SEXUAL SELECTION AND SEX ALLOCATION IN THE GREGARIOUS PARASITOID WASP NASONIA VITRIPENNIS

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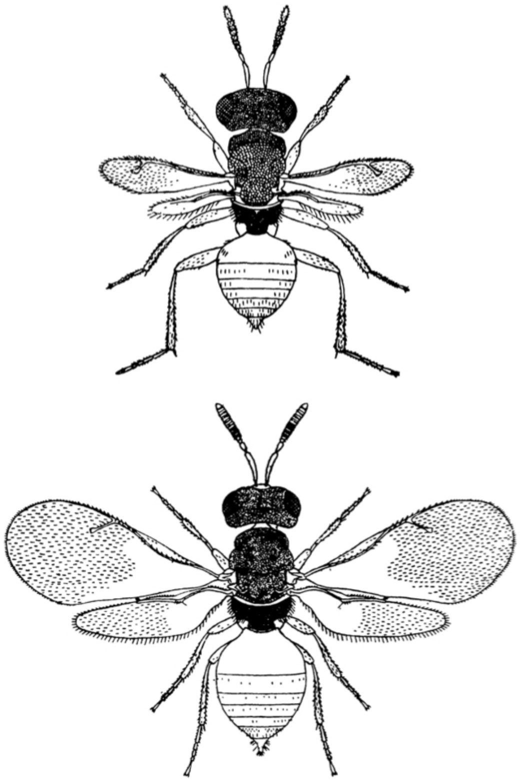
Sexual selection and sex allocation in the gregarious parasitoid wasp *Nasonia vitripennis*



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Submitted By Anna Margaret Moynihan

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Abstract

Sex allocation and sexual selection have been heavily studied, but rarely linked. In this thesis I investigated the interface between them in the gregarious parasitoid wasp *Nasonia vitripennis,* both directly and through their interactions with the mating system and sexual conflict. Chapter 2 investigated sexual selection and mating at the natal site: earlier eclosing males mated more females independently of body size. *Nasonia* follows Local Mate Competition, which describes how a female laying eggs alone on a patch of resources (a so-called single-foundress) should lay an extremely female-biased brood to minimise competition between her sons, yet ensure all her daughters are fertilised. Based on this I predicted that males with with fewer brothers would be better inseminators. Despite finding significant among-strain variation in (1) single-foundress sex ratio, (2) mate competitiveness when alone and (3) when in competition, (4) sperm resources, but not (5) sperm-depletion (Chapters 3 & 4), I did not find the predicted relationship. Conversely males from strains with more brothers had a higher mating success under competition (Chapter 3) leading to the question: does mating success select on sex ratio or vice versa? Either way it is a result of an interaction between sexual selection and sex allocation. Chapter 5 investigated the role of male post-copulatory courtship on female re-mating, and found that among-strain variation in female re-mating was not associated with variation in the duration of the post-copulatory courtship. Chapter 6 reviewed sexual conflict in the Hymenoptera: their haplodiploid genetics, newly sequenced genomes and varied life-histories provides a base for future research to build on. Finally I highlight the novel links between sexual selection, sex allocation, sexual conflict and the mating system found during my studies that will hopefully prompt future research on this topic.

1. Introduction

Sexual selection

The concept of sexual selection started with Darwin's attempt to explain many of the exaggerated traits observed in one sex of a species that could not be explained by his theory of natural selection (Darwin, 1871, Darwin, 1859). He proposed that many traits had evolved as a result of competition not to survive, but to reproduce. Darwin sub-divided sexual selection into two categories: those that were due to inter-sexual selection, or mate choice, and those that arose due to intra-sexual selection or competition between individuals of one sex for mates. Today, we define sexual selection as the non-random variation in mating success. We now think of sexual selection as a component of natural selection, as opposed to Darwin's distinct processes (Kokko et al., 2006b).

The idea of competition between members of the same sex leading to the evolution of weapons like the antlers of a stag, was accepted much earlier than female mate choice (reviewed in Andersson, 1994). Genetic models subsequently showed that if winning depends on the relative, and not absolute size of the trait, intra-sexual competition can lead to exaggeration of the trait (Maynard-Smith & Brown, 1986).

Explaining the mechanisms through which mate choice and ornaments evolve was the challenge for the following decades. The re-discovery of Mendel's work on genetics in the early 20th Century, and the understanding that selection occurs at the level of the allele, revolutionised our approach to sexual selection during the modern synthesis. During this period, a wide range of hypotheses were produced to explain preference and ornamentation, especially when females do not receive any non-genetic benefits from their mate. Fisher's (1930) run-away selection hypothesis proposed that positive feedback could explain the evolution of mate choice preferences and ornaments. For instance, consider that females vary in the extent of a heritable trait for preference of an ornament that has heritable variation in males. Those females with the higher preference will mate males with the bigger ornament, and so the offspring will inherit both the preference and the exaggerated ornament. As the preference and ornament spread in the population, a new benefit to males having big ornaments emerges: these males will have higher mating success because of the female preference. Such a benefit could maintain the ornament at values which may be sub-optimal from a natural selection point of view, i.e. an ornament that is costly in terms of survival.

The main problem with Fisher's hypothesis is that it did not solve the problem of how the preference arose in the first place. Subsequent explanations have tried to tackle this by proposing that the ornaments are indicators of heritable fitness (reviewed in Maynard-Smith, 1991, Kirkpatrick & Ryan, 1991, Kokko et al., 2003, Mead & Arnold, 2004, Andersson & Simmons, 2006, Kokko et al., 2006b). Zahavi, (1975 & 1977) proposed his handicap principle that the traits under mate choice are honest indicators of condition or genetic quality. Hamilton and Zuk (1982) proposed that the traits in question may be honest signals of parasitism, and the degree of resistance to parasites is reflected in the secondary sex traits. The Hamilton and Zuk model also deals with the issue that directional selection on a handicap-style trait will erode genetic variation, as the parasites provide a constantly (co-)evolving selection pressure.

Our understanding of sexual selection was further revolutionised when we began to consider what happens after copulation. Geoffrey Parker started to think about sperm competition after observing female dungflies mating repeatedly (Parker, 1970). Sperm competition occurs whenever the ejaculates of more than one male compete to fertilise the eggs of a female inside her reproductive tract. Once molecular techniques improved with the advances in PCR, widespread polyandry (a pre-requisite to sperm competition) was confirmed in wild populations (reviewed by Jennions & Petrie, 2000). From this point on, the importance of sperm competition was appreciated. A major consequence of sperm competition is post-copulatory sexual selection. Simmons (2001) divided adaptations as a result of sperm competition into two categories: offensive and defensive. Defensive adaptations seek to avoid the risk of sperm competition in the first place, for example by preventing female re-mating, or avoiding non-virgin females. On the other hand, offensive sperm competition adaptations seek to maximise a male’s success within the competition, for example by displacing a rival's sperm or passing a larger ejaculate. These adaptations also include the morphology of the sperm cells themselves (Calhim et al., 2007).

Parker considered sperm competition as an extension of intra-sexual competition. However, we now know that females also influence the outcome of sperm competition. Females have the potential to manipulate sperm transfer, storage and its utilisation in fertilisation (Thornhill, 1983; and reviewed by Eberhard, 1996). In this way a female can bias paternity towards a favoured male. Alternatively females can encourage sperm competition by mating with lots of males and either selecting the desired sperm for fertilisation, or letting the competition between the ejaculates result in only the best sperm fertilising her eggs, ensuring (potentially) good genes for the next generation. The mechanisms through which cryptic female choice occurs are starting to be elucidated (e.g. Qazi, 2002, Rosengrave et al., 2008, Edvardsson & Arnqvist, 2000).

It was also Parker (1979) who first made apparent the importance of the idea of sexual conflict. Sexual or natural selection acts in different ways on traits in males and females. Whenever selection results in different trait optima for the two sexes, sexual conflict occurs (Parker 1979). When the trait is controlled by a single locus, for example human hip-width, then intra-locus conflict may occur. In the hip-width example, bipedalism favours narrower hips for easier movement in both sexes. For females, however, reduced risk of death in childbirth selects for wider hips. As a result, sexually selected dimorphism occurs (Rice & Chippindale, 2001). Intra-locus conflict is assumed to be most commonly resolved through the evolution of sexual dimorphism (Rice & Chippindale, 2001, Cox & Calsbek, 2009). Inter-locus conflict occurs when multiple loci are involved in the expression of the traits in conflict.

These traits often originate through sexual selection, but not always (e.g. parental care is naturally selected and a source of conflict (Houston et al., 2005)). Examples of inter-locus conflicts are the various grasping traits that have evolved in male diving beetles (Coleoptera: Dytiscidae) (Bergsten et al., 2001) and water striders (*Gerris odontogaster*) (Arnqvist, 1989). In insects, males often need to grab hold of the female in order to mate, which she often resists. In order to increase the number of matings, and hence fertilisations the male achieves, males of the diving beetles have evolved suckers to hold onto females, and water striders have evolved elongated abdominal segments. These traits then select for female-counter traits in sexually antagonistic coevolution. The female diving beetles have rough wing covers which make it harder for the suckers to stick (Bergsten et al., 2001), and female water strider's abdominal spines to make it harder for the males to achieve genital contact (Arnqvist & Rowe, 1995). Sexual selection and sexual conflict are therefore tightly associated.

The frame-work of sexual conflict also offered a new explanation for the evolution of mate choice and exaggerated traits: chase-away selection. Imagine a species where the females eat red fruits, and so have a sensory bias to be attracted to the colour red. If a male developed a red secondary sexual trait, he could attract more mates by exploiting her sensory bias. Males are therefore manipulating the female response to a signal. This may lead to chase-away selection, where females raise their threshold of sensitivity to the trait in order to avoid costly sub-optimal mating, which in turn selects for males to increase the trait expression (Holland & Rice, 1998). The current thinking is that all these processes (Fisherian, handicap, good genes and chase-away) work alongside each other to create mating biases and ornaments (Kokko et al., 2003, Kokko et al., 2002).

Recently, the focus of sexual selection research has returned to the issue of sex-roles, as researchers such as Arnold & Duvall (1994) and Kokko et al., (2006b,) Kokko & Jennions, (2008) tackled some of the problems with Trivers' (1972) initial anisogamy argument. Now we are beginning to challenge the view of males competing and females choosing and investigate how sexual selection has acted on females through competition (Clutton-Brock, 2009, Gwynne, 1991) and on both sexes through mutual mate choice (Kraaijeveld et al., 2007).

Another growing area of research is the role of environmental heterogeneity on sexual selection (Cornwallis & Uller, 2010, Kokko & Heubel, 2008, Kokko & Rankin, 2006). Environmental heterogeneity in both time and space can lead to differences in the strength and direction of sexual selection in different circumstances, especially if sexual phenotypes are plastic. For example, selection on growth of the secondary sexually selected horns in Soay sheep depends on the environment throughout the life of the male (Robinson et al., 2008). Despite this growing awareness, there are still very few studies that have actually considered the influence of environmental heterogeneity (Bussière et al., 2008).

Sex allocation

Darwin was not as successful at his attempt in explaining sex ratios as his efforts in the field of sexual selection. He did, however in the first edition of his 1871 book tackle the issue of unbiased sex ratios as a problem for his theories of natural and sexual selection, revising it for the second edition to famously state that "*the whole problem is so intricate that it is safer to leave its solution for the future*" (Darwin, 1874). Despite some attempts to explain the problem, it was ultimately Fisher (1930) re-working earlier work by Darwin (1871) and Düsing (1884), who explained selection for the unbiased sex ratio. Fisher's explanation rests on the observation that for diploids each offspring has a mother and a father, so the total fitness of males and females in a population must be equal; this is known as the Fisher condition. This leads to negative-frequency dependent selection on the sexes produced, and stabilises investment into each of the sexes as equal. Sex allocation is the amount of parental resources invested into each sex (Charnov, 1982). Of course there are assumptions associated with Fisher's model: a stable age distribution, parental control over the sex determination, no cooperative or competitive interactions between relatives and no sex-specific influence of environment on fitness, to name a few.

The consequences of breaking these assumptions to Fisher's model were investigated in 1960s and 1970s. For instance, Hamilton's (1967 & 1979) Local Mate Competition (LMC) describes how local mating leads to competition between a mother's sons for mates. As the number of other females (foundresses) increases, the proportion male of the brood should rise with it, reaching 50% sons (Figure 1.1). If females are ovipositing alone on a patch of resources (as a single-foundress) she should lay a highly female-biased sex ratio, in order to minimise competition between her sons. This facultative sex allocation response to the number of foundresses on a patch has been observed in the lab and the wild in a wide range of organisms (e.g. ambrosia beetles (Biedermann, 2010); parasitoid wasps (Werren, 1980, Werren, 1983); protozoan parasites (Reece et al., 2008); mites (Izraylevich & Gerson, 1996); for a review see West (2009)).

This breaking of assumption was followed up by Trivers and Willard (1973), who showed that the environmental conditions could select for parents to adjust the sex ratio of their offspring. Trivers and Willard describe a species where maternal condition is transmitted to the offspring through increased provisioning. When maternal condition is good, they predict that mothers should overproduce the sex that benefits most from heightened condition. This has subsequently been observed in a range of taxa, most commonly the ungulates (Hewison & Gaillard, 1999, Sheldon & West, 2004).

The next big development in the field of sex allocation was the consideration of genetic conflict. This was first considered as conflict over sex allocation in the eusocial Hymenoptera (Trivers & Hare, 1976). Hymenoptera have haplodiploid genetics, which raises relatedness asymmetries (Figure 1.2). Using Hamilton's (1964) inclusive fitness theory Trivers and Hare (1976) showed how the evolutionary stable strategy (ESS) sex allocation differed for the queens and workers in the eusocial Hymenoptera. There has since been an explosion of interest of such conflicts within insect societies (Ratnieks et al., 2006).

Genetic conflict remained a theme as the discovery and exploration of sex allocation distorters grew. Sex allocation distorters are selfish genetic elements (either nuclear or cytoplasmic) that modify the sex of their host, or the sex ratio produced by the host, in order to increase their own transmission, often at the cost of the host's transmission (Burt & Trivers, 2008). Such sex ratio distorters are now known to be common across plants and animals (Meiklejohn & Tao, 2010, Werren & Beukeboom, 1998, Hurst, 2008).

The interactions between sex allocation and sexual selection

Just as the fields of sexual selection and sex allocation both experienced major developments with the consideration of sexual conflict, I think a key development in the coming years will be the awareness of the influence of facultative sex allocation and sexual selection on each other. In this section, I will outline the interactions between the four key themes of my thesis: sexual selection, sex allocation, sexual conflict and mating systems. The keystone of many of the relationships which link sex allocation and sexual selection is the sex ratio at the time of mating.

Operational sex ratio, sexual selection and sex allocation

The operational sex ratio (OSR) is the ratio of fertilisable females to sexually active males in the population (Emlen, 1976). Several factors can contribute to a biased OSR: sex-specific mortality rates, the time spent between mating and being ready to reproduce again (e.g. parental care, or a post-mating refractory period), and uneven primary sex ratios from biased sex allocation (Parker & Simmons, 1996, Kokko & Jennions, 2008). To truly understand the links between OSR, sex allocation, sexual selection, mating systems and sexual conflict would require some serious modelling. However, here I will draw on the existing literature to begin to understand these relationships.

The role of OSR was first considered within the realm of mating system description (Emlen & Oring, 1977). The mating system of a species describes the number of mates each sex have during a breeding season, and how those mates are acquired. The mating system was thought to be defined by two key variables: the OSR and the environmental potential for polygamy (Emlen & Oring, 1977). The environmental potential for polygamy is how the spatial-temporal distribution of each sex predicts whether or not that sex, or a resource it needs, can be defended and monopolised (Thornhill & Alcock, 1983, Emlen & Oring, 1977). The various mating systems we observe predict the nature of the sexual selection acting on the sexes. Where females can be defended male adaptations for male-male competition or resource defence will be selected. Likewise, if females cannot be defended and female choice evolves, that will select for ornamentation. It was thought that as the OSR became more biased, the intensity of sexual selection on the majority sex would increase (Emlen & Oring, 1977).

Recent models of OSR and sexual selection have demonstrated a feedback loop between OSR and sexual selection. Kokko and Jennions (2008) thoroughly investigated the evolution of sex roles considering the sex ratio, parental investment and sexual selection while controlling for the Fisher condition. Ultimately, the Fisher condition causes frequency dependent selection on the degree of competition experienced by the sexes: if you are unlikely to find a mate quickly when you return to the mating pool, stay and invest more in your current brood (Kokko & Jennions, 2008). This has the effect of also reducing any bias in the OSR. Therefore the OSR does alter the intensity and direction of sexual selection, just not necessarily how we thought. Indeed, many studies have found that at a biased-OSR the intensity of sexual selection can decrease for the over-represented sex. For example, the polygynandrous common lizard *Lacerta vivipara* has stronger selection on male body size at female-biased OSR, and this is thought to be due to the high costs of mating to females (Fitze & Le Galliard, 2008). Similarly, in the fish *Rhodeus sericeus,* the territories of males break down under male-biased OSR leading to groups of males releasing sperm clouds when females spawn, which results in a lower reproductive skew (Mills & Reynolds, 2003). The reliability of OSR is also questioned by a study in *Nephila plumipes* spiders that showed that it varied greatly over the season, and the spatial scale used (Kasumovic et al., 2008).

Since Emlen and Oring's (1977) initial work, we now consider other factors to have contributed to the description of the mating system, but the idea of a measure of the direction and intensity of sexual selection has persisted. Other methods of quantifying the intensity of sexual selection have since been proposed. The potential reproductive rate of each sex (Clutton-Brock & Vincent, 1991, Ahnesjö et al., 2001) describes mate availability, and includes how much time each sex spends "out" of the mating pool, providing parental care, or experiencing a post-reproductive refractory phase (Parker & Simmons, 1996). Shuster and Wade (2003) developed their I_{mates} approach that takes into account temporal and spatial distribution of the sexes when measuring the intensity of sexual selection, whereas the Bateman curves developed by Arnold and Duvall, (1994) use the relationship between the number of mates and fecundity for each sex.

While we now understand the mating system to not to be solely determined by OSR, it does still respond to changes in the OSR. For example, both sexes can care in the blue tit *Parus caeruleus*, and so the offspring raised by pairs have a higher chance of fledging than those cared for by females only (Kempenaers, 1994). In some years a female-biased OSR occurs due to higher predation of males. Such female-biased

OSR leads to polygyny as opposed to social monogamy (Kempenaers, 1994). Similarly, as seen above an increase in the density of males can lead to the resource defence breaking down for the fish *Rhodeus sericeus* (Mills & Reynolds, 2003).

The OSR not only has a feedback loop with sexual selection as described by Kokko & Jennions (2008), but also with sex allocation itself in some situations, mainly when there are overlapping generations. It is often proposed that frequency-dependent Fisherian sexual selection could act on the OSR in order to reduce the bias, however evidence is weak and patchy. Such a response has been observed in some (but not all) pre-industrial populations of humans (Lummaa et al., 1998). One test in skinks (*Eulamprus tympanum*) found that the females did not alter their sex allocation behaviour to compensate for the OSR (Allsop et al., 2006). In another lizard species *Amphibolurus muricatus*, females did manipulate the sex ratio in response to the OSR, however they overproduced the majority sex, which the authors proposed may because they use the OSR as a cue to sex-specific survival probabilities (Warner & Shine, 2007).

Nevertheless, while the debate over which methods are best for calculating the intensity of sexual selection continues, OSR, and the facultative sex allocation that can result in a biased OSR remains an important component of determining the nature and extent of sexual selection.

Mating system and sex allocation

Aside from this interaction between sex allocation, sexual selection and OSR that I described above, there are other connections between sexual selection, mating system and sex allocation. For example the mating system itself can influence the nature of facultative sex allocation. Perhaps the most well-known example of this is Local Mate Competition (LMC) (Hamilton, 1967, Hamilton, 1979). The natal-site mating selects for mothers to manipulate the sex ratio to minimise competition between her sons.

The original LMC models assume that all mating occurs at the natal patch, which has been observed in many species when males do not disperse (for a review see West, 2009). In the case of Bethylid parasitoid wasps, species differ in the extent to which males can disperse, leading to so-called partial LMC (Hardy & Mayhew, 1998). This family of parasitoids meets the prediction that species with higher male dispersal ability will have less intense local mating competition, and so sex ratios will be less female-biased (Hardy & Mayhew, 1998). This finding has also been confirmed in the non-pollinating fig wasps: where males are wingless, the sex ratio is most female biased, becoming less female biased in male wing-dimorphic species, and even less so when males are fully winged (Fellowes et al., 1999, West & Herre, 1998a).

Sexual selection and sex allocation

I have discussed the interactions between sex allocation and sexual selection mediated by OSR. There are also some scenarios where sexual selection and facultative sex allocation are known to influence each other, most commonly in species showing sex allocation through Trivers-Willard effects. Trivers and Willard (1973) described ungulates where maternal condition is usually transmitted to the offspring through higher birth weight and more milk resources and condition can subsequently be a good predictor of the mating success of one sex. For simplicity, we will assume that the fitness of males depends more on condition, however this is not always the case. Trivers-Willard facultative sex allocation therefore depends heavily on the sexual selection that leads to sex-specific condition-dependence. Population genetic models of Trivers-Willard effects showed that sexual selection was crucial to the invasion of a sex allocation allele: there must be a strong genetic covariance between the sex allocation allele and the mating bias of sons across all environments (Wade et al., 2003). Models tackling the extent of the sex allocation bias depend on two key factors: the strength of transmission of the maternal condition, and the importance of condition to a male's mating success (Leimar, 1996, Hewison & Gaillard, 1999). To understand the trade-off between these two parameters, we must consider that mothers are maximising not only the number, but also the reproductive value of their grandchildren. Of course mothers cannot know the condition of their offspring's mates, but can assume that they will have the mean population quality. If the transmission of maternal condition to offspring is stronger, then fewer grand-offspring produced by good-condition daughters could have higher total reproductive value than a greater number of average-condition grand-offspring produced through a son. Likewise, if sexual selection is strong due to a large reproductive skew among males heavily dependent on condition, then a good-condition son can produce many average-condition grandchildren. This prediction has found mixed support (Schino, 2004, Sheldon & West, 2004), but it does demonstrate that sexual selection and sex allocation are linked in Trivers-Willard situations.

An extension of the Trivers-Willard hypothesis is that attractive males should sire more sons than daughters. This was first proposed by Burley (1981) following observations in the zebra finch. Despite this initial study having been contested (e.g. Rutstein et al., 2005), the idea has persisted in many species (West & Sheldon, 2002). This effect of over-producing sons when the father is attractive has been modelled successfully, suggesting it is feasible. It relies on good-gene benefits and the heritability of male attractiveness (Fawcett et al., 2007, Pen & Weissing, 2000).

Study organism: *Nasonia vitripennis*

My study organism throughout this thesis is the gregarious parasitoid wasp *Nasonia vitripennis. Nasonia vitripennis* (Hymenoptera: Chalcidoidea: Pteromalidae) is one of four species within the *Nasonia* genus. It is a small (approximately 2mm long) wasp found throughout the Northern hemisphere (Whiting, 1967). *Nasonia vitripennis* parasitise the pupae of large dipterans, laying their eggs inside the puparium, but on the outside of the fly pupa itself. The host is killed by the female's sting but necrosis is arrested by components of the venom (Rivers & Delinger, 1995). Hence *N. vitripennis* is described as a gregarious (i.e. multiple eggs per host) idiobiont (i.e. host has stopped development) ectoparasitoid (i.e. larvae develop on the outside of the host's body) (Godfray, 1994).

Life cycle of *Nasonia*

Nasonia vitripennis spend around two days as eggs and then hatch into larvae, they feed on the host tissues for about nine days at 25°C, until they begin pupation. Pupation takes a further three days at 25°C, passing through several identifiable pupal stages (Whiting, 1967) which allow for the sexing of pupal wasps. Once the pupae eclose into adults, they chew a hole in the host puparium and exit.

The males tend to eclose and emerge sooner than females, waiting for their virgin sisters to emerge from the hole in the host puparium. The courtship and mating behaviour of *N. vitripennis* is well described (Barrass, 1960, Barrass, 1961, Whiting, 1967). Males deposit a rectally-produced pheromone on the substrate, often on the host puparium's external surface, which is attractive to virgin females (Steiner & Ruther, 2009b, Ruther et al., 2009, Ruther et al., 2007). The male mounts the female, orienting himself over her antennae, where he begins a series of stereotyped head nod cycles. On the first of each of these cycles he emits a dose of pheromone from his mouth-parts (van den Assem et al., 1980); the chemical identity of this pheromone is not yet known. When the male has successfully courted the female, she drops her antennae and moves her abdomen to expose her genital pore. On receiving this signal, the male backs up to copulate with the female. He inserts his aedeagus into her reproductive tract to deposit his ejaculate. Once copulation is complete, the male returns to his original courtship position, where he performs more bouts of the head-nod cycle. This is known as post-copulatory courtship, and is thought to have a role in turning-off female receptivity to future matings (Leonard & Boake, 2008, van den Assem & Visser, 1976). Most females in the wild are singly-mated (Grillenberger et al., 2008), but double mating is observed in the lab and the wild (Burton-Chellew et al., 2007a, Grillenberger et al., 2008, Holmes, 1974, Grant et al., 1980).

One of the purposes of mating is to transfer sperm. A little is known about spermatogenesis in parasitoids and *Nasonia* in particular. *Nasonia* males are proto-spermatogenic, i.e. they produce all their flagellate spermatozoa as pupae, and the production of this is synchronous (Whiting, 1967, Clark et al., 2010, Hogge & King, 1975, Clark et al., 2008, Pennypacker, 1958). The timing of sperm production across the parasitoids is predicted to vary with the nature of their mating opportunities (Boivin et al., 2005). The haplodiploidy shared by all Hymenoptera alters the process of spermatogenesis: the first round of meiosis is not performed due to the male haploidy, leaving all spermatozoa genetically identical (Pennypacker, 1958). The spermatozoa themselves are similar to those of all Chalcidoidea: the head of the spermatozoa consists of an acrosome and a spiral nucleus followed by the flagellate tail packed with mitochondria (Lino-Neto et al., 2000, Lino-Neto & Dolder, 2001, Hogge & King, 1975). After mating the sperm are stored in the female's spermatheca until she uses them for fertilising eggs. The spermatheca consists of a spermathecal capsule where the sperm are stored, the spermathecal gland, and a long duct which connects to the vagina (King, 1962).

Mated females have higher activity levels than virgins (King et al., 2000) and disperse away from the patch, leaving the wingless (brachypterous) males behind. After dispersal females then search for new hosts to oviposit. We know little of the cues used for the host searching in *Nasonia* in the wild, but there is some evidence that olfactory cues of the host and the host-environment are used (Whiting, 1967, Schurmann et al., 2009) along with some cues of the hosts nutritional quality (Blaul & Ruther, 2011). Also a genetic basis for host choice has been established between the species of the *Nasonia* genus (Desjardins et al., 2010).

Before stinging a host and ovipositing, an adult female assesses if the host is suitable for oviposition, including if it has previously been parasitised by another female (Whiting, 1967). Once the female has stung the host, she feeds on the haemolymph, gaining valuable protein which she uses for egg-maturation (Rivero & West, 2005). Depending on the species, size and quality of the host, the female then lays a clutch of eggs (typically 20-50 eggs for the *Calliphora vicina* hosts used in this thesis) using a wide range of cues to determine both the size of her clutch and its sex ratio (see sex allocation in *Nasonia* section below).

The mechanism that an ovipositing female uses to control the sex ratio is thought to be precise fertilisation. Their haplodiploid genetics mean that in general, a fertilised egg will become a daughter and unfertilised egg will become a son. Control of the sex ratio is distinct from sex determination itself. The molecular regulation of sex determination mechanism itself has recently been resolved after decades of confusion reviewed by Beukeboom et al., (2007). After assessing the evidence for various mechanism of sex determination, including maternal effects, genomic imprinting, and ploidy, Beukeboom et al., (2007) proposed a model for sex determination in *Nasonia,* which relied on a combination of genomic imprinting and maternal effects. Recently, the molecular regulation of sex determination was established (Verhulst et al., 2010), and it closely matches the prediction of Beukeboom et al., (2007). Verhulst et al., (2010) found the *transformer* gene in *Nasonia*, that they named *NVtra* for *Nasonia vitripennis transformer.* They discovered that *NVtra* shows sex-specific splicing patterns and through a series of experiments using dsRNA to silence *NVtra* in adult females, they discovered that the *NVtra* is silenced in eggs through maternal imprinting. The eggs are also provisioned with the maternally produced female-spliced mRNA of *NVtra.* Unfertilised eggs develop into males because they cannot produce the female-specific splice forms of *NVtra* once the maternal provision runs out. In fertilised eggs, the paternally inherited *NVtra* locus is not imprinted, and so the fertilised eggs can continue to produce the female-specific splice form of *NVtra* necessary to develop as a female. This is of course, sensitive to some of the endosymbionts that can feminise or masculinise embryos (see sex allocation in *Nasonia* section).

Sexual selection in *Nasonia* and other parasitoids

One of the hall-marks of sexual selection is the sexual dimorphism that results from sex-specific selection on trait values. In general the parasitoid wasps, like most Hymenoptera, show considerable sexual dimorphism (Stubblefield & Seger, 1994). On the whole, females are larger than males, but body sizes do generally overlap (Hurlbutt, 1987b). Fighting adaptations are observed in the males of some species (e.g. *Melittobia acasta* (Innocent et al., 2007); the fig wasp parasitoids (Hamilton, 1979)). Female parasitoids, as aculeates, have an ovipositor which they use to sting the host, and also lay the eggs. Aside from sexual size dimorphism, relatively little is known about sexual selection in the parasitoid wasps. In the fatally-fighting *Melittobia acasta* development time is also under sexual selection: earlier males do best in competition, but if the wasps are age-matched, larger males are more likely to win (Innocent et al., 2007).

The dimorphic external characters of *Nasonia vitripennis* are described in depth in Darling and Werren, (1990), and summarised here. In general *Nasonia* males are smaller than females, but their body sizes do overlap. This reduced male body size is thought to be associated with the evolution of receptivity signalling in many Chalcidoidea, as males cannot detect the female opening her genital pore when they are in the courtship pose (van den Assem, 1976, van den Assem & Jachmann, 1982). The hind tibia of *Nasonia,* and many other species are elongated relative to body length in order to assist males in mating. Male and female *Nasonia* also have different pigmentation patterns, however this has not been investigated in terms of its evolutionary function. Unsurprisingly the shape of the abdomen differs between the sexes, probably as a consequence of the different genitalia within. One striking difference is that males are brachypterous, in that they have vestigial wings, while females have fully developed wings and can fly. This restricted male dispersal ability has implications for the sex allocation strategy of *N. vitripennis.* Males are also protandrous: they tend to develop faster than their sisters, and are ready to mate sooner, although again there is some overlap between the sexes.

Where so few studies have investigated sexual selection in the parasitoids, *Nasonia* is one of the best understood species in this respect. Larger body size provides no advantage to males in competition (Burton-Chellew et al., 2007b), however larger males do have more sperm cells (Clark et al., 2010). Females are more attracted to males that produce larger quantities of the male rectally-produced pheromone, which may be an honest signal for sperm-resources (Ruther et al., 2009, Blaul & Ruther, 2011).

Sex allocation in *Nasonia* and other parasitoids

In contrast to sexual selection, facultative sex allocation has been intensely studied in the parasitoid wasps. Two regimes of sex allocation are most common: local mate competition, and Trivers-Willard effects.

For solitary parasitoid wasps that lay just one egg per host, the size of the host will determine the body size of the adult wasp, and hence contribute to its fitness. In most parasitoid wasps, body size is more important to females than males (Godfray, 1994). Therefore in the solitary parasitoids, it is predicted that under the Trivers-Willard hypothesis (1973) female offspring should be laid on relatively larger hosts. This has been observed in many parasitoid species (West & Sheldon, 2002). For example *Lariophagus distinguendus* females lay daughters in larger hosts and sons in smaller hosts (van den Assem, 1971). Such a sex allocation strategy is easier if the host's development is arrested by the sting of the parasitoid. If the wasp is a koinobiont (i.e. the host continues to develop once parasitised) the initial host size is not a reliable cue for the amount of resource available to the developing larvae.

In the gregarious parasitoids, population structure may lead to competition between brothers for mates, resulting in the sex allocation strategy of Local Mate Competition. This is also possible in the quasi-gregarious parasitoids where only one wasp develops per host, but the hosts are clustered together to form patches. As the number of females ovipositing on a patch (foundresses) increases, the sex ratio a mother lays should increase from an extreme female-bias to equality (Hamilton, 1967, Hamilton, 1979). This response has been observed in many parasitoid species (for a review see West, 2009). For the quasi-gregarious wasps, the size of the egg clumps will alter the intensity of LMC and hence the degree of sex ratio adjustment. This was found to be true across 31 species of the scelionid wasps where the size of host egg masses and the degree of dispersal opportunities for males and females varied (Waage, 1982). The Scelionidae are not the only group where the biology deviates from the assumptions of Hamilton's original LMC models. Numerous extensions have been proposed, and tested in many parasitoid species where some mating occurs away from the patch, or the clutch sizes of females vary, or even the timing of the oviposition. These extensions, and the evidence for them have been recently reviewed by West (2009).

Non-facultative changes in the sex ratio are also observed in some parasitoid species. Aside from those caused by selfish genetic elements and endosymbionts, frequency dependent selection can also play a role. Due to their haplodiploidy, virgin parasitic wasps can lay all-male broods. These virgin, or sperm-depleted, females ovipositing are called constrained females. If the incidence of constrained females in the population is large, this can lead to an excess of males, consequently selecting females that are not constrained to lay slightly more females through frequency-dependent selection (Godfray, 1990, Ode et al., 1997). This effect has been observed in many parasitoid species (for a review see West, 2009) but is not thought to be important for *Nasonia vitripennis* as the incidence of constrained females is low (Beukeboom & Werren, 2000, Grillenberger et al., 2008, King & D'Souza, 2004).

Sex allocation in *Nasonia* follows LMC (Hamilton, 1967). Females assess the number of foundresses on the patch through several cues including the parasitised status of the host (King & Skinner, 1991, Werren, 1980, Shuker & West, 2004); the presence of other females in the patch (Flanagan et al., 1998, Werren, 1983, Shuker & West, 2004); and olfactory cues (Shuker et al., 2007b). The age of the brood a female is about to super-parasitise is also important: female adjust the sex ratio based on the probability that the males from the previous brood will still be alive to compete with her sons (Shuker et al., 2005, Shuker et al., 2006a). Despite predictions that female *N. vitripennis* should alter their sex allocation behaviour dependent on the relatedness of the females in the patch, there is no evidence to suggest they do this (Reece et al., 2004, Shuker et al., 2004, Shuker et al., 2006b). *Nasonia* females can discriminate between species within the genus: females are more likely to reject a host previously parasitised by another species, but if they do accept the host they do not alter the sex ratio as predicted (Ivens et al., 2009). The assumptions of many LMC models and extensions have been tested, and confirmed in wild populations (Grillenberger et al., 2008, Molbo & Parker Jr, 1996, Burton-Chellew et al., 2008).

Factors other than LMC cues can also influence the sex allocation behaviour of *Nasonia* females. Several studies have demonstrated heritable variation in sex allocation behaviours. The narrow sense heritability of single-foundress sex ratio has been measured to be between 0.05-0.15 (Orzack & Gladstone, 1994) and 0.09-0.15 (Parker Jr & Orzack, 1985), while the broad-sense heritability measured at 0.02-0.17 (Pannebakker et al., 2008). Between-strain variation in single-foundress sex ratio has also been established (Orzack et al., 1991, Orzack & Parker Jr, 1990, Orzack, 1990). Artificial selection experiments have also proved successful (Parker Jr & Orzack,

1985) and the mutability of sex ratio was found to be 0.001-0.002, which is consistent with life-history traits in other insects (Pannebakker et al., 2008). This body of evidence for a genetic basis to the sex ratio culminated in the discovery of a Quantitative Trait Locus (QTL) for sex ratio variation on chromosome two (Pannebakker et al., 2011).

Nasonia vitripennis also suffers from infections of selfish sex ratio distorters which skew the sex ratio to benefit their own transmission. Paternal Sex Ratio (*PSR*) B chromosomes cause the paternal chromosomes to condense just after fertilisation (itself surviving), resulting in all-male broods (Beukeboom, 1994, Beukeboom & Werren, 1993, Beukeboom & Werren, 2000, Dobson & Tanouye, 1996, McAllister & Werren, 1997, Werren & Stouthamer, 2003). The endosymbiont *Wolbachia* is wide-spread in *Nasonia* wasps, and causes cytoplasmic incompatibility, which can result in all-male broods if *Wolbachia*-free eggs are fertilised by *Wolbachia*-bearing sperm (Bordenstein et al., 2001, Bordenstein et al., 2003, Bordenstein & Werren, 1998, Breeuwer & Werren, 1995, Perrot-Minnot et al., 1996). The bacterium *Arsenophonus nasoniae* produces all-female wasp broods: it prevents the production of maternal centromeres that are necessary for haploid male production (Ferree et al., 2008). These selfish genetic elements can therefore greatly influence the sex ratio of a brood laid by a mother.

Thesis aims

In this thesis I studied the links between sexual selection, sexual conflict, mating systems and sex allocation in the gregarious parasitoid wasp *Nasonia vitripennis*. My primary focus was sexual selection in this species, asking what makes a good son in *N. vitripennis*, and how does that impact on sex allocation?

Some of these associations have already been established. The link between mating system and sex allocation is well known for the gregarious parasitoid wasp *Nasonia vitripennis*. Hamilton's (1967 & 1979) Local Mate Competition describes how local mating leads to competition between a mother's sons for mates.

The link between sex allocation and sexual conflict has also been previously investigated in *Nasonia vitripennis*. As a result of their haplodiploidy, the optimum sex ratio for *Nasonia* mothers and fathers differs (Figure 1.2). The mothers are selected to follow the predictions of LMC, however the father should favour all the eggs to be fertilised with his sperm, leading to all-female broods irrespective of environmental conditions (Shuker et al., 2009). This leads to the prediction that male *N. vitripennis* will have adaptations to increase the fertilisation, and oviposition rate of their mates. Some evidence for such adaptations has been found as the sex ratio a female laid was partly attributable to the strain of her mate, however the proportion of variance explained was small (Shuker et al., 2006c).

Other forms of sexual conflict have been initially explored in the *Nasonia* genus, using comparisons of con- and heterospecific matings. The four species of *Nasonia* are cross-fertile once they are cured of endosymbionts such as the intra-cellular bacteria *Wolbachia* (Bordenstein et al., 2001). Geuverink et al., (2009) compared females from three of these species mated to con- and heterospecific males. For only one of the species testes (*N. giraulti)* the largely monandrous females were more likely to re-mate if they first mated a heterospecific male and were then presented with a con-specific male than any other combination. This may suggest that *N. giraulti* males are better at preventing re-mating in their con-specific females, than males of other *Nasonia* species. The extent to which this demonstrates sexual conflict is also unclear, as no cost to mating was established for the females.

In this thesis, I have built on these existing studies, to further understand the links between the four themes I have introduced: sex allocation, sexual selection, sexual conflict and the mating system. First I investigated the importance of mating system and sexual selection, through a competition experiment between sons to test how the relationship between body size and development time influenced fitness (Chapter 2). Next, I investigated the direct effects of sexual selection and sex allocation, by investigating the association between mate competitiveness and the single-foundress sex ratio (Chapter 3). I then extended these ideas by examining the among-strain variance in sperm resources and sperm-limitation (Chapter 4). Finally, I investigated the links between sexual conflict and mating system by screening variation in female re-mating rate, and looking for male-adaptations to reduce the re-mating propensity of their mates (Chapter 5). These studies, along with a review into sexual conflict in the Hymenoptera (Chapter 6), have linked together many of the relationships between sexual selection and sex allocation, through mating systems and sexual conflict.

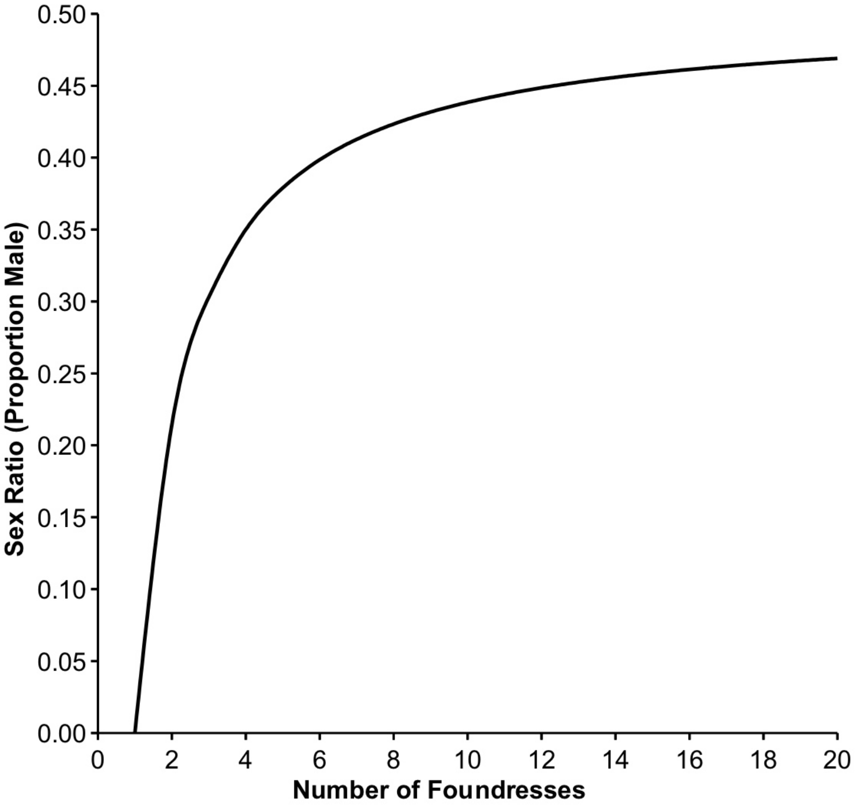
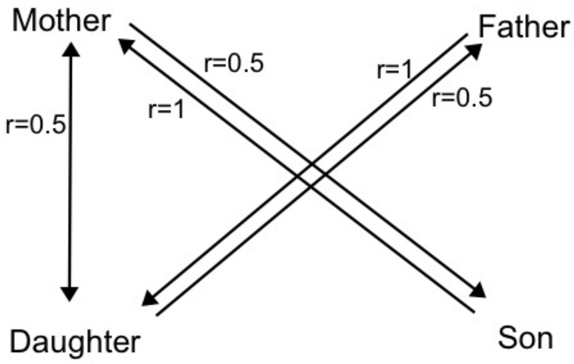


Figure 1.1: The relationship between sex ratio and foundress number under Local Mate Competition (LMC). As the number of foundresses increases, optimal sex ratio predicted by Local Mate Competition increases the proportion male. The line follows the equation for haplodiploids, $s^*=[(n-1)(2n-1)]/[n(4n-1)]$ where s^* is the optimal sex ratio, and n is the number of females ovipositing on the patch, called foundresses.



	Father	Mother	Son	Daughter	Brother	Sister
Female	0.5	0.5	0.5	0.5	0.25	0.75
Male	0	1	0	1	0.5	0.5

Figure 1.2: The relatedness asymmetries created by haplodiploidy. Females are diploid, developing from fertilised eggs. Males are haploid, developing from unfertilised eggs. Double-headed arrows indicate that the relatedness is equal in both directions. Where the relatedness differs from each parties stand-point, single-headed arrows are used. The table demonstrates the relatedness of siblings to each other also.

2. Sexual selection on male development time in the parasitoid wasp *Nasonia vitripennis*

This chapter can be found in Appendix 2 in its published form as: Moynihan, A.M. & Shuker D.M. (2011) Sexual selection on male development time in the parasitoid wasp *Nasonia vitripennis, Journal of Evolutionary Biology* **24**: 2002–2013

Abstract

Mating systems are shaped by a species' ecology, which sets the stage for sexual selection. Males of the gregarious parasitoid wasp *Nasonia vitripennis* compete to mate virgin females at the natal site, before females disperse. Males could increase their fitness by being larger and monopolising female emergence sites, or by emerging earlier pre-empting access to females. I consider sexual selection on male body size and development time in *Nasonia*, and a potential trade-off between the two traits. I explored sex-specific patterns of larval and pupal development, finding that smaller wasps developed slower than their host-mates. Using competition experiments between brothers I found that earlier eclosing males mated more females independently of absolute and relative body size. My data explain the lack of relationship between fitness and body size in male *Nasonia*, and reinforce the importance of protandry in mating systems where access to mates is time-limited.

Keywords: mating success, body size, development time, protandry, trade-off, sexual selection

Introduction

Sexual selection is expected to have acted on male traits that make them good at finding and inseminating females, and then having their sperm used to fertilise eggs (Darwin, 1871, Andersson, 1994). The way in which males achieve this fitness will be shaped by the mating system, which describes the spatial and temporal predictability of females and the extent to which males can gain or monopolise access to females (Emlen & Oring, 1977). If females can be defended, then sexual selection is predicted to have resulted in adaptations, such as weapons or large body size, which increase the chance that a particular male will be successful. If females cannot be defended, this will lead to a different suite of adaptations, to increase a male's chance of locating and courting a female (Andersson, 1994).

Females are easiest to defend if they are clustered together in space or time. If females are clustered together in time, then the life history and development times of males and females may be under sexual selection to ensure that individuals enter the mating population at the appropriate time. Development time is known to be an important component of fitness in many organisms, such as butterflies (Fagerström & Wiklund, 1982), damselflies (Plaistow & Siva-Jothy, 1999), mayflies (Rowe & Ludwig, 1991), mosquitoes (Holzapfel & Bradshaw, 2002) and salamanders (Semlitsch et al., 1988). The importance of development time to fitness is apparent in species where one sex is selected to be ready to mate before the other (Morbey & Ydenberg, 2001). Morbey & Ydenberg (2001) categorise the hypotheses to explain one sex's readiness to mate before the other into two groups (Table 2.1). First, there are those hypotheses in which selection influences the arrival time of each sex into the mating population, but not the relative arrival time of each sex (i.e. the difference between the sexes – the extent of protandry or protogyny – is an indirect outcome of selection). Second, there are those hypotheses that consider selection directly on the relative arrival times of each of the sexes to the mating population. For the latter, the earlier sex (usually males) either gains an advantage through increased mate opportunities, or the later sex minimises the time spent waiting for potential or suitable mates, or can chose a higher quality mate from those that have been in the mating pool for longer. The mating system of a species will play a key role in the selection for either protandry or protogyny, and the seven hypotheses need not be mutually exclusive.

Obtaining and monopolising mates when females are clustered spatially or temporally may therefore involve contest traits (such as body size) as well as developmental traits. Traits like body size and development time may well also trade-off with one another (Stearns, 1992), and it has been shown in a number of species that as development time becomes critical, for example as the end of the season approaches, individuals sacrifice the proposed benefits of a large body size in order to become reproductively mature sooner (model (Rowe & Ludwig, 1991), empirical tests (Semlitsch et al., 1988, Plaistow & Siva-Jothy, 1999)). Many of these studies assume that large body size confers fitness benefits to males and females: this has not always been found to be true when tested though (McLachlan & Allen, 1987, Klingenberg & Spence, 2003, Burton-Chellew et al., 2007b). In any case, the extent to which contest or development time traits are favoured by sexual selection is likely to depend on details of the ecology or mating system of any given species.

For the gregarious parasitoid wasp *Nasonia vitripennis*, the mating system is best described as a mix of scramble competition and defence of the female emergence site. Female *Nasonia* lay a large clutch of eggs on dipteran pupae within the host puparium. Once hatched, the larvae feed on the host tissues, before pupating and then eclosing into adults (Whiting, 1967). Males emerge from the host puparium first, waiting near the exit hole they chewed for newly-eclosed females to emerge; such defence tends to break down if many males are present, with males scrambling to find virgin females. If the patch contains multiple hosts, males may explore other hosts, and can detect if these hosts also contain female pupae soon to eclose (Shuker et al., 2005). As with many parasitoids, *N. vitripennis* has highly structured populations, with sib-mating commonly occurring at the natal site. This can result in so-called Local Mate Competition (LMC) between related males, which selects for female-biased sex ratios (Hamilton, 1967, Werren, 1983, West, 2009). Despite the female-biased sex ratios arising from LMC, males usually have at least one competitor in both the wild and the lab (Grillenberger et al., 2008). However, although we know a lot about some of the consequences of the mating system in parasitoid wasps like *Nasonia* in terms of things like sex ratio selection, we know rather less about the sexual selection that arises under such mating systems (Godfray, 1994).

This mating system combined with the mating behaviour of the wasps allows us to make predictions about the relative importance of body size and development time to male fitness. Most females emerge from the host puparium as virgins (99% Drapeau & Werren, 1999) and then males mount them and begin courtship; most females mate the first male they encounter, and become unreceptive to further matings for several hours (Shuker et al., 2007a, Leonard & Boake, 2008). On the rare occasions that polyandry does occur, there is first-male sperm precedence (Holmes, 1974, Leonard & Boake, 2008). After mating, females disperse away from the patch, to search for oviposition sites leaving the shorter-winged (brachypterous) males behind (King, 1993). The *N. vitripennis* mating system therefore appears to favour protandry in many aspects: access to females is temporally limited (Thornhill & Alcock, 1983) and females disperse after typically mating just once, with first-male sperm precedence (Holmes, 1974, Fagerström & Wiklund, 1982, Leonard & Boake, 2008).

Just as we expect mating system to shape selection on development time, it will also impact on other traits, including body size. The contribution of body size to fitness in this wasp has been investigated for both sexes. Bigger females have higher fecundity in *N. vitripennis* (van den Assem & Jachmann, 1982, West et al., 2001). For males, size could confer an advantage in intra-sex competition to control the emergence site. Anecdotally, the largest male *N. vitripennis* successfully defends the emergence hole in the host puparium and consequently monopolises the females emerging (van den Assem & Vernel, 1979). However, no size advantage has been found in competitive environments in previous experiments (Burton-Chellew et al., 2007a), although larger males have higher sperm resources on eclosion which could confer fitness advantages (Clark et al., 2010). In summary, both body size and development time could contribute towards male fitness in *N. vitripennis.*

In this study I did two things. First, I tested for a relationship between body size and development time in *N. vitripennis*. I performed three experiments in order to test this relationship at pupation, adult eclosion and emergence from the host puparium. The time spent feeding will be a crucial component to any body-size development-time relationship. Observing the timescale of pupation within the context of both eclosion and emergence from the puparium allowed us to see if larger individuals spent more time feeding, and if larval feeding time differed between the sexes. In many parasitoids, including *Nasonia*, there is evidence of adults spending time inside the host puparium and even mating before they emerge (Drapeau & Werren, 1999). To disentangle the relationships between body size and eclosion and emergence time, I opened hosts and removed the pupae to observe the eclosion times for natural clutches of wasps. Second, I tested the fitness benefits of being larger or emerging from the host earlier in a competitive mating situation that mimics the natural mating system. The mating system of *N. vitripennis* led us to predict that earlier males will have more matings than their later competitors, and that this will have a greater impact on fitness than differences in body size. If this is true, and there is a trade-off between body size and development time, I would expect large body size to be sacrificed for development time.

Materials and Methods

Wasps

The experiment used two lab strains of *Nasonia vitripennis*: AsymC and STDR-TET. Both strains are antibiotic-treated and free from *Wolbachia* and other endosymbionts. The strains differ in the allele at the eye-colour locus *S* within the *R-locus* (Whiting, 1961). The AsymC line is homozygous for a black, wild-type eye colour allele; these are referred to here as the wild-type wasps. The STDR-TET wasps are homozygous for a recessive scarlet eye colour allele at the same locus; these are referred to here as red-eye wasps. I kept the wasps under controlled conditions in incubators at 25°C and under a cycle of sixteen hours of light with eight hours of darkness throughout experiments and culture. Under these conditions the generation time is approximately 14 days (also see Results). In both experiments and culture conditions, the hosts used were the pupae of the large dipteran *Calliphora vicina*.

What is the relationship between body size and development time?

To control for maternal effects, I initiated grand-parental generations for my experiments. I took mated two-day-old females from stock tubes to be the grandmothers of the experimental generation and put them through a pre-treatment process. Females are synovigenic: they are born with some immature oocytes, and must host-feed to gain protein for egg maturation (Rivero & West, 2005). To allow this, I started the pre-treatment process with the females on a host for 24 hours. I then removed the host and gave the females a piece of filter paper soaked in honey-water for 24 hours, before a second host is provided for oviposition. The female had this host to oviposit on for 24 hours before she was removed and the parasitised host incubated.

Once the offspring of these grandmothers emerged from the hosts they were allowed to mate with their siblings for 24 hours. I used one mated female from each tube to be an experimental mother. I put these females through the same pre-treatment regime as the grandmothers with one difference: mothers had their second host to oviposit on for only six hours. All females received their host within 45 minutes, and were removed in the same order at the same rate. This short window ensured that any within-brood differences in development time of more than 6 hours cannot be attributed to differences in laying order alone. After oviposition, I removed the females and allocated the parasitised hosts randomly to an experiment and incubated them. In order to test the relationship between body size and development time, I assayed development at three transitions: larvae to pupae; pupae to adults; and adults leaving the host puparium. This allowed us to see whether relationships between development time and body size are due to differences in time spent feeding, pupating or leaving the host puparium.

Experiment One: Pupation time

To investigate the relationship between body size and the onset of pupation, I scored the numbers of wasp larvae and pupae within hosts across a series of time points. I opened 15 hosts of each strain per day from days three to six post-oviposition and counted the number of larvae. As the wasps began to pupate, I scored developmental stage more frequently: on day seven post-oviposition I scored 36 hosts over 3.5 hours, then after a two-hour gap, another 45 hosts were scored in a further 2.5 hour session. On day eight I scored 24 hosts over two hours. The sample size for each strain is therefore n=110, or n=115. Any pupae were carefully removed and kept at 25°C until they eclosed as adults. Individuals were considered pupae once legs and wings were clearly visible under a dissecting microscope, corresponding to the white pupa stage (*sensu* Whiting, 1967). A single observer (AMM) carried out this stage to standardise any subjectivity. Once individuals had eclosed as adults, I used an Olympus microscope and digital camera set-up, using the Olympus proprietary software Cell^D to measure the hind tibia length of the wasps to 0.0031746µm. Hind tibia length is a common measure of body size in parasitoids (Godfray, 1994). Male *N. vitripennis* are often smaller than their female conspecifics, but have longer tibia lengths due to differing allometries (Whiting, 1967, Sykes et al., 2007).

Experiment Two: Eclosion time

In order to observe eclosion, I removed the wasp pupae from their host. To control for any potential impact of this manipulation on development time, I opened the hosts at two points: the yellow eye stage on day nine post-oviposition, and the half-melanised stage on day eleven post-oviposition (Whiting, 1967). I counted any diapause or dead larvae in with the pupae and included this in the final clutch size. The 33 hosts that contained more than five diapausing larvae were excluded from the analysis, as their effect on the development of their siblings is unknown. After the pupae had been removed from the host, they were returned to a glass vial and kept as a sibling-group. I set up and observed n=72 replicates for each strain at each of the two pupal stages, at randomised positions within the incubator.

Adult wasps typically emerge around lights-on in the incubator (Bertossa et al., 2010), which was 07:00 hours. I commenced observations at 10:30 on day thirteen post-oviposition prior to what I expected to be the commencement of emergence on day fourteen. I then started observations at 05:00 hours on days fourteen and fifteen after oviposition. Observations continued until 15:00 hours, by which time very few wasps per hour were eclosing. A total of 26 scans were performed across all three days. The times between scans varied from 23 to 231 minutes, with a mean of 77.07 (SE = 13.06) minutes; this variation was due to differing numbers of tubes requiring handling in each scan, with a desire to maximise the number of scans performed. When adult wasps were found in a tube, I removed and kept them for measuring, recording the replicate number, date and time they were collected. I followed the tibia measuring procedure described for Experiment One. I also measured and recorded any individuals that did not eclose or emerge during the observation period (constituting a 27th time-point).

Experiment Three: Emergence from the host.

Emergence occurs when the adult wasps leave the host puparium, therefore in this experiment I did not manipulate the hosts after oviposition. For each strain I observed n=72 hosts. These hosts were observed, collected and measured under the same conditions, and in the same scans for adult wasps as Experiment Two. Again, the replicates were arranged in a random order in the incubator.

Is there sexual selection on body size and development time?

To test the prediction that males benefit from eclosing earlier and to assess the relative importance of body size and degree of protandry, I observed the eclosion time of groups of two virgin brothers together with ten virgin females and tracked the fitness of each male using a genetic eye-colour marker. For all experimental wasps I controlled maternal effects as before, giving the experimental mothers six hours to oviposit. In order to reduce variation between the competitor males due to factors other than body size and eclosion time (for example those differences in the genetic background of the STDR-TET and AsymC strains, or those from different host environments) I used brothers that differed by genetically-marked eye-colour. Using brothers also allowed me to observe natural, realistic levels of variation between competitors, who in the wild will most commonly be brothers (Grillenberger et al., 2008). To generate these males I mated red-eyed females to black-eye males, so their daughters are heterozygous for the black and scarlet alleles at the eye colour locus.

Half of these females' eggs will carry the scarlet allele and half the black allele due to the random assortment of chromosomes. *Nasonia,* like all Hymenoptera, are haplodiploid: males develop from unfertilised haploid eggs, and females from fertilised eggs. The male pupae in the resulting mixed-sex broods are the experimental males. These males shared a host environment, and have normal relatedness between brothers, but different eye colours. At day eleven post-oviposition, the eye-colour of the pupae can be discriminated. I therefore took one pair of brothers that differed from each other in eye colour from each host. The experimental females were red-eye pupae removed from their hosts on days eight and nine after oviposition, and kept in groups of virgin females with nine other non-sisters. Once all the pupae had been collected, the 60 replicates were assembled in 75 × 10mm glass tubes bunged with cotton wool, with each replicate containing two male pupae and ten female pupae. This set up is close to natural mating conditions: males have a competitor and two males with ten females gives a sex ratio close to those assayed under single-foundress conditions (0.163 proportion male Chapter 3).

I recorded the number of males and females eclosed in each of the tubes every hour for 28 hours. After 28 hours, I removed any individuals that had not yet eclosed and excluded any replicates which did not contain two males and at least eight eclosed females. The remaining (n = 56) replicates had 24 hours to mate. I then put each female on a host for 24 hours, to test which males had inseminated them. Because the scarlet eye colour allele is recessive to the black, the eye colour of the daughters indicates which males, if any, inseminated the female. The hind tibia length of all individuals was recorded using the method previously described for Experiment One.

The use of brothers proved successful in that I removed any strain effects found in the previous experiments, whilst retaining the two eye-colour phenotypes. There was no difference in body size between the eye-colours (mean red-eye male hind tibia length = 1913 microns (SE = 23.02), mean black-eye hind tibia length = 1930 microns (SE = 22.90)). In 19 of the 53 pairs of brothers both males eclosed in the same observation interval. Of the remaining pairs the black-eyed brother eclosed first 21 times and the red-eyed brother 13 times. The overall proportion of females sired by different eye-colours did differ: 39.3% of females laid only red-eye daughters, whereas 51.9% of females laid only black-eyed daughters with the remaining 8.8% of females laying daughters of both eye colours.

Statistical analysis

All statistics were performed in R (R Development Core Team, 2008, Ihaka & Gentelman, 1996). Full, unsimplified, models are reported throughout.

For Experiment One I wanted to analyse the relationship between body size and development. There was a negative relationship between clutch size and wasp body size for both sexes (LM: Male Tibia $t_{1,59} = -2.32$ $p = 0.02$; Female Tibia $t_{1,59} = -3.04$ $p = 0.004$), so to avoid problems associated with co-linearity I analysed body size and clutch size in separate models. I analysed the effect of clutch size and the time of observation on the proportion of the brood pupated, using a generalised linear model with a quasi-binomial error structure. The significance of each term was ascertained using a likelihood ratio test. Due to the destructive sampling for this experiment, I only knew the tibia lengths of those individuals that had already pupated by the given sampling point, and were measured after they had eclosed into adults days later. I therefore analysed the relationship between the body size of those that had pupated and the proportion of the brood that had pupated within each of the hosts. A generalised linear mixed effects model (GLMM) was used to investigate the relationship between pupation times and log tibia length, using only the 81 hosts that contained pupae, with Host ID fitted as a random effect. Sex was fitted as a fixed effect in this model, because males and females have different tibia lengths, and the allometry between body size and tibia length differs between the sexes.

There was also a relationship between clutch size and sex ratio (LM $t_{1,78} = -2.76$, $p = 0.00727$), so again I analysed sex ratio and development time separately. The relationship between proportion of the brood pupated and sex ratio appeared to be non-linear in data exploration, therefore I used a generalized additive model (GAM) with a quasi-binomial error family (Crawley, 2005). Sex ratio was a smoothed term, with strain as a parametric term.

For experiments two and three, the between-host comparisons of eclosion and emergence using clutch size were performed using survival analysis. A Mixed Effect Cox's Proportional Hazards (MECPH) test was used within the R package "coxme" with true time to eclosion of each individual as measured in minutes since midnight on 8th March 2009. Due to the co-linearity between body size, sex ratio of the brood and clutch size, I did not include sex as a fixed effect on eclosion or emergence time. MECPH was also used to analyse the relationship between the time to eclosion or emergence relative to the first eclosed/emerged individual in the host, and the body size and sex of the wasps, with Host fitted as a random effect.

For the competition experiment, I used a Generalised Linear Model (GLM) with quasi-binomial error structure to analyse the proportion of the females inseminated by the wild-type male as a measure of his mating success. This includes any females that were inseminated by both males (i.e. polyandrous females), and the total includes any females that laid no daughters and are presumed virgins. The difference between the hind-tibia length of the wild-type and red-eye males was used as the measure of relative body size in the full linear model. The limited variation in number of females available (8-10) was not associated with male success and not included in the analyses presented below. The significance of each term was tested using a Chi Squared test of the full model compared to a model with the term removed.

Results

What is the relationship between body size and development time?

Experiment One: Pupation time

I opened a total of 225 hosts, of which 81 contained at least one pupa. From those 81 hosts I scored a total of 1736 pupae and 654 larvae. The mean clutch size was 29.88 (SE = 0.77). The remaining 144 hosts contained only larvae, 21 of which were opened on days when other hosts did contain pupae. The time window between the first pupae and last larvae being observed was more than 25 hours, far longer than the six-hour oviposition window (i.e. variation beyond that which could be attributed to variation in time available for oviposition).

At a given point in time, a greater proportion of the brood from clutches with more individuals (i.e. larger clutch sizes) had pupated (Chi Squared test p = 0.005) and there was no effect of strain on this relationship (Chi Squared test p = 0.89). Larger individuals pupated first: I found that the body size of the measured individuals was greater when a smaller proportion of their brood had pupated (Figure 2.1 & Likelihood Ratio test, LR = 8.70, p = 0.013) when controlling for clutch size. There was no effect of strain on this relationship (LR = 2.66, p = 0.26), nor was there a significant interaction (LR = 0.80, p = 0.37). Males pupated before females since as the proportion of the brood that had pupated increased, so the sex ratio of those individuals that had pupated became less male-biased (Figure 2.2 & GAM smooth terms, Estimated d.f. = 1.54, F = 11.24, $p < 0.0001$) and there was no effect of strain (parametric coefficient $t_{1,80} = -1.49$, $p = 0.14$).

Experiment Two: Eclosion time

Over three days I observed a total of 255 hosts, which produced a mean of 27.4 (SE = 0.5) adult wasps each. Of the broods in which all individuals eclosed within the observed period, the mean range of eclosion time was 17 hours 38 minutes. In 44 hosts the entire brood eclosed between two observation points. The least synchronous host took 46 hours and 21 minutes for all individuals to eclose. Thirty wasps eclosed after observations were completed, and these were entered as censored data into the analysis.

Wasps from larger clutch sizes eclosed sooner (MECPH Z = 3.73, $p < 0.0001$). The wild-type wasps eclosed later than the red-eye broods (Z = -7.26, $p < 0.0001$), and broods that were removed from the host on day 9 post-oviposition eclosed later than those removed on day 11 post-oviposition (Z = -5.28, $p < 0.0001$). This shows that opening the host earlier causes the whole brood to decelerate pupation somewhat. The clutch size effect was exaggerated for wild-type wasps (Z = 3.92, $p < 0.0001$).

Within hosts, larger individuals of both sexes eclosed earlier (MECPH Z = 13.26, $p < 0.0001$). Males were protandrous, eclosing earlier than females controlling for tibia length (Z = 4.57 $p < 0.0001$). Males eclosed within a smaller time window than females (Figure 2.3 & 2.4), and there was no significant interaction between body size and sex on eclosion time (Z = -0.57, *p* = 0.64). The bi-modal pattern of eclosion observed (Figure 2.4) is likely to be a result of eclosion peaking around lights-on in the incubator. Strain and the stage at which the wasp pupae were isolated had no significant effect on eclosion time within broods (Strain Z = 1.53, *p* = 0.13; Treatment Z = 0.70, *p* = 0.48).

Experiment Three: Emergence from the host

A total of 144 hosts were observed, of which 134 contained wasps, producing a mean of 28.13 (SE = 0.89) adult wasps each. When I opened the host puparia after the observation period had ended, I found 83 wasps that had not emerged and these were again included as censored data. Of those broods from which all individuals emerged within the observation period, the maximum range of emergence time was 39 hours, 31 minutes. Two broods emerged entirely between two observation points. The mean range of emergence times was eleven hours and nineteen minutes (Figure 2.3).

Individuals from larger clutches emerged sooner (MECPH Z = 4.57, *p* < 0.0001). There is no effect of strain (Z = -1.255, *p* = 0.25), nor an interaction of clutch size with strain (Z = -1.31, *p* = 0.19). Larger individuals of both sexes also emerged sooner (Z = 8.49, *p* < 0.0001). Males of a given size were protandrous, emerging before females (Z = 3.48, *p* = 0.0005), and there was no difference between the strains in emergence time (Z = -1.23, $p = 0.22$). The relationship between body size and emergence time did not differ between the sexes (Z = -1.89, $p = 0.058$).

Is there sexual selection on body size and development time?

The earlier a male eclosed relative to his competitor the higher his mating success (Table 2.2 and Figure 2.5). The mating success of a male was not affected by the eclosion time of the females (Table 2.2). The difference in male size did not predict male fitness in this competitive environment (Table 2.2). This finding is robust: if I remove the minutes until the red-eye competitor male ecloses from the model, none of the effects are significant. Also, if I used the actual tibia lengths of both males instead of the difference, there was still no influence of body size on mating success (GLM $F_{2,10.5} = 0.77$, $p = 0.46$). If I just fit the difference in tibia to the proportion of females inseminated, I find that body size still has no significant effect (GLM, $F_{1,13.9} = 0.58$, $p = 0.45$). I also found no significant quadratic term for the relative eclosion time squared ($F_{1,10.34} = 1.57$, $p = 0.22$) or the difference in body size ($F_{1,10.08} = 0.19$, $p = 0.66$) on male mating success. A wild-type male inseminated 55.7% of the females that laid daughters.

Discussion

Highly structured populations in which mating and competition for mates commonly occurs between kin have been studied in detail in terms of traits such as sex allocation, but much less so in terms of the sexual selection arising from this competition (Hardy et al., 2005b, Hardy et al., 2005a). In *Nasonia vitripennis*, mating populations are not only highly spatially structured, but reproductively receptive females are also extremely clumped temporally, following emergence from their host. Using an experiment that mimics these mating conditions, I have found that the time a male enters the mating population is under sexual selection, resulting in selection for protandry (Table 2.2, Figure 2.5). I propose that male *N. vitripennis* prioritise early arrival to the mating pool over body size, beginning pupation as soon as their host-mates do. The contribution of protandry to male fitness has been under-appreciated in previous parasitoid studies, where body size has been the focus (e.g. Burton-Chellew et al., 2007b, & Reece et al., 2007). An exception to this is the parasitoid wasp *Melittobia acasta*, where eclosion order predicts winning the lethal male-male fights typical of this species to a greater extent than being the larger male (Innocent et al., 2007). When males were age-matched post-eclosion, size played a more important role (Innocent et al., 2007). My study is perhaps the first to clarify that it is the early development, not the body size, that confers the competitive advantage to males, when males emerge both earlier and larger under controlled laboratory conditions.

In theoretical models, reduced male dispersal and female monandry, such as we see in *N. vitripennis,* contributes to the evolution of protandry (Fagerström & Wiklund, 1982). Males can benefit from protandry through many routes (Morbey & Ydenberg, 2001); the relative weight of these benefits will be a product of the mating system.

As discussed above, these benefits fall into two broad groups: those where the arrival of each sex into the breeding pool is under selection independently, and those where the arrival time of one sex relative to the other is under selection. Of the seven hypotheses reviewed by Morbey and Ydenberg (2001), my experiment is less able to explore the effects of relative emergence times between the two sexes central to hypotheses 1-4 (Table 2.1), because I restricted female dispersal. In my experiment, once females had eclosed they spent an average of 35.5 hours with males, which is much higher than I would expect from an observed dispersal rate of one female every 180 seconds from more realistic artificial patches (C.J. Doughty and D.M. Shuker, unpublished observations). This restriction would have removed any disadvantage to males eclosing later than females; the females were unable to disperse un-mated, and as such I found no effect of female eclosion time on male fitness (Table 2.2). Although I have not considered these possibilities here, there is evidence that such direct selection on the relative arrival time of the sexes occurs in species of migratory birds (Coppack et al., 2006).

That said, of the direct selection hypotheses I can perhaps rule out three of them for other reasons. Since *N. vitripennis* commonly mates with siblings as a result of LMC (Grillenberger et al., 2008), in-breeding avoidance (H4, Table 2.1) is an unlikely hypothesis to explain protandry. Mate-choice (H3, Table 2.1) is another unlikely explanation of protandry in *N. vitripennis* as females usually mate with the first male they encounter (Chapter 5), although there is some suggestion of assortative mating by eye-colour (Figueredo & Sage, 2007). Waiting costs for females (H2, Table 2.1) are also unlikely to explain protandry, not least as female *Nasonia* do not need to spend time in a comparatively risky breeding environment in order to gain a mating, as they can gain some fitness as virgins (Steiner & Ruther, 2009a). Also some females eclose before males (Figure 2.3). The mating opportunity hypothesis (H1, Table 2.1) proposes that protandry allows males to maximise their mating opportunity and this is consistent with the mating system of *N. vitripennis*, as earlier males will have more females to mate with. Evidence for the mate opportunity hypothesis has been found in other species, including Waved Albatross (*Phoebastria irrorata*) (Huyvaert et al., 2006).

Of the three remaining (indirect selection) hypotheses, the constraint hypothesis (H7, Table 2.1) can be discounted. If this were explaining protandry in *N. vitripennis* I would expect to have found that later eclosing females were larger, however the opposite relationship was found. The susceptibility hypothesis (H6, Table 2.1) is harder to reject, but is not compelling either: female *Nasonia* disperse soon after their first mating, and so poor conditions in the mating pool are unlikely to influence emergence timing. The rank advantage hypothesis (H5, Table 2.1), which has had the greatest support from migratory bird studies (Kokko et al., 2006a, Sergio et al., 2007), is the most supported by the data presented here. The earlier males will win contests for the best territories. Males emerge and defend a hole in the host puparium, with territory "quality" being determined by gaining access to the earliest females that eclose. Earlier females of *N. vitripennis* and other parasitoid wasps are typically larger and have more oocytes, and therefore represent a more valuable resource for males (van den Assem & Jachmann, 1982, West et al., 2001). Moreover, the first male has sperm precedence on the rare occasions when females mate more than once (Holmes, 1974, Leonard & Boake, 2008), compounding the advantages for an early male. Mechanistically, early-eclosing males will be fully sclerotised with a full complement of pheromones to attract females before they appear (Ruther et al., 2009). The male's rectally-produced female-attract and -arrest pheromone is likely to be important in mate-acquisition (Steiner & Ruther, 2009b), and an earlier male could potentially have more opportunities to deposit pheromone and attract females. Protandry in *N. vitripennis* is therefore favoured by both mate opportunities (H1) and rank advantage (H5) (Table 2.1).

While my data indicate a larger role of development time than previously appreciated, selection is still likely to have influenced male body size. My assay detected no advantage to larger body size within the naturally generated range of sizes observed (Table 2.2), consistent with previous studies (Burton-Chellew et al., 2007b). Selection on male body size may therefore be stabilising, with a lower limit due to the need to produce enough sperm, and few benefits to being very large. Sperm depletion is potentially an important constraint for *Nasonia* male fitness as they do not produce spermatozoa as adults (Hogge & King, 1975, Clark et al., 2010), and can have very high numbers of females available to mate with in the wild (Werren, 1983, Grillenberger et al., 2008). This can be problematic for females too because males could continue to mate after they are sperm depleted, as is the case for the parasitoid *Trichogramma evanescens* (Damiens & Boivin, 2006). Whilst mated females with little or no sperm can gain some fitness in haplodiploids, sperm limitation is nonetheless costly. Male body size may also be influenced by selection on female body size through correlated selection (Lande & Arnold, 1983). Males have a smaller body size overall compared to females, but they can overlap to some extent. The dimorphism between male and female *N. vitripennis* is otherwise large: females are fully-winged so they can disperse to find new hosts, whilst males are brachypterous with vestigial wings that prevent flight (Whiting, 1967). This sexual dimorphism, with different allometries for different body parts, suggests that males and females have evolved in response to different selective pressures on components of body size.

I found a negative relationship between body size and time spent in development for both sexes. This is contrary to the findings of two other parasitoid studies: larger individuals take longer to develop for *Microplitis demolitor* and *Apanteles carpatus* (Harvey et al., 2000) and *Muscidifurax raptorellus* (Harvey & Gols, 1998). However, in other invertebrate orders, a negative relationship like that found here for *N. vitripennis,* has been observed (Gebhardt & Stearns, 1993, Blanckenhorn & Fairbairn, 1995, Castillo & Nunez-Farfan, 1999, Maklakov et al., 2004). Larger individuals were developing faster at all three of the transitions I measured: larvae to pupae, pupae to eclosed adult, and the emergence of adults from the host. This shows that the relationship I have observed here starts with differences in time spent feeding, and then follows through the rest of the developmental schedule.

While my data could support the idea that the body size-development time relationship is under selection in *N. vitripennis,* I cannot rule out several alternative explanations. Differences in individual- or resource-quality could generate such a negative relationship between body size and time spent in development. It has been shown that differences in resource availability during development can result in an absence of predicted trade-offs between life-history traits (van Noordwijk & de Jong, 1986). Such differences in resource quality can arise through several routes. For instance, differing growth rates continuing through size-triggered moult-period results in larger individuals reaching the final instar sooner (Klingenberg & Spence, 2003). Alternatively the resources available for growth can differ: for parasitoids clutch sizes can differ on equal-sized hosts due to differences in the mother's body size and egg availability, or the presence of a previous female's eggs (Hardy et al., 1992, Flanagan et al., 1998). Indeed when we look at relationships between broods I found that individuals from larger clutches are both smaller in body size, and the whole clutch develops faster than smaller broods. This is in stark contrast to the within-clutch relationships where individuals are sharing resources and I found that the larger individuals are the faster developers.

These mechanisms for creating negative relationships between life-history traits might explain the disagreement between my study and those from other parasitoid studies. I propose that small *N. vitripennis* males sacrifice the greater sperm reserves associated with larger body size (Clark et al., 2010), in order to arrive in the mating pool at the same time as their competitors, because early development confers more advantages in competition between host-mates than body size (Table 2.2). The aforementioned *Microplitis demolitor* and *Apanteles carpatus* study (Harvey et al., 2000) looked at solitary parasitoids: such individuals do not share host resources hence I would not expect males to trade-off body size in favour of development time as there are no males to compete against in the host patch. Similarly, the between-host comparisons of Harvey & Gols (1998) with *Muscidifurax raptorellus* would also not show such a trade-off.

Two other studies have measured protandry, body size and fitness. In the grasshopper *Sphenarium purpurascens*, there was a negative relationship between body size and becoming an adult (Castillo & Nunez-Farfan, 1999). For the grasshopper, male fitness depended on both early arrival and large body size, leading Castillo and Nunez-Farfan to suggest that the correlation between development time and body size is under selection. For the spider *Stegodyphus lineatus* larger males were found to reach maturity later: the opposite relationship to that found here in *Nasonia* (Maklakov et al., 2004). *Stegodyphus lineatus* males benefited from early maturity as virgin females are less choosy than previously mated females. Mated females then choose larger males for second mates, leading to benefits in being large. Maklakov et al., (2004) suggest that being early is most important, and if a male cannot be early, he should at least be large to gain fitness as a second male. The spider and grasshopper studies assayed the body size-development time relationship under ambient conditions and as such they could not control for lay-date or temperature effects, and consequently could not rule-out artefacts of different growth-rates explaining the relationships they observe between body size and development time, unlike my study under laboratory-conditions. These two previous studies, and my work here with *N. vitripennis*, represent different outcomes of selection acting on the links between body size, time taken to develop and fitness for males. The spiders either develop early or compensate by spending longer in developing to become larger, whereas the negative relationship between body size and development time I found in *Nasonia* rules out this sort of compensation that could have masked body-size benefits in previous studies that did not control for age. The grasshoppers also appear to be selected to be early and large.

Finally, if development time is so crucial for male fitness, can larvae alter their developmental schedules depending on the developmental stages of other larvae in the host puparium? Although we do not know the mechanism, there is some evidence that individuals can modulate their developmental rate. When more than one female parasitises a host (superparasitism), if the second brood is laid within 48 hours of the first, the two broods will eclose more or less synchronously (Werren, 1980). The cuticular hydrocarbon profile of a wasp (*Vespula germanica*) has been shown to change on pupation (Brown et al., 1991) suggesting a possible mechanism for detection. Experiments that manipulate larval developmental plasticity, and explore how it is controlled, are therefore an obvious next step.

In summary, my experiments have shown that within the size range tested, development time is more important to male fitness than body size. My study highlights the importance of considering details of the mating system when investigating the effects of sexual selection: despite a number of previous experiments focusing on body size in parasitoids, it has proved to be less important than development time when variation in an important aspect of the mating system, protandry, varies among males. As such, I suggest that development time rather than body size is under sexual selection in *Nasonia vitripennis* males. Moreover, male mating behaviour and the selection arising from variation in that behaviour also influences sex allocation in species such as *Nasonia*. When LMC is extreme (i.e. females lay eggs alone) the number of males produced is expected to be influenced by the ability of a given male to fertilise a given number of its sisters before they disperse (Godfray, 1994). However, selection for success in competitive mate acquisition may not be the same as selection for efficient mating of females when mate competition is reduced (under female-biased sex ratios). The extent to which sexual selection and sex allocation therefore interact in *Nasonia* is currently being explored.

Table 2.1: The seven hypotheses for the evolution of protandry reviewed in Morbey & Ydenberg, (2001). The hypotheses are grouped in terms of direct or indirect selection on the difference between the sexes in development time.

Hypothesis		Direct/Indirect	Summary
1	Mate Opportunity	Direct	Direct selection on the relative arrival of the sexes to maximise the mating opportunities of the earlier sex
2	Waiting cost	Direct	Selection on the relative arrival of the sexes in order to minimise the time spent un-mated by the later sex.
3	Mate choice	Direct	Selection on the choosing sex to arrive later in order to have a wider pool to chose from.
4	Out-breeding	Direct	Selection on the relative arrival of the sexes so that related individuals avoid mating.
5	Rank Advantage	Indirect	Selection on the territorial sex to be earlier than their competitors.
6	Susceptibility	Indirect	Selection on the later sex to avoid risky conditions they would be exposed to earlier, for example bad conditions in the mating area.
7	Constraint	Indirect	Stronger selection on a trait correlating with late arrival in one sex, such as prolonged feeding for larger body size.

Table 2.2: The relationship between wild-type male success, body size and eclosion time. The full model is shown here. The significance of each term was calculated using model comparison

	Coefficient	Standard Error	*p*
Intercept	6.659	5.235	
Minutes until the red-eye male ecloses	1.752×10^{-03}	5.905×10^{-04}	0.001829
Minutes until first female ecloses	-2.655×10^{-05}	8.220×10^{-04}	0.9742
Minutes until the last female ecloses	1.232×10^{-04}	8.684×10^{-04}	0.8799
Minutes until median female ecloses	-1.726×10^{-04}	8.461×10^{-04}	0.8383
Mean Female Tibia Length	-1.236×10^{-04}	8.688×10^{-05}	0.1900
Difference in tibia length between the black and red eye male	1.709×10^{-05}	1.977×10^{-05}	0.3865

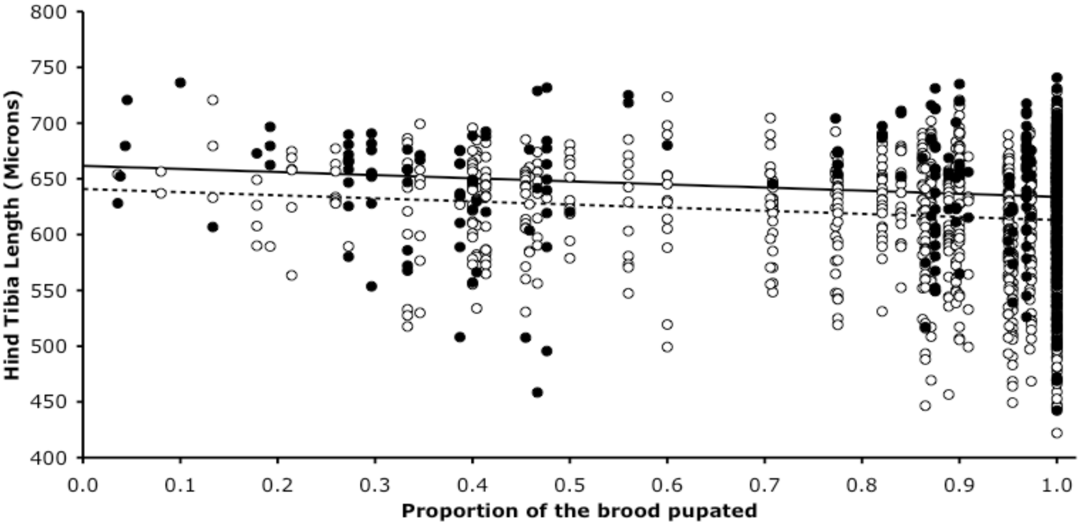


Figure 2.1: Larger individuals pupate earlier generating a negative relationship between body size (measured as hind tibia length) and proportion of the brood pupated when sampled. Strains are combined. Females are represented by open circles, and males by closed circles. The lines shown are the model fits. The fitted line for males is solid, and the fitted line for female is dashed (Male Tibia Length = 661.6462 + (Proportion pupated × -27.8158; Female Tibia Length = 640.8391 + (Proportion pupated × -27.8158)).

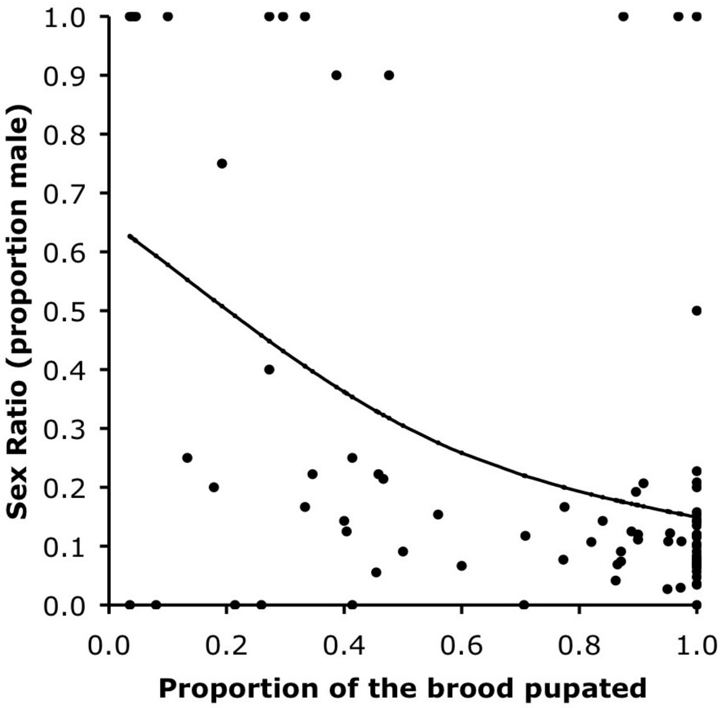


Figure 2.2: Male *Nasonia vitripennis* pupate before females. As the proportion of the brood pupated increased, the sex ratio (measured as proportion male) of those that have pupated becomes less male-biased. The line shown is the model fit from the general additive model (GAM) with the strains pooled. Note that complete brood sex ratios are female-biased.

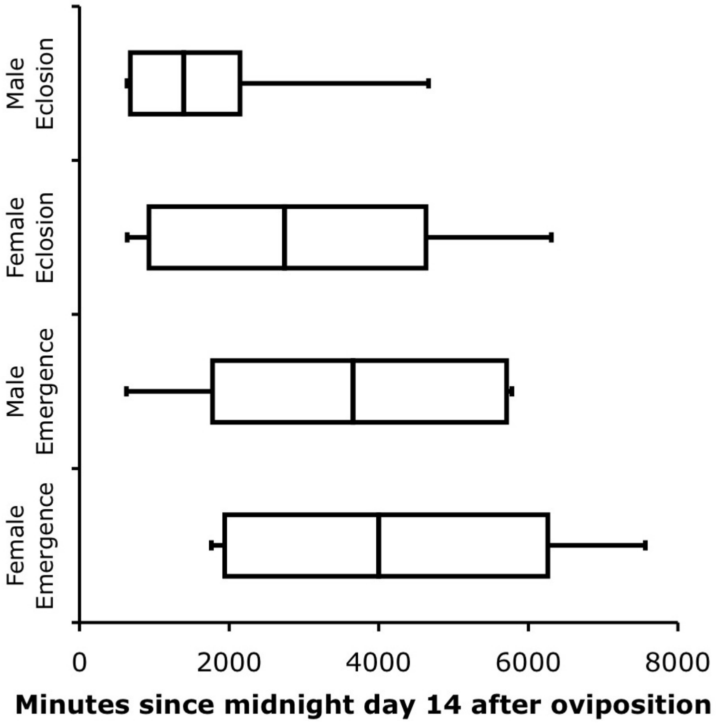


Figure 2.3: Box and whisker plot showing the pattern of eclosion and emergence from the host puparium over true time. The box shows the range of the first and third quartile, with the median marked as a bar. The ends of the whiskers represent the full range of the observations.

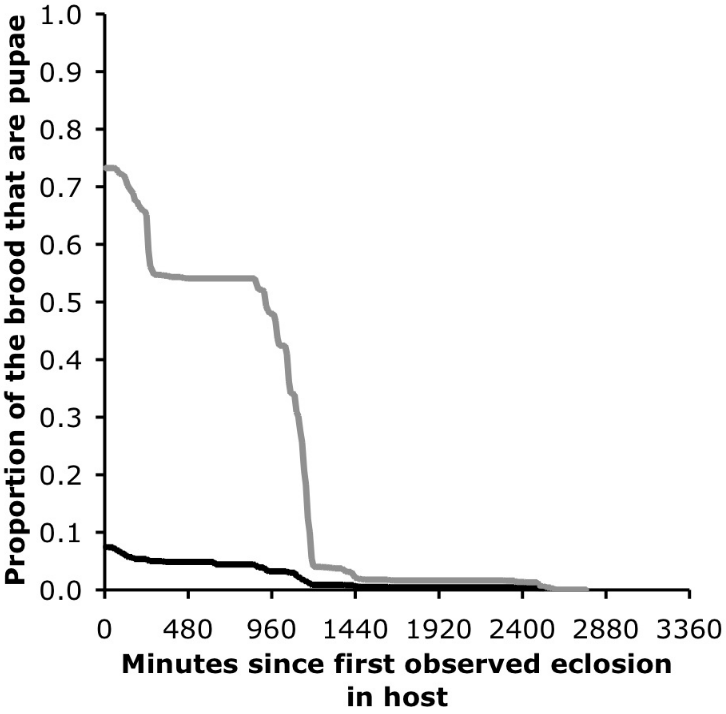


Figure 2.4: Male *Nasonia vitripennis* eclose before females. The black line represents male eclosion (in terms of proportion still pupae), and the grey line female eclosion. Time is measured as the minutes since the first observation of an experimental host.

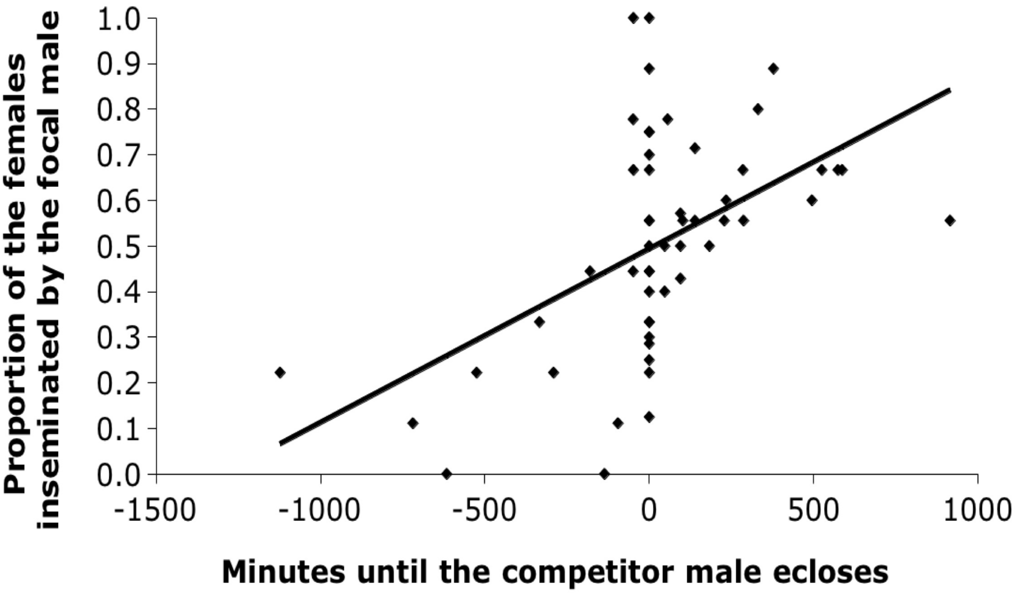


Figure 2.5: Protandry is under sexual selection in *Nasonia vitripennis*. There is a positive relationship between the difference in emergence time of focal and competitor males and the mating success of the focal (wild-type) male. The line shown is the model fit from a GLM with a quasi-binomial structure (Proportion of females inseminated by the focal male = 0.4937+0.0003808 × Minutes until competitor male ecloses).

3. Sex allocation and the competitiveness of sons for mates

A version of this chapter is being prepared for submission to Behavioural Ecology. The authors will be: Anna M. Moynihan, Stephen A. Spencer, Taehyun J. Park, Christopher J. Doughty, David M. Shuker.

SAS and TJP were undergraduate students who assisted helped perform and collect the data for experiment four. CJD was an undergraduate who designed and performed experiment two.

Abstract

Hamilton's theory of Local Mate Competition predicts females ovipositing alone on a resource patch (termed single-foundresses) will lay broods consisting of only daughters. We call this prediction “Hamilton's zero”. This has been interpreted as selecting for the production of the minimum number of sons required, after allowing for developmental mortality and the ability of sons to successfully transfer sufficient sperm and so females usually lay several sons. If this were true, we would expect a negative correlation between the competitiveness of sons for mates (including their insemination capacity) and the sex ratio (proportion male) of the brood a female produces. I tested this in the gregarious parasitoid wasp *Nasonia vitripennis*, using 18 iso-female lines from the same population. I first established among-line variation in single-foundress sex ratios. I then found no evidence that sons from low sex ratio strains were better inseminators when alone or in competition, contradicting my prediction. Instead, sons from high proportion male strains were better competitors. My findings question the current interpretation of Hamilton's zero and suggest under-appreciated links between sex allocation and sexual selection.

Keywords: Local mate competition, fertility insurance, sexual selection, single-foundress sex ratio, insemination capacity.

Introduction

The amount of investment parents allocate to offspring of each sex (sex allocation) has profound effects on the biology of a species. For many species the sex ratio at the brood- and population-levels coincide at the equal sex ratio of 0.5 proportion male. There are however, many scenarios in which individuals may be selected to make facultative changes to the sex ratio of the offspring they produce, which may lead to biased primary sex ratios at fertilisation (West, 2009). Such biased sex allocation can lead to uneven relative abundances of each sex at the time of mating (operational sex ratio, OSR: Emlen, 1976) (Kokko & Jennions, 2008). The OSR is one of many indicators of the direction and intensity of sexual selection in a species (see also Clutton-Brock & Vincent, 1991, Ahnesjö et al., 2001, Parker & Simmons, 1996, Shuster & Wade, 2003, Arnold & Duvall, 1994). Indeed Kokko & Jennions (2008) showed that the OSR plays a key role on the evolution of sex roles and hence sexual selection. Experimental studies have also demonstrated the importance of OSR. In some cases biasing the OSR towards the choosing sex increases the intensity of sexual selection on the competing sex as predicted: for example female-choice in guppies (Jirotkul, 2000) and male choice in sex-role reversed pipefish (Berglund, 1994). However, this is not always the case: a decrease in the intensity of sexual selection at biased OSR has been observed (e.g. the lizard *Lacerta vivipara* (Fitze & Le Galliard, 2008); the fish *Rhodeus sericeus* (Mills & Reynolds, 2003)). Either way, the importance of sex allocation, through the operational sex ratio, on sexual selection is clear.

Aside from the influence of sex allocation on sexual selection mediated by OSR, the interaction between sex allocation and sexual selection is most frequently studied in the context of Trivers-Willard effects. Trivers and Willard (1973) proposed that when mothers are in good condition, they should bias sex allocation in favour of the sex whose fitness depends most heavily on condition, and this has been observed in many ungulate species (Hewison & Gaillard, 1999, Sheldon & West, 2004). In order for a Trivers-Willard sex allocation allele to invade, there must be a strong genetic covariance between the degree of mating bias of sons and the sex allocation allele across all environments (Wade et al., 2003). The sex allocation therefore relies on a large skew in mating success across males, which causes strong sexual selection.

In this study I investigated the interaction between sexual selection and Local Mate Competition (LMC), another set of conditions that may select for facultative sex allocation. Hamilton (1967 & 1979) described how competition for mates between brothers in a structured population can select for female-biased sex allocation. As the number of other mothers laying eggs on that resource patch (foundresses) increases, the sex allocation favoured by LMC become less female-biased (Figure 1.1). This is favoured by the inclusive fitness benefits that arise from the reduction of competition between sons for mates and the increase in the number of sisters for them to mate (Taylor, 1981).

Hamilton (1967) modelled the precise way in which the unbeatable sex ratio for diploids (s*) would vary with the number of foundresses (n), such that s*=(n-1)/2n (Figure 1.1). This model predicts that when there is a single foundress (n=1), the optimal sex ratio is 0 proportion male. This zero (i.e. all female) sex ratio is a mathematical outcome of the model that has limited biological applicability, as typically at least one son is needed in order to inseminate the female brood. Instead, this zero sex ratio, which I term here "Hamilton's zero", is usually interpreted as representing the minimum number of males required to successfully inseminate all the females on the patch, under extreme LMC where females are exclusively at the natal patch (Godfray, 1994, Waage & Sook Ming, 1984, Hardy & Cook, 1995, Heimpel, 1994). This overproduction of sons to ensure that all a mother's daughters are inseminated is known as fertility insurance, and is thought of mainly as a combination of developmental mortality and limited insemination capacity (Dijkstra, 1986, Hardy & Cook, 1995). Sex-specific developmental mortality has been demonstrated to influence the Evolutionary Stable Strategy single-foundress sex ratio for parasitoid wasps (Green et al., 1982, Hartl, 1971, Hardy, 1994, Hardy & Cook, 1995) and fig wasps (West & Herre, 1998b). If the probability of a son dying before adulthood is high, his mother should lay extra sons to make sure that enough survive to adulthood to inseminate all the daughters. Fertility insurance is not limited to single foundress situations however; it has been suggested to explain higher than initially predicted sex ratios in a range of organisms, including *Plasmodium* parasites (Gardner et al., 2003, West et al., 2002) and fig wasps (West & Herre, 1998b).

The logic underpinning Hamilton's zero suggests that the competitiveness of sons for mates, which is under sexual selection, will be correlated with single-foundress sex ratio. In other words, the better able a son is to inseminate his sisters before they disperse, the fewer sons his mother should produce, leading to a genetic correlation between male inseminating capacity and sex ratio. If this were true, I would expect males from genetic backgrounds producing high sex ratios to be worse at mating and inseminating their sisters efficiently. In some gregarious parasitoid species, one male is sufficient to inseminate all his sisters (*Goniozus legneri* (Hardy et al., 2000); *Goniozus nephantidis* (Hardy et al., 1999)), however in other species insemination capacity is lower than the typical number of females in a brood (Table 3.1).

My study species is the gregarious parasitoid wasp *Nasonia vitripennis,* whose sex allocation behaviour under LMC has been well-studied (e.g. Burton-Chellew et al., 2008, Flanagan et al., 1998, Shuker et al., 2005, Shuker & West, 2004, Werren, 1980, Werren, 1983). Single-foundress sex ratios have been assayed numerous times in the lab and the wild, and multiple males are usually produced in single-foundress broods (Table 3.2). Females most commonly oviposit alone in the wild (Molbo & Parker Jr, 1996, Grillenberger et al., 2008), therefore single-foundress sex ratios are expected to be under the strongest selection (West & Herre, 1998b). Given heritable variation in single-foundress sex ratios in *N. vitripennis* (Orzack & Gladstone, 1994, Parker Jr & Orzack, 1985, Pannebakker et al., 2011), we would expect that the single-foundress sex ratio is under natural selection.

The mating capacity of *N. vitripennis* males has been tested before (Table 3.1: Grant et al., 1980), however just one strain was used. Here, I test the predicted relationship between mate competitiveness and single-foundress sex ratio across eighteen strains of *N. vitripennis* collected as iso-female lines from the same population. As such, my study is the first to look at within-population variation. In my first experiment I screened the eighteen strains for their single-foundress sex ratios. For two of the strains I made behavioural observations to describe mate acquisition and processing in more detail when males are alone and in competition. Next I looked at the successful insemination of females by males from each of these strains when males were either alone, or in competition.

Materials and Methods

Wasps and experimental conditions

Nasonia vitripennis is a gregarious parasitoid wasp that lays its eggs on large dipteran pupae. These experiments used eighteen strains that originated as iso-female lines collected from the same population in Cornell, New York, USA by Prof Leo Beukeboom in 2007. These eighteen strains will here be referred to as the Cornell strains. Throughout culture and experiments, I kept the wasps in incubators at 25°C under 16h-8h light-dark cycle and reared them on the pupae of *Calliphora vicina* as hosts. In order to mitigate against any unknown host or maternal effects, and to allow host-feeding to mature eggs (Rivero & West, 2005), I routinely pre-treated females before experiments. Pre-treatment started with isolating mated females and giving them each a host for 24 hours on which to feed. I then removed this host and replaced it with a piece of honey-water-soaked filter paper for a further 24 hours. Finally the female was given a host on which to oviposit for a maximum of 24 hours before I removed the female and incubated the host. Vial positions were always fully randomised inside the incubator.

Like all Hymenoptera *Nasonia vitripennis* are haplodiploid. Daughters are diploid and develop from a fertilised egg, whereas sons are haploid and develop from an unfertilised egg. This genetic system is useful because it allows us to distinguish between mated and un-mated females based on the sex ratio of their brood: all-male broods are laid by virgins, and the presence at least one daughter means the mother was inseminated.

In addition to the focal Cornell strains, for the competition experiment and behavioural observations I also used genetically-marked wasps from the STDR-TET strain. The STDR-TET strain is homozygous for the scarlet allele at the eye colour locus *S* within the *R*-locus, which is recessive to the wild-type black-eye allele (Whiting, 1961), giving the wasps red eyes. I refer to the STDR-TET as red-eye wasps from here. The Cornell strains are homozygous for the dominant black-eye allele at the *S* locus. The red-eye individuals are also cured of *Wolbachia* infection by tetracycline treatment (J.H. Werren personal communication). *Wolbachia* is an endosymbiotic bacterium common to many insects. In *Nasonia*, if eggs do not contain *Wolbachia* they can only be successfully inseminated by sperm that are also free from *Wolbachia* (Breeuwer & Werren, 1995). The wild-type Cornell strains do have *Wolbachia* infections. The result of a Cornell male mating with an STDR-TET female is therefore a brood entirely consisting of red-eye males. I used this fact to assign parentage in the mating competition experiment. If the *Wolbachia* marker were to fail, black-eye daughters would be produced, providing a second level of assurance when scoring paternity (Table 3.3).

The mating behaviour of *N. vitripennis* has also been described (Barrass, 1961). Males approach and mount females before beginning a series of stereotyped head nod cycles. On the first nod of each cycle he emits a pheromone from his mouth-parts (van den Assem et al., 1980). The female signals receptivity to the male by lowering her antennae and bending her abdomen to expose her genital pore. On receiving this signal, the male backs up the female, makes genital contact and the pair copulate. The male performs a short bout of head nod cycles again after copulation (post-copulatory courtship) then dismounts. Females disperse soon after mating, leaving the wingless males behind.

Experiment One: Single-foundress sex ratio screen

In this experiment I allow females to oviposit alone in order to look for variation in single-foundress sex ratio between the eighteen Cornell strains.

Wasps

This experiment used all eighteen Cornell strains described above, with the experimental grandmothers coming from the seventh generation reared in the laboratory. In order to generate the animals for the experiment I took 30 mated females from stock tubes of each of the eighteen Cornell strains and pre-treated them as described above in glass vials that are 75 × 10mm. I gave these females two hosts on which to oviposit in order to generate experimental females. I allowed the resulting offspring to mate and then isolated a single daughter from each vial for the test-oviposition.

Test oviposition

I pre-treated the mated experimental females as described above, however on the day of test oviposition two hours after a single host was provided I added escape tubes to the vials. The escape tubes allowed the female to disperse from the host patch and limited superparasitism (Werren, 1983, Shuker et al., 2005, Pannebakker et al., 2011). After 24 hours I removed any females still in the host chamber. The parasitised hosts were incubated, and I scored the sex ratio of the offspring the females had laid once they emerged and died.

Statistical analysis

Analysis for this and all other experiments present in this chapter was performed in R (R Development Core Team, 2008, Ihaka & Gentelman, 1996).

Seventy-eight mothers did not lay any offspring, therefore no sex ratio could be counted. All male broods were excluded where the female is assumed a virgin (9 broods). This left a total of 343 sex ratios to be analysed, a mean of 19.06 (SE = 6.73) replicates per strain. One strain, COR10, produced only one mixed sex brood, due to a high number of virgins and non-oviposition in this strain. I repeated the analyses with strain COR10 excluded to ensure this did not skew the results.

I fitted a generalised linear model (GLM) using a quasi-binomial error structure to test for among-strain variation in sex ratio. A linear model (LM) was used to analyse the relationship between sex ratio, clutch size and strain. The significance of terms was tested using Likelihood Ratio tests comparing the full model to that with the term of interest removed.

Experiment Two: Male behaviour observations

In this experiment I observed groups of wasps mating, in order to learn more about how any differences in the competitiveness of males for mates might affect Hamilton's zero.

Wasps

Two strains, COR10 and COR29 were chosen for the behavioural observations, and red-eye wasps used as competitor males. I took experimental individuals directly from mass-culture hosts as pupae as before to ensure virginity, and kept them in isolation in the incubator until they eclosed. All experiments used one-day-old wasps.

Mating observations

A biologically reasonable situation was created with one or two males with ten females, generating sex ratios of 0.091 or 0.167 proportion male, which is within the range of single-foundress sex ratios (see Table 3.2 & Figure 3.1). We also know that clutches of wasp eclose synchronously leading to a short window during which mating can occur (Chapter 2; Appendix 2: Moynihan & Shuker, 2011) and so observing mating over a short time is reasonable.

For each treatment, I set up eleven (n = 11) sets of ten females in mating arenas of 70mm diameter × 10mm. There were three treatments: “same strain” which consisted of a focal male with ten females of the same strain, “competitor” which consisted of a focal male with ten females of the same strain and a red-eye competitor male, and “different strain” which consisted of a focal male with ten females of the other strain. Each wasp only experienced one treatment. For each mating trial, I observed the focal male for 10 minutes through a dissecting microscope, recording when he mounted, copulated and dismounted females. If a mating was in progress at the end of the ten minutes it was allowed to finish. Each male-female contact was called an encounter. Encounters were counted, and scored as successful if the pair mated, and unsuccessful if they did not. The time the male spends courting and mating a female is referred to as his female handling time.

Statistical analysis

The total number of mounts, the number of which successfully resulted in mating, and the proportion that were successful was calculated for each male and then compared using an ANOVA, with strain and treatment as fixed factors. For proportion successful, I used a generalised linear model with a quasi-binomial error structure. The handling time for successful encounters was compared between treatments and strains using the subset of successful encounters, with male ID as a random effect. The significance of each term was calculated using F tests to compare the full model to that with the term of interest removed for the number of mounts, the number of matings, and the proportion of success. For handling time, Likelihood Ratio tests were used to compare the full model to one with the term of interest removed.

Experiment Three: Mating capacity

I constructed groups of ten virgin females and one virgin male of the same strain to test his mating capacity, under a time constraint. The measure of mating capacity is the probability that a single, focal, female is inseminated in that time. Note that this is not strictly a measure of insemination capacity as the males were under a time constraint.

Virgin production

I needed to generate a large number of virgin male and female wasps, for this experiment. To generate the virgin males I took 48 female pupae from each of the eighteen Cornell strains from the mass culture tubes at laboratory generation twelve, at days seven to nine post-oviposition. I put the pupae into individual glass vials with a cotton plug. Once the pupae eclosed, I gave the three-day-old adult females three hosts. Males tend to eclose before females (Chapter 2; Appendix 2: Moynihan & Shuker, 2011) and so the delay between between eclosion and oviposition ensured that the virgin males would ensure emergence synchronously with the virgin females.

To produce the virgin females, I took 42 mated females from each strain from mass-culture at laboratory generation twelve and placed them in individual glass vials with three hosts to oviposit on for four days. Seven days after oviposition I opened the host puparium and removed female wasp pupae. A maximum of twenty sister pupae were kept together in a vial until they eclosed.

The virgin male and female wasps were kept for up to four days, and were provided with a piece of honey-water soaked filter paper on days zero, two and four after emergence.

Mating test

I created groups of ten non-sister virgin females of the same strain on the day of the mating trial. In order to avoid female age effects, I used a mix of one- to four-day post-eclosion females, recording mean female age. The virgin female groups had between one and two hours to acclimatise to bench conditions before the trial began. A single two- or three-day-old virgin male of the same strain was introduced to the ten virgin females. I ended each trial after fifteen minutes, interrupting any matings and mixing the females in the vial by gentle tapping. A single female was then removed pseudo-randomly and designated the focal female. The male was left with the other nine females in the test vial. The focal female was given three hosts to oviposit on for 24 hours at incubator conditions, after which she was removed and discarded, and the hosts incubated until the brood emerged and the sex ratio of their brood could be scored. The remaining nine females were left together for a further 24 hours to see if the male was able to inseminate any of the females.

After 24 hours I removed the male, killed him by freezing, and measured the length of the right hind tibia using a stereo dissection microscope at ×100 magnification and an eyepiece micrometer. Hind tibia length is a standard measure of body size in many insects, including parasitoid wasps (Godfray, 1994 and see Experiment Four Statistical Analyses). I kept the nine females together and gave them four hosts to oviposit on for 24 hours, after which I removed the females and incubated the hosts. Once the adults had emerged from these hosts, I scored the presence or absence of daughters. The presence of daughters indicates that at least one female was inseminated and able to fertilise an egg and produce a daughter.

Statistical analysis

I tested 212 males, and excluded three because the male was lost or damaged after mating. I excluded a further sixteen replicates because the focal female did not lay a clutch, preventing us from ascertaining her mated status. The total number of males analysed was therefore 193, with a mean of 10.72 (SE = 0.54) replicates per strain.

Following the methods of Zuur et al., (2010) I checked for collinearity between the proposed independent variables of male age, mean female age, male size. I found that mean female age was positively associated with male age ($F_{1,186} = 7.69$, p = 0.006), and therefore excluded female age from the analysis, as I am more interested in male effects. The mated status of each focal female was analysed as a Linear Mixed Effect (LME) model with binomial errors and strain as a random factor. The mean strain single-foundress sex ratios from experiment one were fitted, as well as male body size and male age. I tested the significance terms by comparing the full model to a model with the term of interest removed in a Chi Squared model comparison test.

Experiment Four: Male competitive ability

In this experiment I tested male inseminating ability when the Cornell males are in competition with a red-eye male to mate ten virgin females in fifteen minutes. The measure of the focal (Cornell) male's success in this experiment is his ability to reduce the number of red-eye daughters his competitor produces. The more fertilisations the focal Cornell male gains, the fewer his competitor achieves.

Wasps

I used the eighteen Cornell strains and the red-eye wasps as the competitor strain. If the focal, Cornell, male inseminated a red-eye female, only male red-eye offspring would be laid. If the *Wolbachia* transmission failed, the eye colour marker ensures that any daughters will be conspicuous with black eyes. However, if the red-eye competitor male inseminates the virgin red-eye females, the female will lay red eye daughters and sons. The outcomes of different matings are summarised in Table 3.3.

Virgin production

In order to produce virgins for testing I took mated females from stock tubes and put them through the pre-treatment process previously described. I then allowed the resulting offspring to mate in the tubes for two days before taking one female per tube and putting them through the pre-treatment process. The broods these females laid were the experimental individuals. I opened hosts between days seven and twelve post-oviposition to sex pupae and remove virgins. I took two males from each mother, keeping them together, and only using one male from each tube, to avoid using brothers yet have a spare male available.

Test mating

The day of eclosion was recorded, and females were grouped into tens and put into large glass vials (75 × 25 mm). I used one-day-old wasps throughout. The ten virgin females were not sisters, nor were they a sibling to the competitor red-eye male. The wasps had an hour to acclimatise to bench conditions before I put a red-eye male and a Cornell male into the tube of virgin females for fifteen minutes. I then removed the males, keeping them for measuring later. Each replicate group of females was given ten hosts to parasitise for 24 hours. After the females had oviposited, they were removed and the parasitised hosts incubated until the brood emerged. Once any offspring had emerged, I counted the sex and eye-colour of the resulting brood. I also estimated body size for both males, recording their hind tibia length, head capsule width and wing length using a digital camera mounted to a dissecting microscope and Cell^D image-analysis software (Olympus). In total 358 focal males across 18 strains were tested.

Statistical analysis

As is common in parasitoids I found significant positive allometries between all three measures of body size (Wing & Tibia Length $r = 0.36$, $t_{1,699} = 10.05$, $p < 0.001$; Head Width & Tibia Length $r = 0.52$, $t_{1,699} = 16.23$, $p < 0.001$; Head Width & Wing Length $r = 0.58$, $t_{1,699} = 18.89$, $p < 0.001$). Therefore I focused on hind tibia length for the subsequent analyses as is common practise in parasitoids.

Any replicates where there were no red eye females were excluded, also if I could not measure the leg length of both of the males. In total, 344 replicates were analysed, with a mean of 19.11 (SE = 3.93) replicates per strain. A total of 520 black eyed daughters were observed in 67 of 344 replicates.

I used a Linear Mixed Effects model to analyse the relationship between the fitness of the red-eye males (the number of daughters he sires) and the strain single-foundress sex ratio of the competitor Cornell male, with the difference in hind tibia length between the Cornell male and the red-eye competitor. The model included random effects of day of testing (n = 3), strain (n = 18), and the experimenter (n = 2). The strain sex ratio used is the single-foundress sex ratio from the screen in experiment two. The significance of each fixed term was tested using Likelihood Ratio tests between models fitted using Maximum Likelihood. Parameter estimates were gained from models fitted using Restricted Maximum Likelihood.

Results

Experiment One: Single-foundress sex ratio screen

Across all the strains the mean sex ratio was 0.163 (SE = 0.039) proportion male (Figure 3.1), and the mean clutch size was 34.68 (SE=12.16). There was significant variation among strains in sex ratio (GLM $F_{17,21.73} = 2.27$, $p = 0.003$). The mean sex ratio did not equal one male per clutch: across all strains the mean number of males was 5.61 (SE = 0.21). For one of the strains (COR10), only one mixed sex brood was produced, if I remove strain 10 from the analysis, the effect of strain is still significant (GLM $F_{16,21.73} = 2.37$, $p = 0.002$).

There was also among-strain variation in clutch size (Chi Sq = -3.04, $p = 0.001$), which was also unaffected by the exclusion of strain 10 from the analysis (significance of strain in model when strain 10 is excluded, Chi Sq = -2.99, $p = 0.0007$). I found no relationship between clutch size and sex ratio when controlling for strain (Chi Sq = -0.19, $p = 0.104$).

Experiment Two: Behavioural observations

Across all strains and treatment groups, the focal males mounted a mean of 10.9 (SE = 2.9) females, of which a mean of 5.9 (SE = 1.5) successfully resulted in a mating. The average time a male spent courting and copulating a female (the handling time) was 48 (SE = 10.5) seconds.

Observations showed that there were differences in male mating behaviour depending on his strain, or the treatment (Table 3.4). The two strains differed in both handling time and the number of mounts. The effect of the treatment group was more pronounced when comparing males in competition than those where males were paired with females of a different strain. While males in competition mounted more females, a smaller proportion of their mounts were successful and when they were successful the handling time was more variable compared to males alone (Table 3.4). The number of successful matings was not predicted by strain or treatment despite these differences in behaviour.

Experiment Three: Mating capacity

Across all strains, 55.44% of focal females were inseminated. The average male mating capacity when given a fixed number of 10 females is therefore 5.54 females across all strains. Strain single-foundress sex ratio did not predict mating success (4.76 ± 4.55, Chi Sq = 1.05, $p = 0.30$) (Figure 3.2). Males in trials on the second day post-adult eclosion were more likely to inseminate a focal female than males in trials on the third day (-1.39 ± 0.7, Chi Sq = 4.00, $p = 0.045$), however, as male age and female age were correlated, this could be an effect of female age. Body size was not significantly associated with mating success (-0.0145 ± 0.0126Chi Sq test = 1.32, $p = 0.25$). Variance component analysis showed that strain explained 20.32% of variance in the full model.

For the analysis above, I have assumed that a randomly chosen focal female that laid at least one daughter was fully inseminated. Males may become sperm-limited as they mate successive females (Chapter 4 and references therein). If this were occurring, I would expect to see intermediate sex ratios (i.e. more sons than expected) laid by sperm-limited females. The mean sex ratio of the focal females that produced at least one daughter was calculated for each strain. A paired t-test compared this sex ratio with the strain average obtained from experiment one, and found that the inseminated focal female sex ratios were less female-biased than the strain sex ratios ($F_{1,17} = 2.33$, $p = 0.032$). This post-hoc analysis of sperm limitation was somewhat limited.

Experiment Four: Male competitive ability

When males are in competition, the sex ratio of the strain the Cornell male comes from significantly predicts the number of daughters sired by his competitor: males from strains with high sex ratios are better competitors ($b = -169.4 \pm 54.85$, Likelihood Ratio test = 8.86, $p = 0.003$) (Figure 3.3). There is no evidence for an effect of body size measured as the difference in tibia length between the males (0.00405 ± 0.00724, L.Ratio = 0.32, $p = 0.57$), and no interaction between body size and strain sex ratio (-0.0229 ± 0.0440, L.Ratio = 0.27, $p = 0.60$). The random effect of strain was not significant associated with of the number of daughters the red-eye male sires in the full model (L.Ratio < 0.01, $p > 0.99$).

Discussion

This study is the first to investigate within-species variation in mate competitiveness alongside variation in the single-foundress sex ratio in a parasitoid wasp. I found among-strain variation in single-foundress clutch size and sex ratio, which would suggest differences in mate competitiveness under our interpretation of Hamilton's zero. This was supported by the different handling times and mounting rates of the two strains whose mating behaviour I observed, and the large portion of variance in mating capacity explained by strain. Despite this, I did not find that males from strains with lower sex ratios (fewer sons) were better inseminators either when males were alone or when in competition (Figures 3.2 & 3.3). The usual interpretation of Hamilton's zero does not therefore explain the among-strain variation in single-foundress sex ratio in *N. vitripennis*. Instead, I found that under competitive conditions, males from higher sex ratio strains were better competitors.

Support for the fertility insurance interpretation of Hamilton's zero has been found in other species exhibiting LMC. Broods of the Ambrosia beetle *Xyleborinus saxesenii,* usually contain just one son who successfully mates up to 80 sisters, suggesting that other factors must constrain sex ratio in this species (e.g. maximum clutch size) (Biedermann, 2010). Similarly, the number of gametes a male gametocyte of malarial blood parasites produces was found to significantly influence the maximum degree of female-bias in the gametocyte sex ratio (Gardner et al., 2003, West et al., 2002).

Among the gregarious parasitoid wasps however, when insemination capacity has been studied it is usually more than the number of sisters per male, but lower than the total number of sisters in the average brood (Table 3.1 species: *Lariophagus distinguendus*, *Nasonia vitripennis*, and *Trichogramma evanescens*). The parasitoid studies in Table 3.1 measure how many females were inseminated, and did not take into account the potential for females to not receive enough sperm. Although my data were not collected with sperm-limitation in mind, I did find some preliminary evidence for sperm limitation. This requires further investigation, as the fertility insurance interpretations of Hamilton's zero would extend to include providing the copulated females with full fertility.

Despite this body of work supporting the fertility insurance interpretation of Hamilton's zero, I have found the opposite correlation in *Nasonia vitripennis* when looking at a different component of fertility insurance: females with more competitive sons lay a higher sex ratio (Figure 3.3). The increased proportion of males mothers lay when their sons are good competitors is reminiscent of Trivers-Willard sex allocation effects. Trivers & Willard (1973) hypothesised that females in a good environment with high resource availability will favour the production of the sex where fitness is most dependent on resource allocation. This has been extended to consider that females mating attractive fathers would lay more sons, first proposed by Burley (1981). While Burley's initial evidence for this in zebra finches has since been contested (Rutstein et al., 2005), the idea has persisted, finding evidence in other species (West & Sheldon, 2002). Evolutionary stable strategy models have demonstrated that when the male's attractiveness or mate competitiveness is heritable and due to good-genes processes, that mothers with attractive mates should produce even sex ratios, and those with unattractive mates female-biased sex ratios, resulting in attractive males siring relatively more sons, however these effects are thought to be weak (Fawcett et al., 2007, Pen & Weissing, 2000). The models of Fawcett et al., (2007) and Pen & Weissing, (2000) rest on the assumption that sons inherit their father's attractiveness of viability advantage. *Nasonia* are haplodiploid, and so sons develop from unfertilised eggs: they therefore do not have fathers, only mothers. This abolishes the proposed advantage to producing more sons. For a mother to benefit from her mate's good genes, she should lay more daughters, and have more attractive grandsons.

Neither Hamilton's zero, nor sexually successful fathers having more sons can explain my finding that males from high sex ratio strains are better competitors. My data are correlative, leaving us an issue of causation: do higher sex ratios select for better competitor males, or do better competitor males select for higher sex ratios? Either way, I have no evidence for good competitors being associated with low sex ratios as predicted (Figures 3.2 & 3.3). The heritable variation in single-foundress sex ratio in *N. vitripennis* (Orzack & Gladstone, 1994, Parker Jr & Orzack, 1985, Pannebakker et al., 2011, & Figure 3.1) would suggest that higher sex ratios could simply have selected for better competitor males. This could be tested with selection experiments with four treatment groups: higher sex ratio or no sex ratio selection, crossed with mating under natural competitive environment, or between a male and female in isolation. After a number of generations of selection, the sex ratio and competitive ability of males should then be tested. If males from the strain selected for higher sex ratios become more competitive, this would suggest that the less female-biased operational sex ratio has increased the intensity on male competitive ability. This could come about rapidly through non-genetic changes if there is phenotypic plasticity. Males that have more brothers may be able to detect the sex ratio of their host-mates and shift their development accordingly to become better competitors, for example by developing faster to gain first-male advantages (Chapter 2; Appendix 2: Moynihan & Shuker, 2011). This is easily tested by comparing males of the same strain from unmated mothers, and hence all male broods, to those that developed with sisters.

This experiment has shed light on other forms of selection in *Nasonia vitripennis* aside from that on sex allocation. Neither of the measures of mate competitiveness depend on his body size nor that of his rival. This supports previous findings from *N, vitripennis* (Chapter 2; Appendix 2: Moynihan & Shuker, 2011, Burton-Chellew et al., 2007b), but is in contrast to *Colpoclypeus florus* where body size positively correlates with male insemination capacity (Dijkstra, 1986).

In this study I have investigated the relationship between sexual selection and sex allocation in *Nasonia vitripennis*. I did not find the expected relationship I had predicted from our interpretation of Hamilton's zero, however, I did find a positive relationship between male competitiveness and the single-foundress sex ratio. While the direction of causation remains to be established, the relationship is a product of the interaction between sex allocation and sexual selection.

Table 3.1: Summary of previous studies into insemination capacity in parasitoid males where the insemination status of females is monitored. Sex Ratio is measured as proportion male. The total number of sisters is calculated as (1-sex ratio)*Clutch Size. Females per male is calculated as the reciprocal of the sex ratio.

Species	Local mating	Number of mates before limitation	Total number available to mate	Polyandry	Sex Ratio	Clutch Size	Sisters in brood	Sisters per male
Colpoclypeus florus	Partial, both sexes disperse after local mating.[b]	6.2[a]	15[a]	Not data	Lab = 0.132 field = 0.23 [b]	Lab = 11.8 Field = 13.6 [b]	Lab = 10.24 Field = 10.47	Lab=7.57 Field =4.3
Cotesia glomerata	Partial, both sexes can disperse before mating.[c]	14.13[d]	20[d]	No[e]	0.328[f]	25.91[f]	17.41	3.05
Lariophagus distinguendus	Yes[g]	13[g]	20[g]	Yes[h]	0.35[i]	Mean number per cluster =49[i]	Total females in cluster =31.8	2.86
Nasonia vitripennis	Yes	5.54[j] 6.795[k]	10[j] 10[k]	No[l]	0.162[j]	19.05[j]	15.9	6.17
Spalangia cameroni	Solitary[m]	36[m]	4 per day, 13 days[m]	No data	solitary[m]	solitary[m]		
Spalangia drosophilidae	Solitary[n]	6[n]	13[n]	No data	solitary[n]	solitary[n]		
Trichogramma evanescens	Yes[o]	18[o] 18[p]	90[o] 20[p]	Yes[o]	0.17[q]	12[q]	9.96	5.88
Uscana semifumipennis	No, outbreeding[r]	6[s]	6[S]	No data	0.33[S]	24.9[S]	16.69	3.03

[a] (Dijkstra, 1986); [b] (Hardy et al., 1998); [c] (Gu & Dorn, 2003); [d] (Tagawa, 2002); [e] (Tagawa & Hidaka, 1982); [f] (Tagawa, 2000); [g] (Steiner et al., 2007); [h] (Ruther et al., 2000); [i] (van den Assem, 1971); [j] this study; [k] (Grant et al., 1980); [l](Grillenberger et al., 2008); [m](King, 2000); [n] (Simmonds, 1953); [o](Jacob & Boivin, 2004); [p] (Damiens & Boivin, 2005); [q] (Waage & Lane, 1984); [r] (Henter, 2003); [s] (Henter, 2004).

Table 3.2: Summary of the single-foundress sex ratios reported from other studies of *Nasonia vitripennis*. I conducted a literature search for studies that measured the single-foundress sex ratio in *Nasonia vitripennis.* The criteria for inclusion were: the wasp strains were not treated with antibiotics; the sex ratio and clutch size are reported; sex ratios were measured over single bouts of oviposition, not life-time measures. Sex ratios are the proportion male. Mean number of males was therefore calculated as the product of Sex Ratio and Clutch Size.

Sex Ratio	**Error**	**Clutch Size**	**Mean Number of Males**	**Reference**	**Population**	**Place of testing**
0.198	0.242	36.9	7.3	(Balas et al., 1996)	US, NY	Lab
0.273	0.408	54.0	14.7	(Burton-Chellew et al., 2008)	Netherlands	Wild
0.144	0.016	78.6	11.3	(Drapeau & Werren, 1999)	US, NY	Lab
0.31	0.02	15.4	4.8	(Flanagan et al., 1998)	US, NY	Lab
0.04	0.004	14.3	0.6	(Grillenberger et al., 2009)	US, NY	Wild
0.16	0.0255	32.0	5.1	(King & Skinner, 1991)	US, OH	Lab
0.218	0.229	22.4	4.9	(Molbo & Parker Jr, 1996)	Sweden	Wild
0.183	0.006	33.7	6.2	(Pannebakker et al., 2008)	Netherlands	Lab
0.185	0.0175	10.4	1.9	(Rivero & West, 2005)	US, NY	Lab
0.14	0.17	19.3	2.7	(Sykes et al., 2007)	Netherlands	Lab
0.1851	0.1138	31.7	5.95	GRAND MEAN		

<u>Table 3.3:</u> Possible outcomes of the matings in Experiment Four.

Parents	Offspring
Black-eye *Wolbachia* positive male (COR) x Red-eye *Wolbachia* negative female (STDR-TET)	Red-eyed sons only
Black-eye *Wolbachia* negative male (COR) x Red-eye *Wolbachia* negative female (STDR-TET)	Red-eyed sons and Black-eyed daughters
Red-eyed *Wolbachia* negative male (STDR-TET) x Red-eye *Wolbachia* negative female (STDR-TET)	Red-eyed sons and Red-eyed daughters.

Table 3.4: Male behaviour in the trials depends on their strain, and also the treatment. The test statistic reported for number of encounter, number of matings, and proportion success, is the F test comparing the full model to that with the term of interest removed. The test statistic reported for handling time is the Likelihood Ratio from comparing the full model to that with the term of interest removed. The factor levels are reported when significant, as the mean ± one standard error of the mean.

	Trait			
	Number of encounters	**Number of matings**	**Proportion success**	**Handling time (seconds)**
Strain				
Test statistic	9.94	0.0067	3.08	6.07
p	**0.0025**	0.94	0.084	**0.014**
10	11.85 ± 3.04			49.6 ± 11.42
29	9.94 ± 1.71			46.4 ± 9.3
Treatment				
Test statistic	7.52	2.97	4.01	6.54
p	**0.0012**	0.059	**0.023**	**0.038**
Same strain	9.31 ± 2.28		0.63 ± 0.25	45.95 ± 8.80
Different strain	11.23 ± 2.50		0.60 ± 0.14	47.82 ± 9.66
Competition	12.14 ± 2.34		0.48 ± 0.15	50.28 ± 12.53
Interaction				
test statistic	0.35	0.50	0.26	0.38
p	0.70	0.61	0.77	0.83

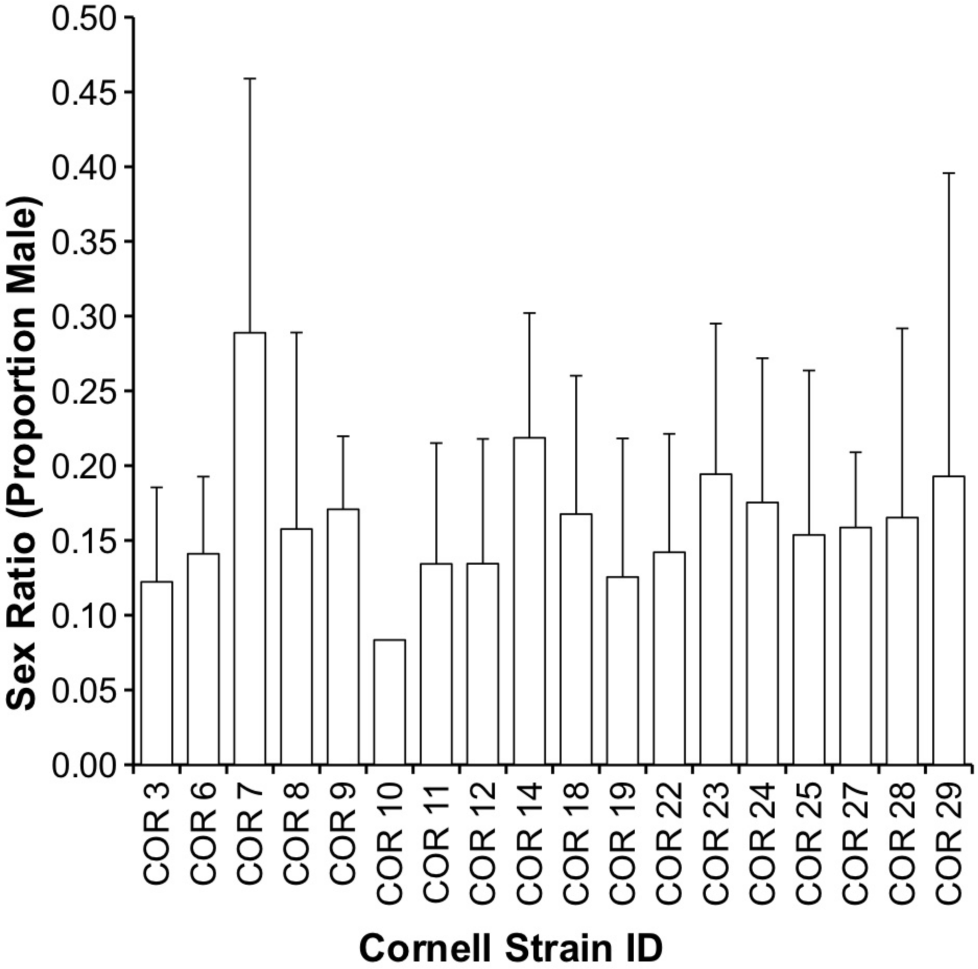


Figure 3.1: Mean single-foundress sex ratio, measured as proportion male for each strain. Error bars are standard error of the mean.

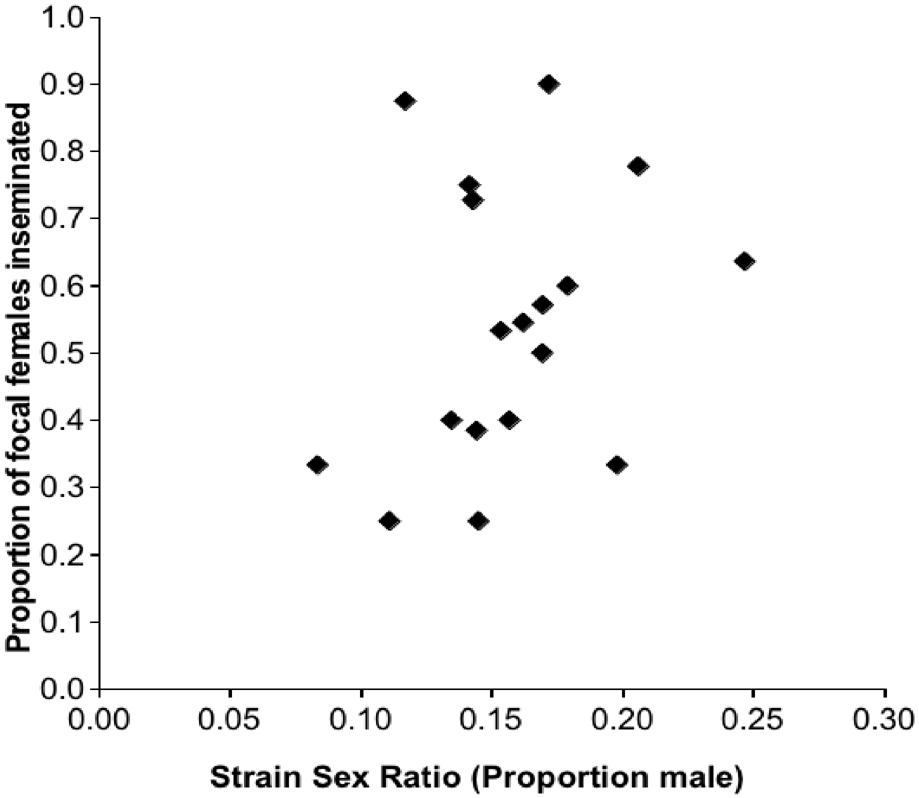


Figure 3.2: There is no correlation between strain sex ratio and the probability a lone son inseminates a focal female. Single-foundress sex ratios from the sex ratio screen are used. Sex ratio is measured as proportion male.

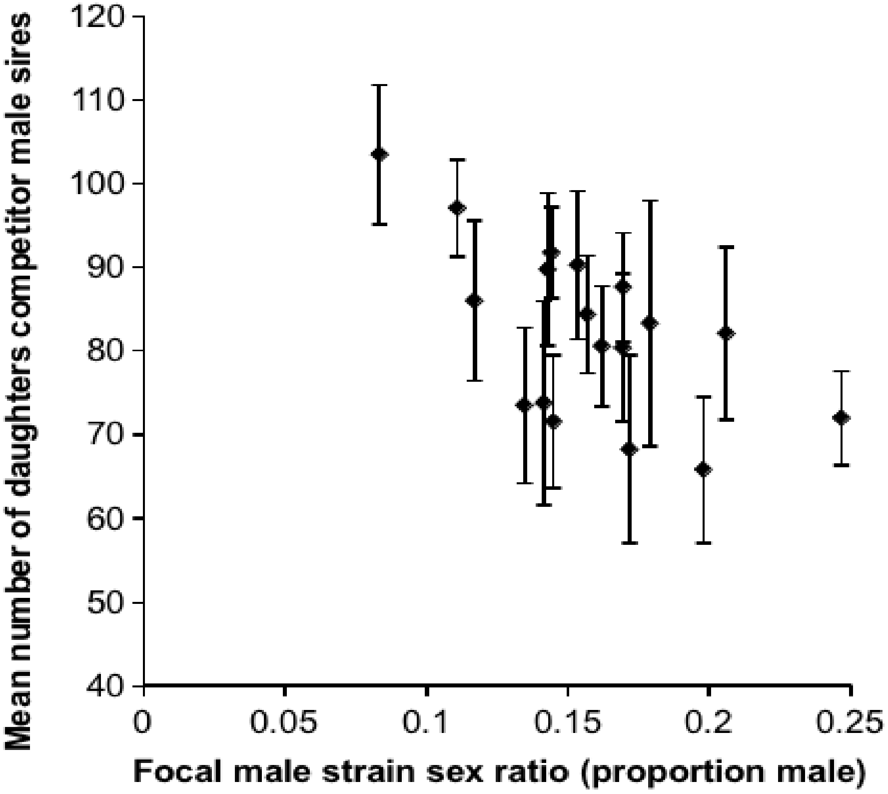


Figure 3.3: Sons from high sex ratio strains are better competitors. As the strain sex ratio of the focal male increases, the mating success of his competitor decreases. Error bars show the standard error on the number of daughters the competitor male sires. Strain sex ratios are the means from the single-foundress sex ratio screen. Sex ratio is proportion male.

4. Sperm resource levels and risk of sperm limitation is not associated with single-foundress sex ratio for a gregarious parasitoid wasp

A version of this chapter is being prepared for submission. The authors will be Anna M. Moynihan and David M. Shuker

Abstract

Hamilton's theory of Local Mate Competition (LMC) predicts that when females are laying eggs alone (as a so-called single-foundress), and their offspring will emerge and mate locally amongst themselves, they should lay a female-biased sex ratio. In fact, the mathematical solution for the optimum sex ratio is to lay only females, but all-female broods are not commonly observed among species known to exhibit LMC in the wild, and there is often more than one son in each brood. An over-abundance of males in single-foundress broods is usually explained through fertility insurance: foundresses lay more sons to ensure that enough reach adulthood to inseminate all their daughters. I extend this fertility insurance idea to include the potential for sperm-depleted sons that do not have enough sperm to provide full-fertility to their sisters. Using eighteen iso-female strains of the gregarious parasitoid wasp *Nasonia vitripennis* I performed the first study of within-species variation in sperm-resources and sperm-limitation in parasitoids. I found that seminal vesicle volume differed between strains, but did not correlate with single-foundress sex ratio, contrary to the fertility-insurance hypothesis. I also found evidence of sperm limitation in later-mated females, but this did not vary with strain. Therefore sperm-limitation is unlikely to be a common occurrence in wild *N. vitripennis*.

Keywords: male insemination capacity, local mate competition, sperm-depletion, *Nasonia vitripennis*, body size, seminal vesicle volume, fertility insurance.

Introduction

One of the best-studied forms of facultative sex allocation is Local Mate Competition (LMC; Hamilton, 1967) which is displayed by a wide-range of organisms including aphids, mites, spiders, barnacles, malaria blood parasites, snakes and fish and most famously the parasitoid wasps (see West, 2009 for a review). Hamilton (1967) described the optimal sex ratio a mother should lay if mating occurs locally on a patch of resource and only one sex disperses. Under LMC mothers should adjust the sex ratio of a brood (measured as the proportion male) in response to the number of other females laying eggs (foundresses) on the oviposition patch. As the number of foundresses (n) increases, the degree of local mating decreases, which makes the production of sons more favourable (Figure 1.1). Hamilton modelled the rise in the unbeatable sex ratio for diploids (s^*) with increasing foundress number (n) such that $s^*=(n-1)/2n$. When a female oviposits alone ($n=1$) the LMC equation solves to predict all female broods. Son-free broods are, however rarely observed in nature (Hamilton, 1967). The few sons that are laid have historically been interpreted as the minimum number of sons required to ensure the fertility of a mother's daughters under extreme LMC, as females will not find alternative mates away from the natal patch (Charnov, 1982, Godfray, 1994, Waage & Sook Ming, 1984, Hardy & Cook, 1995, Heimpel, 1994, Chapter 3). Virgin females can gain some fitness by laying all-male broods: if the virgin female oviposits on a patch with other, mated, females, her sons will have mates available.

Previous attempts to explain higher than predicted sex ratios have focussed on fertility insurance through developmental mortality and male insemination capacity. Models that assumed zero fitness for virgin females have shown that the unbeatable sex ratio a mother should lay increases with the rising probability of male death before mating (Nagelkerke & Hardy, 1994). The number of sons surviving after developmental mortality must be enough to inseminate the brood, which may be more than one if males have a finite insemination capacity that is smaller than the average clutch size (Dijkstra, 1986, Hardy & Cook, 1995). Insemination capacity has found some support in explaining additional sons in single-foundress broods in some parasitoid wasps (reviewed in Chapter 3). The relative importance of male insemination capacity and developmental mortality was compared by Hardy et al. (1998) in three species of parasitoid. In the parasitoid wasp *Colpoclypeus florus*, males can inseminate fifteen females before dispersal, and so the presence of more than one son at clutch sizes less than sixteen is explained by the high developmental mortality in this species. Whereas limited male insemination capacity is more likely as an explanation of additional males in *Goniozus nigrifemu* and *Goniozus legneri* where developmental mortality is low (Hardy et al., 1998). Models of LMC in malaria and other blood parasites have shown that the number of gametes a male gametocyte produces has an impact on the sex ratio observed: fewer potential gametes per gametocyte leads to higher sex ratios (Gardner et al., 2003, West et al., 2002).

Many of the parasitoid studies discussed in Chapter 3, and my own assay of male mating ability in Chapter 3, used the presence or absence of female progeny as the measure of insemination capacity. A male could however, pass an ejaculate to a female that is not enough to give her full fertility over her reproductive lifespan. Here, I extend my consideration of fertility insurance and the single-foundress sex ratio, by looking at variation in sperm resources as a different component of male fertility.

Sperm limitation is is a potentially serious problem for parasitoid wasps. Parasitoid wasps, as Hymenoptera, are haplodiploid, meaning that females develop from fertilised eggs and males from unfertilised eggs. Consequently, a virgin female can lay a brood of sons, but must be mated to produce daughters. In many species, including *N. vitripennis,* males are proto-spermatogenic: they eclose as adults with all their sperm and do not continue spermatogenesis after this point (Clark et al., 2010, Hogge & King, 1975). This fits their extremely local mating pattern. Males are therefore under strong pressure to allocate their sperm resources wisely to maximise their number of offspring (Dewsbury, 1982). Sub-optimal sperm allocation can mean males run out of sperm and yet still have mating opportunities (Wedell et al., 2002). There is much evidence in parasitoid wasps of males continuing to mate once sperm-depleted, passing no sperm to their mates (*Dinarmus basalis* (Bressac et al., 2008); *Trichogramma evanescens* (Jacob & Boivin, 2004, Damiens & Boivin, 2006); *Spalangia drosophilae* (Simmonds, 1953); *Nasonia vitripennis* (van den Assem & Visser, 1976, Grant et al., 1980); *Pachycrepoideus vindemiae* (Nadel & Luck, 1985) and *Spalangia cameroni* (King, 2000)). Of the females that do receive a full ejaculate, we know this is not always enough for full-lifetime fertility, as the proportion of sons in a female's brood has been shown to increase over her lifespan (Bressac & Chevrier, 1998, Geuverink et al., 2009, Jacob & Boivin, 2005). Females can also be partially inseminated, which can be observed either through: (a) counting the spermatozoa in her spermatheca (Nadel & Luck, 1985, Bressac et al., 2008, Dijkstra, 1986, Geuverink et al., 2009); (b) observing an increase in the total offspring sex ratio a female produces the later she is in a sequence of mates for a given male (Ruther et al., 2009, Hurlbutt, 1987a, Sekhar, 1957, Nadel & Luck, 1985, Vevai, 1942, Sandanayaka et al., 2011, Perez-Lachaud, 2010, King & Fischer, 2010, Henter, 2004); (c) observing a change in how an individual female's sex ratio varies across her lifespan with respect to her position in a sequence of matings with a given male (Chevrier & Bressac, 2002, Steiner et al., 2007). The probability of sperm-limited females is compounded by the fact that most parasitoid females mate just once (Ridley, 1993, Gordh & DeBach, 1978, Quicke, 1997) and that they often mate with a male who has probably already mated several other females due to the typically female-biased sex ratio. A female's receptivity to further matings can be turned off by sperm-depleted males in some species through post-copulatory interactions (i.e. without actual sperm transfer: *Nasonia vitripennis* (Barrass, 1964, van den Assem & Visser, 1976); *Spalangia endius* (King & Fischer, 2010); *Lariophagus distinguendus* (Steiner et al., 2007), but not *Trichogramma evanescens* (Damiens & Boivin, 2006)). This leaves females unlikely to mate again when they run out of sperm (Do Thi Khanh et al., 2005, van den Assem et al., 1984). Females of

Trichogramma evanescens run out of sperm over their lifetime, irrespective of how many times they copulated, suggesting that either they can only store a fixed volume of sperm, or their ability to fertilise eggs declines with age (Jacob & Boivin, 2005). All of these factors contribute to the incidence of females in the wild that have no sperm reserves and are effectively virgin (termed "constrained females" in the parasitoid literature (Godfray, 1990, Ode et al., 1997)).

Here I will connect the well-researched fields of sperm-depletion in parasitoids with fertility insurance interpretations of Hamilton's zero. I will look at within-population variation in sperm resources and sperm-limitation, in order to test the prediction that males from strains with lower single-foundress sex ratios will have higher sperm reserves than those with more brothers. Consequently, males from low sperm-resource strains are predicted to become sperm-depleted faster. If this is true, then foundresses with sons that are less able to inseminate all their sisters, might lay additional sons in order to ensure the fertility of her daughters. A preliminary test of focal female sex ratios from the insemination capacity experiment indicated some evidence of sperm limitation (Chapter 3) which I will pursue here. Previous studies have rarely investigated within-species variation in parasitoid sperm resource level and sperm-limitation. Total sperm counts were found to differ for two strains of *Anisopteromalus calandrae* (Bressac et al., 2009, Do Thi Khanh et al., 2005), but body size was not controlled for. Also the identity of the male a female mated with explained some variance in the sex ratio of the resulting brood in both *Nasonia vitripennis* (Shuker et al., 2006c) and *Uscana semifumipennis* (Henter, 2004), but these studies did not rigorously investigate sperm-limitation as a potential mechanism.

I assayed sperm resources across 18 strains of the gregarious parasitoid wasp *Nasonia vitripennis* from the same population. My measure of sperm resource was seminal vesicle volume. Sperm is stored here once produced, and seminal vesicle volume has proved a successful assay of sperm number for *Trichogramma euproctidis* (Martel et al., 2011) and *Spalangia cameroni* (King & King, 1994), a member of the same family as *Nasonia* (Pteromalidae). I then took four of these strains that captured the range of variance in seminal vesicle volume and tested them for sperm limitation. To do this I mated males with 12 females sequentially, putting the first, fourth, eighth, tenth and twelfth females on hosts to count the number of offspring the male sired. As the wasps are haplodiploid, sons have no fathers and daughters result from fertilised eggs. Therefore the number of daughters is the measure of male fertilisation success.

Materials and Methods

Experiment One: Variation in seminal vesicle volume across strains

In this experiment I investigated the allometry between body size and seminal vesicle volume across eighteen strains of Nasonia vitripennis from the same population.

Wasps and pre-treatment

The wasps used for this experiment are the eighteen Cornell strains of *Nasonia vitripennis* used in Chapter 3. The host used throughout culture and experimental conditions were pupae of the large dipteran *Calliphora vicina.* Unless otherwise stated, I kept wasps in the incubator at 25°C, with a 16hrs light to 8hrs darkness light cycle.

The experiment was performed in three blocks. For each block males were of the same generation and eclosed on the same day. Their mothers were collected from stock tubes and put through the pre-treatment process described in Chapter 2 in order to prepare the mothers for oviposition. Pre-treatment begins with an initial host for 24 hours, which the mothers use for host-feeding to gain protein which they use for oocyte-maturation (Rivero & West, 2005). This host was then replaced with honey-water soaked filter-paper for a further 24 hours. Finally the oviposition host is provided for 24 hours. The parasitised hosts were then incubated until day 11 post-oviposition, when I opened the hosts and removed the male wasp pupae inside. Male pupae were then isolated in glass vials of 75 × 25 mm and incubated until they eclosed. Upon eclosion, they were put into an incubator at 6°C with 24 hours of darkness, where they were kept until they were measured. For block A 72 males were measured, 28 for block B and 109 for block C. The total number of replicates for each strain varied from 7 to 18, with the average at 12 males per strain. The range of ages on the day of measurement for each block was as follows: A 10-28; B 8-19; C 3-17 days post-eclosion.

Dissection protocol

Males were dissected in pools of TBST buffer (50mM Tris, 150 mM NaCl, 0.1% Tween, 0.05% NaN3, pH 7.5), following the protocol of (Clark et al., 2010). Under a stereomicroscope the reproductive tract was removed, and the seminal vesicles measured. I took three measurements of both the longitudinal and lateral dimensions of both seminal vesicles at ×126 in Cell^D software (Olympus) using the stereomicroscope (see Figure 4.1). If one seminal vesicle was damaged, I used the remaining intact seminal vesicle. The age of the male in days post-eclosion was recorded, along with his hind tibia length measured at ×20 using photographs through a microscope and in Cell^D imagine software as before.

Analysis

The volume of the seminal vesicle was estimated as an oblate spheroid using $(4\pi ab^2)/3$, where a = mean longitudinal seminal vesicle dimension (microns), and b = mean latitudinal seminal vesicle dimension (microns).

Statistical analysis was performed using R (R Development Core Team, 2008, Ihaka & Gentelman, 1996). Initial exploration of the data showed a cubic relationship between body size and seminal vesicle volume, and so hind tibia length cubed was used. A linear mixed effect model (LME) was used for analysis, with a random effect structure of strain nested within block, with the fixed effects of male age, strain single-foundress sex ratio and body size, and the interaction between body size and strain single-foundress sex ratio. Single-foundress sex ratio data were the strain means measured in Chapter 3.

The significance of both fixed and random effects was tested using Likelihood Ratio tests, comparing a full model and the model with the term of interest removed. The models used in Likelihood Ratio tests were fitted using Maximum Likelihood (ML), whereas the parameter estimates were taken from the full model fitted with Restricted Maximum Likelihood (REML).

To investigate possible different allometries of seminal vesicle volume on body size between the strains, independent of strain sex ratio, I performed a second analysis where strain is included in a second model as both a factor and a random effect, following Crawley (2007). In this LME model, a random effect structure of strain nested within block was used, with male age, hind tibia length, strain and the interaction between strain and hind tibia length as fixed effects. Again the significance of model terms was tested using Likelihood Ratio tests.

Experiment Two: Sperm Limitation

In this experiment I tested four strains of *Nasonia vitripennis* for sperm limitation. Males were mated to twelve females sequentially. A previous study on mate competitiveness in this population had shown some evidence of sperm-limitation occurring when males had mated a mean of 5.6 females under strict time constraints (Chapter 3). A subset from these twelve females were then given hosts to oviposit on, to count the number of daughters they lay.

Wasps

I used the Cornell strains with the biggest (strains 6 & 23) and smallest (strains 18 & 28) seminal vesicle volumes controlling for body size and age as determined from experiment one.

I set up a grand-parental generation to allow us to create staggered blocks and control for maternal effects. I kept females at 18°C with 16hrs of light and 8hrs of darkness with honey-water soaked filter paper every day, then took six from each strain each day and put them through the pre-treatment process described in experiment one on the appropriate day. The pre-treatment from this point was conducted at standard incubator conditions described in experiment one. The females from the resulting broods were allowed to mate and then were pre-treated, this time with two hosts for oviposition.

These hosts were opened on day ten post-oviposition for each of the five blocks. I took at least twelve sister pupae from one host. Two male pupae were collected from another host. This meant that females were groups of sisters, and the male that mates them is from the same strain, but is not their brother.

Mating day

On mating day wasps were allowed to acclimatise for an hour on the bench before matings. Experiment one showed an effect of age on seminal vesicle volume, and so all males were tested at age one day after eclosion. Each mating occurred in a clean tube of dimensions 50 × 10mm.

The male was added to the arena followed by the female for each mating. I observed the matings under a ×10 binocular microscope to ensure genital contact. I recorded the time the female was added to the mating tube, the time of mating and also the time the female is removed after the male had dismounted. After mating the female was removed, isolated in a fresh vial and returned to the incubator. The next female was then added until a male had been observed mating all twelve females. I then kept the male to measure his hind tibia length following the protocol described for experiment one. The average time pairs spent together in a tube was 124.3 (SE = 62.63) seconds, and the lag to the next mating was 32.44 (SE = 44.73) seconds.

In each of the five blocks, four males from each strain were tested with twelve females. On block four, only three males for strain 23, and three males for strain 28 were tested, the lost males were due to death before mating. The total number of males tested per strain is therefore n = 20 for strains 6 and 18, and n = 19 for strains 23 and 28.

One day after mating the first, fourth, eight, tenth and twelfth females (according to mating sequence) were each given four hosts in an isolated vial. I changed these hosts every second day for six days, so a female received three batches of four hosts over her lifetime, referred to as the three oviposition bouts. Due to the extremely adverse weather conditions in the UK at the time, I was unable to reach the lab one of the experimental days. As a result, Block 3 females had their third group of hosts for three days, and block 5 females had their second group of hosts for three days and did not receive a third group of hosts. Each set of hosts was allocated a random ID in order for the sex ratios to be counted blind, therefore each oviposition bout occurred at a random position within the incubator, and the parasitised hosts were incubated at a random position within the incubator. All hosts were incubated until the offspring emerged and died.

Once the clutches had eclosed and died, the number of sons, daughters, dead larvae and diapausing individuals in each tube was counted.

Analysis

I stated in the methods that there was one day in which I was prevented from accessing the lab due to snow. I suspected that females ovipositing in those bouts may have behaved unusually, perhaps with increased levels of self-superparasitism and I therefore repeated the analyses with those oviposition bouts removed from the dataset. Model results were significantly different when the bad weather broods were included; I therefore only present analysis of the reduced dataset.

Females have been predicted to optimise sex ratio and clutch size together (King, 1987, Nagelkerke & Hardy, 1994), so females with the same sex ratio but wildly different clutch size will have used different numbers of sperm cells. Also males could influence clutch size and sex ratio allocation by seminal fluid effects (Shuker et al., 2006c). I therefore analysed the number of females and clutch size rather than the sex ratio.

All analyses were performed in R (R Development Core Team, 2008, Ihaka & Gentelman, 1996). The number of females in each group of hosts was analysed in a LME, with male nested within strain, nested within block as random effects. The fixed effects of male hind tibia length (in microns), the mother's mating sequence number (1, 4, 8, 10, 12), the interaction between male body size and the mother's mating sequence number, and the oviposition bout (1, 2, 3) within female sequence number were included in the model. The significance of terms was tested using Likelihood Ratio tests comparing the full model to a model with the term of interest removed, both fitted using ML methods. Parameter estimates are reported from the full model fitted using REML.

The same analysis was performed for the total number of offspring: the clutch size.

The random effect of strain (within block) on both clutch size and number of daughters in these models was tested using a likelihood ratio test. In order to avoid pseudoreplication, a new random factor was created giving each strain within a block a new, different level, so instead of 4 strains each replicated within 5 blocks, there are now 20 levels designated A-T. The model with the random effect of male, within strain within block was compared against a model with the random effect of male within strain-block. A Variance Components Analysis from the full model was also used to described what proportion of variance is explained by the random effect of strain for clutch size and number of daughters, using the REML estimates.

The relationship between male hind tibia length and the total number of daughters sired was then investigated. Using an LME I fitted the mean number of daughters per oviposition bout, by all females inseminated by the same male, with male hind tibia length (microns) as a fixed effect, and strain nested within block as random effects using maximum likelihood methods. The effect of tibia length was tested with a Likelihood Ratio test.

Results

Experiment One: Variation in seminal vesicle volume across strains

A total of 219 males were measured across three blocks. The mean hind tibia length was 151.2 microns (SE = 19.0). The mean volume of each seminal vesicle was 2.27×10^{-2} microlitres (SE = 9.53×10^{-3}) cubic microns.

Across the strains, larger males had a higher seminal vesicle volume (LR test, Tibia (Microns) Cubed, b= 3.49 ± 1.72, L.Ratio = 33.52, $p = 0.040$) (Figure 4.5), as did older males ($b = 1.02\times10^{9} \pm 8.17\times10^{8}$, L.Ratio = 33.52, $p < 0.0001$). Seminal vesicle volume was not however associated with strain sex ratio when controlling for male body size and age ($b = 6.22\times10^{9} \pm 2.71\times10^{10}$, L.Ratio = 0.008, $p = 0.93$) (Figure 4.2) nor was there an interaction between hind tibia length and strain sex ratio ($b = -0.692 \pm 10.0$ L.Ratio = 0.006, $p = 0.94$).

There was also no evidence for different allometries of hind tibia length with seminal vesicle volume across the strains. When strain was fitted as a fixed effect, it was not significant as main effect (LR test, L. Ratio = 10.79, $p = 0.87$), or interacting with hind tibia length (L. Ratio = 14.36, $p = 0.64$).

Experiment Two: Sperm limitation

Across all females the mean clutch size was 270.0 (SE = 7.52) and the mean sex ratio (proportion male) was 0.25 (SE = 0.21). The 78 males tested copulated with twelve females each. Five females per male (the first, fourth, eighth tenth and twelfth) were kept to measure the number of daughters they produce, however five females died before oviposition, so the total number of females analysed was therefore 385. Twelve of those females laid no daughters on any oviposition bout and are therefore considered constrained females. One constrained female occurred in strain 6; she was the first mated by her male. Three constrained females were found in strain 18; the fourth, eighth and tenth mated by their males. Just one constrained female was found in strain 23; the fourth mated by her male. Seven constrained females were found in strain 28, a first, eighth, tenth and four twelfth mated females. There is evidence from other parasitoid studies that males can mate, but not inseminate one female, but then go on to successfully inseminate a subsequent female (*Spalangia cameroni* (King, 2000); *Spalangia drosophilae* (Simmonds, 1953)). I therefore did not exclude males that had a failed copulation with one female but later successful copulations.

Number of daughters

There was evidence for sperm limitation: females who were mated later in the sequence laid fewer daughters, however the effect is not very strong ($b = 7.03 \pm 3.03$, LR Test, L. Ratio = 5.39, $p = 0.02$) (Figure 4.3). This sperm limitation was not seen across a female's three oviposition bouts though ($b = 0.026 \pm 0.068$, L. Ratio = 0.15, *p* = 0.70) so it does not appear that females are running out of sperm as they lay more eggs. Male body size did not influence the number of daughters a female laid (*b* = 0.063 ± 0.035, L. Ratio = 3.34, *p* = 0.068), but it did interact with female mating sequence number so that females mated earlier in the sequence by larger males laid more daughters (*b* = -0.008 ± 0.003, L. Ratio = 6.80, *p* = 0.009) (Figure 4.4). Strain explained 7.74% of variance in daughter number, however this was not significant (L. Ratio = 1.46×10^{-6}, *p* = 0.999).

Larger males do not have a higher fitness overall: the total number of daughters a male sired is not predicted by his body size (*b* = 0.0038 ± 0.027, L.Ratio = 0.13, *p* = 0.72) when controlling for the number of oviposition bouts the females he mated with survived to perform.

Clutch Size

There was no effect of sperm limitation on the total number of offspring laid. The clutch size was not affected by male body size (*b* = 0.0098 ± 0.0.26, LR Test, L.Ratio = 0.14, *p* = 0.70), female mating sequence number (*b* = -0.41 ± 2.64, L.Ratio = 0.02, *p* = 0.88) or their interaction (*b* = $-7.2\text{x}10^{-4}$ ± 0.0026, L.Ratio = 0.07, *p* = 0.79). There was, however a significant effect of oviposition bout within female mating sequence number (*b* = 0.45 ± 0.06, L.Ratio = 57.44, *p* < 0.0001) (Figure 4.5): females laid larger clutches on their second and third oviposition bouts.

The random effect of strain explained 2.60% of variance in clutch size, and the effect was not significant in model comparison (L.Ratio = 1.37×10^{-6}, $p = 0.9991$).

Discussion

This study is the first to investigate the relationship between within-species variation in sperm resources and sperm-limitation, and differences in single-foundress sex ratio. I predicted that males from strains with a higher sex ratio (proportion male) would have lower sperm resources, and would therefore become sperm-limited faster. I found no evidence that males from strains with higher sex ratios had lower sperm resources (Figure 4.2), as measured by the volume of their seminal vesicles. My findings here support those of my previous study (Chapter 3) that the differences in the minimum number of males required to inseminate all the females in the brood is unlikely to explain between-strain heritable variation in single-foundress sex ratio in *Nasonia vitripennis*.

The females in this experiment did become sperm limited: those mated later in the sequence laid fewer daughters (Figure 4.3). While sperm limitation did occur, there were very few constrained females laying all-male broods, indicating that when *Nasonia vitripennis* males are provided females sequentially they are able to (at least partially) inseminate more females than previously thought (see Grant et al., 1980). The females who laid fewer daughters did not produce a pattern consistent with running out of sperm over successive oviposition bouts. It is possible that the females can assess their sperm resources and produce fewer daughters over a given number of hosts, as there is no indication they reduce clutch size (Figure 4.5). The strong selection on single-foundress sex ratios relative to multi-foundress scenarios (West & Herre, 1998b, Shuker et al., 2005), could result in females saving sperm to produce the best possible single-foundress sex ratio in case they later encounter an unparasitised host alone. Grillenberger et al., (2008) found that in the wild six of 49 females oviposited on more than one patch of hosts, so multiple oviposition opportunities exist in nature. This hypothesis that females could conserve sperm for future oviposition events, would benefit greatly from a theoretical treatment, in which female mortality should also be included.

Larger males did not overall sire more daughters, however the females earlier in the mating sequence (first to eighth) mated by a larger male did produce more daughters (Figure 4.4). Seminal vesicle volume, my assay of sperm resources, did significantly differ between strains when controlling for age and body size, however the strains did not differ in their allometries. Despite choosing four strains to represent the greatest variance in seminal vesicle volume for the sperm-limitation study, I did not find an effect of strain on sperm limitation. I had predicted that strains with a higher single-foundress sex ratio would have lower sperm resources and become sperm limited faster, analogous to the relationship between the sex ratio in malaria blood parasites and the number of gametes a male gametocyte can produce (Gardner et al., 2003).

The influence of a male on the sex ratio his mate produces is proposed to be through the number of spermatozoa he transfers to her in his ejaculate. Ejaculates are not just spermatozoa; they consist of other factors that are known to increase oviposition rate in other insects (Eberhard, 1996, Chapman, 2001). If seminal fluid becomes depleted along with sperm numbers, I would expect to observe a reduction in their effect too. I found no main effect of female mating sequence number or male body size on clutch size, suggesting that this seminal-fluid depletion is not occurring. It is possible that a seminal fluid component could increase fertilisation rate rather than oviposition rate. If such a component existed in a limited quantity, the depletion of such a resource could be an alternative explanation to the decline in the number of daughters laid with mating sequence number I observed (Figure 4.3), and also the lack of decline in daughter number across host bouts. I note though that the presence of such ejaculate components remain speculative. Strain had no significant effect on clutch size here, despite clutch size showing significant among-strain variation in the single-foundress sex ratio screen of Chapter 3. A previous study of male influence on sex ratio in *Nasonia vitripennis* also did not find an effect of male influence on the clutch size his mates laid (Shuker et al., 2006c). Instead, females lay larger clutch sizes with successive oviposition bouts (Figure 4.5), perhaps due to the ongoing host-feeding and egg maturation that limits the size of the first clutch (Rivero & West, 2005).

While larger *N. vitripennis* males did not sire more daughters in total, they did have higher seminal vesicle volumes (Figure 4.6), and sired more daughters with their earlier mates (Figure 4.4). Previous studies in *N. vitripennis* have found no advantage to increased male body size when males are alone or in competition (Chapter 3, Chapter 2; Appendix 2: Moynihan & Shuker, 2011, Burton-Chellew et al., 2007b). Here I have confirmed the previous findings of (Grant et al., 1980, Clark et al., 2010) that larger *N. vitripennis* males have larger sperm resources. This supports my proposed lower limit to male body size outlined in Chapter 2. A recent *N. vitripennis* study has shown that sperm limitation might be higher when males are in poor nutritional condition (Blaul & Ruther, 2011). Very few of the papers investigating sperm limitation of male insemination capacity have taken male body size into consideration, although the positive relationship between body size and sperm resources found here is consistent with studies in *Colpoclypeus florus* (Dijkstra, 1986); *Uscana semifumipennis* (Henter, 2004); *Dinarmus basalis* (Lacoume et al., 2006) and *Spalangia cameroni* (King & King, 1994).

The interaction between male body size and female mating sequence number on the number of daughters a female lays, could be due to sperm allocation differences. While individual spermatozoa are considered to be relatively cheap, a whole ejaculate may represent a significant proportion of a limited resource (Dewsbury, 1982), leading to the prediction that males should allocate their sperm-resources strategically. Across insects, males of different body sizes, have been shown to employ different strategies for ejaculate allocation, particularly when body size predicts mating frequency (reviewed in Wedell et al., 2002). Some of the best studied cues for strategic allocation are perceived risk of sperm competition and female quality (e.g. Gage & Barnard, 1996 and Bretman et al., 2009). While I controlled for the perceived risk of sperm competition by keeping males in isolation before mating and preventing access to other males during matings, I did not measure female body size, the best predictor of female condition. It would be reasonable to predict that males should allocate a larger ejaculate to big female, because the females have a higher fecundity as they lay more eggs (van den Assem & Jachmann, 1982), and could also store more sperm. However, as females were both sisters and chosen randomly for the place in the mating sequence, it is unlikely that larger females were consistently placed earlier in the mating sequence.

Nasonia vitripennis males are known to be proto-spermatogenic: they do not continue to synthesise sperm as adults (Hogge & King, 1975, Clark et al., 2010). They are wingless and do not disperse far from the patch, so once the females at their patch are mated, future matings are unlikely, and so it seems beneficial to have as much sperm ready as soon as possible (Boivin et al., 2005). Despite this, I found a positive correlation between age and seminal vesicle volume. This would suggest that either sperm continue to migrate to the seminal vesicles in the days after eclosion, or the seminal vesicles swell with some other, non-spermatozoa factor. As I did not count the number of spermatozoa in the seminal vesicles, I am unable to distinguish between these explanations without further experimentation. This finding contrasts with the negative effect of age on insemination capacity found in Chapter 3, which could be due to other age-associated factors. For instance, male age correlated with female age in that experiment, and so the pattern observed in Chapter 3 could be due to female age. The increasing seminal vesicle volume with age is probably of little importance in the wild, as males are unlikely to survive past a few days, and most of their matings will occur in the first 48 hours after eclosion. Note that in the sperm limitation experiment described here, male age was controlled to a 24 hour window to minimise the influence of this effect. Increasing sperm supplies with age have been found in *Anisopteromalus calandrae* (Bressac et al., 2009). This species does exhibit LMC along with a strong host-quality effect (Nishimura & Jahn, 1996), however the extent of male dispersal is unknown. If males could disperse to find other mates after mating their sisters at the natal patch, continued sperm production would be beneficial.

The importance of sperm-limitation to females in the wild falls into two categories. There are those females that have some but not enough sperm, and those that are constrained and have no sperm. Females could end up with no sperm reserves through failed copulations, like the twelve (out of 385) females observed here. Failed copulations could be caused by the genitals not engaging properly, female rejection of sperm, or males not having the sperm ready to ejaculate. Whichever the route, failed copulations are probably more common than is usually appreciated (Eberhard, 1996). There has been considerable discussion in the parasitoid literature about females with no sperm reserves, the so-called constrained females. Most of this has focussed on the prediction that foundresses should lay a more female-biased sex ratio when there are constrained females in their population (who produce only male offspring), through negative frequency-dependent selection (Godfray, 1990). Populations of *N. vitripennis* have been found to have a low incidence of constrained females (Beukeboom & Werren, 2000, Grillenberger et al., 2008), which would predict a sex ratio shift of less than 0.01 towards fewer sons (Godfray, 1990). The low reported incidence of constrained females combined with the small shifts on sex ratio observed in unconstrained females (King & D'Souza, 2004), means that the predictions of constrained sex allocation theory, if met, will have a small effect on the observed sex ratio in *Nasonia*. Consequently, constrained females will have low fitness, but will not exert a strong selection pressure on the rest of the population.

The partial-insemination which I have seen in this study is unlikely to occur very often in the wild. We are seeing the first signs of sperm limitation through a reduction in the number of daughters a foundress lays when her mate had 11 previous copulations. Wild estimates of *N. vitripennis* clutch size vary greatly, but the sex ratio is around 0.2-0.3 proportion male (Chapter 3). Even under the extremely female-biased sex ratios, a male is unlikely to receive more than 11 mating opportunities from these generally monandrous females (Holmes, 1974), unless the clutch sizes are very large.

This is the first study to investigate within-population differences in sperm resources and sperm-limitation and associate this with differences in the single-foundress sex ratio through fertility insurance. While I did find between-strain differences in sperm resource, measured through seminal vesicle volume, I did not find differences in sperm-limitation due to strain. My data further reinforces previous studies suggesting differences in single-foundress sex ratio are not due to differences in male insemination capacity. Instead, I did find more evidence of a body-size advantage in the form of higher seminal vesicle volume, and also more daughters sired from early-mated females.

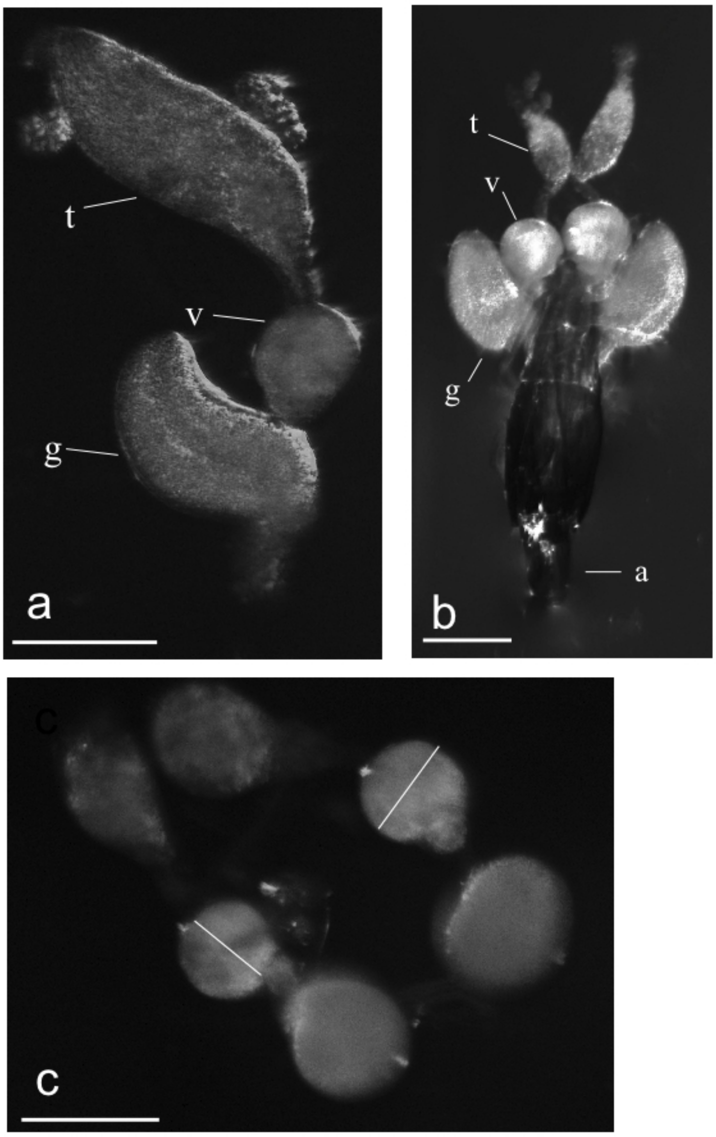


Figure 4.1 The dissected *Nasonia vitripennis* male reproductive tract. **1a**) One half of the reproductive tract. **1b**) The complete tract with aedeagus. **1c**) Both sides of the reproductive tract with the aedeagus removed. The white line on the left hand-side shows the position of the longitudinal measurement, and the line on the right hand side seminal vesicle shows the latitudinal measurement. In all images white scale bar in the bottom left hand side is 250µm, and the following labels apply (t) testes, (v) spheroid seminal vesicles, (g) accessory gland and (a) aedeagus.

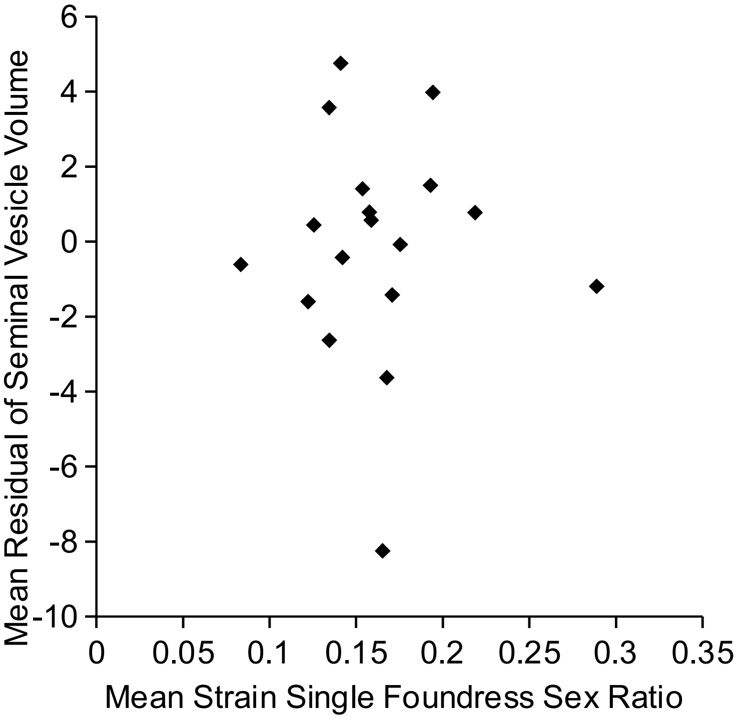


Figure 4.2: There is no relationship between seminal vesicle volume and the strain single-foundress sex ratio. The strain single-foundress sex ratios were established in the screen in Chapter 3, and are measured as proportion male. The mean seminal vesicle volume residual $\times 10^{-7}$ for each strain is presented. The residuals were calculated from a linear mixed effects model, of seminal vesicle volume explained by the fixed effects of hind tibia length cubed and male age in days after eclosion, and the random effect of block.

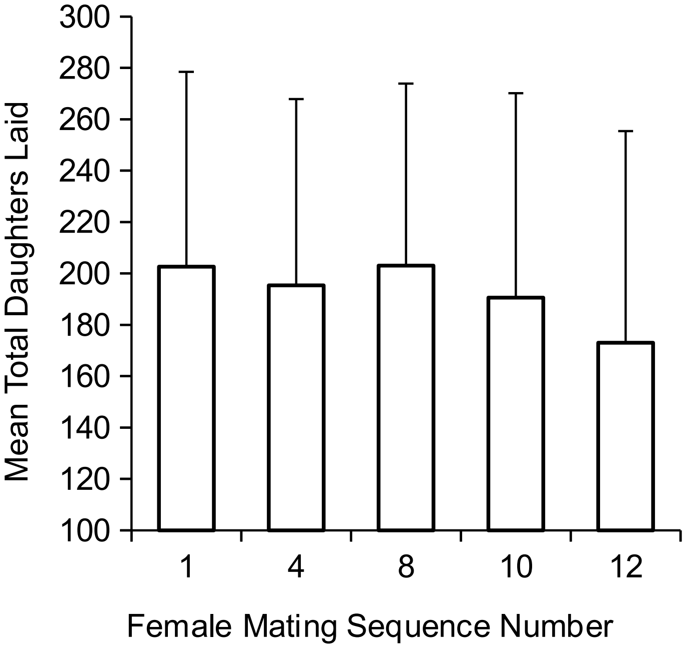


Figure 4.3: The number of daughters laid by a female decreases as the number of mates her male has previously inseminated increases. Sums across all hosts are shown. Error bars are one standard error of the mean.

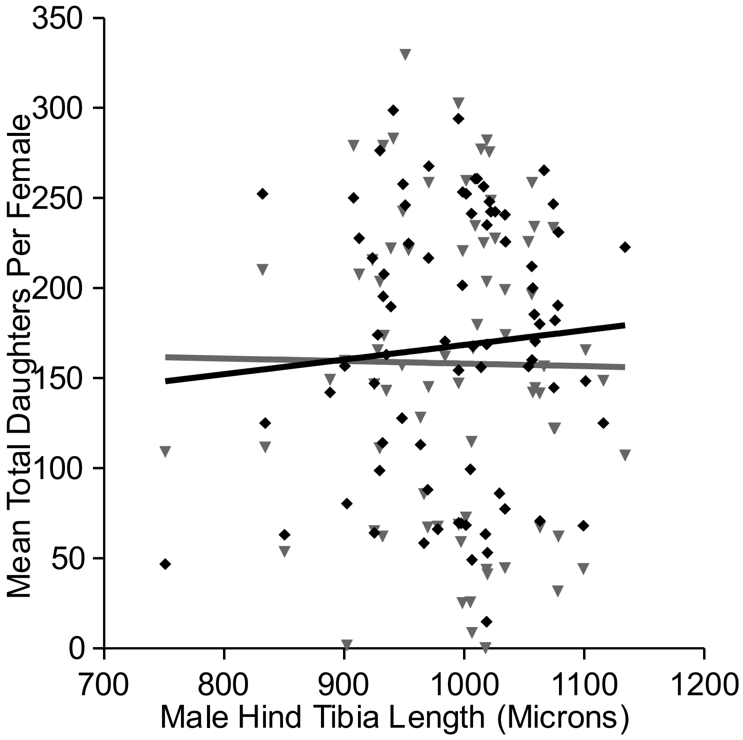


Figure 4.4: The number of daughters a female lays depends on both the size of the male who mated her, and the previous number of females he copulated. This graph shows the interaction between male body size and the female mating sequence number categorised as early (1st, 4th and 8th females) in black lines and data points, and late (10th and 12th females) in grey lines and data points. This early-late division is purely for graphical purposes, the data was analysed with female mating sequence number as a covariate. The y-axis shows the mean number of daughters laid by the females mated by that male within the female mating sequence category.

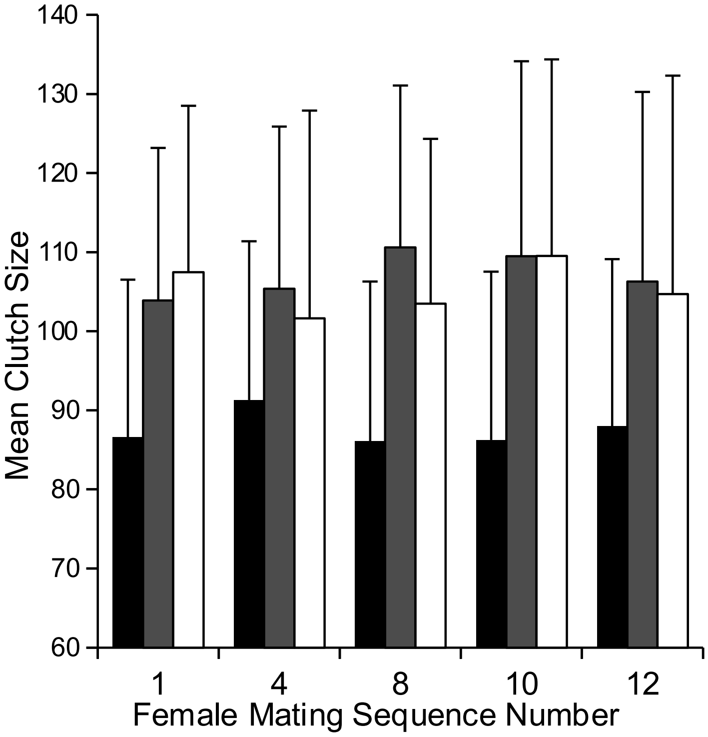


Figure 4.5: Clutch size increases with host oviposition bout, and female mating sequence number. Black bars are for the first host bout, the second host bout is in grey, and the third in white bars. Error bars are one standard error of the mean.

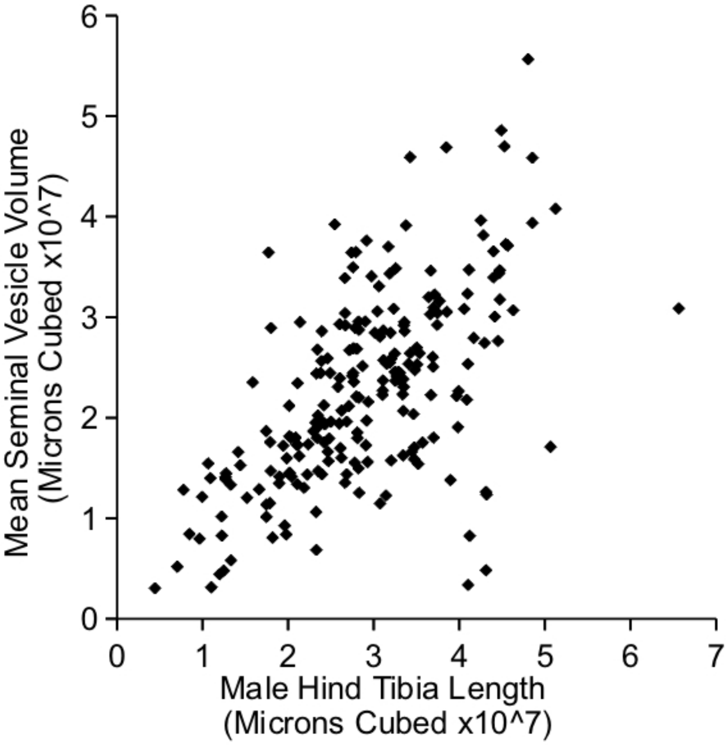


Figure 4.6: Seminal vesicle volume increases with the cube of body size. The measure of body size is hind tibia length measured in microns.

5. Variation in post-copulatory courtship is not associated with female re-mating rate in the parasitoid wasp *Nasonia vitripennis*

A version of this chapter is being prepared for submission to the Journal of Insect Behaviour. The authors will be: Rebekah Watt, Anna M. Moynihan, Taehyun J. Park, and David M. Shuker.

I designed and performed the experiment with RW, we contributed equally. TJP assisted in performing the experiment. The analysis and interpretation shown here was performed independently from RW.

Abstract

If there is any chance that a female will mate more than one male, selection will have acted on males traits to reduce the probability that females will re-mate, in order to protect their paternity. In the gregarious parasitoid wasp *Nasonia vitripennis* a second bout of courtship performed by the male after mating is thought to prevent female re-mating by switching off her receptivity. Here, I investigated how variation in this post-copulatory courtship and other courtship components was associated with female re-mating across nine strains of *Nasonia* from the same population. I found significant among-strain variation in female re-mating probability, but this was not associated with post-copulatory courtship duration. Instead, females that received more pre-copulatory courtship on their first mating were less likely to re-mate. I interpret this as females having an overall level of receptivity, and those with low receptivity require more courtship in the first mating, and then are unlikely to re-mate on the second trial. Rather than the duration of post-copulatory courtship being key, instead there could be a threshold amount of post-copulatory courtship required to reduce receptivity, perhaps related to the release of male pheromones.

Keywords: receptivity, post-copulatory courtship, polyandry, sexual conflict, parasitoid wasp.

Introduction

Sexual and natural selection have acted on the ability of each of the sexes to maximise the number and quality of their offspring, leading to many differences between the two sexes, beginning with anisogamy (Trivers, 1972). This has often given rise to different trait optima for males and females, resulting in sexual conflict (Parker, 1979, Chapman et al., 2003). The two sexes are in conflict over a wide-range of traits including mating duration, sex allocation, parental investment, and the number of mates (reviewed by Arnqvist & Rowe, 2005).

If the balance of costs and benefits to additional mates is different for the two sexes, they will “disagree” in evolutionary terms on the optimal number of mates. Females can incur costs to mating through lost opportunities for feeding, energetic costs or damage during mating itself (Chapman et al., 1995, Daly, 1978, Bell & Koufopanou, 1985, Fowler & Partridge, 1989, Arnqvist & Nilsson, 2000). Females are thought to increase their fitness from multiple matings through direct benefits like access to resources, or indirect benefits like genetically fitter offspring (good-genes), more genetically attractive offspring (Fisherian sexual selection) or through a genetically diverse or compatible brood (Thornhill & Alcock, 1983, Andersson, 1994, Colegrave et al., 2002). For males however, providing the risks to mating are not large, and there is no paternal care, each additional mating could lead to a gain in paternity (Bateman, 1948). If there is any chance that a female may re-mate and reduce the fitness of her original mate, selection will favour traits to protect his paternity (Simmons, 2001).

Sperm competition occurs whenever the ejaculates of more than one male compete to fertilise a female's eggs (Parker, 1970). Male adaptations to improve their chance of success in this competition fall into two categories: offensive and defensive. Offensive adaptations are concerned with reducing the success of males that have previously inseminated the female a male copulates, for example sperm displacement (Arnqvist, 1988), or damaging a rival male's sperm (den Boer et al., 2010). Defensive adaptations aim to prevent females re-mating or using sperm from subsequent males, and examples include mate guarding (Arnqvist, 1988), mating plugs (Baer et al., 2001) and physiological effects on female receptivity (Chapman, 2001).

Females may also have adaptations to manipulate the outcome of sperm competition to maximise their fitness (Eberhard, 1996). One method thought to be especially powerful is cryptic female choice where females can bias which sperm fertilises their eggs (Eberhard, 1996, Thornhill, 1983). The mechanisms through which this is achieved are rarely elucidated despite a wide range of taxa where cryptic female choice has been proposed (Eberhard, 1996, Rosengrave et al., 2008, Pizzari & Birkhead, 2000, Qazi, 2002). An exception to this is the red flour beetle *Tribolium castaneum*: during copulation males rub the female's elytra with the tarsi of his legs, which influences his success in sperm competition (Edvardsson & Arnqvist, 2000). Edvardsson and Arnqvist (2000) removed the tarsi of some males and showed that while this manipulation did not influence the number of sperm transferred, it did reduce the fertilisation success of the males. Moreover, they found that the rate of leg-rubbing increased fertilisation success for the unmanipulated males only, therefore this male copulatory behaviour is used by females as a cue to bias paternity.

Here, I have investigated female re-mating rate and male adaptations to prevent female re-mating in the wasp *Nasonia vitripennis,* a gregarious parasitoid of large dipteran fly pupae. When a brood of wasps emerge as adults, they mate at the natal patch before the females disperse, leaving the wingless males behind. The population and patch sex ratios are usually female biased in the wild (0.2 - 0.3 proportion male, Burton-Chellew et al., 2008, Grillenberger et al., 2008, Molbo & Parker Jr, 1996, Chapter 3) as a result of facultative sex allocation under Local Mate Competition (LMC) (Hamilton, 1967). Like many parasitoid wasps, *N. vitripennis* is mostly monandrous (Ridley, 1993, Gordh & DeBach, 1978), but double matings have been recorded in the wild (4% Grillenberger et al., 2008) and the lab (3% Grant et al., 1980; 53% Holmes, 1974; 8.8% Chapter 2) resulting in mixed-paternity broods. It has also been shown that polyandry will evolve under lab conditions (Burton-Chellew et al., 2007a), and has a heritable genetic component (Shuker et al., 2007a).

While the existence of polyandry in *N. vitripennis* is well established, the costs and benefits to males and females of polyandry are less well investigated. Female *N. vitripennis* could benefit from multiple matings by increasing their sperm resources. Sperm-limitation is known to be a problem in many parasitoid species including *N. vitripennis* (Chapter 4, Beukeboom & Werren, 2000, Grillenberger et al., 2008), however there is no evidence that females with low sperm resources are more likely to mate again, since sperm-depleted males successfully switch-off female receptivity (van den Assem & Visser, 1976, Barrass, 1964). Alternatively, the multiple cytoplasmic factors that can cause incompatibility between males and females in *N. vitripennis* add to the potential benefits of multiple mates (Breeuwer & Werren, 1995, Bordenstein et al., 2001, Beukeboom & Werren, 1993). In terms of costs of re-mating, a preliminary investigation into sexual conflict in *Nasonia* species failed to find a survival cost to mating (Geuverink et al., 2009). While female re-mating is at a low frequency in *N. vitripennis*, males nonetheless have some incentive to protect their paternity, and as such we might expect to find adaptations in males to reduce female re-mating. The pattern we see may be the result of successful and pervasive male defensive sperm competition adaptations.

Adaptations for the prevention of female re-mating have been observed in many hymenopteran species (reviewed in Chapter 6 & Kraaijeveld, 2009). In *Nasonia vitripennis*, males initially court females after mounting, performing a series of “head-nod” displays while positioned over the female's head and antennae, releasing pheromones from their mouthparts (van den Assem et al., 1981, van den Assem et al., 1980). After successfully copulating, the male *N. vitripennis* moves back to this initial courtship position over the female's antennae and performs another series of head-nod cycles (Whiting, 1967, Barrass, 1960). Behavioural observations and experiments that prevent it have suggested that this post-copulatory courtship is key to males preventing female re-mating and protecting paternity in *Nasonia vitripennis* (van den Assem & Visser, 1976). Post-copulatory courtship has also been demonstrated to reduce female receptivity in several other parasitoid wasp species (King & Fischer, 2005, Gordh & DeBach, 1978, Allen et al., 1994, King & Fischer, 2010) and the bee *Centris pallida* (Alcock & Buchmann, 1985). Moreover, sperm-depleted *N. vitripennis* males can successfully prevent females they have copulated with from re-mating, suggesting that the presence of sperm is unlikely to be the signal (van den Assem & Visser, 1976, Barrass, 1964), although we know little about depletion or otherwise of other ejaculate components in *Nasonia* that may influence receptivity (Gillott, 2003). The reduction of post-mating receptivity has some species-specificity within the *Nasonia* genus: conspecific males were better at preventing female re-mating than heterospecific males for *N. giraulti* females, but males of both species reduced receptivity with equal success in *N. vitripennis* females (Geuverink et al., 2009).

In order to investigate the influence of differing durations of courtship components on the probability of a female re-mating, I screened nine genotypes of *N. vitripennis* for variation in female re-mating rate and recorded the natural courtship patterns of the first and second males with each female. Following previous work on the role of post-copulatory courtship in *Nasonia* and other insects, I predict that the longer the post-copulatory courtship a female receives from her first mating, the less likely she is to take a second mate. Understanding the influence of courtship behaviours on female re-mating probability may shed light on the strategies employed by male *N. vitripennis* to gain and protect paternity.

Methods

Wasps and pre-treatment

My study species is *Nasonia vitripennis* (Hymenoptera: Pteromalidae), a gregarious parasitoid of large dipteran pupae such as the Calliphoridae and Sarcophagidae (Whiting, 1967). The host species for stock rearing and the experiment are *Calliphora vicina* pupae. The wasps were cultured at 25°C in incubators with a 16L:8D cycle throughout stock maintenance and experiments.

I used nine strains from the Cornell group used in Chapters 3 & 4, that were collected as iso-female lines from nest boxes in Cornell by Professor Leo Beukeboom, NY: 3, 8, 12, 18, 19, 22, 23, 24 and 28. The experiment was performed in five blocks, to ensure that age of experimental subjects could be reliably controlled. Previous experiments have shown that polyandry can evolve in the lab (Burton-Chellew et al., 2007a) and so in order to control for generation effects within in each block, all wasps from all nine strains were drawn from the same lab generation (24, 28 and 30).

To control for any maternal or host effects, I put the wasps through a preparatory generation. I took females that had spent two-days mating in a stock tube after adult emergence and isolated them in a 75×10mm glass tubes with cotton wool bungs. The isolated females were given a single host for 24 hours, which they feed on to gain protein to mature their eggs (Rivero & West, 2005). The host was replaced with a small piece of filter paper soaked in honey-water for the next 24 hours. I then gave the females one host to oviposit on for six hours. The female was removed from the tube and the parasitised hosts incubated for ten days.

In order to acquire virgins for the experiment I opened the hosts after these ten days and removed the wasp pupae. Two males and two females from each host were isolated in fresh vials and I recorded the ID of their natal host in order to avoid brother-sister matings, and the use of two males or females from the same brood. The sexed wasp pupae were returned to the incubator until they eclosed as virgin adults four days later.

Behavioural Observations

Wasps had an hour on the lab bench to acclimatise before testing. Pairs were set up so that one-day-old virgin females were paired with a one-day-old virgin non-brother male from the same strain. The male and female were observed in a mating arena of a glass tube 50×10mm under a binocular dissecting microscope at ×20 magnification.

A clean glass tube and cotton wool bung was used for each observation. The pairs were observed until they had copulated and the male dismounted, or until ten minutes had passed. Genital contact was observed through the binocular dissecting microscope to confirm copulation. If a courtship was in progress at the end of the ten minutes, I allowed it to continue until the male had dismounted.

The courtship and mating behaviour of *N. vitripennis* has been well described (Barrass, 1961, van den Assem & Visser, 1976, van den Assem et al., 1981, van den Assem & Werren, 1994, van den Assem & Vernel, 1979, Jachmann & van den Assem, 1996). After approaching and mounting a female, the male begins his courtship positioned over her antennae. He performs a series of stereotyped head nod cycles, each of which begins with a pheromone emission from his mouth-parts (van den Assem et al., 1980, Ruther et al., 2010). The female drops her antennae and opens her abdomen to expose her genital pore, which is the signal of her receptivity to the male. The male backs up the female, makes genital contact and the pair copulate as he transfers his ejaculate. After copulation the male returns to his initial position over the female's antennae to perform another bout of courtship: the post-copulatory courtship. The female will drop her antennae to signal receptivity again, only this time the male dismounts.

While females of *Nasonia* are more likely to signal receptivity to con-specific than a male from another (Bordenstein et al., 2000), there is no evidence for pre-copulatory female choice within species (e.g. Shuker et al., 2006c, & see results).

I recorded the time that the following events occurred in seconds after the male and female were introduced into the mating arena: mounting; the beginning of courtship; the beginning of copulation; the beginning of post-copulatory courtship; dismounting (Figure 5.1). Mounting and the onset of courtship occurred within 1s of each other, therefore I used a single time point. Courtship duration was used as a proxy for the number of head nod cycles a male performs (van den Assem & Visser, 1976, van den Assem et al., 1981). If a courtship bout was in progress at the end of the ten minutes, I observed them until the male dismounted. Repeated bouts of courtship not resulting in copulation were allowed and recorded.

The females that mated on day one were given another male 24 hours later, for the second test. The second male is a two-day-old virgin male that is neither the brother of the female nor her previous mate. On the second test I carried out the same observation protocol as the previous day.

A total of 256 females were tested over five blocks, by three experimenters. Each experimenter had a random sub-sample of the wasps for testing that block. Each female wasp was observed by the same experimenter for both tests. The number of replicates per strain tested ranged from 19 to 36, with a mean of 31.67 (SE = 7.91).

Of the 256 females tested, only 200 were included in the analysis. Nine females died between the first and second trial. Eighteen females did not mate on the first trial: thirteen of which were courted but never signalled receptivity, the remaining five were never courted by a male. This shows the low pre-copulation rejection rate of *N. vitripennis* females. Two females were injured during transfer into the trial arena on the second day. Twenty-seven females were not courted by the male on the second trial. The number of females included in the analysis per strain ranged from 9 to 36, with a mean of 22.22 (SE = 7.71) females per strain.

Analysis

The behavioural observations allowed us to calculate the following traits of interest across the two trials: latency to mount (s); pre-copulatory courtship duration (s); copulation duration (s); post-copulatory courtship duration (s); and number of courtship bouts (see Figure 5.1).

Initial inspection of the data detected one female (from strain COR19 in the fifth block) as a potential outlier as she received 114 seconds of pre-copulatory courtship on trial one. When all the analyses described below were repeated with the potential outlier removed, the significance of none of the terms in none of the models was altered. Therefore, the analyses presented here include this data point.

On the second day males often mounted, courted and dismounted females several times without her signalling receptivity. Females also sometimes required more than one courtship bout before signalling receptivity. As a result of this, the courtship measures for the second trial are pooled into a “total courtship” measure across multiple mounts. For both trials the variable “handling time” was also created as a measure of the total time it takes for a male to process a female in terms of the sum of pre-copulatory courtship, copulation, and post-copulatory courtship.

Differences between the duration of courtship components between the two trials were investigated. For the pre-copulatory components latency to mount, pre-copulatory courtship duration and bout number I used all the females in the analysis. Latency to mount was analysed in a Linear Mixed Effects (LME) model fitted using Maximum Likelihood (ML) methods after a square-root transformation. The fixed effects of trial (1 or 2) and if the female mated on the second trial (re-mating) were included in the model, along with the random effects of female nested within experimenter nested within strain nested within block. This same model was used for courtship duration, however courtship duration required a natural log transformation. The significance of each term was tested by Likelihood Ratio tests, comparing the full model to a model with the term of interest removed. The full model, fitted using

Restricted Maximum Likelihood methods (REML) was used for reporting parameter estimates. The number of courtship bouts used a generalised mixed effects model, with a poisson distribution. The fixed and random effects were the same as those described for the latency to mount and courtship duration models. The significance of model terms was tested using Chi Square tests on the change in deviance between the full model and one with the term of interest removed.

The difference in copulation duration and post-copulatory courtship between the two trials was analysed using only the 111 females that mated on both days. The difference in copulation duration between trials was modelled using an LME with ML methods, after a square-root transformation. The fixed effect of trial was included in the model along with the same random effects as above. The same model was fitted for post-copulatory courtship duration, which required a log-transformation. The significance of terms of interest and the parameter estimates were calculated using the likelihood ratio test.

To test the variation in courtship components due to differences between the strains, I fitted models that contained just the random effects of female within experimenter within strain within block to each of the courtship components, using the subsets of data and transformations described above. In order to test for the effect of strain without pseudoreplication I created a new random effect, strain-block, that generated a new factor level for each strain-block combination. Likelihood ratio tests or Chi

Square tests were then used to compare the model with the fully nested random effects and that containing strain-block, as appropriate.

Whether or not a female re-mated, was modelled as a binomial trait in a generalised linear mixed effects model. The random effects of experimenter within strain within block were included in the model, along with the fixed effects of the following courtship components: latency to mount trial one; courtship duration trial one; copulation duration trial one; post-copulatory courtship duration trial one; latency to mount trial two; courtship duration trial two. The significance of the random effect of strain was tested using the creation of a new random effect, strain-block as previously described.

Subsequent analyses generated new fixed effects. In order to test for the potential effect of the total courtship received on trial one, the sum of trial one courtship and post-copulatory courtship duration was fitted instead of their separate effects. Handling time was also investigated, in this case the sum of courtship, copulation duration and post-copulatory courtship on the first day was fitted in place of their separate effects. The same random effects were modelled as before, and the significance of terms of interest was tested using Chi-squared tests on the change in deviance as described previously on ML models. All statistical analysis was performed in R (Ihaka & Gentelman, 1996, R Development Core Team, 2008).

Results

The average male took 83.3 (SE = 6.85) seconds to mount the female on her first trial. After 14.46 (SE = 0.81) seconds of courtship in 1.01 (SE = 0.007) bouts, copulation occurred and took 15.83 (SE = 0.51) seconds and was followed by 16.36 (SE = 0.72) seconds of post-copulatory courtship. On the second trial the male mounted the female after 113.34 (SE = 7.23) seconds, and performed 89.73 (SE = 5.84) seconds of courtship in 2.03 (SE = 0.12) bouts. Overall 55.45% of females mated again on the second trial. Of those that did mate, the mean copulation duration was 16.04 (SE = 0.33) seconds, and the post-copulatory courtship duration was 20.66 (SE = 4.19) seconds.

There were a number of differences in courtship and copulation behaviour between the two trials. First, males took longer to mount females in the second trial (Latency to mount on trial one = 83.34 (SE = 6.85) seconds, trial two = 113.34 (SE = 7.23) seconds; LR test, L.Ratio = 12.09, $p = 0.0005$). Whether or not a female re-mated in trial two was not associated with latency to mount (L.Ratio = 0.76, $p = 0.38$), and this did not differ across the two trials (L.Ratio = 1.48, $p = 0.22$).

Second, pre-copulatory courtship duration was significantly longer in the second trial (LR test, L.Ratio = 339.3, $p < 0.0001$). Females that re-mated in trial two received less pre-copulation courtship in both trials than those that did not re-mate (L.Ratio = 9.92, $p = 0.0016$) (Figure 5.2). There was a significant interaction between re-mating and trial: the increase in pre-copulatory courtship on trial two was higher for the females that did not re-mate (L.Ratio = 55.58, $p < 0.0001$) (Figure 5.2).

Similarly, there were fewer bouts of pre-copulatory courtship on the first trial (Chi Sq test = 104.3, $p < 0.0001$) and for females that re-mated in trial two (Chi Sq test = 11.45, $p = 0.0007$). All females regardless of re-mating received similar number of courtship bouts during trial one (Figure 5.3), leading to significant interaction between re-mating status and trial (Chi Sq test = 34.99, $p < 0.0001$). Therefore both the duration of pre-copulatory courtship and the number of bouts varied primarily due to female willingness to mate in trial two.

In contrast, copulation duration and post-copulatory courtship duration did not vary across trials one and two (Copulation duration: LR test, L.Ratio = 3.66, $p = 0.056$; Post-copulatory courtship: L.Ratio = 0.006, $p = 0.94$).

Generally the nine strains did not differ much in their courtship behaviour. There was no significant variation among strains in latency to mount (LR test, L.Ratio = 2.4, $p = 0.12$), pre-copulatory courtship duration (L.Ratio < 0.001, $p > 0.99$), the number of courtship bouts (Chi Sq test < 0.001 $p > 0.99$) or post-copulatory courtship duration (LR test, L.Ratio = 1.83, $p = 0.18$). However the strains did significantly vary in terms of copulation duration (L.Ratio = 4.98, $p = 0.03$) (Figure 5.4).

Contrary to my prediction, female re-mating in trial two was not associated with the duration of post-copulatory in trial one (*b* = 0.01 ± 0.03, Chi Sq test = 0.16, *p* = 0.69). Similarly female re-mating was not associated with latency to mount on day one (*b* = 0.005 ± 0.003, Chi Sq test = 1.86, *p* = 0.17) or copulation duration in trial one (*b* = -0.07 ± 0.06, Chi Sq test = 1.57, *p* = 0.21). First, the longer the pre-copulatory courtship in trial one and in trial two the less likely a female was re-mate (trial one: *b* = -0.13 ± 0.05, Chi Sq test = 9.71, *p* = 0.0018; trial two *b* = -0.064 ± 0.011, Chi Sq test = 98.61, *p* < 0.0001) (Figure 5.5). The pattern in trial two is probably due to males continuing to mount unreceptive females for longer in this trial. Second, females that re-mated were mounted more rapidly in trial two (latency to mount in trial two: *b* = -0.014 ± 0.004, Chi Sq test = 16.61, *p* < 0.0001) (Figure 5.6). There was also significant among-strain variance in polyandry (ranging from less that 10% to 100% of females re-mating; Chi Sq test = 12.26, *p* = 0.0004) (Figure 5.7).

Using the sum of courtship from trial one paints a similar picture. The sum of pre- and post-copulatory courtship a female received on the first trial was significantly associated with female re-mating in the second trial: the less courtship needed in the first trial, the more likely a female was to be polyandrous (*b* = -0.051 ± 0.020, Chi Sq test = 9.66, *p* = 0.0019) (Figure 5.8). In this model, latency to mount in trial two remained significant (*b* = -0.010 ± 0.0028, Chi Sq test = 16.85, *p* < 0.0001), as did pre-copulatory courtship duration in the second trial (*b* = -0.049 ± 0.008, Chi Sq test = 98.97, *p* < 0.0001). Latency to mount and copulation duration in the first trial were again not significantly associated with female re-mating (Latency to mount Chi Sq test = 1.82, $p = 0.18$; Copulation duration Chi Sq test = 1.47 $p = 0.23$).

Handling time was not associated with female re-mating however (Chi Sq test = 3.39, $p = 0.065$). As with previous analyses, the latency to mount on the first trial was not significant (Chi Sq test = 1.24, $p = 0.28$), but the latency to mount on the second trial ($b = -0.011 \pm 0.003$, Chi Sq test = 12.17, $p = 0.0005$) and the pre-copulation courtship on the second trial were significantly associated with re-mating ($b = -0.057 \pm 0.010$, Chi Sq test = 99.59, $p < 0.0001$).

Discussion

Males in many species influence the patterns of future female receptivity during mating, either through their behaviour or the production of pheromones or molecules in their ejaculates (Gillott, 2003, Eberhard, 1996, Chapman et al., 1995, Wolfner, 2002, Moore et al., 2003). In *Nasonia vitripennis* previous studies suggested that male post-copulatory courtship is a signal associated with female moving from receptivity (van den Assem & Visser, 1976). Here I explored whether variation in the duration of post-copulatory courtship was associated with female re-mating rate, using nine iso-female strains of wasps from the same Cornell population. I found among-strain variation in the proportion of females re-mating. This confirms previous evidence for genetic variation in this trait in what is a mostly monandrous species (Shuker et al., 2007a, Burton-Chellew et al., 2007a). The amount of post-copulatory courtship a female received after her first mating did not influence the probability of her mating again, contrary to my prediction. Other courtship components did correlate with female re-mating: females that signalled receptivity earlier in their first trial were also more likely to mate in their second trial. Similarly, the females that were mounted and courted more rapidly in their second trial were also the females more likely to be receptive and re-mate. These observations suggest two things. First, initial receptivity may be correlated with the propensity to be polyandrous, perhaps through an overall higher level of receptivity, suggesting a shared underlying mechanism. Second, unreceptive females may be less attractive, or better at avoiding males in subsequent encounters, leading to a longer latency to be mounted and courted. Unsurprisingly, pre-copulatory courtship in the second trial is associated with re-mating by females, as males fruitlessly keep courting unreceptive females for longer than males able to mate with their polyandrous partners.

Although post-copulatory courtship has previously been shown to switch-off female receptivity in *Nasonia vitripennis* (van den Assem & Visser, 1976), my study was unable to link the length of that post-copulatory courtship with female polyandry. There is, however an important difference between my study and that of (van den Assem & Visser, 1976): I measured variation in post-copulatory courtship, where as van den Assem & Visser (1976) prevented post-copulatory courtship by removing the male as soon as genital contact was broken. Therefore, it might be that the presence of some small amount of post-copulatory courtship is crucial, and most of the males in my study provided that threshold amount and so successfully reduced female receptivity. This does leave unanswered questions as to why males provide more than some threshold level of post-copulatory courtship. It might be that given the generally low levels of polyandry in the wild (perhaps associated with limited encounters with males once females have dispersed from the natal patch) there is rather weak selection on male post-copulatory courtship. However, it may be that duration *per se* does not capture the important aspects of this interaction: males may remain with a female not necessarily because they need more courtship to become unreceptive, but instead stay with females until that behavioural switch has taken place (signalled by females with their second lowering of their antennae). In this way it may represent post-copulatory guarding. As such the length of post-copulatory courtship may be as much driven by females as males. The timings and changes in female gene expression associated with the switch from receptivity to unreceptivity are currently under investigation.

My data on latency to mount and court in the second trial suggests that perhaps females on the second trial are less attractive because they are one day older or no longer virgins, supporting previous studies with *N. vitripennis* (Jachmann & van den Assem, 1996, Leonard & Boake, 2008). Contact with a male can render a female unattractive through changes in her odour in several other insect species (King & Fischer, 2005, Ayasse et al., 2001). If such a pheromonal tag exists, it could build up with time spent in contact with a male, in which case those females courted for longer should smell less attractive, and so the male would take longer to mount the female. The female sex pheromone is already characterised and known to be attractive to males (Steiner et al., 2006). Olfactory experiments could test this, using a bioassay to compare the attractiveness of females that have been courted only, courted and copulated, and those that were courted, copulated, and received post-copulatory courtship.

In my experiment, even the females that did re-mate in their second trial required more pre-copulatory courtship than virgin females through both increased bout numbers and bout durations (Figures 5.2 & 5.3), in line with the findings of Leonard & Boake (2008). This result is contrary to that of van den Assem & Visser (1976) who found that *N. vitripennis* males spent less time courting non-virgin females. This suggests that females do experience a drop in receptivity after mating, even those that do re-mate. My finding here is consistent with patterns found in other parasitoid wasps. For instance, mated female *Dinarmus basalis* required more courtship on their second mating (Chevrier & Bressac, 2002), and males of the parasitoid wasp *Aphytis melinus* will persistently court a mated un-receptive female, suggesting that he cannot detect her mated status (Allen et al., 1994).

While the associations between latency to mount and pre-copulatory courtship in the second trial and re-mating appears to be a consequence of female reduced receptivity, the influence of pre-copulatory courtship during the first trial could affect female re-mating rate. The longer a female is courted on the first trial the less likely she is to re-mate (Figure 5.5), consistent with previous findings in *N. vitripennis* (Leonard &

Boake, 2008). Leonard and Boake (2008) hypothesised that the increased pre-copulatory courtship of the first male increases the amount of time the second male has to court to induce receptivity. While I cannot determine the causality of this relationship from my experiment, there are several hints that this may not be case. We know that females who have been courted but not copulated are still receptive to mating more than 24 hours later, suggesting pre-copulatory courtship does not play a role in switching-off receptivity (van den Assem & Visser, 1976). Pre-copulatory courtship does influence some post-mating responses, for instance by reducing female attraction to the male sex-attractant pheromone (Ruther et al., 2010). It is possible that longer courtship reduces the probability of a female re-mating, but perhaps it is most likely that females who are less likely to re-mate the second day (i.e. generally have lower receptivity) require more courtship on the first day, as I observed (Figure 5.2).

In addition to among-strain variance in polyandry, I also observed significant among-strain variation in copulation duration. This finding may be relevant to insemination ability and sperm limitation because copulation duration has been used as an assay for the amount of sperm a female receives in several species, but has yet to be explicitly tested in parasitoids. Copulation duration and ejaculate transfer may not simply correlate with the number of sperm, but also the amount of seminal fluid. Seminal fluid has been suggested to be associated with other potential manipulations of female physiology and behaviour as well receptivity (Gillott, 2003, Chapman, 2001). For instance, *Nasonia vitripennis* is predicted to be in sexual conflict over sex allocation: females are selected to follow the predictions of Local Mate Competition, whereas due to their haplodiploidy, males are only related to the daughters, not the sons in the brood and so gain more fitness from an all-female brood (Appendix 1: Shuker et al., 2009). Male *N. vitripennis* have been shown to influence sex allocation behaviour of the female they mate and one possible (although currently untested) mechanism is through components of the ejaculate (Shuker et al., 2006c).

In this study I have found further evidence of genetic variation in the probability that a female will re-mate. Variation in the amount of post-copulatory courtship was not associated with re-mating, despite previous studies which prevented post-copulatory courtship suggesting it was key to switching off female-receptivity. Here I have proposed that females have different overall receptivities, and that those that have lower receptivity in general require more pre-copulatory courtship on their first mating, and are then less likely to re-mate. This suggests that post-copulatory courtship may still be a defensive adaptation to sperm-competition, however my data provide no explanation for the variance in this trait. My data also hint that males can discriminate between virgin and mated females using olfactory cues which warrant further study. The mechanisms of the receptivity-switch in *Nasonia* are currently being studied at the gene-expression level (R. Watt and D.M. Shuker personal communication), which may provide more insights into the role of post-copulatory courtship as a defensive adaptation against sperm-competition.

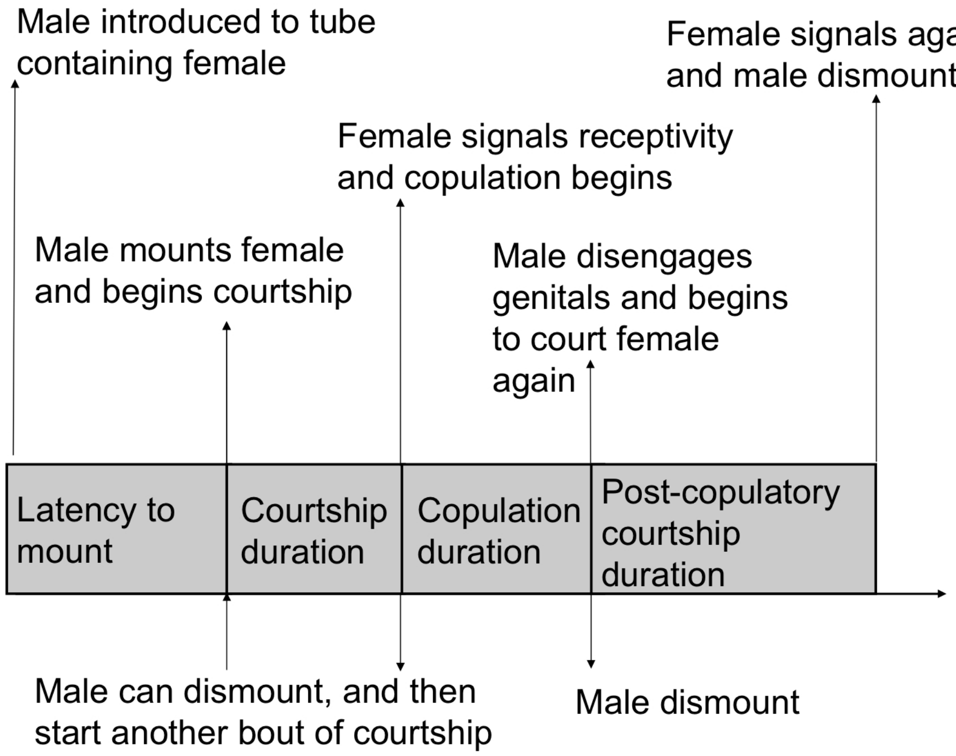


Figure 5.1: The pathway of copulation components in *Nasonia vitripennis*. The grey boxes show the courtship components measured in the experiments. Arrows pointing up from this time line show the criteria used to break the copulation into the four components. Arrows pointing downwards demonstrate how there can be several bouts of courtship before copulation, or indeed no copulation at all.

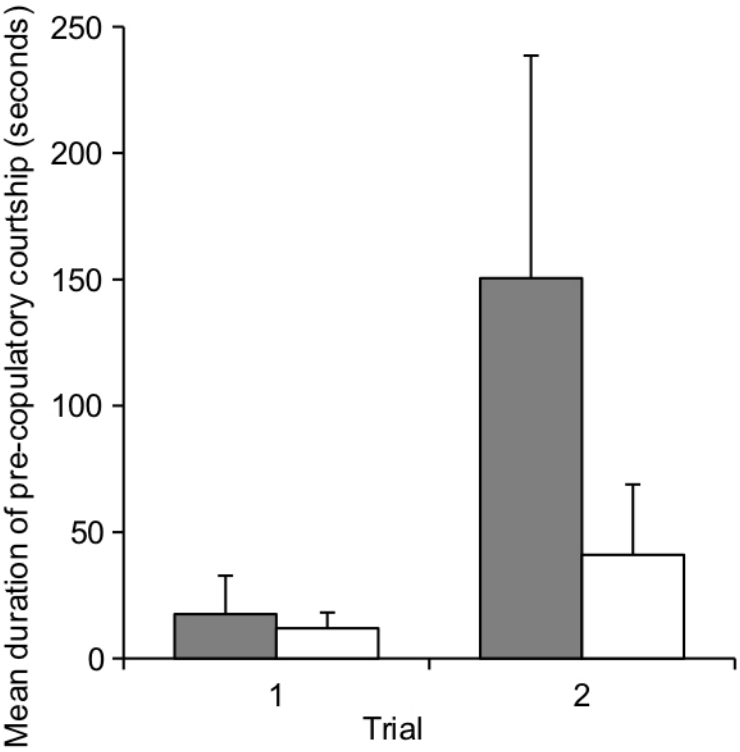


Figure 5.2: The interaction between courtship duration by trial day and re-mating on the second trial. Error bars are ± one standard error. Polyandrous females who mated on the second day are white bars, whereas those females that did not mate on the second day are in the grey bars.

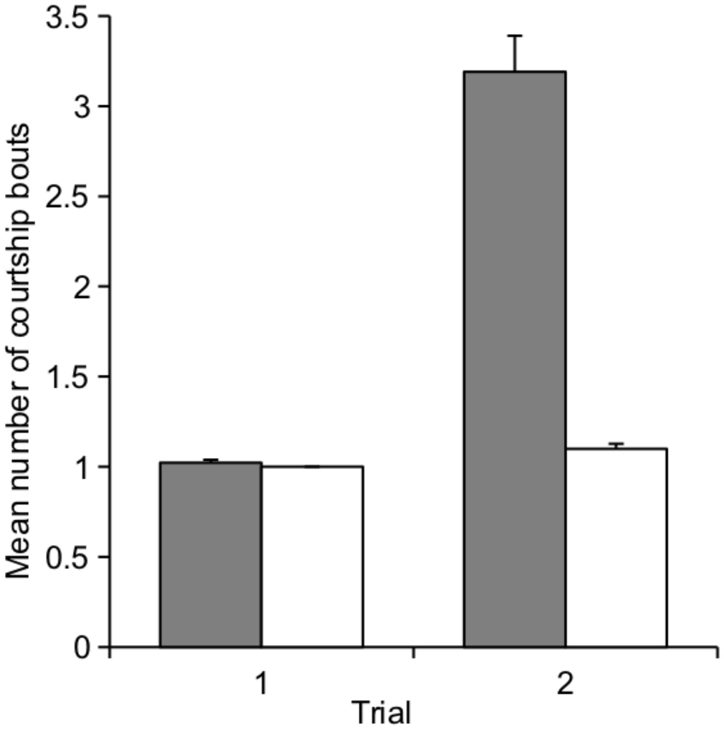


Figure 5.3: Number of courtship bouts varies across the two trials and whether or not the females re-mates. Error bars are ± one standard error. Grey bars represent females that did not mate on the second trial, whereas white bars represent those females that did mate on the second trial.

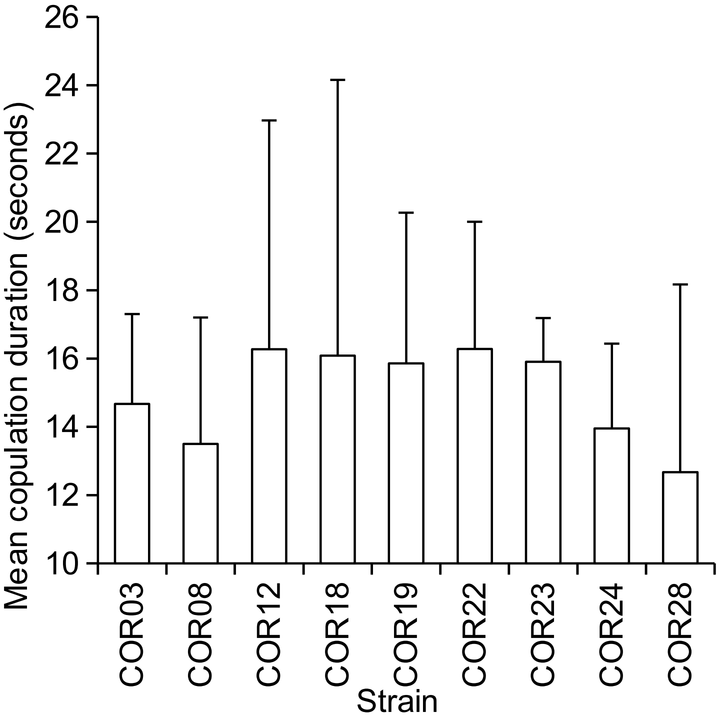


Figure 5.4: The variation in copulation duration from the first trial between the nine iso-female strains. Error bars are ± one standard error of the mean.

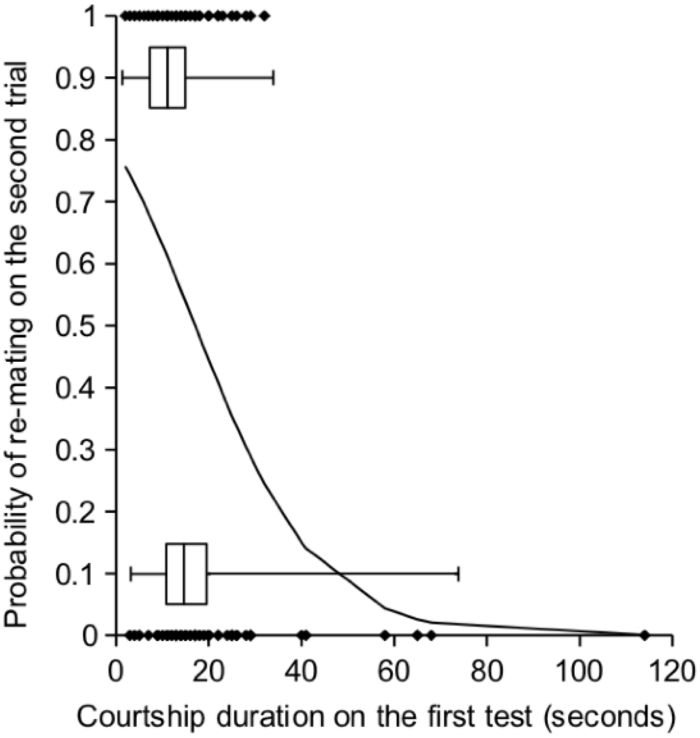


Figure 5.5: The length of pre-copulatory courtship a female receives during the first trial decreases her probability of mating on the second trial. The line is the logistic regression. The box and whisker plots show the distribution of the data points for females that did re-mate on the second trial (upper plot) and those that did not (lower plot). The box shows the range of the first and third quartile, with the median marked as a bar. The ends of the whiskers represent the full range of observations, excluding the female that received 114s of courtship.

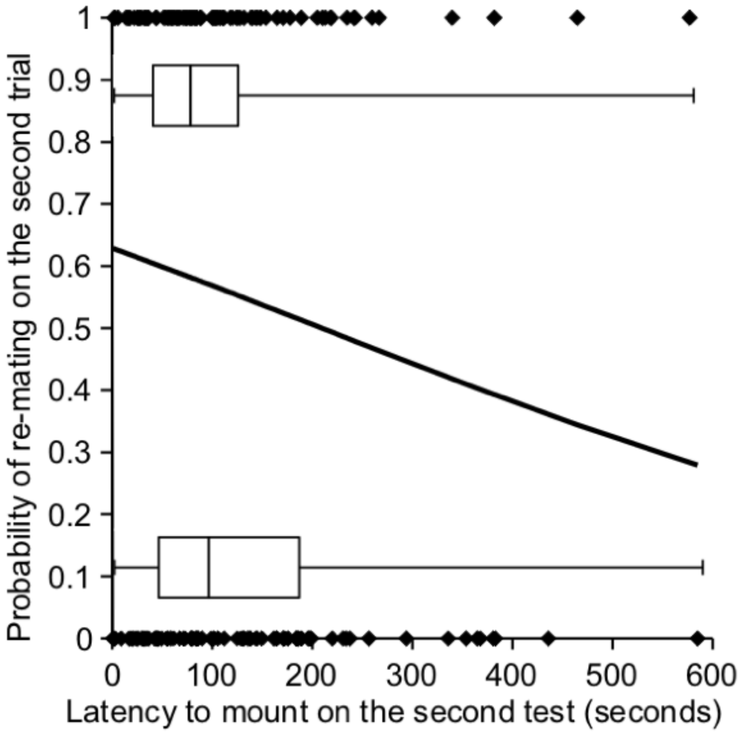


Figure 5.6: Polyandrous females are mounted sooner in the second trial. The line is the logistic regression. The box and whisker plots show the distribution of the data points for females that did re-mate on the second trial (upper plot) and those that did not (lower plot). The box shows the range of the first and third quartile, with the median marked as a bar. The ends of the whiskers represent the full range of observations.

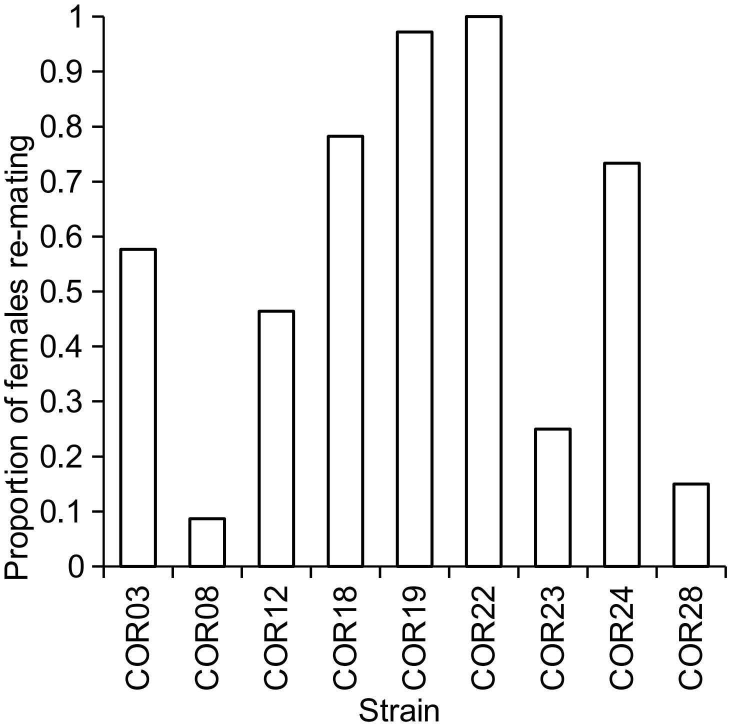


Figure 5.7: There is significant among strain variation in polyandry.

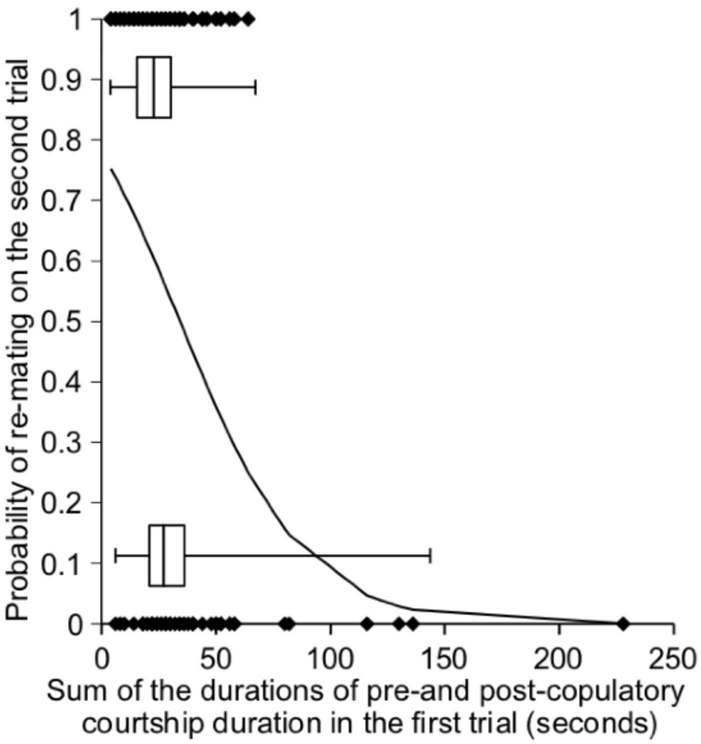


Figure 5.8: Polyandrous females receive less pre- and post-copulatory courtship in total than females that do not re-mate. The line is the logistic regression. The box and whisker plots show the distribution of the data points for females that did re-mate on the second trial (upper plot) and those that did not (lower plot). The box shows the range of the first and third quartile, with the median marked as a bar. The ends of the whiskers represent the full range of observations, excluding the female that received 114s of courtship.

6. Sexual conflict in the Hymenoptera: an order of haplodiploid insects

A version of this chapter is being prepared for submission to Biological Reviews. The authorship will be Anna M. Moynihan and David M. Shuker.

Abstract

Sexual conflict arises from differences in the evolutionary interests of males and females, and therefore characterises reproductive interactions between the two sexes. The genetics of sexual conflict are usually modelled in diploid organisms, but in this review, I focus on sexual conflict in the Hymenoptera, an order of insects with haplodiploid genetics containing the ants, bees and wasps. In species with haplodiploidy, diploid females develop from fertilised eggs and haploid males from unfertilised eggs. Although haplodiploidy does not predict more or less conflict overall then diploidy, the literature suggests that genetic details do matter to the precise predictions of sexual conflict models. I examine this literature in the present review. Future research should continue to exploit this extensive knowledge of the reproductive biology of Hymenoptera and the newly sequenced genomes to extend our understanding of sexually antagonistic coevolution by parameterising and testing the predictions of the population genetic models.

Keywords: haplodiploidy, sexual conflict, arrhenotoky, sperm competition, mating, sexual dimorphism, sex allocation.

Sexual Conflict

Sexual conflict arises when females and males have different optima for one or more traits (Parker, 1979, Chapman et al., 2003). This is predicted to occur frequently as natural and sexual selection have acted on males and females to maximise their own individual fitness, which can come at the expense of their mate's fitness, especially if they are unrelated. The sexes can be in conflict over any trait, from morphologies such as limb length to more derived traits like offspring sex ratio and genital morphology. While it is now widely recognised as a phenomenon that can potentially affect any trait, research on sexual conflict usually focuses on reproductive behaviours and decisions. As a result of this research, sexual conflict is thought to be an important component of reproductive behaviour in sexually reproducing species (Arnqvist & Rowe, 2005).

Sexual conflict can drive evolutionary change through sexually antagonistic coevolution (Arnqvist & Rowe, 2005). It arises from conflicting sex-specific selection pressures on each sex for a particular trait. If an allele in one sex is successful in this, that sex moves the value of the trait in conflict further away from the optimum of the other sex. This leads to an arms race between the sexes through sexually antagonistic coevolution. One of the biggest impacts on the extent to which traits are able to evolve along sex-specific trajectories in such arms races is the genetic basis of the trait. This is encapsulated in the literature as the distinction between intra- and inter-locus sexual conflict, on traits that are based at single- or multiple loci respectively.

Intra-locus conflicts occur when males and females have different optima for a trait they both express, whereas inter-locus conflicts occur when the traits involved in the conflict are controlled by many loci. Of course, most traits will be influenced by many loci, but this device is still useful. Intra-locus conflict include those over shared morphological traits, for example hip-width in humans (Rice & Chippindale, 2001). Narrower hips are advantageous for increased bipedal mobility, whereas the risk of death in childbirth selects for wider hips in females (Rice & Chippindale, 2001). In contrast inter-locus conflicts include the grasping traits males in the water strider genus *Gerris* need in order to grab hold of females, and the anti-grasping traits females have to limit this (Arnqvist & Rowe, 2002). Males in some *Gerris* species have evolved grasping adaptations that include the elongation of the genital and pregenital segments of the abdomen to assist them in grabbing a female to copulate, whereas females have evolve erect abdominal spines and a genital tip that makes the females less accessible (Arnqvist, 1989, Arnqvist & Rowe, 1995, Arnqvist & Rowe, 2002). Intra- and inter-locus conflict are resolved at the genetic level through different mechanisms. It is important to note that resolution does not mean that the potential for sexual conflict is necessarily removed, simply that the expression of the conflict is reduced (see Chapman, 2006).

Intra-locus conflict over the value of a trait at a single locus can be resolved through several routes: a mutation that leads to duplication and sex-limited expression, a modifier that alters the effect of the allele in each sex, or sex-linkage (Rice & Chippindale, 2001). It is through these mechanisms of sexual conflict resolution that the sexual-dimorphism in many animals and plants is thought to have evolved (Cox & Calsbek, 2009). Until the conflict is resolved at the genomic level in this way, polymorphism at the loci under conflict will be maintained, leading to a persistence of the conflict itself (Rice & Chippindale, 2001).

Inter-locus conflicts can be resolved by a mutation at a different locus to those causing conflict (Chapman, 2006). For example, a mutation could make a female less sensitive to a signal trait expressed by males to manipulate her into sub-optimal mating (Rowe et al., 2005). Alternatively, under monogamous conditions, males could evolve to harm their mates less (Holland & Rice, 1999), or females could evolve resistance to the mating costs imposed by males (Wigby & Chapman, 2004). In order for a new trait to resolve the conflict in this way, linkage disequilibrium must evolve with the alleles at the other loci involved in the conflict (Chapman et al., 2003, Cameron et al., 2003).

In this review I consider sexually antagonistic evolution in the Hymenoptera. I take the Hymenoptera as a case study for four main reasons. First, the asymmetric genetic system of haplodiploidy (also called arrhenotoky) possessed by all Hymenoptera has created the basis for many new conflicts. Indeed, there has been recent interest in how genetic system and ecological circumstances interact to affect genetic conflict (Appendix 1: Shuker et al., 2009, Ross et al., 2010). Second, the Hymenoptera have a wide range of life-histories and ecologies, which have inspired much empirical work. Third, the wealth of sexual dimorphism and sexual conflict already identified and studied across the Hymenoptera, in particular the intensive study of the eusocial Hymenoptera, provides an ample resource for study. Finally, the key limitation of nearly all studies of sexual conflict is the lack of empirical evidence for the shape of the fitness surfaces of a given trait for males and females. I also suffer from this problem. However, the biology of the Hymenoptera do suggest some novel costs and benefits underlying otherwise familiar sexual conflicts that may facilitate progress on the more general issues.

Now that I have outlined the basics of sexual conflict theory, in the rest of this review I will describe the biology of the Hymenoptera. I will discuss how their genetic system of haplodiploidy is predicted to alter the genetics of sexual conflict resolution, and with those predictions in mind, I will review the evidence for sexual conflicts in the Hymenoptera by following the life-cycle. Finally I will discuss promising areas for future research.

The Hymenoptera

The Hymenoptera are an insect order consisting of the Symphyta (the sawflies) and the Apocrita (the ants, bees and wasps). With over 100,000 described species, the Hymenoptera is one of the larger insect orders (Stubblefield & Seger, 1994). Among this diversity are economically important insects, including the sawfly pests, and the ants which are major drivers of ecological community function. The value of pollinating bees including domesticated bees is estimated to be worth $17.67 billion to the USA alone (Losey & Vaughan, 2006, Morse & Calerdone, 2000), and native parasitic wasps, which control population densities of many insect pests have an estimated value $13.6 billion to the USA (Losey & Vaughan, 2006). Another study found that $20 billion of crop loss is prevented in the USA by parasitoid wasps (Gadau et al., 2008).

The economic impact of the Hymenoptera has inspired much research. The vast sexual dimorphism observed in the Hymenoptera suggests an evolutionary past rich in sexually antagonistic evolution. Male and female Hymenoptera lead very different lives, especially as adults (reviewed by Stubblefield & Seger, 1994). Generally, females mate in early adult life and then find food resources for themselves and their offspring, either laying eggs on that resource, or retrieving it to a nest they constructed. This nest may be a social environment. Indeed, many of the most famous hymenopterans are social, ranging from colonial nesters to the eusocial ants, bees and wasps with sterile castes of female workers. In contrast to these long, often complex lives of adult females, males typically lead shorter adult lives. Males often compete with other males for matings often under extreme violence, and then play no role in parental care.

Despite being able to draw a general picture of the lives of male and female Hymenoptera, there are a wide-range of life-histories and ecologies represented in this group. The most obvious division is that between the social and non-social species, however there is great diversity even within those groups. The social species vary from communal nesters through to the eusocial species with sterile worker castes. Social species include herbivorous bees collecting nectar and pollen, carnivorous wasps, and ants which farm aphids or even fungus. Within the non-social species there are the solitary pollinating bees, parasitoid wasps which lay their eggs on other arthropods, and mason wasps which are solitary nest builders.

The Hymenoptera may differ greatly between species in life-history, and within species across castes and sexes, but they are unified by the final important aspect of their biology: their haplodiploidy. The typical pattern of haplodiploidy in the Hymenoptera is that females develop from fertilised eggs as diploids, and males from unfertilised eggs as haploids. This has impacts the biology of the species, most clearly through the relatedness asymmetries that arise (Figure 1.2), but also through changes in the nature of selection.

In the next section I will discuss the implication of haplodiploidy for sexual conflict resolution, before continuing on to explore the evidence for such conflicts in the Hymenoptera.

Sexual conflict resolution in haplodiploids

Most of the theoretical work on sexually antagonistic coevolution is modelled in a diploid system, where each individual has two copies of the same chromosome, one inherited from each parent (e.g. Rice & Chippindale, 2001, Chapman et al., 2003). Haplodiploidy is characterised by haploid males developing from unfertilised eggs, and diploid females developing from fertilised eggs. However, depending on the species, the sex determination mechanism itself can be based on zygosity, or the ploidy of one or more sex-determining loci (single, or multi-loci Complimentary Sex Determination) (Cook & Crozier, 1995). Haplodiploidy itself may be a consequence of past sexual conflict. Sex determination mechanisms are thought to have evolved under conflict between entities: cyto-nuclear conflict, sexual conflict, parent-offspring conflict, sex chromosome and B-chromosome drive (Werren & Beukeboom, 1998). Genetic conflict is the main culprit for the evolution of haplodiploidy (Normark, 2004 & 2006). Paternal genome elimination is thought to be a precursor to haplodiploidy (Brown, 1964, Bull, 1979). Brown (1964) and Bull (1979) showed that maternal genes which eliminate the paternal genome gain an advantage through increased transmission in the next generation because there is no reduction in meiosis through their haploid sons. This maternal advantage of the elimination of the paternal genome only holds if the fitness of a haploid son is at least half that of a diploid son.

Not only is haplodiploidy a consequence of past sexual conflict, but it also alters the circumstances under which sexual selection and sexually antagonistic coevolution will occur. The study of the genetics of sex conflict in haplodiploids can draw on the literature considering diploids if we think of all haploid chromosomes as analogous to the X-chromosome in diploids with male heterogameity (Kraaijeveld, 2009). Diploid heterogametic males have only one copy of the X-chromosome which does not recombine with the Y so they are hemizygous for the X, just as haplodiploid males are hemizygous for all chromosomes. Several studies have modelled the fate of X-linked loci in diploids, and so can be interpreted as models for haplodiploids too (for example see Hartl, 1971, Hedrick & Parker, 1997, Crow & Kimura, 1970, Hartl, 1972, Brückner, 1978, Lester & Selander, 1979, Pamilo, 1979, Curtsinger, 1980, Avery, 1984, Owen, 1986, Werren & Beukeboom, 1993, Liu & Smith, 2000, Hedrick, 2007).

Population genetic models of the resolution of sexual conflict have to take into account several changes when considering haplodiploids. First, as a result of the male hemizygosity, all expressed alleles are effectively dominant in males. Second, alleles do not spend an equal amount of time in male and female genetic backgrounds: at a population sex ratio of 0.5, an allele spends two-thirds of its time in females, and a third in males. This has important consequences for effective population size. Third, a gene involved in male function always finds itself in a female in the next generation. This is in contrast to diploid systems, where sex chromosomes are a common method of sex determination. As a result, genes that have sex-specific function in diploids can lie on a sex chromosome in the heterogametic sex, but this is not possible for haplodiploids.

These consequences of haplodiploidy to population genetic models of the resolution of sexually antagonistic coevolution apply to both intra- and inter-locus conflicts. Inter-locus conflicts are modelled through multi-loci population genetics, although there have been few attempts for haplodiploids so far (but see Owen, 1988, and Liu & Smith, 2000). An allele in a male that reduces the fitness of his mate can go to fixation faster in haplodiploids than a diploid autosome, because it spends less time exposed to selection in the haploid males (Andrés & Morrow, 2003). Further, the epistasis required to maintain linkage disequilibrium between two alleles is less likely to occur in haplodiploids, due in part to the lack of recombination in haploid males (Owen, 1988, Andrés & Morrow, 2003, Hedrick & Parker, 1997). Such epistasis and linkage disequilibrium is needed to reduce the expression of a conflict (Chapman et al., 2003, Cameron et al., 2003). Therefore resolution is slower to evolve for inter-locus conflicts on haplodiploid chromosomes than diploid autosomes.

Models of the genetic resolution of intra-locus conflicts for haplodiploids must compensate for the haploidy of the males. The mean fitness of males and females in a population with a mean sex ratio of 0.5 will be equal. Therefore we have to compensate for the fitness of hemizygous males expressing the same phenotype as heterozygous females (Hartl, 1972). Depending on the extent of this dosage compensation, there could be less scope for the maintenance of polymorphisms (Lester & Selander, 1979), leading to a greater probability that a given intra-locus conflict will be resolved (Curtsinger, 1980). One mechanism through which these intra-locus conflicts could be resolved is sex-limited expression, such as that observed in the wasp *Vespula squamosa* (Hoffman & Goodisman, 2007). There were 52 expressed sequence tags (EST) that differed in expression level between sexes, castes and developmental stages in *V. squamosa*, of which 4 out of 40 of the sex-specific ESTs were expressed only in males (Hoffman & Goodisman, 2007). Haploid males can evolve faster then their diploid sisters due to the increased exposure of alleles to selection (Hartl, 1972). This has been supported by the finding that genes expressed only in males were less similar to known sequences than those expressed only in female *Vespula squamosa* (Hoffman & Goodisman, 2007).

While there has been little modelling of haplodiploid population genetics, we can infer that intra-locus sexual conflict will be resolved quickly, perhaps favouring sexual dimorphism. Conversely, inter-locus sexual conflict will be resolved slower than for a diploid species, resulting in the maintenance of the conflict for longer.

With these predictions in mind, I will now proceed to review the evidence for expressed conflicts in the Hymenoptera.

Evidence for the expression of sexual conflict in the Hymenoptera

In my review of the evidence for the expression of sexual conflict, I will follow the life-cycle of the individuals, to show how pervasive sexual conflict is. Where to place the conflict in the life-cycle is of course somewhat arbitrary in many cases, however I hope to have made informative and helpful decisions in this regard. As I mentioned earlier, any trait can be the basis of sexual conflict if males and females have different optima, but there is a study bias towards traits involved in reproductive behaviours and decisions, which is reflected in the examples below.

Development

There is great potential for sexual conflict over traits expressed during the pre-adult phase of an insect. Before they are adults, insects spend most of their time feeding to grow and develop, it is during this time that many life-history sexual conflicts arise. Life-history traits are a product of the allocation of resources to survival, growth and reproduction, which captures the basis of many sexual conflicts: how much individuals invest in current reproduction compared to future reproduction with (potentially) a different mate (Wedell et al., 2006). It is during the pre-adult period that the sexual dimorphism I previously mentioned will develop, resulting in the myriad of differences between males and females we observe (Stubblefield & Seger, 1994, Starr, 1984).

Sex-specific morphological adaptations in females are observed for female-specific roles like nest building and provisioning. Nest building specialisations include the foretarsal rakes and pygidial plates found in some digger wasps to move the soil or sand (e.g. the solitary wasp *Tachysphex inconspicuus* (Kurczewski et al., 2010)). Prey capture and transport adaptations are also seen in many species of wasp (Evans, 1962), and the pollen carrying hairs, called scopa, found on Megachilid bees are only found on females, who use them to aid nest provisioning (Neff, 2008). Another obvious difference between males and females is the presence of the ovipositor and the venom associated with it. The ovipositor has in some species diverged further and become a specialised sting in female Aculeates, where eggs no longer travel down the ovipositor but are deposited at the base (Stubblefield & Seger, 1994).

In contrast to female nesting and provisioning adaptations, male adaptations are focused on acquiring mates. Solitary bees of the genus *Nomia* have expanded tibia to hold on to females during matings (Wcislo et al., 1992), whereas the expansion of tibia in many Apoidea are thought to be sexually selected displays (Low & Wcislo, 1992). Fighting adaptations are also present in many wasp species, for example the fig wasps, where males fight to the death for the privilege of mating the females

(Hamilton, 1979, Cook et al., 1997). The fig wasps have, like many Hymenoptera, also evolved winglessness in one of the sexes (Cook et al., 1997).

Even after the growth and development of sex-specific adaptations are completed, the timing of the transition to adulthood is also commonly sexually dimorphic. Protandry, where males arrive at the mating arena before the females, is most commonly recorded. This has been frequently observed in the parasitoid wasps and solitary bees (see "locating mates" below and Chapter 2) as well as many social species (Bulmer, 1983). Protandry is not the rule however; protogyny (where females are ready to mate first) is also documented in social species, usually when the female reproductives are produced first as part of a sex allocation strategy (Strassmann, 1984, Tsuchida & Suzuki, 2006, Beekman & van Stratum, 1998, Suzuki, 1986, Bull & Schwartz, 2001).

This short tour of sex differences in the Hymenoptera shows that there has been sex-specific selection in evolutionary past of the Hymenoptera. The constraints imposed by sharing a genome have not prevented the evolution of these very different morphologies, life-histories and behaviours.

Mate location

Once adult, the focus is no longer on investing resources in growth and development, but instead spending those resources on reproduction and survival. In order to reproduce in the internally fertilising Hymenoptera you must first find a mate.

In gregarious species, potential mates can be located at the natal site, and so mating at the emergence site is therefore commonly observed. This is especially common in gregarious parasitoid wasps, where the females will typically mate once and then disperse, possibly taking a second mate at a different location (reviewed by Ridley, 1990). This can become quite extreme in some species where mating occurs within the host puparium (*Trichogramma papilionis* (Suzuki & Hiehata, 1985); *Nasonia longicornis* & *Nasonia giraulti* (Drapeau & Werren, 1999)). Males of the braconid wasp *Alabagrus texanus* are solitary, and so do not become adults at a natal site surrounded by potential mates. However, *A. texanus* males can detect pupae that are about to emerge, but cannot discriminate the sex of the pupae. A swarm of males will locate the pupae about to emerge and if it is female there will be scramble competition to mate with it. Females disperse soon afterwards, having generally mated just once (Goh & Morse, 2010). While there is little information on the costs and benefits to each sex of searching for mates at emergence, it could pose a cost as it can remove the potential for female choice before copulation.

If the emergence time and location cannot be reliably predicted, it may be that a resource needed by females can be located and defended. Some solitary bees are known to enter into convenience polyandry in order to gain access to a food source. Male *Anthophora plumipes* defend the food source of comfrey flowers (*Symphytum orientale*) with such vigour that females are forced into sub-optimal foraging in order to avoid harassment (Stone, 1995). Similarly, *Anthidium maculosum* females receive enough sperm for full fertility from a single copulation, yet accept further matings after vigorous resistance in order to gain access to a food source (Alcock et al., 1977, Severinghaus et al., 1981). Females of the alfalfa leaf-cutting bee *Megachile rotundata* will resist mating with the males that harass them on the food source, despite this harassment resulting in a loss of female fecundity (Rossi et al., 2010). Similarly *Osmia rufa* bees also mate at the food source (Seidelmann, 1999), creating the potential for resource defence polygyny and the associated conflict over mating.

In contrast to the food-resource defence observed in the solitary bees, the males of many species form leks by aggregating on or over an area that does not contain essential resources for females. Inside the lek, there will either be male-male aggression, or courtship of females (Shelly & Whittier, 1997). There are four main hypotheses for the evolution of lekking, reviewed in Field et al., (2002) and Shelly & Whittier (1997). Predation avoidance capitalises on the reduction in *per capita* predation risk in a group. The hotspot hypothesis proposes that males cluster in areas where females are at higher density, but do not defend any resource a female needs. On the other hand, males are proposed to benefit from clustering around a super-attractive male in the hotshot hypothesis. Finally, females could also prefer males in leks as it increased her opportunity to compare males.

Lekking has been observed in all of the major life-history groups of Hymenoptera: ants (e.g. Hölldobler, 1976); eusocial bees (e.g. O'Neill et al., 1991); eusocial wasps (e.g. Beani et al., 1992); non-social bees (e.g. Kimsey, 1980, Watmough, 1974); and non-social wasps (e.g. Kroiss et al., 2010, Alcock, 1975). The airborne mating flights of *Apis mellifera* (Strang, 1970, Michener, 1974) are perhaps the best known example of lekking in the Hymenoptera, and the ant *Acromyrmex versicolor* shows a lekking mating system where females have been observed to re-enter the mating swarm to take additional mates (Reichardt & Wheeler, 1996). Males in the substrate-based leks of the ant *Nemka viduata* can pick up wingless females and fly them to a secluded location for mating, suggesting there is little scope for female choice before mating (Tormos et al., 2010). Nevertheless, female *N. viduata* can still exert control in this situation, as they must exude their sting in order for mating to occur.

Lekking may be so common in the social insects because it is the interest of the workers for their queen to mate only once. A worker in a colony with a singly-mated queen will have a higher average relatedness to the reproductives they raise (see the sperm usage section for further discussion). Workers have therefore been selected to reduce the number of mating opportunities for their queen, which has perhaps contributed to the evolution of the short-mating window common to many eusocial hymenopterans (Couvillon et al., 2010, Boomsma, 2007, Strassmann, 2001). This is supported by observations in some ants that the workers control the timing of the mating-flights for their queens (Markl et al., 1977, Marikovsky, 1961). Such a worker-enforced short mating time would enhance the advantages to lekking for a eusocial queen.

In summary, the process of mate location therefore offers many opportunities for females to enter into sub-optimal mating and therefore conflict, by removing her opportunities for female choice.

Mating

The focus of research on sexual conflict is often the mating interaction itself, in particular the number of mates each sex has. As such investigating the costs and benefits to multiple mating has been a major research focus.

The costs and benefits to multiple mating are well investigated in the eusocial Hymenoptera. There has been considerable interest in the benefits associated with greater colony genetic diversity through reduced parasite load and improved colony productivity (Table 6.1) (Keller & Reeve, 1994, Schmid-Hempel, 1994, Sherman et al., 1988, Kraus & Page, 1998). Notably, polyandry reduces the relatedness between colony members, which has important fitness consequences for the queens (see sperm usage section), so she benefits from multiple mating through many routes.

As such, it has been predicted that the costs associated with multiple mating are lower in the social insects (Bourke & Franks, 1995, Parker et al., 1993, Ratnieks, 1990, Ratnieks & Boomsma, 1995). Despite this, longevity costs to polyandry and mating have been observed in social insects (Table 6.2). Another form of cost, sexually transmitted disease, is however unlikely to contribute to the costs of mating in social insects due to their long generation time, short copulation window and long-term sperm-storage requirements (Boomsma et al., 2004).

A significant component of the cost to mating can be the duration of the mating encounter itself as males and females are more exposed to predation and unable to forage or perform other activities during this time. The short copulation window is also thought to reduce direct costs to mating, such as increased predation risk and lost foraging opportunities in social insects (Strassmann, 2001). In contrast to this generalisation, the bumble bee *Bombus terrestris* has a copulation duration of up to 40 minutes (Brown & Baer, 2005). In the course of this long copulation sperm transfer is relatively quick, but 30 minutes are needed to form a mating plug (Duvoisin et al., 1999), which generally prevents further matings in this species. Additionally, the shorter the duration of a queen's first copulation, the more likely she is to re-mate (Brown et al., 2002). This could be due to two factors: shorter copulations could reflect fewer sperm cells being transferred and so females re-mate to top-up their sperm supply, or a shorter copulation could reflect a smaller dose of the ejaculate compounds known to reduce female receptivity (Baer et al., 2001). However, there is a positive relationship between copulation duration and female mating frequency across four species of *Bombus* bees (Foster, 1992), which may be due to increased investment into mating plug transfer in the polyandrous species. This is directly against the prediction of Brown and Baer (2005) that mate guarding is ineffective in polyandrous species, so males should be selected to reduce their investment in individual matings.

Of the non-social species, there are fewer studies investigating the costs and benefits of mating. There was no evidence for a cost to polyandry in the parasitoid *Trichogramma evanescens*; in the presence of hosts females with multiple mates lived longer (Jacob & Boivin, 2005). This is in contrast to species of parasitoid where mated, or multiply mated females have a shorter lifespan (*Pteromalus cerealellae* (Onagbola et al., 2006); *Bathyplectes curculionis* (Jacob & Evans, 2000); *Trichogramma minutum* (Li et al., 1993)).

One of the sex determination mechanisms used by the Hymenoptera may contribute to the advantages of polyandry and indeed mate choice: many hymenopterans use single-locus Complementary Sex Determination (sl-CSD). Here individuals heterozygous at the locus become female, whereas individuals hemi- or homozygous at the locus become males. If a female and her mate share a CSD allele, only half of the subsequent fertilised eggs will become daughters; the other half will develop as sterile diploid males. This can introduce a huge cost to mating, especially for males (Cook & Crozier, 1995). As a result, reducing diploid male production is one of the hypothesised benefits to multiple matings for both males and females (Tarpy & Page, 2002). Despite this, there is little evidence that diploid male production poses a large cost to social species (Ratnieks, 1990, Pearcy et al., 2009). For the solitary wasp *Cotesia glomerata* however, which shows sl-CSD, polyandry may be the mechanism for avoiding inbreeding costs. Although female *C. glomerata* do not avoid mating with males that share CSD alleles, disassortative mating arises through female dispersal and/or multiple mating instead, reducing the consequences of inbreeding (Ruf et al., 2010).

Similarly, the various endosymbionts documented in some species would also confer advantages to multiple mating. Endosymbionts, such as *Wolbachia* can lead to cytoplasmic incompatibility, and are found in many Hymenoptera species (see Heimpel & de Boer, 2008 and references therein). If both sexes mate more than once, they can increase the probability that they find cytoplasmically compatible gametes (Bordenstein et al., 2001).

Given these various benefits to polyandry, we would expect that females sometimes mate multiply, which in turn can give rise to new conflicts. Whenever females mate more than once, males will be selected to prevent this in order to protect their paternity. Mating has been shown to successfully reduce female receptivity in several species (*Aphytis lingnanensis* (Gordh & DeBach, 1976); *Aphytis melinus* (Allen et al., 1994); *Bracon hebetor* (Ode et al., 1997); *Nasonia vitripennis* (van den Assem & Jachmann, 1999); *Anisopteromalus calandrae* (Do Thi Khanh et al., 2005);

Lariophagus distinguendus (Steiner et al., 2007); multiple parasitoid wasp species (Ridley, 1993); *Bombus terrestris* (Baer, 2003, Sauter et al., 2001); *Centris pallida* (Alcock & Buchmann, 1985)).

As we saw above, in some social insect species, female re-mating is reduced by males producing a "mating plug", which attempts to block the female's reproductive tract. These plugs are produced mainly from accessory gland secretions, and are successful at preventing female re-mating in some species (*Solenopsis invicta* (Mikheyev, 2003); fungus-growing ants (Mikheyev, 2004); *Bombus terrestris* (Baer et al., 2001)) but not others (*Bombus hypnorum* (Brown et al., 2002); *Carebara vidua* (Robertson, 1995)). The mechanism of preventing re-mating is not necessarily mechanical; a specific fatty acid in the mating plug is known to reduce female receptivity in the bumblebee (Baer et al., 2001). In some species the mating plug includes the male's external genitalia, resulting in his death (*Atta* sp. (Hölldobler & Wilson, 1990); *Pogonomyrmex formicidae* (Markl et al., 1977); *Formica rufa* (Marikovsky, 1961); stingless bees (Sakagami, 1982, Paxton, 2005); *Apis mellifera* (Koeniger, 1984)).

Male accessory gland secretions can influence female re-mating through mechanisms other than the production of the mating plug though. While there is little direct evidence of accessory gland secretions altering the female's physiology to reduce her re-mating rate (Baer, 2003, Boomsma et al., 2005), it is generally assumed that ejaculate components have been selected to alter female behaviour to benefit the male (Gillott, 2003, Avila et al., 2011). Two studies have looked at the links between male reproductive traits and polyandry in the fungus-growing ants. Mikheyev (2004) found a negative relationship between mating rate and accessory gland size across a phylogeny of fungus-growing ants, contrary to what they expected. Mikheyev proposed that this findings could be explained by the benefits of their daughters coming from genetically diverse colonies (Table 6.1) overcoming the loss of paternity to each male from polyandry, however this seems unlikely. On the other hand, Baer and Boomsma (2004) found an increase in testes investment in more polyandrous species of fungus-growing ants.

Aside from the physiological and physical mechanisms previously described, there are also other mechanisms through which males reduce female re-mating. Mated females of *Lasioglossum malachurum* (Ayasse, 1994) and *Centris adani* (Frankie et al., 1980) are marked with anti-aphrodisiacs once mated (Ayasse et al., 2001) in order to make the female less attractive and thus reduce her probability of re-mating. There is, however, no evidence of such an effect in other species (*Nasonia vitripennis* (Ruther et al., 2010); Chapter 5). Behavioural processes reduce receptivity in other species. After copulation, the males of several species are observed to return to their original courtship position over the female's antennae to perform another round of courtship which reduces female re-mating rate (*Spalangia endius* (King & Fischer, 2005, King & Fischer, 2010); *Aphytis melinus* (Allen et al., 1994)); *Nasonia vitripennis* (van den Assem & Visser, 1976, Leonard & Boake,

2008) (Chapter 5); *Aphytis lingnanensis* (Gordh & DeBach, 1978); and the bee *Centris pallida* (Alcock & Buchmann, 1985)).

Despite the many outbreeding advantages associated with polyandry, many parasitoids show extreme inbreeding for many generations (Godfray, 1994). The high, but asymmetric relatedness between mates in this situation (Figure 1.2) begs the question of how much a male should harm his sister. It is important to remember that males and females cannot "win" sexual conflict. Each offspring has two parents, so all else being equal, the sexes must have equal total fitnesses. "Winning" can only occur at the level of the allele: a new invasive allele can "win" sexual conflict by spreading and reducing the fitness of another allele. This distinction is particularly important in the following scenario. In such unusual circumstances with strong inbreeding, we would predict that in order to maximise its inclusive fitness, a new mutation causing female-harm in a male should be selected to harm their sisters less than an unrelated female. However, due to the common presence of multiple females ovipositing on a patch of host resources, parasitoid males will also often mate with non-sisters. High-relatedness between individuals in a local-mating scenario has influenced the outcome of several models, including those of another form of conflict: parent-offspring conflict over sex allocation (Werren & Hatcher, 2000, Pen, 2006). There is however, a lack of evidence to suggest that parasitoids can detect relatedness (Reece et al., 2004). In support of this brothers are known to reduce the fitness of their sisters during development in the parasitoid wasp *Nasonia vitripennis* through asymmetric larval competition: at large clutch sizes the number of brothers in the clutch reduces female body size, and consequently fitness (Sykes et al., 2007).

There is plenty of evidence for the expression of sexual conflict over mating, often arising from female re-mating and male adaptations to prevent it. It is important to note however, that forced copulations are improbable in the Hymenoptera because the female must move her sting before copulation can begin (Page, 1986). So while females can be coerced into more copulations than required (e.g. copulating with sub-optimal males) or prevented from additional copulations, they often cannot be physically forced into mating.

Sperm usage

Once the male has transferred his ejaculate and it is stored in the female's spermatheca, there is still potential for conflict. Females are under natural selection to use this sperm resource to lay the optimum clutch size and sex ratio for their particular set of conditions. However, males are under selection to persuade the female to use as much of their sperm as possible in order to maximise the male's fitness. These two optimal strategies may differ in that females may be selected to leave some eggs unfertilised to produce sons, whereas the optimal strategy for males will be to fertilise as many eggs as possible. This difference in optimal sperm use is exacerbated if the female mated several males, because the ejaculates of those males will be experiencing sperm competition. Polyandry, and therefore sperm competition is widespread in nature (reviewed by Jennions & Petrie, 2000 and Simmons, 2001). Males and females will therefore both be selected to have adaptations to achieve their sex-specific optimal sperm use strategy.

Sperm competition will select for two broad categories of male adaptation: those that prevent female re-mating, and those that improve his success in competition against her previous mates. Many of the male adaptations that prevent female re-mating in the first place have been previously discussed in the mating section; here I will discuss the adaptations focused on increasing his fertilisation success in competition against the female's previous mates. Sperm displacement resulting in last male precedence in sperm competition is a common adaptation in other insects (Walker, 1980), but has not yet been found in the Hymenoptera (Page, 1986, Quicke, 1997, Simmons, 2001, Damiens & Boivin, 2006, King, 1962, Allen et al., 1994, Franck et al., 2002, Holmes, 1974, Wilkes, 1965, Wilkes, 1966, Lopes et al., 2003, El Algoze et al., 1995, Metcalf & Whitt, 1977). This may be due to the fine control females have over the release of sperm from the spermatheca for fertilisation preventing a large outflow of sperm. Another potential mechanism for males reducing the success of other ejaculates is through damaging the rival spermatozoa directly. Non-self seminal fluid has a detrimental effect on sperm survival in the polyandrous eusocial hymenopterans *Apis mellifera*, *Acromyrmex echinatior* and *Atta colombica* (den Boer et al., 2010). The female cannot top-up her sperm supplies and in some species it must survive for many years to produce a successful colony, and so destruction of sperm by males is not in the evolutionary interest of the female.

Many of the observed advantages to polyandry focus on the benefits of a genetically diverse brood (Table 6.1). In order for females to reap these benefits, they must preserve the variation in the multiple ejaculates they store. Adaptations to maintain this variation have been observed in many species. Female spermathecal secretions reduce the effect of sperm destruction observed in *Apis mellifera*, *Acromyrmex echinatior* and *Atta colombica* as noted above (den Boer et al., 2010). Similarly, females are proposed to mix the sperm within the spermatheca of several species to ensure they have a diverse colony at every stage (El Algoze et al., 1995). *Apis mellifera* queens store only 5% of the sperm they receive, ensuring a mix of ejaculates are retained (Franck et al., 2002).

Conflict over sperm usage goes further than sperm competition in the Hymenoptera due to the link between fertilisation and sex determination. The relatedness asymmetries generated by haplodiploidy set up the potential for sexual conflict over sex allocation: mothers are equally related to their sons and daughters, but fathers are only related to daughters (Figure 1.2) (Trivers & Hare, 1976). We would predict therefore that males will be under selection to increase the proportion female of the brood laid by their mate (Appendix 1: Shuker et al., 2009, Wild & West, 2009, West, 2009, Ratnieks et al., 2006). The sex allocation strategy of the female is, however, predicted by other factors (for a review see West, 2009): many of the inbreeding parasitoids experience Local Mate Competition (Hamilton, 1967, Charnov, 1982), and split sex ratios can evolve in the social species (Meunier et al., 2008), whereas other species without such inbreeding are predicted to use a more equal sex allocation.

Despite being predicted on a number of occasions, male manipulation of the sex ratio has been rarely tested. In several parasitoid wasps the sex ratio a female produced was significantly affected by the identity of the male that inseminated her (*Dinarmus basalis* (Chevrier & Bressac, 2002); *Uscana semifumipennis* (Henter, 2004); *Nasonia vitripennis* (Shuker et al., 2006c)), however sperm-limitation effects cannot be definitively excluded from these studies. This suggests that males have adaptations to manipulate female sex allocation behaviour, perhaps via accessory gland proteins. In a study of double and single mated parasitoid wasps *Dinarmus basalis,* doubly mated females laid more daughters (Chevrier & Bressac, 2002). This would be expected if each male's seminal fluid manipulated females to lay more daughters. However this study does not allow us to rule out additional matings simply reducing the potential for sperm limitation. Adaptations in males to increase the usage of their sperm, by the female laying more daughters, are also seen in the mud-daubing wasps. Males of the mud-daubing wasp *Trypoxylon politum* sometimes guard the active nest of the female they copulated with against usurpation (Brockmann & Grafen, 1989). This nest-guarding behaviour allows the females more time to collect the larger provisioning items needed to rear daughters. Nests that are guarded by males therefore have more daughters, causing a guarding male to increase the use of his sperm and therefore his fitness (Brockmann & Grafen, 1989). Observations in the closely related *Trypoxylon lactitarse* found males always guarded the nest, however, and no effect on the sex ratio of the resulting offspring was observed (Buschini, 2007).

Sexual conflict over sex allocation in the social Hymenoptera is a more complicated matter. The queen and workers often have different optimal sex ratios, due to the relatedness asymmetries set up by haplodiploidy (Figure 1.2) (Tsuji, 1996, Ratnieks et al., 2006). The queen controls the sex ratio at fertilisation, but the sex ratio of the adult reproductives is under worker control because they can selectively kill offspring of the less desirable sex (Moritz, 1985, Ratnieks & Boomsma, 1995, Boomsma, 1996). The extent of the conflict for the fathers, queens, and workers depends on the number of times the queen mated, as this alters the relatedness between her offspring. When a queen has mated once, her optimal sex ratio of reproductives is 0.5 proportion male, whereas for the workers it is 0.25 proportion male, and for the father all female-reproductives is optimal to ensure maximum usage of his sperm. As the female becomes increasingly polyandrous, the queen's optimal sex ratio remains the same, and the worker's rises to meet hers as the mean relatedness between a randomly-sired worker and a randomly-sired female reproductive falls (Boomsma, 2007). As a result, conflict over sex ratio in the eusocial Hymenoptera is closely linked to conflict over the number of mates a queen has (Tsuji, 1996, Werren & Beukeboom, 1998).

Worker-control of the sex ratio therefore adds to the advantages of multiple mating for eusocial Hymenoptera queens, because the sex ratio of reproductives is closer to her evolutionary stable strategy (ESS) sex ratio. For the males that inseminate these queens, each additional mate she takes not only reduces the proportion of the female reproductives that he sires, but also the total number as a product of worker-manipulation of the sex ratio. This leads to a non-linear loss in paternity, which is unlikely to be compensated by the benefits of having his daughters raised in a genetically diverse colony (Table 6.1) (Strassmann, 2001). In light of this we would expect strong selection for adaptations to reduce re-mating in social insect males (Boomsma & Ratnieks, 1996).

Beyond the adaptations to prevent female re-mating already discussed, post-copulatory tactics will also play a crucial role. If queens are multiply-mated the ejaculates of each male shares an interest in clumping into sire-specific groups within the spermatheca. Clumping is favoured to keep the relatedness asymmetry and hence daughter production high (Boomsma, 1996). Hymenopteran males are haploid, so there is no recombination before gametes are formed, resulting in clonal sperm so cooperation between a male's spermatozoa should be strong. Evidence for sperm-clumping is mixed, finding support in some species (*Formica sp.* (Sundstrom & Boomsma, 2000); *Apis mellifera* (Page & Metcalf, 1982); *Pogonomyrmex badius* (Rheindt et al., 2004)) but not others (*Apis mellifera* (Franck et al., 2002); *Paravespula maculifrons* & *Vespula squamosa* (Ross, 1986); *Acromyrmex versicolor* (Reichardt & Wheeler, 1996)). Overall, the importance of sperm clumping has been downplayed (Cole, 1983, Crozier & Brückner, 1981). Female counter adaptations to sperm clumping would include the sperm mixing previously discussed.

In summary, sexual conflict can arise from sperm competition adaptations (Stockley, 1997). The scope for conflict over sperm usage in the hymenopterans is exacerbated by the coinciding conflict over sex allocation. But not all interactions need to be agonistic, indeed seminal fluid is known to prolong female lifespan in some long-lived eusocial species (Schrempf & Heinze, 2008, Schrempf et al., 2005).

Offspring care

Many species in the Hymenoptera engage in some form of provisioning or parental care either before or after mating. This includes the social species with elaborate parental or worker provided care, and the provisioning of nests with prey or pollen in some wasps and bees, along with the parasitoid mothers stinging and laying their eggs on or in their host.

In many species there is sexual conflict over offspring care (reviewed by Houston et al., 2005). If there is investment beyond the gametes we would predict that each sex is selected to exploit the other into investing more, while they reduce their own level of investment (Trivers, 1972). As a generalisation, the sexes will therefore be in conflict over the amount of parental investment each provides.

In haplodiploids, where fathers are unrelated to any males in the brood, this conflict over parental care could be even stronger. An allele promoting caring behaviour in males will only spread if the male cares for individuals it is related to. At first appearance, haplodiploidy reduces the benefits to caring. However, if the brood has an equal sex ratio and there is only one sire, the mean relatedness for males to the brood is the same if they are diploid or haplodiploid (see Figure 1.2). That said, the mean relatedness of a father to the brood increases with the proportion of daughters in the brood, and will decrease with the number of patrilines in the brood. Therefore haplodiploidy itself is not a barrier to the evolution of paternal care, as illustrated by the haplodiploid thrips, where paternal care has evolved in a number of species (Kudô et al., 1992, Tallamy, 2001).

The best known examples of parental care occur in the colonies of the social Hymenoptera. The eusocial male often dies after the brief nuptial flight living on as only sperm in a queen’s spermatheca, so they are unable to partake in care as fathers. There is consequently no exclusive paternal care in the eusocial Hymenoptera (Zeh & Smith, 1985, Tallamy, 2001). There is the possibility of drones caring for their siblings in a number of social wasp species, and observations are biased to those species where behaviour is open to view (*Polistes ferreri* (Sinzato et al., 2003); *Polistes jadwigae* (Makino, 1993); *Polistes metricus & Polistes fuscatus* (Hunt & Noonan, 1979); *Mischocyttarus mastigophora* (O'Donnell, 1999); *Ropalidia marginata* (Sen & Gadagkar, 2006)). Male are therefore capable, but perhaps lacking in opportunities for offspring care. The evolutionary consequences (for males, females and workers) of these instances of male parental care remain to be fully explored.

Amongst the non-social hymenopterans, social care is sometimes observed in the mud-daubing wasps. As discussed earlier, males of the wasp *Trypoxylon politum* guard the active nest against usurpation, allowing females more time to collect the larger provisioning items needed to rear daughters (Brockmann & Grafen, 1989). Observations in the closely related *Trypoxylon lactitarse* found males always guarded the nest (Buschini, 2007). The mud-daubing wasp *Oxybelus* shows some nest-guarding by males, which results in less kleptoparasitism of prey from flies (Peckham, 1977). These adaptations are usually interpreted as males providing care to manipulate the sex ratio a female lays, especially in *T. politum* where this strategy is successful.

Sexual conflict can arise during offspring care over reasons other than the parental investment. Just as we saw males defending female feeding sites in order to force females into convenience polyandry, similar harassment can occur if males defend nesting or oviposition sites. Males of the beewolf *Philanthus bicinctus* set up territories around female nest sites at the onset of nesting. As there are usually more males than there are nesting sites, unreceptive females are commonly harassed by males (Gwynne, 1978, Gwynne, 1980). The females of *Abispa ephippium* show little resistance to males that catch and mate them as they build their mud nests (Thornhill & Alcock, 1983). The cost and benefits to such polyandry for *A. ephippium* is not known, however, the mud is a crucial resource for nest-building and so the avoidance costs would be high, potentially favouring convenience polyandry.

Sexual conflict over offspring provisioning is atypical in the Hymenoptera as a result of their lifestyles and haplodiploidy. If the brood sex ratio is male-biased, the payoff to caring for a father can be lower than that for diploid males at the same degree of polyandry. Care by males is observed in some Hymenoptera, and in the non-social species studied to date is associated with sex allocation manipulation.

Future directions

I have reviewed the evidence for sexual conflict in the Hymenoptera, and the consequences of haplodiploidy on the genetic mechanisms of resolution. In this section I will review the directions for future research.

Genetics of conflict resolution

Future research should aim to test the predictions of population genetic models of sexually antagonistic coevolution at X-linked loci in diploid species (Kraaijeveld, 2009). Overall, my review of the implications of haplodiploidy showed that intra-locus conflicts are more likely to be resolved relatively faster, whereas inter-locus conflicts would be more likely to be resolved relatively slower, when compared to a diploid system.

Intra-locus conflict is usually resolved through sex-limited expression (Rice & Chippindale, 2001), resulting in sexual dimorphism (Cox & Calsbek, 2009), so we would predict more dimorphism. Strong sexual dimorphism has been observed across the Hymenoptera (Stubblefield & Seger, 1994, Starr, 1984), and the growing number of Hymenoptera genome sequences should encourage more expression studies like that of *Vespula squamosa* to identify sex-limited expression (Hoffman & Goodisman, 2007). Ultimately, a comparison of the proportion of genes expressed only in one sex in diploid and haplodiploid insects would be informative. Similarly, a comparison of the divergence of male-expressed and female-expressed genes compared to genes expressed in both sexes will test the prediction that sex-specific expressed genes in haploid males evolve faster than their diploid sisters (Hartl, 1972).

Sex-limited expression could come about in haplodiploids through genomic imprinting, which is the differential methylation of genes based on their parent of origin. Haig was the first to predict that the haplodiploid genetic system predisposes a species to genomic-imprinting-mediated conflicts, in line with his kinship theory of imprinting (Haig, 1992, Haig, 2000). Models have demonstrated that conflict between alleles inherited from each parent (matrigenes and patrigenes) would pervade the females in eusocial hymenopteran societies (Queller, 2003, Kronauer, 2008, Wild & West, 2009, Day & Bonduriansky, 2004).

The parent of origin genomic imprinting these models suggest could only be found in female hymenopterans, as the haploid males have only one parent. Males could however label their own gametes with parent of origin information, and also experience within generation methylation of alleles. For example, it would be beneficial for males to imprint loci that would encourage their sterile worker daughters to manipulate the sex ratio in their father's favour and produce sons of their own. As Queller, (2003) points out, females would have to wait for selection to balance the risk between imprinting alleles to improve the fitness of the matrigenes in sons and the potential of reducing the fitness of the same matrigene in a daughter.

While there are few known loci where parent-of-origin genomic imprinting occurs in hymenopterans, there is evidence that differential methylation occurs. The full complement of imprinting genetic machinery has been found in *Apis mellifera* (Wang et al., 2006) and also *Nasonia vitripennis* (The *Nasonia* Genome Working Group, 2010). When *Dnmt3*, a locus associated with imprinting is silenced using RNAi in honeybee larvae, the developmental pathway is changed and almost three times as many larvae develop into fully fertile queens than controls (Kucharski et al., 2008). Methylation has also been demonstrated in eleven other social hymenopterans (Kronforst et al., 2008). Genomic imprinting is known to be crucial to the mechanism of sex determination in the parasitoid *Nasonia vitripennis* (Dobson &

Tanouye, 1998, Trent et al., 2006, Verhulst et al., 2010). In addition, two studies have demonstrated grand-paternal effects (hypothesised to be caused by genomic imprinting (Beukeboom & van den Assem, 2001, Guzman-Novoa et al., 2005)). Males could manipulate the use of genomic imprinting in sex- and caste-determination to ensure that his sperm are used to fertilise eggs that will become reproductives (new queens) and not sterile workers, and future research should investigate the role of parent-of-origin imprinted loci in caste development to test these ideas.

Further to my proposed investigation of intra-locus conflict resolution, future models of sexually antagonistic coevolution in haplodiploids should also consider multi-loci conflicts, as there is a relative paucity of theory in this area. Quantitative trait loci (QTL) studies are now commonplace in Hymenoptera (e.g. Packer & Owen, 1990, Volny & Gordon, 2002, Packer & Owen, 1994, Jensen et al., 2002, Pannebakker et al., 2011, Rütten et al., 2004), which should encourage further research into quantitative genetic models. These same molecular ecological techniques will allow future researchers to quantify many of the sensitive parameters in the population genetic models, such as measures of polymorphism, effective population size, and the level of dosage compensation for fitness in males and the population sex ratio (Lester & Selander, 1979).

The inter-locus conflict models for haplodiploids predict that resolution will be slower than for diploid autosomes, as the linkage equilibrium necessary is less likely to occur (Owen, 1988, Andrés & Morrow, 2003, Hedrick & Parker, 1997). Linkage disequilibrium has been successfully identified in several hymenopteran species (e.g. Packer & Owen, 1990, Volny & Gordon, 2002, Packer & Owen, 1994, Jensen et al., 2002), however not for traits known to be involved in currently-expressed sexual conflicts.

Evidence of the expression of conflict

The Hymenoptera exhibit a range of life-histories, providing different situations where males and females are in control of mating frequency. Despite this there are few scenarios where the actual costs and benefits are shown. Here, I suggest areas for future research to build on this existing knowledge.

The costs and benefits to additional matings have been researched in the eusocial insects, yet despite many promising observations, they are lacking in the non-social species. The non-social bees where males guard food resources provide a particularly tractable example of an area where the costs and benefits to multiple mating can be calculated. The parasitoid wasps are another group where there is patchy evidence of costs to mating, and some observations of male-adaptations to prevent female re-mating.

The accessory gland proteins are particularly promising as a target for future research, following up the sexually antagonistic coevolution unearthed in the diploid *Drosophila melanogaster* (Fricke et al., 2009, Chapman, 2001, Wigby & Chapman, 2004, Wigby & Chapman, 2005, Wigby et al., 2009, Yapici et al., 2007). Indeed the seminal fluid proteins of honey bees have been sequenced and their functions predicted, however no attempt has been made to compare honeybee seminal fluid proteins with known female-manipulating proteins (Collins et al., 2006, Collins et al., 2004). Tantalisingly, despite the sex-peptide receptor that is widely conserved across the insects (Yapici et al., 2007), it has not been found in the *Apis* or *Nasonia* sequenced genomes (The *Nasonia* Genome Working Group, 2010), nor was the sex peptide itself found (Hauser et al., 2010). Sex-peptide is not the only component of the *Drosophila* ejaculate to alter a female's behaviour after mating though. Future researchers should characterise the ejaculate proteome to search for suitable candidates. The increasing number of genomes sequenced in the Hymenoptera provide us with an excellent opportunity to do this in many species (The Honeybee Genome Sequencing Consortium, 2006, The *Nasonia* Genome Working Group, 2010, Smith et al., 2011a, Suen et al., 2011, Bonasio et al., 2010, Wurm et al., 2011, Smith et al., 2011b). RNAi knock-out techniques have been developed for each of the major groups of hymenopterans: wasps (e.g. Lynch & Desplan, 2006), bees (e.g. Belles, 2010), sawflies (e.g. Sumitani, 2005) and ants (e.g. Lu et al., 2009). This technique could then be used to test the function of the knocked-down ejaculate components in these species. This could be followed up with studies like that of

Kocher et al., (2008 & 2010) comparing the change in transcription of queens that were mated by wild-type and knock-down males.

Summary

This review has drawn together the varied research on sexual conflict in the Hymenoptera for the first time. I have summarised the genetic theory of conflict resolution in haplodiploids, finding that intra-locus conflicts may become resolved faster in the haplodiploids, whereas inter-locus conflicts could be slower to resolve at the genetic level. In some cases, haplodiploidy itself creates new conflicts as a result of the relatedness asymmetry. By combining the wealth of research about the reproductive biology of the Hymenoptera with the new genomic techniques, we can extend our understanding of the nature of sexual conflict.

Table 6.1: Benefits to polyandry in three species of eusocial Hymenoptera

Species (Source)	Benefit
Bombus terrestris	
(Baer & Schmid-Hempel, 1999)	Parasite load is lower in colonies that have higher number of sperm donors.
(Baer & Schmid-Hempel, 2001)	Intermediate level of relatedness in a colony (>1 patriline) associated with lower parasitism.
(Shykoff & Schmid-Hempel, 1991)	Parasite transmission was more likely between more closely related individuals.
(Liersch & Schmid-Hempel, 1998)	Genetically heterogeneous colonies had lower parasite prevalence, species richness and load.
Pogonomyrmex occidentalis	
(Cole & Wiernasz, 1999)	Polyandrous queens had larger colonies, with higher reproductive output.
Apis mellifera	
(Tarpy, 2003)	More genetically diverse colonies, grew faster with less disease.
(Jones et al., 2004)	More diverse colonies are better at keeping the hive temperature constant.
(Tarpy & Page, 2002)	Colonies with increased diversity (measured as the number of sex determining alleles in the drones) have reduced worker infection.
(Fuchs & Schade, 1994)	Single patriline colonies had a lower reproductive output and food source collection.

Table 6.2: Examples of costs to multiple mating in the eusocial Hymenoptera

Species (Source)	Cost
Pogonomyrmex formicidae	
(Markl et al., 1977)	Harassment during mating flights leads to females produce distress calls to workers.
Atta colombica	
(Baer et al., 2006)	Queens that had a higher number of sperm cells stored, from a greater number of sires, invested less in their immune response.
Apis mellifera	
(Ruttner, 1980)	15-30% queen mortality on mating flight
(Ratnieks, 1990)	Mark-recapture during mating flight showed 4% mortality.
Bombus terrestris	
(Baer & Schmid-Hempel, 2005)	Negative correlation between the number of mates and the queen's survival during hibernation.
(Greeff & Schmid-Hempel, 2008)	Presence of sperm reduces female longevity controlling for body size.

7. General Discussion

Sexual selection and sex allocation

My thesis aim was to establish what makes a "good son" for an *N. vitripennis* mother by studying sexual selection in this species, and to establish how this influences her sex allocation behaviour. Males will gain fitness by developing earlier (Chapter 2; Appendix 2: Moynihan & Shuker, 2011), where larger body size is not a direct advantage in pre-copulatory competition (Chapters 2 & 3; Appendix 2: Moynihan & Shuker, 2011), but larger body size did result in more sperm reserves and a higher number of daughters from their earlier mates (Chapter 4). Variation in the courtship routine of a male did not influence the probability of his losing fitness through a female he copulated re-mating (Chapter 5).

The patterns of sexual selection I observed are often a product of the local mating environment which also selects for the facultative sex allocation in *N. vitripennis.* Local Mate Competition (LMC), predicts an extremely female biased broods when a female is laying eggs alone on a patch (as a single-foundress) and so her sons are competing only with each other (Hamilton, 1967, Hamilton, 1979). In such a single-foundress situation, females are predicted to lay as few sons as possible to inseminate all her daughters, ensuring their fertility (Heimpel, 1994, Hardy & Cook, 1995, Waage & Sook Ming, 1984). Mechanisms to explain why there is often more than a single son include developmental mortality and limited male insemination capacity (Hardy & Cook, 1995, Hardy et al., 1998, Nagelkerke & Hardy, 1994). The extremely female biased sex ratios produced under LMC creates strong selection pressures on males to able to inseminate large numbers of females. If males are not able to inseminate all the females in their brood, then I predicted that mothers would lay more sons to ensure the fertility of their brood. In this way, the single-foundress sex ratio is a product of sexual selection on males to be good inseminators, and natural selection on females to lay the optimal sex ratio. Sexual selection and sex allocation should therefore be linked, and I would predict the build-up of linkage disequilibria between sex allocation and mating ability genes.

I screened the single-foundress sex ratio of eighteen strains of *N. vitripennis* and found significant between-strain variation in sex ratio, which was not explained by differences in the mating ability of sons (Chapter 3), or their total sperm resource (Chapter 4). There were no differences between strains in the sperm-limitation experienced by females who were inseminated by males that had copulated a number of times before (Chapter 4). This sequential presentation of females demonstrated that male *N. vitripennis* are capable of mating many more females than found in Chapter 3, and Grant et al. (1980) where strict time constraints were in place. These experiments did not show the effects of sexual selection on sex allocation that I had predicted.

I did nevertheless find evidence of sexual selection and sex allocation interacting: males from strains where broods are less female-biased (i.e. more males in the brood) were better competitors (Chapter 3). The causality of this remains to be established:

males from strains with higher sex ratios could have more experience with competitors and thus have been under stronger selection to be a good competitor, alternatively females could lay more sons when they will be better competitors. Either explanation is a result of the relationship between sex allocation and sexual selection.

Sexual conflict in *Nasonia vitripennis*

Mating frequency and subsequent female re-mating is a source of sexual conflict in many species (see Chapter 6 for a review in the Hymenoptera). Many factors influence the expression of conflict over this trait, however in most species there is the potential for conflict to occur (Arnqvist & Rowe, 2005). There has been little investigation into the costs and benefits of multiple mating in *N. vitripennis.* Despite this some aspects of *N. vitripennis* biology allows us to predict that such a conflict could arise. We know that there is first-male precedence in sperm-competition, but also that second males to mate do gain some paternity (Holmes, 1974). Hence males could lose fitness if a female they inseminated accepts a second mate. Females may benefit from second matings through increased sperm reserves, especially since sperm-limitation can be a problem in *N. vitripennis* (Chapters 3 & 4); alternatively, the problem of cytoplasmic incompatibilities may lead to advantages to multiple mating (Beukeboom & Werren, 1993, Bordenstein et al., 2001, Breeuwer & Werren, 1995). A small study did show a strain-specific benefit to higher fecundity in double-mated *N. vitripennis* females (Burton-Chellew, 2007). In an initial study no survival cost to mating was demonstrated for females in heterospecific crosses within the *Nasonia* genus (Geuverink et al., 2009), however further investigation is required.

There was significant between strain variation in female re-mating rate in *N. vitripennis* (Chapter 5). I recorded the duration of several courtship components, predicting that the post-copulatory courtship duration would influence female re-mating rate, based on the prior observations of van den Assem & Visser (1976) and Leonard & Boake (2008). However, the probability that a female re-mates was not associated with the duration of the post-copulatory courtship she received from her first mate (Chapter 5). Instead, females who needed more courtship before signalling receptivity on the first trial were less likely to mate on the second trial, suggesting that re-mating propensity is related to some form of overall "receptivity" and so is a property of the female. As such, although male post-copulatory courtship does appear to limit re-mating, the effects appear qualitative and not quantitative.

Sexual conflict over female re-mating rate in *N. vitripennis* warrants further investigation. Once the costs and benefits to multiple mating for females have been established, the mechanisms of male and female counter-adaptations predicted by sexually antagonistic coevolution can be investigated. The gene expression changes associated with the switching-off of receptivity are being studied in *Nasonia* (R. Watt & D.M. Shuker personal communication). To study the male adaptations in *Nasonia* we could utilise the newly sequenced *Nasonia* genome (The *Nasonia* Genome

Working Group, 2010) to begin to characterise possible components of the ejaculate that may influence female physiology (Chapter 6). The effects of seminal fluid could be tested by comparing the sex ratio and clutch size produced by females that mated once, and twice. Sperm-depleted males could be included in such an experiment to disentangle the effects of additional doses of sperm, and doses of just seminal fluid. The consequences of double mating with non-depleted males was tested in *Trichogramma evanescens* (Jacob & Boivin, 2005) and *Anisopteromalus calandrae* (Do Thi Khanh et al., 2005), and no difference in sex ratio with singly and doubly mated females, suggesting that the effect of seminal fluid components may not be particularly strong in those species.

Genetic variation and selection

Throughout this thesis I have found significant among-strain variation in a number of traits: single-foundress sex ratio, single-foundress clutch size, mating capacity (Chapter 3); seminal vesicle volume (Chapter 4); and copulation duration and re-mating probability (Chapter 5). This suggests heritable variation in these traits, and in the case of single-foundress sex ratio and clutch size gene regions associated with such variation have been identified (Pannebakker et al., 2011). By demonstrating such between-strain variation in nine or more strains collected from the same population at the same time, we can begin to ask questions about the evolution of these traits. Between nine and eighteen strains is not a large amount of genetic variation, certainly it is insufficient by quantitative genetic standards, however it is an improvement compared to so many studies that use just one or two strains. Heritable variation is the substrate of evolution, and is therefore crucial to our interpretation of these findings. We cannot draw conclusions about sexual selection if we cannot demonstrate heritable variation.

Males and the sex ratio

Most sex allocation theory focuses on female sex allocation behaviour, because the mother, or in the case of social insects the female workers, are usually in control (Wild & West, 2009). Little thought has been given to the role of males in this process. In this thesis I have considered two ways in which males can influence the sex allocation behaviour of a female: as both sons and fathers.

Fathers

The role of males in sex allocation is rarely thought of, as sex allocation is usually under female control in most taxa (Wild & West, 2009). Where male influence in sex allocation is considered, it is usually as his role of father in sexual conflict over sex allocation. The relatedness asymmetry set up by haplodiploidy (Figure 1.2) results in *Nasonia* mothers favouring the sex ratio predicted by LMC and fathers always preferring a more female-biased sex ratio. This leads to a sexual conflict over sex allocation (Chapter 6) (Appendix 1: Shuker et al., 2009). This is because fathers have only daughters and no sons, and sons have no fathers, only mothers (Figure 1.2).

This conflict predicts that males will have adaptations and females counter-adaptations to move the brood sex ratio closer to their own sex-specific optimum. The sex ratio female *N. vitripennis* lay was significantly (but weakly) affected by the strain of the male who mated her (Shuker et al., 2006c), suggesting that males can manipulate the sex allocation behaviour of females. One potential mechanism for this is through components of the ejaculate manipulating female fertilisation rate.

A male can also affect the sex allocation behaviour of his mate by not providing her with enough sperm. Here, males are acting very directly as a constraint on adaptive sex allocation by females. Female Hymenoptera can lay broods of all-sons when they have no sperm; as we have seen such females with no sperm reserves are known as constrained females (Godfray, 1990). These constrained females are unable to produce daughters, and so lay all-male broods. The sons in these all-male broods can find mates if another, mated, female lays daughters on the same patch. If there is a high enough proportion of constrained females in the population there will be an excess of males, and models predict that negative frequency-dependent sex ratio selection will favour females laying more daughters if they are mated (Godfray, 1990). This is not thought to be especially important in *N. vitripennis*, because the incidence of virgin females appears low in the wild (Beukeboom & Werren, 2000, Grillenberger et al., 2008).

Constrained-females could lack sperm for two reasons: either because they were never copulated, or they were copulated but not enough sperm was passed in order for them to have full fertility. Such females will also be constrained in their sex allocation behaviour by their mates.

I found significant variation in sperm resources between eighteen strains of *N. vitripennis* (Chapter 4). This suggests that the probability of a female becoming constrained after receiving a small ejaculate could depend on the strain (i.e. genotype) of the male that mated her. However in my experiment (Chapter 4), that did not lead to differences in sperm-limitation between the strains. It is still feasible that we would observe strain differences in sperm-limitation and the probability a female will become constrained, if the males had more than twelve sequential mates.

Seminal vesicle volume has been used as a measure of sperm resource in parasitoids such as *Trichogramma euproctidis* (Martel et al., 2011) and *Spalangia cameroni* (King & King, 1994). However the exact relationship between sperm count and seminal vesicle volume should also be established for *N. vitripennis*. Individual sperm cells are often thought of as relatively cheap, however an entire ejaculate can be costly (Dewsbury, 1982), leading to the prediction that males should use their sperm resource prudently and perform strategic ejaculation in response to sperm competition and female quality cues (reviewed by Kelly & Jennions, 2011). Strategic ejaculation has been observed in another parasitoid wasp in response to the threat of sperm competition: fewer sperm were transferred to females when males were kept in groups with other males prior to mating in the polyandrous wasp *Trichogramma turkestanica* (Martel et al., 2008). The potential for such strategic ejaculation should be investigated in *Nasonia.* For example, males from strains with a smaller total resource could be more prudent with their sperm, providing a greater number of females with full fertility than a male with a larger sperm resource that is less prudent.

Just as males may use strategic ejaculation to reduce the risk of sperm competition, males could have adaptations to encourage a female to use his sperm during sperm competition. In haplodiploids these adaptations may also be co-adaptations for manipulating his mate's sex allocation behaviour. A male that can encourage a female to use his sperm over another male's sperm may also be successful in persuading a female to use his sperm over no sperm to produce daughters rather than sons. This could be tested by repeating the experimental design of Shuker et al. (2006c) with several of the Cornell strains against a females from a "standard" where the mean sex ratio is closest to average the for all strains. The sperm precedence of males from these strains when first or second to mate a standard female strain could also be tested. If adaptations for sperm-competition are co-adaptations for manipulating sex allocation, I would predict a correlation between strain sex ratio manipulation and sperm-competition success.

This is reminiscent of the relationship between sex allocation conflict and sperm competition traits I discussed in the social insects in Chapter 6. There, males lose fitness in a non-linear manner when worker-control of the sex ratio reduces the female-bias in the sex ratio as the queen has more mates (Strassmann, 2001, Ratnieks et al., 2006). This leads to strong selection on males to prevent queens re-mating, and also on success in sperm competition.

Sons

Sons could alter the sex allocation behaviour of their mothers if variation in their competitive ability influences how good they will be at inseminating her daughters. When females are laying eggs alone on a patch of resources, Hamilton's LMC equation solves to predict all-female broods (Hamilton, 1967, Hamilton, 1979). This has usually been interpreted as meaning the minimum number of sons required to ensure the fertility of all the daughters in the brood (Heimpel, 1994, Hardy & Cook, 1995, Waage & Sook Ming, 1984). If a mother's sons are not very good at inseminating her daughters, I should expect that she will have to lay a higher proportion of sons to ensure that her daughters are all sufficiently inseminated.

My single-foundress sex ratio screen showed no relationship between clutch size and sex ratio (Chapter 3) demonstrating that one male is not enough to inseminate a whole brood, and therefore multiple males are required. If one males was enough to inseminate a brood of any size, I would expect to see a reciprocal relationship between sex ratio and clutch size. In this way, sons are affecting the sex allocation behaviour of their mothers. However, I found no evidence for males from strains with higher proportion male sex ratios having a lower mating capacity (Chapter 3), or a lower total sperm resource (Chapter 4) as predicted by the fertility insurance interpretations of the single-foundress sex ratio. This suggests that differences in male mating capacity is not explaining between-strain variation in single-foundress sex ratio.

Throughout this thesis, I have measured and discussed the sex ratio of the brood when they reach adulthood, the secondary sex ratio. Sex allocation behaviour occurs at the primary sex ratio. The outcome of this in terms of fitness benefits is influenced by sex-specific developmental mortality to reach the secondary (adult) sex ratio. Much of the work on sex-specific developmental mortality has been to address the fertility insurance explanations of a mother laying more than one son per brood (Green et al., 1982, Nagelkerke & Hardy, 1994), but such patterns of mortality can also influence sex allocation more generally, for instance through asymmetric larval competition. Asymmetric larval competition has been detected in *N. vitripennis*, however models suggest that the effect it will have on sex ratio optima is small (Sykes et al., 2007). There is little evidence to date for larval mortality when the preferred host species (calliphorid or sarcophagid fly pupae) are used (Werren, 1984, Velthuis et al., 1965, Walker, 1967, Werren, 1980).

A screen of the primary and secondary clutch size and sex ratio of several strains of *N. vitripennis* would maybe contribute further to our understanding of how sons influence sex allocation behaviour, although the effects are probably limited. Such a screen would require techniques such as flow cytometry to sex the eggs or very early embryos, such as those developed for the ants (De Menten et al., 2003). A higher developmental mortality in either (or even both) sexes in a particular strain, would act as an influence on the foundress' sex allocation behaviour, in that she must allocate more eggs as sons in order to ensure there are enough sons to fertilise all her daughters. Those extra sons are eggs which then cannot be fertilised and laid as daughters. In this way, developmental mortality is another route through which sons, more so than their sisters, can influence the sex allocation of their mother.

Another route through which sons could potentially influence the sex allocation behaviour of their mother is parent-offspring conflict over sex allocation. This is best described in social insects, where the offspring (workers) can control the secondary sex ratio (Ratnieks et al., 2006). Queens can combat worker control by laying male offspring before female reproductives in order to force the workers to raise more brothers than is optimal for them, as seen in bumble bees (Bourke, 1997, Bourke & Ratnieks, 2001). Sex allocation is usually interpreted from the point of view of the parents: however any situation resulting in biased sex ratios in a non-social species will lead to conflict between parents and offspring over the sex ratio. At its simplest, offspring should always rather be the rarer sex, as this sex will have a higher reproductive value (Werren & Beukeboom, 1998).

In species exhibiting LMC, which so often results in female-biased sex ratios, I would expect the offspring's optimal sex ratio to be less biased than that of their mother. When modelled, the structured population, female-biased dispersal and inbreeding lead to the prediction that offspring actually favour a more female-biased sex ratio than their mother in models of diploid species (Werren & Hatcher, 2000, Pen, 2006). In particular, the probability of a mother's offspring competing with their siblings, and paternal-half siblings leads to a more female-biased sex ratio being favoured. This problem is yet to be modelled in haplodiploids. However the relatedness asymmetries generated by haplodiploidy (Figure 1.2) are likely to extend the inclusive fitness benefits to offspring of being part of a more female-biased brood. Existing models assume that both the parents and the offspring have some influence over sex allocation, however there is little evidence for this in *N. vitripennis* (Verhulst et al., 2010). Sex determination mechanisms are thought to have evolved under conflict over sex allocation, so perhaps in the evolutionary past offspring had a greater role in their sex determination than they do today (Werren & Beukeboom, 1998, Werren & Hatcher, 2000).

Conclusions

In this thesis I have studied sexual selection in *N. vitripennis,* establishing sexual selection on several traits that have not previously been investigated (Figure 7.1). While I did not find the evidence for a role of sexual selection in sex allocation that I had expected, I did find that sex allocation may have influenced sexual selection, as males from high sex-ratio strains were better competitors (Chapter 3). My findings have complemented existing work in *N. vitripennis* to provide a more complete picture of sexual selection in this species so commonly studied for its sex allocation behaviour. I used multiple genotypes in Chapters 3-5, testing and finding between-strain variation in a number of traits. Demonstrating heritable variation in a trait is key to discussing selection acting on that trait, and builds the contribution of this thesis towards the work investigating how sexual selection and sex allocation interact as a species evolves. As a result of these investigations into sexual selection and sex allocation, I found evidence for new routes through which males can contribute to the sex allocation behaviour of females: as fathers through providing insufficient sperm, or through manipulating the sex ratio their mate lays. The role of males in sex allocation is a field which requires further exploration. The body of work here establishes the role of sexual selection, which is often closely linked with sexual conflict. Future work should focus on sexual conflict in *Nasonia vitripennis*, particularly over sex allocation, to balance the huge body of work investigating conflict over sex allocation in social Hymenoptera (Chapter 6).

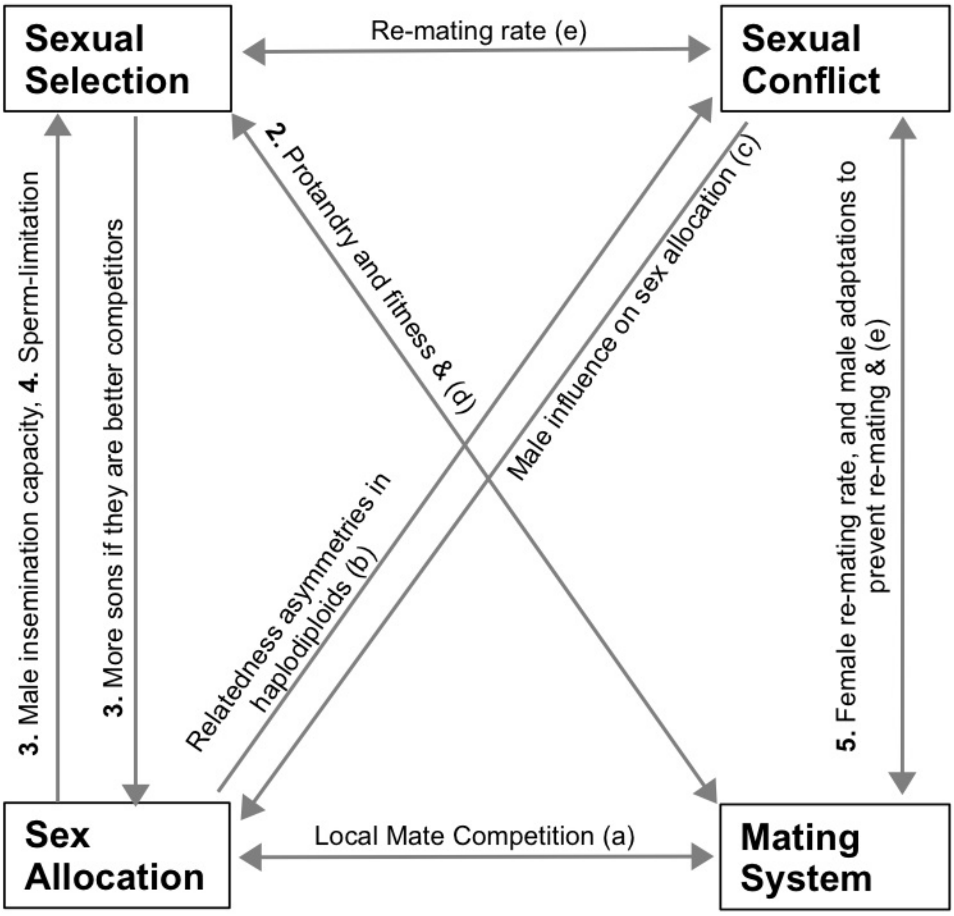


Figure 7.1: The links between sexual selection and sex allocation involve mating system and sexual conflict in *Nasonia vitripennis*. Numbers in bold refer to chapters. Letters are references to external sources: a) (Hamilton, 1967, Werren, 1980, Werren, 1983); b) (Shuker et al., 2009); c) (Shuker et al., 2006c); d) (Burton-Chellew et al., 2007b, Blaul & Ruther, 2011, Ruther et al., 2009); e) (Geuverink et al., 2009).

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Appendix 1: Shuker, D. M., Moynihan, A. M. & Ross, L. 2009. Sexual conflict, sex allocation and the genetic system. *Biology Letters* 5: 682–685.

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Appendix 2: Moynihan, A.M. & Shuker D.M. (2011) Sexual selection on male development time in the parasitoid wasp *Nasonia vitripennis, Journal of Evolutionary Biology* **24**: 2002–2013

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