The ecology and evolution of female-specific ornamentation in the dance flies (Diptera: Empidinae)

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Abstract

Elaborate morphological ornaments can evolve if they increase the reproductive success of the bearer during competition for mates. However, ornament evolution is incredibly rare in females, and the type and intensity of selection required to develop female-specific ornamentation is poorly understood. The main goals of my thesis are to clarify the relationship between the type and intensity of sexual selection that drives the evolution of female ornamentation, and investigate alternative hypotheses that might be limiting or contributing to the development of female ornaments. I investigated the ecology and evolution of female-specific ornaments within and between species of dance flies from the subfamily Empidinae (Diptera: Empididae). The dance flies display incredible mating system diversity including those with elaborate female-specific ornaments, lek-like mating swarms, aerial copulation and nuptial gift giving.

To elucidate the form of sexual selection involved in female-ornament evolution, I experimentally investigated the role of sexual conflict in the evolution of multiple female-specific ornaments in the species *Rhamphomyia longicauda*. Through manipulative field experiments, I found that variation in the attractiveness of two ornaments displayed by females indicates that sexual conflict, causing a coevolutionary arms race, is an important force in the evolution of multiple extravagant female ornaments. Using *R. longicauda* again, I tested for a role of functional load-lifting constraints on the aerial mating ability of males who paired with females displaying multiple large ornaments. I found no evidence of functional constraints influencing the mating opportunities of elaborately ornate females, but instead discovered a relationship consistent with positive assortative mating for mass.

Biased sex ratios are predicted to increase the intensity of sexual selection in a population, which in turn, is predicted to influence the evolution of ornamentation. I measured the incidence and prevalence of vertically transmitted symbiotic bacteria that has been observed to distort the sex ratio in other Dipteran hosts. While my survey revealed that symbionts occur at high incidence and variable prevalence across dance fly hosts, I found no effect of symbiont infection levels on population sex ratios, or female-specific ornament evolution. Further investigation into the relationship between sex ratios and female-ornament evolution using the comparative method revealed that the operational sex ratio (OSR) of a population did not predict continuous measures of female ornamentation across species. However, female-ornament evolution did predict male relative testis investment across species indicating that female ornaments likely indicate increased levels of polyandry.

My thesis reveals that sexual selection theory developed to describe male-specific ornament evolution cannot readily be translated to apply to females. I show that male mate choice, rather than functional constraints or ecological associations with bacteria, is likely driving the evolution of female-specific ornaments. I also identify sexual conflict as an important selective force in the evolution of female-specific ornaments.

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Chapter 1

General introduction

Sexual selection theory

Sexual selection results from differential reproductive success between individuals (Darwin, 1871). Many of the most extravagant morphological and behavioural traits in nature arise from selection on the ability of individuals to compete for and attract mates. Traits in the form of armaments, typically for competing with members of your own sex, and ornaments, which increase the attractiveness to the opposite sex, can evolve if they increase fitness despite costs on viability. Differential reproductive investment in gametes and parental care between males and females alter the strength and form of sexual selection (Trivers, 1972). In general, females, (who typically invest more heavily in gametes), are predicted to wait longer between reproductive bouts and therefore have a lower potential reproductive rate relative to males (Clutton-Brock & Vincent, 1991; Clutton-Brock & Parker, 1992; Kokko et al., 2012). Ultimately, this imbalance can lead to conditions where males are limited in their access to females (Emlen & Oring, 1977), resulting in contests that lead to stronger sexual selection in males.

Sexual selection can act through two main mechanisms: mate choice (intersexual selection), or intrasexual selection via contests. Mate choice occurs when the limiting sex favours certain phenotypic trait classes among the opposite sex when choosing a mating partner. Why particular traits are the target of mate choice, and how the mating preferences in the opposite sex were derived and are maintained has been the subject of extensive sexual selection research (Andersson & Iwasa, 1996; Arnqvist & Rowe, 2005). Mate choice typically results in the choosy sex (typically the limiting sex) receiving benefits, either directly (e.g. nutrition via a nuptial gift (Vahed, 1998; Lewis et al., 2014) or a high quality territory/habitat (Bensch & Hasselquist, 1992)) or indirectly (e.g. high quality genes and/or attractive offspring). Intrasexual selection occurs when there is competition (typically in the non-limiting sex) for access to mates or resources that might improve reproductive success. Within this context, the sexually selected traits in the non-limiting sex can improve their bearer's reproductive success by providing an advantage in the intrasexual contests.

While evidence of sexual selection acting on both sexes is widespread (Clutton-Brock, 2009), it is typically stronger in males than it is in females. However, there are cases where sexual selection acts more strongly on the female of a species compared to the male (Gwynne, 1990). Because female gametes (eggs) are more costly to produce than male gametes (sperm), females typically invest more in reproduction than males and are usually the limiting sex (Trivers, 1972). For sexual selection to be stronger in females there needs to be high variation in female quality (Parker, 1983; Owens & Thompson, 1994; Johnstone et al., 1996; Kvarnemo & Simmons, 1999) or males need to incur a high cost associated with mating (Kokko & Monaghan, 2001; Kokko & Johnstone, 2002). Males can experience high mating costs if they provide a nuptial gift, perform long or elaborate courtship behaviors, or if engaging in mate searching or copulation significantly reduces their future reproductive encounters. From the male perspective, female quality can vary by fecundity (Kvarnemo & Simmons, 1999), the stage of egg development (Funk &

Tallamy, 2000; LeBas et al., 2003) or by the likelihood of achieving paternity (Parker & Pizzari, 2010). In species where there is a high risk or intensity of sperm competition, males might engage in mate choice for females based on their perceived likelihood of fertilizing a female's eggs (Bonduriansky, 2001); a male might reject a female who has recently mated if there is first-male sperm precedence (Simmons, 2001) or a female with immature eggs if there is last-male sperm precedence (Parker, 1970). Indeed, where there are high breeding costs to males or high variation in female quality, the conventional mating roles can be reversed (Herridge et al., *in press*), and females become the target of sexual selection experiencing both mate choice (by males) and female-female competition (Gwynne, 1991).

Male mate choice has been documented in a number of taxa (Bonduriansky, 2001) with males demonstrating preferences for fecund females (Pitafi et al., 1995) or for correlates of fecundity such as female body size (Funk & Tallamy, 2000) or mass (Gwynne, 1991; Byrne & Rice, 2006). The risk of sperm competition has also been shown to influence a male's mating decisions (Schwagmeyer & Parker, 1990) and to affect male reproductive morphology (Pitnick, 1996; Vahed & Parker, 2011). In species where males have ample opportunity to mate, female phenotypic quality (e.g. fecundity) is presumed to be more important than female genetic quality. This is because, compared to the fitness effects of offspring quality, a male's reproductive success is typically more heavily linked to the number of offspring he sires (Bonduriansky, 2001). However, in some relatively rare cases, when the number of females available for insemination is low (e.g. in monogamous mating systems), female genetic quality can become an important factor in a male's mate choice decision (Roulin et al., 2000). For most species, though, a female's phenotypic quality is the focus of male choice, and this typically manifests as mate choice for fecund females (Bonduriansky, 2001).

Sexual selection can also act on females through intrasexual competition. Intrasexual competition among females is expected to be high when the operational sex ratio (OSR, the number of males and females available to mate at a given time) is female-biased (Emlen & Oring, 1977). Females might compete with one another for access to mates, or the resources provided by mates, by interrupting copulation (Bro-Jorgensen, 2008) or fighting against one another (Petrie, 1983). Some taxa display both male mate choice and female-female competition (Gwynne & Simmons, 1990) and these two processes can interact to influence mating behaviour (Santangelo & Itzkowitz, 2006). However, mating roles need not completely reverse, and both female-female competition and female (Owens & Thompson, 1994) or mutual mate choice (Johnstone et al., 1996) can occur within a species.

A biased OSR, which indicates the intensity of intrasexual competition in a population, can develop because of the life history trade-offs in the organism (Kokko et al., 2012), or because of biases in the primary and adult sex ratios (i.e. fewer of one sex in the population). Biases in the primary and adult sex ratios can result from environmental effects (Bowers et al., 2015), differential sex-specific larval survival (Wellings et al., 1986), variation in the sex-specific risk of predation (Gwynne & Bussière, 2002) or because of sex-ratio distorting endosymbionts (Hurst & Frost, 2015). Endosymbionts are incredibly common across arthropod species (Weinert et al., 2015) and certain symbiont species are well-known to cause wide-spread sex ratio distortion in their hosts. Sex ratio distortion typically occurs because the endosymbiont causes male-specific mortality in the their hosts, resulting in female-biased sex ratios. The sex ratio biases caused by symbionts can occur rapidly and have important consequences for the behaviour and reproduction of the host, including a consistently female-biased OSR (Hurst & Frost, 2015).

Female ornamentation

While sexual selection is well-documented in females (Clutton-Brock, 2009), reports of female ornamentation are scarce, and female weaponry is virtually unknown (Bro-Jorgensen, 2008). Female ornaments may in some cases be similar to traits possessed by males of the same taxon, a phenomenon called mutual ornamentation (Amundsen, 2000). Typically the sexes differ in their expression of mutual ornaments, and many hypotheses exist as to why mutual ornamentation persists (Kraaijeveld et al., 2007). One possibility is that females evolve ornaments through correlated evolution because they share genes with males (Lande, 1980; Rice, 1984; Lande, 1987). In some taxa, females adopt the male phenotype and mimic male sexual displays, including ornamentation, to avoid harassment by males (Burley, 1983). Mutual sexual selection can give rise to mutual ornamentation both through mutual mate choice (Jones & Hunter, 1993; Jones & Hunter, 1999), and mutual intrasexual competition (Moore, 1997). Mutual social competition can also give rise to mutual ornaments (West-Eberhard, 1979; West-Eberhard, 1983), particularly if both sexes are competing for some non-sexual resources such as a higher quality territory (Rohwer, 1975).

While even more rare than mutual ornamentation, female-specific ornaments have been documented in a number of different taxa including birds (Heinsohn et al., 2005), fish (Amundsen & Forsgren, 2001; Berglund & Rosenqvist, 2008), lizards (Weiss, 2002), and insects (Funk & Tallamy, 2000; LeBas et al., 2003). Most explanations for the evolution of mutual ornaments cannot be used to justify female-specific ornaments. Typically sexual or social selection is invoked to explain the evolution of female-specific ornaments. Indeed, sexual selection for female displays can arise through male mating preferences (Amundsen & Forsgren, 2001), and female-female competition can also select for female-specific ornaments (Heinsohn et al., 2005). The benefits males receive from mating ornamented females remains unclear, although they may have to do with detecting variation in female quality or fecundity. Indeed, female ornament expression has been found to positively covary with fecundity (Berglund & Rosenqvist, 2008), egg carotenoid concentration (Svensson & Petersson, 2000) and female condition (Weiss, 2002). However, there have also been reports that female ornament expression can negatively covary with egg quality (Nordeide et al., 2006) and condition (Nordeide et al., 2008).

One possible explanation for negative covariance between ornaments and offspring number is that females might face a trade-off between fecundity and ornament expression (Fitzpatrick et al., 1995); the finite amount of resources that a female has to invest means that any investment in ornament expression diverts resources away from other aspects of life history, including fecundity and maternal care. This potential trade-off is problematic for both sexes, but females might overcome any costs to ornaments if they are able to gain resources at each mating, for example through male-provided nuptial gifts. However, males should not choose females displaying elaborate ornaments if those ornaments also signal a diversion of resources away from fecundity. One theoretical model of female ornament evolution suggests that female displays are important for signaling fecundity when direct mate assessment is difficult, even if females are at risk of incurring a fecundity cost (Chenoweth et al., 2006). This model predicts stabilizing selection on female ornaments, such that females who display intermediate ornament expression should have the highest mating success, a pattern that has also been observed empirically (Wheeler et al., 2012). In this thesis I investigate the importance of classic predictors of sexual selection leading to male-specific ornaments (sexual conflict and OSR) in the context of female-specific ornament evolution. I also test two alternative hypotheses, functional constraints and reproductive parasitism, to clarify how conflicting selection pressures within females might contribute to patterns of female-specific ornamentation. I carried out my studies across a group of taxa that vary in the type and intensity of female-specific ornaments they display to potential mates.

The study system

Dance fly biology

The Empidinae dance flies display a number of interesting mating traits; males typically provide the female with a nuptial gift at the time of mating, many species form lek-like mating swarms, and roughly one third of the species from *Rhamphomyia* and *Empis* genera display female-specific ornaments that are entirely absent in the male (Cumming, 1994). Female dance flies can display several sex-specific ornament types: inflatable abdominal sacs, pinnate leg scales and darkened or enlarged wings (Collin, 1961; Cumming, 1994). The degree and type of ornamentation varies substantially across the group, with some species displaying almost no sexual dimorphism, and others that display multiple female-specific ornaments (Funk & Tallamy, 2000).

Prey-item nuptial gifts are an important resource to female dance flies because the protein she derives from them seems to be required for oocyte maturation (Downes, 1969). However, in many species males have evolved the ability to exploit the female willingness to trade sex for food, and will 'cheat' her by providing non-nutritious substitutes at the time of copulation (LeBas & Hockham, 2005). These non-nutritious nuptial gifts can consist of debris from the environment (twigs, stones, leaves), male secretions (such as a silk balloon) or the empty exoskeletons of previously consumed prey item (Kessel, 1955; LeBas & Hockham, 2005).

Mating swarms in the dance flies can vary substantially by species in terms of density, sex ratio and timing. Many species form mating swarms at a 'swarm marker' and return to the same site annually with specific individuals revisiting the swarm for the duration of the mating season (Svensson & Petersson, 2000). For most species, the mating swarm sex ratio (or OSR) varies temporally throughout a swarming season (Funk & Tallamy, 2000; Svensson & Petersson, 2000; Wheeler, 2008). However, the mean OSR for the season varies quite substantially across species from female-biased (Funk & Tallamy, 2000) to male-biased (Chapter 5).

Across the Empidinae subfamily, patterns of sexual selection on female-specific ornaments and male preferences for them remain unclear. Previous studies of sexual selection across the group have shown that female-specific pinnate leg scales (LeBas et al., 2003) and enlarged (Svensson & Petersson, 1987) or patterned wings (Svensson, 1997) are honest indicators of fecundity, such that increases in ornament displays correspond with increased fecundity. However, a recent study showed that both inflatable abdominal sacs and pinnate leg scales were under stabilizing selection in a multiply-ornamented species, such that females that displayed an intermediate level of ornamentation were more likely to mate (Wheeler et al., 2012). Therefore, while female-specific ornaments might typically serve to indicate honestly a female's quality, in some species males might be discriminating against those females who over-invest in ornaments relative to fecundity. Intriguingly, in species for which multiple female-specific ornaments exist, it remains unclear how these traits are interacting – do multiple ornaments act as independent signals, or contribute to some overall phenotype that is under selection as a whole?

The long-tailed dance fly, Rhamphomyia longicauda

Female long-tailed dance flies display two sex-specific ornaments: inflatable abdominal sacs and pinnate leg scales (Figure 1.1). A female inflates her abdominal sacs by swallowing air and can increase her abdominal area by three to four times its deflated size (Funk & Tallamy, 2000). Pinnate leg scales occur on the femora and tibia of all six of a female's legs and, like the abdominal sacs, are completely absent in males. The leg scales extend laterally along a female's legs and give the appearance of her having broader legs than males. A female positions her legs alongside her inflated abdomen to display both ornaments in a mating swarm, presumably to exaggerate her body size as perceived by males.

R. longicauda mating swarms form annually in June in North Eastern North America and occur at dusk and dawn in wooded areas where there are gaps in the tree canopy. The mating swarms are typically female-biased (Funk & Tallamy, 2000), but the sex ratio can vary temporally and spatially (Steyskal, 1941; Downes, 1969; Wheeler, 2008). Males

photos by Gil Wizen

Figure 1.1. Female-specific ornamentation in *Rhamphomyia longicauda*. The female (left) is displaying two ornaments, inflatable abdominal sacs and pinnate leg scales, during a mating swarm. Both ornaments are completely absent in the male (right).

enter the mating swarm from below carrying a nuptial gift (typically a winged insect prey item). Females within the mating swarm vary substantially in the number of mature oocytes they carry; all levels of egg maturity have been observed, even in specimens collected late in the season (Funk & Tallamy, 2000). Considering both the variation in female quality and the presumed cost to males of providing a nutritious nuptial gift, *R. longicauda* might experience female-female competition and male mate choice. Thus, females are predicted to be the target of sexual selection in the long-tailed dance fly.

Previous studies of *R. longicauda* support sexual selection as being important for the evolution of female-specific ornaments in this species. In an experimental field study, Funk and Tallamy (2000) showed that males were more attracted to larger plastic models of displaying females. Wheeler (2008) improved on this study by controlling for body size and showed that males were more attracted to female silhouettes if they displayed large ornaments compared to small ornaments. Funk and Tallamy (2000) also showed a positive covariance between egg size and inflated abdomen area that explained 23% of the variation in abdomen size. However, this result was interpreted as an indication that the abdominal ornament in *R. longicauda* was deceptive, because in a related non-ornamented species, *R. sociabilis*, egg size explained 72% of abdomen area.

In a recent selection analysis, wild *R. longicauda* females that were caught in copula were compared with unpaired females from the mating swarm, which showed that both inflatable abdomens and pinnate leg scales are under stabilizing selection (Wheeler et al., 2012); females displaying intermediate levels of ornamentation were most likely to be mated. This pattern could indicate that females are being discriminated against by males if they over- or under-invest in ornamentation. However, an alternative hypothesis is that while males may be discriminating against females displaying small ornaments, it might be functional constraints rather than mate choice that are limiting the pairing success of females displaying the largest ornaments. Indeed, in other dance fly species that engage in aerial copulation similar to that observed in *R. longicauda*, males have been shown to be at their load-lifting capacity (Marden, 1989); males paired with the most massive female they could carry given the load-lift constraints placed on their wings by their own body mass and the mass of the nuptial gift they were already carrying. Crucially, a formal test of the load-lifting functional constraint hypothesis in *R. longicauda* has not been performed.

Research objectives

The main objective of my thesis is to provide novel insights into the interactions of the ecological and evolutionary forces that shape female ornamentation. I have studied dance flies from the subfamily Empidinae for two main purposes. First, I investigate two hypotheses that have been shown to correlate with male-specific ornament evolution but remain untested in female-specific ornament evolution. I tested these hypotheses in the multiply ornamented species *Rhamphomyia longicauda*.

Chapter 2. Sexual conflict causing an antagonistic arms race between the sexes has been shown theoretically and empirically to result in multiple male-specific deceptive display traits that are differentially attractive to females. Given that there are vast differences in how each sex allocates resources to life history traits, it currently remains unclear whether sexual conflict could influence female-specific ornament evolution in the same way. In this chapter I test for evidence of sexual conflict in the evolution of multiple female-specific ornaments by investigating the attractive value of each ornament type. Based on theoretical predictions about the development of male-specific ornaments, I expected to see variation in the attractiveness of each female-specific ornament being displayed. Specifically, I hypothesized that the more recently evolved ornament should be more attractive to males than the more basal ornament if female ornaments are deceptive, as predicted by sexual conflict theory.

Chapter 3. Functional constraints in taxa where males carry females during copulation can cause non-random mating patterns that are not selected for by classic mate choice theory. In *R. longicauda*, males carry females during aerial copulation and previous research has shown that female ornaments in this species are under stabilizing selection: females displaying intermediately sized ornaments had the highest mating success. However, classic theories of mate choice for ornaments predict that selection on display traits should be directional. One untested hypothesis for why stabilizing selection is observed for *R. longicauda* ornaments is that functional constraints are limiting the size or mass of female that a male can lift during aerial copulation even though male choice should be for the largest ornaments. In this chapter I test for a load-lift constraint on a male's ability to carry females displaying the largest ornaments. I hypothesized that females displaying the largest ornaments should pair only with the largest males, and female mass should trade-off with a male's own mass and the mass of the nuptial gift he carries.

Second, I quantified variation across dance fly species to ask questions about the selective pressures contributing to female-specific ornaments in multiple lineages.

Chapter 4. Endosymbionts that act as reproductive parasites to their hosts have been shown to manipulate host sex ratios to be female-biased. Theory (developed to understand male-specific ornament evolution) predicts that bias in the sex ratio can cause sex-specific ornamentation in the sex in excess. I tested to see if endosymbiont infection prevalence was related to sex ratio biases across dance fly species. I predicted that if endosymbionts were causing sex ratio distortion in species with female-specific ornaments, that there should be a pattern of female bias in the adult sex ratio (ASR) and female-biased infection prevalences in species with female ornamentation.

Chapter 5. The operational sex ratio (OSR) has been shown to accurately predict the intensity of sexual selection leading to male-specific ornamentation. In this chapter I tested if female-specific ornament evolution could be predicted by population OSR measures. I predicted that species that have a more strongly female-biased OSR should also display higher degrees of female-specific ornamentation. Further, I predicted that female-specific ornaments should indicate that females are mating with more than one male, and therefore there should be a correlated response in the reproductive anatomy of males from species with more female ornamentation.

My work focuses on the evolution of female ornaments that challenge conventional theory about the costs and payoffs of investing in ornamental traits. Thanks to a combination of detailed hypothesis tests within a single relatively well-studied species (Chapters 2 & 3) and comparative analyses across a diverse and relatively poorly known group of species (Chapters 4 & 5), I hope to clarify the conditions that favour ornament evolution, both for the fascinating and unusual taxa that feature female ornaments, as well as for more classic mating systems.

Chapter 2

The role of sexual conflict in the evolution of multiple sex-specific female ornaments

Abstract

Sexually selected ornaments rarely develop in females, in part because male choice for adorned females is difficult to sustain, especially if attractive females mate more frequently and therefore impose high risks or intensities of sperm competition. However many dance flies have extravagant female ornaments, and some species even have multiple ornaments, which are difficult to explain using classic adaptive mate choice models. Alternatively, sexual conflict might favour deceptively seductive traits in females, which allow them to secure nutritious nuptial gifts from males in spite of preferences for gravid or fecund females, as well as antagonistic responses in males that help them resist female seduction. The resulting arms race would produce stronger selection on recent rather than ancestral ornaments. We compared the effect on attractiveness of two ornaments in an extravagantly adorned dance fly, *Rhamphomyia longicauda*, by manipulating the appearance of plastic silhouettes of females suspended in a wild swarm. We found significant directional preferences for larger ornaments of both types, but variation in the more derived ornament was much more effective at improving attractiveness. Our results support the possible role of sexual conflict in generating the many and diverse elaborate female ornaments among dance flies.

Introduction

Sexually selected ornaments are among the most fantastic and bizarre traits found in nature. While these extravagant ornaments undoubtedly improve an individual's reproductive success (Darwin, 1871), they might nevertheless trade off with other important life history traits. Benefits from increased reproductive success must, therefore, outweigh any costs in order for an ornament to persist. The trade-off between sexual displays and other characters may be particularly important in explaining the rarity of female ornaments, especially among taxa in which females experience strong sexual selection in the form of sexual competition (Clutton-Brock, 2009; Shuker, 2010). Compared to males, female reproductive fitness is typically more resource limited, so investing in costly sexual traits might decrease fecundity rather than honestly advertise it (Fitzpatrick et al., 1995).

Given their potential costs, the persistence of female ornaments in a few rare taxa is puzzling (Funk & Tallamy, 2000; Heinsohn et al., 2005; Tobias et al., 2012; Flanagan et al., 2014). One possible explanation is that what appear to be ornaments have evolved for reasons other than improving sexual attraction (West-Eberhard, 1979; West-Eberhard, 1983; Heinsohn, 2008; Tobias et al., 2012); in such cases the costs of investing in elaborate traits are outweighed by advantages in other aspects of life history. For example, female ornaments might evolve primarily for the purposes of intrasexual competition for resources rather than for access to mates (Heinsohn et al., 2005; LeBas, 2006; Heinsohn, 2008; Flanagan et al., 2014).

If, by contrast, female ornaments arise through classic mate choice, they must not only compensate for any direct resource costs required for their construction, but they must also honestly provide information to males about female reproductive value (LeBas et al., 2003; Wheeler et al., 2012; Flanagan et al., 2014). The potential trade-off between ornaments and offspring poses one challenge to the signal value of ornamental traits, but this challenge can be overcome, if, for example, resources used to construct ornaments are not limiting for offspring production (Fitzpatrick et al., 1995). A more serious constraint on the signal value of ornaments occurs when females store sperm from multiple partners. In such cases, the expected benefit of ornamentation for females (in terms of heightened attractiveness) is probably almost always a cost to males, who should generally prefer relatively unattractive but monandrous mates over attractive ones that present higher risks or intensities of sperm competition (Fitzpatrick et al., 1995; Amundsen, 2000; Amundsen & Forsgren, 2001). In other words, the heightened attractiveness of adorned females dilutes the share of her eggs to which any individual mate has access, and therefore should theoretically disfavor male preferences for elaborately ornate females.

An alternative explanation is that female ornaments can arise through sexually antagonistic coevolution instead of classic mate choice models (Holland & Rice, 1998; Arnqvist & Rowe, 2005; Arnqvist, 2006). In this scenario, the primary function of female ornaments is to deceive mates rather than honestly signal quality (Funk & Tallamy, 2000; Arnqvist, 2006), which relieves this hypothesis of the burden to adaptively explain male preferences. To illustrate how this could occur, imagine that females experience intense competition for access to males (perhaps because males provide valuable goods or services to their mates, such as nutritious “nuptial gifts” (Lewis et al., 2014)). Presented with competing offers from courting females, males should begin to discriminate among potential mates in favour of particularly fecund or gravid individuals, especially if the last male to mate before oviposition obtains the highest share of paternity in a clutch (Parker, 1970; Birkhead & Moller, 1998; Simmons, 2001). To the extent that such male choice might limit female access to male-provisioned resources, selection will favour any female traits that exploit male preferences for characters signaling high fecundity, and make females appear especially gravid or fecund (even if such traits provide males with less information on female fecundity or gravidity). From the female perspective, the direct advantages of acquiring nuptial gifts could compensate for any resource costs of ornament investment. From the male perspective, costs of mating with deceptively underfecund or particularly promiscuous females could incite sexually antagonistic coevolution, and lead to an arms race (Parker, 1979; Holland & Rice, 1998; Gavrilets et al., 2001) in which females attempt to seduce choosy males with more and more elaborate disguises of their fecundity, and males attempt to resist the deceptions of females. Arms races arising from sexual conflict are well described for species with "conventional" sex roles, in which males typically develop traits that harm female fitness, while females evolve resistance to male manipulation (Holland & Rice, 1998; Rowe et al., 2005).

The same coevolutionary theory also predicts cyclical patterns of male resistance to deceptive female ornaments followed by novel ornamental innovations that overcome developing male resistance. Ultimately, antagonistic coevolution can result in multiple and elaborate female ornaments even if male preferences for such traits are not necessarily adaptive. The crucial distinctive feature of sexually antagonistic arms races, that novel traits are more effective at manipulating the opposite sex than more ancestral ones, provides an opportunity to distinguish sexual conflict from other models of ornament evolution (Holland & Rice, 1998).

Dance flies from the subfamily Empidinae (Diptera: Empididae) display considerable interspecific variation in mating system. One third of species from the group feature some of the most extravagant female-specific ornaments yet described (Cumming, 1994; Funk & Tallamy, 2000; LeBas et al., 2003) including darkened, patterned or enlarged wings, feathery "pinnate" leg scales and, more rarely, inflatable abdominal (pleural) sacs (Collin, 1961; Cumming, 1994). Males often provide direct benefits to females by providing a prey item as a nuptial gift during copulation. In most species, females are not known to hunt as adults, and seem to rely on protein from male-provisioned gifts to supplement their metabolic protein reserves (Newkirk, 1970). While the value of ornaments to female dance flies seems straightforward (they have almost certainly evolved in the context of competition for access to male courtship gifts), the adaptive significance of male preferences for ornaments are much less clear. As is typical for insects, female dance flies can store sperm from many males, and eggs are only fertilized immediately prior to oviposition. Furthermore, previous work suggests that ornaments may in fact disguise rather than exaggerate fecundity (Funk & Tallamy, 2000), which implies they have limited signal value to choosy males. In this chapter, we describe a manipulative field experiment that measures selection on two ornamental traits to discern whether an intersexual arms race may help explain the prevalence of dance fly ornaments without requiring adaptive male choice.

Females of the long-tailed dance fly, *Rhamphomyia longicauda*, possess two extravagant ornaments: pinnate scales over the length of all femora and tibia, and pleural sacs that are inflated just prior to swarming. Few dance fly species have abdominal ornaments, and when present they co-occur with pinnate leg ornaments (Collin, 1961; Cumming, 1994) suggesting that inflatable abdomens are recent innovations compared to pinnate scales (Chapter 5). Both ornaments appear to exaggerate a female's apparent fecundity and to improve female attractiveness (Funk & Tallamy, 2000) in the highly competitive context of *R. longicauda* mating swarms (which are usually heavily female-biased; (Funk & Tallamy, 2000; Gwynne & Bussière, 2002; Wheeler, 2008)).

R. longicauda males carrying prey items enter the swarm from below, and appear to assess potential mates by hovering below a female (perhaps to observe her ornaments more closely) before presenting her with a nutritious nuptial gift. Previous work using plastic models of females within the mating swarm showed that *R. longicauda* males are more attracted to larger females, consistent with directional selection on ornament size (Funk & Tallamy, 2000). However, Wheeler et al. (2012) found that females with intermediate levels of ornamentation were more likely to mate than either extreme. This apparent contrast could arise because of sexual conflict. If large ornaments are good at initially attracting males, but less effective during subsequent examination of prospective mates (when males might attempt to see past the disguises of females), one might observe both linear selection on ornaments during initial attraction and stabilizing selection on ornaments for mating success.

We experimentally manipulated the relative size of both leg and abdominal ornaments using plastic models similar to those employed by Funk and Tallamy (2000) in order to quantify the independent effect of each trait on attractiveness. This experiment allowed us to assess whether, as predicted by sexual conflict, selection on abdomens is stronger than selection on leg scales, or whether (as predicted by more classic honesty models of ornament evolution) the two traits contribute equally (in terms of phenotypic variation or in terms of relative surface area) to providing a single signal.

Methods

Study system

In Northeastern North America, courtship swarms of *R. longicauda* form annually along riverbanks and occur from the end of May until the beginning of July (Steyskal, 1941; Newkirk, 1970). Swarms are crepuscular, forming exclusively at dusk and dawn, beneath gaps in the forest canopy and are typically strongly female-biased (Cumming, 1994; Funk & Tallamy, 2000; Gwynne et al., 2007) although the sex ratio can vary spatially and temporally (Wheeler, 2008). Before they enter the swarm, female *R. longicauda* swallow air to inflate abdominal sacs that exaggerate their body size. Within the swarm, females fly parallel to the ground and position their pinnate-scaled legs laterally around their inflated abdomen, which further exaggerates their size when they are viewed from below.

Males carrying insect prey items as nuptial gifts enter the swarm from below the displaying females. From this position, females appear as silhouettes against the backdrop of the dawn or dusk sky. A male approaches a female and hovers below her, presumably to better inspect a potential mate. During pair formation, the male transfers the nuptial gift to the female and the pair exits the display swarm to copulate ‘on the wing’ where a male carries a female while she feeds on the nuptial gift (See fig. 8 from Funk & Tallamy, 2000)).

Female silhouette creation

We independently manipulated both female ornaments and mating swarm position in order to disentangle the partial effects of selection for attractiveness on each of the ornamental modules (abdomens and leg scales). There is strong natural covariance between both ornament types in wild female flies (because females who accumulate more resources as larvae are likely to invest heavily in both ornaments; (Van Noordwijk & de Jong), which makes separating the effects of selection on each ornament in real specimens difficult (Wheeler et al., 2012). Our artificial silhouettes break apart the natural covariance, and display combinations of ornaments that do not exist in nature to improve our ability to visualize the whole fitness landscape, as well as to improve our statistical power for measuring partial selection on each module.

We created 25 models of female silhouettes (Figure 2.1) using a template provided by David Funk (See fig 3. Funk & Tallamy, 2000). We manipulated the abdomen size independently of leg scales such that we had five different abdomen widths: mean +/-2SD, mean +/-SD, and population mean (estimates of population means and standard deviations come from Wheeler et al. (2012). Although we initially attempted to similarly restrict our models' pinnate scales to the range of natural variation, we could not precisely control the apparent size of the legs, which made such fine scale variation impractical. Consequently for leg scales we included a larger range of sizes including legs similar to males (the ancestral condition) and legs twice as large as the largest found in nature: mean +/-10.8SD, mean +/-5.4SD, and population mean.

We printed the artificial silhouettes on plastic transparencies and attached each of them to a 30cm length of fishing line. To simulate the positioning of female dance flies in the mating swarm, we fastened fishing weights above the models to keep the silhouettes

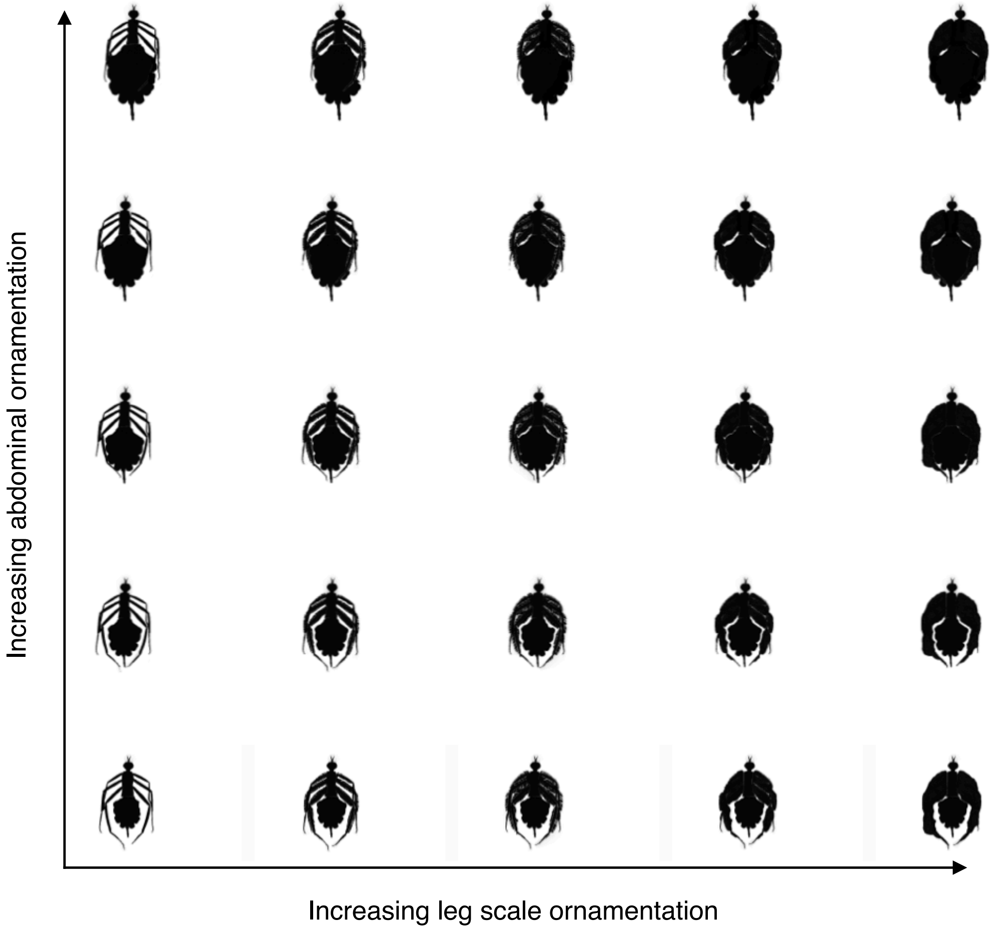

Figure 2.1 Silhouettes of 25 plastic models of females created to display to males within the mating swarm. Silhouettes vary in the amount of two female-specific ornaments on display. Abdomen ornament sizes are population mean, mean ± 1.5 SD and mean ± 2.5 SD. Leg scale ornament sizes are population mean, mean ± 5.4 SD and mean ± 10.8 SD.

parallel with the ground. We placed a stake on either side of the swarm site (1.5m apart) with a piece of fishing line stretched between them 1m above the ground. We then chose five silhouettes at random and spaced them 15cm apart across the centre of the line such that the flanking models were approximately 37cm from a stake.

Experimental set up

We carried out male mate choice trials from June 12-22, 2012 at the study site, which has been used for previous studies of this species (Gwynne et al., 2007; Bussière et al., 2008; Wheeler et al., 2012), located near Glen Williams, Ontario, Canada on an island in the Credit River (43$_o$41’11”N, 79$_o$55’34”W). A trial consisted of a single swarming event at dawn (roughly 4:45am) lasting approximately one hour. We set up female silhouettes the evening before a trial began (at least eight hours in advance) so that they were already *in situ* when the swarm started. Each trial began when a male first approached one of the female models. We recorded an approach to a model when a male fly carrying a nuptial gift hovered less than 5cm beneath a female silhouette for more then 3 seconds. Swarm position, ornament sizes and number of male approaches were tallied for each silhouette on each date. We concluded a trial when five minutes passed without observing a male approach (typically between 5:45 and 6am).

Statistical Analyses

To investigate the relationship between female silhouette morphology and male attraction, we fit all models described below with the number of approaches by courting males as a response and ornament expression levels and the swarm position of silhouettes (distance from the centre of the swarm) as predictor variables using R statistical software (R Core Team, 2014). We standardized each of the morphological predictors to facilitate comparisons between traits. However, our standardized coefficients are not strictly equivalent to standardized selection gradients because attraction is only the first stage of mating success, and therefore cannot be straightforwardly translated into fitness (Wheeler et al., 2012).

The nature of selection varies as a function of swarm composition, which can differ substantially from day to day (Wheeler, 2008). Therefore we built a generalized linear mixed model with Poisson error and log-link (because attraction is measured in male approaches and is a count variable), including "date" as a random effect in the lme4 package in R (Bates et al., 2014). Pinnate leg scales, abdomen inflation and position within swarm were fitted as fixed effects. We included each predictor variable as well as its square and interactions in order to assess curvilinear and correlational effects of morphology on attractiveness. We illustrate the partial effects of each ornamental trait by plotting the fitted effects after fixing the other covariates at their mean value.

To test the hypothesis that the two ornamental modules reinforce one another, combining to provide a single impression of size to males, we performed another analysis in which we regressed male approaches on the total area (mm^2) contributed by each ornament type to the silhouette area (instead of the standardized trait size). If the two ornaments both contribute to an overall impression of size, we expect to see similar improvements in attractiveness for an additional unit of female area, regardless of whether that increase in area comes from leg scales or abdominal inflation. By contrast, if the abdominal ornament is an evolutionary innovation brought about by increasing levels of male resistance to deceptive leg pinnation, we predict that abdominal ornaments should be more effective at improving attractiveness than leg ornaments, whether these ornaments are computed in terms of absolute area or phenotypic standard deviations.

Results

We recorded a total of 1479 male approaches over the course of ten mating swarms. Males preferentially approached female silhouettes displaying larger ornaments, and males were more likely to approach and court a female silhouette if it was positioned near the centre rather than the periphery of the swarm (Table 2.1, Figure 2.2).

Figure 2.3 illustrates the partial effects of abdomen and leg scale size on numbers of male approaches. We have superimposed the raw data to assist with visualization, but note that much of the variation in attractiveness is explained by orthogonal dimensions of the silhouette phenotype that are accounted for in the estimation of the partial effects. We also note that although the effects illustrated in Figure 2.3 appear curvilinear, this is due to the back-transformation from log-linear phenotypic space; there was no evidence of significant quadratic selection on either of the ornaments (Table 2.1). Instead, both ornamental traits appeared to improve attractiveness in a linear fashion: males are more likely to approach female silhouettes with larger pinnate leg scales (Table 2.1, Figure 2.3A) and larger abdomens (Table 2.1, Figure 2.3B). Although the variation in silhouette pinnate leg scales was larger than variation in abdominal ornamentation, (+/- 10.8SD compared with +/- 2SD, respectively), male dance flies responded more strongly to variation in abdomens than in pinnate leg scales (Figure 2.3).

We also found a significant negative coefficient associated with the term describing an interaction between abdominal and leg ornamentation (Table 2.1). Figure 2.4 helps to illustrate this nonlinear correlational effect: the convex curvature near the apex shows that the two ornaments combine in a less than additive way. Furthermore, while abdominal ornaments are always important for attracting mates, pinnate leg scales are only important if a female’s abdomen is small.

In order to compare the effects of both ornaments on attractiveness as a function of overall size (rather than in terms of wild phenotypic variation), we performed the same analysis as that described in Table 2.1 except using the area (in mm^2) of each ornament as a predictor. Table 2.2 illustrates that abdominal area still has a stronger effect on

Table 2.1. Parameter estimates for a generalized linear mixed effects model describing how standardized ornamentation and swarm position traits affect female attractiveness.

	trait	**estimate**	**standard error**	**z value**	**p value**
linear	swarm position	-2.94	0.216	-13.6	<0.0001
	leg scale pinnation	1.35	0.379	3.57	<0.0001
	abdomen size	3.72	0.438	8.50	<0.0001
quadratic	swarm position	-1.02	0.187	-5.43	<0.0001
	leg scale pinnation	-0.278	0.252	-1.11	0.269
	abdomen size	-0.0731	0.433	-0.169	0.866
interaction	swarm position * leg scale	0.0078	0.0067	1.16	0.246
	swarm position * abdomen	-0.0196	0.0294	-0.667	0.505
	leg scale * abdomen	-0.0136	0.0031	-4.36	<0.0001

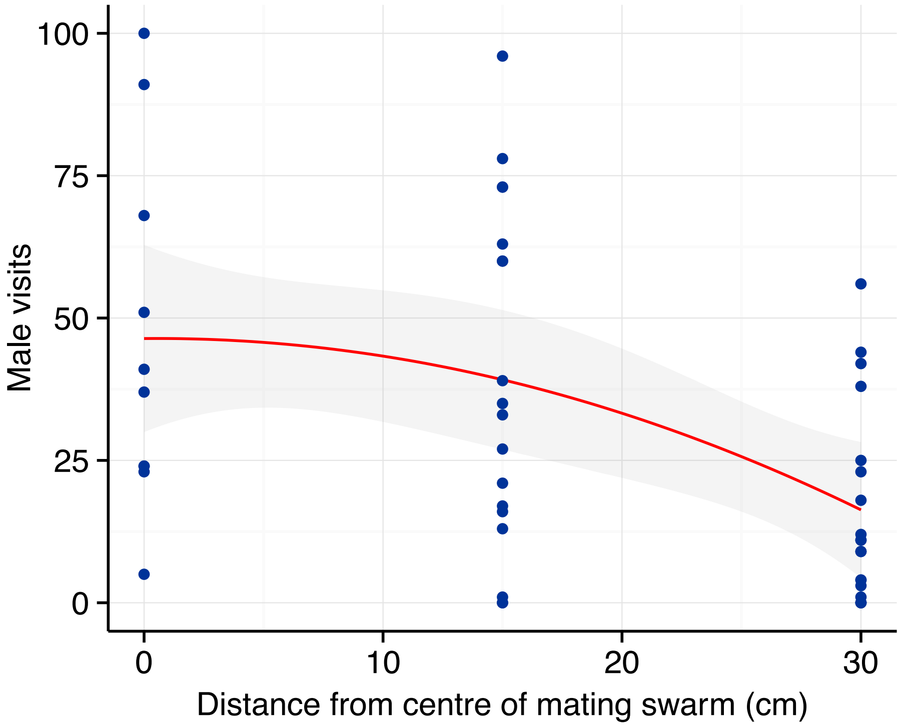

Figure 2.2. The partial quadratic effect of female swarm position on male visitation from a linear mixed effects model. Swarm position was measured as the distance from the centre of the mating swarm in increments of 15cm. The shaded area represents the standard error around the measure, values for the mode are reported in Table 2.1.

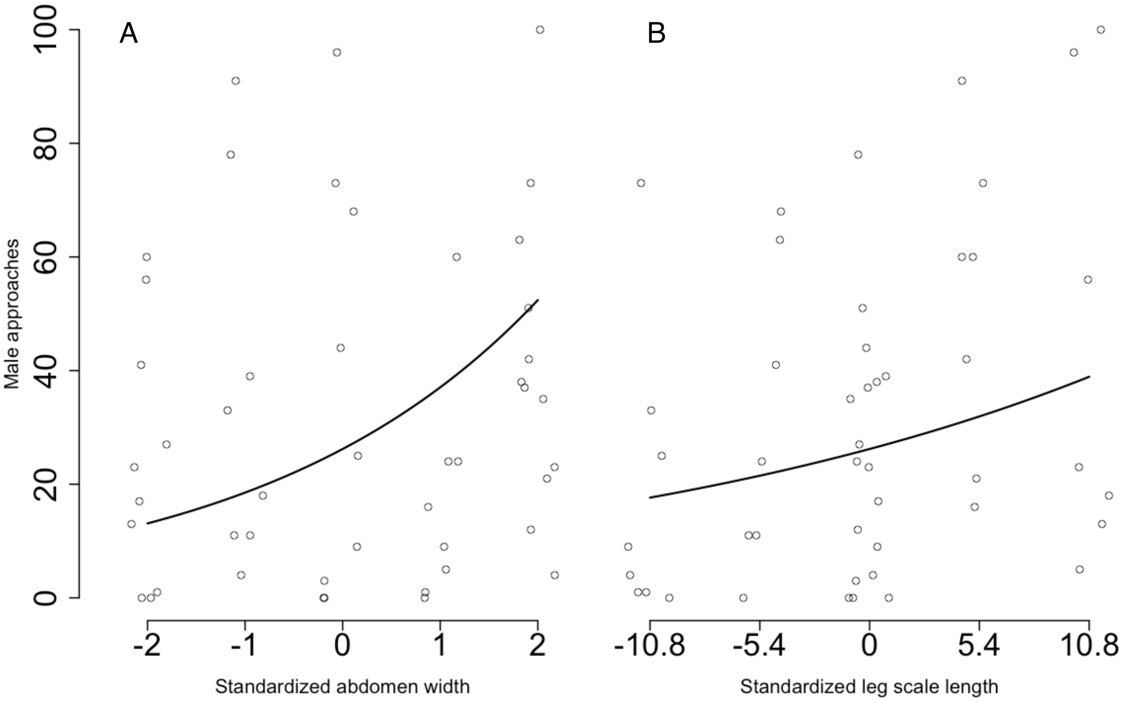

Figure 2.3. The partial effect of manipulated female ornamentation (abdomen width and pinnate leg scale length) on male courtship attempts overlaid on the raw data. Abdomen size was partitioned from the population's natural size variation ranging from two standard above and below the mean population size (-2 to 2 on the x-axis). The leg scale length was manipulated such that males were presented with ornament sizes from outside the natural population range (no scales (mean male leg size), 0.5×, 1.5× and 2× mean female scale length).

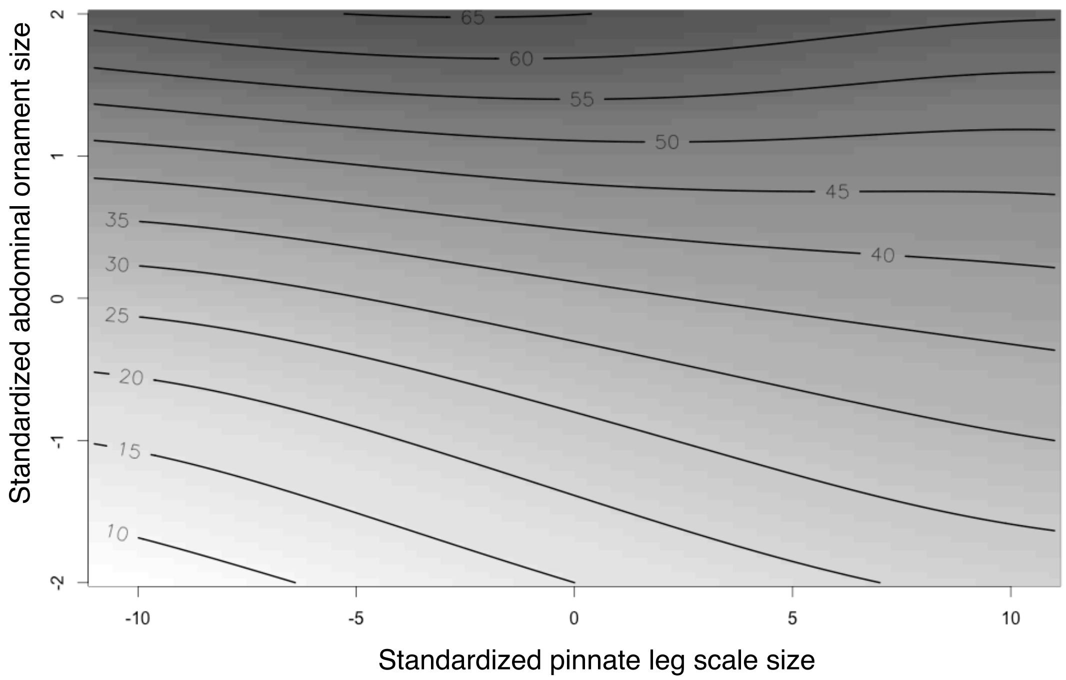

Figure 2.4. The mate attraction landscape incorporating linear and correlational attraction on abdominal ornaments and pinnate leg scales in *R. longicauda* females. Values on contour lines indicate the average number of male visitors for the trait space that each line occupies.

Table 2.2. Parameter estimates for a generalized linear mixed effects model describing how area (mm^2) of ornamentation and swarm position affect female attractiveness

	trait	**estimate**	**standard error**	**z value**	**p value**
linear	swarm position	-4.39	2.44	-1.8	0.0725
	leg scale pinnation	6.98	1.59	4.38	<0.0001
	abdomen size	11.29	1.86	6.07	<0.0001
quadratic	swarm position	-1.00	0.188	-5.32	<0.0001
	leg scale pinnation	-0.273	0.235	1.161	0.246
	abdomen size	-0.0894	0.43	-0.208	0.835
interaction	swarm position * leg scale	0.114	0.118	0.966	0.339
	swarm position * abdomen	-0.0284	0.069	-0.412	0.68
	leg scale * abdomen size	-0.532	0.119	-4.47	<0.0001

attractiveness than leg scales; a given unit of silhouette area is twice as effective at improving attractiveness if it contributes to the abdomen rather than the leg area.

Discussion

We measured male attraction to two female-specific ornaments in *Rhamphomyia longicauda* and show that males are attracted to both ornaments, but that variation in inflatable abdominal sacs has a stronger effect on attractiveness than variation in pinnate leg scales, a pattern predicted by sexual conflict theory. This difference in effectiveness is sustained whether we regress attractiveness on units of ornament area or phenotypic standard deviations, which suggests that the two ornaments are not simply acting together as a single reinforced signal. Below we discuss the implications of our study for the role of sexual conflict in female ornament evolution.

Evidence of sexually antagonistic coevolution

The extent to which active male choice can favour extravagant female ornaments is theoretically constrained by both potential resource trade-offs between ornaments and offspring, as well as by heightened sperm competition that might occur in attractive, polyandrous females (Bonduriansky, 2001). We predicted that an arms race resulting from sexual conflict (Arnqvist & Rowe, 2005) might explain the presence of two extravagant female ornaments in *R. longicauda* without requiring adaptive male choice. Instead, female ornaments are favoured because they improve female access to male-provisioned nuptial gifts, while males might try to resist seduction by deceptive females by more closely inspecting mates prior to passing over prey. Cyclic bouts of sexually antagonistic coevolution (Holland & Rice, 1998) (in which females develop deceptive ornaments, and males evolve to resist them) would result in weaker selection on more ancestral forms of ornamentation (such as the pinnate leg scales of *R. longicauda*) compared to more recently derived ornaments (such as the inflatable abdominal sacs)

(Murray unpublished data). Consistent with the antagonistic coevolution prediction, we found that variation in abdominal ornaments is more important for attractiveness than variation in pinnate leg scales (Figure 2.3). Moreover, pinnate leg scales are only effective at increasing attractiveness when the abdominal ornament is small; when abdominal sacs are large there is no discernible effect of pinnate leg scales (Table 2.1, Figure 2.4).

While there are many examples of males preferring large females, it is rare to find male choice specifically for female ornaments (Amundsen, 2000; Bonduriansky, 2001), (but see LeBas et al., 2003). In the case of *R. longicauda*, males appear to receive less information about a female's fecundity through her ornaments than they would if there were no ornaments at all (Funk & Tallamy, 2000). Our experimental design allowed us to disentangle a female's display modules and compare the effect of each on male attraction. We were also able to assess how the ornaments combined to improve attractiveness. Both the combined effect of ornaments in standardized phenotypic space (Table 2.1, Figure 2.4) and our analyses of ornament area (Table 2.2) suggest that males are not simply attending to the overall size of a silhouette. This observation is not consistent with an alternative hypothesis for multiple ornament evolution, that the ornaments act together as single reinforced signal (Candolin, 2003).

Stronger male attraction to abdominal ornaments compared to pinnate leg scales may be a function of how mate assessment occurs within the mating swarm. Males approach potential mates from below and inspect females against the backdrop of the sky. During this assessment, males hover below their female of interest, perhaps in order to inspect her more closely. Our results indicate that males can differentiate between the contributions of ornamental leg scales and inflated abdomens to silhouette size. Perhaps sexual conflict over male choice for fecund females has selected for males that attend more closely to the details of a female's phenotype. We predict that inspections prior to pair formation (such as are observed in *R. longicauda*) should be especially prolonged in taxa featuring female ornaments that might otherwise disguise a female's ovarian condition. Similarly, any trait (such as visual acuity) that allows males to better distinguish between deceptive ornaments and cues of actual fecundity should be favoured, and increase selection for novel ornaments that circumvent male resistance. The inflatable abdomens found in *R. longicauda* (and which may have evolved independently in a few other dance flies) may be more effective disguises because they are less easily detected by male sensory systems. It may also be easier to differentiate a deceptive fecundity signal that evolves on the legs compared to the abdomen simply because abdomen size is more closely associated with fecundity (Bonduriansky, 2001).

While our experiment did not directly measure mating success, we were able to quantify the attractiveness of each ornamental trait displayed by *R. longicauda* females in the mating swarm, which has been shown to be an important contributor of mating success in many taxa (Candolin, 2003). Following male attraction, however, a female might undergo further assessment by her mate, resulting in discrimination against (overinvesting) females displaying the largest ornaments that are a greater cost to fecundity (Fitzpatrick et al., 1995; Chenoweth et al., 2006). In fact, Wheeler et al. (2012) showed that *R. longicauda* females displaying the largest ornaments are less likely than intermediately ornamented females to mate. An alternative explanation for the intermediate female advantage predicted by Chenoweth et al. (2006) and observed by Wheeler et al. (2012) is that females displaying the largest ornaments are actually too heavy or cumbersome for some males to carry while flying united. Load-lifting constraints compromising mating success of the largest or most ornamented females would predict size- or weight-assortative mating, with the largest females pairing only with males that have greater load-bearing capacities (Marden, 1989). Previous research found no evidence of size-assortative mating in *R. longicauda* (Bussière et al., 2008), but further study quantifying the total load a male carries (female and nuptial gift) and traits related to a male's own load-lifting ability (e.g. wing load, aspect ratio) is required to fully rule out load-lifting constraints in the evolution of female ornaments (Marden, 1989).

The effect of mating swarm position on female attractiveness

Our results show that a female silhouette is more likely to attract courting males if it is displayed closer to the centre of the lek-like mating swarm (Figure 2.2). This finding is consistent with previous work on another lekking insect species, *Ceratitus capitata*, which found that lek position was an important indicator of attractiveness (Niyazi et al., 2008). Further, many studies investigating diverse taxa with lek mating systems have shown that centrally positioned males are the most attractive (Fiske et al., 1998; Kokko et al., 1998; Bro-Jorgensen, 2008). In many male leks, intra- as well as intersexual selection for a central position is described, however, in *R. longicauda* mating swarms, while we have compelling evidence for intersexual selection on swarm position (Figure 2.2), there is no evidence that females physically engage with one another (Bussière et al., 2008; Wheeler, 2008). Previous work on *R. longicauda* swarm position (Bussière et al., 2008) showed that female flies at the bottom of the swarm (where males enter) were larger than females higher up in the swarm. Perhaps males enter the mating swarm from beneath at its centre, and both our study and Bussière et al. (2008) have captured different spatial dimensions of the same phenomenon: females competing for access to males (and their gifts) when they first enter the swarm. Additionally or alternatively, being close to the periphery of the swarm might make individuals more vulnerable to predation. *Tetragnatha* spiders build webs around the periphery of *R. longicauda* mating swarms and dance flies are frequently preyed upon (Gwynne et al., in review). It is likely that being in the centre of the swarm means that both sexes are safer from spider predation, which could confer an advantage to centrally positioned silhouettes in the absence of any intraspecific competition for position.

Conclusions

Female-specific ornaments are rare among animals, and almost never as extravagant as those found in males. Their prevalence and exorbitance among dance flies is not easily explained by classical theories of ornament evolution. Here, we adopt sexual conflict theory, as it has been applied to taxa featuring more typical sex roles, and find that it predicts the difference in effect of two ornaments as well as explaining the presence of both. We therefore suggest that sexual conflict may be the key to explaining the curious and fantastic diversity of female ornaments among dance flies.

Chapter 3

The role of functional constraints in non-random mating patterns for a dance fly with female ornaments

Abstract

Mate choice is typically invoked to explain non-random mating patterns, particularly in species that display elaborate ornaments. However, conflicting selection pressures on traits can result in functional constraints that also contribute to non-random mating patterns. We tested for functional load-lifting constraints in *R. longicauda*, a species that displays multiple female-specific ornaments that are under stabilizing selection. *R. longicauda* males provide females with a nuptial gift before engaging in aerial mating, where the male bears the entire weight of the female and nuptial gift for the duration of the copulation. We tested to see if wild-caught males collected during copulation were experiencing load-lift limitations. We found no evidence that functional constraints are contributing to non-random mating patterns in *R. longicauda*. However, we did find evidence suggesting mass-assortative mating may occur. We suggest that male mate choice for intermediate female-specific ornamentation in *R. longicauda* would be a valuable future avenue of research.

Introduction

Mate choice is typically invoked to explain mating patterns, but there are other limitations on mating that can influence who an individual mates with. For example, the ability to evaluate accurately a potential mate's quality (Chapter 2; Endler & Basolo, 1998; Funk & Tallamy, 2000; Candolin, 2003) or functional limitations to an individual's mating success (Marden, 1989; Almbro & Kullberg, 2008) can both result in non-random mating patterns. Difficulty in accurately assessing potential mates can result in maladaptive mate choice (Candolin, 2003; Dubois et al., 2011) and functional or physical constraints can limit the pool of individuals available for an individual to mate with regardless of mating preferences (Schluter et al., 1991; Peckarsky et al., 2002). Functional constraints are frequently important for the mating ecology of flying animals (McLachlan & Allen, 1987; Marden, 1989; Sawadogo et al., 2013), and physiological constraints on flight performance might particularly influence mating patterns in taxa that engage in aerial copulation (Marden, 1989; Peckarsky et al., 2002). Functional lift constraints may be especially important among insects that engage in aerial copulation because female size is frequently used as an indicator of fecundity with the result that male mate choice for large females is widespread across the group (Bonduriansky, 2001). If load-lifting limits constrain a male's ability to mate with the most attractive females (because they are also the heaviest females), mate choice alone will not be able to account for resulting mating patterns. Indeed, loading constraints that impact the reproductive biology of a species are likely, not only to change observed mating patterns, but also bring about important ecological and evolutionary consequences.

In some insect taxa that engage in aerial copulation, males provide females with a nuptial gift (Thornhill & Alcock, 1983) before supporting the entire combined load during mating (Kessel, 1955; Marden, 1989; Funk & Tallamy, 2000). Loading must be particularly intense in these species because loadings from both foraging and mating are combined. For a wide variety of insects there is a positive correlation between nuptial gift size and reproductive success (Thornhill, 1979; Svensson et al., 1990; Vahed, 1998), therefore load-lift limitations in species that both provide nuptial gifts and engage in aerial copulation are likely to have functional constraints that impact their reproductive success. Sexual selection should favour males that offer large nuptial gifts to the largest females. However, males who are at the upper limit of their load-carrying capacity may face a trade-off between the size of nuptial gift they offer, and the size of female the mate with.

Dance flies from the subfamily Empidinae (Diptera: Empididae) are a group with several species that exhibit mating behaviour in which males provide nuptial gifts to females and then carry the combined load of female and nuptial gift during aerial copulation (Cumming, 1994). Indeed, patterns consistent with load-lift constraints have been previously observed in a dance fly (Marden, 1989). However, one particularly well-known feature of dance flies is that several species have evolved female-specific ornaments (Collin, 1961; Cumming, 1994; LeBas et al., 2003; Wheeler et al., 2012), and while these are hypothesized to influence on mating patterns (Bussière et al., 2008; Wheeler et al., 2012), no study has yet investigated loading constraints in a dance fly with female-specific ornamentation. Selection on ornaments is likely to be an important contributor to observed mating patterns in dance flies with female-specific displays. Therefore, by investigating functional constraints for dance flies that exhibit both female ornamentation and load-lifting mating behaviour (nuptial gift giving and aerial copulation), we can contrast our findings with previous results in non-ornamented dance flies (Marden, 1989) to inform our understanding of the interaction between sexual selection and functional constraints in the evolution of mating patterns. Here, we use *Rhamphomyia longicauda,* a dance fly with female-specific ornaments, to investigate the role of functional constraints in the evolution of non-random mating patterns.

R. longicauda forms mating swarms at dusk and dawn in which females display two sex-specific ornaments: pinnate leg scales and inflatable abdominal sacs (Newkirk, 1970; Funk & Tallamy, 2000). Males enter the mating swarm carrying a prey item as a nuptial gift and approach females from below. When mated pairs first form, they engage in the apparently cumbersome task of transferring the nuptial gift from the male to the female as they exit the mating swarm. Some initial attempts at pairing and gift transfers are unsuccessful, and the male and female separately return to the mating swarm. For pairs that manage to successfully transfer the nuptial gift, copulation occurs on the wing, with the male carrying the female while she feeds on the nuptial gift he has provided (Funk & Tallamy, 2000).

Previous studies have shown that *R. longicauda* females with the largest ornaments are more likely to attract a male (Chapter 2; Funk & Tallamy, 2000; Wheeler, 2008) suggesting positive directional sexual selection on ornamentation. However, a cross-sectional comparison of the phenotypic traits of successful (paired) and unsuccessful (unpaired) females found no evidence for directional selection on either of two ornamental traits (Wheeler et al., 2012). Instead, both ornaments were under stabilizing selection; females displaying intermediate-sized ornaments were more likely to be paired than rivals with either small or large ornaments. The difference in success during attraction versus pair formation may reflect differences in selection acting on separate episodes in the *R. longicauda* mate pairing process: while large ornaments are important for initially attracting males to potential mates within the *R. longicuada* mating swarm, the actual mating success of a female may be mediated by behavioural interactions that follow the initial attractiveness stage, and which disproportionately affect the most ornate females.

Why might females that are most attractive initially (with the largest ornaments (Chapter 2 ; Funk & Tallamy, 2000)) have depressed mating success relative to more intermediately ornate rivals (Wheeler et al., 2012)? One hypothesis is that there may be functional constraints on the pairing process (Bussière et al., 2008; Wheeler et al., 2012). If females displaying the largest ornaments are also the heaviest (because large ornaments probably covary with increased mass), it is possible that high-mass females, while attractive, are more difficult to carry during aerial copulation. If female mass constrains pairing success, then we might expect to see a positive relationship between female mass and male wing traits; males with larger wings are able to carry heavier females. Further, load-lift limitations are likely to produce a negative relationship between female mass and a male's own relative wing loading measure (i.e. male mass/wing area). Loading-constraints are also predicted to produce a trade-off between nuptial gift and female mass (Marden, 1989). Finally, if heavy females can only be borne by males with above average load-carrying abilities, we expect the total wing loads (total mass per unit surface area of wing) observed within the population to be lighter than those from a null distribution assuming random mating.

Methods

Sample Collection

Samples were collected near Glen Williams, Ontario on the banks of the Credit River in July 2012. This site has been used for several previous studies on *R. longicauda* (Gwynne & Bussière, 2002; Gwynne et al., 2007; Bussière et al., 2008; Wheeler et al., 2012). At each swarming event, we collected mated pairs from nuptial flight using an entomological sweep net. We placed pairs and their nuptial gifts individually in collection tubes. It was not possible to retrieve all nuptial gifts from each mated pair because the female will sometimes drop the gift when she is caught in the net. All samples were frozen at -20C immediately following collection.

Morphological Measurements

Morphological measurements were taken using a dissecting microscope connected to a camera and analyzed using ImageJ digital imaging software (Abràmoff et al., 2004). We took the following morphological measurements: femora and tibia length, wing length, thorax length, leg area (including pinnate leg scales in the female), wing area, number of eggs and the egg length of five eggs per individual. For paired characters we measured both right and left sides and took the mean. When this was not possible because of damage to one side, we measured only the undamaged side. We also dissected the spermathecae and recorded the presence or absence of sperm to determine whether or not the female had mated previously (we note that a female may have received sperm from the male she is currently paired with, and consequently we may be underestimating the number of females that were virgins prior to mating). We recorded male mass, female mass and, when possible, nuptial gift mass within 24 hours of collection.

We also computed two indices of the load on an insect's wings arising from its own body mass: wing load is the ratio of male mass to wing area (the load already being carried by the male's wings) and aspect ratio is the ratio of the squared wingspan to wing area (Berwaerts et al., 2002; Gyulavári et al., 2014).

Statistical Analyses

Area measurements (legs and wings) were square root transformed and mass measurements were cube root transformed prior to inclusion in any statistical models. All independent variables were also z-transformed before analyses. Because *R. longicauda* swarm composition can vary temporally (Wheeler, 2008), for each set of statistical models we tested for an effect of "date" that each sample was collected as a random effect in a mixed model but found the results from mixed and fixed models were qualitatively the same. For simplicity, the results we report here all come from fixed models.

In order for a loading constraint to contribute to the observed stabilizing selection pattern on *R. longicauda* ornaments (Wheeler et al., 2012), female mass must positively covary with female ornaments. We performed a linear regression of female leg ornamentation on female mass. In order to assess whether female mass was an accurate measure of fecundity in *R. longicauda*, we also performed a linear regression of oocyte count and size against female mass.

High positive correlations between phenotypic traits are expected whenever groups of traits are functionally related. To test for collinearity in our models we examined variance inflation factors (VIF) using the ‘vif’ function in the car package (Fox, 2011). If any VIF in our linear model exceeded three we removed the variable with the highest VIF value and reran the model until all VIF values were below three. We note that this is a conservative VIF cut-off (Zuur et al., 2010), but given the potential for collinearity between the wing traits used as predictor variables, we wanted to limit correlations between phenotypic traits as much as possible.

We performed a series of analyses to test for evidence of loading constraints in *R. longicauda* mated pairs. First, we wanted to investigate the relationship between the load a male carries during aerial copulation and male wing traits. We performed general linear models on both of our datasets (with and without the nuptial gift measure) that included predictor variables that have previously been shown to impact loading constraints and insect flight performance e.g. (Berwaerts et al., 2002; Peckarsky et al., 2002; Gyulavári et al., 2014). We fit the load a male lifts during aerial copulation as the response, and morphological measures for male thorax length, male wing load (male mass/wing area), wing aspect ratio (wingspan^2/wing area) and wing length as predictor variables. Because female dance flies feed on the nuptial gift during copulation, the mass of the gift will decrease in proportion to how much the female has fed. Similarly, the female’s mass will increase as she feeds. Therefore, the combined mass of both the female and the nuptial gift is a superior measure of the estimated load being lifted by the male for testing the constraint hypothesis.

We next wanted to test whether the observed mass that a male carries during aerial copulation was less than expected by chance (i.e. if mating was random). We used resampling methods to assess the null expectations for the total mass supported per mm^2 of male wing area under the assumption of random pairing. For every iteration of our resampling procedure, we randomly paired each male in our dataset with a female and nuptial gift, and then recomputed the mean ratio of total load mass to male wing area. This exercise produced 9999 means from the null distribution, where female mass, gift mass, and male wing area had no bearing on pair formation. We added our observed mean to these 9999 trials, and assessed its rank among the total distribution of 10000 as the probability that our collected samples could have arisen through random pairing in nature. We repeated this procedure but omitting nuptial gift mass to take advantage of the more numerous samples collected without nuptial gifts. If load-lifting constraints are important for the non-random mating patterns observed in *R. longicauda*, then we would expect that the load-lift ratios from our actual dataset to fall within the lower 2.5% (for a significance cut off of $p<0.05$) of the random distribution of loading ratios (i.e. indicating that the mass being lifted by males is less than would be expected under random pairing).

Results

We collected 112 *R. longicauda* mated pairs from June 5-22, 2012. For 57 of those pairs we also collected their nuptial gifts, however, the remaining 55 pairs dropped the nuptial gift during collection. Summaries of our morphological measurements for female and male traits can be found in Table 3.1. Only 3.5% of the females had no sperm in their spermatheca, and while we recognize that this might be an underestimate, we can only be sure that four females were collected during their first mating event. Because of the small fraction of our samples that were definitely collected during their first mating, we did not include this index of previous mating activity in any further analyses.

We first wanted to test whether or not female mass covaried with female fecundity, oocyte size, or ornamentation. In insects, a female's mass frequently predicts her fecundity or egg quality (Bonduriansky, 2001), and ornamentation in *R. longicauda* covaries positively with body size (Wheeler, 2008). We regressed female ornamentation against female

Table 3.1. Mean morphological trait values for paired *R. longicauda* females and males (N=112).

trait	**mean**	**standard error**
females		
thorax length (mm)	1.60	0.012
squareroot wing area (mm)	3.98	0.021
wing length (mm)	6.74	0.040
mass (mg)	4.63	0.13
hind leg length (femora and tibia: mm)	6.46	0.36
squareroot hind leg ornament area (mm)	2.23	0.014
fecundity (oocyte count)	66.3	1.67
oocyte size (mm)	0.31	0.01
males		
thorax length (mm)	1.57	0.012
squareroot wing area (mm)	3.71	0.023
wing length (mm)	6.31	0.039
mass (mg)	2.68	0.085
hind leg length (femora and tibia: mm)	5.77	0.033
squareroot hind leg area (mm)	1.29	0.008
nuptial gift mass (mg)*	0.80	0.068

*N=57

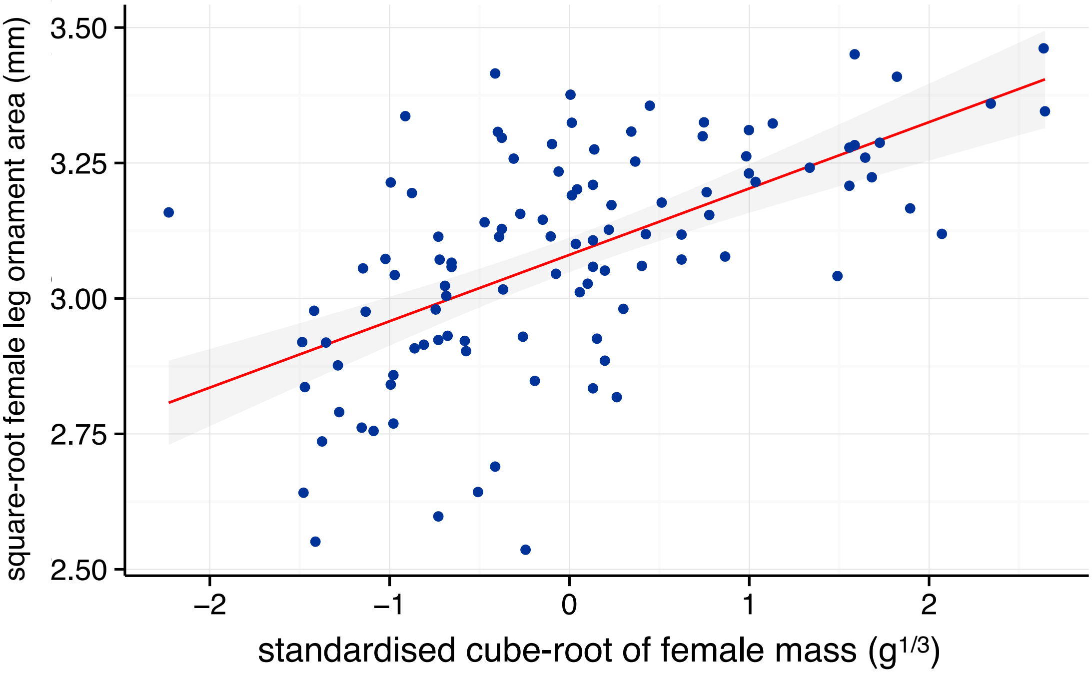

Figure 3.1. Linear regression of square-rooted female leg ornament area (mm) on standardised cube-rooted female mass ($g^{1/3}$). The shaded area represents the standard error measure around the linear estimate (shown in red). (β=0.12, R^2=0.35, $F_{(1, 105)}$=58.35, $P<0.001$).

mass and found that, as expected, there is a positive relationship (Figure 3.1; β=0.12, R^2=0.35, $F_{(1, 105)}$=58.35, P<0.001). Next, we regressed measures of female fecundity and oocyte size against female mass. We found that female mass positively covaries with female fecundity (Figure 3.2; β= 6.029, R^2=0.11, $F_{(1, 105)}$=14.33, P<0.001) but not oocyte size (β= 8.45, R^2=0.01, $F_{(1, 105)}$=1.035, P=0.31).

The load being lifted by the male during in-flight copulation was calculated as the combined mass of the nuptial gift and the female (Marden, 1989). The proportion of the load that the nuptial gift makes up ranged from 4-40% (mean: 14.26 ± 0.97%; Table 3.1). If males experience a load-lift constraint during aerial copulation, they may have to trade-off nuptial gift mass with female mass; i.e. we expected to see a negative relationship between nuptial gift and female mass. However, we found the opposite pattern, that nuptial gift mass positively covaries with female mass in mated pairs (β= 0.91, R^2=0.09, $F_{(1, 56)}$=6.85, P=0.01).

In the event of a load-lift constraint, we predicted that a male's own wing load (mass/wing area) would negatively covary with the mass of the load he lifts during aerial copulation; males already burdened by a high wing load should be constrained by their own mass and only able to carry (and copulate with) females with relatively low mass. However, in the event of a load-lift constraint we expected a positive relationship, between the mass of the load being lifted by the male and male wing size traits (length and aspect ratio); larger wings should be able to carry more mass. We found that the only morphological trait that predicted the mass being lifted during aerial copulation was a male's wing load (male mass/wing area) for both datasets (with and without nuptial gift; Tables 3.2 and 3.3). Contrary to the load-lift constraint predictions, we found that males that had a high wing load paired with heavier females (in the absence of nuptial gift measure; Figure 3.3; β=0.00062, R^2=0.20, $F_{(1, 105)}$=26.95, P<0.001) and were found with a heavier combined mass (when nuptial gift measures were available; Figure 3.4; β=0.00086, R^2=0.25, $F_{(1,56)}$=19.6, P<0.001).

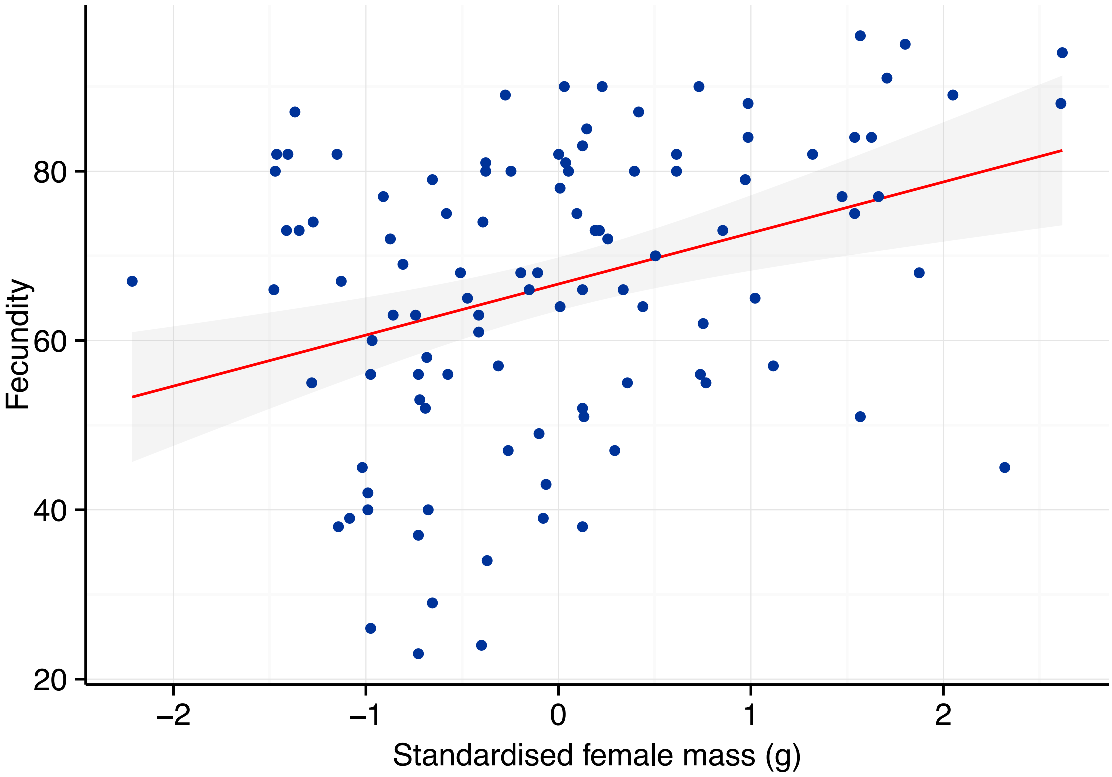

Figure 3.2. Linear regression of female fecundity (measured as oocyte count) against standardised female body mass. The shaded area represents the standard error measure around the linear estimate of the relationship (shown in red). (β= 6.029, R^2=0.11, $F_{(1,105)}$=14.33, $P<0.001$).

Table 3.2. Male wing traits that predict the mass carried by a male during copulation in *R. longicauda*.Estimates are standardised coefficients of a multiple regression. Mass carried is measured as combined mass of female and nuptial gift (g).

trait	**estimate**	**standard error**	**z**	**p**
intercept	0.0046	0.00012	35.06	<0.001
male wing length	0.000051	0.00020	0.28	0.79
male wing load (mass/wing area)	0.00079	0.00021	3.82	<0.001
aspect ratio (wingspan^2/wing area)	0.00025	0.00020	1.23	0.23

Table 3.3. Male wing traits that predict the mass carried by a male during copulation in *R. longicauda*. Estimates are standardised coefficients of a multiple regression. Mass carried is female mass (g).

trait	**estimate**	**standard error**	**z**	**p**
intercept	0.0056	0.00019	28.82	<0.001
male wing length	-0.000056	0.00013	4.76	0.68
male wing load (mass/wing area)	0.00062	0.00013	2.39	<0.001
aspect ratio (wingspan^2/wing area)	0.00018	0.00012	1.15	0.14

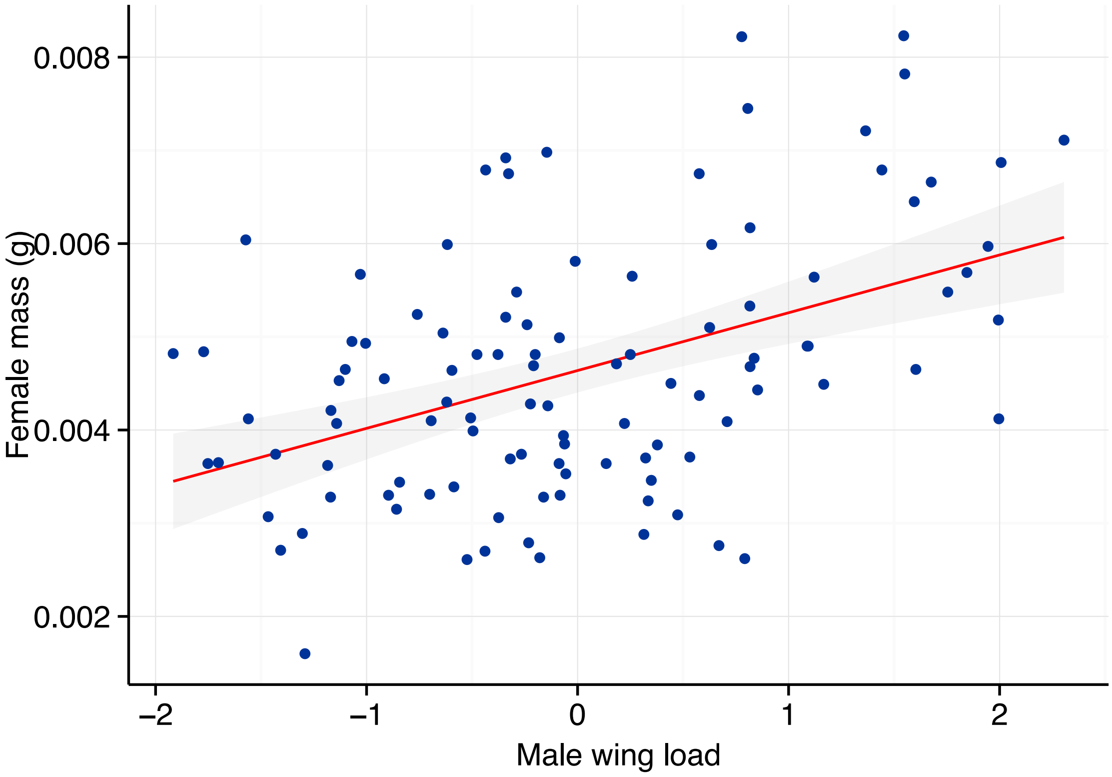

Figure 3.3. Linear regression of the female mass (g) on standardised male wing load in *R. longicauda* mated pairs. Wing load is calculated as mass/wing area (see text for details). The shaded area represents the standard error measure around the linear estimate (shown in red). (β=0.00062, R^2=0.20, $F_{(1,\ 105)}$=26.95, $P<0.001$).

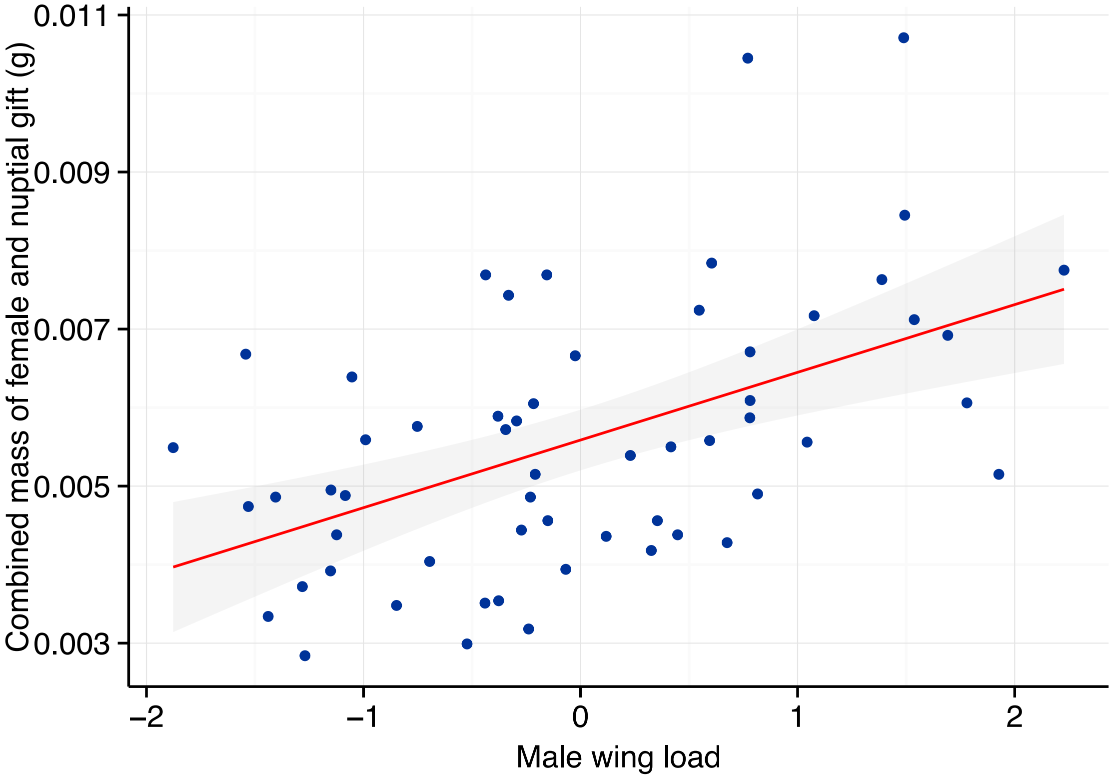

Figure 3.4. Linear regression of the combined mass of the female and nuptial gift on standardised male wing load in *R. longicauda* mated pairs. Wing load is measured as load male mass/wing area. The shaded area represents the standard error measure around the linear estimate (shown in red). (β=0.00086, R^2=0.25, $F_{(1,56)}$=19.6, P<0.001).

We performed a permutation test to determine the null distribution of mean loads a male might carry if foraging and mating were completely random. We calculated the load-lift ratio as the total mass (g) that a male carries per mm^2 of male wing area. The total mass for the load-lift ratio was calculated as the combined mass (female + nuptial gift + male, g) divided by the male's wing area (mm^2). Male mass and wing area were coupled for all tests because these traits will necessarily be correlated and are not independent. If males are constrained by the mass of the load they carry during copulation, then we expect the observed mean load-lift ratio to be significantly smaller than the null distribution (because males are carrying loads with a lighter mass than expected under random mating). We performed 9999 iterations without resampling and found a null distribution of load-lift ratios that had a mean of 0.000607 ± 0.000016 g/mm^2 (Figure 3.5). The mean load-lift ratio we observed in nature is 0.000606 ± 0.000022 g/mm^2, which was not significantly smaller than expected by our null distribution ($P = 0.24$). We performed a similar permutation test without nuptial gifts included in the load measurement (so that the potential sample to draw from was larger) and retrieved the same answer: the null distribution of wing loads had a mean of 0.000538 ± 0.000014 g/mm^2 (Figure 3.6) while our data had a mean of 0.000535 ± 0.000013 g/mm^2 ($P=0.13$).

Discussion

We tested wild-caught *R. longicauda* mated pairs for a load-lifting functional constraint that might be contributing to non-random mating patterns. We found that male wing load, but no other male wing traits, covaried with the mass of the load a male carried, but in the opposite direction of that predicted by a load-lift constraint. We also performed permutation tests to assess the null distribution of load-lift ratios when random foraging and mating are assumed. We found that our observed load-lift ratio did not differ from the null distribution providing further evidence that load-lifting functional constraints are not contributing to the non-random mating patterns observed in *R. longicauda*.

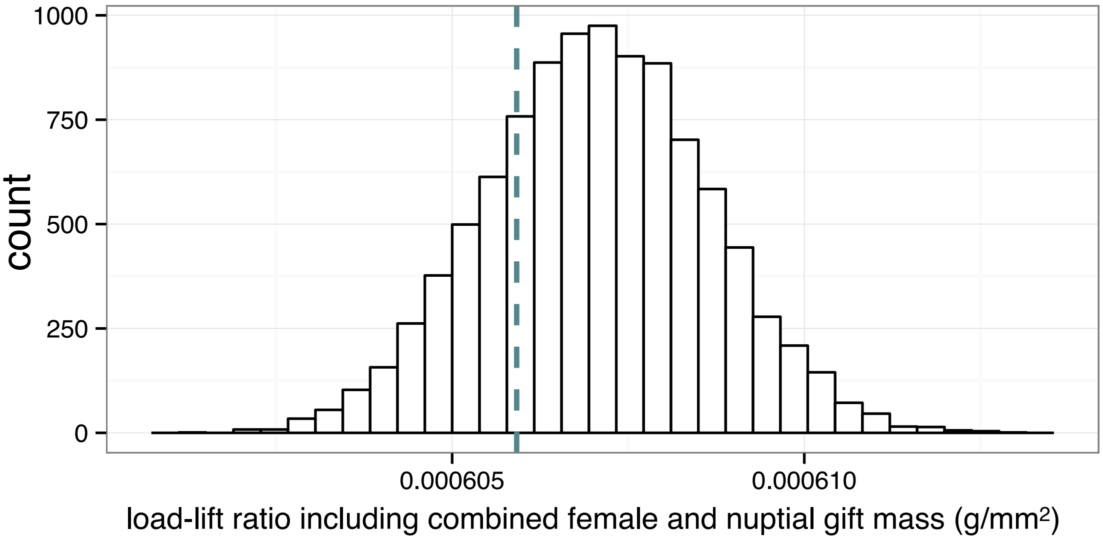

Figure 3.5. Null distribution of the load-lift ratio from a permutation test with 10000 iterations assuming random mating and male foraging. Load lift ratio is the total mass carried by male wings(g) / wing area (mm^2). The mass for the mean load carried by the male is made up of randomised female and nuptial gift mass values and non-randomised male mass values. The observed mean of the load-lift ratio from our data is displayed as the dashed line (p=0.24).

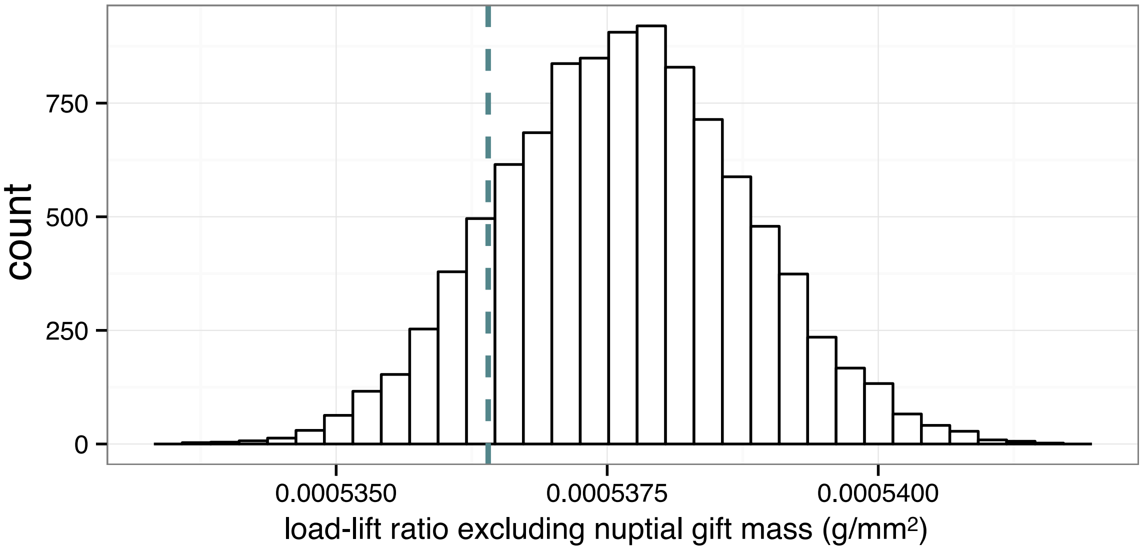

Figure 3.6. Null distribution of the load-lift ratio excluding nuptial gift mass assuming random mating from a permutation test with 10000 iterations. Load-lift ratio is calculated as total mass carried by male wings excluding nuptial gift (g)/ wing area (mm^2). The mass for the load carried by the male is made up of randomised female mass values and non-randomised male mass values. The observed mean of the load-lift ratio from our data is displayed as the dashed line (p=0.13).

Testing for load-lift constraints

R. longicauda mated pairs copulate in-flight with the male carrying the female while she feeds on the nuptial gift he provides. It is unlikely that the female is able to contribute to flight during copulation, leaving the male to bear the weight of the entire load (Marden, 1989). We performed three analyses to test for load-lift functional constraints in *R. longicauda*. First, we tested for a negative relationship between the female mass and nuptial gift mass that a male carries. We hypothesized that a male experiencing a loading constraint might have to trade-off female mass with nuptial gift mass. Second, we tested for a relationship between the load a male carries and a male's wing traits. We predicted that female mass would positively covary with wing size traits (aspect ratio and wing length), but negatively covary with a male's wing load measure (mass/wing area); males that have relatively small wings for their mass are most likely to experience a load-lift constraint. Third, we performed a permutation test to create a null distribution of mean load-lift ratios (total load/wing area) assuming random mating and foraging. If males experience a load-lift constraint, we expected our observed mean load-lift ratio to be less than the null distribution of mean load-lift ratios; if males are constrained by the mass of the female and nuptial gift they can carry, then we expected our observed mean load-lift ratio to be significantly less than the null distribution. We found no evidence for a load-lifting constraint in *R. longicauda* (Tables 3.2, 3.3, Figures 3.3-3.6); female mass and nuptial gift mass did not covary negatively, a male's wing load did not trade-off with the mass of the female he paired with, and our observed load-lift ratios did not differ from null distributions.

Previous work on load-lifting constraints in a different dance fly species suggested that males do experience a load-lift constraint during in-flight copulation. Marden (Andersson, 1989) found a negative relationship between female mass and nuptial gift mass, and a significant difference in the observed load-lift ratio compared to a simulation of random pairings. Marden (1989) concluded that a similar functional constraint might be an important influence of mating patterns in other taxa with where the male carried the female during copulation. However, instead of the constraint predicted by Marden (1989), we found evidence for assortative mating by mass in *R. longicauda*; males with a high mass for their wing area paired with heavier females (Tables 3.2, 3.3, Figures 3.3, 3.4).

Both *R. longicauda* and *Hilara sp.* (Marden, 1989) display elaborate mating behaviour that is common across diverse dance fly taxa, including lek-like mating swarms, nuptial gift giving, and in-flight copulation (Marden, 1989; Cumming, 1994; Funk & Tallamy, 2000). However, there are two striking differences between the mating system of *Hilara sp.,* where load-lifting functional constraints were observed, and that of *R. longicauda*. First, species from the *Hilara* genus do not have female-specific ornaments (Collin, 1961; Cumming, 1994) while *R. longicauda* display multiple female-specific ornaments (Chapter 2 ; Funk & Tallamy, 2000). Second, Marden (1989) describes a male-biased operational sex ratio (OSR) in *Hilara sp.*, while *R. longicauda* has a female-biased OSR (Chapter 5 ; Funk & Tallamy, 2000). These two aspects of mating system variation indicate that sexual selection and mate choice in *Hilara sp.* and *R. longicauda* are operating very differently (Emlen & Oring, 1977; Clutton-Brock, 2009). If we assume that the selection pressures that cause functional constraints and sexual selection are both contributing to non-random mating patterns, it seems less surprising that we see a load-lift constraint in *Hilara sp.* but not in *R. longicauda*.

The presence of female-specific ornaments and a female-biased OSR in *R. longicauda* suggests that females in this species are competing for access to mating opportunities (Emlen & Oring, 1977; Kokko et al., 2012). Similarly, the male-biased OSR and lack of female ornaments in *Hilara* that experience load-lifting constraints (Marden, 1989) suggests that males are competing more strongly for access to mates. How does a difference in the sex that competes for access to mates relate with other selection pressures to create a load-lifting functional constraint? One hypothesis is that selection pressures that lead to functional constraints might conflict with sexual selection by limiting in-flight copulation duration (Schluter et al., 1991). Longer in-flight copulation duration will cause higher energy expenditure from the male because he has to carry the mass of the female and the nuptial gift for the duration (Marden, 1989). However, longer aerial copulation is also likely to covary positively with higher numbers of sperm transferred (Thornhill, 1979; Simmons & Siva-Jothy, 1998; Simmons, 2001), and variation in the amount of sperm transferred can impact the number of offspring a male sires (Parker & Pizzari, 2010). Therefore, in species where males can be choosy about the female they mate with (e.g. species with female-biased OSR) males might be less likely to mate with a female that is at the upper limits of his load-lifting abilities if it means he will be unable to transfer as much sperm as necessary to ensure paternity. However, if males are not the choosy sex, such as in *Hilara sp.*, mating with a heavy female for a shorter copulation duration (and potentially transferring a small number of sperm) is better than not mating at all if mating opportunities are limited.

Assortative mating by mass

While a previous study found no size-assortative mating in *R. longicauda* (Bussière et al., 2008), our results imply a pattern of assortative-mating by mass across mated pairs (Figures 3.3, 3.4). However, these findings need not be contradictory because paired females can be heavier but not larger in linear dimensions than unpaired females (Woodhead, 1981). Assortative-mating by body mass has been observed in diverse taxa (Ridley & Thompson, 1979; Alcock & Gwynne, 1987; Speakman et al., 2007), however, it is a difficult pattern to interpret (Crespi, 1989); insect body mass can vary because of environmental factors (Alcock & Hadley, 1987), and mass is often associated with an individual's condition (McLain & Boromisa, 1987). Interestingly, loading constraints have been proposed as a possible mechanism for patterns of assortative-mating by mass in species where males carry females. However, following a comparative study across diverse arthropod taxa, Crespi (1989) concluded that functional constraints were generally far less likely to explain observed assortative-mating patterns compared to mate choice.

Given Crespi's (1989) findings, (and the lack of evidence for loading constraints observed in our study), it seems much more likely that mate choice is responsible for observed patterns of mating in *R. longicauda* that we observed in this study. Because mass is often associated with physiological or reproductive condition in insects, it may be that the assortative mating observed in this study is for mate quality. We show that, as expected, mass covaries positively with ornamentation (Figure 1.3) and fecundity (Figure 3.2) in *R. longicauda.* Therefore, high-condition males could be using female fecundity when evaluating and choosing a mate. While there is variation around the linear estimates for both ornamentation and fecundity regressed on female mass (arguably two measures of female quality), on average choosing an ornamented female will also result in a heavier, more fecund female but see (Chapter 2 ; Funk & Tallamy, 2000). However, the results found in this study support a pattern of directional selection for female ornaments, but do not help to explain the pattern of stabilizing selection observed by Wheeler et al (2012).

Male mate choice for female-specific ornaments

We searched for a load-lift constraint using three different tests, but found no evidence that *R. longicauda* males are limited by the mass of the load they can carry during aerial copulation. This finding allows future studies investigating mating patterns in *R. longicauda* to focus on male mate choice as a reasonable explanation for why patterns of stabilizing selection are observed (Wheeler et al., 2012). Indeed, if males choose females with intermediate ornamentation in *R. longicauda*, it would confirm previous predictions made about the evolution of female-specific ornaments: first, males should not choose to mate with females who overinvest in ornaments at the expense of fecundity (Fitzpatrick et al., 1995). Second, when males cannot accurately assess female fecundity, such as during aerial courtship, adaptive male mate choice for female displays can evolve and result in stabilizing selection on female ornaments (Chenoweth et al., 2006).

Conclusions

We set out to test the hypothesis that functional loading constraints were contributing to non-random mating patterns in *R. longicauda*, a hypothesis brought forward by two previous studies measuring selection on female ornaments in this species (Bussière et al., 2008; Wheeler et al., 2012). While we found no evidence for a load-lift constraint, we do have results that imply a pattern of assortative mating for mass in *R. longicauda*. Further, by ruling out loading constraints as a contributing force to the evolution of non-random mating patterns for *R. longicauda*, future studies can focus on alternative explanations, such as the intriguing possibility of male mate choice for intermediately sized ornaments.

Chapter 4

Do sex ratio distorting endosymbionts influence the evolution of female ornamentation in the Empidinae dance flies?

Abstract

Dance flies from the subfamily Empidinae are well known for their unusual reproductive biology that includes female-specific ornamentation and female biased swarming behaviour. The cause of the repeated evolution of multiple types of female ornaments in this group remains unknown. One hypothesis that has not yet been tested is that infections by reproductive parasites might be causing changes in the sex ratios of the dance flies and driving the evolution of female-specific ornaments. Indeed, vertically transmitted reproductive parasites can have varied effects on their host's biology including sex ratio distortion resulting in female-biased population sex ratios. We investigated the Empidinae dance fly species for infection incidence and prevalence for three symbionts that are well-known to act as reproductive parasites in several Diptera host lineages: *Wolbachia*, *Rickettsia* and *Spiroplasma*. We also measured the adult sex ratio (ASR) using two separate sampling methods in dance fly host species and related symbiont infection prevalence to sex ratio bias across taxa. We found widespread infection incidence by all three symbionts tests across dance fly species and variation in prevalence between species and sexes. We found no relationship between infection prevalence and ASR measures and no evidence that symbiont infections were influencing the evolution of female-specific ornaments in dance fly taxa.

Introduction

Vertically transmitted symbiotic bacteria that are transferred from mother to offspring are common across many arthropod lineages. Symbionts can have several different effects on their host's biology, including long-term associations as nutritional symbionts that are required for a host's survival (Chen et al., 1999), increased immune function to aid in elevated parasite defense (Oliver et al., 2003; Chrostek et al., 2013) and altering a host's reproductive biology to facilitate population invasion and elevate prevalence (Yen & Barr, 1971; Hackett et al., 1986). Generally, symbionts fall into one of three categories that vary with respect to their effect on the host's expected sex ratio: mutualists which elevate host fitness, where prevalence is expected to be equal in both sexes; reproductive symbionts that induce cytoplasmic incompatibility, where prevalence is also expected to be equal in both sexes; and sex ratio distorting reproductive symbionts, where the prevalence is expected to be lower in males compared to females (Hurst & Frost, 2015).

Sex ratio distortion, which usually increases the proportion of female offspring produced by a host, is beneficial to a reproductive parasite because females alone transfer maternally inherited symbionts to the next generation. Reproductive parasitism, as the name implies, is generally thought to be detrimental to the host because the reproductive phenotypes produced are deleterious (Hurst & Frost, 2015). However, reproductive parasites are widespread and their reproductive phenotypes can also be associated with other beneficial impacts on their host (Hamilton & Perlman, 2013). There are several different mechanisms that can be employed by sex ratio distorting symbionts to increase the proportion of females in the offspring of their host, including male killing (Hurst et al., 1999b), feminization (Bouchon et al., 1998), and parthenogenesis induction (Stouthamer et al., 1993). In some rare cases, reproductive parasites can create strong population wide sex ratio biases, generating selection pressures that can cause changes in host reproduction and mating strategies (Jiggins et al., 2000b; Charlat et al., 2007).

One widespread group of symbionts that is well known for altering its hosts' reproductive biology is *Wolbachia* (Stouthamer et al., 1999; Engelstadter & Hurst, 2009; Goodacre & Martin, 2012). *Wolbachia* generally have a high incidence across taxa, and are predicted to infect up to two thirds of insect species (Hilgenboecker et al., 2008; Weinert et al., 2015). However, the prevalence of *Wolbachia* is highly variable (occasionally only a small proportion of a host population is infected (Weinert et al., 2007)), making it difficult to screen for across many host species. In addition to *Wolbachia*, other symbiont taxa are also reproductive parasites and potential sex ratio distorters, including *Rickettsia* and *Spiroplasma* bacteria (Duron et al., 2008; Hurst & Frost, 2015). Both *Rickettsia* and *Spiroplasma* can infect diverse arthropod hosts (Weinert et al., 2015) including spiders (Goodacre et al., 2006), ladybirds (Weinert et al., 2007), *Drosophila* (Clark et al., 2005; Mateos et al., 2006) and the Diptera superfamilies Muscoidea (Martin et al., 2012) and Empidoidea (Martin et al., 2013a). Furthermore, all three symbionts (including *Wolbachia*) have been shown to cause female-biased sex ratios in diverse host taxa, and *Wolbachia* and *Rickettsia* can do so using multiple distortion mechanisms (Duron et al., 2008; Hurst & Frost, 2015).

In populations affected by sex ratio distorting symbionts, uninfected females produce equal numbers of males and females, whereas infected individuals only rarely produce males (due to occasional inefficient vertical transmission). Therefore, most males originate from uninfected females and do not carry the symbiont, whereas many females originate from infected mothers and are themselves infected. Consequently, the prevalence of the infection for a sex-ratio distorting symbiont should be higher in females compared to males. Further, if a population's infection prevalence is high, the unequal vertical transfer of infection between the sexes can cause a female-biased ASR (Hurst & Frost, 2015).

A strong female bias in the sex ratio may profoundly alter the dynamics of sexual selection (Jiggins et al., 2000b). When males become scarce, competition between females increases, potentially resulting in changes in the strength and direction of intrasexual competition, sexual conflict and mate choice (Emlen & Oring, 1977; Arnqvist & Rowe, 2005; Andersson & Simmons, 2006). In butterflies, symbiont-induced sex ratio distortion can cause sex-role reversal (Jiggins et al., 2000b), increases in polyandry and associated decreases in male ejaculate and nuptial gift investment per mating (Charlat et al., 2007). In this study, we investigate whether sex ratio distorting symbionts help promote the evolution of female-specific ornaments in the Empidinae subfamily of dance flies (Diptera: Empididae).

Study system: dance flies (Diptera: Empididae: Empidinae)

Dance flies exhibit incredible mating system diversity, the evolution of which remains largely unexplained. For example, dance flies exhibit variation in courtship behaviour, sexual dimorphism and sex roles; several species display multiple female-ornaments, mating behaviour often involves the transfer of nuptial gifts during copulation, and lek-like mating swarms are often female-biased (e.g. Steyskal, 1941; Downes, 1969; Cumming, 1994; Svensson, 1997; Funk & Tallamy, 2000; Gwynne & Bussière, 2002; LeBas et al., 2003). One potential possibility is that reproductive parasites contribute to mating system variation within this group (Martin et al., 2013a). Some species exhibit highly female-biased operational sex ratios (OSR) (Svensson, 1997; Funk & Tallamy, 2000), which may arise due to aspects of their mating behaviour (Emlen & Oring, 1977; Kokko et al., 2012), but female-biased OSRs could also result from biases in the adult sex ratio (ASR) caused by symbiont infections (Jiggins et al., 2000b). Given the potential for symbionts to influence sexually selected behaviours such as mate choice (Moreau et al., 2001; Markov et al., 2009), their effects on sexual selection among taxa need clarifying.

In this study we measure the incidence of symbiont infection across dance fly species, investigate which infections may be sex ratio distorters, and test whether the presence of sex ratio distorters correlates with the evolution of costly sexually selected traits in females. We specifically focus on the subfamily Empidinae (Diptera: Empididae), which contains three genera (*Empis, Hilara* and *Rhamphomyia*) with substantial mating system variation (Marden, 1989; Funk & Tallamy, 2000; Svensson & Petersson, 2000). The Empidinae offer an excellent opportunity to explore patterns of symbiont incidence, check for sex ratio distorting symbionts in a new group of insect hosts, and test for a relationship between potentially sex ratio distorting symbionts and the evolution of female-specific ornaments.

Methods

Sample collection and morphology measures

We collected dance fly specimens using sweep nets from April-August of 2011 and 2012 from the locations listed in Table 4.1. We also used Malaise trap sampling to collect an additional estimate of ASR for several sites near the Scottish Centre for Ecology and the Natural Environment (SCENE) on the eastern shore of Loch Lomond, Scotland, UK (GPS coordinates; 56°09′06.35″N, 004°38′36.20″W). Operational sex ratio measurements were taken from Chapter 5. Sample sizes varied based on species abundance at the time and location of sampling. We aimed to collect at least 20 females per species so that we would have a 90% chance of detecting infection rates of 12% prevalence or higher. However, there was high variation in the number of individuals collected for each species group, resulting in some samples having smaller sample sizes than others (Table 4.1).

Adult sex ratio measures were calculated from specimens collected using both Malaise traps and vegetation sweeps. We used two sampling methods to increase the number of species collected as well as to attempt to correct for any biases associated with the sampling method (e.g. Aguiar & Santos, 2010). Malaise trap samples were stored in 70%

Table 4.1. Adult sex ratios (ASR) calculated from two different sampling techniques and operational sex ratio (OSR) displayed for 20 dance fly species. ASR and OSR values shown are the proportion of males and deviations from 1:1 were calculated using an exact binomial goodness of fit test. OSR values are taken from Chapter 5.

Species	**Location**	**Vegetation sweep ASR**	**Vegetation N**	**Malaise trap ASR**	**Malaise N**	**OSR**[a]
E. aestiva	SCENE, UK	0.54	55	0.48	365	0.34
E. borealis	Aviemore, UK	0.54	41	NA	NA	0.44
E. grisea	SCENE, UK	0.25	8	0.54	37	NA
E. nigripes	SCENE, UK	0.49	397	0.64***	339	0.46
E. stercorea	SCENE, UK	0.35	20	0.44	64	NA
E. tessellata	SCENE, UK	0.23*	13	0.60	70	0.71
H. chorica	SCENE, UK	NA	1	NA	2	0.54
H. interstincta	SCENE, UK	NA	1	NA	0	0.82
H. litorea	Edinburgh, UK	NA	0	NA	0	0.64
H. maura	SCENE, UK	NA	2	NA	0	0.62
R. albohirta	SCENE, UK	0.50	8	0.59	42	NA
R. crassirostris	SCENE, UK	0.83	12	0.60	75	0.34
R. dentipes	SCENE, UK	0.73	11	0.17**	23	NA
R. longicauda	Glen WIlliams, ON, Canada	0.54	56	NA	4	0.24
R. longipes	SCENE, UK	0.40**	693	0.55***	1896	0.71
R. nigripennis	SCENE, UK	0.47	121	0.70	10	0.87
R. stigmosa	SCENE, UK	0.48	29	0.33	9	0.57
R. sulcata	SCENE, UK	NA	1	0.33	6	0.63
R. tibiella	SCENE, UK	0.63	38	0.40*	160	0.41
R. umbripennis	SCENE, UK	0.50	8	0.50	91	NA

SCENE = Scottish Centre for Ecology and the Natural Environment, see text for detailed location.
*p<0.05, **p<0.01, ***p<0.001
[a] OSR measures reported in Chapter 5

ethanol, while sweep net samples stored at -20C. When testing for the presence of endosymbionts, we avoided specimens stored in alcohol to prevent potential contamination from hosts being stored together. If we did not collect enough vegetation sweep net samples (fewer than 20 of each sex) for a particular species, we supplemented wherever possible with samples from mating swarms that were used to calculate the operational sex ratio (OSR) in Chapter 5. Because we wanted to avoid biases due to behavioural differences unrelated to reproductive parasites, we did not include counts of specimens from mating swarms (which contribute to the OSR) in our estimates of the ASR. We report all sex ratios as the proportion of males in a population.

Empidinae dance flies are known to display several female-specific ornaments: darkened or enlarged wings, pinnate leg scales and inflatable abdominal sacs (Cumming, 1994). We classified species as ornamented (1) if females displayed at least one of these traits, and unornamented (0) if females had none of these traits (Table 4.2).

Testing for endosymbiont prevalence

We extracted DNA using DNeasy animal tissue extraction kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. Samples were individually crushed using a mortar and pestle and then a small sample of each fly was removed for extraction while the remainder of the specimen was stored at -20C. We pooled flies in groups of five individuals of the same sex and species. The mitochondrial CO1 region of all samples was amplified to confirm successful DNA extraction using primers LCO1490 and HCO2198 (Folmer et al., 1994). To test for the presence of three different symbionts, we PCR amplified each sample using the following primers, *Wolbachia* (wsp81f – wsp691r) (Zhou et al., 1998), *Rickettsia* (R1 - R2) (von der Schulenburg et al., 2001), and *Spiroplasma* (27F - MGSO/SP-ITS-N2) (Van Kuppeveld et al., 1992; Jiggins et al., 2000a). PCR amplifications were carried out in 20μL reactions with 6.3μL ddH$_2$O, 4μL 5X Taq Polymerase Buffer, 2.0μL of 25 mM MgCl$_2$, 0.5μL of each primer (10μM), 1μL of 10 mM

Table 4.2. Symbiont infections by *Wolbachia*, *Rickettsia* and *Spiroplasma* for 20 dance fly species is shown along with the presence of female-specific ornamentation displayed by each host species. Sex ratios shown are the proportion of males. Deviations from 1:1 were calculated using an exact binomial goodness of fit test.

Species	N	symbiont	sampled sex ratio[b]	prevalence in females[c]	prevalence in males	female ornamentation[d]
E. aestiva	56		0.50			1
E. borealis	20	*Spiroplasma*	0.50	0.00	0.30	1
E. grisea	8	*Spiroplasma*	0.75	0.00	0.16	0
E. nigripes	84	*Wolbachia*	0.50	0.71**	0.40	1
		Rickettsia		0.07	0.00	
		Spiroplasma		0.12	0.00	
E. stercorea	*15*	*Rickettsia*	0.53	0.43	0.00	0
		Spiroplasma		0.57*	0.00	
E. tessellata	*35*	*Rickettsia*	0.60	0.00	0.33	0
		Spiroplasma		0.00***	0.71	
H. chorica	*40*	*Wolbachia*	0.50	0.15	0.00	0
		Spiroplasma		0.00	0.10	
H. interstincta	*40*	*Spiroplasma*	0.50	0.15	0.10	0
H. litorea	*40*		0.50			0
H. maura	*48*	*Rickettsia*	0.50	0.04	0.00	0
		Spiroplasma		0.09	0.32	
R. albohirta	*8*		0.50			1
R. crassirostris	*40*	*Rickettsia*	0.50	0.10	0.00	0
		Spiroplasma		0.55	0.25	
R. dentipes	*8*		0.50			0
R. longicauda	*40*	*Rickettsia*	0.50	0.20	0.00	1
		Spiroplasma		0.85***	0.00	
R. longipes	*56*	*Spiroplasma*	0.50	0.04	0.00	1
R. nigripennis	*54*	*Rickettsia*	0.50	0.18	0.00	1
		Spiroplasma		0.39***	0.00	
R. stigmosa	*24*	*Rickettsia*	0.50	0.08	0.00	0
R. sulcata	*40*	*Rickettsia*	0.50	0.00**	0.50	0
		Spiroplasma		0.00	0.15	
R. tibiella	*33*		0.52			1
R. umbripennis	*30*		0.67			0

*p<0.05, **p<0.01, ***p<0.001

[b] proportion of males in the sample of hosts tested for symbionts for each species

[c] prevalence tests were conducted on both sexes, but significance values are assigned to measures in the ‘prevalence in females’ column only regardless of the direction of bias

[d] for female-specific ornamentation, 0=absent, 1=present

dNTPs, 1 unit of GoTaq DNA polymerase (Promega Cat. No. M830b), and 2-4μL of template DNA. We used a standard PCR protocol (2 min denaturation at 95°C followed by 35 cycles of 95°C for 60s, 54°C for 30s, 72°C for 30s and a final elongation at 72°C for 5m). We then visualized PCR amplicons on 1% agarose gels to score the presence or absence of a band. For each DNA sample we ran a positive control PCR using Cytochrome Oxidase Subunit I (COI) primers run simultaneously with the endosymbiont PCR assay with the same PCR conditions. Where PCR amplification indicated a symbiont infection, we extracted DNA from each individual that made up the positive pooled sample and PCR amplified the specimens to get a measure of symbiont infection status for each individual.

Statistical analyses

All analyses were carried out using R (R Core Development Team, 2014). For each sampling method estimating ASR, we performed a Pearson chi-square contingency table analysis to test for independence in the measures of individuals collected by sex and species. Next, we tested for sex ratio bias in each species using the results from both sampling methods separately. We also performed a Fisher's exact test for each sex ratio to determine it differed from the null of 1:1. In order to accurately assess variation between the sexes in symbiont prevalence and ASR, we used the "p.adjust" function from the stats package allowing for a 10% false discovery rate to correct for multiple tests (Benjamini & Hochberg, 1995).

In order to test for sex ratio distorting symbionts in the species that were infected, we quantified the infection prevalence in both sexes to see if one sex or the other was over-represented. We used a Fisher's exact test to assess differences in the infection prevalence in males and females. If bacteria are responsible for skewing the sex ratio then we expected to see a female-bias in the prevalence of infected hosts. If the sex ratio distorting symbiont is at high enough prevalence in the host population, we might also see female-biased ASR estimates. If, however, the ASR is female-biased but infected animals are equally likely to be male or female, other factors than symbionts we screened are probably influencing the sex ratio.

Finally, to test whether symbiont infection prevalence predicted female-specific ornamentation across dance fly species, we fit a binomial generalized linear mixed effects model using the lme4 package (Bates et al., 2014), including "species" as a random effect. Our binomial index of female ornamentation (present or absent) was our response variable, and the prevalence of infection, type of infection (*Rickettsia*, *Spiroplasma* or *Wolbachia*) and prevalence sex-bias were included as fixed effects. We included interactions between all fixed effects in our maximal model. We simplified the model by sequentially removing non-significant terms and testing (using a chi-square test) to compare the new model to the previous one. The more complex model was retained whenever simplification resulted in significant increase in model deviance.

Results

Dance fly sex ratio measures

We measured the adult sex ratio (ASR) in two different ways to capture measures for as many species as possible, as well as to calculate a more accurate estimate of Empidinae ASR (Table 4.1; Longino et al., 2002; Aguiar & Santos, 2010). The number of species collected, the sample size per species, and the direction of sex ratio bias differed depending on the sampling method. Vegetation sweep counts ranged from 0 to 693 individuals per species and Malaise trap counts ranged from 0 to 1896 individuals per species. Tests performed on each sampling method ASR results revealed that sex ratios do differ significantly across species (p<0.001, for both measures). For ASR estimates from sweeping vegetation, most species did not differ from a 1:1 sex ratio except for *E. tessellata* and *R. longipes*, which both had female-biased sex ratios. For Malaise trap samples, most species also did not differ from 1:1, but we found male-biased sex ratios for *E. nigripes* and *R. longipes*, and female-biased sex ratios for *R. dentipes* and *R. tibiella*.

Symbiont prevalence

We tested 719 individuals from 20 dance fly species for the presence of three commonly occurring and potentially sex ratio distorting symbionts, *Rickettsia*, *Spiroplasma* and *Wolbachia* (Table 4.2). More than half of the host species tested (14 out of 20) were infected with at least one symbiont. *Wolbachia* was found in two host species, *Rickettsia* in nine and *Spiroplasma* in thirteen. We found nine host species that were infected with more than one symbiont, eight that were infected with two symbionts, and one, *Empis nigripes*, in which we successfully amplified PCR products for *Wolbachia*, *Rickettsia* and *Spiroplasma* bacteria.

The bacterial prevalence across host species was variable, ranging from 0-56% for *Wolbachia*, 0-25% for *Rickettsia*, and 0-43% for *Spiroplasma*. The majority of the infections (20 out of 24) occurred in only one sex: seven of the infections occurred only in males, and thirteen were found only in females. This large number of male-only infections differs from previous reports of many female-only infections and very few male-only infections in other insect host groups (Weinert et al., 2007). Allowing a false discovery rate of 10% to account for multiple tests (Benjamini & Hochberg, 1995), out of the 14 infected species, two had a significantly higher infection prevalence in males compared to females and four had a significantly higher infection prevalence in females compared to males (Table 4.2).

Sex ratios and infection status

Having identified candidate sex ratio distorting symbionts, we asked whether the species they infected had biased ASR estimates. We found female-biased infection prevalence for symbionts in four host species, suggesting that these symbionts may be sex ratio distorting reproductive parasites: *E. nigripes* infected with *Wolbachia*, and *E. stercorea*, *R. longicauda* and *R. nigripennis* infected with *Spiroplasma* (Table 4.2). None of these host species with female-biased infections had a corresponding female-biased ASR estimated from either sampling method (Table 4.1). The sample size for the *E. stercorea* sample was not large (N=15; eight males and seven females).

R. longipes and *R. tibiella* were the only host species for which we found a female-biased ASR estimated from a large sample size (N>100; Table 4.1). However, there was no corresponding female-biased infection prevalence in either species to indicate that sex ratio distorting symbionts might be responsible for these biases. We found no symbiont infections in *R. tibiella*, while *R. longipes* had a very low prevalence, albeit female-only, *Spiroplasma* infection (Table 4.2).

Symbionts and female-specific ornaments

To investigate the effect of symbiont prevalence on the evolution of female-specific ornaments we fit a binomial generalized linear mixed effects model with ornamentation as the response, and species as the random effect. We fit the prevalence of infection, type of symbiont (*Rickettsia*, *Spiroplasma* or *Wolbachia*) and prevalence sex-bias as fixed effects including all possible interactions between fixed effects in the maximal model. If symbiont prevalence is influencing the evolution of female-specific ornaments, we expected a significant interaction between prevalence and sex-bias; high prevalence symbiont infections that are sex-biased should be the most likely predictors of female-specific ornaments. However, our simplified model did not include any interaction terms, and there was no evidence that symbiont prevalence related to the evolution of female-specific ornaments (Table 4.3).

Table 4.3. Results from a binomial generalised linear mixed model investigating the effect of individual symbiont prevalence, symbiont taxa (*Rickettsia*, *Spiroplasma* or *Wolbachia*), and prevalence sex-bias on the evolution of female-specific ornaments. Species was fit as random effect. *Wolbachia* is used as a reference level (intercept) in the model.

	estimate	**se**	**z**	**p**
intercept	-3.27	1.79	-1.83	0.10
Rickettsia	0.25	0.66	-0.83	0.41
Spiroplasma	0.05	0.70	0.47	0.64
prevalence	-3.36	3.64	-0.102	0.92
bias	5.63	5.52	0.202	0.84

Discussion

Dance flies from the subfamily Empidinae show remarkable variation in reproductive behaviour, with repeated transitions in mating systems among closely related lineages (Chapter 5). While the focus of previous interest in this group has been on courtship feeding behavior, and its potential role in increasing sexual selection on females, reproductive parasites present an important and as yet untested alternative explanation for the prevalence of unusually high levels of competition between females and male choice in this group. We used PCR-based screens to survey the incidence of three bacterial symbionts in 20 species from the Empidinae subfamily of dance flies. We related the sex-specific prevalence of symbionts to species-level sex ratio estimates and female-specific ornamentation. Specifically, we tested for evidence of sex ratio distorting symbionts that might be causing female-biased adult sex ratios in host species of dance flies.

We found that *Rickettsia*, *Spiroplasma* and *Wolbachia* bacteria all occur in Empidinae dance flies, but vary by incidence and prevalence across host species. This is the first study that has thoroughly investigated the incidence of potential sex-ratio distorting symbionts in this subfamily, and our results suggest that all three symbionts assayed have high incidence, but varied prevalence in natural populations of the Empidinae. We show that symbiont prevalence, while unlikely to be the cause of observed ASR biases or female ornamentation, varies by sex and across species. We identify four different host species infected with symbionts that exhibit female-biased infection prevalence that might be sex ratio distorting reproductive parasites.

Dance fly sex ratio estimates

We estimated the ASR using two sampling methods and revealed variation in the number of species collected, the sample size per species and the direction of sex ratio bias between the two sampling methods (Table 4.1). The high variation in sample size and species diversity between collection methods is likely caused by several factors including differences in the ecology and behaviour between species (Collin, 1961; Cumming, 1994) and bias in where sampling occurred. For example, we collected very few samples from the *Hilara* genus using both vegetation sweep netting and the Malaise traps, presumably because many *Hilara* species are often found in and around freshwater (Collin, 1961) rather than in the forested conditions where most collections took place. Species phenology may also contribute to variation in sample sizes across species collected using sweep netting and Malaise traps. Our sampling efforts were concentrated in May-July when the majority of British Empidinae species are active (Collin, 1961). However, some species' flight periods begin earlier or later than our main sampling window, which could have resulted in poor sampling for at least one of our measures. For example, *E. borealis* typically emerges in March and is in flight until mid to late April (Collin, 1961; Svensson & Petersson, 2000). Thus, it is not surprising that we did not collect any specimens in any of our Malaise traps that were set up from May through July.

While the majority of our ASR estimates reveal a sex ratio that is not significantly different from 1:1, two of the sex ratios from sweep net collections were uneven, (*R. longipes* and *E. tessellata* were female-biased), and four of our Malaise trap ASR estimates revealed significant deviations from a unity sex ratio (*R. dentipes* and *R. tibiella* were female-biased, while *E. nigripes* and *R. longipes* were male-biased; Table 4.1). *R. longipes* had the largest sample size for both collection methods and was the only species that revealed sex ratio bias from both measures, however, the bias occurred in opposite directions. This finding suggests that sex differences in behaviour affect the proportion of males and females we collect using each sampling method. We found a female-bias in the ASR measured from sweep netting vegetation, which could suggest sex differences in nectivorous feeding.

The Empidinae have recently been recognized as an important group of pollinators for diverse plant species (Lefebvre et al., 2014). Female dance flies are thought to have lost the ability to hunt for prey items (Downes, 1969). While they do not forage as predators, it is possible that females spend more time foraging on flowers and other vegetation than males. In contrast, males need to hunt for prey presumably both for their own nutrition as well as to provide nuptial gifts to females (Cumming, 1994). Consequently, males might spend more time in flight searching for prey, which could explain why we found more males than females were collected in Malaise traps for some species (Aguiar & Santos, 2010).

There is no clear association between the expression of female ornaments and biased ASR measurements. We collected sex ratio estimates for 8 ornamented and 12 unornamented species, and found two female-biased and two male-biased ASR measures from ornamented species, and one female-biased and one male-biased ASR from unornamented species. Taken together, our findings further demonstrate the well-established importance of sampling method in the estimation of ASR from natural populations (Darwin, 1871; Ream & Ream, 1966; Longino et al., 2002; Donald, 2007; Aguiar & Santos, 2010).

Symbiont prevalence

Many of the symbionts we found only infect a small proportion of the population (less than 3% of individuals; Table 4.2). At such low frequency, these infections may have been missed in populations that were less well sampled. It is difficult to accurately test the proportion of potential host species that are infected by symbionts. Although symbionts are detectable with PCR-based screens, they vary in their infection prevalence and low-density infections may be difficult to detect. It is also impossible to prove that a population is entirely uninfected. Therefore, our study may have underestimated the symbiont prevalence and diversity in the Empidinae. We also did not test for all known symbiont taxa (e.g. Flavobacteria species (Hurst et al., 1999a) and *Cardinium* (Martin et al., 2013b)). *Cardinium* bacteria, in particular, are commonly occurring potential sex ratio distorters for a variety of host taxa (Duron et al., 2008; Hurst & Frost, 2015; Weinert et al., 2015). It seems possible that a PCR-based screen for *Cardinium* would reveal further infections in our sampled host taxa and might be contributing to observed sex ratio biases. For example, *R. tibiella* had an estimated ASR (Malaise trap) that was female-biased from a sample size of 160 individuals (Table 4.1), but no symbiont infections were detected (Table 4.2). Given that *R. tibiella* has a female-biased OSR, female-biased ASR (Table 4.1) and multiple female-specific ornaments (Collin, 1961), investigation into infection by other potentially sex ratio distorting symbiont taxa, particularly *Cardinium*, in *R. tibiella* would be useful.

Interestingly, we found several infections that occurred in males but were completely absent in females, including two that showed significant male-biased infection prevalence: *Spiroplasma* in *E. tessellata* and *Rickettsia* in *R. sulcata* (Table 4.2). If the symbionts we tested for are indeed maternally-inherited, then they must be present in the females of their host species as well. One potential explanation for why we found no infection in females is that symbionts infect the sexes at different densities. It may be that within females, the symbionts are at very low densities that did not turn up on a PCR screen, while in males the symbionts proliferate to higher densities that are more easily detectable. This pattern might be observed if the symbiont is harmful to its host; we might see strong selection for decreased virulence in females (the sex that transfers them to the next generation), while no selection on the symbiont to be less virulent in males. Alternatively, it is possible that the symbionts we detected at male-biased prevalence are not maternally inherited. The biased measures could come from sex-based differences in male and female diets, if for example male dance flies are more likely to consume symbiont-infected prey items than females.

The taxa we tested for in our screens are all known to be vertical, maternally-inherited symbionts (Hurst & Frost, 2015). However, we cannot rule out the possibility of contamination and horizontal transfer between species because of dance fly mating behaviour. The prey items that males use as nuptial gifts are frequently other Diptera, and occasionally other species of dance flies (Cumming, 1994). Therefore, the dance flies we screened may have ingested infected prey items. Because flies were crushed prior to DNA extraction, it is possible that symbionts from ingested prey items may have resulted in positive screens for uncontaminated individuals.

To test whether these male-biased infections are maternally-inherited we could test for an association between symbiont genotypes and a host's mtDNA. If symbionts are maternally-inherited then we would expect particular symbiont lineages to be associated with specific mtDNA haplotypes. However, if symbionts are not maternally-inherited, and instead contamination through prey consumption explains the male-biased patterns, then symbiont genotypes will be random with respect to mtDNA haplotypes.

Dance flies and symbionts

A previous study (Martin et al., 2013a) that screened for *Rickettsia*, *Spiroplasma* and *Wolbachia* taxa across host species from the Empidoidea superfamily (to which the Empidinae subfamily belongs) included two species that overlap with our sample. Martin et al. (Martin et al., 2013a) found an individual from each of *H. interstincta* and *E. nigripes* to be infected with *Rickettsia*. In our study, we found *E. nigripes* infections involving all three symbiont taxa, while *H. interstincta* was only infected with *Spiroplasma* (Table 4.2). Their study screened hundreds of host species but in many cases, only a single individual represented each species. While most symbionts exhibit relatively stable prevalences, the infection prevalence of a population can change rapidly (Weeks et al., 2007; Werren et al., 2008; Himler et al., 2011), which means that any single survey is merely a snapshot of infection levels that fluctuate over time. Further screens investigating spatial and temporal patterns in dance fly symbiont incidence and prevalence would be necessary to determine the dynamics of this system.

Sexual selection and symbiont infections

Reproductive parasites, such as *Rickettsia*, *Spiroplasma* and *Wolbachia* bacteria, can have drastic effects on sexual selection in their hosts (Jiggins et al., 2000b; Charlat et al., 2007). The female-biased sex ratios produced by reproductive parasites are hypothesized to influence the intensity and direction of sexual conflict in host species (Hurst & Frost, 2015). With a relative excess of females, male manipulations to female reproductive physiology, and the corresponding female resistance traits should both decrease. Sexual conflict is known to cause exaggerated ornamental traits that may exploit sensory biases in the more choosy sex (Chapter 2; Holland & Rice, 1998). Here, we hypothesized that female-biased sex ratios resulting from infection with reproductive parasites might lead to a reversal in the direction of sexual conflict and the evolution of female-specific ornaments in the dance flies. We tested the effect of symbiont prevalence for three taxa that are known to cause host sex ratio distortion. We controlled for species in our analysis and found that symbiont prevalence does not predict female-specific ornaments in the dance flies (Table 4.3).

One explanation for the lack of association between symbiont infection prevalence and ornamentation is that symbiont-host associations are relatively short-lived on an evolutionary timescale. Indeed, there is very little correlation observed between reproductive parasite phylogenies and the phylogenies of their hosts (Stouthamer et al., 1999). This pattern contrasts with many mutualists, such as *Buchnera*, that exhibit very close co-speciation with congruent host and symbiont phylogenies (Clark et al., 2000; Hosokawa et al., 2006). Therefore, relative to the time it takes for ornament evolution, any infection by reproductive parasites in the dance flies will be brief.

Conclusions

We tested whether sex ratio distorting reproductive parasites were causing female-specific ornament evolution in the Empidinae. We found no evidence that reproductive parasites systematically affect sex ratios or ornament expression in the dance fly taxa sampled. However, our study revealed widespread infections by three potentially sex ratio distorting symbionts. We identify four host species that showed a female-biased symbiont prevalence, which could indicate infection by reproductive parasites. Although we find no evidence that symbionts are distorting sex ratios, nor associated with the repeated evolution of ornaments in Empidinae dances flies, it is difficult to dismiss the possibility that they play a role in reproductive or life history strategies. More directed investigation into the detrimental or beneficial effects of host-symbiont relationships might yet uncover a role of symbionts in dance fly evolution.

Chapter 5

Female-specific ornaments, operational sex ratio and relative testis size: a comparative study in the Empidinae dance flies

Abstract

Sexually selected ornaments are highly variable, even between closely related species, and the factors that drive ornament evolution remain unclear. Recent studies have questioned the importance of the intensity of sexual competition relative to other factors such as the fitness benefits of remating, and the time between consecutive mating contests. Species with female-specific ornaments may help enlighten this debate. Because female ornaments arise only rarely, the taxa in which they do occur may clarify what factors control exaggerated ornament expression. We investigated the relationship between the intensity of intrasexual competition (as estimated by the operational sex ratio, OSR) and the evolution of female-specific ornaments across Empidinae dance fly species. We also measured relative testis size across species to test whether female ornamentation covaried with polyandry, which should select for greater sperm production in males. To control for shared ancestry in our analyses, we created a phylogeny that revealed multiple independent origins of female-specific ornaments across the dance flies. We used our phylogeny to perform a comparative analysis of the covariance between the OSR and female ornamentation, and found no significant association. We did find that more ornate species had relatively larger testes, however, suggesting that high levels of ornamentation occur in more polyandrous taxa. The fact that high levels of ornamentation and polyandry are not easily predicted by OSR supports assertions of the importance of considering other measures of sexual selection when predicting the intensity of intrasexual competition among females.

Introduction

The evolution of elaborate sexual ornaments can arise if their presence increases the reproductive success of their bearer (Darwin, 1871). There is striking diversity in the size and shape of sexual ornaments, often even between closely related species (Ord & Stuart-Fox, 2006; Pomfret & Knell, 2008). The evolution of variation in interspecific ornamentation can be independent of phylogeny and is frequently driven by transitions in mating system (Andersson & Iwasa, 1996). For example, Darwin (1871) noted the connection between ornaments and mating system when he realized that sexual dimorphism in plumage was exaggerated in more highly polygynous birds, while monogamous birds were generally more similar in colouration.

The evolution of exaggerated secondary sexual traits is heavily favoured when variation in mating success among individuals in a population is high (Andersson & Iwasa, 1996). Mating success can vary depending on access to receptive mates (Emlen & Oring, 1977). One measure that has been identified as an important indicator of pre-mating competition for mates is the operational sex ratio (OSR), the relative proportion of receptive males and females (Emlen & Oring, 1977; Kvarnemo & Ahnesjo, 1996). The intensity of both intra- and intersexual selection is expected to increase as the OSR becomes more biased toward one sex or the other (Emlen & Oring, 1977; Gwynne, 1990; Clutton-Brock & Parker, 1992). A biased OSR can lead to intense intrasexual competition for mates, which has been shown in some cases to drive sexual selection for secondary sexual traits (Johnstone et al., 1996; Kvarnemo & Ahnesjo, 1996; Kvarnemo & Ahnesjo, 2002). However, the OSR has come under scrutiny for its effectiveness as a measure of sexual selection (Shuster & Wade, 2003; Klug et al., 2010; Kokko et al., 2012). First, the OSR does not directly measure selection, but the intensity of the contest faced by each sex. The OSR does not, therefore, account for the fitness gain per mating within each sex (the Bateman gradient; (Bateman, 1948)). Second, one assumption related to the OSR's ability to reliably indicate the intensity of sexual selection, is that an increase in the bias of the OSR increases mate monopolization by the more common sex; the greater the imbalance in the OSR, the greater the expected variance among members of the limited sex (Emlen & Oring, 1977). It is the increased variation in mating success that provides the opportunity for selection, rather than the OSR itself. However, this argument is very one-sided; while mate monopolization will be important for male reproductive success, female reproductive success will be largely unaffected by monopolizing a male to gain access to his sperm. Third, the variation in time spent away from the 'mating pool' between the sexes can interact with the OSR to influence the opportunity for selection. For example, if females spend more time than males investing in parental care and therefore away from the 'mating pool', when they do decide to mate, females are unlikely to have to wait long to find a receptive male regardless of the OSR. Therefore, there is unlikely to be strong selection on traits that improve a female's access to mates even when the OSR is highly female-biased (fig. 6C from Kokko et al., 2012). Despite these caveats related to the effectiveness of the OSR as a predictor of sexual selection (Shuster & Wade, 2003; Klug et al., 2010; Kokko et al., 2012) several studies have shown that the OSR is still a valuable measure of the potential levels of intrasexual competition faced by individuals (Jirotkul, 1999; Berglund & Rosenqvist, 2008; Silva et al., 2010; Monteiro & Lyons, 2012; Monteiro & Vieira, 2013).

Bias in the OSR has been shown to arise depending on the mating system of the organism combined with variation in resource availability and parental investment (Gwynne, 1990). Given that access to mates is generally a limiting factor for reproductive success in males, but not females, (Trivers, 1972), there are typically many more receptive males than females in a population. When the OSR is male-biased and females are scarce selection will typically favour traits that improve a male's access to females. However, there is evidence in many species that the OSR can consistently be female-biased (Funk & Tallamy, 2000; Charlat et al., 2007; Liker et al., 2013). The selection pressures faced by females when they are the sex in 'excess' cannot be assumed to directly mirror the situation faced by males in a male-biased OSR (Gwynne & Simmons, 1990; Forsgren et al., 2004; Silva et al., 2010). Because eggs are more costly to produce than sperm, female reproductive success is typically limited by gamete production rather than access to mates (Trivers, 1972). Thus, despite several reports of female-biased OSR in the literature, it remains unclear whether a female-biased OSR will influence the intensity of intrasexual competition. One way to test for a relationship between OSR and the intensity of intrasexual competition is to investigate the relationship between OSR and female-specific ornament evolution. Because female-specific ornament evolution is rare and only occurs under limited conditions (see below), investigating the conditions allowing for ornament expression in females would help clarify the impact on the OSR for sexual trait evolution more generally.

Female-specific ornament evolution

Sexual selection in females is a well-documented phenomenon (Clutton-Brock, 2009). However, heightened sexual selection that results in female-specific ornament evolution remains uncommon, even among taxa in which females experience relatively strong sexual selection (Amundsen, 2000). In the rare cases when female-specific ornaments do evolve, they are hypothesized to serve the same purpose that they do in males: attract mates and/or to aid in intrasexual competition (Amundsen, 2000). Unlike in males, however, female gamete production is typically very costly (for a review of variation in spermatogenesis costs see Parker & Pizzari, 2010), so the expression of exaggerated ornamental traits could come at a cost to fecundity (Fitzpatrick et al., 1995). Therefore, if female ornaments evolve to attract mates, the act of displaying the ornament could undermine its signaling value to males if it advertises a female’s lack of investment in eggs. Females might avoid the potential ornament-offspring trade-off if they receive direct benefits from mating, such as nutritious nuptial gifts (Vahed, 1998; South & Lewis, 2012) that compensate for resource investment in ornament expression. However, males should prefer females who invest less in ornamentation if these females are more fecund

(Fitzpatrick et al., 1995), especially if ornate females, by mating more frequently, provide a smaller share of paternity to their mates compared to less ornamented females. However, if males cannot accurately assess female fecundity, such as when courtship occurs in flight (Funk & Tallamy, 2000; LeBas et al., 2003), they might need to rely on indirect fecundity cues, such as ornaments, during mate choice. Therefore, adaptive male choice for female ornaments can evolve regardless of potential fecundity costs (Chenoweth et al., 2006).

In species where males provide resources, such as nuptial gifts, to their mates, female polyandry might increase if access to resources is only gained through increased matings (Gwynne, 1990). An increase in female polyandry because of increased resource acquisition could result in a female-biased OSR. Male mate choice is likely to evolve if mating is costly (because of nuptial gift provisioning) and there are many females to choose from (Bonduriansky, 2001). If females face intense intra- and inter-sexual selection, they may develop traits that exaggerate their fecundity to attract male mates (Chenoweth et al., 2006). Therefore, if competition for direct benefits offered by males is strong, and displaying ornaments improves a female's access to direct benefits through increased mating opportunities, then we might predict female-biased OSR measures to positively covary with female-specific ornamentation.

Increased levels of polyandry within a species should produce predictable changes in the reproductive morphology of males, including increased relative testis investment (Simmons, 2001). Relative testis size has been shown to reliably covary with rates of female polyandry across diverse taxa (Parker et al., 1997; Vahed et al., 2011). Two complementary hypotheses have emerged in the literature to explain the positive relationship between testis size and polyandry: numerical sperm competition (Gay et al., 2009) and male mating rate (Parker & Ball, 2005), (for a review see Vahed & Parker, 2011). However, the cause-and-effect relationship between polyandry and testis size may not be as straightforward as originally thought (Simmons, 2001); population traits, such as the sex ratio, can influence how a male allocates resources during copulation as well as his relative testis size (Reuter et al., 2008). Therefore, given the complexity of the link between polyandry and relative testis investment, and the dearth of empirical studies about the relationship between female-specific ornaments and polyandry, predictions about the relationship between female display traits and relative testis investment in males remain unclear. Indeed, while evidence from studies on individual species can provide insight into biological processes, to properly elucidate how patterns of female-biased OSR and female-specific ornamentation relate to one another and manifest in male traits, we need comparative evidence from multiple lineages that vary in female-specific ornament expression.

In the present study we investigate the relationship between OSR and female-specific ornamentation in Empidinae dance flies (Diptera: Empididae), a group that displays incredible mating system diversity. Species range from those that exhibit almost no sexual dimorphism, to those that display multiple female-specific ornaments (Collin, 1961; Cumming, 1994). Mating in many dance fly taxa occurs in lek-like mating swarms that reliably occur at specific ‘swarm markers’ throughout the breeding season (Funk & Tallamy, 2000; Svensson & Petersson, 2000). The presence of mating swarms in dance fly taxa allows for multiple OSR measures to be taken throughout the breeding season. We can then relate measures of a species’ potential for intrasexual competition (OSR) with female-specific ornament expression and test for evidence of polyandry by measuring male relative testis investment. By investigating these relationships in a phylogenetic framework, we can account for the similarity observed between species because of shared ancestry to answer questions about the relationship between OSR bias and female-specific ornament evolution. We can also test whether female ornaments indicate increased polyandry, by measuring the relative male testis investment across species. Finally, we can estimate the number of independent origins of female-specific ornamentation across the Empidinae.

Methods

Study Species

Dance flies from the subfamily Empidinae (Diptera: Empididae) are known for displaying remarkable mating behaviour. In many species, mating swarms form at reliable markers that the population returns to daily. In some species, females have evolved sex-specific ornaments that they display within the mating swarm. Males enter the swarm carrying a nuptial gift (often a prey item) and approach a female to mate with her. When males and females pair off, they often leave the mating swarm while transferring the nuptial gift from the male to the female, and engaging in copulation. Mating often takes place either in flight (Funk & Tallamy, 2000) or hanging from a substrate (LeBas & Hockham, 2005). If the nuptial gift is a food item, the female will consume the prey for the duration of the copulation.

Approximately one third of species from the genera *Rhamphomyia* and *Empis* display female-specific ornamentation (Cumming, 1994). Within ornamented species, there are three main categories of traits that females display: pinnate leg scales, inflatable abdominal sacs and darkened or enlarged wings. The pinnate leg scales extend laterally on a female's legs and are completely absent in male dance flies (Figure 5.1). Pinnate leg scales can vary in size and the number of legs on which they occur. Inflatable abdominal sacs are more rare and only known within a few described species. Sexual dimorphism in wings is common and highly variable across the dance fly lineage. The best-studied examples of exaggerated female wing ornamentation are the enlarged and patterned female wings of *Rhamphomyia marginata* (Svensson, 1997) and the enlarged and darkened wings of *Empis borealis* (Figure 5.2; Svensson & Petersson, 2000).

Figure 5.1. Female-specific pinnate leg scale ornamentation for hind, mid and front legs of *Rhamphomyia longicauda.*

Figure 5.2. Sexual dimorphism in wing size morphology for *Empis borealis*.

Sample collections for phylogenetic inference

All samples were collected using entomological sweep nets. *Empis borealis* samples were collected in the Cairngorms National Park near Aviemore, UK in March 2012. *Hilara litorea* samples were collected in Edinburgh, UK in July and August 2012. All other species from the UK were collected from May-July 2011 from the woodland and farmland along the West Highland Way on the eastern side of Loch Lomond between the Scottish Centre for Ecology and the Natural Environment (SCENE) and Rowardennan, Scotland. *Rhamphomyia longicauda* samples were collected in June 2012 from an island in the Credit River near Glen Williams, Ontario, Canada. A complete list of sampling sites can be found in Table 5.1.

Morphological measurements

Morphological measurements were taken using a dissecting microscope connected to a camera and analyzed using ImageJ (version 1.48) digital imaging software (Abràmoff et al., 2004). In order to quantify continuous variation in leg and wing dimorphism across species, we took the following external morphological measurements: femora and tibia length, wing length, thorax length, femora and tibia area, and wing area. To estimate the amount of leg dimorphism within each species we took the square root of total leg area (femora and tibia) for each leg (hind, mid and front) and divided by total leg length. We summed the relative leg dimorphism values for hind, mid and front legs in each sex. We then subtracted the male value from the female value such that higher positive values of leg dimorphism indicate more pinnation on female legs for that species. To quantify wing dimorphism within each species, we took the square root of wing area and divided by wing length in each species. We then subtracted the male value from the female value to compute an index of wing dimorphism for each species. In order to estimate a correlated response to mating system variation in males, we dissected and measured the two dimensional area of the testes for ten males per species, then

Table 5.1. Sample collection locations for Empidinae dance flies.

species	GPS coordinates	collection locations
Empis aestiva	56°09′06.35″N, 004°38′36.20″W	SCENE, UK
Empis borealis	57°14′38.20″N, 003°41′42.55″W	Aviemore, UK
Empis nigripes	56°09′06.35″N, 004°38′36.20″W	SCENE, UK
Empis stercorea	56°09′06.35″N, 004°38′36.20″W	SCENE, UK
Empis tessellata	56°09′06.35″N, 004°38′36.20″W	SCENE, UK
Hilara chorica	56°09′06.35″N, 004°38′36.20″W	SCENE, UK
Hilara litorea	55°55′22.28″N, 003°10′34.04″W	Edinburgh, UK
Hilara maura	56°09′06.35″N, 004°38′36.20″W	SCENE, UK
Rhamphomyia crassirostris	56°09′06.35″N, 004°38′36.20″W	SCENE, UK
Rhamphomyia dentipes	56°09′06.35″N, 004°38′36.20″W	SCENE, UK
Rhamphomyia longicauda	43°41′11.00″N, 079°55′34.00″W	Glen Williams, Canada
Rhamphomyia longipes	56°09′06.35″N, 004°38′36.20″W	SCENE, UK
Rhamphomyia nigripennis	56°09′06.35″N, 004°38′36.20″W	SCENE, UK
Rhamphomyia stigmosa	56°09′06.35″N, 004°38′36.20″W	SCENE, UK
Rhamphomyia sulcata	56°09′06.35″N, 004°38′36.20″W	SCENE, UK
Rhamphomyia tibiella	56°09′06.35″N, 004°38′36.20″W	SCENE, UK

standardized mean testis area by dividing by an individual male's thorax length to arrive at a relative testis size measure per species. For paired characters we measured both right and left sides and took the mean. When this was not possible because of damage to one side, we measured only the undamaged side.

OSR measurements

In order to predict the intensity of intrasexual competition, we estimated the OSR across dance fly species. We used an entomological sweep net to collect a sample of the individuals attending a mating swarm at a single swarming event. We calculated the OSR as the number of males swarming divided by the total swarm population. Because mating swarms are often predictable and occur at the same swarm marker for the duration of a swarming season (Funk & Tallamy, 2000), we returned to the same mating swarm areas and collected multiple mating swarm events to more accurately reflect the OSR as it changes through the breeding season for a given species. Previous work in individual dance fly species has shown that the mating swarm OSR can vary both spatially and temporally (Svensson & Petersson, 2000; Wheeler, 2008) (Funk & Tallamy, 2000), so we took the total number of swarming OSR tallies across each swarming event to get an estimate of the OSR for each species.

Sequencing of CAD

To estimate the evolutionary relationships amongst the 16 flies of interest for this study, we chose the phylogenetic marker gene CAD (Moulton and Wiegmann 2004). CAD is a fusion protein encoding the first three enzymes of the *de novo* pyrimidine biosynthetic pathway. This gene has proven useful for resolving phylogenetic relationships in Diptera (and particularly in the superfamily Empidoidea) because it is single-copy, relatively intron-free, and possesses moderate levels of non-synonymous divergence (Moulton & Wiegmann, 2004; Moulton & Wiegmann, 2007). DNA was isolated from individual flies using DNeasy animal tissue extraction kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. We amplified a ~1200bp partial coding sequence from the carbomoylphosphate synthase (CPS) domain of CAD using Empididae degenerate PCR primers obtained through personal communication with Brian Cassel from the Wiegmann research group at North Carolina State University (empCAD292F: AGYAATGGNCCNGGHGATCC and empCAD695R: GGRTCYARRTTYTCCATRTTRCA). PCR amplifications were carried out in 20μL reactions with 4.0μL ddH$_2$O, 4μL 5X Taq Polymerase Buffer, 1.8–2.1μL of 25 mM MgCl$_2$, 2 μL of each primer (2.5μM), 1μL of 10 mM dNTPs, 1 unit of Taq polymerase, and 2-4μL of template DNA. All reactions were carried out using a 3-step touchdown PCR modified from Moulton and Wiegmann (Moulton & Wiegmann, 2004) (4 min denaturation at 94°C followed by 4 cycles of 94°C for 30s, 52°C for 30s, 72°C for 2m, 6 cycles of 94°C for 30s, 51°C for 30s, 72°C for 2m, and 36 cycles of 94°C for 30s, 45°C for 20s, 72 °C for 2m30s). PCR amplicons were visualised on 1% agarose gels to ensure that the PCR was successful and generated only a single band. Each PCR fragment was directly sequenced on both strands on an ABI 3730 capillary Sanger sequencing instrument at the Edinburgh Genomics Sequencing facility (Edinburgh, UK). We assembled the forward and reverse strands using Sequencher 4.7 and edited chromatograms manually to ensure that all base calls, and variant sites were reliably scored. We also included the partial CAD sequence of *Hilara lugubris*, which is the only Empidinae species in NCBI Genbank identified to the species level (accession number: DQ369299.1). As an outgroup we used *Heterophlebus versabilis* (accession number: HM062728.1) from the related Empididae subfamily, Trichopezinae. This outgroup was chosen because it is closely related to Empidinae based on previous phylogenetic work on the Empidoidea (Moulton & Wiegmann, 2007). In addition, the uncorrected pairwise genetic distance between *Heterophlebus versabilis* and each ingroup sequence was always greater than the genetic distance between any pair of ingroup sequences. We aligned all the sequences using their translated amino acid sequences in MUSCLE v 3.8.31 (Edgar, 2004) before back converting to a DNA alignment.

Phylogenetic Inference

We conducted a Bayesian MCMC phylogenetic analysis of CAD sequences in MrBayes v 3.2.4 (Ronquist et al., 2012). Because the CAD sequence we analysed is protein coding, we used a ‘codon’ model of evolution to capture the heterogeneity of mutation rate and selective constraint on sites across the sequence. We also ran simpler models to ensure that we had not over-parameterised the model; we ran one model in which the first, second and third codon positions were partitioned (parameter estimation for each partition was unlinked) and another model where there was no partitioning of sites. For each model we allowed MrBayes to select the best base substitution scheme with a reversible jump MCMC (nst =mixed). We compared the fit of the models by approximating the marginal likelihood with the stepping stone estimator; these marginal-likelihoods were then evaluated using Bayes factors to assess the fit of the data to the three models. For each model we ran three independent runs for 3.5 million cycles, each with four Markov chains. We allowed for a burnin such that the average standard deviation of split frequencies dropped below 0.01 before we began sampling (burnin = 0.5-1.5 million). To ensure convergence we checked that Potential Scale Reduction Factor (PSRF) for all parameters converged to 1.0 and that the average Estimated Sample Size (ESS) for all parameters exceeded 200. To account for uncertainty in the phylogenetic tree in our statistical analysis, we randomly sampled 1300 topologies from the posterior probability of trees (see below).

For simplicity, we created binary characters (presence or absence) for each of the four types of female-specific ornaments displayed by each Empidinae species that we sampled: wing colour dimorphism, wing size dimorphism, pinnate leg scales and abdominal sacs (Table 5.2). We then mapped each trait onto the consensus tree by delineating the most parsimonious transitions in character states. We note that this is an

Table 5.2. Summary table of morphological traits and operational sex ratio (OSR) across 16 Empidinae dance fly species. Continuous traits are displayed as trait mean ± standard error. OSR is measured as the proportion of males. N=10 for leg, wing and testis measures.

species	**OSR**	**leg dimorphism**	**wing dimorphism**	**relative testis size**	**discrete ornaments**
E. aestiva	0.34±0.05	0.178±0.011	-0.057±0.021	0.360±0.018	leg scales wing colour
E. borealis	0.44±0.12	-0.060±0.021	0.058±0.008	0.147±0.004	wing size
E. nigripes	0.46±0.16	0.094±0.021	-0.025±0.007	0.232±0.015	leg scales wing colour
E. stercorea	0.51±0.19	0.036±0.031	-0.0029±0.0008	0.225±0.019	none
E. tessellata	0.71±0.10	0.032±0.008	0.025±0.001	0.221±0.021	none
H. chorica	0.54±0.02	0.030±0.011	0.017±0.006	0.326±0.011	none
H. litorea	0.64±0.02	-0.009±0.007	0.011±0.002	0.252±0.012	none
H. maura	0.62±0.17	-0.050±0.023	0.0073±0.0001	0.260±0.011	none
R. crassirostris	0.34±0.05	-0.008±0.008	0.0096±0.0006	0.100±0.010	none
R. dentipes	0.75±0.33	-0.065±0.009	-0.068±0.009	0.195±0.014	none
R. longicauda	0.24±0.04	0.226±0.008	0.0066±0.001	0.339±0.007	leg scales abdominal sacs
R. longipes	0.71±0.04	0.135±0.027	0.012±0.008	0.253±0.031	leg scales
R. nigripennis	0.87±0.33	0.011±0.011	0.000064±0.000008	0.240±0.022	wing colour
R. stigmosa	0.57±0.21	0.021±0.009	0.017±0.002	0.219±0.006	none
R. sulcata	0.63±0.09	-0.035±0.008	-0.0043±0.0007	0.249±0.012	none
R. tibiella	0.59±0.15	0.070±0.004	-0.014±0.004	0.312±0.017	leg scales abdominal sacs

oversimplification of the data because even between closely related species that share the same ‘type’ of ornament, there is obvious variation in ornamentation.

Statistical analysis

All statistical analyses were carried out in R (R Core Development R Core Development Team, 2014). Within each species, all continuous morphological traits were standardized (by subtracting the mean) and scaled (by dividing by two standard deviations) so that each trait was measured on a common scale (Gelman & Hill, 2007). The OSR was measured as the arcsine-square root transformed ratio of the proportion of males in the mating swarm.

We employed a standard comparative method (Felsenstein, 1985; Harvey & Pagel, 1991) to test for an effect of OSR on female ornamentation and whether the degree of female ornament expression predicted a correlated response in male relative testis investment. We used the pic function from the APE package (Paradis et al., 2004) to compute phylogenetically independent contrasts. In order to visually characterize mating system variation across species and reduce the dimensionality of our multiple continuous measures, we then subjected our independent measures to a principal components analysis (PCA). The analysis was carried out on the correlation matrix of the phylogenetically independent contrasts.

We used MCMCglmm (Hadfield, 2010) to perform comparative analyses using phylogenetic mixed models. We fit two models: one with continuous measures of female ornamentation as a response and OSR as the fixed effect, and one that fit testis size as the response predicted by continuous measures of female ornaments and their interaction. We simplified our models by dropping non-significant terms from the full model sequentially and using the Deviance Information Criterion (DIC) value to compare between the fit of the old and new model. Wherever model simplification resulted in an increased DIC we retained the more complex model. To test for the effect of phylogenetic ancestry we calculated the phylogenetic heritability (an analogue to Pagel's lambda), which estimates the proportion of between species variance explained by the phylogeny (Hadfield, 2010).

To correct for uncertainty in the phylogeny during our comparative analysis, we marginalized over the posterior distribution of trees created during phylogenetic inference above. We sampled a tree at iteration t, ran 1000 iterations of the MCMC comparative analysis and then saved the last MCMC sample. The values from the variance components in the saved MCMC sample were then used in the analysis for starting values at iteration t+1 and a new tree from the posterior distribution was taken. This process was repeated 1300 times (i.e. using 1300 trees randomly sampled from the posterior probability of trees) and the first 300 iterations were discarded as burn-in as in (Ross et al., 2013) while retaining a sample size of 1000.

Our MCMCglmm models assumed a Brownian model on the logit probability scale for the phylogenetic effects (Hadfield, 2010). We corrected for phylogenetic non-independence by using the CAD phylogeny and the tree sampling method described above. For all models we used a weakly informative parameter-expanded prior. We report the significance of our fixed effects as pMCMC, which is twice the posterior probability that the estimate is positive or negative (whichever is smallest), and can be considered the equivalent of the frequentist p value (Hadfield, 2010).

Results

Morphological traits and operational sex ratio

We measured the operational sex ratio, two measures of female ornamentation and relative testis size across 16 Empidinae dance fly species and report our findings as mean trait value ± standard error for each species (Table 5.2). The sampled populations for the OSR estimates varied by species, and this variation is reflected in the standard error reported for each species' mean value.

Empidinae phylogeny

We successfully amplified and sequenced the partial CAD coding sequence for all species included in this study. The chromatograms of *E. storcorea* and *R. stigmosa* were truncated and therefore only partial sequences were included (478bp and 734bp, respectively). All ambiguous bases were marked with an 'N' to avoid poor quality nucleotide calls influencing the phylogeny. The alignment of sequences was straightforward with a single 6bp deletion in the ancestor of the *Hilara* species included in our study. We assessed three models of sequence evolution and found the data fit the 'codon' model better (ln(marginal likelihood) = -5988.72) than both simpler models: three codon positions partitioned (ln(marginal likelihood) = -6162.19), and no partitioning (ln(marginal likelihood) = -6516.24). Bayes factor (BF) calculations indicated strong support for the codon model over the partitioned (BF = 2.2×10^{75}) and unpartitioned (BF = 1.3×10^{229}) models.

The phylogeny inferred using CAD included 22 species, five *Empis*, six *Hilara*, 10 *Rhamphomyia* and the outgroup *Heterophlebus versabilis*. The outgroup rooted the tree on the branch connecting *Hilara* to *Empis* and *Rhamphomyia*, consistent with Moulton and Wiegmann (2007). The consensus tree displayed in Figure 5.3 was well resolved: 15 of 19 nodes had a posterior probability >0.95, and only two nodes were unresolved (<0.5), which created a polytomy among *R. crassirostris, R. longicauda* and the well-supported sister pair *R. stigmosa* and *R. sulcata*. Some of these ambiguities could be resolved by including more sequence from CAD and other phylogenetic markers or by sampling more species. However, uncertainty in the exact topology of the unresolved nodes in our CAD tree was accounted for by marginalizing over the posterior probability of tree topologies in our statistical analysis (see below).

Our mapping of binary character states, while conservative, does estimate multiple origins of female ornament evolution even within our sample of dance fly species (Figure 5.3).

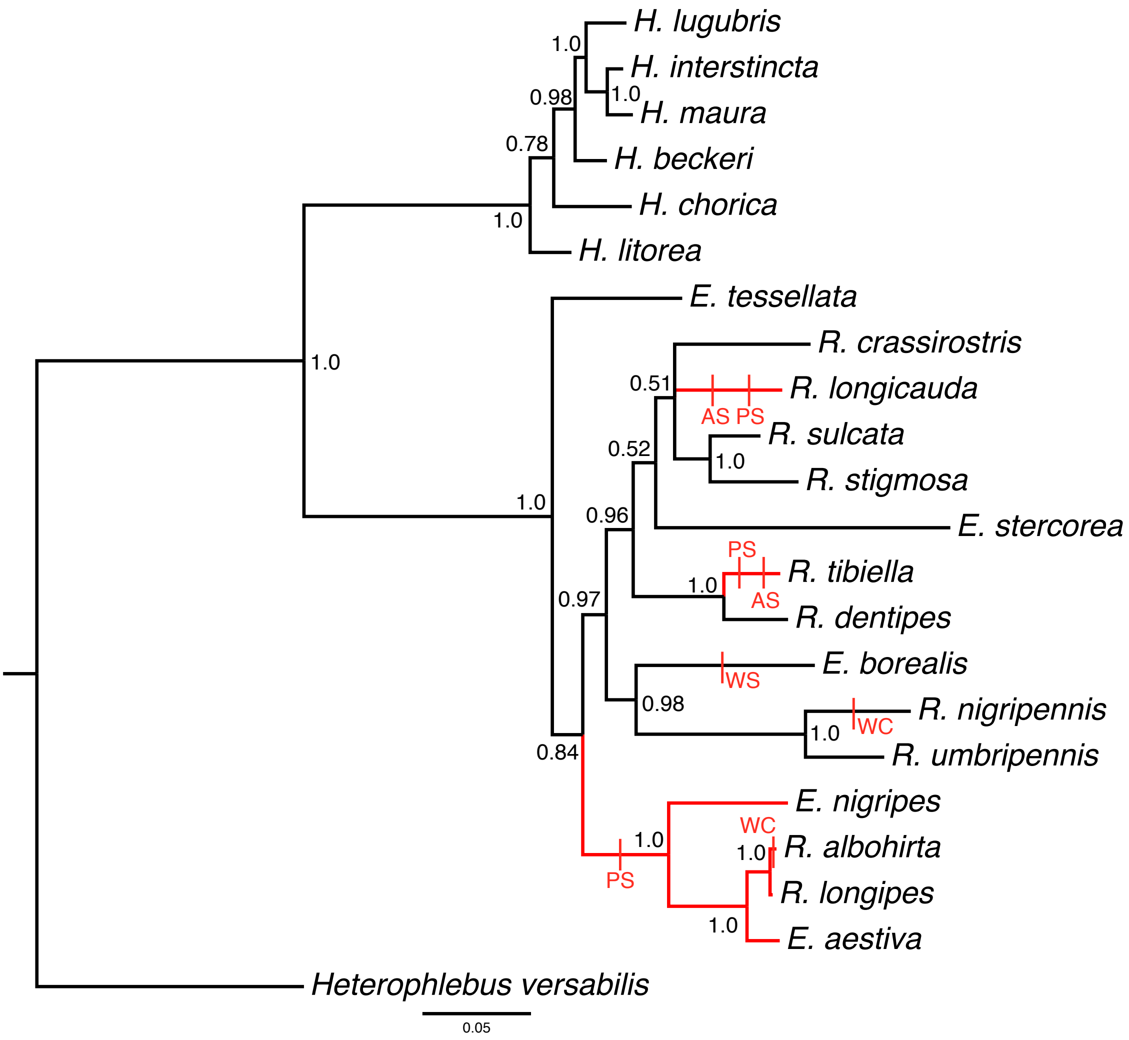

Figure 5.3. Consensus Bayesian phylogenetic tree of 21 Empidinae species inferred using partial protein coding sequence of CAD. Estimates of the evolutionary relationships between species from the genera *Empis*, *Hilara*, and *Rhamphomyia* are displayed with the outgroup Heterophlebus versabilis. Node labels indicate the posterior probability of each split, and nodes with less than 0.50 posterior probability are displayed as polytomies. Branches coloured red represent species or clades with female-specific ornamentation in the form of pinnate scales. Red vertical hashes indicate the branch on which different female ornaments are inferred to have arisen. Four ornaments are shown, pinnate scales (PS), inflatable abdominal sacs (AS), wing colour dimorphism (WC) and wing size dimorphism (WS). Each transition in character state was inferred using parsimony.

Pinnate leg scales show three independent origins, wing colour dimorphism and abdominal sacs show two origins and wing size dimorphism shows a single origin.

Principal components analysis

To visualize the relationship between species in our study, we performed a PCA on phylogenetically independent contrasts of female leg and wing ornaments, relative testis size and OSR across dance fly species (Table 5.3; Figure 5.4). We found that the first two principal components, PC1 and PC2, explained over 80% of the variation in our data. PC1 loads positively on relative testis size and leg dimorphism and negatively on wing dimorphism and OSR, while PC2 loads positively on legs and OSR, but negatively on wings and testes (Table 5.3). The relative positioning of each species in principal component space can be seen in Figure 5.4.

Comparative analysis

If the evolution of female-specific ornaments is indicative of competition for access to males (or their nuptial gifts), we predicted that increased female ornamentation should positively covary with an increase in female-biased OSR. To test for an effect of OSR on female ornamentation, we fit a generalized linear mixed effects model. Total ornamentation (the sum of wing and leg dimorphism for each species) was fit as the response, OSR was fit as the fixed effect and the phylogeny was fit as a random effect using the MCMCglmm package. We found no effect of OSR on the degree of ornamentation in a species after correcting for phylogeny (pMCMC=0.154; Table 5.4). We calculated the phylogenetic heritability (analogue to Pagel's lambda) as 0.25, indicating a low degree of phylogenetic structure in ornamentation after accounting for variation in OSR measures.

We wanted to test for an effect of female ornaments on male testis size. If ornament evolution accompanies increased polyandry within a mating system, then we expect a positive relationship between female ornament expression and male relative testis size.

Table 5.3. Principle components analysis loadings from phylogenetically independent contrasts of 16 Empidinae dance fly species. Collectively the two principle components explain over 80% of the variation in the data (57.4% and 25.2%, respectively). OSR was measured as the proportion of males.

trait	**PC1**	**PC2**
relative testis size	0.512	-0.065
leg dimorphism	0.620	0.049
wing dimorphism	-0.121	-0.980
OSR	-0.582	0.198

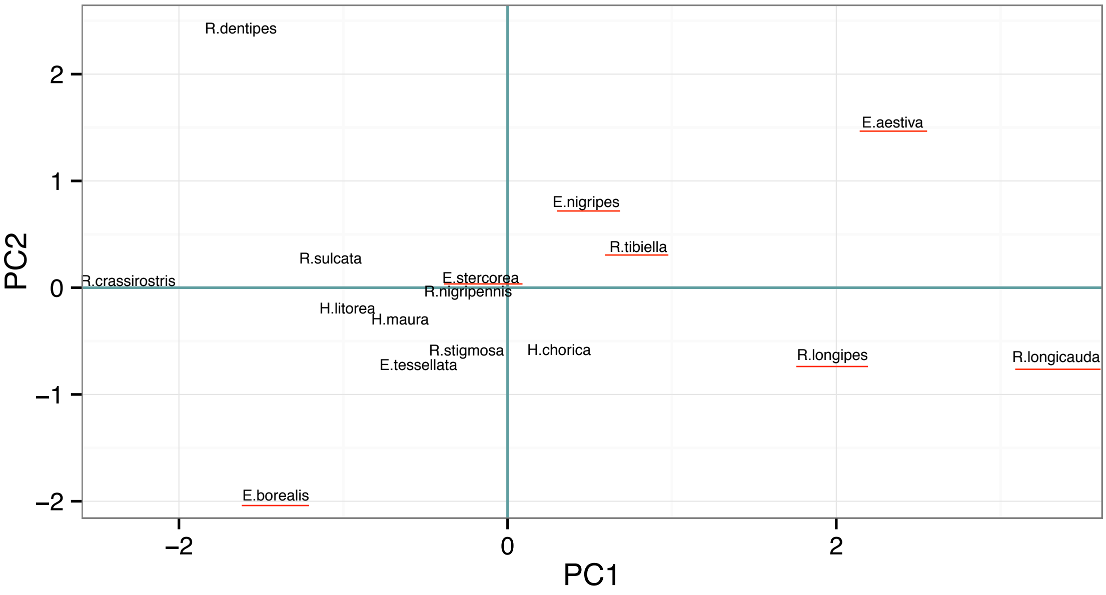

Figure 5.4. Ordination of 16 Empidinae species along the first two principle components of a PCA based on phylogenetically independent contrasts of morphological and behavioural traits.Species that display female-specific leg or wing ornamentation are underlined in red. (See Table 5.3 for loadings).

Table 5.4. Estimates from a phylogenetically-controlled analysis of female ornamentation (leg dimorphism + wing dimorphism) predicted by operational sex ratio (OSR) estimates across Empidinae species. The model used a “Gaussian” distribution as specified in MCMCglmm. Values were generated using the summary function in the MCMCglmm package in R.

	posterior mean	**L-95% CI**	**U-95% CI**	**eff. samples**	**pMCMC**
Intercept	0.172	-0.025	0.371	1000	0.089
OSR	-0.160	-0.375	0.071	1000	0.154

We fit a MCMCglmm mixed model with relative testis size as the response, the presence of female leg and wing ornaments as two discrete factors along with the interaction between them as fixed effects, and the phylogeny as a random effect. We found that relative testis size was best described by a simplified model (Table 5.5) with a significant positive linear effect of leg dimorphism on relative testis size (pMCMC<0.001). Wing dimorphism did not predict relative testis size (pMCMC=0.422), and the interaction between leg and wing dimorphism was also non-significant (pMCMC=0.078). However, although the interaction term is non-significant, removing the effect caused an increase in the DIC value and therefore we retained the interaction term in our simplified model. To illustrate the effect of the ornament interaction, we show the linear effect of leg dimorphism on relative male testis investment for high and low wing dimorphism in Figure 5.5. We calculated the posterior probability of phylogenetic signal (mixed model equivalent of Pagel's lambda) as 0.28, indicating a low degree of phylogenetic structure in relative testis size after accounting for variation in dimorphism measures.

Discussion

Dance flies from the subfamily Empidinae display extreme mating system variation from species with multiple female-specific ornaments (Fgure 5.1, Figure 5.2) to those that display very little sexual dimorphism. To estimate the evolutionary relationships between species, we created a phylogeny of 22 dance flies. Our phylogeny showed multiple origins of female-specific ornamentation across species (Figure 5.3). We used the phylogeny to control for shared ancestry and plotted the relationships between species from a PCA of our four measures of mating system diversity (Table 5.3, Figure 5.4). Next we performed a comparative analysis to investigate the relationship between OSR and continuous measures of female-specific ornamentation. We also tested for a relationship between the intensity of female-specific ornamentation and male relative testis investment. We found that OSR did not predict female ornamentation (Table 5.4), but that

Table 5.5. Estimates from a phylogenetically-controlled analysis of female ornaments on the relative testis investment by males across Empidinae species. The model incorporated a “Gaussian” error distribution. Values were generated using the summary function in the MCMCglmm package in R. (leg=leg dimorphism; wing=wing dimorphism).

	posterior mean	L-95% CI	U-95% CI	eff. samples	pMCMC
intercept	0.251	0.214	0.297	1000	<0.001
leg	0.042	0.020	0.066	1000	<0.001
wing	0.009	-0.012	0.033	1000	0.422
leg*wing	0.013	-0.001	0.031	1000	0.078

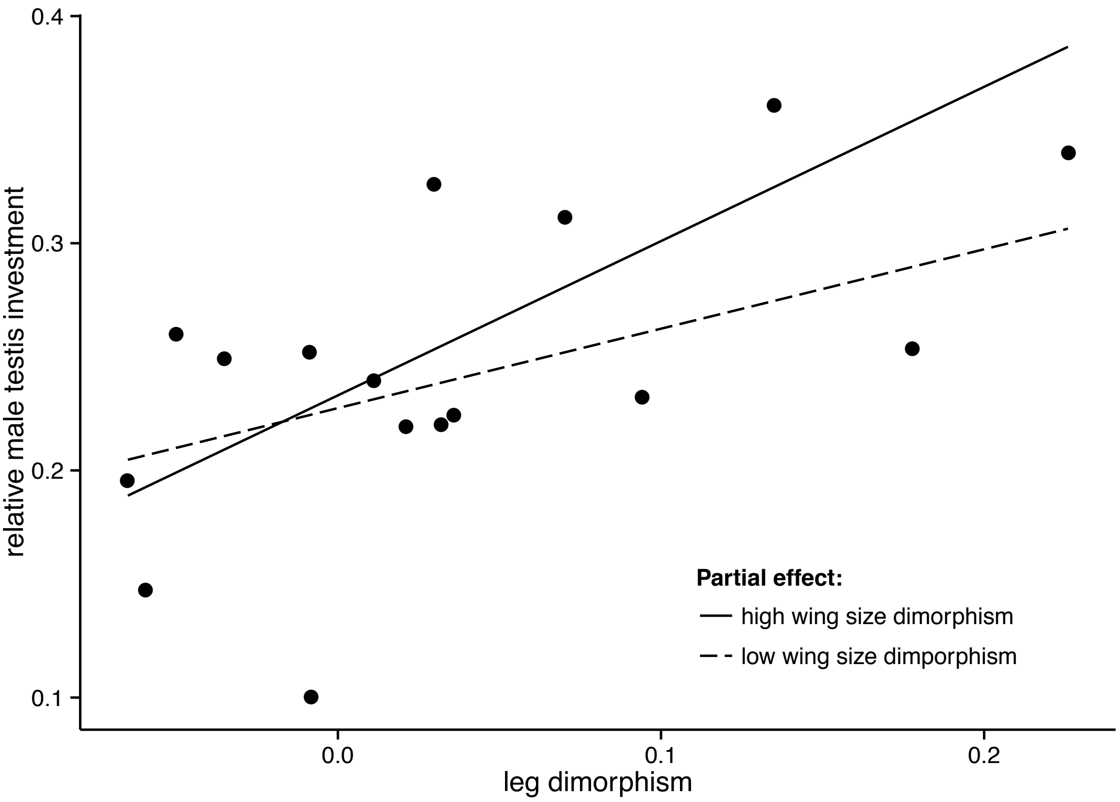

Figure 5.5. Predicted linear slopes from MCMCglmm model showing the partial effect of wing dimorphism (high and low) on the relationship between leg dimorphism and relative testis size.Solid line represents high wing size sexual dimorphism; dashed line shows low wing size sexual dimorphism.

increased female-specific ornamentation positively covaried with relative male testis investment (Table 5.5, Figure 5.5).

Empidinae phylogeny

Our phylogeny revealed that all species in the genus *Hilara* are members of a well-supported monophyletic clade. In contrast, the genera *Rhampomyia* and *Empis* are not monophyletic, but are instead mixed together in larger clade distinct from *Hilara*. Assuming parsimony, we mapped the evolution of female ornamentation onto the phylogeny (see Figure 5.3). Pinnate leg scales appear to have evolved three times in the *Empis*/*Rhamphomyia* clade; once in *E. aestiva, E. nigripes*, *R. longipes* and *R. albohirta*, which form a single well-supported clade with all species displaying female-specific pinnate scales, once in *R. longicauda* and once in *R. tibiella.* Our phylogeny suggests that *R. longicauda* and *R. tibiella* each appear to have independently evolved pinnate scales and both species also display inflatable abdominal sacs in the mating swarm. Wing sexual dimorphism also appears to have distinct origins on the phylogeny; *R*. *albohrita* and *R*. *nigripennis* have separately evolved wing colour dimorphism, and *E*. *borealis* females have evolved larger wings. It is important to note that this tree represents only a small fraction of the species diversity in the Empidinae and more complete taxonomic sampling could very well alter the mapping of these traits and our inferences of how many times ornamentation has evolved. However, given that we report four distinct sexually dimorphic traits, it seems clear that ornamentation has arisen independently in multiple lineages. In addition, the pinnate scales of *R*. *longicauda* and *R*. *tibiella,* which appear as distinct transitions, are morphologically distinct and unlikely to be homologous traits. Indeed, even for *R. longipes and R. albohirta*, which our tree identifies as sister species that share the same origin of leg pinnation (Figure 5.3), the degree of ornamentation is different; *R. longipes* displays hind leg female-specific pinnation, while *R. albohirta* females have pinnation on their hind and mid legs (Collin, 1961). Therefore, by simplifying continuous ornamental traits into binary characters on the phylogeny we are being conservative with our estimates of the number of times that female ornaments arise in the dance flies. A complete comparative analysis with more thorough sampling of the *Empis* and *Rhamphomyia* clade would provide valuable insights into the evolution of different forms of female-specific ornamentation.

Female-biased OSR and female ornamentation

We did not find a relationship between the OSR and the amount of female-specific ornamentation (Figure 5.4, Table 5.3, Table 5.4). A PCA revealed that leg dimorphism loaded positively, while OSR loaded negatively on the first principal component (PC1), which accounted for more than 50% of the variation in our data (Table 5.3). When we formally tested to see if female-specific ornaments could be predicted from female-biased OSRs, a pattern that is superficially similar to those observed in males (Pomfret & Knell, 2008), we found that there was no relationship (Table 5.4). The fact that we see no relationship between OSR bias and ornamentation could mean that it is not an appropriate measure for predicting sexual selection across dance fly taxa, or perhaps that the constraints placed on female ornament expression (Fitzpatrick et al., 1995) mean that the relationship is more complicated in females than it is in males.

One of the criticisms of the OSR as an estimate of the strength of sexual selection is that it does not directly measure selection for a population (Klug et al., 2010). While the OSR is likely an indicator of the intensity of the contest for mates, it does not account for differences in the relative increase in fitness per mating between the sexes (Bateman gradient; (Bateman, 1948)). Further, one assumption related to the OSR's ability to reliably indicate the intensity of sexual selection, is that an increase in the bias of the OSR increases mate monopolization by the more common sex (Emlen & Oring, 1977). Mate monopolization, while very important for male reproductive success, is unlikely to be tightly linked to female reproductive success. Instead, females are more likely to be limited by access to resources than sperm (Trivers, 1972). Finally, the OSR might not account for female ornamentation in our measures because we have not considered the influence of conditions that might cause selection to favour traits that reduce the time between matings; i.e. the time spent by each sex engaging in other activities besides mating can differentially impact the likelihood of secondary sexual traits evolving, even with a biased OSR (Kokko et al., 2012). Therefore, it seems likely that for dance flies the OSR is an indicator of the intensity of the contest for access to mates, but the actual intensity of sexual selection is much more complex. Our finding is in line with previous criticism about the OSR as a measure of sexual selection; to accurately measure the intensity of selection on males and females we need to calculate OSR measures and account for variation in the Bateman gradient (Bateman, 1948), the degree of mate monopolization (Klug et al., 2010) and differences in the time spent attempting to mate compared with other activities (Kokko et al., 2012).

OSR measures have been shown to vary spatially and temporally in dance fly species (Svensson, 1997; Funk & Tallamy, 2000; Svensson & Petersson, 2000; Wheeler, 2008). One limitation to our measures of OSR is that we did not capture multiple populations of the same species and therefore cannot account for spatial variation in OSR measures within species. A previous study showed that between two populations of the same species, the OSR was able to accurately predict different levels of sexual selection (Monteiro & Lyons, 2012). Therefore, despite our attempts to estimate the OSR for entire species, we may still be falling short if we do not account for variation across populations, especially in flying insects. Importantly, however, we were able to capture temporal variation in OSR within a species by returning to the same swarm and sampling the OSR repeatedly throughout the breeding season (Svensson & Petersson, 2000).

Female-specific ornamentation and male relative testis investment

We found that the leg sexual dimorphism had a positive relationship with male relative testis investment across species (pMCMC=0.002), however wing size dimorphism across species did not predict male testis investment (pMCMC=0.400), but the interaction between leg and wing dimorphism was only marginally non-significant (pMCMC=0.078; Table 5.5, Figure 5.5). The positive interaction effect between leg and wing dimorphism could indicate that the effect of wing dimorphism depends on leg dimorphism, and we have illustrated this pattern in Figure 5.5. The difference in the significance of the two female ornaments could partly result from the taxa that we sampled here. Many of the species measured (6 out of 10) have female ornamentation on their legs that is obvious even in the field, and are described by Collin (1961) as having 'distinct pinnate leg scales'. However, only one of our species, *Empis borealis*, has obvious wing size dimorphism and is described by Collin (1961). It is possible that wing dimorphism could serve another purpose in the dance flies as well as ornamentation. For example, sexually dimorphic pigmentation in damselfly wings is related to thermoregulation across species (Svensson & Waller, 2013), and wing size dimorphism has been shown to trade-off with other life history traits differently in males and females (Guerra, 2011). Therefore, while it is possible that selection on wing dimorphism is different from selection on leg dimorphism, it is also possible that wing ornamentation was not accurately represented in our samples.

One of the most consistently observed patterns relating to increased testis size is positive covariance with polyandry; to be more specific, testis size increases when females mate with more than one male (Pitnick, 1996; Pitcher et al., 2005; Montgomerie & Fitzpatrick, 2009; Soulsbury, 2010; Vahed et al., 2011). Two hypotheses that may be interrelated have been used to account for this pattern, the numerical sperm competition hypothesis and the male mating rate hypothesis reviewed in Vahed and Parker (2011). The numerical sperm competition hypothesis posits that larger testes will allow males to produce more sperm per ejaculate to compete more effectively in numerical sperm competition (Parker et al., 1997), while the male mating rate hypothesis predicts that males with larger testes will be able to increase the number of copulations they engage in, potentially with smaller ejaculate investment per mating. Our results suggest that relative to unornamented species, ornamented female dance flies are more likely to be polyandrous (Table 5.5, Figure 5.5). However, the relationship between polyandry and relative testis investment is not necessarily linear and can depend on both the rate of polyandry and the degree of sperm competition (Parker et al., 1996; Parker & Pizzari, 2010). Therefore, in order to distinguish between, or relatively weight, the importance of the numerical sperm competition hypothesis and the male mating rate hypothesis, we would have to directly measure rates of polyandry across dance fly taxa that vary in their amount of female-specific ornamentation.

Conclusions

We inferred a phylogeny of the Empidinae species that shows, for the first time, that female-specific ornaments have evolved multiple times in the dance flies. We next performed a comparative analysis to estimate the role of intrasexual competition (measured as OSR) in predicting female-specific ornament evolution across dance fly species. We also tested for a correlated response to female ornament evolution by measuring male relative testis size across taxa. We found that female-specific ornamentation could not be predicted by bias in the OSR and hypothesize that other metrics indicating the intensity of sexual selection might be important (Bateman gradient, degree of mate monopolization and time spent attempting to mate), particularly when assessing the relationship between female-biased OSR and female-specific ornaments. We did, however, find that female ornamentation positively covaries with relative testis investment across taxa indicating that in species that display female-specific ornamentation, females are mating more than once. Both the lack of relationship between OSR and ornamentation and the positive relationship between ornaments and testis investment suggest that future work investigating polyandry rates across dance fly taxa would be a necessary undertaking to further clarify the relationship between mating system variation and female-specific ornament evolution.

Chapter 6

General discussion

In this thesis, I investigated the causes and consequences of female-specific ornamentation both within and between species of dance flies (Diptera Empididae: Empidinae). I reconstructed evolutionary relationships between species and analyzed the intensity of intrasexual competition (as estimated by the OSR) as a predictor of female ornamentation in a comparative phylogenetic framework. I investigated symbiont infections as a cause for ornament evolution, tested for functional constraints in the evolution of non-random mating patterns, and assessed the role of sexual conflict in the evolution of multiple female-specific ornaments. In this final chapter of my thesis I summarise the main conclusions of each of the preceding four chapters and outline the broad contributions that my thesis work has made to our understanding of female ornament evolution and mating system variation. I also provide suggestions for future research on female-specific ornament evolution in the dance flies that might be profitably undertaken.

Summary of thesis chapters

Chapter 2: *The role of sexual conflict in the evolution of multiple sex-specific female ornaments.*

Sexual conflict causing a coevolutionary arms race between the sexes has been shown to result in multiple manipulative male-specific ornaments in some species. In this chapter I performed a manipulative field experiment to test a key prediction of sexual conflict in the evolution of multiple deceptive female-specific ornaments. I found that both ornaments displayed by *R. longicauda* females showed patterns consistent with directional selection. However, the effect size and statistical interaction between the two display traits demonstrated that the ornament types (leg scales vs. abdomens) differed in their ability to attract male mates. Consistent with sexual conflict theory for male-specific ornament evolution, I found that the evolutionarily more derived ornament, abdominal sacs, is better at attracting potential mates, while the basal display trait, pinnate leg scales, is only important when abdominal sacs are small. These findings provide the first evidence that an antagonistic coevolutionary arms race between the sexes is driving the evolution of multiple female-specific ornaments.

Chapter 3: *The role of functional constraints in non-random mating patterns for a dance fly with female ornaments.*

Mate choice is frequently invoked to explain non-random mating patterns, however alternative explanations, such as functional constraints, can also contribute to observed variation in mating success. In this chapter I tested for a role of functional constraints in the observation of stabilizing selection on two female-specific ornaments in *Rhamphomyia longicauda*; I tested whether heavy females with large ornaments suffer lower than expected mating success because many males cannot carry them during aerial copulation, which is an alternative to mate choice against highly ornate individuals. In the presence of a functional load-lift constraint on males during aerial copulations, I expected to see (1) a trade-off between female mass and nuptial gift mass, (2) a negative relationship between the mass of the female a male mates with and the wing loading a male experienced because of his own mass, and (3) that the mean observed load lifted by males should be lighter than the majority of observations from a randomly sampled null distribution of potential loads. I found no evidence for a load-lift constraint in *R. longicauda* from either (1), (2) or (3) above. Instead, I found a significant positive correlation between a male's own wing loading measure (mass/wing area) and the mass of the female he mated with. Thus, rather than discovering a trade-off related to a load-lift constraint, I uncovered what appears to be positive assortative mating for mass in *R. longicauda*. Although I was not able to address the still puzzling finding that *R. longicauda* females displaying the largest ornaments have lower mating success than intermediately ornamented females, my findings here suggest that rather than being constrained functionally, males might be discriminating against females who over-invest in female ornaments at the expense of fecundity.

Chapter 4: *Do sex ratio distorting endosymbionts influence the evolution of female ornamentation in the Empidinae dance flies?*

Biased sex ratios are predicted to indicate the intensity of intrasexual competition in a population, a metric that is well-known to influence mating system evolution. In this chapter I investigated the potential role of three maternally-inherited symbiont taxa in distorting sex ratios among dance flies leading to mating system variation and female-specific ornament evolution. I used PCR screens to identify hosts that were infected with symbionts and used sex-biased prevalence measures to identify taxa that might be infected with sex ratio distorting symbionts. I did not find a relationship between infection prevalence and ornament evolution. I also tested for an effect of symbiont prevalence on adult sex ratio (ASR) estimates across dance fly host species but found no effect. However, I did find high incidence and variable prevalence measures across a novel group of insect hosts for three well-known symbiont taxa. Importantly, from this study I was able to rule out the possibility that widespread sex ratio distorting symbiont infections are a major cause of mating system diversity and female-specific ornament evolution in dance flies.

Chapter 5: *Female-specific ornaments, operational sex ratio and the testis size: a comparative study in the Empidinae dance flies.*

The intensity of intrasexual competition (as estimated by the bias of the OSR) is known to cause male-specific ornament evolution. However, it is not clear whether the relationship between OSR bias and female-specific ornaments should mirror the situation in males. In this chapter I used a comparative analysis to investigate the influence of OSR on mating system variation and the evolution of female-specific ornamentation across Empidinae dance fly species. I discussed why theory may or may not predict the evolution of female ornamentation from female-biased OSRs and tested male reproductive traits for a correlated response to female ornamentation. I showed that female-biased OSRs did not predict the evolution of female-specific ornaments across dance fly taxa; however, the evolution of female ornaments positively covaried with a corresponding increase in male relative testis size. Correlated evolution of male testis size with female ornaments likely indicates that in species that display female-specific ornamentation there is increased polyandry which in turn causes increased male investment in relative testis size. This study provided the first comparative test of the impact of female-biased OSR on female ornament evolution, and provides further evidence that the OSR is an emergent property of the mating system that acts as an indicator of the potential for sexual selection, rather than a cause of sexual selection.

My thesis has demonstrated that a classic predictor of the intensity for sexual selection does not predict female ornament expression across dance fly species (Chapter 5). This finding is in contrast to relationships between male ornamentation and male-biased OSRs that have been previously observed (Pomfret & Knell, 2008). It is possible that the contrast observed in the relationship between the sexes is illustrative of the fundamental difference between male and female gamete investment. Because males do not need to preserve resources for costly gamete production (but see Pitnick & Markow, 1994), there are likely much broader circumstances under which ornament evolution can occur in response to intrasexual competition as indicated by sex ratio bias. Given the reproductive constraints placed on females because of relatively costly gamete production, it is possible that the OSR, while still indicative of sexual selection on females, is less likely to result in the evolution of costly ornaments.

Consistent with previous scrutiny about the OSR as a measure of sexual selection, (Shuster & Wade, 2003; Klug et al., 2010; Kokko et al., 2012), I show that female intrasexual competition across dance fly taxa cannot be effectively predicted by the OSR alone. However, I present three caveats that are likely to have influenced the ability of the OSR to act as a signal for female-specific ornament evolution: (1) the OSR can only measure the intensity of the contest for mates, not the actual fitness gains per mating (Bateman gradient; Bateman, 1948); (2) mate monopolization is unlikely to increase female fitness, and therefore, a part of OSR theory that was created under the assumption of male-biased OSRs, cannot be directly translated to indicate intrasexual competition when the OSR is female-biased; (3) the OSR cannot account for sex differences in selection for traits that decrease the time an individual must wait for a mating event. Further investigations that account for variation in the fitness gains associated with remating and the time that individuals spend away from the mating swarms across varied mating systems may yet show that the OSR is an effective indicator of intrasexual selection in female dance flies. However, until we are able to address the questions arising from my thesis, the predictive power of the OSR as an indicator of the intensity of sexual selection on females that might drive female-specific ornament evolution remains unclear.

Unresolved questions

My thesis has addressed a number of questions about to the consequences of mating system variation as it relates to the evolution of female-specific ornaments. In addition, I have generated a valuable resource, a phylogeny containing some commonly occurring dance fly species, that might aid future in-depth studies on the evolutionary transitions of female-specific ornaments. Next I briefly outline potential future research questions related to the evolution of female-specific ornaments in the Empidinae dance flies that carry on from the research that I have presented in this thesis.

Female ornamentation and nuptial gift evolution

In females, ornamentation is expected to trade-off with fecundity (Fitzpatrick et al., 1995). Females might overcome this trade-off if they receive direct benefits from mating, such as nutritious nuptial gifts, that make-up for any costs associated with investment into ornamentation. One major unknown variable in the evolution of female ornamentation in the dance flies is the importance of nuptial gifts. Some potential questions that might be addressed in the future include: How do nuptial gifts in ornamented and non-ornamented dance fly species vary? Compared to unornamented species, are gifts in ornamented taxa bigger or more nutritious to overcome any potential costs to fecundity? Do mating rates in ornamented species increase so severely that they tax a male’s ability to provide nutritious prey items? Are female-ornamented species more likely to experience an invasion of cheats whereby males are providing easier to obtain non-nutritious nuptial gifts in response to increased mating rates?

Nuptial gift evolution in the dance flies

In Chapter 5 I found a positive covariance between female-specific ornaments and relative testis size, which I concluded was indicative of a general trend that species with female-specific ornaments experience increased polyandry. Further, I hypothesized that increased polyandrous behaviour occurs because females receive direct nutritional benefits at every mating and an increased mating rate might correspond to increased female reproductive success. However, if females are displaying ornaments to deceive males about their fecundity (Chapter 2; Funk & Tallamy, 2000) and increasing their mating rate to receive proteinacious nuptial gifts (Chapter 5) it seems likely that males would also evolve adaptations to resist being exploited by females. One potential way that males might resist female deceptive traits is by ‘cheating’ and presenting females with non-nutritious nuptial gifts (LeBas & Hockham, 2005). Indeed, males of many species of dance flies have been observed engaging in ‘cheating’ behaviour related to nuptial gift giving by presenting potential mates with non-nutritious gifts that include empty carcasses of prey items that have already been consumed, debris from the surrounding area including twigs, stones, leaves or willowseed fluff, and silk balloons (Kessel, 1955; Cumming, 1994; Preston-Mafham, 1999).

Regardless of the type of non-nutritious gift that males attempt to present, it seems likely that costly deception must have been involved in the transition from nutritious to non-nutritious nuptial gifts in the dance flies. A formal comparative analysis of the evolution of mating systems and nuptial gifts in the Empidinae would be very interesting. Of particular of interest, and expanding on some of the work from my thesis, we could test for a link between the prevalence of a species to use cheating nuptial gifts and the rates of polyandry. We could quantify the size and nutritional value of gifts males carry as they enter the mating swarm and ask questions about variation in nuptial gift quality and mating system variation. For example, is there a relationship between nuptial gift nutritional value and ornamentation? The majority of studies investigating the evolution of nuptial feeding in the dance flies to date have occurred in non-ornamented species (Marden, 1989; Preston-Mafham, 1999). For species that exhibit female-specific ornamentation, gifts are potentially of higher importance because females might require more or better nuptial gifts to make up for the resources spent on ornament expression (Fitzpatrick et al., 1995). Presumably, if males and females are engaged in an antagonistic coevolutionary arms race (Chapter 5), deceptive female ornaments that manipulate males into increasing their mating rate (and therefore the number of gifts they provide) might result in more overall matings/nuptial gift transfers, but less nutritious gifts supplied at each mating. Indeed if this is true, then we would expect to see species with no ornaments providing more nutritious gifts at each mating, engaging in fewer matings and potentially copulating for longer when they did mate.

Sexual selection on genital morphology

One of the most interesting findings of my thesis is evidence for the role of sexual conflict in the evolution of multiple female ornaments (Chapter 2). A coevolutionary arms race between the sexes has previously been proposed to have a role in the evolution of males giving non-nutritious nuptial gifts in the dance flies (Arnqvist & Rowe, 2005). However, sexual conflict resulting in multiple female-specific ornaments has not previously been shown. The implications for this finding are two-fold for potential future research into sexual conflict in the dance flies: (1) sexual conflict predicts that we should see coevolving male resistance traits in response to female deceptive ornaments, and (2) if sexual conflict is strong enough to be driving the evolution of female ornamentation in the dance flies, it is also likely to be an important force in other aspects of dance fly biology, such as male genitalia/sperm traits and female reproductive tract coevolution (Tatarnic & Cassis, 2010). By testing for male resistance traits that have evolved in response to the evolution of deceptive female ornaments, we could confirm our findings indicating evidence of sexual conflict, and more definitively demonstrate that there is a coevolutionary arms race between the sexes in *R. longicauda*. Similarly, the sexual conflict described in Chapter 2 is likely to be reflected in the genital morphology and reproductive tract in *R. longicauda*. This is particularly true because female ornaments are predicted to enable a female to increase her mating rate in order to receive more nuptial gifts (Chapter 5). Sexual conflict over mating rate has been shown to result in rapid coevolutionary changes in the reproductive morphology of males and females (Rowe & Arnqvist, 2011).

Mating system diversity has been shown to covary with sexual selection on genital morphology. For example, genital shape divergence and complexity is related to increased polyandry in insects (Arnqvist, 1998; Kuntner et al., 2009; Rowe & Arnqvist, 2011) and correlated evolution in male and female genital complexity has been observed across related species (Tatarnic & Cassis, 2010).

The dance flies, like most insects, display remarkable diversity in genital structures that are frequently used in identification (Collin, 1961). It would be interesting to relate measures of mating system diversity in the dance flies, such as female ornamentation and species-level polyandry estimates, to relative genital divergence and complexity. Previously, measures comparing between genital and non-genital morphology showed that relative divergence should be higher in species with increased polyandry (Arnqvist, 1998). However, sexual selection has been shown to differentially impact genital structures depending on whether they are intromittent or non-intromittent (Rowe & Arnqvist, 2011). Therefore, there is potential for variation in genital morphology across dance fly species to quantify the intensity of pre- and post-copulatory sexual selection related to other mating system traits.

We could measure the strength, (and potentially the direction), of sexual conflict by testing for correlated evolution in male and female reproductive structures across species (Tatarnic & Cassis, 2010). If males and females are engaged in a coevolutionary arms race over mating rates, then we might expect to see highly correlated genital divergence, as traits in one sex evolve to manipulate, and traits in the other sex evolve quickly to resist those manipulations (Holland & Rice, 1998). Because my work and other studies (Funk & Tallamy, 2000; LeBas & Hockham, 2005) support a role for sexual deception during attraction and pairing, one might predict genital structures to help partners secure (in the case of deceptive partners) or escape from (in the case of deceived partners) copulations after initial contact. Therefore, we would expect to see an increase in genital complexity and relative divergence (compared to non-genital traits), for species that experience stronger sexual selection (Rowe & Arnqvist, 2011), such as those species engaged in a sexual conflict arms race. Of particular interest in the dance flies, would be determining the direction that sexual conflict was operating in. However, while we would likely be able to identify candidate species (from highly correlated, highly complex genitalia) that were engaged in a sexual conflict arms race, it might be difficult to identify the direction of sexual conflict (i.e. which sex is manipulating vs. resisting). Given that we observe sexual conflict acting in the opposite direction from that described in conventional models (Holland & Rice, 1998) (i.e. *R. longicauda* showed exaggeration of female traits and males resistance; Chapter 2), it is possible that female-mediated increases in polyandry are again leading to manipulative traits in females and resistance traits in males.

Conclusion

Mating system diversity and mating behaviour in the dance flies has been under investigation for more than 100 years (Hamm, 1908; Hamm, 1909). The highly diverse, and charismatic mating behaviour of this group has attracted attention from natural historians since the turn of the century. Several species have been singled out and used as model systems in which to investigate diverse questions about behavioural ecology, natural history and the evolution of alternative mating strategies (Svensson & Petersson, 1987; Svensson, 1997; Funk & Tallamy, 2000; Gwynne & Bussière, 2002; Bussière et al., 2008). Future studies building on the work presented in this thesis would provide further insights into the strength of sexual selection, mate choice and relative importance of sexual conflict in driving changes in mating systems specifically related to female-specific ornament evolution. I hope that by initiating the first comparative analysis investigating female-specific ornament evolution in Empidinae dance flies my thesis, and the publications that arise from it, will invigorate and broaden the past century of research on a fascinating group of insects.

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