to analyze whether HGG males were quicker to court females than were MAO males (experiment 2), and whether tagged females evaded males which had not tagged them (experiment 6). In experiment 8, a repeated measures Poisson regression and a repeated measures logistic regression were used to determine any fitness consequences to females that mated with multiply-mated males. Models were fitted using an AR(1) covariance structure for observations within males. A test for linear trend in the effect of number of matings on number of progeny, and on the proportion of daughters, was also conducted. Statistical tests were run with PASW version 18 (Chicago, IL, U.S.A.) and PROC GLIMMIX of SAS® Systems (Cary, NC, U.S.A.), with α = 0.05.
2.4. Results

2.4.1. Laboratory experiments

Mating behavior of males in a non-competitive setting

Males courted and engaged only in the MAO tactic whereby upon encounter a male mated at once each single female (experiment 1a: binomial test proportion 0.50, \( n=15, \ p < 0.0001 \)) and each female in groups of five (experiment 1b: binomial test proportion 0.50, \( n=15, \ p < 0.0001 \)). In experiments 1a and 1b, the courting and mating sequence entailed a brief (ca. 4 s) pre-copulatory ritual, copulation, and a post-copulatory ritual which lasted between 15-67 s. All females alone or in groups readily mated with the male.

Mating behavior of males in a competitive setting

Males placed in a competitive setting exhibited the MAO and the HGG tactic. MAO males invariably and immediately mated any receptive female they encountered and never used the HGG tactic. Seventy-one percent of HGG males mated the first receptive female they encountered by engaging her in the same mating sequence as described in a non-competitive setting. All HGG males engaged in tagging of non-tagged virgins (see above), after which they returned and mated with them. HGG males were faster than MAO males in making contact with the first female (Paired t test: \( t_9 = -2.931, \ p = 0.017 \)). On average, 22.9 s (SD = 16.52) and 44.96 s (SD = 28.37) elapsed before HGG males and MAO males, respectively, contacted the first female. HGG males tagged the first and second female for their harem on average 49 s (31.7 < \( \mu < 66.3 \)) and 43.3 s (30.2 < \( \mu < 56.6 \)), respectively, later. Females tagged by an HGG male mated the HGG male that had tagged them (Chi-square test: \( \chi^2 = 17.286, \ p < 0.0001 \)) (Figure 2.1). Thirty percent of non-tagged females readily mated a MAO male.

Prevalence of specific alternative reproductive tactics

In a competitive setting, 60% of males engaged in both the MAO and HGG tactic, whereas 40% of males exhibited only one of the two tactics (13% HGG; 27% MAO). Overall, MAO males mated with 25 females (42%), whereas HGG males mated with 35 females (58%).
Mechanisms of HGG tactic: Antennation between male and female, microstructure of antennomeres involved, and exploration of pheromone transfer

Antennation is the mechanism mediating the HGG tactic. Recordings of the exceedingly fast (<1-s) tagging behavior invariably revealed the same sequence of events. First, the HGG male approaches and orients himself perpendicular to the female’s thorax, rarely making physical contact at this stage. He then attempts to assume a head-to-head position with the female. While approaching from the left side of the female, he brings the apical antennomere of his right antenna into contact with the apical antennomere of her left antenna (Figure 2.2) (see Supplementary Video). When the male and female are in a head-to-head position, he may repeat the apical antennomere contact procedure with the other antenna.

The antennae of males and females are geniculate and dimorphic (Figure 2.3). They comprise 10 and 12 antennomeres, respectively. The males’ apical antennomere (ca. 132 µm in length) is significantly longer than each of all other antennomeres; it is also 3× longer and more slender than the females’ apical antennomere (ca. 48 µm).

Pheromone is transferred from an HGG male to a female when he tags her. The tagged female, with the tagging pheromone masked, and the untagged female were equally often selected as a first mate by the male that had tagged the female (Chi-square test: \( \chi^2 = 0.059, n = 22, p = 0.670 \)) (Figure 2.4; experiment 4). In contrast, the tagged female, without the tagging pheromone masked, was selected as a first mate by the male that had tagged her significantly more often than the untagged female (Chi-square test: \( \chi^2 = 4.545, n = 22, p = 0.033 \)) (Figure 2.4; experiment 5).

Harem guarding function

Pheromone-tagging functions as a form of in-absentia mate guarding. Males avoided making contact with the female tagged by the other male, and did not mate with her (Chi-square test: \( \chi^2 = 12.80, n = 20, p = 0.001 \)) (Figure 2.5, top). Pheromone-tagged females, in turn, resisted mating with the other male (which had not tagged them) ~12 times longer (Paired sample t-test: \( t_1 = -5.351, n=20, p=0.008 \)) (Figure 2.5, middle), and exhibited bouts of evasive behavior ~4 times more often (Paired sample t-test: \( t_1 = 2.847, n= 20, p = 0.025 \)) (Figure 2.5, bottom) than did females not previously tagged.
Collectively, these results indicate that pheromone-tagging serves as a form of *in-absentia* mate-guarding.

**Harem gathering function**

The strategy of tagging females increased mating opportunities for males. #2-Males which had tagged #2-females mated them only after they had mated the untagged #1-females (Chi-square test: $\chi^2 = 8.067, n = 15, p = 0.005$) (Figure 2.6, top), thereby effectively doubling their number of mates. Moreover, HGG males secured more untagged #1-females as mates than the sexually inexperienced #1-males (Chi-square test: $\chi^2 = 5.4, n = 15, p = 0.02$) (Figure 2.6, bottom). The few #1-males that did mate, exhibited only the MAO tactic. The few #1-females which mated with a sexually inexperienced #1-male exhibited no apparent evasion behavior.

**Fitness cost to females mated with multiply-mated males**

Females’ reproductive output was not affected by mating with multiply-mated males within this context. There was no significant effect of the number of matings of a male on the total number of progeny his mates produced (Poisson regression: $F_{5,72} = 0.69, p = 0.631$) (Table 2.1, top), and no significant linear increase or decrease ($F_{1,72} = 1.97, p = 0.165$) in the effect of number of matings on numbers of progeny. There was also no significant overall effect of number of matings of a male on the proportion of daughters his mates produced (Logistic regression: $F_{5,69} = 0.46, p = 0.802$) (Table 2.1, bottom), and no significant linear increase or decrease ($F_{1,69} = 1.00, p = 0.320$) in the effect of number of matings on proportion of daughters, revealing no fitness effect on females’ reproductive output based on their choice of singly- or multiply-mated males.

**2.5. Discussion**

In a non-competitive setting, *O. kuvanae* males invariably and immediately mated any untagged female they encountered (MAO tactic), whether or not she was on her own or in a group. Conversely, under the conditions of a competitive setting, males mostly exhibited two reproductive tactics, a harem-gathering and -guarding (HGG) tactic and a mate-at-once (MAO) tactic, the former yielding a higher fitness. The HGG tactic of
O. kuvanae males was not only based on the condition of a competitive setting, but also status-dependent, as demonstrated by results that HGG males located virgins two times quicker than did MAO males. Their superior speed was not due to a large body size, or a larger body size relative to a competitor’s as shown in the parasitoid wasp Nasonia vitripennis (Burton-Chellew et al., 2007); their speed could be correlated to their health, which – in turn - could be contingent on host-quality and possibly the egg-laying strategy of a mother. Also, dimorphic traits (e.g., winged vs. wingless) that correlate to mating tactics in most chalcidid male wasps (reviewed by Brockmann, 2008) were absent in O. kuvanae males.

Contrary to our prediction, O. kuvanae males in a competitive setting occasionally exhibited the same tactic (e.g., MAO only), or the same sequence of tactics (e.g., MAO followed by HGG) suggesting that tactics may be heritable (Gross, 1996).

However, choice of tactic can vary, regardless of genotype, whereby males implement different tactics or the same tactic (Gross, 1996). For example, in an O. kuvanae population with many HGG males that might have identical pheromone tags, the MAO tactic could be relatively common as the HGG tactic in this context could be costly for males in terms of overall fitness and energy. A large representation of HGG males could be influenced by the females’ timing of receptivity, which can be contingent on their emergence in synchrony (Werren, 1980) or asynchrony (Shuker et al., 2005). In the latter case, the HGG tactic could function to secure more asynchronously than synchronously receptive females for a harem because asynchrony provides a succession of receptive females in close proximity, and males are less likely to lose reproductive opportunities while guarding (Shuster and Wade, 2003). However, this consideration does not apply to HGG males of O. kuvanae that effectively guard a harem of synchronously receptive females in absentia.

Only HGG males switch tactics. When they are in the presence of competitors, they perform the MAO tactic first and then shift to, and then retain, the HGG tactic. Their ability to engage in both tactics could be based on a genetic threshold model in which the tactic (threshold traits) is under polygenic control that itself is environmentally sensitive and often condition-dependent (Moczek and Nijhout, 2003). Not implementing the HGG tactic initially would be advantageous as a male can transfer enough sperm to
fertilize all of a female’s eggs, negating the need to mate many females in order to pass on his genes through his daughters (Shuster and Wade, 2003); therefore, the MAO tactic could be a type of “insurance” mating in case a male is outcompeted by other HGG males, and is left without a harem. Initially engaging in the MAO tactic could also provide the time needed for a male to assess the environment (including the number of females and competing males), thereby determining whether it is advantageous to subsequently engage in the HGG tactic.

The MAO tactic of _O. kuvanae_ males is based on their ability to efficiently detect and immediately mate all untagged females, which fits the model prediction (Shuster and Wade, 2003) that the MAO tactic should exist when females are spatially clustered and synchronously receptive. The HGG tactic of _O. kuvanae_ males, which the model predicts to coexist but to be less prevalent when females are spatially clustered and synchronously receptive, corroborates the theory that mating tactics can coexist when male-male competition over numerous synchronously receptive females is intense (Shuster, 2010).

Monopolization of females can be reproductively costly for a male as he may lose time guarding receptive females, instead of mating females (Thornhill and Alcock, 1983; Shuster and Wade, 2003). This does not apply to _O. kuvanae_ HGG males which attain multiple mates by guarding receptive females _in absentia_ (Figure 2.6). There were no detectable fitness costs to females mating with multiply-mated males; however, whether tagged females that mate with a MAO male experience adverse fitness consequences remains unknown. Five consecutive matings of males with virgin females reduced neither the total number of offspring nor the proportion of daughters they sired (Table 2.1), indicating no effect due to sperm depletion. Therefore, the time between tagging and mating a female is likely not the time a male needs to replenish his sperm (Boivin et al., 2005), but rather to encounter and secure more untagged females.

When females exercise some control over mating, males may use courtship to secure mating opportunities through behavioral, psychological, or physiological means (Brown et al., 1997). That an untagged _O. kuvanae_ female readily mated with an HGG or MAO male indicates that a female may indirectly choose a mate who is the fastest, and thus the first male to contact her (Wiley and Poston, 1996). The degree of relatedness
between females and HGG and MAO males were not accounted for in this study, because *O. kuvanae* does not recognize kin (K.A., unpubl. data). Therefore, a female’s initial choice is indirect and based on the male’s speed to locate her; however, once a female was tagged, she demonstrated direct choice for the male that had tagged her (Figure 2.1). The odds of a female being tagged by a brother lie in the egg-laying strategy of their mother which will have produced greater proportions of sons with increasing numbers of ovipositing females on the same egg mass (Somjee et al., 2011). As a result, HGG siblings could have a better chance of outcompeting HGG non-siblings, provided that sibling and non-sibling HGG males have different pheromone tags. As soon as the tagging-pheromone is identified, and its absolute configuration known for each bioassay male, it will become clear which of these possible interpretations apply to our data.

The tagging pheromone in *O. kuvanae* appears to have two functions. It masks the attractiveness of tagged virgin females and it alters the behaviour of females in that they avoid all courting males except the one male that tagged them (Figure 2.5). Such pheromones are known to occur in mating systems where females that mate quickly with the first male that reaches them are not susceptible to takeovers by other males, and after copulation become completely non-receptive (Thornhill and Alcock, 1983). In these systems, males strategically transfer pheromone that (*i*) counteracts the attractant signal associated with receptive females, (*ii*) creates a learned association with deterrents, (*iii*) arrests competing males, (*iv*) carries messages that are understood only by the female, and/or (*v*) induces resistance behaviour in females towards “unfamiliar” males (Siegel and Hall, 1979; Wyatt, 2003; Widemo and Johansson, 2006; Johansson and Jones, 2007).

Females that await the return of, and mate with, the HGG male that tagged them, and therefore avoid mating attempts by other males in the interim, could attain a fitness advantage. They will mate with a male that was the fastest to have found and tagged them. Assuming that his genotype is superior to that of his competitors, and is an inherited trait, he might bestow a fitness advantage on his mate(s); he gains fitness benefits as he quickly increases his harem size.
O. kuvanae females rarely mated with an HGG male that did not tag them (Figure 2.1). In the few incidences they did, the pheromone transfer by the tagging male was likely not complete or successful because other males were not deterred by these females, and these females did not avoid other males. Alternatively, a few males had identical pheromone tags. This implies that each male typically has a unique odour that the female recognizes. The ability to recognize the unique odour of a specific prospective mate has been reported for badgers, shrews, and Eurasian deer. For territorial marking or countermarking, males deposit their unique odour which correlates with a specific number and type of chemical constituents (reviewed by Johansson and Jones, 2007). However, whether females indeed recognize unique odours based on these correlations is yet to be confirmed by testing blends of synthetic chemicals.

Unlike mammals, insects are known thus far to recognize “merely” kin, colony or nestmates based on their cuticular hydrocarbons (Lloyd, 1981; Ayasse et al., 2001; Johansson and Jones, 2007). In O. kuvanae, the uniqueness of each male’s tagging pheromone may rest with two methylated cuticular hydrocarbons which are more abundant in males than in females, and which apparently have sex-specific absolute configuration with potentially numerous and unique blends of stereoisomers (Ablard et al., 2012).

Among parasitoids, antennation is an underlying mechanism of courtship or sex pheromone-mediated mating (Quicke, 1997; Ayasse et al., 2001). Antennae are sexually dimorphic and house pheromone glands and pheromone receptors, as shown in Amitus spiniferus Brèthes (Isidoro and Bin, 1995), Anaphes spp. (van Baaren et al., 1999), Epidinocarsis lopezi De Santis (van Baaren et al., 2007), and Trichopia drosophilae Perkins (Romani et al., 2008). As yet unknown was that antennae also play the key role in the tagging of female O. kuvanae, a behavior during which the male brings an apical antennomere into brief contact with an apical antennomere of the female (Figure 2.2). This brief physical contact is needed to tag a female, indicating that the mode of tagging must be chemical (see discussion above). Any mechanical alteration of the female’s distal antennomere(s) during tagging would be rather unlikely, and would not be sufficiently unique to enable males to reject females tagged by other males (Figure 2.5, top), and to recognize their own tag on females. This interpretation is supported by experimental results that males failed to recognize their tag when it was
masked (Figure 2.4). The concept of chemical transfer during tagging is also supported by images showing that the males’ distal antennomere is much longer than all others (Figure 2.3). Its great surface area might facilitate efficient transfer of pheromone to a female within a <1-s time frame. Also present on male, but apparently not female, apical antennomeres were microstructures (marked by arrow in Figure 2.3c) that resemble chemosensory sensilla which function in contact reception of non-volatile chemicals (van Baaren et al., 2007).

The pheromone tag a female receives is likely to have low volatility as commonly shown for recognition pheromones (Billen and Morgan, 1998; Wyatt, 2003). There were also porous structures analogous to placoid sensilla (marked by arrows in Figure 2.3e), which in Cotesia spp. are suggested to facilitate mate recognition in males and long-range stimuli detection in females (Bleeker et al., 2004; van Baaren et al., 2007). There was no evidence for pheromone-producing tyloid glands as reported in Syrphoctonous tarsatorius (Steiner et al., 2010) and Pimpla turionellae (Bin et al., 1999). Transmission electron microscopy and ultrastructural exploration are needed to pinpoint the location and microstructure of pheromone glands (Isidoro and Bin, 1995; Romani et al., 2008).

2.6. Conclusion

Experimental manipulation of courting wasps enabled us to explore and address some of the theories and outstanding questions about ARTs in insect mating systems including, but not limited to, the functional design of traits related to sexual interactions, such as courtship behavior and pheromones that affect male or female control (Alexander et al., 1997; Johansson and Jones, 2007). In the mating system of O. kuvanae, competing males deploy ARTs and exhibit traits in the early phase of courtship that put them in control. Through speed and unique pheromone with which HGG males tag virgin females, they acquire a harem, with females using these traits to recognize a specific male. This tagging promotes selection of other traits such as male preference for untagged virgin females, and female preference for tagger-males. In a later phase of the courtship sequence, females exert choice by resisting mating with unfamiliar males, a type of coercion behavior that appears to stabilize this intricate mating system. We show that a mating system is not necessarily controlled by either the male or the female, but
that either sex can be in control by expressing different traits at different phases of the courtship sequence. We further show that pheromones mediate not only specific mate recognition but also mate assessment.

Figure 2.1. **Evidence for a harem-gathering and guarding (HGG) strategy.** This is indicated by the 89% of females that mated the HGG male (♂) which had tagged them rather than another male (♂) which had not. Bars with different letters are significantly different from one another. Note: (1) Female and male symbols in parenthesis represent the number and status of insects tested per replicate; (2) prior to the experiment, the four females (♀♀♀♀) had no contact with any male, and the two males (♂♂) had no contact with any female.
Figure 2.2. Graphical illustration of pheromone-tagging behavior. During the process, the male brings an apical antennomere into brief contact with an apical antennomere of the female (see also Supplementary Video).
Figure 2.3. Morphology and microstructures of antennae. Focus-stacked images of male (a) and female (b) antennae (apical antennomeres marked by white bar), and environmental scanning electron micrographs of apical antennomeres of male (c) and female (d) antennae, and placoid-type sensilla (arrows) on the apical antennomere of a male antenna (e), with cuticular pores evident in higher magnification (f). Note: (1) microstructure marked by arrow in c resembles a chemosensory sensillum which functions in contact reception of non-volatile chemicals in the egg parasitoid wasps Anaphes victus Huber and A. listronoti Huber (van Baaren et al., 1999); (2) placoid sensilla (see e) in Cotesia spp. are suggested to facilitate mate recognition in males and long-range stimuli detection in females (Bleeker et al., 2004; van Baaren et al., 2007).
Figure 2.4. Evidence for deployment of tagging pheromone. Percent of tagged virgin females, with the pheromone tag masked (♀️) (Exp. 4), or not masked (♀️) (Exp. 5), and of untagged virgin females (♀️) that were selected as the first mate by the male (♂️) which had tagged the female. Bars with different letters are significantly different from one another. Female and male symbols in parenthesis represent the number and status of insects tested per replicate.
Figure 2.5. Pheromone-tagging serves as a form of mate guarding. Percent of virgin females not previously tagged (♀), or tagged (♂), that were selected as the first mate by two males without any prior sexual (tagging) experience (♂♀) (top); time elapsed until females mated (middle); and number of evasive bouts made by females in response to the courting males (bottom). In each pair of bars, bars with different letters are significantly different. Mated females (♀) did not re-mate and were ignored by males. Female and male symbols in parenthesis represent the number and status of insects tested per replicate.
Figure 2.6. The strategy of tagging females increases mating opportunities for males. Percent of virgin females [one tagged (♀), one not (♀)] mated first by the tagging (harem–gathering and -guarding) male (♂) (top); percent of males [one with tagging experience (♂), one without (♂)] mated with females not yet tagged (♀) (bottom). In each pair of bars, bars with different letters are significantly different from one another. Female and male symbols in parenthesis represent the number and status of insects tested per replicate. Mated females (♀) did not re-mate.
Table 2.1. Estimated means, standard error (SE), and 95% confidence limits (CL) for the total number of progeny (top), and proportion of daughters (bottom), produced by females that mated with a male which had mated previously 0-5 times.

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2.7. Acknowledgements

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2.8. Literature cited


3. Does the stereochemistry of methylated cuticular hydrocarbons contribute to mate recognition in the egg parasitoid wasp *Ooencyrtus kuvanae*?²

3.1. Abstract

Close-range sexual communication of the egg parasitoid wasp *Ooencyrtus kuvanae* takes place on host gypsy moth, *Lymantria dispar*, egg masses. We tested the hypothesis that mate recognition is mediated, in part, by low-volatility cuticular hydrocarbon (CHC) pheromone components. Gas chromatographic and GC-mass spectrometric analyses of body surface extracts of males and females revealed no sex-specific components, but two components, 5-methylheptacosane (5-me-27Hy) and 5,17-dimethylheptacosane (5,17-dime-27Hy), were consistently more abundant in extracts of males. The ratio of 5-me-27Hy and 5,17-dime-27Hy was similar in extracts of males and females, and quantitative differences on their own seemed insufficient to impart sex-specific CHC profiles. Therefore, we further hypothesized that the absolute configuration of 5-me-27Hy and 5,17-dime-27Hy contributes to mate recognition or attraction. As the stereoisomers of 5-me-27Hy and 5,17-dime-27Hy currently cannot be separated chromatographically, we could not determine the stereochemistry of the insect-produced components. Instead, we synthesized all stereoisomers and bioassayed synthetic blends in laboratory experiments. Of eight 2-component blends, each blend containing one of the two enantiomers of 5-me-27Hy and one of the four stereoisomers of 5,17-dime-27Hy, the blend of (5S)-methylheptacosane and (5R,17S)-dimethylheptacosane attracted males, whereas the blend of (5R)-methylheptacosane and (5R,17R)-dimethylheptacosane repelled males. Apparent recognition of both pheromone

components and pheromone antagonists by males supports the hypothesis that the stereochemistry of 5-me-27Hy and 5,17-dime-27Hy, and possibly other methylated CHCs, may differ between male and female O. kuvanae, and that these differences may serve in mate attraction and recognition.

3.2. Introduction

Sex pheromones play a critical role in sexual communication systems of social and solitary hymenopterans (Ayasse et al., 2001), such as parasitoids (Godfray, 1994). Female parasitoids produce sex pheromones that comprise components of high and/or low volatility (Ardeh et al., 2004). Volatile components enable mate finding by attracting males over a long range, as shown in species of the Ichneumonidae (Eller et al., 1984), Braconidae (DeLury et al., 1999), Aphelinidae (Fauvergue et al., 1995), and Aphididae (Decker et al., 1993). In quasi-gregarious parasitoids which mate at the site of emergence, close-range sex pheromones are expected to prevail (Godfray, 1994), and to attract or help locate nearby mates (Ayasse et al., 2001), as reported in species of the Braconidae (Syvertsen et al., 1995), Chalcididae (Mohamed & Coppel, 1987), Trichogrammatidae (Pompanon et al., 1997), Pteromalidae (Nichols et al., 2010; Sullivan, 2002), Ichneumonidae (Shu and Jones, 1993), and Aphididae (McClure et al., 2007). Close-range and contact sex pheromones can also contribute to the process of mate choice (Ayasse et al., 2001), and function to distinguish between conspecifics and heterospecifics (Singer, 1998), their sex (Steiner et al., 2006), receptivity and reproductive status (Ayasse et al., 2001), or they can coordinate sexual behaviour during courtship (Takahashi and Sugai, 1982; Johansson and Jones, 2007).

Close-range sex pheromones often comprise cuticular hydrocarbons (CHCs) which originate from exocrine glands associated with the cuticle (Ayasse et al., 2001). Cuticular hydrocarbon sex pheromones of female parasitoid wasps help attract mates within a close range (Ayasse et al., 2001; Sullivan, 2002), serve in mate recognition (Singer, 1998) or mate assessment (reviewed by Johansson and Jones, 2007), and coordinate courtship (Steiner et al., 2006; Ruther et al., 2011).
Culticular hydrocarbon pheromone components are typically long-chain aliphatic and methyl-branched alkanes and alkenes which differ within and between species in the composition, absolute abundance or relative proportion of components, sometimes based on the reproductive status of individuals. For examples, males of the vinegar fly *Drosophila grimshawi* discriminate between qualitative differences in pheromone streaks deposited by competitors, and then deposit more pheromone that attracts females to their leks (Widemo and Johansson, 2006). Male *D. virilis* and *D. melanogaster* initiate courtship behavior in response to (Z)-11-pentacosene and (Z,Z)-7,11-heptacosadiene, respectively (Oguma et al., 1992). In the pteromalid parasitoid wasp *Dibrachys cavus*, levels of 3-methylnonacosane and 3-methylhentriacontane increase on the cuticle of females when they became sexually attractive (Ruther et al., 2011). Finally, in the pteromalid parasitoid wasp *Nasonia vitripennis*, the same CHCs occur on males and females, but they differ in their relative abundance. Females have relatively more 9-, 11-, 13-, and 15-methylalkanes, and 9,x-, 11,x-, 13,x-, and 15,x-dimethylalkanes than males, but males have more 5- and 7-methylalkanes and 3,x-, 5,x-, and 7,x-dimethylalkanes (Steiner et al., 2006).

The absolute configuration of methylated CHC pheromone components has rarely been determined. This is surprising because stereoisomers alone or in combination can elicit very different behavioral responses. For example, in a study of scale-derived contact pheromone of female peach twig borer moths, *Anarsia lineatella*, males contacted female decoys baited with a blend of (R)-11-methyltricosane, (S)-11-methyltricosane and octadecyl acetate in the presence of gland-derived sex pheromone (Schlamp et al., 2005). However, the blend with the S-enantiomer alone did not elicit a behavioural response, and it was inhibitory with the R-enantiomer alone (Schlamp et al., 2005). Males of the longhorned beetle *Tetropium fuscum*, copulated with females treated with (S)-11-methylheptacosane, but their mating behavior (mounting and copulation) was reduced by (R)-11-methylheptacosane (Silk et al., 2011).

Mate location and elaborate courtship of the quasi-gregarious egg parasitoid wasp *Ooencyrtus kuvanae* take place on host gypsy moth, *Lymantria dispar*, egg masses which measure 2–3 cm across and contain several hundred eggs covered in setae (Brown, 1984). Those in the uppermost layer can be parasitized by female *O. kuvanae* that insert a single egg into each accessible host egg. Females can choose to
fertilize an egg and produce a daughter, or not to fertilize an egg and produce a son. Within four weeks, wasps complete development inside host eggs and emerge en masse as sexually mature adults, females one day later and twice as numerous as males. Males search for a female and then engage her in a mating sequence that consists of a brief (ca. 4 s) pre-copulatory ritual, copulation, and post-copulatory ritual, which may last 15-67 s; both rituals entail extensive antennation (Ablard et al. 2011).

Females mate only once in their lifetime, whereas males mate multiply. Mated females disperse within 24 h, seeking new discrete patches of L. dispar egg masses to parasitize (Brown 1984), whereas males remain on the host egg mass as long as there are mating opportunities (K.A., unpibl. data)

If multiple female foundresses oviposit concurrently on a gypsy moth egg mass, they produce proportionately more sons (Somjee et al., 2011), implying that females recognize the presence of other females. Moreover, mate-seeking males on a gypsy moth egg mass orient toward females, but not toward males (K.A., unpibl. data.), again implying that males and females differ in their visual and/or olfactory characteristics. We tested the hypotheses that close-range mate attraction or recognition in O. kuvanae is mediated, in part, by close-range low-volatility CHC pheromone components.

3.3. Methods and Material

3.3.1. Experimental Insects

A new colony of O. kuvanae was started every six months with specimens field collected from Mt. Gretna, Pennsylvania (40°14´N, 76°27´W), or near the town of North East, Maryland (39°36´N, 75°55´W) (USA). All insects were reared under a 16:8 h light:dark regime at 22-25 °C and a 50-70% relative humidity (RH) (Hofstetter and Raffa, 1997) in the Global Forest Quarantine Facility of Simon Fraser University on eggs of gypsy moth (which were separated out from egg masses covered in setae) supplied by the U.S. Forest Service (Hamden, Connecticut, USA). They were contained in Plexiglass cages (40 × 40 × 30 cm) and provisioned with cotton wicks (1 × 10 cm; Richmond Dental, Charlotte, NC, USA) soaked in a 30% honey water solution every two days.
Wasps were used in all experiments within one day of emergence to avoid adverse effects associated with aging (e.g., delayed mating response) (van den Assem, 1996). Every 21 days, 10 gypsy moth egg masses were introduced to be parasitized by female wasps. Fourteen days later, parasitized eggs were removed and 1000 eggs were placed singly into translucent plastic cups (103.5 ml) secured with a lid. Emergent insects were provisioned with a honey water-soaked cotton wick (1 × 1.3 cm) and separated by sex under a microscope.

3.3.2. **Body Surface Extractions**

Fifty males and 50 females were placed into separate vials on dry ice containing 100 µl of hexane and (9E)-octadecenyl acetate as an internal standard. Samples to be bioassayed did not contain an internal standard. After 10 min of extraction at room temperature, the supernatant was transferred to separate vials, and cold-stored (4°C). Aliquots of concentrated samples were analyzed by gas chromatography (GC) and coupled gas chromatography-mass spectrometry (GC-MS) (see below). Prior to bioassays, body surface extracts were warmed to room temperature for circa 15 min, and then concentrated to 25 µl under a stream of nitrogen.

3.3.3. **Analyses of Body Surface Extracts**

Extracts were analyzed by GC-MS using both a Varian Saturn 2000 Ion Trap coupled with a workstation version 6.9.1 (Agilent Technologies Inc.) and equipped with a DB-5 column (30 m × 0.25 mm ID), and an HP quadrupole instrument operated at 70eV (Ion Trap) and equipped with a HP-5 column (30 m × 0.25 mm I.D). The quadrupole instrument was employed because it is most suitable to determine methyl branch positions in molecules. GC-MS samples were analyzed in splitless mode, using helium as the carrier gas and the following temperature program: 50 °C (2 min) then 20 °C/min to 280 °C (isothermal 15 min). No compounds of interest eluted at higher temperatures, as determined in preliminary analyses.
3.3.4. Preparation of Synthetic Blends

All blends of synthetic compounds to be tested in bioassays were analyzed by GC, using a Zebron-5 column (30 m × 0.25 mm ID; Phenomenex, Torrance, CA, USA) in a Hewlett Packard (HP) 5890 Series II gas chromatograph, splitless mode injection, helium as the carrier gas, and the following temperature program: 160 °C (1 min), 20 °C/min to 280 °C (isothermal for 15 min).

3.3.5. General Methods and Instrumentation for Syntheses

Tetrahydrofuran (THF) was dried by standard methods. Oven-dried glassware was assembled hot under Ar flow, and maintained under Ar; liquids were transferred by cannula under Ar pressure. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. Nuclear magnetic resonance (NMR) spectroscopy of synthetic compounds was conducted on a Bruker BioSpin-400 spectrometer (Bruker, Rheinstetten Germany) (at 400 MHz for 1H, 101 MHz for 13C) with CDCl3 as solvent; chemical shifts are reported in ppm relative to TMS (1H, δ 0.00) and CDCl3 (13C, δ 77.00).

Synthesis of Racemic 5,17-Dimethylheptacosane (6a in Figure 3.1)

2,14-Pentadecanediol (1a) obtained by Grignard coupling of dicuprate derived from 1,9-dibromononane (Aldrich, Milwaukee, WI, 53201, USA) and propylene oxide (Aldrich) (Tadaaki et al., 1996) was oxidized to 2,14-pentadecadione (2a) with PCC (Aldrich) (Matsuyama and Mori, 1994) in 92.0% yield. Wittig reaction of 2a with one equivalent of butyldiphenylphosphorane produced a mixture of two isomers (E and Z) of unsaturated ketone 3a, three isomers of diene 4a, and some unreacted 2a. The isomers of 3a (29% yield) were easily separated by flash chromatography (on silica, eluent hexane/ether 9:1 v/v) from starting material and hydrocarbon 4a, and reacted with n-decylidenetriphenylphosphorane producing all four isomers of 5,17-dimethylheptacosa-4,17-diene (5a). A partially purified (flash chromatography on silica, hexane as eluent) mixture of these dienes was subjected to hydrogenation in hexane with 10% Pd/C as a catalyst. Final purification of the hydrogenated material afforded hydrocarbon 6a in 56.5% yield (15.0% overall yield). 5,17-Dimethylheptacosane (6a) had identical mass spectra, NMR spectra, and GC retention times as the stereoisomers (see
below). Racemic 5,10-dimethylheptacosane (6b) was obtained by the same sequence of reactions (Figure 3.1).

Racemic 5-methylheptacosane was synthesized by Grignard coupling of 2-bromohexane (TCI America, Portland, OR 97203, USA) (via dicuprate) with 1-bromodocosane (Aldrich) in 42% yield.

(S)- and (R)-1-Iodo-2-methyl-dodecanes (11 and 14 in Figure 3.2)

tert-Butyldimethylsilylchloride (DMTBSCL) (14.75 g; 1.05 equiv.) and imidazole (6.66 g; 1.05 equiv.) were added to methyl (R)-3-hydroxy-2-methyl propanoate (7; 11.00 g; 93.2 mmol; Aldrich) dissolved in dimethylformamide (50 ml). After stirring overnight at room temperature (rt), methyl (R)-3-tert-butyldimethylsilyloxy-2-methylpropanoate was obtained in quantitative yield. Borane reduction of this silyl ether with 46.6 ml 2.0 M BH$_3$·(CH$_3$)$_2$S (Aldrich) (93.2 mmol) in THF under argon yielded known (S)-2-methyl-3-(tert-butyldimethylsilyloxy)-1-propanol (8) (King et al., 1995; Schlamp et al. 2005) in quantitative yield after 48 h of stirring at ambient temperature. After quenching the reaction mixture with concentrated aq. K$_2$CO$_3$, the product was extracted with ether/hexane (1:1 v/v, 3 x 100 ml), the solution was dried (MgSO$_4$), and the solvent was removed in vacuo. A portion (15.34 g; 82.0 mmol) of the monosilyl ether 8 (> 98% pure, GC) was converted to (R)-3-tert-butyldimethylsilyloxy-2-methylpropyl iodide (9) (Marshall et al., 1987; Nakamura and Mori, 2000) by stirring with triphenylphosphine (26.3 g; 100 mmol) and imidazole (7.82 g, 115 mmol) at 0 °C in dichloromethane and by adding to this mixture crystalline iodine (25.4 g, 100 mmol) in three portions. After 2 h of stirring, the reaction mixture was quenched with 50% aqueous methanol, and the product was extracted with hexane (3 x 50 ml). Extracts were combined, washed with water and brine, and dried (MgSO$_4$), and the solvent was removed in vacuo. The (R)-iodide (9) was purified (98.4 %) by flash chromatography using hexane as eluent. The yield was 24.45 g (91.5% based on starting ester 7).

CuI (1.06 g; 5.58 mmol) was added at -30 °C to freshly prepared nonylmagnesium chloride [refluxing 9.05 g (55.8 mmol) of nonyl chloride with 2.70 g Mg in 200 ml THF for 4 h]. After stirring the suspension for 10 min, the iodide 9 (11.4 g, 36.3 mmol) was added and the reaction mixture was stirred for another 60 min at -23 °C, allowed to warm to rt, and then quenched with saturated aqueous NH$_4$Cl. The resulting
(S)-1-tert-butylsilyloxy-2-methyl-dodecane (Schlamp et al., 2005) was extracted with hexane (3 x 75 ml); the extracts were washed with water and brine, dried and evaporated. Without further purification, the residue was dissolved in THF (150 ml) and tetrabutylammonium fluoride hydrate (12 g; Aldrich) and H₂O (1 ml) was added. The mixture was stirred overnight. Work-up and chromatographic purification afforded 6.51 g (32.5 mmol, 98.5% pure, 89.5% yield) of (S)-2-methyl-dodecan-1-ol (10) (Schlamp et al., 2005).

Alcohol 10 (6.51 g, 32.5 mmol) was converted to the corresponding (S)-1-iodo-2-methyl-dodecane (11) (Marsden and Newton, 2007) with triphenylphosphine (10.23 g), imidazole (2.88 g), and iodine (9.90 g), using the iodination procedure described above. The yield after chromatographic purification (50 g silica, hexane as eluent) was 9.05 g (29.2 mmol, >97% pure, 89.7% yield) [α]D20 = + 2.06 (c 1.53; CHCl₃). ¹H NMR (CDCl₃), δ (ppm): 0.88 (t, 3H, J = 6.8 Hz), 0.97 (d, 3H, J = 6.5 Hz), 1.16-1.49 (m, 19H), 3.15 (dd, 1H, J = 6.0, 9.6 Hz), 3.23 (dd, 1H, J = 4.6, 9.6 Hz). ¹³C NMR (CDCl₃) δ (ppm): 14.11, 17.95, 20.60, 22.68, 26.92, 29.33, 29.58, 29.61, 29.62, 29.67, 31.90, 34.75, 36.46.

(R)-1-iodo-2-methyl-dodecane (14) was obtained by the same procedure, starting with (S)-3-hydroxy-2-methyl propanoate (12) via (S)-3-tert-butylidimethylsilyloxy-2-methylpropyl iodide (13) (Marshall et al., 1987, Nakamura and Mori, 2000) in 72% overall yield. GC retention times and NMR data matched those of the (S)-enantiomer (11). Optical rotation for iodide 14: [α]D20 = - 2.04 (c 2.35; CHCl₃).

(S,S)-2,14-Dimethyltricosane-1-ol (16 in Figure 3.2)

CuI (0.38 g) was added under Argon at -30 °C to a freshly prepared stirred Grignard solution of 1,9-nonadimagnesium bromide [from 2.03 ml 1,9-dibromononane (10 mmol) and magnesium turnings (0.97 g)]. After stirring 20 min, a solution of the iodides 9 and 11 (10 mmol each) in 50 ml THF was added. The reaction mixture was kept at -20 °C for 1 h, and then warmed to rt and quenched with saturated aqueous NH₄Cl. The products were extracted (3 x 50 ml) with hexane/ether (1:1), the extracts washed with brine, dried (MgSO₄) and concentrated in vacuo. Monosilyl ether 15 constituted 20% of the evaporation residue (GC/MS). Filtering through silica (10 g) with hexane afforded a non-polar fraction which was concentrated and treated with 40 mmol of tetrabutylammonium fluoride hydrate in 100 ml THF at rt (72 h stirring). Regular work-
up and two flash chromatography runs (50 g of silica each with 10% ether in hexane as eluent) afforded 499 mg of pure alcohol 16 [1.3 mmol in 13% yield based on 3 or 5, 50% (i.e., 5 mmol) theoretical yield]. \([\alpha]_D^{20} = -3.8\) (c 0.21; CHCl₃). \(^1^H\) NMR (CDCl₃), \(\delta\) (ppm): 0.83 (d, 3H, \(J = 6.6\) Hz), 0.88 (t, 3H, \(J = 6.9\) Hz), 0.91 (d, 3H, \(J = 6.8\) Hz), 1.18-1.49 (m, 19H), 1.61 (m, 1H), 3.41 (dd, 1H, \(J = 6.6, 10.5\) Hz), 3.51 (dd, 1H, \(J = 5.8, 10.5\) Hz). \(^1^C\) NMR (CDCl₃), \(\delta\) (ppm): 14.10, 16.57, 19.71, 22.69, 26.99, 27.09, 29.36, 29.66 (2), 29.67, 29.69 (unresolved), 29.70 (2), 29.73, 29.95, 30.04, 31.93, 32.75, 33.15, 35.76, 37.10, 68.41. HRMS (ESI) calcd. for C₂₆H₅₃ ([M+H⁺]-H₂O): 365.4147, found 365.4140.

\((R,S)-5,17-\text{Dimethylheptacosane} (17 \text{ in Figure 3.2})\)

CuI (0.38 g) was added under Argon at -30°C to a freshly prepared stirred solution of Grignard reagent (from 1.50 ml of 1-bromopropane and 0.49 g of Mg). To this mixture the mesylate of alcohol 16 [freshly prepared from alcohol 16 (240 mg; 0.63 mmol)], mesyl chloride (0.50 ml) and triethylamine (1 ml) in dichloromethane (15 ml) at 30°C/30 min in 5 ml of THF was added dropwise in 10 min. The reaction mixture was stirred at -23°C for 30 min, allowed to warm to rt, and quenched with aqueous NH₄Cl. \((R,S)-5,17-\text{Dimethylheptacosane} (17)\) was extracted with hexane (3 x 30 ml). Extracts were dried (MgSO₄) and concentrated in vacuo. Hydrocarbon 17 was purified by flash chromatography on 20 g of silica with hexane as eluent, affording 170 mg of pure 17 (0.42 mmol, 66.7% yield, 8.7% overall yield based on 9 or 11). Anal. calculated for C₂₉H₆₀ (%): C 85.21, H 14.79; found: C 85.28, H 14.60. \(^1^H\) NMR (CDCl₃), \(\delta\) (ppm): 0.83 (d, 3H, \(J = 6.5\) Hz), 0.84 (d, 3H, \(J = 6.5\) Hz), 0.88 (t, 3H, \(J = 6.9\) Hz), 0.89 (t, 3H, \(J = 6.8\) Hz), 1.18-1.40 (m, 48H). \(^1^C\) NMR (CDCl₃), \(\delta\) (ppm): 14.11, 14.17, 19.73, 22.69, 23.05, 27.09, 29.34, 29.36, 29.67 (unresolved), 29.70, 29.74, 30.04, 31.93, 32.74, 32.75, 36.79, 37.10, 37.11.

\((R,R)-5,17-\text{Dimethylheptacosane} (18 \text{ in Figure 3.2})\)

Hydrocarbon 18 was obtained in a similar way from the iodides 9 and 14 via intermediate \((S,R)-2,14-\text{dimethyltetraicosane}-1\)-ol \([\alpha]_D^{20} = -3.9\) (c 1.41; CHCl₃), GC retention times and NMR data matched those of alcohol 16]. Overall yield of 18 based on 9 or 14 was 10.9%. Anal. calculated for C₂₉H₆₀ (%): C 85.21, H 14.79; found: C 85.10, H 14.45. GC retention times and NMR data matched those of 17.
(S,S)-5,17-Dimethylheptacosane (19 in Figure 3.2)

Hydrocarbon 19 was obtained in a similar way from the iodides 11 and 13 via intermediate (R,S)-2,14-dimethyltetrasacose-1-ol \([\alpha]_D^{20} = +4.1 \text{ (c 1.06; CHCl}_3)\), GC retention times and NMR data matched those of alcohol 16. Overall yield of 19 based on 5 or 7 was 34.3%. Anal. calculated for C_{29}H_{60} (%): C 85.21, H 14.79; found: C 85.08, H 14.66. GC retention times and NMR data matched those of alcohol 17.

(S,R)-5,17-Dimethylheptacosane (20 in Figure 3.2)

Hydrocarbon 20 was obtained in a similar way from the iodides 13 and 14 via intermediate (R,R)-2,14-dimethyltetrasacose-1-ol \([\alpha]_D^{20} = +4.9 \text{ (c 0.59; CHCl}_3)\), GC retention times and NMR data matched those of alcohol 10. Overall yield of 20 based on 13 or 14 was 12.9%. Anal. calculated for C_{29}H_{60} (%): C 85.21, H 14.79; found: C 84.88, H 14.53. GC retention times and NMR data matched those of hydrocarbon 17.

Syntheses of the known enantiomers 24 and 25 of 5-methylheptacosanes (Fig. 3; Marukawa et al., 2001) were carried out using the same chiral precursors 7 and 12 we employed for the synthesis of stereoisomers of 5,17-dimethylheptacosane.

(R)-5-Methylheptacosane (24 in Figure 3.3)

Silylation of the propanoate 7 followed by borane reduction and Sworn oxidation afforded (R)-tert-butylidemethylsilyloxy-2-methyl-propanal (21) (Burke et al. 1985). Using a Wittig reaction, the aldehyde 21 was immediately converted to the olefins 22 (Z/E ratio = 10:1) which were separated from polar impurities on silica with hexane as eluent. Without additional purification, the olefins were subjected to consecutive desilylation (with Bu₄NF) and hydrogenation (with H₂/5% Pd/C) leading to (S)-2-methyl-tetrasacose-1-ol (23) (Mori and Jiang, 1992) (m.p. 62-63 °C; 21.7 % yield based on 7). Mesylation of 23 followed by reaction with propylmagnesium bromide (via dicuprate) afforded (R)-5-methylheptacosane (24) (Marukawa et al., 2001) in 45% yield (9.8% overall yield). \(^1\)H NMR (CDCl₃), \(\delta\) (ppm): 0.84 (d, 3H, \(J = 6.6 \text{ Hz}\)), 0.88 (two t, 6H, \(J = 6.9 \text{ Hz}\), \(J = 6.9 \text{ Hz}\)), 1.15-1.40 (m, 49H). \(^{13}\)C NMR (CDCl₃), \(\delta\) (ppm): 14.11, 14.16, 19.72, 22.69, 23.05, 27.09, 29.34, 29.37, 29.66, 29.70 (unresolved), 29.74, 29.70, 30.04, 31.93, 32.74, 36.78, 37.41. Spectral data of 24 and synthetic intermediates matched those reported in the literature.
(S)-5-Methylheptacosane (25 in Figure 3.3)

Compound 25 was synthesized in the same way from methyl (S)-3-hydroxy-2-methylpropanoate 12 via (S)-tert-butyldimethylsilyloxy-2-methyl propanal (Thomas and Whitehead, 1989), and corresponding (R)-2-methyltetrasane-1-ol (Mori and Jiang, 1992). The spectral data of 25 and synthetic intermediate compounds matched those of their respective enantiomers 22, 23, and 24. Overall yield of 25 was 8.3%.

3.3.6. General Design of Experiments

As visual signals between prospective mates play an important role in the sexual communication system of parasitoids, two O. kuvanae females were chosen as a vehicle for stimuli application in each bioassay. To eliminate mate choice by females (van den Assem, 1986), females were killed on dry ice and randomly assigned a treatment or control stimulus. Aliquots of test stimuli [treatment: body surface extract (1 insect equivalent or synthetic compounds in 0.5 µl of solvent; control: 0.5 µl of solvent] were applied through a drawn-out micro-capillary tube to the females’ antennae. Stimuli were applied to antennae because close-range pheromones are transferred primarily via antennation (Ayasse et al., 2001), antennal tips are the point of contact between courting O. kuvanae males and females (Ablard et al., 2011), and the CHC profile of the females’ antennae does not differ from that of other body parts (data not shown). The two females were placed 1 cm apart under a Petri dish lid (30 mm diam) similar in size to a gypsy moth egg mass, and thus conducive to study close-range mate searches by males. Two sexually inexperienced males were then placed under the Petri dish lid to record which female they first sought and approached (close-range attraction), and antennated. A bioassay was terminated when one of the males had contacted a female. When males approached a female but then turned away without making antennal contact, their behavior was recorded as repulsion.

3.3.7. Specific Experiments

We predicted that the body surface extract of males, but not of females, when applied to females would deter males from contacting such females. In two-choice
experiments 1 and 2, we thus applied 0.5 µl aliquots of hexane (solvent control) or body surface extract of females (treatment) (Exp. 1) or males (Exp. 2) to a dead female.

With males avoiding females treated with body surface extract of males (see Results and Discussion), and with 5-methylheptacosane (5-me-27Hy) and 5,17-dimethylheptacosane (5,17-dime-27Hy) being more abundant in body surface extracts of males than of females (see Results and Discussion), we further predicted that the absolute configuration of 5-me-27Hy and 5,17-dime-27Hy contributes to mate recognition. Specifically, we tested in experiments 3-10 (N=20 each) eight 2-component blends, each blend containing one of the two enantiomers of 5-me-27Hy (at 3 ng) and one of the four stereoisomers of 5,17-dime-27Hy (at 6 ng) as follows: experiment 3: 5R-me-27Hy plus 5S,17S-dime-27Hy; experiment 4: 5R-me-27Hy plus 5R,17S-dime-27Hy; experiment 5: 5R-me-27Hy plus 5S,17R-dime-27Hy; experiment 6: 5R-me-27Hy plus 5R,17R-dime-27Hy; experiment 7: 5S-me-27Hy plus 5S,17S-dime-27Hy; experiment 8: 5S-me-27Hy plus 5R,17S-dime-27Hy; experiment 9: 5S-me-27Hy plus 5S,17R-dime-27Hy; experiment 10: 5S-me-27Hy plus 5R,17R-diMe-27Hy. The 1:2 blend ratio of synthetic compounds was based on a 1:2 to 1:4 blend ratio of 5-me-27Hy and 5,17-dime-27Hy in body surface extracts of males and females. All eight experiments were run in parallel, completing one or two replicates of each experiment on the same day.

Taking into account that the blend of 5S-me-27Hy plus 5R,17S-diMe-27Hy attracted males in experiment 8, and that these two components may constitute sex pheromone components of the female, we then tested 5S-me-27Hy (6 ng) and 5R,17S-diMe-27Hy (6 ng) singly (experiments 11 and 12) and in combination (experiment 13) to determine whether both components are essential in attracting males. As they were (see Results and Discussion), we re-tested the blend of 5S-me-27Hy (3 ng) and 5R,17S-dime-27Hy (6 ng) in experiment 14 as a positive control, and in parallel experiment 15 tested whether the blend’s attractiveness was compromised in the presence of other stereoisomers, as implicated by the results of experiments 1 and 2, and 3-10. Specifically, in experiment 15 we tested the blend of racemic 5-me-27Hy (6 ng, 5S- and 5R-me-27Hy) and racemic 5,17-dime-27Hy (24 ng, 5R,17S-, 5S,17S-, 5S,17R-, and 5R,17R-dime-27Hy) versus a solvent control.
In each of experiments 11 and 12, the synthetic standard was tested at the same amount (6 ng) to eliminate otherwise uneven compound quantity as a response variable to bioassay males. In experiment 13, a 1:2 ratio of 5S-me-27Hy and 5R,17S-diMe-27Hy, instead of the 1:1 ratio we tested, would have better approximated the ratio found in body surface extracts. In experiment 14, we thus retested 5S-me-27Hy and 5R,17S-diMe-27Hy at a 1:2 ratio. In parallel experiment 15, we doubled the amount of racemic 5-me-27Hy (6 ng) and quadrupled the amount of racemic 5,17-dime-27Hy (24 ng) to ensure that 5S-me-27Hy and 5R,17S-diMe-27Hy were present at the same amount and ratio as in experiment 14.

3.3.8. Data analysis

Data of all experiments were analyzed by a chi-square test in PASW v. 18.0 (International Business Machines Corp.)

3.4. Results and Discussion

Comparative gas chromatograms of body surface extracts of male and female O. kuvanae revealed no sex-specific components, yet males avoided females treated with body surface extract of males (Figure 3.4), suggesting that the CHC profiles of males and females differ qualitatively. Two components (A and B in Figure 3.5) were consistently more abundant in extracts of males than in extracts of females, suggesting that they could play a role in mate recognition or attraction. The retention indices (RI) (van den Dool and Kratz 1963) of component A (2748) and B (2781) on a DB-5 column differed from those of straight-chain aliphatic alkanes, indicating that both components were either unsaturated and/or contained one or more methyl branches. El and Cl mass spectra of both components showed no evidence for double bonds, but revealed that the molecular weights (MW) of A (394) and B (408) differed by one carbon. Based on our RI library of methylated alkanes (e.g., Gries et al., 1993a,b, 1994, 1997, 2005), we could infer that A was likely a mono-methylheptacosane. Moreover, its mass spectrum revealed enhanced fragmentions m/z 84, 85 and 337 (Figure 3.5), indicative of a methyl branch at C5. Drawing further on evidence that the mass spectral cleavage of methylated long-chain hydrocarbons occurs at the carbon carrying the methyl branch.
(e.g., Schlamp et al., 2005), we hypothesized that A was 5-me-27Hy. Synthetic 5-me-27Hy had identical retention and mass spectral characteristics as insect-produced A, and a mass spectrum as previously reported for this compound (Gulias Gomes et al., 2008; Mullen et al., 2008), thus confirming the structural assignment.

Component B, with a 14-dalton higher molecular weight but only a 40-unit greater RI than component A, was hypothesized to be a dimethylheptacosane. Its mass spectrum with fragment ions m/z 84, 85, 168 and 267 suggested that one branch was again at C5. We inferred the position of the second branch based on the fragment ions m/z 168 and 267. The former indicated a methyl branch at C10 or C17. We favored C17 because of the diagnostic ion m/z 267 (MW minus 141; see Figure 3.5), but synthesized both 5,10- and 5,17-dimethylheptacosane. Retention time and mass spectral characteristics only of 5,17-dimethylheptacosane matched those of insect-produced B. Its mass spectrum was consistent with previous reports (Gulias Gomes et al., 2008).

The ratio of 5-me-27Hy and 5,17-dime-27Hy (1:2 to 1:4) was similar in extracts of males and females. Moreover, even though these two components were more abundant in body surface extracts of males, this difference seemed insufficient for a reliable, gender-specific CHC profile. We thus hypothesized that the CHC profile of females differs from that of males, in part, in the absolute configurations of 5-me-27Hy and 5,17-dime-27Hy, and possibly other methylated CHCs. Determining the stereochemistry of 5-me-27Hy and 5,17-dime-27Hy produced by males and females proved impossible. There is no chiral GC column available yet that separates stereoisomers of long-chain methylated alkanes. Thus, we decided to synthesize all possible stereoisomers and to bioassay the insects’ response to them. As males search for newly eclosed virgin females as prospective mates, we bioassayed the response of males to blends of synthetic stereoisomers applied to females.

Of eight 2-component synthetic blends, each containing one of the two enantiomers of 5-me-27Hy and one of the four stereoisomers of 5,17-dime-27Hy, the blend of 5S-me-27Hy and 5R,17S-dime-27Hy was attractive to males, and the blend of 5R-me-27Hy and 5R,17R-dime-27Hy was repellent (Figure 3.6). All other blends had no behaviour-modifying effect (Figure 3.6). 5S-Me-27Hy and 5R,17S-dime 27Hy in
combination, but not singly, attracted males (Figure 3.7), indicating that both are essential close-range pheromone components.

The blend of 5S-me-27Hy and 5R,17S-dime-27Hy was not attractive in the presence of other stereoisomers. This became apparent when we tested the blend of racemic 5-me-27Hy and 5,17-dime-27Hy, and the blend of 5S-me-27Hy and 5R,17S-dime-27Hy, in parallel-run experiments 14 and 15 (Figure 3.8). The racemic blend in experiment 15 likely had no behavioural activity because the attractiveness of the pheromone components was masked by the repellency of 5R-me-27Hy and 5R,17R-dime-27Hy (see experiment 6; Figure 3.6).

We cannot rule out the possibility that other components in the CHC profile of females are part of the sex pheromone. Aliquots of synthetic components were applied to freshly killed females enhancing, or interfering with, the effect of other CHCs on the integument. We attempted to strip CHCs off the integument of females prior to applying synthetic blends, but learned that even consecutive body surface extracts of the same females still contained significant amounts of CHCs (data not shown). Baited decoys instead of baited dead females proved less effective in inducing attraction of males (see also Carlson et al., 2005), indicating that males respond best to a combination of olfactory and visual signals and/or that the female sex pheromone contains additional as yet unknown components. These may even be specific triacylglycerides, which have just been identified as sex pheromone components in body surface extracts of the parasitic wasp Lariophagus distinguendus (Kuhbander et al., 2012).

Our findings that O. kuvanae males (1) responded equally to females treated, or not, with body surface extract of females (Figure 3.4, experiment 1), (2) oriented toward females treated with a blend of 5S-me-27Hy and 5R,17S-dime-27Hy (Figure 3.6, experiment 8 ), and (3) avoided females treated with body surface extract of males (Figure 3.4, experiment 2) or the blend of 5R-me-27Hy and 5R, 17R-dime-27Hy (Figure 3.6, experiment 6), all indicate that the olfactory system of males is capable not only of recognizing pheromone components but also of detecting pheromone antagonists that differ in absolute configuration. Such discriminatory ability of olfactory receptors is likely to evolve if these antagonists had a functional role in the sexual communication system of O. kuvanae.
The stereochemistry of optically-active sex pheromone components imparts species-specific communication channels in many insects. Channels vary in that only one optical isomer is attractive and the antipode is not active (e.g., Tóth et al., 1989; King et al., 1995; Duff et al. 2001), two isomers are synergistic (e.g., Borden et al., 1976; Millar et al., 1991; Gries et al., 1999, 2003), or non-pheromonal isomers are antagonistic (e.g., Klimetzek et al., 1976; Cardé et al., 1977; Miller et al., 1977; Plimmer et al., 1977). Antagonists can also be pheromone components of heterospecifics that compete for the same communication channel (e.g., Oehlschlager et al., 1987; Szöcs et al., 1993; Leal, 1996). This does not apply to O. kuvanae because sexual communication takes place on L. dispar egg masses when the wasps emerge en masse, which is not likely to coincide with emergent periods of other less common egg parasitoids of L. dispar, such as Anastatus disparis. Thus, the males’ avoidance of females treated with body surface extract of males (Figure 3.4, experiment 2), or of females treated with the blend of 5R-me-27Hy and 5R,17R-dime-27Hy (Figure 3.6, experiment 6), could not have been due to recognition of heterospecific pheromone components. Instead, it may have been due to recognition of CHCs that are gender-specific and conceivably produced by males.

Cuticular hydrocarbons have been implicated or documented to contribute to gender or mate recognition in many insect species (Howard and Blomquist, 2005). Males or females may produce distinctive components not produced by the other gender (Trabalon et al., 1992; reviewed by Howard and Blomquist, 2005) or they may produce the same components at gender-specific ratios (Jallon and David, 1987; Steiner et al., 2006). The interpretation of data is sometimes difficult because the reported qualitative or quantitative difference in the CHC profile of males or females may or may not play a critical role in gender recognition, and relatively few studies have linked specific CHCs with behavioral responses by the insects (e.g., Ginzel et al., 2003; Carlson et al., 2005).

When there is no apparent difference in the CHC profile of males and females, like in various species of parasitoid wasps (Howard, 1992; Howard and Liang, 1993), or when differences seem subtle, like in O. kuvanae, the stereochemistry of methylated alkanes or alkenes could provide a mechanism that may facilitate gender or mate recognition. Although appealing, this concept will be difficult to prove and will depend upon the development of analytical technology (see above) that allows unequivocal assignment of the specific stereoisomers produced by males or females. Once
developed, such technology could help determine whether specific stereoisomers play a role in mate recognition, or in species recognition in closely-related congeners that share one or more methylated CHCs, as shown in the cerambycid beetles *Tetropium fuscum* and *T. cinnamopterum* (Silk et al., 2011). Until then, support for these concepts will rely solely on bioassays of stereospecific methylated alkanes or alkenes.

![figure with chemical reactions](image)

**Figure 3.1.** Synthesis of racemic 5,17-dimethylheptacosane.

1-6 (a): n = 11, m = 10; 1-6 (b): n = 4, m = 17;
Figure 3.2. Syntheses of \((R,R)\)-, \((R,S)\)-, \((S,R)\)-, and \((S,S)\)-5,17-dimethylheptacosanes.
Figure 3.3. Syntheses of (R)- and (S)-5-methylheptacosanes.

Figure 3.4. Percent male *Ooencyrtus kuvanae* responding in experiments 1 and 2 to two recently killed conspecific females treated either with a hexane control stimulus (0.5 µl) or body surface extract of females (experiment 1) or males (experiment 2) at 1 insect equivalent (1 IE). Both experiments (N = 20 each) were run in parallel. In each experiment, bars labeled with different letters are statistically different. Note: 1 IE = amount of compounds extracted from the body surface of one insect during 10 minutes.
Figure 3.5. Gas chromatograms (see Methods and Material for details) of body surface extracts of male and female *Ooencyrtus kuvanae*, and mass spectra of cuticular hydrocarbon components A and B which were consistently more abundant in extract of males.
Table 3.6. Percent male Ooencyrtus kuvanae responding in experiments 3-10 (N = 20 each) to two recently killed conspecific females treated either with a pentane control (0.5 µl) or with a 2-component synthetic blend in pentane, each blend containing one of the two enantiomers of 5-methylheptacosane (5-me-27Hy) and one of the four stereoisomers of 5,17-dimethylheptacosane. All eight experiments were run in parallel. In each experiment, bars labeled with different letters are significantly different.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>χ²</th>
<th>P</th>
<th>Stimuli tested</th>
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<td>3</td>
<td>0.2</td>
<td>0.65</td>
<td>5R-me-27Hy (3 ng) 5S,17S-dime-27Hy (6 ng) Solvent control</td>
</tr>
<tr>
<td>4</td>
<td>0.2</td>
<td>0.65</td>
<td>5R-me-27Hy (3 ng) 5S,17S-dime-27Hy (6 ng) Solvent control</td>
</tr>
<tr>
<td>5</td>
<td>0.8</td>
<td>0.37</td>
<td>5R-me-27Hy (3 ng) 5S,17R-dime-27Hy (6 ng) Solvent control</td>
</tr>
<tr>
<td>6</td>
<td>7.2</td>
<td>0.007</td>
<td>5R-me-27Hy (3 ng) 5R,17R-dime-27Hy (6 ng) Solvent control</td>
</tr>
<tr>
<td>7</td>
<td>0.8</td>
<td>0.37</td>
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<tr>
<td>8</td>
<td>7.2</td>
<td>0.007</td>
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</tr>
<tr>
<td>9</td>
<td>0.0</td>
<td>1.0</td>
<td>5S-me-27Hy (3 ng) 5S,17R-dime-27Hy (6 ng) Solvent control</td>
</tr>
<tr>
<td>10</td>
<td>0.0</td>
<td>1.0</td>
<td>5S-me-27Hy (3 ng) 5R,17R-dime-27Hy (6 ng) Solvent control</td>
</tr>
</tbody>
</table>

Stimuli tested  | % females first contacted by males
<table>
<thead>
<tr>
<th>Exp. 11</th>
<th>$\chi^2 = 1.0; P = 1.0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5S-me-27Hy (6 ng)</td>
<td>a</td>
</tr>
<tr>
<td>Solvent control</td>
<td>a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exp. 12</th>
<th>$\chi^2 = 0.8; P = 0.8$</th>
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</thead>
<tbody>
<tr>
<td>5R,17S-dime-27Hy (6 ng)</td>
<td>a</td>
</tr>
<tr>
<td>Solvent control</td>
<td>a</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Exp. 13</th>
<th>$\chi^2 = 5.0; P = 0.025$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5S-me-27Hy (6 ng)</td>
<td>a</td>
</tr>
<tr>
<td>5R,17S-dime-27Hy (6 ng)</td>
<td>b</td>
</tr>
<tr>
<td>Solvent control</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3.7.** Percent male *Ooencyrtus kuvanae* responding in experiments 11-13 ($N = 20$ each) to two recently killed conspecific females treated with a pentane control (0.5 µl) or with synthetic (5S)-methylheptacosane (5S-me-27Hy), (5R,17S)-dimethylheptacosane (5R,17S-dime-27Hy) or both, all diluted in pentane. All three experiments were run in parallel. In each experiment, bars labeled with different letters are statistically significant.
Figure 3.8. Percent male *Ooencyrtus kuvanae* responding in experiments 14-15 (*N = 20 each*) to two recently killed conspecific females treated with a pentane control (0.5 µl) or with a 2-component blend of (5S)-methylheptacosane (5S-me-27Hy) and (5R,17S)-dimethylheptacosane (5R,17S-dime-27Hy) or racemic 5-methylheptacosane (5-me-27Hy) and 5,17-dimethylheptacosane (5,17-dime-27Hy), all diluted in pentane. The two experiments were run in parallel. In each experiment, bars labeled with different letters are statistically significant.

### 3.5. Acknowledgements

We thank G. Andersen, M. Andersen, H. Bottomley, U. Somjee, and O. Moeri for technical assistance; E. Kiehlmann for constructive comments, and S. DeMuth for graphical illustrations. 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 (GF-18-2007-226; GF-18-2007-227) as sponsors.
3.6. Literature cited


4. Mechanisms, functions, and fitness consequences of pre- and post-copulatory rituals of the parasitoid wasp *Ooencyrtus kuvanae*³

4.1. Abstract

Males and females of the parasitoid wasp *Ooencyrtus kuvanae* (Howard) (Hymenoptera: Encyrtidae) emerge en masse from gypsy moth, *Lymantria dispar dispar* (L.) (Lepidoptera: Noctuidae: Lymantriinae), host egg masses. Males engage females in a brief pre-copulatory ritual, mate, and then execute a post-copulatory ritual. We investigated mechanisms, functions, and fitness consequences of the pre- and post-copulatory ritual by high-speed cinematography, gas chromatographic-mass spectrometric analyses of volatile constituents on the insects' integument, and behavioral assays. Our data indicate that the mechanisms of the pre- and post-copulatory ritual are physical interactions rather than pheromone transfer. During the pre-copulatory ritual, the males put females into a trance-like state that persists for some time after copulation. Males attained a mating with in-trance females 9.5 times faster than with females that had come out of trance. Mated females with post-copulatory ritual experience did not remate, whereas females lacking that experience did. The total number of offspring and daughters did not differ between females with or without post-copulatory ritual experience or in relation to the duration of that ritual. The post-copulatory ritual functions as a form of mate guarding in that the male accelerates awakening of the in-trance female, which then rejects mating attempts by other males, ensuring his paternity.

4.2. Introduction

Mating systems of parasitoid wasps are diverse and thus ideal to study insect courtship behavior (Godfray, 1994). They vary in their degree of conflict among males, female choice, spatial and temporal occurrence of females and their reproductive biology, and in operational sex ratio (Bradbury & Vehrencamp, 1977; Emlen & Oring, 1977; Thornhill & Alcock, 1983; Godfray, 1994).

In most polygynous female-defense mating systems of quasi-gregarious parasitoid wasps (solitary parasitoids whose host is in clumped and discrete patches), females can mate multiply, control the sex of their offspring by haplodiploid sex determination, and produce a daughter-biased sex ratio, whereas males remain at the emergence site, compete over virgin females, and often mate with their sisters (Emlen & Oring, 1977; Thornhill & Alcock, 1983; Godfray, 1994; Godfray & Cook, 1997). A daughter-biased sex ratio is thought to reduce competition among sons for mating opportunities with their sisters, because only one or a couple of sons can mate multiple sisters; however, this has rarely been demonstrated (Hamilton, 1967; Taylor, 1981; Abe et al., 2007; Somjee et al., 2011). Instead of not competing, males might enter a scramble competition by quickly courting virgin females and then by guarding them through a post-copulatory ritual (van den Assem, 1986; Allen et al., 1994; Andersson, 1994; Godfray, 1994; Godfray & Cook, 1997; Quicke, 1997; Shuster & Wade, 2003).

Most described mating systems in arthropods entail either a pre- or a post-copulatory ritual. Pre-copulatory rituals occur, among others, in the windscorpion, *Eremobates pallipes* (Say), the desert spider *Agelenopsis aperta* (Gertsch), *Ancylocmetes* spiders, camel spiders of the order Solifugae (Comstock, 1980; Tomasinelli, 2003; Becker et al., 2005), and parasitoids such as *Stenomalina liparoe* (Giraud) (van den Assem, 1986). These rituals entail routine repetitive, stereotypic strikes of the female by the courting male (Bastock, 1967; van den Assem, 1986; Eberhard, 1996) or aphrodisiacs that place the female into a trance-like state (from here on 'trance') and/or receptive state prior to, and during, copulation (van den Assem, 1970; Khasimuddin & DeBach, 1975; Bin et al., 1999; Becker et al., 2005).
If a pre-copulatory ritual puts females into trance, mated females could remain receptive to additional matings. This should favor selection for post-copulatory mechanisms that ensure the reproductive success and increased fitness of the first male mate. These mechanisms may entail (1) physical guarding of the mated females (Viggiani & Mazzone, 1980; Thornhill & Alcock, 1983; Suzuki & Hiehata, 1985; Field & Keller, 1993), (2) sperm precedence (Allen et al., 1994) that renders a mated female unreceptive to further matings (Gordh & DeBach, 1978; Alcock & Buchmann, 1985; van den Assem, 1986; van den Assem & Werren, 1994; Brown et al., 1997), and rarely (3) pheromone transfer (King & Fischer, 2005). The post-copulatory ritual could confer a fitness advantage on the male if it ensures his paternity.

The post-copulatory ritual could also confer a fitness advantage on the female, allowing her to reassess her mate, gain the time needed for sperm transfer, or utilize sperm in favor of producing daughters, compared with females that mated with males that could not complete the post-copulatory ritual (Kajita, 1986; Simmons, 2001). In systems of haplodiploid sex determination and potential inbreeding, as in *Ooencyrtus kuvanae* (Howard) (Hymenoptera: Encyrtidae), inbreeding causes mothers to share more genes with their daughters than with their sons, favoring the production of daughters (Boomsma & Grafen, 1991). This prediction has been supported by a study of fig wasps (Herre, 1985), revealing that in those species where female wasps are more likely to mate with a relative they adjust the sex ratio of their offspring toward daughters.

Females rendered unreceptive as a result of the post-copulatory ritual, as shown for *Aphytis melinus* (DeBach) (Allen et al., 1994), may benefit further from the post-copulatory ritual if it prevents additional matings, which could make females adjust the sex ratio of her offspring toward more or only sons (Flanagan et al., 1998; Jacob & Boivin, 2005; Santolamzza-Carone & Pestana, 2010).

Mating systems with both a pre- and a post-copulatory ritual are known to occur only in a few parasitoid species (Bastock, 1967; van den Assem, 1986; Eberhard, 1996) and the underlying mechanism and function of these rituals when they occur in tandem are not well understood (Brown et al., 1997; Quicke, 1997). The quasi-gregarious, 2-mm egg parasitoid wasp *O. kuvanae* exhibits both pre- and post-copulatory rituals. The courtship rituals take place on egg masses of host gypsy moth,
Lymantria dispar dispar (L.) (Lepidoptera: Noctuidae: Lymatriinae). The uppermost layer of an egg mass, which measures 2–3 cm across and contains several hundred eggs covered in setae (Brown, 1984), is parasitized by female wasps that insert a single egg into each accessible host egg. Ooenycruts kuvanae have a haplodiploid sex determination system whereby diploid daughters are the result of fertilized eggs, and haploid sons are the result of unfertilized eggs. Within 3-4 weeks, wasps complete development inside host eggs and related offspring emerge as sexually mature adults that can live 4–6 weeks. Males emerge 1 day earlier than females, and are half as numerous as females. Males typically remain on the egg mass until there are no more mating opportunities, whereas mated females disperse within 24 h (Brown, 1984). In this competitive mating system, an MAO or HGG male engages a female in a brief (ca. 4 s) pre-copulatory ritual, then mates, and immediately performs a post-copulatory ritual, which may last 15–67 s. Pheromonal communication during courtship has yet to be demonstrated.

Our objectives were to unravel the mechanisms, functions, and fitness consequences of pre- and post-copulatory rituals of O. kuvanae, predicting that both rituals are functionally linked and confer a fitness advantage on one or both mates.

4.3. Materials and Methods

4.3.1. Insects

A new colony of O. kuvanae was started every 12 months with specimens field-collected from Mt. Gretna, Pennsylvania (40°14´N, 76°27´W), or North East, Maryland (39°36´N, 75°55´W) (USA). All insects were reared under a L16:D8 photoperiod at 22–25 °C and 50–70% r.h. (Hofstetter & Raffa, 1997) in the Global Forest Quarantine Facility of Simon Fraser University on eggs of gypsy moth supplied by the US Forest Service (Hamden, CT, USA). They were contained in Plexiglas cages (40 x 40 x 30 cm) and provisioned with cotton wicks (1 x 10 cm; Richmond Dental, Charlotte, NC, USA) soaked in a 30% (wt/vol) honey water solution every 2 days. Insects were used in all experiments within 1-3 days after emergence to avoid adverse effects associated with aging (van den Assem, 1996). Every 21 days, 10 gypsy moth egg masses were
introduced to be parasitized by female wasps. Fourteen days later, parasitized eggs were removed, and at least 800 eggs were placed singly into translucent plastic cups (103.5 ml) and secured with a lid. Emergent insects were provisioned with a honey water-soaked cotton wick (1 x 1.3 cm) and separated by sex and size under a microscope.

4.3.2. **Mechanisms of the pre- and post-copulatory ritual**

**Physical interactions between a male and a female during pre- and post-copulatory rituals**

Experiment 1 sought evidence in both the pre- and post-copulatory rituals (N = 10 each) for stereotypic strikes of the female by the courting male that could put her into trance prior to copulation or awaken her from it after copulation. The exceedingly fast rituals were filmed using the Fastec imaging camera IN1000M2GB, equipped with the Fastec imaging software version 3.0.4 (Fastec Imaging, San Diego, CA, USA). Footage was obtained at rates of 250 or 500 frames per second, with a resolution of 440 x 330 and 320 x 240, respectively. In each replicate, a female and a male were placed on filter paper and retained in a glass Petri dish on scale to the field of view of the camera lens (12 mm). Frame by frame analysis of footage with MiDAS player software version 5.0.0.3 (Xcitex, Cambridge, MA, USA) determined the male’s and female’s body parts coming in contact with another.

**Do body washes of wasps reveal pheromone transfer during pre- and post-copulatory rituals?**

Experiment 2 was designed to reveal evidence for potential pheromone transfer during pre- and post-copulatory rituals. Groups of five males or five females without any prior contact with potential mates, groups with just pre-copulatory ritual experience, and those with both pre- and post-copulatory ritual experience (N = 6 each) were placed into six separate vials, each vial containing 30 µl of hexane and (9E)-octa-decen-1-yl acetate as a generic internal standard. Individuals with ritual experience were placed into vials within 30 s of obtaining it. Aliquots of samples were analyzed by coupled gas chromatography-mass spectrometry (GC-MS), using a Varian Saturn 2000 Ion Trap GC-MS and a Varian workstation version 6.9.1 (Agilent Technologies, Mississauga, ON,
Canada). The GC-MS was fitted with a DB-5 column (30 m x 0.25 mm inner diameter), employing the following temperature program: 2 min at 50 °C and 20 °C per min to 280 °C.

Do behavioral responses of wasps reveal evidence of pheromone transfer during the post-copulatory ritual?

Experiment 3 (N = 20) was designed to explore whether females with post-copulatory ritual experience are less attractive to males than females without that experience. A virgin male without prior contact with a female was confined for 10 min in a Petri dish (30 mm diameter) with two females, each of which had mated within the preceding 1 min. One female had experienced the post-copulatory ritual, whereas the other had not. The latter type of female had been obtained by allowing her to mate with a male and by removing him before he could perform the post-copulatory ritual. The time the virgin male spent approaching or contacting each female was recorded.

4.3.3. Functions of the pre- and post-copulatory ritual

Does the pre-copulatory ritual put females into a trance?

Experiment 4 explored whether the pre-copulatory ritual has the effect to put a female into trance. A male was confined with a virgin female in a Petri dish (30 mm diameter). The male was removed immediately following completion of the pre-copulatory ritual (N = 20), following the pre-copulatory ritual and copulation (N = 20), and following the post-copulatory ritual (N = 20). In each replicate, the female’s behavior was recorded, for up to 5 min following removal of the male.

Do in-trance females allow mating more quickly than those not in a trance?

Experiment 5 was designed to investigate whether in-trance females allow mating more quickly than those not in a trance. A male was confined with a virgin female in a Petri dish (30 mm diameter). After he had completed the pre-copulatory ritual with the female, he was removed. In treatment 1 (N = 20), immediately following removal of the first male, a new male was introduced and confined with the in-trance female. In treatment 2 (N = 20), the new male was introduced 3.5 min after removal of the first
male, when the female appeared to have come out of the trance (see Experiment 4). In both treatments, the time elapsed until the female was mated was recorded.

4.3.4. **Fitness consequences of the post-copulatory ritual**

Do males gain a fitness advantage by engaging females in the post-copulatory ritual?

Experiments 6 and 7 (N = 20 each) were designed to investigate whether the post-copulatory ritual serves as a form of ‘in absentia’ mate guarding by males following mating. In experiment 6, a male without prior contact with a female was confined for 10 min in a Petri dish (30 mm diameter) with two females that had mated within the preceding 1 min. One female had experienced the post-copulatory ritual, whereas the other had not. The latter type female had been obtained by allowing her to mate and by removing the male before he could perform the post-copulatory ritual. We recorded which of the two females remated.

Experiment 7 was identical in design but tested whether mated females with post-copulatory ritual experience remated in their lifetime. Such females were placed and kept singly in plastic cups containing 100 gypsy moth eggs and a cotton wick soaked in a 30% honey water solution. Every day throughout the female’s lifetime, a virgin male was confined with her for 30 min. We recorded whether the female remated.

Do females gain a fitness advantage by engaging in the post- copulatory ritual?

Experiment 8 was designed to explore whether the post-copulatory ritual is not just a form of mate guarding, but also a ritual that increases a female’s fitness through the total number of offspring, total number of daughters, and the sex ratio of her offspring. Two treatments were tested in parallel. In treatment 1 (N = 20), a virgin female and male were confined in a Petri dish (30 mm diameter) and separated after they had mated and completed the post-copulatory ritual. In treatment 2 (N = 20), the male was removed immediately after mating, allowing the female to come out of trance on her own. Females of both treatments were placed and kept singly for 14 days in plastic cups (103.5 ml) containing 100 gypsy moth eggs and a cotton wick soaked in a 30% honey
water solution. The total number of offspring, total number of daughters, and the sex ratio of offspring were recorded.

**Does the duration of the post-copulatory ritual affect the total number of offspring and number of daughters?**

Experiment 9 \((N = 42)\) was carried out to test whether the duration of the post-copulatory ritual affects the total number of offspring and number of daughters. A male and female were confined in a Petri dish (30 mm diameter), and mating and duration of their post-copulatory ritual were recorded. Females were then placed and kept singly for 14 days in plastic cups (103.5 ml) containing 100 gypsy moth eggs and a cotton wick soaked in a 30% honey water solution. Both the total number of offspring and number of daughters were recorded.

**4.3.5. Data analyses**

All data were analyzed using PASW v.18.0 (SPSS, Chicago, IL, USA). In experiment 3, the time males spent contacting females with or without post-copulatory ritual experience was analyzed by the paired sample \(t\)-test. Data (normally-distributed) of experiment 4 were analyzed by descriptive statistics. In experiment 5, time elapsed until 'in-trance' or 'post-trance' females mated with an unfamiliar male was analyzed by the independent samples \(t\)-test. In experiment 6, the numbers of females with or without post-copulatory ritual experience remating or not with an unfamiliar male were analyzed by the Pearson’s correlation coefficient \(\chi^2\) test. In experiment 7, the numbers of females that remated over their lifetime were analyzed with a binomial test. In experiment 8, the total numbers of daughters, sons, or both produced by females with or without post-copulatory ritual experience were analyzed by ANOVA. In experiment 9, the total number of offspring and of daughters produced by females in relation to the duration of the post-copulatory ritual they experienced was analyzed by Pearson’s correlation.
4.4. Results

4.4.1. Mechanisms of the pre- and post-copulatory ritual

Physical interactions between a male and a female during pre- and post-copulatory rituals.

In experiment 1, recordings of the 4- to 23-s pre-copulatory ritual invariably revealed the same physical interactions between a male and a female (Figure 4.1). First, the male interlocks the female’s antennae with his antennae. Subsequently, her antennae drop pointing straight down. The male then rapidly and repeatedly strikes them with his forelegs. Stereotypically, four synchronous strikes with both legs precede five alternating strikes with the left and right leg. Throughout the ritual, the female does not exhibit any physical behavior toward the male.

Similar to the pre-copulatory ritual, the male begins the 15- to 67-s post-copulatory ritual by interlocking the female’s antennae with his and then proceeds to strike her antennae with his legs. In contrast to the pre-copulatory ritual, the male uses his forelegs in random rather than repetitive or synchronous patterns of strikes. Occasionally, he also uses his head to assertively push the female up and onto the posterior tip of her abdomen. The female then strikes back at the male with her forelegs and/or midlegs. If her actions are interrupted, she may become motionless; that, in turn, induces new actions by the male.

Do body washes of wasps reveal evidence for pheromone transfer during pre- and post-copulatory rituals?

In experiment 2, there was no evidence for pheromone transfer during pre- and post-copulatory rituals. There was no difference in the quantity and quality of volatile constituents found in body washes of (1) females, or males, without any prior contact with a potential mate and females, or males, with pre-copulatory ritual experience (chromatograms not shown), (2) females, or males, with just the pre-copulatory ritual experience, and those with both pre- and post-copulatory ritual experience (Figure 4.2), implying that males, or females, do not transfer any type of marker pheromone during either type of ritual.
Do behavioral responses of wasps reveal evidence for pheromone transfer during the post-copulatory ritual?

In experiment 3, the time a male spent approaching or contacting a female with or without post-copulatory ritual experience did not significantly differ (paired sample t-test: \( t = 0.197, \text{ d.f.} = 19, P = 0.85 \)) (Figure 4.3). These data provide evidence that pheromones are not transferred to females during the post-copulatory ritual and that pheromones do not deter males from courting mated females.

4.4.2. Functions of the pre- and post-copulatory ritual

Does the pre-copulatory ritual put females into trance?

In experiment 4, after the pre-copulatory ritual had taken place, or the pre-copulatory ritual + copulation, females remained in a completely motionless state for (mean ± SE) 40.8 ± 3.52 and 47.9 ± 3.78 s, respectively. Conversely, when females engaged in the post-copulatory ritual, they began on average after 4.8 ± 0.42 s to physically strike the male, and they continued to do so until the ritual was completed. Thereafter, they remained active for the entire 5-min observation period. These data suggest that the male during the pre-copulatory ritual puts the female into a trance, which persists for nearly a minute following copulation, unless the female engages in the post-copulatory ritual that awakens her from the trance within seconds.

Do in-trance females allow mating more quickly than those not in a trance?

In experiment 5, males attained a mating with in-trance females 9.5 times quicker than with females that apparently had come out of trance (independent samples t-test: \( t = -5.461, \text{ d.f.} = 38, P<0.001 \)) (Figure 4.4).

4.4.3. Fitness consequences of the post-copulatory ritual

Do males gain a fitness advantage by engaging females in the post-copulatory ritual?

In experiment 6, there was a significant correlation between females with or without post-copulatory ritual experience and their probability to remate (Pearson’s correlation coefficient \( \chi^2 \): \( r = 7.06, \text{ d.f.} = 1, P = 0.007 \)) (Figure 4.5). Females with post-
copulatory ritual experience did not remate, whereas those lacking that experience did, indicating that the post-copulatory ritual serves as a form of 'in absentia' mate guarding. In experiment 7, mated females with post-copulatory ritual experience never remated in their lifetime (binomial test: proportion = 0.50, 95%, P<0.0001). Females lived 21.5 ± 1.12 days (mean ± SE), which is typical in laboratory populations of *O.kuvanae* females (Brown, 1984).

**Do females gain a fitness advantage by engaging in the post-copulatory ritual?**

In experiment 8, there was no difference in the total number of offspring (ANOVA: F<sub>1,38</sub> = 1.564, P = 0.22), the number of daughters (F<sub>1,38</sub> = 2.614, P = 0.11), and the number of sons (F<sub>1,38</sub> = 0.693, P = 0.41) that females produced with or without post-copulatory ritual experience (Figure 4.6), indicating that females without post-copulatory ritual experience still transfer, store, and utilize sperm.

**Does the duration of the post-copulatory ritual affect the total number of offspring and number of female offspring?**

In experiment 9, the duration of the post-copulatory ritual ranged between 15 and 67 s and lasted on average 26 s. The duration did not correlate to the females' total number of offspring (Pearson’s correlation: r = 0.149, d.f. = 42, P = 0.35) or the number of daughters (r = 0.134, d.f. = 42, P = 0.40), implying that it is neither indicative of the complement of sperm that is transferred or utilized nor indicative of the female reassessing her mate, as she did not adjust a male’s inclusive fitness in favour of producing more daughters, according to the duration (his performance) during this ritual.

**4.5. Discussion**

Our data support the conclusion that (1) the mechanisms of the pre- and post-copulatory ritual in *O. kuvanae* entail repetitive physical action or interaction between a male and a female and that there is no pheromone transfer during these rituals; (2) the pre-copulatory ritual puts the female into a trance-like state (‘trance’) that renders her receptive to mating; (3) the post-copulatory ritual accelerates the awakening of the female from the trance, which renders her unreceptive to mating with other males; and
(4) the post-copulatory ritual ensures the male’s paternity but has no quantitative fitness advantage or disadvantage for a singly mated female or male, because neither the total number of offspring nor the number of daughters differed between females with or without the post-copulatory ritual or in relation to its duration.

The pre-copulatory ritual proceeds at such a high speed that essential behavioral elements could be revealed only by slow-motion cinematography and thus may have been missed in previous studies (Alzofon, 1984). After the male interlocks his antennae with the female’s, he rapidly and repeatedly strikes her antennae with his forelegs in a stereotypical sequence of behavioral elements (Figure 4.1). With no analytical (gas chromatographic) evidence for pheromone transfer during the pre-copulatory ritual in *O. kuvanae*, it appears to be this repetitive physical action that helps a female enter a motionless and receptive state. Some form of mechanical stimulation also takes place in the pre-copulatory behavior of pteromalid, chalcidoid, and eucyitid wasps (Barrass, 1979; van den Assem, 1986; King & Fischer, 2005), but the outcome is not necessarily immobility and receptiveness of the female. Females of the chalcidoid wasp *Nasonia* spp., e.g., remain unreceptive if the male is removed just prior to copulation. In other species, like *Aphytis* spp., males emit an ‘arrestant’ pheromone that keeps the female quiescent prior to and during copulation (Khasimuddin & DeBach, 1975). In ichneumonid, platygastrid, and crabonid wasps, the male mounts the female, strikes or taps her antennae with his, and transfers pheromone in the process (Isidoro & Bin, 1995; Bin et al., 1999; Steiner et al., 2010; C. Holliday, pers. comm.).

Physical interactions during the post-copulatory ritual of *O. kuvanae* differ from those of the pre-copulatory ritual in that the male uses his forelegs, and sometimes his head, to push the female up and onto the posterior tip of her abdomen, and in that the female ‘strikes back’ at the male. The female striking against the male may signal to him that she is coming out of trance because he will induce new actions if she again becomes motionless. Analogous to the pre-copulatory ritual, there is no analytical or behavioral evidence that pheromones play any role in the post-copulatory ritual. This conclusion is based on results (1) that males spent similar amounts of time pursuing and contacting mated females with or without post-copulatory ritual experience (Figure 4.3) and (2) that there was no difference in chemical constituents of body washes of females, and of males, with or without post-copulatory ritual experience (Figure 4.2).
We concluded that following the pre-copulatory ritual *O. kuvanae* females enter into a trance. After that ritual and even after mating, in-trance females remained motionless for some time and allowed males to attain a mating 9.5 times faster than females that had apparently come out of trance (Figure 4.4). The post-copulatory ritual then plays a key role in accelerating the awakening of a female from her trance and receptive state. This conclusion is supported in that females with post-copulatory ritual experience did not remate over the course of their lifetime, but some females lacking that experience did (Figure 4.5). Forms of mechanical interaction or physical contact during the post-copulatory ritual that render mated females unresponsive and/or unattractive to competing males occur also in other species of the Hymenoptera and Diptera (Thornhill & Alcock, 1983; Alcock & Buchmann, 1985; King & Fischer, 2005). The post-copulatory ritual may not only awaken a female from her trance, but it also may be a mechanism by which a female controls whether she mates singly or multiply. It may also help prevent physical takeover from sneaker males (Thornhill & Alcock, 1983), who attempt to copulate with a female while she is engaged in the post-copulatory ritual with the first male to mate. Often, this sneaker tactic is a tactic used by MAO males (K. Ablard, pers.obs.). For example, when an *O. kuvanae* male pushes a mated female onto the posterior tip of her abdomen, sneaker males, which bypass the pre-copulatory ritual and attempt to copulate with in-trance females, are physically unable to mount a female (K. Ablard, pers. obs.). Such distinctive guarding behavior by non-sneaker males may have been selected for in response to sneaker males to minimize sperm competition (Sivinski, 1980; Alexander et al., 1997).

There is an obvious fitness advantage of the post-copulatory ritual for males. The male that successfully completes the ritual with his female ensures his paternity because she will never remate (Figure 4.5) over her lifetime and all her daughters will carry his genes. Singly mated females, in turn, do not appear to gain or lose a fitness advantage dependent upon the post-copulatory ritual. Both the total number of offspring and number of daughters were nearly the same for females with or without post-copulatory ritual experience (Figure 4.6). In contrast, females of the aphelinid wasps *Encarsia* spp. and *Prospalptella* spp. with post-copulatory ritual experience produce daughters unlike their counterparts lacking that experience (Kajita, 1986). That female *O. kuvanae* did not adjust the total number or sex ratio of their offspring to favour daughters, may be
because of the fact that the post-copulatory ritual is an obligatory element of their mating system and has not evolved to ensure sperm utilization directly affecting the inclusive fitness of the female.

If the post-copulatory ritual were an obligatory element of a mating system, and females were to reassess a male’s courting ability during that ritual, they could produce more daughters to favor a high-quality mate (Alcock & Buchmann, 1985; Alexander et al., 1997). If we were to gauge the courting ability of a male O. kuvanae by, e.g., the duration of his post-copulatory ritual as a means to physically guard his mate, then males with an extended post-copulatory courtship ritual should influence the sex ratio adjustment of females in favour of relatively more daughters. That this was not the case in O. kuvanae substantiates our conclusion that female O. kuvanae exhibit no form of mate choice based solely on the duration of the post-copulatory ritual. Rather, the length of the post-copulatory ritual may reflect the time the male needs to render a female unreceptive to further matings, as shown in the pteromalid parasitoid wasp Spalangia endius Walker (King, 2010). Moreover, post-copulatory rituals rarely occur in parasitoid wasps (van den Assem, 1986), and females need to mate only once to fertilize the complete complement of their eggs, negating the need for preferential mate choice, unless females mate multiply (Brown et al., 1997; Godfray & Cook, 1997; G. Boivin, pers. comm. 2007).

Remating by female O. kuvanae is rare but does occur when a male could not complete the post-copulatory ritual or when a ‘sneaker male’ mates with a female while she is engaging in the post-copulatory ritual with her first mate, with both males then competing assertively over post-copulatory rites. Having developed the microsatellite libraries and primers for paternity testing, we will be able to determine whether by cryptic choice a female adjusts the proportion of offspring sired by the first and second mate. Evidence that the post-copulatory ritual is independent of sperm precedence, as shown for the parasitoid wasp A. melinus (Allen et al., 1994), will substantiate the conclusion that this ritual serves as a means to awaken the in-trance female after mating and render her unreceptive to mating attempts by other males.
Figure 4.1. Drawings illustrating the stereotypic physical interactions between a male and a female Ooencyrtus kuvanae during the pre-copulatory ritual: (A) male interlocks female’s antennae (green) with his antennae; (B) female’s antennae point down and male strikes them with his forelegs (red) in four synchronous strikes; (C) male’s left leg strikes female’s right antenna; and (D) male’s right leg strikes female’s left antenna. Throughout the ritual, the female does not exhibit any physical behaviour toward the male.
Exp. 2

A  Females with pre-copulatory ritual

Females with pre- and post-copulatory ritual

B  Males with pre-copulatory ritual

Males with pre- and post-copulatory ritual

Figure 4.2.  Representative, 5-specimen-equivalent gas chromatograms each of body washes of Ooencyrtus kuvanae (A) females, and (B) males, with just pre-copulatory ritual experience and with both pre- and post-copulatory experience.
Figure 4.3. Mean (+ SE) time *Ooencyrtus kuvanae* males spent contacting mated females with or without post-copulatory ritual experience.

Figure 4.4 Mean (+ SE) time elapsed until in-trance or post-trance *Ooencyrtus kuvanae* females mated with an unfamiliar male.
**Figure 4.5.** Number of *Ooencyrtus kuvanae* females with or without post-copulatory ritual experience remating or not with an unfamiliar male.

**Figure 4.6.** Mean (+ SE) percent of sons and daughters produced by *Ooencyrtus kuvanae* females with or without post-copulatory ritual experience.

### 4.6. Acknowledgements

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4.7. Literature cited


5. Local mate competition in the solitary parasitoid wasp *Ooencyrtus kuvanae*\(^4\)

5.1. Abstract

Local mate competition (LMC) occurs when brothers compete with each other for mating opportunities, resulting in selection for a female-biased sex ratio within local groups. If multiple females oviposit in the same patch, their sons compete for mating opportunities with non-brothers. Females, in the presence of other females, should thus produce relatively more sons. Sex ratio theory also predicts a more female-biased sex ratio when ovipositing females are genetically related, and sex-ratio responses to foundress size if it differentially affects fitness gains from sons versus daughters. The mating system of the parasitoid wasp *Ooencyrtus kuvanae* meets assumptions of LMC. Females insert a single egg into each accessible egg of gypsy moth, *Lymantria dispar*, host egg masses. Wasps complete development inside host eggs and emerge en masse, as sexually mature adults, resulting in intense competition among brothers. We tested the hypothesis that *O. kuvanae* exhibits LMC by manipulating the number of wasp foundresses on egg masses with identical numbers of eggs. As predicted by LMC theory, with increasing numbers of wasp foundresses on an egg mass, the proportions of emerging sons increased. In contrast, the presence of a sibling compared to a non-sibling female during oviposition, or the size of a female, did not affect the number or sex ratio of offspring produced. The *O. kuvanae* system differs from others in that larvae do not compete for local resources and thus do not distort the sex ratio in favor of sons. With no resource competition among *O. kuvanae* larvae, the sex ratio of emergent son and daughter wasps is due entirely to the sex allocation by ovipositing wasp foundresses on host egg masses.

5.2. Introduction

Fitness consequences associated with controlling the sex ratio of offspring have been considered since Darwin (1871). In randomly mating populations, the evolutionarily stable strategy is to invest equally in sons and daughters (Fisher 1930). In contrast, in spatially structured populations with mating or competition among siblings, a strongly biased sex ratio can be favoured (Hamilton 1967).

Local mate competition (LMC) contributes to a female-biased sex ratio (Hamilton 1967). In species exhibiting LMC, brothers remain in their natal patch and compete with each other for mating access to females. In this context, a mother will maximize her fitness by producing relatively few sons so as to limit competition among them, resulting in a daughter-biased sex ratio (Taylor 1981). Inbreeding also affects sex ratio in a way independent of LMC. In haplodiploids, inbreeding causes mothers to share more genes with their daughters than with their sons, favoring the production of daughters (Boomsma and Grafen 1991). This prediction has been supported by a study of fig wasps (Herre 1985), revealing that in those species, where female wasps are more likely to mate with a relative, they adjust the sex ratio of their offspring towards daughters.

Many species exhibiting female-biased sex ratios are Hymenoptera with a haplodiploid sex-determination system, enabling females to control the sex of their offspring. Females that allow sperm to fertilize an egg, produce a daughter, and by not fertilizing it, they produce a son. Haploid sons derive their entire genome from their mother, whereas daughters are diploid and receive genes from both parents. This simple mechanism of controlling the sex ratio of offspring has facilitated studies that investigated how females adaptively adjust sex ratio based on various factors, including clutch size, number of foundresses, and host quality (Hardy et al. 1992; Kjellberg et al. 2005; Pereira and Prado 2006). Studies of sex ratio adjustment by parasitoid wasps have revealed some of the most conclusive examples of adaptive behavior in animals (Godfray 1994).

Sex ratio theory predicts that if multiple females oviposit in the same patch, their sons will compete for mating opportunities not only among themselves but also among non-siblings. This makes it adaptive for females to produce relatively more sons in the
presence of another ovipositing mated female. As the number of ovipositing females (n) in a patch increases, the optimal sex ratio (r % males) should increase according to the equation \( r = (n - 1)/2n \) (Hamilton 1967). This equation predicts a daughter-biased sex ratio for low numbers of foundresses, with increasing proportions of sons, as the number of foundresses in a patch increases, reaching an asymptote at a 1:1 sex ratio. Hamilton’s (1967) original equation was later modified to incorporate effects of haplodiploidy \([r = (n - 1) (2n - 1)/n(4n - 1)]\) (Hamilton 1979; Taylor and Bulmer 1980). For some parasitoid wasp species, notably Nasonia vitripennis (Werren 1983; King and Skinner 1991b), experimental manipulations of foundress numbers have confirmed predictions from theory regarding LMC.

Several additional selective forces are also predicted to affect optimal sex ratios. First, sex ratio theory predicts that if females can recognize sisters ovipositing on the same patch, they should produce a lower proportion of sons (Frank 1985; Taylor and Crespi 1994; Shuker et al. 2004a). Of five species tested to date for sex ratio adjustment in response to breeding with kin versus non-kin, none has demonstrated such abilities (Shuker et al. 2004a), for reasons that remain unclear.

Secondly, competition for host resources among developing larvae may also alter sex ratio. When several female N. vitripennis lay eggs in the same host, larval sons and daughters engage in local resource competition (LRC), often causing greater mortality of daughters (Godfray 1994; Santolamazza-Carbone and Rivera 2003; Suzuki et al. 1984). The effect of LRC among larvae is one of the most important confounding factors in experimental studies of LMC (Godfray 1994).

Thirdly, the size of a female parasitoid may affect her clutch size and the sex ratio of her offspring. Larger females tend to hold more eggs and produce larger clutch sizes than smaller females (Visser 1994; Sagarra et al. 2001; Santolamazza-Carbone et al. 2007); larger clutch sizes engender a higher proportion of daughters in fig wasps (Kjellberg et al. 2005), apparently due to stronger effects from LMC. Similarly, smaller N. vitripennis foundresses, which contribute a relatively low proportion of offspring to a patch, produce proportionally more sons, because their sons are not likely to compete among themselves for mates and thus they are less affected by LMC (Werren 1980).
With the sex ratio of haplodiploid wasps affected by inbreeding, LMC, LRC, foundress relatedness, and/or the size of females, we have searched for a study organism that would allow us to isolate the effect of each one of these factors. The solitary parasitoid wasp *Ooencyrtus kuvanae* appeared to be a useful model organism for such studies. This 2-mm long haplodiploid, egg parasitoid wasp oviposits singly into eggs of its host (gypsy moth, *Lymantria dispar*), which are found in large, discrete patches which measure 2–3 cm across. Although a gypsy moth egg mass contains several hundred eggs, only those in the uppermost layer (15–20%) are parasitized by one to multiple female wasps. A second egg that is inserted into a parasitized host egg is not likely to develop; superparasitism is uncommon and invariably yields smaller-than-normal offspring (Hofstetter 1996). Within 3–4 weeks, son and daughter wasps complete development inside the host eggs and emerge as sexually mature adults, females slightly later and about twice as numerous as males (Brown 1984), resulting in intense competition among males (see Chapter 2). There is no LRC among *O. kuvanae* larvae because a single larva completes development to a sexually mature adult wasp within each host egg (Hofstetter 1996). Moreover, the number, size, and relatedness of females on a gypsy moth egg mass can readily be experimentally controlled.

Working with *O. kuvanae* females, our objectives were to test whether: (1) increasing numbers of wasp foundresses on a gypsy moth egg mass produce proportionately more sons, as predicted by LMC (Hamilton 1967); (2) two sibling foundresses produce relatively fewer sons than the two non-sibling foundresses; and (3) the size of a single female affects her absolute clutch size and/or the sex ratio of her offspring.

### 5.3. Materials and methods

#### 5.3.1. Study insects

In July 2009 and May 2010, we collected gypsy moth egg masses parasitized by *O. kuvanae* from central Pennsylvania and from Maryland (USA), respectively. These field sites were ~266 km apart and consisted of *Quercus* hardwood forests lightly populated with *L. dispar*. The resulting laboratory populations A and B each originated
from ~10 egg masses. Populations were kept in the Global Forest Quarantine Facility at
Simon Fraser University. They were reared under a 16-L: 8D photoperiod at 22–25°C
and 50–70% RH (Hoffstetter and Raffa 1998). Females of each population were
provided with gypsy moth eggs supplied by the US Forest Service (Hamden,
Connecticut, U.S.A.). Two weeks later, 120 parasitized eggs from each population were
isolated. Emergent wasps were given a honey–water-imbued cotton wick (1 × 1.3 cm),
identified by sex based on the translucent antennae of males and opaque antennae of
females (Brown 1984), and allowed to mate with a wasp from the same population. All
matings were observed under a Petri dish lid (30 mm diameter) similar in size to a gypsy
moth egg mass. Mated females were immediately separated and introduced to 65 gypsy
moth eggs. Each parasitized egg was then transferred to, and kept in a separate
translucent Solo plastic cup (104 ml) until the adult wasps emerged and could be used in
experiments.

5.3.2. General protocols

Within 3 days of emergence, virgin females were paired with a non-sibling male
from the same population under a Petri-dish (30 mm diameter) where mating was
observed. Mated females were assigned to specific treatment groups (see experiments
1–3), placed in a glass jar with 100 gypsy moth eggs, and allowed 14 days to parasitize
them, after which they were removed, and offspring were allowed to emerge. Every day
for 14 days, each jar was checked to ensure parent females were alive, and every 3
days, the honey-imbued cotton wick (1 × 1.3 cm), which sustained the female(s) was
replaced. Jars (7%) that contained a dead parent female were excluded from data
analyses. In total, 96% of females survived for the duration of the experiments, and all
females assigned a ‘large’ or ‘small’ body size in experiment 3 remained alive for the
duration of that experiment. After 14 days, parent females were removed, and emergent
sons and daughters were counted. To ensure all offspring were accounted for, eggs
were kept for an additional 4 weeks after the parent females had been removed.
5.3.3. Experimental methods

Experiment 1: Effect of number of foundresses on sex ratio and number of offspring

Mated females were assigned to one of five treatment groups: (1) 1 female (n = 5); (2) 2 females (n = 6); (3) 3 females (n = 5); (4) 4 females (n = 5); and (5) 10 females (n = 5). In each replicate, the treatment group was then placed in a glass jar with 100 gypsy moth eggs and allowed 14 days to parasitize them. Emergent offspring in each jar were counted and identified by sex.

Experiment 2: Effect of presence of a sibling or non-sibling female on sex ratio and number of offspring

Mated females were divided into three groups; (1) single female (n= 11) [A=6, B=5], (2) two female siblings (n=10) [A= 6, B= 4], and (3) two female non-siblings (n=10) [A= 4, B = 6]. Siblings and non-siblings from each population were ensured by separating those offspring from controlled mating pairs. Each group of females was then placed in a glass jar with 100 gypsy moth eggs and allowed 14 days to parasitize them. Emergent offspring in each jar were counted and identified by sex.

Experiment 3: Effect of female size on number and sex ratio of her offspring

Within 3 days of emergence, virgin females were classed as ‘small’ (n =10) or ‘large’ (n = 9). Each mated female was then placed into a glass jar with 100 gypsy moth eggs and allowed 14 days to parasitize them, after which she was removed. Emergent offspring in each jar were counted and identified by sex. Post mortem, the head width and left hind tibia length of the parent female were measured under a microscope using a micrometer (Joyce et al. 2009). Females whose equated measurements averaged > 2.9 µm were classified as large.

5.3.4. Data analyses

In experiment 1, the statistical relationship between the number of foundresses in a patch and the proportion of sons emerging from it was analyzed by the Pearson's correlation coefficient. To test the proportion of males recorded in our study versus the
proportion predicted by Hamilton's theory, a generalized linear mixed model (GLMM) and an omnibus test were applied. The GLMM tested the estimated probability of the proportion of sons (logit scale) in each foundress class compared to the predicted probability, assuming that the actual numbers of sons recorded followed a binomial distribution and incorporating overdispersion among the replicate trials within each foundress class. The omnibus test then determined whether all five mean proportions matched with the predicted proportions. Total numbers of offspring per foundress class were analyzed by One-way ANOVA followed by Tukey's HSD test. The mean per foundress number of offspring among foundress classes was analyzed by One-way ANOVA. In experiment 2, proportion of sons and the total number of offspring by females in the presence of a sibling or non-sibling were analyzed by One-way ANOVA, followed by Tukey's HSD test. In experiment 3, sizes of 'large' and 'small' females were compared by a two-sample t-test. SPSS version 18.0 was used for all data analyses. The confidence interval for all tests was set at 95%.

5.4. Results

Experiment 1: Effect of number of foundresses on sex ratio and number of offspring

In experiment 1, with increasing numbers of foundresses in a patch, the proportion of sons emerging from it significantly increased [Pearson's correlation r =0.650, n=26, P <0.0001] (Figure 5.1, top). For each foundress class, and for all classes combined, there was a significantly greater mean proportion of sons recorded than predicted by Hamilton's theory [F_{5,21} =12.55, P <0.001] (Table 5.1). Both the mean total number and the mean per foundress number of offspring varied significantly among foundress classes [F_{4,21} = 6.733, P= 0.001; F_{4,21} = 108.218, P < 0.0001] (Figure 5.1, bottom).

Experiment 2: Effect of presence of a sibling or non-sibling female on sex ratio and number of offspring

In experiment 2, there was a significant difference in the proportion of sons between treatments [F_{2,28} = 3.135, P=0.05]. Single females produced a significantly
lower proportion of sons than females paired with a sibling foundress (P= 0.013) or a non-sibling foundress (P= 0.002) (Figure 5.2, top). The proportion of sons produced by paired sibling and non-sibling females did not significantly differ [F$_{1,18}$ = 0.744, P= 0.400]. There was also no significant difference in the number of total offspring produced by single or paired sibling or non-sibling females [F$_{2,28}$ = 0.890, P= 0.422] (Figure 5.2, bottom) and by paired sibling and non-sibling females [F$_{1,18}$ = 0.806, P= 0.381].

**Experiment 3:**
**Effect of female size on number and sex ratio of her offspring**

In experiment 3, pre-mated females that were assigned to groups considered 'large' or 'small' in body size, and that were measured post-oviposition and post mortem, significantly differed in body size [df = 18, t = 0.6990, P= <0.001] (Figure 5.3, top). There were no significant differences in the proportion of sons [F$_{1,18}$ = 2.623, P= 0.123] (Figure 5.3, middle) and in the total number of offspring [F$_{1,18}$ = 0.003, P= 0.958] (Figure 5.3, bottom) produced by large or small females.

**5.5. Discussion**

Our findings that two female *O. kuvanae* foundresses on a gypsy moth egg mass produce a higher proportion of sons than a single female (Figure 5.2, top), and that the proportion of sons increases with increasing numbers of foundresses present on the egg mass (Figure 5.1, top), fit with the pattern of sex ratio adjustment predicted by Hamilton (1967), and suggest that *O. kuvanae* exhibits sex ratio effects from LMC. Similar proportions of sons produced by females ovipositing in the presence of a sibling or a non-sibling female (Figure 5.2, top), indicate that females either do not recognize, or do not respond to the presence of kin. Finally, similar numbers of offspring and proportions of sons produced by small or large females that oviposit in isolation (Figure 5.3) indicate that the size of a female does not affect her absolute reproductive output and the sex ratio of her offspring. While our data are in general agreement with the LMC theory, the proportion of sons in our study exceeded that predicted by Hamilton's (1967) theory. This deviation could not be attributed to competition among larvae, or the size or relatedness of female foundresses. Instead, the higher than predicted proportion of sons
indicates that there may be some deviation from the strict assumptions of the LMC theory, and that some sons may disperse and mate away from their natal patch with non-sibling females. This hypothesis is supported by observations that host egg masses can be within centimeters of each other, particularly during *L. dispar* population outbreaks (PWS and GG, personal observations). Close proximity of host egg masses and dispersal of sons would decrease LMC on an egg mass and select for foundresses to produce a higher proportion of sons than predicted by Hamilton’s model, which assumes complete male philopatry (Debout et al. 2002; West and Herre 1998).

Equal clutch size laid by foundresses, and simultaneous oviposition, are additional assumptions implicit in Hamilton’s original model (Hamilton 1967). We could not follow focal individuals and control for potential differences in clutch size, or the order in which foundresses sampled the patch and oviposited. These factors could have modulated the sex ratio of offspring, as previously shown in *N. vitripennis* (Shuker et al. 2006), but they would not be expected to generate the direction of deviations from theory that we observed.

The effect of larval LRC on sex ratio has been an important complicating factor in previous studies on LMC (Godfray 1994; King and Skinner 1991a; Werren 1983). While (asymmetrical) larval competition in *N. vitripennis* was predicted to have a minimal effect on LMC (Sykes et al. 2007), we provide the first experimental evidence for LMC in a system entirely decoupled from LRC among larvae.

A suite of studies has demonstrated that female parasitoids adjust the sex ratio of their offspring in relation to foundress number (Werren 1983; Frank 1985; Burton-Chellew et al. 2008; King 1987; King and Skinner 1991a,b) but the mechanisms mediating such adjustments are little understood. Female *N. vitripennis* apparently adjust the sex ratio of their offspring not only in response to the presence of another female but also in response to her size, possibly as a proxy for fecundity (Flanagan et al. 1998). In other parasitoid wasps with potentially multiple foundresses on a patch, each foundress laid proportionally fewer eggs than she would on her own, and thus more male eggs are laid first (Green et al. 1982; Griffiths and Godfray 1988), resulting in a higher proportion of sons (Debout et al. 2002; Kjellberg et al. 2005; Raja et al. 2008; van Welzen and Waage 1987). Our study shows that the number of offspring per foundress
decreases with an increasing number of co-foundresses, as also found by Hoffstetter and Raffa (1998). In these studies, the question remains whether females adjusted the sex ratio in response to other females on a patch or simply as a result of laying fewer and thus more male eggs. As the *O. kuvanae* reproductive system exhibits LMC without larval LRC, it is well suited for testing mechanisms of adaptive sex ratio adjustment.

The size of female *O. kuvanae* affected neither the clutch size nor the proportion of sons (Figure 5.3). These results contrast those of other studies showing that large female parasitoids produce larger clutch sizes and relatively more daughters than do small female parasitoids (Hardy et al. 1992; Kjellberg et al. 2005; Sagarra et al. 2001). The effect of female size on reproductive output may vary between species and may depend upon their reproductive strategy or life history traits; for example, wasp size may be constrained by host egg size in egg parasitoids such as *O. kuvanae*. Within the context of LMC, the size of a female may also affect the sex ratio of her offspring. A foundress may compare her size to that of others and adjust the number and sex ratio of her offspring accordingly (Flanagan et al. 1998). Whether this also applies to *O. kuvanae* cannot be inferred from our data. In experiment 1, the multiple females present on the same gypsy moth egg mass were similar in size, and in experiment 3 large and small females oviposited in isolation.

The relatedness of ovipositing *O. kuvanae* foundresses did not affect the number or sex ratio of their offspring (Figure 5.2). These results parallel those of experimental studies, and meta-analysis, of parasitoid wasps, ants, fig wasps, and spider mites which show that species exhibiting LMC fail to recognize, or respond to, foundress kin (Shuker et al. 2004a, b). This conclusion, however, does not apply to the thrips *H. pedicularius* which adjusts sex ratio in accordance with whether or not a female has dispersed (and breeds with non-relatives), as opposed to breeding with relatives in her natal colony (Taylor and Crespi 1994); this form of sex ratio adaptation thus need not involve kin recognition per se. More generally, the apparent lack of foundress kin recognition in *O. kuvanae*, and in other species (Shuker et al. 2004a,b), may be due to some combination of a lack of suitable kin-recognition cues, a general absence of interactions between related females after dispersal, or weak selection compared to other factors (Shuker et al. 2004a). These possible interpretations would need to be addressed in further studies.
In conclusion, the *O. kuvanae* mating system allowed us to isolate and study the effect of various factors that engender changes in the sex ratio of offspring. It differs from others in that larvae do not compete for local resources, and thus, do not distort the sex ratio in favor of sons. With no resource competition among larvae, the sex ratio of emergent son and daughter wasps is due entirely to the sex allocation by ovipositing wasp foundresses on host egg masses. With increasing numbers of wasp foundresses, the proportions of emerging sons increased as predicted by the LMC theory (Hamilton 1967).
5.6. Tables and Figures

**Table 5.1.** Statistical analyses of the mean proportions of sons recorded in our study and predicted by Hamilton’s theory for each foundress class and for all five classes combined

<table>
<thead>
<tr>
<th>Number of foundresses</th>
<th>Recorded [sons]</th>
<th>Predicted [sons]</th>
<th>GLMM: $P$ (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.18</td>
<td>0.10</td>
<td>0.0044 (0.2083)</td>
</tr>
<tr>
<td>2</td>
<td>0.31</td>
<td>0.21</td>
<td>0.0086 (0.1771)</td>
</tr>
<tr>
<td>3</td>
<td>0.52</td>
<td>0.30</td>
<td>&lt;0.0001 (0.1874)</td>
</tr>
<tr>
<td>4</td>
<td>0.51</td>
<td>0.35</td>
<td>0.0028 (0.1913)</td>
</tr>
<tr>
<td>10</td>
<td>0.58</td>
<td>0.44</td>
<td>0.011 (0.2049)</td>
</tr>
<tr>
<td>Omnibus test:</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*sons* Proportion of sons; *Predicted* Proportion of sons predicted by Hamilton’s model; *GLMM* Generalized linear mixed model
Figure 5.1. Correlation between the number of female Ooencyrtus kuvanae foundresses on a gypsy moth egg mass and the proportion of sons (top; Pearson’s $r = 0.650$, $n = 26$, $P < 0.001$) and the total number of offspring (bottom) produced. The confidence intervals for the mean proportion of sons are depicted in red. Hamilton’s (1979) theoretical prediction is drawn for comparison. For each foundress class, and for all classes combined, there was a greater mean proportion of sons recorded in our study than predicted by Hamilton’s theory [$F_{5,21} = 12.55$, $P < 0.0001$] (Table 5.1). The mean total number of offspring of ten foundresses was significantly smaller than that of any other number of foundresses; One-way ANOVA followed by Tukey’s HSD test, $P< 0.05$. 
Figure 5.2. Proportion of sons (top) and the total number of offspring (bottom) produced by females of the parasitoid wasp Ooencyrtus kuvanae when they oviposited singly (n = 11) or in the presence of a sibling foundress (n = 10) or a non-sibling foundress (n = 10). The proportion of sons was significantly lower for single females but was nearly identical between sibling and non-sibling pairs; One-way ANOVA followed by Tukey’s HSD test, $P < 0.05$. There was no difference in the total number of offspring produced by females of all treatment groups; One-way ANOVA, $P > 0.05$. 
Figure 5.3. (top) Size (presented as mean between head width and hind tibia length) of female Ooencyrtus kuvanae classed as ‘large’ (n = 9) or ‘small’ (n = 10) prior to the experiment and in post mortem measurements; (middle and bottom) proportion of sons and the total number of offspring produced by large or small females (n = 10 each) of O. kuvanae. The size of large and small females differed significantly (independent sample t-test, $P < 0.05$), but the total number and proportion of sons did not (One-way ANOVA, $P > 0.05$).
5.7. Acknowledgments

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6. First-male sperm precedence and the post-copulatory ritual in the parasitoid wasp *Ooencyrtus kuvanae* (Hymenoptera: Encyrtidae)

6.1. Abstract

Sperm competition generates selection for male adaptations to prevent it. The adaptations subject to selection among species where males compete for monandrous virgins who are receptive for a short time remain unclear. Males of the egg parasitoid wasp *Ooencyrtus kuvanae* compete over females following emergence from gypsy moth, *Lymantria dispar*, egg masses. Males engage virgins in a brief pre-copulatory ritual, mate, and then immediately perform a lengthy post-copulatory ritual. Following the pre-copulatory ritual, females enter a receptive state that persists until the male completes the post-copulatory ritual. Sneaker (*P*₂) males sometimes copulate with a female while she is *in copula* with her first mate (*P*₁ male), or while she is engaged in the post-copulatory ritual, and they also perform the post-copulatory ritual. The existence of a sneaker tactic, and competition of *P*₁ with *P*₂ males over post-copulatory ritual rites, imply sperm precedence for *P*₂ males. However, if a *P*₁ male’s reproductive success were contingent on his performance of both the pre- and post-copulatory ritual, then *P*₁ males could gain sperm precedence. We investigated *i*) paternity of *P*₁ and *P*₂ males using DNA microsatellite analysis, *ii*) copulation and post-copulatory behavior of both males, and *iii*) potential morphological adaptations of the males’ aedeagus for sperm removal. Our data indicate a preponderance of first male sperm precedence, and suggest that the post-copulatory ritual represents a male adaptation to help prevent sperm competition.

6.2. Introduction

Sperm competition, the process by which spermatozoa of two or more males compete to fertilize the egg(s) of a lone female, occurs when females mate with multiple males in a single breeding bout (Parker, 1970; Simmons, 2001). Evolutionary responses that help males avoid or cope with sperm competition include morphological, physiological and behavioral adaptations (Knowlton and Greenwell, 1984; Simmons, 2001).

In species with internal fertilization, the female may store sperm from competing males in her reproductive tract or sperm storage organ (e.g., spermatheca) for some time before fertilization, thus setting the stage for intense and potentially prolonged sperm competition. In response, males may achieve first-male sperm precedence if they reduce, or prevent, competition from rival sperm by (i) depositing copulatory plugs to prevent female re-mating (Simmons, 2001), (ii) reducing a female’s attractiveness to other males through substances in the seminal fluid (Simmons, 2001), (iii) removing sperm from the female’s sperm-storing organ (Gromko et al., 1984), (iv) prolonging the duration, or increasing the frequency, of copulations (Thornhill, 1984), and (v) by engaging in post-copulatory interactions such as grasping or guarding the mated female (Gwynne, 1984; Alcock, 1994). Pre-copulatory mate guarding also favors first-male sperm precedence (Brown et al., 1997), but post-copulatory rituals may represent an adaptation to sperm competition if mated females would otherwise remain receptive (Alcock, 1994).

Prolonged interactions, whereby a male will stay near, or remain in contact with, a receptive female following insemination, may evolve in response to direct competition from rival males attempting to re-mate with that female (Alcock, 1994). Under this scenario, post-copulatory interactions are expected to take longer when receptive females are limited and difficult to secure (Parker, 1974; Alcock, 1994; Simmons, 2001), and to intensify when the second or last male to mate (henceforth $P_2$ male) is likely to deposit sperm that will be used to fertilize a greater proportion of eggs than sperm from the first male (henceforth $P_1$ male), due to last-male sperm advantage (Boorman and Parker, 1976; Simmons, 2001).
Post-copulatory interactions may affect fertilization in species with cryptic mate choice, whereby females manipulate sperm storage and select sperm from particular partners for egg fertilization (Gromko et al., 1984; Eberhard, 1996). Such interactions may also help advertise a male’s quality prior to egg fertilization (Simmons, 1990), or help ensure that mated females are less receptive to other males (Eberhard, 2009).

A specific form of post-copulatory interaction, post-copulatory rituals, occurs mainly in some species of parasitoid wasps (Mackauer, 1969). Such rituals generally resemble courtship interactions, involving more or less stereotypical behavior directed towards females by males, but they occur only after mating, and their adaptive significance has remained largely enigmatic (Viggiani and Battaglia, 1983; van den Assem, 1986; King and Fischer, 2005).

Several hypotheses have been proposed to help explain the evolution of post-copulatory rituals. In some parasitoid wasps, post-copulatory rituals may have evolved in response to selection pressure from rival males (van den Assem et al., 1980) that attempt to mate with a female while she is still in a receptive state from her interaction with the first-mating male. These rituals then function as a form of mate guarding, by reducing the efficacy of mating attempts by rival males and/or by leading to reduced female receptivity (Allen et al., 1994; King and Fischer, 2005). For example, following copulation, males of the parasitoid Pteromalus puparum continuously move the female’s abdomen, apparently to better detect a rival’s attempt at copulating with her (Thornhill and Alcock, 1983), and males of the parasitoid Cotesia rubecula implement female mimicry to distract their rivals (Field and Keller, 1993).

Rival males that are not detected or distracted as a result of a post-copulatory ritual could possibly re-mate a female. In such circumstances, some males may attempt to increase the chances that their sperm is used by strategically repositioning or removing rival sperm from entering the female’s storage organ (Gromko et al., 1984; Thornhill, 1984; Simmons, 2001) via prolonged duration in copula, multiple copulatory bouts, and/or specialized morphological structures (Thornhill and Alcock, 1983). Generally, successful sperm removers and sperm repositioners require between 7 s and 20 min of copulation time (Waage, 1984); most parasitoids do not devote that much time
to the copulatory stage within a mating sequence (Gordh and DeBach, 1978; Allen et al., 1994; King and Fischer, 2005).

Males that reposition and/or remove sperm tend to achieve last-male sperm precedence. Last-male sperm precedence is rare among parasitoids and the mechanism is ambiguous. For example, males of the parasitoid wasp *Diachasmimorpha longicaudata* gain sperm precedence when they re-mate a female after 24 h; the underlying mechanism could be the result of a loss of *P*₁ sperm from the female’s storage organ (Martinez-Martinez et al., 1993), which is often associated with extremely long time periods between copulations (Simmons, 2001). It is also unclear whether *D. longicaudata* males who achieve first-male sperm precedence also perform a pre-copulatory ritual, and/or a post-copulatory ritual that functions as a form of mate guarding, as evidenced in males of the parasitoid wasps *Nasonia vitripennis*, *Aphytis melinus* and *Lariophagus distinguendus* (Holmes, 1974; van den Assem, 1989; Allen et al., 1994).

Both pre- and post-copulatory rituals exist in the quasi-gregarious (with one offspring per host), 2-mm egg parasitoid wasp *Ooencyrtus kuvanae* (Howard). Courtship and mating take place on egg masses of host gypsy moth, *Lymantria dispar dispar*. An egg mass measures 2–3 cm across and contains several hundred eggs covered in setae (Brown, 1984). Eggs in the uppermost layer are parasitized by female wasps that insert a single egg into each accessible host egg. By fertilizing an egg, females produce a daughter, and by not fertilizing it, they produce a son. Haploid sons derive their entire genome from their mother, whereas daughters are diploid and receive genes from both parents. Wasps complete development inside host eggs after 3-4 weeks and emerge *en masse* as sexually mature adults that can live between 4–6 weeks. Females emerge up to 1 d later, are immediately receptive to mating, and are about twice as numerous as males overall. However, a local, adult male-biased sex ratio occurs frequently among non-siblings (Somjee et al., 2011) because males typically remain on the host egg mass as long as there are mating opportunities, whereas mated females disperse within 24 h, seeking new gypsy moth egg masses (Brown, 1984). Prior to mating, the male engages a female in a brief (ca. 4 s) pre-copulatory ritual, then mate (4–9 s), and immediately performs a relatively long (15–67 s) post-copulatory ritual (Ablard et al., 2011). During the pre-copulatory ritual, the females enter into a "trance-like" (unmoving,
unresponsive) state (henceforth “trance”) that persists for some time after copulation (Ablard et al., 2011). It is likely that females control this stage in the mating sequence because if a male does not perform the pre-copulatory ritual, a female will not become receptive. The behavioral mechanisms underlying the post-copulatory ritual resemble those of the pre-copulatory ritual; the male interlocks the female’s antennae with his and then proceeds to strike her antennae with his legs. In contrast to the pre-copulatory ritual, he uses his forelegs in a random rather than repetitive or synchronous pattern of strikes. The female then strikes back at the male with her forelegs. If she is interrupted and becomes motionless, the male resumes his strikes. Thus, the post-copulatory ritual may function as a form of mating guarding to accelerate the ‘awakening’ of the entranced female, who then rejects all mating attempts by other males over the course of her lifetime, ensuring paternity of the $P_1$ male (Ablard et al., 2011).

'Sneaker' ($P_2$) males of *O. kuvanae* do not directly compete for mating opportunities (K. Ablard, personal observation). Instead, they expend less energy than $P_1$ males by not engaging in a pre-copulatory ritual, and by copulating with an in-trance female, when she is either *in copula* with a $P_1$ male, or when she is coming out of the trance while engaged in the post-copulatory ritual with the $P_1$ male. The $P_2$ male then directly competes with the $P_1$ male for post-copulatory rites (K. Ablard, personal observation), thus possibly siring some or all of the female’s daughters.

Interpretation of the adaptive significance of male and female mating behavior in *O. kuvanae*, and other species with post-copulatory rituals, depends critically on patterns of sperm precedence and use. In this study, we investigated the presence of sperm competition in *O. kuvanae* by testing paternity of $P_1$ and $P_2$ males using DNA microsatellite analysis. We predicted that (1) there is first-male sperm precedence, as reported in parasitoids where - as in *O. kuvanae* - some males perform a pre- and a post-copulatory ritual, and mated females remain receptive briefly; (2) male adaptations to sperm competition do not include multiple or lengthy copulatory bouts which are associated with long periods of receptivity in females and last-male sperm precedence, considering that *O. kuvanae* males copulate briefly and females are receptive briefly post copulation, and not receptive following the post-copulatory ritual; (3) the post-copulatory ritual represents a male adaptation to reduce sperm competition, and (4) that males do not possess morphological adaptations for removal of rival male sperm.
because such adaptations are associated with lengthy, rather than brief (see above), copulatory bouts.

6.3. Materials and methods

6.3.1. Experimental insects

A new colony of *O. kuvanae* was started with specimens field collected from *Quercus* hardwood forests in the city of North East, MD. (39°36´N, 75°55´W). All insects were reared under a L16:D8 photoperiod at 22-25 °C and 50-70% RH (Hofstetter and Raffa, 1997) in the Global Forest Quarantine Facility of Simon Fraser University. They were contained in Plexiglass cages (40 × 40 × 30 cm) and provided with cotton wicks (1 × 10 cm; Richmond Dental, Charlotte, NC) soaked in a 30% honey water solution every 2 days. Ten gypsy moth egg masses, supplied by the U.S. Forest Service (Hamden, CT), were introduced to be parasitized by female wasps. Fourteen days later, parasitized egg masses were removed and 1000 eggs were placed singly into translucent plastic cups (103.5 ml) and secured with a lid. Emergent insects were separated by sex and size under a microscope and used in the experiment within 1 d of emergence to avoid adverse effects associated with aging (van den Assem, 1996).

6.3.2. DNA library construction, screening, and enrichment

Methods for DNA library construction, enrichment and screening are published elsewhere (Jones et al., 2002) and were applied by Genetic Identification Services (GIS, Chatsworth, CA). Genomic DNA was partially restricted with a cocktail of seven blunt-end cutting enzymes. Fragments that ranged between 300-700 bp in length were adapted and subjected to magnetic bead capture (CPG, Lincoln Park, NJ), using biotinylated capture molecules. Libraries were prepared in parallel, using Biotin-CA(15), -AAG(12), -AAT(12) and -ATG(12) as capture molecules in a protocol provided by CPG (Lincoln Park, NJ). Captured molecules were amplified and restricted with HindIII to remove the adapters. The resulting fragments were ligated into the HindIII site of pUC19. Recombinant molecules were electroporated into *E. coli* DH5alpha. Recombinant clones were selected at random for sequencing, and enrichment levels were expressed as the
fraction of sequences that contained a microsatellite. Sequences were obtained on an
ABI 377 or an ABI 3730, using ABI Prism Taq dye terminator cycle sequencing
methodology. Microsatellite-containing sequences were identified by inspection, PCR
primers were designed using DesignerPCR version 1.03 (Research Genetics Inc,
Huntsville, AL), and they were purchased from Integrated DNA Technologies (Coralville,
IA). The optimal amplification reaction mix for all primer pairs consisted of 1× Biolase©
Buffer from a 10× stock solution supplied by Bioline (Taunton, MA), 2 mM MgCl₂, 0.2
mM of each dNTPs, 6 µM of each primer, 0.025 U/µl Biolase DNA Polymerase (Bioline
USA, Taunton, MA), and 0.2 ng/µl template DNA in a 50-µl final reaction
volume. Samples were amplified in a Perkin-Elmer-Cetus thermal cycler by an initial
denaturation at 94°C (180 s), followed by 35 cycles of 94°C (40 s), 55°C (40 s), and
72°C (30 s), with a final extension of 72°C (240 s). DNA was extracted using the DNeasy
Blood and Tissue® kit (QIAGEN, Germantown, MD) according to the manufacturer’s
protocol. Microsatellite loci were amplified in 10-µl reactions in the following reaction mix:
MgCl₂, 2 mM; dNTPs (premixed), 0.2 mM each; primers, 0.3 µM each; Biolase DNA
Polymerase® (Bioline USA, Taunton, MA), 0.025 U/µl; template DNA, 0.2 ng/µl. PCR
was conducted in a RoboCycler Gradient 96® thermocycler (Stratagene, La Jolla, CA),
using the same protocol as above. PCR products were separated on 3.5% agarose
gels, and stained with ethidium bromide to identify polymorphic loci; six loci were
polymorphic (A1, A3, A106a, A107, B105, and D106) and limited to this study.

6.3.3. DNA extraction

Frozen-stored specimens were transferred to a bed of ice prior to being crushed
with a sterile plastic micropipette tip. Immediately post-crushing, DNA was extracted
using the microLysis®-Plus kit (Gel, San Francisco, CA) following the manufacturer’s
protocol except that 40 instead of 20 µl of microLysis®-Plus were used.

6.3.4. Polymerase chain reaction (PCR)

DNA paternity analyses were based on four microsatellite loci. Genomic DNA
was amplified with PCR blends that contained 5.15 µl ddH₂O, 1.0 µl of 10× enzyme
buffer, 1.0 µl of 25 mM of MgCl₂, 0.8 µl of 2.5 mM of dNTP mix, 0.3 µl of 10 mM forward-
labeled [700 series] (Integrated DNA Technologies, Coralville, IA) using unlabeled forward primer, 0.3 µl of reverse primer, 0.05 µl of Taq DNA Polymerase (GenScript, Piscataway, NJ), and 1 µl of 2 ng/µl template DNA. The sequences of the designed primers were as follows: A1-F: 5´-CCC GTA TTA TAG ACG TTC GTA C-3´; A1-R: 5´-GCA AAA TTG CAC ATA TAC ACA G-3´; A106a-F: 5´-AGA GCA TAA GCC GTC GTC-3´; A106a-R: 5´-GCG AAG CAC ACA CAA CTG -3´; A107-F: 5´-TTG GTC TCT CTT TCT CGC TTG -3´; A107-R: 5´-GCA GTG CTG TTG CTG TTA C-3´; B105-F: 5´-TCG TCT CGC TTG TTC -3´; B105-R: 5´-AGT TGG TCA GGA GGG TGA G-3´. PCR reactions were denatured at 94 °C (180 s), followed by 30 cycles of 94 °C (40 s), 58 °C (40 s), 72° C (30 s), and a final extension step of 72 °C (320 s). We added 2 µl of formamide and bromophenol blue loading dye to PCR mixtures that were electrophoresed through a 10-% polyacrylamide gel with a 1× TBE buffer at 1500 V and 45 °C for 1.5–2 h on a LI-COR 4300 genetic analyser (Lincoln, NE). Products were visualized for paternity analysis on LI-COR gel images. Parents and offspring were run with a positive control generated from the initial testing of the primers.

Replicates were limited to n = 10 given the challenges of (i) developing a novel and effective experimental protocol for the testing of paternity in an egg parasitoid wasp using DNA microsatellite markers, (ii) attaining and tracking the twice-mated female and mated males within a highly male-competitive setting, and (iii) extracting DNA from extremely small and delicate specimens. Replicates (n = 10) consisted of 30 parents (10 females and 20 males) and 127 daughters, totaling 157 wasps, which were genotyped. This large dataset proved sufficient for the application of robust statistical analyses (see below).

6.3.5. Attaining twice-mated females

To produce twice-mated females, four males without prior contact with a female were confined with one virgin female (n = 10) in a Petri dish (30 mm diam). This competitive setting increased the likelihood that the female would be mated by a sneaker male while she was still in the trance and receptive state following the pre-copulatory ritual and copulation, and prior to the completion of the post-copulatory ritual with the P₁ male. Immediately following the completion of the post-copulatory ritual by the sneaker male, the two males and the twice-mated female were removed from the arena; two
males which did not mate were discarded. The mating order of the two males that mated, and the duration and number of copulations and post-copulatory rituals, were tracked by two observers and by a digital voice recorder equipped with a time tracker. Whether sneaker males were MAO or HGG males could not be determined in this competitive setting. Females were placed singly in glass jars provisioned with food and 40 gypsy moth eggs to parasitize. After 21 d, the females were removed and the 40 eggs were placed singly into plastic cups to prevent mating between offspring. Emergent daughters and sons were counted. Parents and daughters were stored singly in 2.0 ml QIAGEN® sterile microcentrifuge tubes (Toronto, Ontario) at -80° C until DNA extraction.

6.3.6. Microstructure of the males’ aedeagus

The microstructure of the males’ aedeagus was examined by means of photomicrographic imaging and environmental scanning electron microscopy (ESEM). Aedeagi of freshly-killed, 1-d-old virgins (n = 4) protruded without force. Photomicrographic images were obtained with a Nikon Microphot-FX EPI microscope (Japan) and SPOT software v. 4.6 (SPOT Imaging Solutions, Sterling Heights, MI). ESEM images were obtained by mounting insects onto a peg (12.7 mm diam) covered with a conductive carbon adhesive tab, and by imaging with the ESEM FEI Quanta FEG 4000 (FP Innovations, Hillsboro, OR) at magnifications of 1500×, 2500×, and ≥5000× within a chamber kept at ambient temperature, using 1.5 Torr pressure, an accelerating voltage of 15 kV, and a Gaseous Secondary Electron Detector with a 1 mm aperture.

6.3.7. Data analyses

A paired t-test of the normally distributed data was used to compare the mean number of copulations, the mean duration in copula, and the mean duration of the post-copulatory ritual recorded from $P_1$ and $P_2$ males. A Pearson’s correlation was used to test for a linear relationship between (i) the duration of copulation and the number of daughters sired by $P_1$ and $P_2$ males, and (ii) the duration of the post-copulatory ritual and the number of daughters sired by $P_1$ and $P_2$ males.
Fragment sizes (base pair) were scored from LI-COR gel images and assigned paternity probabilities with the computer program COLONY v 2.0 (Jones and Wang, 2010), which assigns paternity based on maximum-likelihood. To accurately assign paternity, COLONY requires additional information regarding the mating and genetic system of the species. For these analyses, females were considered polygamous because they could mate with more than one male, while males were considered monogamous because they were constrained to mate with only one female in our experiment. This program also allowed us to specify the genetic background of the species, which is haplodiploid. Other parameters were constrained to reflect the facts that the female in the experiment was the only possible mother and that each male had a 50% chance of being the sire of the offspring. In addition, we specified a low genotyping error rate (0.00001) and indicated that inbreeding may occur in this species.

A male was assigned paternity for each daughter within a brood if the COLONY-issued probability was 1.000, except for replicates 3, 8, and 10 where paternity was assigned to a total of 14 males whose overall probability of paternity did not equal 1.000, but averaged 0.70. Replicate 3 resulted in an average probability of 0.50 for four daughters; replicate 8 resulted in an average probability of 0.80 for eight daughters; and replicate 10 resulted in an average probability of 0.70 for two daughters. All probabilities were individually tested for each replicate using a binomial distribution. For each replicate, exclusion probabilities were calculated in Microsoft Excel on alleles of an individual locus; a mean was then calculated for all loci. Descriptive statistics of normally-distributed data were used to calculate \( P_2 \) values, and a paired t-test was used to compare the mean number of offspring sired by \( P_1 \) and \( P_2 \) males. Non-paternity assignment analyses were run with PASW v. 18.0 software. The confidence interval for all tests was set at 95%.

6.4. Results

6.4.1. Paternity assignment

Nearly all (98%) daughters were assigned to sires. The exclusion probability (probability that potential sires were excluded on genetic incompatibility alone) averaged
84% over all replicates (Table 6.1). $P_1$ males sired more daughters than $P_2$ males ($P_1$: $\bar{X} = 11.60 \pm 1.899$, $P_2$: $\bar{X} = 1.10 \pm 0.823$; $t_9 = 4.426$, $p=0.002$). Mixed paternity was inferred for 2 of 10 broods, resulting in an overall low $P_2$ value (Table 6.2).

### 6.4.2. Copulation and post-copulatory ritual behavior of $P_1$ and $P_2$ males

Eight out of 10 $P_2$ males copulated with the female while the $P_1$ male was engaging her in the post-copulatory ritual. Two $P_2$ males copulated with the female shortly after the $P_1$ male had began copulating. The number of copulations $P_1$ and $P_2$ males attained did not differ ($P_1$: $\bar{X} = 1.40 \pm 0.221$, $P_2$: $\bar{X} = 1.30 \pm 0.153$; $t_9 = 1.000$, $p = 0.343$).

Mixed paternity occurred in replicates 3 and 10 (Table 6.2). In replicate 3, the $P_1$ male began to copulate first and within a fraction of a second was joined by the $P_2$ male, who copulated simultaneously. When the $P_1$ male had stopped copulating and started the post-copulatory ritual, the $P_2$ male quickly repositioned himself, copulated again, and then engaged in the post-copulatory ritual. In replicate 10, both males copulated once, $P_1$ prior to $P_2$, and in the same order engaged the female in the post-copulatory ritual. The behaviour of $P_1$ and $P_2$ males in replicates 3 and 10 did not differ in any particular way from that of males in the other eight replicates.

Copulation durations of $P_1$ and $P_2$ males did not differ ($P_1$: $\bar{X} = 9.40 \pm 1.869$ s, $P_2$: $\bar{X} = 10.30 \pm 1.309$ s; $t_9 = -0.462$, $p = 0.655$). There was no correlation between (i) the mean copulation duration and the mean number of offspring sired by $P_1$ males ($r = -0.199$, $p = 0.582$), and (ii) the mean duration of copulation and the mean number of offspring sired by $P_2$ males ($r = 0.018$, $P = 0.962$). There was also no correlation ($n = 4$, $r = -4.64$, $p = 0.536$) between the mean copulation duration and the mean number of offspring sired by $P_1$ and $P_2$ males who shared paternity in replicates 3 and 10. Seventy-percent of $P_2$ males also engaged the female in a post-copulatory ritual, either by performing the ritual concurrently with $P_1$ males, or after they physically prevented the ritual of $P_1$ males. The time $P_1$ and $P_2$ spent engaged in the post-copulatory ritual differed ($P_1$: $\bar{X} = 18.30 \pm 2.825$ s, $P_2$: $\bar{X} = 5.90 \pm 1.574$ s; $t_9 = 3.730$, $p = 0.005$).
6.4.3. Microstructure of the males’ aedeagus

The aedeagus (ca. 7 µm in length) of males has no morphological characteristics indicative of a function in sperm removal or displacement. The pointed, rather than arched, tip lacks hooks and spines (Figure 6.1a). The grappling hooks (Figure 6.1b) are likely clamping organs that help grasp the female during copulation.

6.5. Discussion

6.5.1. First-male sperm precedence

In the O. kuvanae mating system, the low $P_2$ value (0.09) of sneaker males is suggestive of strong first-male sperm precedence, with fertilization success of $P_1$ males not due to their number of copulations or time spent *in copula*, or the inability of $P_2$ males to remove sperm. Low $P_2$ values, in general, stem from female preference for a $P_1$ male and other adaptations to first-male sperm usage, unsuccessful copulations due to poor performance, or effective post-copulatory guarding by $P_1$ males (Simmons, 2001; Shuster and Wade, 2003). As a form of post-copulatory guarding, $P_1$ males of O. kuvanae engage the mated female in a post-copulatory ritual which is associated with her not re-mating (Ablard et al., 2011).

Our observations and microsatellite data show that $P_2$ males may copulate successfully, but still sire few offspring (low $P_2$ value). Explanations for this phenomenon may include morphological attributes of the females’ reproductive tract, cryptic sperm choice by females, and/or ineffective post-copulatory guarding by $P_1$ males.

The shape and elasticity of the spermatheca in many insects play a key role in sperm movement and storage (Walker, 1980). The spherical chitinous spermatheca of the wasp Dahlbominus fuscipennis, for example, resists any change in storage capacity. During the first copulation, motile sperm accumulates at the opening of the spermatheca, thereby impeding entry of new sperm in favor of first-male sperm precedence (Wilkes, 1966). As a result, frequent or long copulatory bouts by $P_2$ males decrease their fitness due to loss of alternative mating opportunities.
6.5.2. **Copulation behaviour of \( P_1 \) and \( P_2 \) males**

In our study, \( P_1 \) and \( P_2 \) males of *O. kuvanae* performed the copulatory act equally often, suggesting that \( P_2 \) males could not, or did not, deliver more sperm than \( P_1 \) males. Males of the fly *Dryomyza anilis* and of the scorpionfly *Panorpa germanica*, in contrast, increase their fertilization success with the number of copulatory bouts (Otronen, 1994; Kock and Sauer, 2007). When both \( P_1 \) and \( P_2 \) males of *O. kuvanae* copulated with the same female twice in each of replicates 3 and 9, and for circa the same duration each time, one \( P_2 \) male shared paternity and sired more daughters than the \( P_1 \) male (replicate 3) (Table 6.2).

The duration of a male’s copulatory bout can increase the number of offspring he sires (Simmons, 2001) but this consideration does not apply to male *O. kuvanae*; \( P_1 \) males sired circa 10 times more offspring than \( P_2 \) males, yet \( P_2 \) males remained in copula for circa 0.5 s longer than \( P_1 \) males.

Prolonged duration of a copulatory bout may provide the time needed for sneaker males to remove \( P_1 \) male sperm and deliver their own (Simmons, 2001). In the *O. kuvanae* mating system, removal of \( P_1 \) male sperm by \( P_2 \) males is not likely because the aedeagus of *O. kuvanae* males lacks any attributes which could facilitate sperm removal or displacement. In contrast, in mating systems with last-male sperm precedence, the males’ aedeagus of some species assumes a unique shape or is fitted with spines or hooks capable of displacing a competitor’s sperm (Thornhill and Alcock, 1983). For example, in the dragonfly *Sympetrum rubicundilum*, two long and coiled structures of the males’ aedeagus fit into a paired spermatheca and push \( P_2 \) sperm deeper into the spermatheca while flushing out \( P_1 \) male sperm, resulting in last-male sperm precedence (Thornhill and Alcock, 1983).

6.5.3. **Mate guarding**

As the reproductive success of \( P_1 \) and \( P_2 \) *O. kuvanae* males was not coupled to the number and duration of copulations with the same female, or aedeagus morphology, the underlying mechanisms of first-male sperm precedence in *O. kuvanae* appear to entail both pre- and post-copulatory mate guarding as adaptations to sperm competition. The pre-copulatory ritual is associated with the female entering a ‘trance’ and receptive
state (Ablard et al., 2011). Effectively, a female exhibits indirect mate choice by engaging in the pre-copulatory ritual with the first male to contact her. He proceeds to mate with her and then immediately performs the post-copulatory ritual which results in her exit from the trance and becoming unresponsive (Ablard et al., 2011). Such a form of female mate choice for males that first encounter a female may be favored in species such as *O. kuvanae* that engage in intense, time-limited scramble competition among males for matings. Potential sperm choice by females would favor, by default, the first male to perform the post-copulatory ritual, which typically is the same male to have contacted and engaged her in the pre-copulatory ritual. Whether *P₁* males would sire most offspring had they not performed the post-copulatory ritual first has yet to be determined.

Most *P₂* males who did not sire daughters did not perform the pre-copulatory ritual. In seven out of 10 replicates, the *P₁* male first engaged a female in the pre- and post-copulatory ritual, and sired all of the daughters (Table 6.2). Interestingly, the one *P₁* male which performed the pre-copulatory ritual, but failed to initiate the post-copulatory ritual before the *P₂* male did, had shared paternity with the *P₂* male; this result suggests that the onset of the post-copulatory ritual may function, by default, to accelerate sperm storage (Thornhill and Alcock, 1983) by a female coming out of trance (Ablard et al., 2011).

Theoretical models of mating systems predict that males should abandon their mates immediately after mating, if there is strong first-male sperm precedence (Simmons, 2001). Such behavior negates post-copulatory rituals in species where male adaptations to sperm competition could rely solely on pre-copulatory ritual performance, or chemical substances in seminal fluid of the first male to mate, which immediately inhibit female receptivity (Simmons 2001). In the absence or the presence of competitors, male *O. kuvanae* never abandon their receptive mate immediately after copulation, and complete the post-copulatory ritual even if they then forego mating opportunities with other females. This behavior corroborates the importance of the post-copulatory ritual as a form of mate guarding that functions to ensure paternity in the context of sperm competition (Simmons 2001).
6.5.4. Conclusion

In summary, our study demonstrates that first-male sperm precedence is most prevalent in *O. kuvanae*, but that 'sneaker' males are also capable of achieving successful paternity. The underlying mechanisms do not entail more frequent or prolonged copulatory bouts, or morphological characteristics of the males’ aedeagus. Instead, our data suggest that the post-copulatory ritual may function as an adaptation to sperm competition, whereby the ritual accelerates the awakening of an in-trance female, thereby effectively and quickly closing her window of receptivity.
6.6. Tables and Figures

Table 6.1. **Loci, alleles of parents, daughter genotypes and their proportions, p-value representing probability that the first male to mate (P₁ male) sired each daughter (n), and mean exclusion probability (EP) proportion for each replicate.**

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Loci</th>
<th>Mother</th>
<th>Sire A</th>
<th>Sire B</th>
<th>Daughter genotypes (genotype %)</th>
<th>P value, EP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n=12)</td>
<td>A106a</td>
<td>238</td>
<td>238</td>
<td>250</td>
<td>238</td>
<td>p&lt; 0.0001, 0.87</td>
</tr>
<tr>
<td></td>
<td>A107</td>
<td>192</td>
<td>192</td>
<td>192</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B105</td>
<td>249</td>
<td>249</td>
<td>259</td>
<td>249</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>259, 249 (100)</td>
<td></td>
</tr>
<tr>
<td>2 (n=7)</td>
<td>A106a</td>
<td>254</td>
<td>254</td>
<td>254</td>
<td>238</td>
<td>p = 0.016, 0.83</td>
</tr>
<tr>
<td></td>
<td>A107</td>
<td>200</td>
<td>204</td>
<td>194</td>
<td>204, 200 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B105</td>
<td>240</td>
<td>244</td>
<td>244</td>
<td>240, 240 (100)</td>
<td></td>
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<tr>
<td>3 (n=4)</td>
<td>A107</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>184</td>
<td>p = 0.625, 0.91</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>200, 200 (25); 184, 200 (75)</td>
<td></td>
</tr>
<tr>
<td>4 (n=8)</td>
<td>A106a</td>
<td>246</td>
<td>246</td>
<td>254</td>
<td>246, 254 (100)</td>
<td>p = 0.008, 0.86</td>
</tr>
<tr>
<td></td>
<td>A107</td>
<td>180</td>
<td>254</td>
<td>180</td>
<td>254</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>198, 180 (100)</td>
<td></td>
</tr>
<tr>
<td>5 (n=13)</td>
<td>A106a</td>
<td>252</td>
<td>240</td>
<td>240</td>
<td>252</td>
<td>p &lt; 0.0001, 0.72</td>
</tr>
<tr>
<td></td>
<td>A107</td>
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<td>198</td>
<td>198</td>
<td>192, 206 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B105</td>
<td>247</td>
<td>244</td>
<td>247</td>
<td>247, 244 (100)</td>
<td></td>
</tr>
<tr>
<td>6 (n=16)</td>
<td>A1</td>
<td>242</td>
<td>242</td>
<td>242</td>
<td>242, 248 (100)</td>
<td>p &lt; 0.0001, 0.85</td>
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<tr>
<td></td>
<td>A106a</td>
<td>254</td>
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<td></td>
<td>B105</td>
<td>251</td>
<td>251</td>
<td>251</td>
<td>251, 251 (100)</td>
<td></td>
</tr>
<tr>
<td>7 (n=21)</td>
<td>A106a</td>
<td>246</td>
<td>246</td>
<td>246</td>
<td>238</td>
<td>p &lt; 0.0001, 0.86</td>
</tr>
<tr>
<td></td>
<td>A107</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>184</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>200, 200 (100)</td>
<td></td>
</tr>
<tr>
<td>8 (n=19)</td>
<td>A1</td>
<td>254</td>
<td>246</td>
<td>246</td>
<td>240</td>
<td>p &lt; 0.0001, 0.80</td>
</tr>
<tr>
<td></td>
<td>A106a</td>
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<td>252</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>252, 252 (58); 252, 244 (42)</td>
<td></td>
</tr>
<tr>
<td>9 (n=11)</td>
<td>A106a</td>
<td>242</td>
<td>242</td>
<td>242</td>
<td>252</td>
<td>p = 0.001, 0.88</td>
</tr>
<tr>
<td></td>
<td>A107</td>
<td>188</td>
<td>188</td>
<td>200</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>200, 188 (100)</td>
<td></td>
</tr>
<tr>
<td>10 (n=16)</td>
<td>A106a</td>
<td>242</td>
<td>242</td>
<td>242</td>
<td>250</td>
<td>p = 1.000, 0.84</td>
</tr>
<tr>
<td></td>
<td>A107</td>
<td>188</td>
<td>204</td>
<td>206</td>
<td>198</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>198, 204 (25); 188, 188 (6); 198, 188 (13); 206, 188 (38); 204, 204 (6); 206, 204 (6); 198, 200 (6)</td>
<td></td>
</tr>
</tbody>
</table>
Table 6.2. Number of daughters sired by the first male ($P_1$) and the second male ($P_2$) to mate, $P_2$ values (proportions of offspring sired by $P_2$ male), 95% confidence interval (CI) limits, and list of key courtship and mating behavior ($pcr$ = post-copulatory ritual).

<table>
<thead>
<tr>
<th>Replicate #</th>
<th>Daughters sired $P_1$</th>
<th>Daughters sired $P_2$</th>
<th>$P_2$ values (95% CI limits)</th>
<th>List of key behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>0</td>
<td>0 (0.0)</td>
<td>$P_1$ performed $pcr$ first.</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>0</td>
<td>0 (0.0)</td>
<td>$P_1$ performed $pcr$ first.</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0.75 (0.2194, 0.9868)</td>
<td>$P_1$ and $P_2$ copulated with the same female 2 times. $P_1$ was in copula 2 s longer than $P_2$. $P_2$ performed $pcr$ first.</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>0</td>
<td>0 (0.0)</td>
<td>$P_1$ performed $pcr$ first.</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>0</td>
<td>0 (0.0)</td>
<td>$P_1$ performed $pcr$ first.</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>0</td>
<td>0 (0.0)</td>
<td>$P_1$ performed $pcr$ first.</td>
</tr>
<tr>
<td>7</td>
<td>21</td>
<td>0</td>
<td>0 (0.0)</td>
<td>$P_1$ performed $pcr$ first.</td>
</tr>
<tr>
<td>8</td>
<td>19</td>
<td>0</td>
<td>0 (0.0)</td>
<td>$P_1$ performed $pcr$ first.</td>
</tr>
<tr>
<td>9</td>
<td>11</td>
<td>0</td>
<td>0 (0.0)</td>
<td>$P_1$ and $P_2$ copulated with the same female 2 times and for circa the same duration. $P_2$ performed $pcr$ first, but $P_1$ engaged female in $pcr$ 3 additional times.</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>8</td>
<td>0.5 (0.2551, 0.7449)</td>
<td>$P_2$ was in copula 6 s longer than $P_1$, but $P_1$ performed $pcr$ first.</td>
</tr>
</tbody>
</table>

| Total       | 116                   | 11                     | 0.086 (0.0462, 0.1532)      |                      |
Figure 6.1. Environmental scanning electron micrograph (a) and photomicrographic image (b) of the proximal tip of a representative aedeagus of a male Ooencyrtus kuvanae. Note the absence of spines which could serve in sperm removal.

6.7. Acknowledgements

We thank M. Todd, G. Sadowski, F. Fernando, and K. Jones (Genetic Identification Services) for technical assistance with the DNA library construction, screening, and enrichment; U. Somjee for technical assistance with the experiments; J. Drummond for ESEM and photomicrographic imaging; M. Christie for his exclusion probability program; J. Wolfe for formatting the tables, and D. Stover for helpful suggestions. This work was supported 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, and Global Forest Science (GF-18-2007-226; GF-18-2007-227) as sponsors.
6.8. Literature cited


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7. Conclusions and Future Work

7.1. Conclusions

In my thesis, I have presented an interdisciplinary analysis of local mate competition, and the mechanisms, functions, and fitness consequences of the pre- and post-copulatory ritual in *O. kuvanae*. Prior to my research, there was only a modicum of data on these processes in *O. kuvanae*. My findings have enhanced the knowledge base of these various processes and have allowed alternative interpretations of previous research results. My data have provided a segue for future research, not only on *O. kuvanae*, but on parasitoids in general, specifically in the context of local mate competition, courtship and mating behaviour.

Courtship and/or mating behaviour as discrete forms of ARTs are known to be common in male insects. Theoretical models predict that at least two ARTs can coexist when males intensely compete for monandrous females that are numerous, spatially clustered, and synchronously receptive for a short period of time (Shuster and Wade, 2003). In Chapter 2, I have shown that under these conditions *O. kuvanae* males exhibited at least two male ARTs (HGG and MAO). Unlike the MAO tactic whereby males mate with a female immediately upon encounter, the HGG tactic relies on speed, pheromone-tagging (mediated by antennation) a harem of virgins to mate with at a later time, and on a female’s avoidance of males except for the male that pheromone-tagged her. I also show that females had no adverse fitness effect, as measured by their reproductive output, mating with multiply-mated males.

As male and female *O. kuvanae* discriminate between pheromone-tags (Chapter 2), and as males search and compete for females that emerge from hosts on a discrete patch, the presence of a mate-attraction and -recognition pheromone is likely. It should have low volatility and function as a close-range pheromone (Godfray, 1994). In Chapter 3, I show, that close-range, mate-attraction or -recognition in *O. kuvanae* is
mediated, in part, by close-range, low-volatility CHC pheromone components, and that males are capable not only of recognizing these pheromone components but also of detecting pheromone antagonists that differ in absolute configuration. This could be an underlying mechanism of specimen-specific pheromone (Chapter 2) which, to my knowledge, has not been demonstrated in other insects.

Sex pheromones that mediate mate attraction and recognition may not always be the underlying mechanism of mating stages such as pre- and post-copulatory rituals within a mating sequence. In Chapter 4, I investigated the mechanisms, functions, and fitness consequences of pre- and post-copulatory rituals of *O. kuvanae*. Similar to other parasitoids, the pre- and the post-copulatory ritual entail stereotypical and non-stereotypical mechanical stimulation, respectively. There was no evidence of pheromone transfer during these rituals, which is not surprising given that a sex pheromone plays a key role prior to the pre-copulatory ritual (Chapters 2 and 3). Further, I show that the pre-copulatory ritual has the effect of placing a female into a quiescent state, which persists at least 48 (s) post-copulation unless the female engages in the post-copulatory ritual. Conversely, the post-copulatory ritual functions to accelerate the awakening of a female from the quiescent state; once a female has engaged in this ritual, she never re-mates during her lifetime, thus ensuring paternity for the one male that mated with her and completed the ritual. Females with or without post-copulatory ritual experience, and the duration of the post-copulatory ritual, had no effect on a female’s total number of offspring, daughters, and sons. This implies that the post-copulatory ritual is neither indicative of the complement of sperm that is transferred or utilized, nor indicative of the female re-assessing her mate, because she did not adjust a male’s inclusive fitness according to the length of time he committed to the post-copulatory ritual.

Female parasitoids are known to adjust the sex ratio of their offspring by producing more sons as there are increasing numbers of wasp foundresses ovipositing on the same patch, as predicted by LMC (Hamilton, 1967). In Chapter 5, I showed that this prediction holds true for female *O. kuvanae*. I have also shown that *O. kuvanae* females seemingly do not respond to the presence of kin, because they produced similar proportions of sons whether they oviposited in the presence of a sibling or a non-sibling. These data corroborate other data (see Appendix B) that *O. kuvanae* females likely do not discriminate between kin, and that their size does not affect their absolute
reproductive output and the sex ratio of their offspring. To my knowledge, this is the first study on LMC in a parasitoid whereby larvae do not compete for local resources, and thus do not distort the sex ratio in favor of sons (Godfray, 1994).

My observations that a mated female *O. kuvanae* can be susceptible to copulation by a sneaker male immediately prior to, or while engaged in, the post-copulatory ritual with her first mate, implied the presence of sperm competition. In Chapter 6, I used DNA microsatellite analysis, a method not before implemented to test paternity in an egg parasitoid, to determine first- or second-male sperm precedence as well as to explore male behavioural and morphological adaptations to sperm competition. I found (i) first-male sperm precedence, (ii) male adaptations to sperm competition are not associated with copulation behaviors, (iii) males do not possess morphological adaptations for removal of rival male sperm, and (iv) the post-copulatory ritual represents a male adaptation to reduce sperm competition. I also show that single- or twice-mated *O. kuvanae* females produce similar numbers of sons (Appendix A), compared to the egg parasitoid *Anaphes nitens* where twice-mated females produce more sons. It has been suggested that female *A. nitens* may have limited storage capacity of the spermatheca which could result in sperm flushing or sperm ejection. The increase in the proportions of sons may also be due to incomplete sperm transfer, sperm accumulation or sperm plugs (see Santolamazza-Carbonea and Pestaña, 2010).

An overview of the mating stages within the mating sequence of *O. kuvanae* reveals that pair formation within a competitive setting relies on some degree of control by both sexes. Females readily mate with any male that implements the MAO tactic; however pheromone-tagged females only mate with the male who tagged them. In this respect, the tagging pheromone puts both sexes in control during pair formation. That males must engage a female in a pre-copulatory ritual before she is receptive to copulation suggests that females control this stage in the mating sequence. However, sneaker males with forced copulations take control away from a quiescent female, and from the male who courted and typically mated her first. The post-copulatory ritual as a form of mate guarding likely provides a platform for males to retain, or regain, control thus preventing or reducing sperm competition. Control during this stage is given back to those females who have not “properly” engaged in the post-copulatory ritual and therefore can re-mate.
7.2. Future Work

My thesis has laid the groundwork for many more lines of research. I have demonstrated that two ARTs coexist in a competitive setting. Whether these phenotypes remain fixed throughout repeated and dynamic competitive settings deserves further attention. HGG males are much faster than MAO males but the underlying mechanism of this remains unclear. Age and overall health of males could affect their speed which, in turn, may affect the strategy they select. The overall quality and developmental time of males could be affected by host quality. If so, additional experiments could test whether a foundress that oviposits in the presence of another foundress, strategically produces MAO or HGG males. If so, this would affect the design of studies that test sex ratio theory. Moreover, if females can discriminate between a tagged and a non-tagged female, one could predict that a foundress with a pheromone-tag from an HGG male produces more daughters, and that she would produce fewer HGG sons in the presence of a foundress without a pheromone-tag mated by an MAO male.

I have demonstrated that HGG males use their antennae to pheromone-tag females and that the pheromone mediates mate recognition and mate assessment; however, the location and microstructure of an antennal pheromone gland remain unclear. Future research also needs to address: 1) the molecular structure of the tagging pheromone; 2) the means by which each male can have a unique tagging pheromone; 3) the tagging pheromone of haploid brothers; and 4) the biosyntheses giving rise to unique pheromone tags.

I have shown that the blend of $5R$-me-27Hy and $5R,17R$-diMe-27Hy deters males but other components in the CHC profile of males may contribute to the deterrent effect. If additional components are revealed, they may help deduce the identity of the tagging-pheromone.

I show that the mating system of *O. kuvanae* meets assumptions of LMC. My current data indicate that the size of a female does not affect the number or sex ratio of her offspring but each female in pertinent experiments oviposited in isolation. Testing whether an ovipositing foundress will assess the size of other foundresses in the same
patch, and adjust her sex ratio accordingly, could reveal additional information that may affect sex allocation behaviour in female *O. kuvanae*.

There is strong first-male sperm precedence in *O. kuvanae*. Data supporting this conclusion suggest that the post-copulatory ritual is a male adaptation to sperm competition; however, further experiments are still necessary to demonstrate conclusively that first-male sperm precedence and the post-copulatory ritual are linked. Whether the shape and elasticity of the spermatheca of female *O. kuvanae* resist any change in storage capacity in favor of first-male sperm precedence is yet to be explored.

Overall, my dissertation has focused on laboratory-based experiments. The readily available laboratory-reared insects coupled with a controlled environment were compulsory to successfully execute this type of research. Now that the data are in place and certain methods are established, *in situ* observations of *O. kuvanae* behaviour and *in situ* experiments appear feasible, and may lead to new research questions.
Appendices
8. Appendix A

8.1. Sons produced by once- and twice-mated *O. kuvanae* females

8.2. Methods and materials

8.2.1. *Experimental insects (see section 4.3.1.)*

8.2.2. *Experiments*

Within 1 day of emergence, a virgin female was paired and mated with a virgin male (experiment 1) under a Petri-dish (30 mm diameter) (*n* = 10). On the same day, a virgin female was paired with four virgin males (experiment 2) under a Petri-dish (30 mm diameter (*n* = 10); these conditions triggered the sneaker male tactic which resulted in twice-mated females. Once- and twice-mated females were individually placed in a glass jar with 60 gypsy moth eggs, and allowed 14 days to parasitize them. Every day for 14 days, each jar was checked to ensure the parent female was alive, and every 3 days, the honey-imbued cotton wick (1 x 1.3 cm), which sustained the female, was replaced. In total, 100% of females survived for 14 days. After 14 days, the parent female was removed, and eggs were left untouched until offspring emerged at which point emergent sons were counted. To ensure all sons were accounted for, eggs were kept for an additional 4 weeks after the parent females had been removed.

8.2.3. *Data analysis*

Data were analyzed by an independent t-test test in PASW v. 18.0 (International Business Machines Corp.).
8.3. Results

There was no significant difference between the number of sons produced by a single-mated female ($\bar{X} = 1.60$, SD = 2.221) and twice-mated female ($\bar{X} = 5.00$, SD = 4.830) ($t = -2.022$, d.f. = 18, $P = 0.06$).

8.4. Conclusion

My findings contrast results obtained in a study with the egg parasitoid *Anaphes nitens* (Santolamazza-Carbonea and Pestaña, 2010), where multiple-mated females produced more sons. The production of more sons was suggested to be due to difficulties in sperm transfer, sperm plugs or sperm displacement. These interpretations would not apply to *O. kuvanae* because sperm was successfully transferred by both males that mated with the same female (see Chapter 6), and that there was no evidence of sperm plugs or sperm displacement.

8.5. Literature cited

9. Appendix B

9.1. Kin recognition

Parasitoid wasps have been shown not to recognize kin. Kin recognition could take the form of choosing not to mate with non-siblings, but instead to prefer siblings as mates. The ability to discriminate between kin is an underlying factor of kin selection theory.

Kin selection theory predicts that the high relatedness between sisters means that a female will modify her courtship behavior to favor a full sibling female. Specifically, a female may benefit a brother by partaking in a short post-copulatory ritual, thus providing him with more time to locate and mate more females. Alternatively, a female may benefit a sister by lengthening the post–copulatory ritual with a non-brother, thus increasing the likelihood that a sister will be mated by a brother.

The following experiments were run to test the hypotheses that male *O. kuvanae* do not recognize kin, and that female *O. kuvanae* do not modify their courtship behaviour to benefit sisters.

9.2. Materials and methods

9.2.1. *Experimental insects and general protocol*

Two populations of *O. kuvanae* were used in the experiments. Population (A) specimens originated from gypsy moth egg masses field-collected from Newark, Delaware (U.S.A.). Population (B) specimens originated from gypsy moth egg masses field-collected near the town of North East, Maryland (U.S.A). Both populations were collected between April to October, 2009. Colonies were established and reared in the
Global Forest Quarantine Facility at Simon Fraser University under a 16L: 8D photoperiod at 22-25°C and 50-70%RH (Hofstetter and Raffa, 1997).

Females from each population were provided with gypsy moth egg masses supplied by the U.S. Forest Service (Hamden, Connecticut, U.S.A.). After 14 days, 120 parasitized eggs were collected from population A and population B, and isolated individually in Solo plastic cups. Emergent wasps were separated by sex and provisioned with a 1 x 1.3 cm honey-water imbued cotton wick. Males and females from the same population were mated under a Petri dish lid (30 mm diam.). Following a completed mating sequence, females were immediately separated and each female was introduced to ca. 65 gypsy moth eggs. Parasitized eggs were separated [one egg per translucent Solo plastic cup (103.5 ml) secured with a lid] either after a female had died or 14 days had passed. In total, 23 females, 11 from population A and 12 from population B, served as parents of all the offspring used in experiments to ensure definitive siblings and non-siblings. Offspring used in experiments had no previous sexual experience and were no more than three days old. All replicates were run under a Petri dish lid (30 mm diam.). First choices of mates by males and females, and the duration of the post-copulatory ritual, were recorded by two observers.

9.2.2. Experiments

In experiment 1, one full sibling female and male from population A were confined with a non-sibling male from population B (n=10).

In experiment 2, one full sibling female and male from population A were confined with a non-sibling female from population B (n=10).

In experiment 3, one full sibling female and male from population B were confined with a non-sibling male from population A (n=10).

In experiment 4, one full sibling female and male from population B were confined with a non-sibling female from population A (n=10).

In experiment 5, two full sibling females and one male from population A were confined with a non-sibling male from population B (n=20).
In experiment 6, one full sibling female and male from population A were confined with one full sibling female and male from population B ($n=19$).

### 9.2.3. Data analyses

In experiments 1–5, data on whether sibling or non-siblings mated, were analyzed with a binomial test (test proportion = 0.50%). In experiments 6 and 7, a Chi-square test was used to analyze mate choice, and a One-way ANOVA was used to analyze the duration of the post-copulatory ritual. All data were analyzed using PASW v. 18.0. The confidence interval for all tests was set at 95% ($P<0.05$).

### 9.3. Results

#### 9.3.1. Males and females did not prefer a sibling or non-sibling as a mate.

Experiment 1: Females from population A did not prefer their siblings as a mate (selected mates: sibling: $n=5$, non-sibling: $n=5$; $P=1.000$).

Experiment 2: Males from population A did not prefer their siblings as a mate (selected mates: sibling: $n=6$, non-sibling: $n=4$; $P=0.754$).

Experiment 3: Females from population B did not prefer their siblings as a mate (selected mates: sibling: $n=7$, non-sibling: $n=3$; $P=0.344$).

Experiment 4: Males from population B did not prefer their siblings as a mate (selected mates: sibling: $n=4$, non-sibling: $n=6$; $P=0.754$).

The pooled data of Experiments 1 – 4 reveal no significant preferential mating with a sibling or a non-sibling (selected mates: sibling: $n=22$, non-sibling: $n=18$, $\chi^2 = 0.400$, d.f. = 1, $P = 0.527$).
9.3.2. **Males and females did not prefer to mate with either a sibling or a non-sibling when they were in the presence of a sibling female or a non-sibling female.**

Experiment 5: The presence of a sibling female did not induce preferential mating with a sibling or non-sibling (selected mates: sibling: n = 13, non-sibling: n = 7; \( \chi^2 = 1.800, \text{d.f.} = 1, P = 0.180 \)).

Experiment 6: The presence of a non-sibling female did not induce preferential mating with a sibling or non-sibling (selected mates: sibling: n = 7, non-sibling: n = 12; \( \chi^2 = 1.316, \text{d.f.} = 1, P = 0.251 \)).

9.3.3. **Modification of post-copulatory ritual duration**

The duration of the post-copulatory ritual was not affected by the presence of a sibling or a non-sibling (\( F_{35:38} = 2.086, P = 0.120 \)). In the presence of a sibling, the duration of the post-copulatory ritual of sibling mates (\( \bar{X} = 34.77 \text{ s, SE} = 3.638 \)) or of non-sibling mates (\( \bar{X} = 36.43 \text{ s, SE} = 5.295 \)) was similar (\( P = 0.996 \)). In the presence of a non-sibling, the post-copulatory ritual of sibling mates was shorter (\( \bar{X} = 24.43 \text{ s, SE} = 4.353 \)) than that of non-sibling mates (\( \bar{X} = 43.67 \text{ s, SE} = 6.317 \)), but the difference was not significant (\( P = 0.081 \)).

9.4. **Discussion**

My preliminary findings reveal that *O. kuvanae* is not likely to recognize kin; a larger sample size is needed in order to state otherwise. These preliminary findings are consistent with other robust data on parasitoids where relatives are known to mate (Keller, 1997; Reece et al., 2004; Shuker et al., 2004; Bourdais and Hance, 2009) and inbreeding reduces the ability of kin recognition.

The possible lack of kin recognition, and thus kin selection, in *O. kuvanae* could be due to high levels of inbreeding and mate competition among relatives. In spatially clustered groups, competing individuals are likely to be related to some degree.
Competition among relatives can remove the selective advantage of altruism because an altruistic act toward one relative will not benefit other relatives (West et al., 2002).

Also as predicted, female *O. kuvanae* do not modify their post-copulatory behavior to seemingly favor kin, which is inconsistent with kin discrimination predicted by kin selection theory (Reinhold, 2003). It is likely that females don’t modify their post-copulatory behavior to favor kin because the post-copulatory ritual functions to accelerate the awakening of an in-trance female (Chapter 4), and it may be a male adaptation to sperm competition (Chapter 6).

If kin discrimination does occur at some level in *O. kuvanae*, it may not necessarily be within the framework of kin selection theory. Theoretical models, such as ‘prior association’, ‘green-beard effect’, and ‘phenotype-matching’, could give insight into the potential underlying mechanism(s).

The ‘prior association’ theory states that an individual learns the phenotypes of its relatives early in development; phenotypes are subsequently used to discriminate between conspecifics (Tang-Martinez, 2000). This model has been shown in paper wasps, where a newly emerged wasp can learn the chemical phenotypes of its relatives in 5 hours (Dapporto et al., 2006). As *O. kuvanae* females mate within minutes after emergence, and only once in their lifetime (Ablard et al., 2011), ‘prior association’ is an unlikely model to test for kin recognition in *O. kuvanae*.

The ‘green-beard’ effect is a theory of kin recognition whereby one allele codes for key traits that include sending a recognition signal, reception of that signal by relatives, and subsequent non-discriminatory behaviours towards recognized relatives. This underlying mechanism is rare, has been documented only in a few species (Tang-Martinez, 2000), and is highly unlikely to apply to *O. kuvanae*.

In the ‘phenotype matching’ model, as referred to in Chapter 1, an individual learns its own phenotype and compares it against the phenotype of a conspecific to determine relatedness (Tang-Martinez, 2000). The sweat bee, *Lasioglossum zephyrum*, recognizes kin and the degree of relatedness to kin by ‘phenotype matching’ of chemical profiles. This capability is thought to be due to a genetic component of chemical recognition, primarily in social hymenopterans (Greenberg, 1979). If kin discrimination
exists occurs by genetically determined chemicals in *O. kuvanae*, inbreeding would need to select for a very accurate phenotype-matching system. However, a phenotype-matching system with an inbreeding or outbreeding mechanism can be challenging to tease apart even in systems where 'phenotype matching' has been demonstrated (Greenberg, 1988).

9.5. Literature cited


