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The role of polyandry in sexual selection among dance flies

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General abstract

Elaborate sexual ornaments evolve because mate choice exerts strong sexual selection favouring individuals with high levels of ornament expression. Consequently, even at evolutionary equilibrium, life history theory predicts that ornamental traits should be under directional sexual selection that opposes contrasting selection to reduce the costs associated with their maintenance. Otherwise, the resources used to maintain ornaments should be used to improve other life history functions. Elaborate female ornaments have only evolved in a few species, despite females commonly experiencing strong sexual selection. One explanation for this rarity is that male preferences for female ornaments may be self-limiting: females with higher mating success become less attractive because of the lower paternity share they provide to mates with every additional sperm competitor. The unusual species in which female ornaments do occur can provide rare insight into how selection can favour the expression of expensive characters in females despite their costs. The main goal of my thesis was to determine how sexual selection acts on exaggerated sexual ornaments, and give new insight into how these ornaments may have evolved, in spite of the self-limiting nature of selection on male preferences.

To determine the strength of sexual selection acting on female ornamentation in dance flies, we developed new microsatellite markers to assess polyandry rates by genotyping stored sperm in wild female dance flies. We first used polyandry rates to determine whether ornament expression was associated with higher mating success in female *Rhamphomyia longicauda*, a species that has evolved two distinct and exaggerated female ornaments. Contrary to our predictions, we found no evidence that females with larger ornaments enjoy higher mating success. We then compared polyandry rates in *R. longicauda* to those of two other species of dance fly, one (*Empis aestiva*) that has

independently evolved female ornaments on its legs, and another (*E. tessellata*) that does not possess any discernable female ornaments. We also estimated the opportunity for sexual selection, which we found to be similar and relatively low in all three species. Moreover, the standardized sexual selection gradients for ornaments were weak and non-significant in all three species. Females with more elaborate ornaments, in both within- and cross-species comparisons, therefore did not enjoy higher mating success. Overall, these results suggested that sexual selection operates rather differently in females compared to males, potentially explaining the general rarity of female ornaments.

Our amplifications of stored sperm were able to reveal more than just mate numbers. We developed new methods to study patterns of sperm storage in wild female dance flies. We investigated how the skew in sperm genotypes from mixed sperm stores changed with varying levels of polyandry. Our data suggested that sperm stores were dominated by a single male in *R. longicauda*, and that the proportion of sperm contributed by this dominant male was largely independent of the number of rival males' sperm present in the spermatheca. These results were consistent with the expectation of males using sperm 'offence strategies' in sperm competition and that the most successful male is likely to be the female's last partner before oviposition.

As a whole, my thesis contributed new molecular resources for an understudied and fascinating group of organisms. It exploited these new resources to provide the first estimates of lifetime mating success in several related species, and suggested that the general prediction that ornament expression should covary with sexual selection intensity does not seem to hold in this group. Instead, both the unusual prevalence of

ornaments and the inconsistent evidence for sexual selection that sustains them in dance flies may owe their existence to the confluence of two important factors. First, the conditions under which sperm competition occurs: as last male precedence is likely, males are selected to prefer the most gravid females to secure a high fraction of her offspring's paternity as they are unlikely to mate again before oviposition. Second, potent sexually antagonistic coevolution between hungry females and discerning males: females have evolved ornaments to disguise their stage of egg maturity to receive the benefits of nuptial gifts, while males face the challenge of distinguishing between gravidity and ornamentation in females.

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Chapter 1

General introduction

Natural selection favours heritable traits that serve to increase an individual's fitness(Darwin, 1859; Fisher, 1930). Nevertheless, even traits which covary strongly with fitness, such as body size, immune responses, fertility and sexual ornaments, exhibit profound diversity within and between species. Evolutionary biologists strive to explain the mechanisms that create and maintain such enormous diversity in trait expression. This is often accomplished using experimental studies, which can tease apart alternative evolutionary explanations for patterns in trait expression. At the same time, we also recognize the limitations on much laboratory research, which often cannot reflect the conditions experienced by wild populations. If we are to provide a comprehensive account for the amazing diversity of organisms, we need to incorporate measures of the form and magnitude of selection on phenotypic traits under natural conditions.

Among the most diverse and spectacular phenotypic traits are sexual ornaments, which have evolved in many taxa in order to attract mates. In species in which sexual ornaments have evolved, they are usually borne by males, and males with the largest ornaments generally achieve the highest mating success (Darwin, 1871; Andersson, 1994). The female preferences that lead to high mating success for these males are generally sustained because females tend to have higher fitness when pairing with those males (Andersson, 1982). In some species, however, it is the females that possess ornaments. In those cases, the benefits to males for preferring ornamented mates are less clear. Males do not necessarily gain from mating with heavily adorned females, since female ornaments could require resources that would otherwise be invested in a male's offspring (Fitzpatrick *et al.*, 1995). In addition, males who mate with attractive females are likely to face greater sperm competition from other males (Simmons, 2001). It therefore remains unclear how sexual selection acts on female ornaments and the male preferences that are presumed to engender them.

Polyandry

In many animal taxa, males are selected to mate frequently because their fitness depends more directly on mating success than that of females. This sex difference in the nature of selection on mating frequency is driven by parental investment, starting with unequal investment in gametes, known as anisogamy (Bateman, 1948; Trivers, 1972). Because males produce numerous small sperm and females produce fewer large eggs, female fitness (unlike that of males) is rarely limited by access to mates, but instead depends on factors that constrain the number of offspring they can produce. Consequently, most females can maximize their fitness after just one or two matings (Bateman, 1948). With such apparently diminishing returns on mating, why do females

in some groups, for example the insects, often mate more often than they would need in order to simply secure male gametes?

The processes that explain variation in rates of polyandry (multiple mating by females) across species are not fully understood. Part of the difficulty is undoubtedly because some female remating is not specifically because of advantages to females, but rather because selection on males is so strong, and resisting male advances can be costly (Arnqvist & Nilsson, 2000). Some instances of polyandry therefore represent correlated responses in females to strong selection on male multiple mating (Kokko & Jennions, 2003): because every male mating must inevitably involve a female, the average mating frequencies of males and females must be equal as long as the adult sex ratio is not skewed. In spite of this requirement, the amount of variation in mating frequency among individuals within a sex can differ dramatically between the two sexes (i.e., there is often much higher variation in male mating success than among females (Bateman, 1948). Such a scenario is most likely when the optimal mating rates of the two sexes differ, which makes the rarer sex (and the rarer sex's gametes) a valuable commodity over which there can be intense competition (Emlen & Oring, 1977).

While polyandry may sometimes arise thanks to selection on males, there are several situations that can favour heightened female mating rates in addition to correlated selection. These adaptive explanations for polyandry include both indirect and direct benefits of mating more than once. Indirect benefits are achieved not by females directly, but rather through a female's offspring. These typically fall into two broad categories of genetic benefits: 'good genes' and 'sexy genes' (Yasui, 1998). Good genes are general viability genes passed from fathers to their offspring, and polyandry

can potentially facilitate their acquisition if females trade-up (mating again when they encounter a male of higher genetic quality than previously acquired) or if gamete fusion is more likely when two gametes are genetically compatible (Curtsinger, 1991; Watson, 1991; Keller & Reeve, 1995; Zeh & Zeh, 1996, 1997, Yasui, 1997, 1998). Sexy genes are indirect benefits that provide an advantage in sexual contests, and so are mainly accrued by their expression in a female's sons (Curtsinger, 1991; Keller & Reeve, 1995). As an example, any genetic variants that specifically enhance a male's ability in sperm competition should only be expressed by males, and therefore can only provide an indirect benefit to females (García-González & Simmons, 2005). In such cases, however, high levels of polyandry could be particularly beneficial to females by encouraging intense post-copulatory contests that favour particularly highly performing sperm (García-González & Simmons, 2007). In contrast, direct benefits are so-categorized because they improve the fitness of a female herself, for example because mating can increase a female's fecundity, her survival, or reduce predation risk. The mechanisms that elicit direct benefits are varied, but include nutritional 'nuptial gifts' provided by males during courtship or mating (Gwynne, 1988); high quality territories that a female gains access to when paired with certain males (Howard, 1978; Pleszczynska, 1978); defence against predators provided by males or the habitats males monopolize (e.g., a burrow in which to hide) (Searcy, 1979), and even antipredator compounds the males pass to the females (González *et al.*, 1999).

In contrast to the scarce evidence that polyandry is promoted by indirect benefits (Zeh & Zeh, 1996; Jones *et al.*, 1998; McNamara *et al.*, 2014), there are many examples that suggest females mate multiple times to acquire direct benefits. In species with clear direct benefits associated with mating, there can be intense contests between females

for the valuable goods and services acquired during mating (for example, females in many species can compete for nutritious nuptial gifts provided by males (Gwynne, 1988, 2001). In fact, environmental food shortages can even alter mating roles by changing the value of male nutritional contributions, and initiating intrasexual contests among females and choice by males when food resources are scarce (Gwynne & Simmons, 1990).

Female ornaments

Sexual selection on females is reasonably common (Clutton-Brock, 2007), but examples of sex-specific female ornaments or displays are scarce when compared to males (Amundsen, 2000; Funk & Tallamy, 2000; Amundsen & Forsgren, 2001; Emlen & Wrege, 2004). This is true even for species exhibiting unconventional sex-roles, in which females compete for access to males, whereas males are choosy. The prevailing explanation is that costly female ornaments require a diversion of resources away from eggs, and therefore undermine the presumed benefits of male mate choice: all else being equal, a female with elaborate costly ornaments should be less fecund (and therefore less preferred) relative to a rival who invests less in ornaments and retains more resources for eggs (Berglund, 1994; Fitzpatrick *et al.*, 1995; LeBas *et al.*, 2003). The few taxa in which female ornaments do exist therefore present a unique opportunity to study how trade-offs in life history investment affect the origin and maintenance of costly secondary sexual characters.

Study system - Dance flies

Dance flies of the subfamily Empidinae (Diptera: Empididae) are well known for their interesting mating biology. Mating generally occurs in aerial swarms, and males

typically provide females with a nutritious nuptial gift during mating (Cumming, 1994). Often these swarms form at landmarks, such as trees, and remain in the same location throughout the adult flight period. Swarms have even been found to persist at the same location over multiple years (Svensson & Petersson, 2000).

Dance flies have highly diverse mating systems; with females of closely related species showing strikingly varied levels of sexually selected ornamentation (Collin, 1961; Cumming, 1994). Female ornamentation is common within the genera *Empis* and *Rhamphomyia* (Cumming, 1994) and includes enlarged and/or darkened wings, pennate scales on legs and inflatable abdominal sacs, all of which are absent in the males (figure 1.1). The adaptive significance of interspecific variation in ornamentation is unknown, but one possibility is that it relates to the loss of the ability of females to hunt and their subsequent reliance on males for protein-rich food items which enable females to mature their eggs (Downes, 1970; Cumming, 1994) (figure 1.2). If nuptial-gift resources are rare, and females exploit mating opportunities to feed, males may become the limiting sex (Cumming, 1994). The ratio of sexually receptive males and females, known as the operation sex ratio (OSR) can vary both within and between species, both spatially and temporally over the course of an individual flight period (Funk & Tallamy, 2000; Svensson & Petersson, 2000; Murray, 2015) (figure 1.3). When males are in short supply they are expected to become 'choosy' about which females earn the valuable nuptial gifts they have to offer. Males of some species appear to have exploited a female's willingness to mate in exchange for food by cheating females with token gifts of no direct value to females, including leaf fragments, twigs, seeds or silk balloons (Kessel, 1955; Cumming, 1994; Preston-Mafham, 1999). In theory, males should focus their choice on finding particularly fecund or gravid females (since those

females might provide especially high paternity shares). Females, in turn, might therefore face selection (imposed by male choice) to exaggerate their fecundity or gravidity (Funk & Tallamy, 2000), in a bid to win the food resources that are the main prize in contests for access to male dance flies (Downes, 1970; Cumming, 1994) .

Figure 1.1. Female-specific ornamentation in *Rhamphomyia longicauda*. Females possess pennate leg scales on hind, mid and front legs as well as inflatable abdominal sacs, all of which are entirely absent in males. Photo by Heather Proctor.

Figure 1.2. Nuptial feeding in *Empis tessellata*. The male (top) holds onto vegetation and the female (middle) during copulation while she feeds of a prey-item (lower) nuptial gift. Photo by Tom Houslay.

Figure 1.3. Female-biased mating swarm in *Rhamphomyia longicauda*. Females can be seen displaying inflated abdominal sacs and pennate leg scales, while a substantially smaller single male (that lacks the inflatable abdominal sacs) can be seen just out of focus in the centre holding a prey-item nuptial gift. Photo by John Alcock.

Sexual selection in female dance flies

The evolution of exaggerated female ornaments appears to have occurred multiple times throughout the dance fly lineage (Murray, 2015). Among the species that do exhibit female ornamentation, the form and magnitude of sexual selection on female ornaments seems not to be consistent. Previous studies measuring sexual selection on pennate leg scales in the species *R. tarsata* found leg scales to be under escalating sexual selection. In this species, ornament expression covaried with egg number and size, suggesting that ornaments may honestly signal female fecundity to choosing males (LeBas *et al.*, 2003). In the species *R. longicauda*, females possess inflatable abdominal sacs in addition to pennate leg scales, which might conceivably exaggerate female

fecundity and be preferred by males. Two experimental studies on this species tested male preferences by suspending plastic model females of varying sizes within the mating swarm, and found that males were more attracted to larger silhouettes (Funk & Tallamy, 2000; Murray, 2015). However, a cross-sectional selection analysis of *R. longicauda* that assessed the role of ornaments on mating success found no support for directional selection on ornamentation (Wheeler *et al.*, 2012). Together, these results suggest that while larger ornaments may serve to initially attract males, upon closer inspection males apparently do not prefer to mate with more ornamented females. However, another possibility is that this cross-sectional study missed an important dimension of sexual selection by measuring only instantaneous mating success rather than lifetime mate number. Evaluating this alternative will require the creative use of new molecular resources, because dance flies do not culture well in the laboratory, and field populations are too large to effectively monitor wild individuals for long periods of time.

Research objectives

The main objective of my thesis was to quantify how sexual selection acts on exaggerated sexual ornaments, and thereby provide new insight into how these ornaments may have evolved in spite of the expectation that males should not prefer females that advertise high mating success. To achieve this aim, we developed new molecular tools in order to assess polyandry rates and patterns of sperm storage in wild female dance flies. The detailed objectives of each chapter in my thesis are outlined below.

Chapter 2: The sexes usually differ in their behaviour and morphology in predictable ways: males typically compete for mates and females are often choosy. However, these sex differences, known as the 'sex roles', can vary substantially among and within species. In this chapter, we summarize the main characters that comprise sex roles, review the evolutionary causes of general patterns of sex differences, and explore how systems that deviate from these patterns can provide strong tests of sexual selection theory.

Chapter 3: Documenting polyandry rates is difficult in wild systems, and this is especially true for flying insects with large population sizes, as tracking individual females and using behavioural observations to record each mating event is not feasible. In chapter 3, our aim was to develop microsatellite markers that would allow genotyping of DNA extracted from mixed sperm stores in wild female dance flies of several species. Although we initially attempted to develop consensus primers that would work for many different species, technical obstacles enforced an alternative approach involving the design of specific primers in a smaller number of taxa.

Chapter 4: Life history theory predicts that elaborate ornamental traits should be under strong directional sexual selection because of costs associated with their maintenance and potential trade-offs with other life history traits. These costs may be particularly acute for female ornaments, which may help explain why so few species have them, even when females experience strong sexual selection. In the species *R. longicauda*, females possess two elaborate ornamental traits, but previous work has suggested contrasting relationships between ornaments and mating success depending on the methodological approach. In this chapter, our aim was to document natural

polyandry rates and test for an association between ornament expression and lifetime mating success in wild female *R. longicauda*. In addition we sought to consider the effect of nuptial feeding on egg size or egg number. We expected high rates of polyandry in this system, and that females with larger ornaments might gain higher mating success. We also expected female egg traits to be positively associated with mating frequency.

Chapter 5: In this chapter, we sought to comparatively test the hypothesis that more exaggerated sexual ornaments should usually be under stronger directional sexual selection, by assessing the strength of sexual selection in females of three species of dance fly that varied continuously in their expression of ornamentation. We combined the data from *R. longicauda* collected in chapter 4 with new data on two other species: *Empis aestiva*, which have independently evolved pennate leg scales, and *E. tessellata*, which does not exhibit discernible female ornamentation. We then computed polyandry rates, the opportunity for sexual selection and the standardized sexual selection gradients in all three species. Based on the prediction that stronger selection is required to create more exaggerated ornaments, we expected to find the highest polyandry rates and the strongest sexual selection in the most ornamented species, *R. longicauda*, and the weakest sexual selection in its unadorned relative, *E. tessellata*.

Chapter 6: In systems where females mate multiply and store sperm of multiple males, the benefits of male choice will be mediated by sperm competition. To reduce sperm competition intensity, males may deploy offensive or defensive strategies. We developed new methods in order to study patterns of sperm storage and sperm competition adaptations in wild caught females dance flies. We aimed to study how the

relative contributions of males to mixed sperm stores changed with varying levels of polyandry. We expected that male choice for gravid females may have allowed deceptive female ornaments to evolve across dance flies, but only if the last male to mate with a female had the highest fertilisation success (i.e., if males employed strong offensive strategies to displace previous rivals' sperm). We consequently expected to see high skew in stored sperm genotypes, which was insensitive to the number of rival sperm competitors: both of these features would be consistent with the operation of strong sperm offence traits.

Chapter 2

Diversity in mating and parental sex roles

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Synopsis:

The sexes usually differ in their behaviour and morphology in predictable ways: males typically compete for mates and females are often choosy. However, these sex differences, known as the ‘sex roles’, can vary substantially among and within species. We summarize the main characters that comprise sex roles, review the evolutionary causes of general patterns of sex differences, and explore how systems that deviate from these patterns can provide strong tests of sexual selection theory.

Glossary	
Anisogamy	Unequal investment in gametes between the sexes.
Armaments	Morphological characters used to gain an advantage over rivals in contests for access to mates.
Bateman gradient	The slope of the regression of reproductive success on mate number for a given sex.
Direct benefits (material benefits)	Processes that directly improve the fitness of an animal as a result of an additional mating event or a particular mate choice; direct benefits include nutritional resources provided by a mating partner, access to a territory or refuge, or parental care.
Indirect benefits (genetic benefits)	Processes that improve the genetic quality of an animal's offspring (either their viability, attractiveness, or diversity) as a result of an additional mating event or a particular choice of mate.
Mate choice	A preference for some phenotypic classes of mate over others.
Monogamy	A mating system in which an individual mates with only one partner.
Ornament	A morphological character used to attract the opposite sex during episodes of mate choice.
Operational sex ratio	The relative number of sexually receptive males to females; usually expressed as a proportion (sexually receptive males

	/(sexually receptive males + sexually receptive females)).
Parental investment	Investment in an offspring at a cost of investing in other components of parental fitness.
Parental care	Parental investment that increases an offspring's chance of survival after fertilisation.
Polyandry	A mating system in which females mate with multiple partners.
Sex role reversal	A controversial designation of deviation from the conventional sex roles (see Box 1).
Sex roles	Aspects of mating receptivity, mate choice and parental care that generally differ between males and females; typically masculine sex roles involve high levels of sexual receptivity and low levels of choice and care, while feminine sex roles are less receptive to mating, and have high levels of choice and care.
Social selection	Differences in survival or reproductive success linked to variation in success in social competition for resources; sexual selection is a subset of social selection, in the form of social competition for mates.

Introduction

The sexes usually differ in morphology and behaviour, but the degree of difference varies dramatically across species. Males typically compete for access to female mates, whereas females rarely compete for males. Instead, females tend to invest considerably

more in offspring and choosing mates than males do. The sex difference in contest intensity over mates has selected for weapons and extravagant ornaments in males of many species, while females are rarely armed or adorned in this way. General differences in mating receptivity, mate choice and parental care are collectively known as ***sex roles*** (for definitions of terms in ***bold italics***, see Glossary), and species that deviate markedly from the general patterns (e.g., when females compete for mates, and males are choosy or provide care), are often described as ***sex role reversed*** (a controversial designation; see Box 1).

Box 1: Sex Role Reversal

Ah-King and Ahnesjö (2013) critique the use of the term "sex role reversal" in part because it reduces variation in behaviour and morphology into two discrete categories, which obscures the tremendous variation in natural sex roles (including variation within species across different traits and ecological contexts – see section on Variation in Mating and Parental Roles). These problems have particular resonance for an evolutionary perspective that is motivated to explain diversity. The phrase is unlikely to disappear entirely, in part because its concise form is useful for instantly evoking in an audience something unconventional about a focal mating system. Nevertheless, we agree that it evokes different ideas in different audience members, and therefore endorse Ah-King and Ahnesjö's (2013) recommendation that authors should provide operational descriptions of the specific phenotypic features that are being referred to in any focal case.

Here, we focus primarily on animals with separate sexes (sexual selection theory has fascinating implications for hermaphroditic organisms, but these are beyond the scope of our article). We will explore the causes of sexual differences in both mating and parental behaviour, and illustrate how some unusual systems deviate from conventional sex roles. We also highlight how many of these unusual systems provide strong tests of sexual selection theory, and suggest some directions for future work that may help clarify unresolved questions about the diversity of sex roles among animals.

What causes sexual differences in mating and parental roles?

There are behavioural and morphological traits that distinguish the sexes in many animal species. Typical sex differences are thought to have evolved because the sexes experience disparate forms and intensities of selection. For most species this sex difference in selection is ultimately a consequence of unequal ***parental investment*** in gametes (***anisogamy***); spermatozoa in males are small and relatively plentiful while eggs in females are large and relatively few (Bateman, 1948; Trivers, 1972; Williams, 1975). Females are typically constrained from producing more eggs because of the substantial cost of each of them, and this constraint has important consequences for sex differences in both mating and parental behaviour.

Mating roles

One consequence of greater female parental investment is that males can potentially produce many more offspring than females by parasitizing the substantial investment in gametes of many mates. A male's fitness is therefore often directly related to his ability to secure mates. By contrast, females sometimes gain little fitness by remating,

especially if a male's only contribution during mating is sperm. The fact that males can gain much from remating, while females gain relatively little, means that selection for acquiring mates tends to be stronger on males. This contrast in how mating success covaries with fitness is central to sexual selection theory. The empirical measurement of the regression of fitness on mating success is known as the ***Bateman gradient***, acknowledging Angus Bateman's (1948) work on *Drosophila* that first highlighted the sex differences in relative fitness gains from mating (figure 2.1).

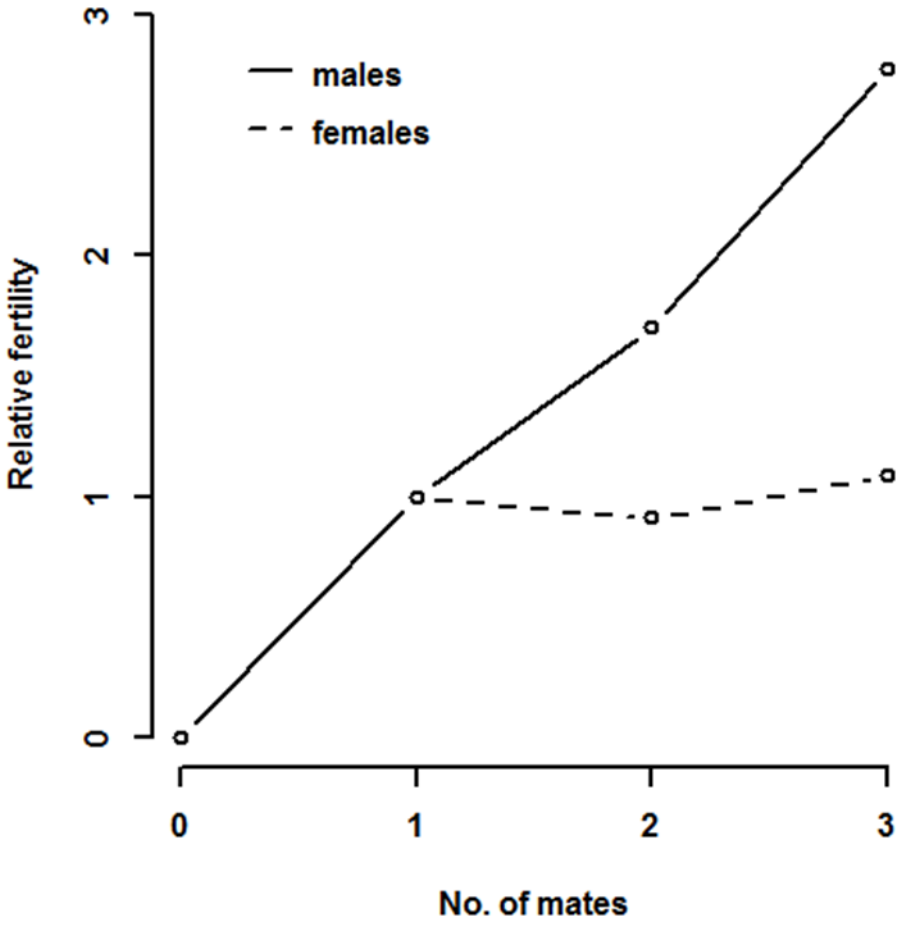

Figure 2.1. Bateman's (1948) *Drosophila* experiments first highlighted that the relationship between fitness (measured as 'relative fertility') and mating success differed between the sexes, with males gaining more fitness by remating than females. Here we reproduce his most famous figure of series 5 and 6 combined. Males are represented by a solid line, and females are represented by a dashed line. Adapted from Bateman, A.J. 1948. Intra-sexual selection in *Drosophila*. *Heredity* 2: 349–368, with permission Macmillan Publishers Ltd.

Although males may gain more fitness by remating than females, the two sexes must on average mate equally frequently, because every mating requires an individual of each sex. Consequently, there are often many more sexually receptive males than females (if female fitness does not covary strongly with mating success, females should prefer to spend most of their time in activities other than mate seeking, such as foraging or caring for young). This resulting bias in the operational sex ratio (Emlen and Oring, 1977) often selects for investment in secondary sexual characters in males (Enders, 1993; Jirotkul, 1999) that help them to find, win, and guard mates from current rivals, and to displace ejaculates of previous rivals stored within females; such contests are responsible for the impressive array of male ***armaments***, fighting (Emlen, 2008) and, during copulation, penile traits that remove sperm (Simmons, 2001). When there is an excess in the number of sexually receptive males, females typically improve their reproductive success by carefully selecting from many willing potential partners (***mate choice***) rather than by mating more frequently. The preferred mate can improve a female's fitness in several ways (Jennions & Petrie, 1997). The careful attention of choosy females in turn selects for the expression of numerous ***ornaments*** that appeal to discriminating females (Houde, 2001).

Parental roles

In addition to the general sex differences in mating roles described above, there are also typical parental roles involving the provision of post-zygotic care: in most animals the female invests more than the male in ***parental care***. The explanation rests in both the costs and benefits of deserting (i.e., failing to care for) the current brood (Trivers, 1972). The considerable investment required by females to produce ova prior to mating means that a female who abandons her current brood (e.g., to seek a better mate) may be unable to replace the brood using her metabolic reserves: she may be better off caring for the current brood

(even if she cares alone, and even if the offspring are of below-average quality) than trying to start all over again with a new partner. In contrast, males can usually easily start over with another mate, because the cost of the initial contribution to mating is so much less, and therefore more easily reproduced from metabolic reserves.

Moreover, relative to females, males can usually gain substantially by deserting and acquiring further mates. First, males typically gain more by deserting due to an inherent risk of cuckoldry (where an individual provides care in another individual's offspring); male confidence of parentage is lower than that of females (especially in species with internal fertilization). With few exceptions, females have strong confidence that the offspring they invest in are their own. As the risk of cuckoldry increases, so should selection favouring male abandonment of offspring in favour of new mating opportunities, such as in the brood-rearing bluegill sunfish (*Lepomis macrochirus*) (Neff & Gross, 2001; but see Alonzo & Klug, 2012 for discussion). In addition, investing time and effort in male care comes at a cost to other opportunities to gain fitness, such as by pursuing further mates.

Variation in mating and parental roles

There are many exceptions to the typical patterns of sex differences in behaviour and morphology that we have described. Exceptional taxa showing unusual mating and/or parental roles provide fascinating examples of the diversity of natural systems. These exceptions have also provided strong tests of the putative causes of sexual differences highlighted above. Although most of the species mentioned below feature rather dramatic departures from the conventional sex roles described above, we note that sexual selection theory predicts (and empirical research supports) continuous variation in mating and parental behaviour, with many taxa occupying the middle ground between the conventional

stereotypes above and the departures listed below (Jones & Hunter, 1999; South & Arnqvist, 2008; South *et al.*, 2009).

Box 2

When males limit the reproductive success of females, females can compete with each other for males. Gwynne and Simmons used an experimental approach to illustrate how environmental food availability altered the mating roles for *Kawanaphila nartee* bushcrickets (Gwynne & Simmons, 1990). Like many bushcrickets (Orthoptera: Tettigoniidae), *K. nartee* males provide nutritious spermatophylax gifts attached to their sperm packets (figure 2.2). In *K. nartee*, these gifts comprise 10 percent of a male's weight and take 5 days to produce (Vahed, 2007). There is a reversal in the mating roles in nature when hungry females compete sexually for matings (male gifts). In experiments with *Kawanaphila* and other tettigoniid species, decreasing proteinaceous food causes a relative increase in male parental investment (gift nutrients in eggs increases), decreases the number of males able to mate (produce gifts) and (in field enclosures) causes a reversal in the mating roles by greatly increasing male choice and female-female competition for mates (Gwynne, 2001). Furthermore, there was even evidence of context-dependent sexual selection on female morphology: the heaviest females had a mating advantage in the control treatments but not food-supplemented treatments. Experimental role reversal also affects other aspects of life history apart from mating itself: as relative male parental investment increases, so does male immune activity, whereas sexually competing females have reduced immune function as paternal investment increases (Vincent & Gwynne, 2014).

Figure 2.2. *Kawanaphila nartee* bushcricket female eating a spermatophore gift. Photo by Darryl Gwynne.

Most studies of unusual sex roles have supported the links between relative investment by the sexes, the operational sex ratio, sexual selection and sexual differences. In a few cases there is plasticity in mating roles that allow tests of factors controlling sexual differences (Box 2). For example, in certain environments males provide large material benefits to females in the form of nutritious "nuptial gifts". The cost of providing these material donations may constrain male ability to remate, while simultaneously increasing the value of remating for females. If male investment is sufficiently important to female fitness, females may be under so much selection to acquire male donations that the operational sex ratio becomes female-biased. Males in turn may then become more choosy than females about their mates (see Box 2). Unusual mating roles are not always directly driven by parental investment, however: in one exceptional case (in a butterfly) a female bias in mate availability is caused by extremely

elevated male mortality (due to *Wolbachia* infection), which skews the sex ratio to such a degree that females form mating leks (Jiggins *et al.*, 2000).

Polyandrous females

As we have seen, the relationship between mate number and fitness is usually strongest in males, whereas females often gain much less by remating (Bateman, 1948). The explanation for this sex difference lies both in the costs and the benefits of remating for females. Female mating costs include mate search and mate-assessment costs. Mating may also heighten the risk of predation (Arnqvist, 1989), or decrease lifespan, for example due to injuries sustained during coupling (Crudgington & Siva-Jothy, 2000) or to damaging effects of seminal fluid (Chapman *et al.*, 1995). While males may also suffer costs such as mate searching, female costs appear to be generally greater. For example, females rarely transfer secretions to males that are costly to the partner's reproductive physiology (female Zeus bugs produce a glandular secretion thought to reduce costs of kleptoparasitism by males, but whether this secretion decreases or improves male fitness is unclear; see Arnqvist, Jones and Elgar, 2006).

Given these costs, what do females gain by remating? Most empirical studies point to *"**direct benefits**"* of multiple mating, to replenish sperm supplies, acquire goods and services from males, or to avoid the costs of resisting harassment in male mating attempts. In contrast, there is substantially less evidence for *"**indirect benefits**"*, including the acquisition of genetically superior or more compatible sperm, or more diverse offspring (Arnqvist and Nilsson, 2000); Slatyer et al., 2012). The benefits of multiple mating for males always involve the direct benefits of fertilizing female ova. Consequently, even when sexually receptive females outnumber males, the covariance between male mating success and fitness will usually be

positive. In such cases, male fitness may be constrained more by other aspects of their biology than by access to mates (e.g., if each mating requires substantial male investment).

Armed and ornamented females

When females are so eager to mate that they outnumber sexually receptive males, we expect females to compete sexually, but even in cases of unusual mating roles, females rarely use weaponry to compete or ornaments to attract mates. Examples of female armaments that function in mate competition are virtually unknown. One explanation is that the cost of expressing weaponry includes structural and metabolic costs as well as the risk of injury during fights; any substantial investment in weaponry could therefore come at a direct cost to a female's own fecundity, and undermine the benefits of winning competition for mates (Berglund, 2013). In fact, in most examples, female armaments appear to have evolved in the context of direct competition with other females for resources rather than mates. Because female reproductive fitness is closely related to maximising their own fecundity (even in systems in which females are relatively polyandrous), females are more likely than males to compete for resources that will benefit the development and production of their offspring (Clutton-Brock, 2007, 2009). The intensity of the reproductive competition between females, and the development of secondary sexual characters, is therefore closely associated with such resource acquisition. In acknowledging the importance of female competition for material resources essential to reproduction (including competition between potential reproductives (queens) in eusocial animals), some authors have advocated developing a broader perspective of ***social selection*** (which emphasizes competition for all resources, not just mates, in social interactions) to facilitate comparisons of secondary sexual traits that arose in differing contexts (West-Eberhard, 1979; Tobias, Montgomerie and Lyon, 2012; but see discussion by Shuker, 2010 and Clutton-Brock and Huchard, 2013).

Examples of female ornaments that function in attracting mates crop up in fishes, birds, and some insects (Tobias *et al.*, 2012). As with female weapons, investment in ornaments may come at a direct cost to a female's own fecundity, which may explain their rarity in spite of the fact that sexual selection on females is relatively common (Bonduriansky, 2001). Ornament evolution is also constrained because it is mediated by male preferences. Any potential trade-off between investment in ornaments and offspring is unlikely to be favoured by choosy males, who would presumably prefer an unadorned but highly fecund mate (Fitzpatrick *et al.*, 1995). Furthermore, selection for mate attraction by females could be self-limiting in another respect: seductive females who attract more mates probably provide smaller paternity shares for focal males than relatively unpopular rivals that offer a lower risk or intensity of sperm competition (Wheeler *et al.*, 2012).

Choosy males

Male choice is generally constrained by the opportunity cost of mate searching and assessment: if males can best gain fitness by acquiring additional mates instead of choosing among them, then choice seems unlikely to evolve. However, if sexually receptive females outnumber receptive males, female quality is variable (e.g., if many receptive females are not yet gravid) and males invest heavily in each mating, males tend to favour traits in females that directly increase their fertilisation success (Bonduriansky, 2001). In taxa where female egg number varies substantially (e.g. invertebrates and fish), males generally prefer traits that signal high fecundity, such as large body size. For taxa where females have less variable fecundity (e.g. mammals and birds), males instead tend to focus on traits that signal reduced sperm competition, such as female mating status or age (Bonduriansky, 2001).

Caring males

For male care to evolve, the benefit to the fitness of his offspring should be greater than the cost incurred by lost mating opportunities (Clutton-Brock, 1991). Relative parental investment is central to sexual selection theory because investment in offspring is both the ultimate cause of sex differences and one of its consequences (by shaping the respective care strategies for males and females). However, discerning cause and consequence can be difficult, as Trivers (1972) first noted (but see experiments in Box 2).

Although the circumstances described in the section on parental roles, above, suggest general sex differences in the likelihood of investing in care, male care is not uncommon. It may be that low certainty of paternity limits care, or alternatively care can enhance paternity (Kvarnemo, 2006).Whatever the cause of the association, high paternity confidence should usually be assured in species with male care (Smith, 1979; Møller & Birkhead, 1993). For example, in the water bug, *Abedus herberti,* males brood eggs that gravid females oviposit on their backs. Although females of this species can store sperm, males reduce the risk associated with uncertain paternity by copulating frequently with the female (approximately every second egg laid) during oviposition, which can last up to two days (Smith, 1979). A second factor selecting for male care is that care *per se* attracts additional mates as in certain fishes. Thus caring males can achieve higher mating success than non-caring males (Tallamy, 2000). This is more likely to enhance male than female fitness, because reproductive rate is less limited in males (Smiseth, 2014). In some systems, such female preferences lead to competition between females for males that can provide the best care (Petrie, 1983; Owens *et al.*, 1994).

Figure 2.3. Female-specific armaments in *Onthophagus sagittarius*. Females are armoured with a pronotal horn absent in males. Photo by Doug Emlen.

Unresolved questions about sex roles

The detailed causes of differences in sexual selection are not yet clear. The complex relationships between investment costs and mating opportunities make distinguishing between causes and effects difficult. Empirical tests (Gwynne & Simmons, 1990) have shown that varying parental investment can reverse mating roles. However, determining general drivers of patterns across diverse species is complicated (Borg *et al.*, 2002) because we still do not know the extent to which differences in parental care are a cause of the general sex role syndromes (the fact that males can avoid care may be what allows them to maximize fitness by mating repeatedly), or rather an ultimate consequence of differences in sexual selection (males may generally avoid care because they gain more by pursuing more mates than by improving the fitness of their existing offspring) (Kokko & Jennions, 2008). Similarly, there is ongoing controversy concerning whether the ratio of sexually receptive males and females determines sexual trait expression by controlling sexual selection

intensity, or whether it emerges as a consequence of differences in sexual selection intensity (Kokko *et al.*, 2014).

These questions are compounded by ongoing debates about how to compare the conditions that affect mating and parental roles across sexes and species. For example, Kokko, Klug and Jennions (2014) suggest that while operational sex ratios and Bateman gradients typically covary, they can provide different but complementary information on fitness benefits of investing in secondary sexual characters. For example, if obtaining new mates becomes more difficult for males, and fitness benefits of mate seeking decrease, there may be selection for paternal care, which affirms Trivers' (1972) insight that parental investment is both a cause and a consequence of differences in selection on the sexes. There have been similar debates about the best metrics for sexual selection intensity between the sexes and across species (measuring selection accurately is a prerequisite for explaining diversity in mating systems). Some authors contend that metrics based on variance in reproductive and mating success (such as the Bateman gradient and the opportunity for sexual selection (Wade, 1979) are better predictors of sex differences in behaviour and morphology across species than trait-specific measures of sexual selection (e.g. the covariance between reproductive success and body size)(Fritzsche & Arnqvist, 2013). One reason for using measures unrelated to phenotypic traits is that traits under strong directional selection can have depleted genetic variance (Prokuda & Roff, 2014), which can lead to lower trait-based estimates of sexual selection. Other authors have demonstrated that variance based measures are only good predictors of sexual selection intensity in very limited circumstances, such as when the potential for mate monopolization is high (Klug *et al.*, 2010; Jennions *et al.*, 2012).

Figure 2.4. Female-specific ornaments in *Rhamphomyia longicauda*. Females are adorned with pinnate scales on their legs and inflatable abdominal sacs to exaggerate their size in mating swarms. Photo by Dave Funk.

Although what ultimately causes sexual differences, and how best to measure them, remains unresolved, there are some promising research directions involving species with unusual mating systems. We have already noted how such taxa have been instrumental in testing some of the key predictions of sexual selection theory, and they promise new insights thanks to some as yet understudied aspects of their biology. For example, one of the major ongoing questions in sexual selection concerns the relative importance of direct and indirect benefits in driving the evolution of mate choice. Species in which males provide substantial nutritional investment have already been deployed to study this problem (e.g. Fedorka & Mousseau, 2002; Iyengar & Eisner, 2004). Species with unusual sex roles may also shed light on general questions concerning life history, because the life history consequences of investing in costly weapons or ornaments are rather different for males than for females (Houslay & Bussière, 2012). Although the theoretical reasons for this rarity have been compellingly documented, there still remain some unexploited opportunities to test these arguments among taxa that possess impressive female armaments and ornaments. Rare examples of such work include that on female-specific armaments in *Onthophagus sagittarius* (figure 2.3)(Simmons & Emlen, 2008) and female-specific ornaments in

Rhamphomyia longicauda (figure 2.4)(Funk & Tallamy, 2000). Such tests continue the tradition established by early work on species with unconventional mating systems of using exceptions to prove the rules.

Chapter 3

Polymorphic microsatellite loci in three species of dance fly

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Abstract

We describe polymorphic microsatellite loci successfully developed for three species of dance fly, *Rhamphomyia longicauda*, *Empis tessellata* and *Empis aestiva* using 454 whole genome shotgun sequence data. We tested 101 markers developed separately for seven dance fly species initially, and selected 27 markers from three species for further testing allelic variation in 18-24 individuals. The within-population heterozygosity for each species ranged from 0.12 to 0.889 for *R. longicauda*, 0.329 to 0.862 for *E. tessellata*, and 0.105 to 0.778 for *E. aestiva*. The number of amplifying alleles per locus ranged from 2 to 18 for *R. longicauda*, 4 to 12 for *E. tessellata*; and 2 to 4 for *E. aestiva*. We discuss the suitability of these markers for assessing mating success and sexual selection in wild female dance flies, which should help clarify why the group exhibits so much variation in mating behaviour.

Introduction

Dance flies (Diptera: Empididae: Empidinae) have diverse mating systems, and in some species females have evolved elaborate sexual ornaments, which are otherwise rare among animals. In order to study why such ornaments evolve, we need markers that might reveal the mating success of different female phenotypes, ideally from several closely related species of dance flies that vary in their mating system. We attempted to design markers with cross-species utility but the primers we designed failed to produce useful markers (See box 1). Nevertheless, we were able to exploit these attempts and below describe individually developed polymorphic microsatellite markers for three species of dance flies from 454 sequence data.

Methods & Results

We collected wild adult females from seven species of dance flies, with at least one species from each of the three genera of the subfamily Empidinae. Each species was collected from a single population: *Rhamphomyia longicauda* were collected from a natural population on the banks of the Credit River, Ontario, Canada (GPS coordinates: 43°41'11.00"N, 0.79°55'34.00"W); *Empis aestiva*, *E. tessellata*, *Hilaria maura*, *R. crassirostris*, *R. longipes* and *R. tibiella* were collected at the Scottish Centre for Ecology and the Natural Environment (SCENE), Scotland (GPS coordinates: 56°09'06.35"N, 004°38'36.20"W). SCENE is a field station situated in the Loch Lomond and the Trossachs National Park and all dance flies were collected from natural populations living in the surrounding woodland. All samples were kept frozen at -20°C prior to DNA extraction.

We extracted DNA from the heads of 10 individuals (five female and five male) of each species using the DNeasy Blood and Tissue Kit (Qiagen), and quantified DNA using a

spectrophotometer (NanoDrop, ND-1000). We pooled the genomic DNA of 10 individuals (5 female and 5 male) before sequencing. We sequenced all species on a 454 Genome Sequencer FLX with Titanium chemistry (454 Life Sciences, Roche) using partial genome (shotgun) sequencing. For the first run using on *R. longicauda* we opted for 1/8th run, while for the remaining species we opted for 1/16th run.

Using the un-enriched 454 sequence data for seven species of dance fly, we searched for repeat regions (microsatellites) that had a minimum of 40 bases either side of the repeat; these sequences had the potential for successful microsatellite primer design. From these sequences we chose potential microsatellite markers for which we designed primer pairs. We designed 101 primer sets using PRIMER3 v4.0.0 (Rozen & Skaletsky, 2000). Of these 101 markers (23 *R. longicauda*; 19 *E. tessellata*; 19 *E. aestiva*; 32 *R. crassirostris*; eight *R. longipes;* four *R. tibiella*) were initially tested for PCR amplification on six individuals from the species they were designed for. We then selected 16 *R. longicauda*, eleven *E. aestival* and nine *E. tessellata* markers that amplified at close to the correct product size and appeared polymorphic for further testing across more individuals.

The PCR reaction volumes were 2µl, with 1µl (air-dried) DNA, 1µl primer mix (forward and reverse fluoro-labelled primers at 0.2 mM) and 1µl Qiagen Mulitplex Master Mix. Reactions were multiplexed when possible. We amplified products under the following PCR conditions: For *R. longicauda*, 95 °C for 15 min, followed by 44 cycles of 94 °C for 30 s, 56 °C for 90 s, 72 °C for 90 s and finally 72 °C for 10 min; for *E. tessellata* and *E. aestiva,* 95 °C for 15 min, followed by 44 cycles of 94 °C for 30 s, 57 °C for 90 s, 72 °C for 90 s and finally 72 °C for 10 min.

Box 1. Markers designed for cross-species utility

Initially we designed markers with the potential for cross-species utility using the un-enriched 454 sequence data from seven common species. We searched the un-enriched sequence data from 7 dance fly species (*E. aestiva, E. tessellata, H. maura, R. crassirostris, R. longicauda, R. longipes, and R. tibiella)* across three genera for repeat regions (microsatellites) that had a minimum of 40 bases either side of the repeat. These sequences had the potential for successful microsatellite primer design. We found 12 'primer designable' regions that aligned in two or more species. Primers were designed using the program Primer3 and tested across 11 species. Of the 10 markers, 9 amplified in two or more species (four markers amplified across three genera and a further three markers across two genera), but unfortunately none of these was polymorphic and therefore none were useful molecular markers for studying mating success.

Using enriched sequence data

To increase the chance of obtaining conserved regions for which we could design markers, we Illumina sequenced a further four common dance fly species (*H. chorica*, *E. stercorea*, *R. nigripennis* and *E. nigripes*), this time enriching for repeat sequences. This produced a higher yield of sequences containing repeat regions to hopefully give us more potential markers with high-cross-species utility. We repeated the same method described earlier, and after aligning sequences that contained repeat regions (and >40 bases each side), we found more than 100 alignments of two or more species. From these consensus sequences, 26 markers were chosen and primers again designed for each marker using Primer3. All 26 primers were designed to have an annealing temperature of between 59 °C and 61°C, a

maximum temperature difference of 0.5°C, a maximum allowable length of a mononucleotide repeat (Max Poly-X) of 3, and when possible a GC clamp. These strict parameters for primer design were used to aid multiplex construction later.

Attempts to develop markers using enriched libraries failed twice, producing spurious results that were not polymorphic. Determining the cause of failure to produce useful markers from enriched sequence data was beyond the scope of this study. However, it is possible that some of this failure may be due partly to the fact that insects have a high proportion of microsatellites clustering into families with similar flanking regions, and an association between these microsatellite families and transposable elements (Meglécz *et al.*, 2007; Gardner *et al.*, 2011).

Table 3.1 Characterisation of 14 *Rhamphomyia longicauda* microsatellite loci in the Credit River population, Ontario, Canada. Markers were combined where possible into seven multiplex reactions.

Locus	Primer sequences and fluoro label (5'-3')		Repeat motif	Multiplex	n	Allele size range (bp)	Na	HE	HO	HWE *p* value	Est. f(null)
RL1AXKU5	F	[6-FAM]CCACGGTATACCTTAATATCCTTTG	(TA)9	4	22	203-268	18	0.818	0.879	0.4983	0.0255
	R	TTTGTACCATTAGCTCCACAGC									
RL1B4R5S	F	[6-FAM]TGATTCCTCCGGCGTATAAC	(AT)10	6	12	371-410	3	0.083	0.163	0.0460	0.4534
	R	TCGGTCCTTCGGAACAATAC									
RL1B5KYR	F	[6-FAM]ACTCAGTCAGACAAAGCACAAATC	(TA)9	5	20	199-235	7	0.65	0.762	0.1374	0.0737
	R	CGTATTACCAGTCCCGTTCTAGTC									
RL1B6362	F	[HEX]TTAATTAGTTATGCGGGTTGGTC	(CA)9	7	22	121-132	4	0.636	0.525	0.9153	-0.1226
	R	TTGTCATATAAAGGAAAGTATGAGTGC									
RL1BHDMD	F	[HEX]GGCAACATATTGACTGGAATCAT	(AT)9	4	23	178-202	5	0.478	0.518	0.1397	0.0369
	R	CCAGAGACTGGAGCAGGAGTA									
RL1BWMXW	F	[HEX]AGGATGAAGTGCAGAAGATCG	(TA)11	6	18	176-242	15	0.889	0.908	0.4796	-0.0102
	R	TTCCATTGGATCATTATGTTAGTTG									
RL1BYZOG	F	[6-FAM]GTGTGCGGTACGGGTAGTG	(AT)9	2	24	118-120	2	0.042	0.12	0.0656	0.4372
	R	GTGATGGTGTTGATGCGAAC									
RL1C4XBN	F	[6-FAM]TTGTTGTTAATGATGCTAATGCTG	(TA)9	3	22	312-316	3	0.636	0.673	1.0000	0.0176
	R	AATATCACCAAGTTCATCATTTATGG									
RL1C9ZMG	F	[HEX]TCGAATAACCTGAATCGTGTAGG	(TA)10	-	22	221-269	9	0.545	0.695	0.1971	0.0821
	R	GCATATACAAGAAACGAACGAATG									
RL1CHUVB	F	[HEX]CGAAACATGTTCGGGTACAG	(TA)11	5	21	224-229	4	0.571	0.519	0.7035	-0.0939
	R	GTGCCACTATAATCACGAATATCTATG									
RL1CUOWO	F	[HEX]TGGCACGCACAGGTATGTA	(AT)9	3	22	262-270	4	0.636	0.527	0.3045	-0.0987
	R	CGCATGCTCTAAGGAAGATCTAA									
RL2F2Z0L	F	[6-FAM]TCTATCCGGGACTCTTGAGC	(AT)9	7	19	206-214	13	0.316	0.408	0.1240	0.1334
	R	AGGTGTTCACTGAAGCAGTAATTG									
RL2H7JXQ	F	[HEX]AAATCCAAAGTCAATCATTTATACCAC	(TA)9	7	22	288-327	9	0.818	0.773	**0.1447**	-0.0402
	R	ATAGGGTGGTTTGGTGGATG									
RL2HRQBP	F	[HEX]GTGTTAAATCTCAATGTGGTGTCC	(AT)9	2	18	142-149	4	0.333	0.617	**0.0029**	0.3015
	R	TATCCAATTGTGCTTCATCAGG									

F, forward; R, reverse; n, total individuals genotyped; bp, base pairs; Na, total alleles observed; H_E, expected heterozygosity; H_O, observed heterozygosity; HWE, Hardy-Weinberg equilibrium; *p*, probability value; Est. F(null), frequency of null alleles

***significant deviation from HWE** as estimated by the Markov chain algorithms (Guo & Thompson, 1992)

Table 3.2 Details of *9 Rhamphomyia longicauda* microsatellite loci rejected after the initial amplification stage.

Locus	Primer sequences and fluoro label (5'-3')		Repeat motif	Expected allele size (bp)
RL1A1ZJS	F	[6-FAM]CAAGAAATGATCTTGGTAAATGCTC	(TTTA)9	185
	R	TTTGCCGCCTTAGACAACTC		
RL1AEFQ1	F	[6-FAM]TGTATTACTCAGTTCCCTTAGAACC	(TA)11	128
	R	GATGGCGACAAATAGAGAATG		
RL1B8QVS	F	[6-FAM]ACACGTGTTTGACACAATACTGAAC	(GT)10	221
	R	AATAATCCATACACACGCGGTAG		
RL1BWSO6	F	[HEX]TCAGGTTCAAACTTGTCAATTACC	(AT)10	317
	R	CGATAGGGTTTATATCATTGATGAAC		
RL1CKNTD	F	[HEX]ACCAGTTAAACCATGATCACTCAC	(TA)11	374
	R	CTAGGCGCCACAACGTATC		
RL1CMAMM	F	[HEX]AAATGTTGACGTCCATTTCCTAC	(TA)9	332
	R	TGTTCGTCTGAAGATTATGAAACC		
RL1EBLLP	F	[6-FAM]TTGTCTATTGTTTGGTGGCCTA	(AT)11	206
	R	CCTTCCGGAGGACCTTAAAT		
RL1ECZKK	F	[6-FAM]GTGTGTCTCCTCCTCACATCC	(TA)11	190
	R	CACAAATAGAATAGACATCCTCACG		
RL2F667Z	F	[6-FAM]TCGTCCGTAATCAACATTTCAC	(AT)9	323
	R	TCATCCTTGTACGGCCTTG		

F, forward; R, reverse; bp, base pairs

Table 3.3 Characterisation of seven *Empis tessellata* microsatellite loci in the Loch Lomond population, Scotland, UK. Markers were combined where possible into seven multiplex reactions.

Locus		Primer sequences and fluoro label (5'-3')	Repeat motif	Multiplex set	n	Allele size range (bp)	Na	H_E	H_O	HWE *p* value	Est. f(null)
ET_1AJNTC	F	[HEX]TGAGAATGTCCACACGATCC	(AC)7	1	26	194-200	4	0.577	0.506	1.000	-0.070
	R	ACGCAACAGACAACTTTAACAAAC									
ET_1BGBFS	F	[HEX]CATTAGGTGGTGGACGAAATC	(CAA)8	2	27	239-246	4	0.333	0.329	0.048	0.000
	R	TCATACCACCACCATTTATACAATTAC									
ET_1EVD1P	F	[HEX]CATGCAACAACATTCACTTCAC	(TAA)8	2	26	143-167	5	0.769	0.621	0.437	-0.168
	R	GAGAACGCGGTCTACTATTTGAG									
ET_2FTSP7	F	[HEX]GATGGTATTACTGGTGCTGGTG	(TGG)7	1	26	79-101	6	0.500	0.750	0.039	0.186
	R	CATCAAATCTACCTGTTCAACAAAC									
ET_2FZRSR	F	[HEX]AGGCTGAACAATTTGAGATTGAG	(GTT)6	-	27	224-259	12	0.815	0.862	0.356	0.022
	R	TGCTCGTACTGATGGTATGGAC									
ET_2HP1XX	F	[6FAM]TTGTCATGTGATGTCGGATGT	(GTT)4	2	27	236-251	8	0.407	0.516	0.318	0.073
	R	CACAATTATATTCACGGCATGTTT									
ET_2JTXTO	F	[6FAM]CGTGATCTTGTTATTGGTGAATATG	(TAT)6	1	24	161-175	6	0.583	0.736	0.157	0.099
	R	TCATTAGGTGGTACAAGTTATAATAGTGG									

F, forward; R, reverse; n, total individuals genotyped; bp, base pairs; Na, total alleles observed; H_E, expected heterozygosity; H_O, observed heterozygosity; HWE, Hardy-Weinberg equilibrium; *p*, probability value; Est. F(null), frequency of null alleles

Table 3.4 Details of 12 *Empis tessellata* microsatellite loci rejected after the initial amplification stage.

Locus	Primer sequences and fluoro label (5'-3')		Repeat motif	Expected allele size (bp)
ET1CNKDB	F	[HEX]AAATTTGAATGTAATATTTGTGGTGGT	(TGA)7	84
	R	ACGTTCATCATCAAATCGTCAA		
ET1B2ORW	F	[6FAM]GACGTGATCTACCACGAAAGG	(TAT)7	86
	R	AATCCAGGCCATGAAACAAG		
ET1DT5XU	F	[6FAM]TTATGAAATTGTTGATTGTTAATTTGG	(GTT)9	127
	R	CAAATTCAACTGTTAATGGTGGTT		
ET1EBT2M	F	[HEX]TGTGTTCCCAAATCGCTTC	(TA)7	132
	R	ACGGAGGCAACAGGTAAGAG		
ET2FVQYC	F	[6FAM]ACCACCATTACCAACACCTTG	(TAT)6	134
	R	AAGCTGATAAACAAGCTAGAAATGC		
ET1DIY59	F	[HEX]AATCACAACATGATGCCAATG	(TA)7	144
	R	CGAAACCCATAATAATTACATGAGAG		
ET2JDRMY	F	[6FAM]CGCACACCTTGTGTAAATTGTC	(TA)7	155
	R	GCGCTTTATTCATAAATTACATCTGTC		
ET2FX076	F	[6FAM]TATTGGACGTGATCCACCTG	(ATT)6	161
	R	TGGAAACGGTTGATACACAAAC		
ET1BABW6	F	[HEX]GGCCTATTGTGGTACCCTTG	(AT)7	174
	R	TGCGAGGATTTCGTTTAACA		
ET2JLCA4	F	[HEX]GTCATCACGCAACTCCATTATC	(TTG)10	197
	R	TTGTAGTTAGTGCATGGTAATTTGC		
ET1AYWD3	F	[6FAM]TTTGATGTTCAATGACCTCCAC	(AT)8	199
	R	TGCACGATTATCCTTGTTTCTG		
ET2GVSSY	F	[6FAM]TGATAAAGGTGGTTATCATCAAGG	(TGG)7	200
	R	GTTCACTTCGATCACGTTCTAAAG		

F, forward; R, reverse; bp, base pairs

Table 3.5 Characterisation of 6 *Empis aestiva* microsatellite loci in the Loch Lomond population, Scotland, UK.

Locus	Primer sequences and fluoro label (5'-3')		Repeat motif	n	Allele size range (bp)	Na	H_E	H_O	HWE *p* value	Est. f(null)
AE1EEONZ	F	[HEX]CCAATTATCGACAATATCACTTCG	(CAA)8	18	96-107	4	0.778	0.649	0.170	-0.107
	R	CAATTGTAATTGGTCCCGTTG								
AE1BTDUZ	F	[6FAM]GAAATATTGATGATGGCCTAAATTC	(AAT)7	19	151-154	2	0.316	0.273	1.000	-0.083
	R	TGATGCATTTGTTGATGCTG								
AE1DGSN4	F	[6FAM]GTCCAACCCGAACAACAAC	(ATT)9	19	164-167	3	0.368	0.568	0.074	0.187
	R	AGCATTAACACAATTAGTATGTTCACC								
AE2J1RZH	F	[6FAM]CACACAACTTCAATCTGACACC	(TA)6	19	187-192	3	0.316	0.284	1.000	-0.079
	R	AACATGGTCCCTGTCTGATG								
AE1EO5W8	F	[HEX]GAACGTCAACCCGGAATTAG	(ACA)8	19	88-96	2	0.105	0.341	**0.010***	0.518
	R	TCCATGTTAGCAATTACCTCAATC								
AE2IR84C	F	[6FAM]CACCACCCACTCATAATAATCG	(CAA)8	16	127-139	3	0.188	0.454	**0.013***	0.394
	R	ATAGCCACGAATTGCTGATG								

F, forward; R, reverse; n, total individuals genotyped; bp, base pairs; Na, total alleles observed; H_E, expected heterozygosity; H_O, observed heterozygosity; HWE, Hardy-Weinberg equilibrium; *p*, probability value; Est. F(null), frequency of null alleles

***significant deviation from HWE** as estimated by the Markov chain algorithms (Guo & Thompson, 1992)

Table 3.6 Details of 13 *Empis aestiva* microsatellite loci rejected after the initial amplification stage.

Locus	Primer sequences and fluoro label (5'-3')		Repeat motif	Expected allele size (bp)
AE1CVPY7	F	[6FAM]CACAGATGTTTTATATGTTTTCTAGG	(GT)13	102
	R	AACTACAATCCTATCCATGTGC		
AE1BBAY4	F	[6FAM]CATCAAATGCAAACAGTTCAATC	(CAA)8	132
	R	TTGTGTTGGCTGTTGTTGAAG		
AE2HNFNE	F	[HEX]TGCATGTTTGTAAAAGTTTGTGG	(ACA)10	138
	R	TTTGGAGTATGTGGGGTTGAG		
AE1BP9M0	F	[HEX]GCAACAACGTCAACATCAGC	(CAA)9	145
	R	GAGCTGGACGTTCTTGGTTC		
AE2H9RLV	F	[6FAM]TGACGTTCTTGTTTAACAGTAGCTTG	(TAT)9	146
	R	GGCCAAGGACATACCCTACC		
AE2GW7JA	F	[HEX]ACTGGCACAACAGTAACATCAAC	(TAA)10	148
	R	CTTCTTCATTGAAATAATCATCACG		
AE2HXSRD	F	[HEX]TGTCTTCCACCTCTTCAACATC	(AGT)6	158
	R	TGAGGAACTTTTATATTGAGGACAAG		
AE1AU08C	F	[HEX]CCAAAATTGCGCATGTCA	(TC)11	187
	R	AAATTTTATTCCTGGGCTCTCC		
AE2GCB9A	F	[HEX]TCGCCACACAATCATTTAGG	(CAA)7	194
	R	ACGTTTGATTGGTAACAATAGTGG		
AE1A3MYF	F	[6FAM]AACCTGAGATTATTAAACGAAGCTG	(TTA)7	200
	R	ACAATGGAGAACCGAAATCC		
AE2HXZK0	F	[HEX]GCAACATAATCATCGACATCAAG	(TAA)6	237
	R	TCTGAACTAGTTTGTTCACCTTCAG		
AE2I35UF	F	[6FAM]CAACAATCCACTACAGAATCTAAAGC	(TAA)5	252
	R	TGATGAAGTTGGCGGTAATG		
AE2IYBLF	F	[HEX]CCGGGTATTAGTTCCACATTATC	(TAA)7	299
	R	GCACCAACTAATGGACTATTTCC		

F, forward; R, reverse; bp, base pairs

We genotyped the resulting PCR products on an ABI 3730 48-well capillary DNA Analyser using GeneScan ROX 500 size standard, and scored the alleles using GENEMAPPER v3.7 software. For the markers that amplified reliably close to the expected product size we tested the genotype frequencies for evidence of deviations from Hardy-Weinberg equilibrium, linkage disequilibrium, and estimated the frequency of null alleles (tables 3.1, 3.3 and 3.5). The observed and expected heterozygosities were calculated using CERVUS v3.0.3 (Marshall *et al.*, 1998). We tested for deviations from Hardy-Weinberg equilibrium (HWE) as well as linkage disequilibrium (LD) using GENEPOP v4.2 (Raymond and Rousset 1995). We dropped markers that did not amplify consistently, or showed a very large deviation from HWE (tables 3.2, 3.4 and 3.6). However, we did keep four markers that significantly deviated ($P<0.05$) from HWE as we recognised that the population we tested the markers may violate some of the Hardy-Weinberg assumptions. For example, the populations of dance flies we tested the markers on may not be mating at random and selection may be occurring but these markers would still be useful for allele counting estimates of mate number.

This procedure produced fourteen *R. longicauda*, seven *E. tessellata* and six *E. aestiva* microsatellite markers. The observed heterozygosity of the single population for each species ranged from 0.12 to 0.889 for *R. longicauda* (table 3.1); 0.329 to 0.862 for *E. tessellata* (table 3.3); and 0.105 to 0.778 for *E. aestiva* (table 3.5). The number of alleles per locus ranged from 2 to 18 for *R. longicauda* (table 3.1); 4 to 12 for *E. tessellata* (table 3.3); and 2 to 4 for *E. aestiva* (table 3.5). These novel microsatellite markers have the potential to capture the full mating history of females from mixed DNA extracted from sperm stores.

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Chapter 4

Sexual selection on seductive female characters

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Abstract

Sexually dimorphic ornaments arise because mate choice exerts strong sexual selection favouring individuals with high levels of ornament expression. Life history theory predicts that elaborate ornamental traits should be under strong directional sexual selection because of costs associated with their maintenance and potential trade-offs with other life history traits. In fact the potential costs of ornament expression are thought to help explain why so few species have female ornaments, even when females experience strong sexual selection. Unusual cases in which female ornaments do occur can therefore provide rare insight into how selection can favour expression of expensive characters despite their costs. We examined how mating success covaried with sexual ornament expression in a species of dance fly with elaborate female sexual ornaments. We captured wild female flies and used molecular markers to estimates mate number from stored sperm. We found no support for the prediction that females with larger ornaments should achieve higher mating success. These data suggest that sexual selection on female ornaments may operate rather differently than it does on male ornaments, which may further explain both the general rarity of female adornments and the conditions that favour their expression in rare species exhibiting elaborate female sexual traits.

4.1. Introduction

Typical sex differences are thought to have evolved due to disparity in the nature and intensity of selection between the sexes. These general sex differences in selection are the consequence of unequal parental investment in gametes, with females having few, large eggs and males having small, but numerous spermatozoa (Bateman, 1948; Trivers, 1972; Williams, 1975). Greater parental investment by females means that males usually gain more from remating than females, and sexually receptive males typically outnumber sexually receptive females. This bias in the operational sex ratio can select for the evolution of traits that improve mating success for the superabundant sex (Emlen & Oring, 1977), and is presumed to account for the evolution of sexual ornaments in males of many species.

The directional selection required to create dimorphic ornaments should lead to exaggerated expression of ornamental traits until the sexual selection benefit is balanced by costs, either because of a premium on resources used to construct the focal trait (metabolic costs), or because the trait depresses fitness in other life history contexts (e.g., when trying to evade predators) (Houslay & Bussière, 2012). When the costs and benefits equalize, we expect the population to remain at equilibrium, with no further trait exaggeration expected in spite of a continued sexual selection benefit for the most ornate individuals. Importantly, although the net fitness consequences of trait expression are neutral at equilibrium, the advantage in sexual contests is expected to persist, and acts to maintain trait expression at a level substantially higher than in the opposite sex. In the absence of directional sexual selection, the trait's costs (either metabolic or in other life history contexts) would select against trait expression, and favour a return to sexual monomorphism. Consequently, whenever we see dimorphic ornaments, we expect to find directional sexual selection favouring the ornamental traits,

even if the net fitness consequence of trait expression is equalized thanks to other aspects of the life history.

Most studies of sexual selection on ornamental traits have been on males, since ornaments occur predominantly in males and females are usually the 'choosy' sex. Meanwhile, examples of elaborate female ornamentation are rare. Exceptions include sexual coloration in female pipefish (*Syngnathus typhle*)(Berglund *et al.*, 1997), the colourful plumage of phalaropes (Reynolds *et al.*, 1986), the fleshy facial ornament of wattled Jacana (*Jacana jacana*) (Emlen & Wrege, 2004), and the pennate leg scales and inflatable abdominal sacs of many species female dance fly (Cumming, 1994; Funk & Tallamy, 2000; LeBas *et al.*, 2003). Sexual selection on females is reasonably common (Clutton-Brock, 2007, 2009) and is more likely to occur when males are in limited supply and females experience competition for mates (Gwynne & Simmons, 1990). Meanwhile, sexual selection on female ornaments has rarely been documented. An exception is the pipefish *S. typhle*, in which females displaying the most contrasting striped coloration honestly signal fecundity and have highest mating success (Berglund *et al.*, 1997).

The rarity of female ornamentation, even in systems where females are subject to sexual selection, is due in part to the costs of developing and maintaining sexually selected traits (Fitzpatrick *et al.*, 1995). Costly sexually selected traits such as elaborate ornaments might reduce the resources available for reproduction, lowering reproductive success. As such, we might expect selection against the most extravagantly ornamented females based on female interests alone. In addition, the evolution of elaborate ornaments is typically mediated by mate preference, and therefore ornaments are expected to benefit not only the females that express them but also the males that are attracted to them. Consistent with this expectation, in

species where male choice is observed, male preference is usually for traits associated with high fecundity or reduced sperm competition intensity, as male fitness is very tightly linked to female fecundity (Bonduriansky, 2001). Consequently any resource cost of ornaments is also a problem for males, who should avoid females that incur fecundity costs of investing in elaborate traits. In addition, although highly ornamented females may be honestly signalling their reproductive potential, the fact that they mate multiply may also present prospective mates with heightened sperm competition. Males may therefore not benefit by choosing a female that is likely to attract many suitors if it means each mate is likely to get only a small share of the paternity of her offspring.

Given the above-mentioned problems, how can we explain species where female ornaments do exist? We might predict female ornamentation is most likely to be observed in systems where females gain direct benefits from mating with multiple males, such as through nuptial feeding. In such systems, among-female competition for matings can be intense (Gwynne & Bussière, 2002) and therefore investment in traits that give females an advantage in competition for males may be favoured.. The rare species where elaborate female ornaments do exist present an opportunity to study the nature of sexual selection on females. We use estimates of wild polyandry rates of female long-tailed dance flies, to examine how sexual selection acts on elaborate female ornamentation.

Dance flies of the subfamily Empidinae (Diptera: Empididae) have remarkably diverse mating systems, with closely related species showing strikingly variable levels of female sexual ornamentation (Cumming, 1994). In most species, adult females have not been observed hunting, and are thought to rely heavily upon protein-rich gifts from males during mating to mature their eggs (Downes, 1970). It is thought that competition among females

for these gifts has led to the evolution of elaborate female ornaments multiple times throughout the lineage (Downes, 1970; Cumming, 1994). Like many insects, female dance flies store sperm. Highly polyandrous females could therefore present mates with high levels of sperm competition. Females with high mating success should theoretically therefore become less attractive to future potential mates thanks to the lower paternity share they provide with every additional sperm competitor.

The long-tailed dance fly, *Rhamphomyia longicauda* is sexually dimorphic, with females of this species possessing elaborate ornamentation in the form of pennate leg scales and inflatable abdominal sacs that they display in female-biased mating swarms (Funk & Tallamy, 2000). These ornaments increase the size of a female's silhouette in a mating swarm, and apparently improve their attractiveness to the choosing males (Funk & Tallamy, 2000). Experimental studies using artificial female flies found that males were attracted to larger models more often (Funk & Tallamy, 2000), and that these preferences are specifically directed to the sexually dimorphic traits as opposed to overall size (Murray, 2015).

Both previously-mentioned choice experiments (Funk & Tallamy, 2000; Murray, 2015) focussed on the attraction phase of mate choice in *R. longicauda*. Given the choice, males have been shown to prefer larger or more ornamented females, but it is unclear as to whether this translates into increased mating success for more ornamented females. Meanwhile, two studies of wild dance flies in species featuring female ornaments have studied the link between ornament expression and mating success, as measured by whether or not a female was caught while paired with a male. One study found no evidence that *R. longicauda* females with the largest ornaments were more likely to be paired (Wheeler *et al.*, 2012). while, a second found that *R. tarsata* females with larger ornaments were most likely to be

paired, reflecting positive directional selection (LeBas *et al.*, 2003). Both studies acknowledged the possibility that some unpaired females, which were scored as 'unsuccessful', could have stored sperm and were actually 'successful' in terms of having some mating success. Indeed, one of these studies did record stored sperm in unmated females (Wheeler *et al.*, 2012). However, it is unclear how the cross-sectional contrast between mated and unmated females could fail to reveal true strong difference in mating success as a function of ornamentation.

One solution is to exploit newly developed molecular markers (chapter 3) to estimate the longitudinal mating success of different classes of females. We extracted and amplified DNA from stored sperm collected from wild *R. longicauda* females in order to estimate female mate number as a measure of mating success. By quantifying the association between ornament size and mate number, we were able to determine the direction and magnitude of sexual selection on female ornaments, and thereby test whether the contrasting results for initial attractiveness and pairing success from previous work on this species represented an artefact or a true difference in selection intensity across different parts of the pairing process.

4.2. Methods

Sample collection

We collected female *Rhamphomyia longicauda* from a mating swarm located on the banks of the Credit River, Ontario, Canada (location coordinates: 43°41'11.00"N, 079°55'34.00"W) during their swarming season in June, 2012. We sampled 53 females over several dates throughout the swarming season, which occurs between the end of May and the beginning of July: 11 females on 7th June (day 159); 27 on 17th June (day 169); and 15 on 22nd June (day

174). Samples were collected using an entomological sweep net and then frozen and stored at -20°C.

Morphological measurements

We photographed individuals through a dissecting microscope (Leica MZ 12 5), with camera (Panasonic Lumix DMC-G10) attachment. We used the digital imaging program Image J (Rashband, 2008) to take measurements of the wing length, thorax scutellum length, hind femora length, hind tibia length, and hind pennate scale area (total area of the hind femora and tibia including scales). It was not possible to take measurements of the abdominal sacs since these deflated following capture and storage. If possible, both right and left body parts were measured and a mean of the two values taken. When one of the body parts was damaged we used a single measure of the undamaged side. We also counted the developing eggs within the abdomen, and measured the longest axis of five eggs in each female to give us a mean egg length.

Spermathecal dissections

We removed the female reproductive tract and transferred it to 100% ethanol for a minimum of 12 hours to dehydrate the contents as described in Tripet et al., 2001. The head of each female was also removed and stored in 100% ethanol for later DNA extraction. This period in ethanol causes the sperm contents inside the spermatheca to coagulate into a pellet, allowing us to remove the sperm pellets after rupturing the spermatheca (Bussière et al., 2010). Each sperm pellet was then transferred to 180µL of buffer solution (ATL buffer from the QIAamp DNA Micro Kit, Qiagen), where it was stored at -20°C until DNA extraction.

Extraction, amplification and microsatellite analysis of DNA

We extracted DNA from the stored sperm using column-based kits designed specifically for extracting DNA from small samples (QIAamp DNA Micro Kits, Qiagen). We followed the protocol outlined in Bussière *et al.* (2010), including the addition of 1µl carrier RNA to 200µL buffer AL and eluting using the smallest volume of elution buffer AE recommended (30µL). To increase DNA yield from sperm, we also added 12 µL DTT to each sample then lysed overnight at 56°C.

We also extracted DNA from the head of each female previously stored in 100% ethanol using an ammonium acetate method based on Bruford et al. (1992). We ground the samples using a pestle before digesting overnight at 56°C. As DNA yield was low, we dissolved the purified DNA product in 50µL of low EDTA TE buffer.

We genotyped all females sampled as well as the sperm stored in their spermatheca using 13 microsatellite markers (described in Chapter 3). To reduce the number of PCR reactions, when possible we multiplexed primer sets (2 or 3 loci amplified in one reaction). For each PCR, reaction volume was 2µL, which included either 1 µL DNA (dried) from female tissue samples or 2 µL DNA (dried) from spermethecal samples, 1 µL primer mix (both forward and reverse primers were at 0.2 mM concentration) and 1 µL Qiagen Multipex PCR Master Mix. We amplified the DNA under the following PCR profile: 95 °C for 15 min, followed by 44 cycles of 94 °C for 30 s, 56 °C for 90 s, 72 °C for 90 s and finally 72 °C for 10 min. PCR products were genotyped on an ABI 3730 48-well capillary DNA Analyser using GeneScan ROX 500 size standard. We scored the alleles using GENEMAPPER v3.7 software.

We derived two separate estimates of mating history: a minimum estimate and a less conservative estimate that exploited information on allele frequencies in the population. To get a conservative estimate of mate number, we first checked for contamination of amplified sperm DNA from female tissue; if female alleles were present in the array we discounted them. We then divided the count of the remaining alleles by two, as each male could be heterozygous, and then rounded up the answer to the nearest integer in order to gain a minimum mate number estimate. Estimates based on allele counting are likely to be very conservative, so we used information on the background allele frequencies in the population to estimate the most probable mate number given the occurrence of alleles in an array. We used population allele frequencies to obtain probable estimates of mate number given the array of alleles detected from genotyped stored sperm, a technique described in Bretman & Tregenza (2005) and which we briefly outline here and in Box 1. To estimate the probable mate number, the product of the probabilities of both observing and not observing alleles in the array is calculated. The probability of not observing an allele, $P_{(not)}$, is calculated using the allele frequency, $f(a)$, and the number of trials, t, of attempting to observe the allele as:

$$P_{(not)} = [1-f(a)]^t$$

As each male has two alleles for a given locus, t is twice the number of males sampled. The probability of observing the allele is calculated as:

$$P_{(obs)} = 1 - P_{(not)}$$

The product of $P_{(not)}$ and $P_{(obs)}$ was calculated for 2 to 70 t which equates to 1 to 35 males. A range of 1 to 35 males was chosen in line with a previous study which probabilistically

estimated polyandry rates in wild field crickets (*Gryllus bimaculatus*; Bretman & Tregenza, 2005), in which 35 was deemed to be sufficiently high to exceed the maximum number of males a female could have mated with or could be detected. The number of trials with the highest probability for a given array of alleles gives us the probabilistic mate number estimate. Our probabilistic mate number estimate for each female was then calculated using the most polymorphic locus, which was not always the same locus for all females.

Box 1: An example of estimating probable mate number

To estimate the probable number of males genotyped from stored sperm we used population allele frequencies as well as the presence and absence of alleles at a locus to calculate the probability of observing those alleles if the DNA of a given number of males were present. The number of males (equal to half the number of trials, *t*) with the highest probability of observing the genotype array was considered the most probable mate number. First, using individual genotype data from a subset of the population of interest, the relative frequency of the alleles *f*(a) was calculated as the number of times a given allele was observed (frequency) divided by the total number of alleles observed for that locus. Below is an example of calculated allele frequencies for one locus using genotype data from 20 individuals, where six alleles of different sizes were observed:

Allele	frequency	*f*(a)
220	2	0.05
224	17	0.425
228	7	0.175
232	7	0.175
236	6	0.15
240	1	0.025
total	40	1

Using *f*(a), we then calculated the probability of not observing the allele $P_{(not)}$ as well as the probability of observing the§ allele $P_{(obs)}$ for a given number of trials, *t*. Each male can have

two alleles, so for one male the number of trials, $t = 2$, for two males $t = 4$. The probability of not observing an allele is calculated as $P_{(not)} = [1\text{-}f(a)]^t$. For example, using allele 220, the probability of not observing the allele in two trials is $P_{(not)} = [1 - 0.05]^2$. The probability of observing an allele is then calculated as $P_{(obs)} = 1 - P_{(not)}$. Both $P_{(not)}$ and $P_{(obs)}$ are calculated for each of the six alleles present for this locus for two to 70 trials (one to 35 males). Once these probabilities are calculated for each allele, they are then used to estimate the probable mate number given the observed arrays of genotypes from amplified stored sperm in females. If, for example, an array had three alleles present, 220, 228, 232, then the product of $P_{(obs)}$ for these alleles and $P_{(not)}$ for those alleles not observed (in this case alleles 224, 236, 240) is then calculated for 2 – 70 t (1 – 35 males). The number of males with the highest overall probability of observing that array is the probable mate number.

Statistical analysis

We performed all statistical analyses in R version 3.2.1 (R Development Core team, 2015). To improve the interpretability of regression coefficients, we ensured predictor variables were on a common scale and dimension for both linear models and generalised linear models. Numeric predictor variables such as thorax scutellum length or hind leg femora length were standardised by centring (subtracting the mean) and scaling (dividing by 2 standard deviations), and predictor variables that were counts were merely centred (Schielzeth, 2010). To ensure all numeric predictor were the same dimension, we square root transformed the variable hind leg scale area to match the dimensionality of the other morphological trait measures prior to scaling and centring. We validated models by visually assessing plots of model residuals for homoscedasticity and normality of residuals.

Testing for directional sexual selection on female ornamentation

To test for positive directional sexual selection on female ornamentation in *R. longicauda*, we quantified the association between ornament expression and female lifetime mating success. We constructed generalised linear models with a Poisson family error structure and log link function. We assessed models for overdispersion by checking whether the ratio of the residual deviance to the degrees of freedom was greater than one; in all cases the ratio was lower than one indicating a lack of overdispersion. We used two estimates of female lifetime mating success, minimum mate number and probable mate number, as our response variables. To quantify the effect of ornament expression separate from body size, we constructed models including both hind leg scale area, a measure of ornament expression, and wing length, a measure of body size, as predictor variables. As females were likely to accumulate mates throughout the flight period, we also included sampling day a continuous predictor. To test that our quantification of the effect of ornament expression on female lifetime mating success was not sensitive to our choice of body size measure, we repeated the analysis, but this time including thorax scutellum length as a body size measure.

To further test whether our model predicting lifetime mating success was sensitive to parameter choice, we constructed a maximal model to include all morphological traits measured as well as fecundity measures. We included the fecundity measures, egg number and mean egg length as predictors in the model, as we recognise that female fecundity may predict some of a female's lifetime mating success. We checked for any problems associated with collinearity between traits measures by checking for variance inflation.

We also wanted to test for any association between mating success and reproductive traits, mean egg length and egg number, so we also constructed a larger model which contained all

morphological traits measured as well as mean both reproductive traits, egg length and egg number. We simplified the model by sequentially removing predictors with the highest P values. At each simplification stage we measured the Akaike information criterion (AIC) to compare models (Burnham & Anderson, 2002). We considered models with a difference in AIC of less than two to be the same, and always kept the simpler model. If the removal of predictor variable in the model resulted in the AIC decreasing by more than two, we kept the term in the model.

Quantifying the direct benefits of nuptial feeding

To explore the potentially direct benefits of feeding on nuptial gifts, we constructed linear models to predict both the number and size of the fraction of eggs that had not yet been oviposited at the time of death on lifetime mating success, using our probable mate number estimate. As body size is often associated with female fecundity, we included wing length as a predictor variable. We included sample day as a factor in each model to allow for any nonlinear effects of date (e.g., if eggs increased in size until a certain date and then laying occurred, causing a drop in the size of eggs still in the ovary). To check our results were not sensitive to our choice of body size measures, we also repeated the analysis using thorax scutellum length instead of wing length.

4.3. Results

We found that all 53 of the wild-caught females had mated with at least one male, as they each had sperm present in their spermatheca. Using microsatellite genotyping, we found that overall 85% had mated more than once. The average number of mates per females was 2.5 ± 0.15 (mean ± SE) using our minimum mate estimate (range 1-6; figure 4.1a), and 2.9 ± 0.19 males using our probabilistic mate estimate (range 1-7; figure 4.1b).

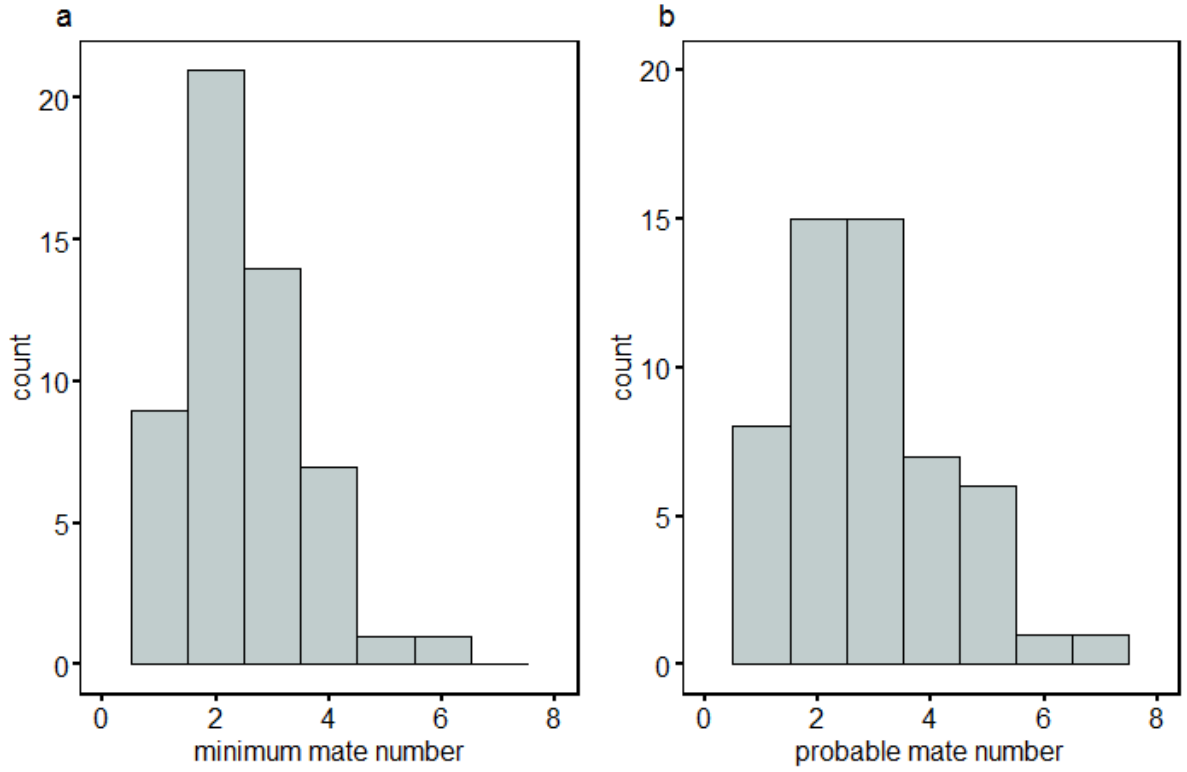

Figure 4.1 Frequency histogram of (a) minimum mate number and (b) probable mate number estimates of 53 *Rhamphomyia longicauda* females.

We found that both minimum mate number and probable mate number tended to slightly increase over the flight period (figure 4.2). However the effect of sampling day was not significant for either minimum mate ($\beta = 0.043$, SE= 0.029, T = 1.497, P = 0.141) or probable mate estimate ($\beta = 0.042$, SE = 0.037, T = 1.157, P = 0.253).

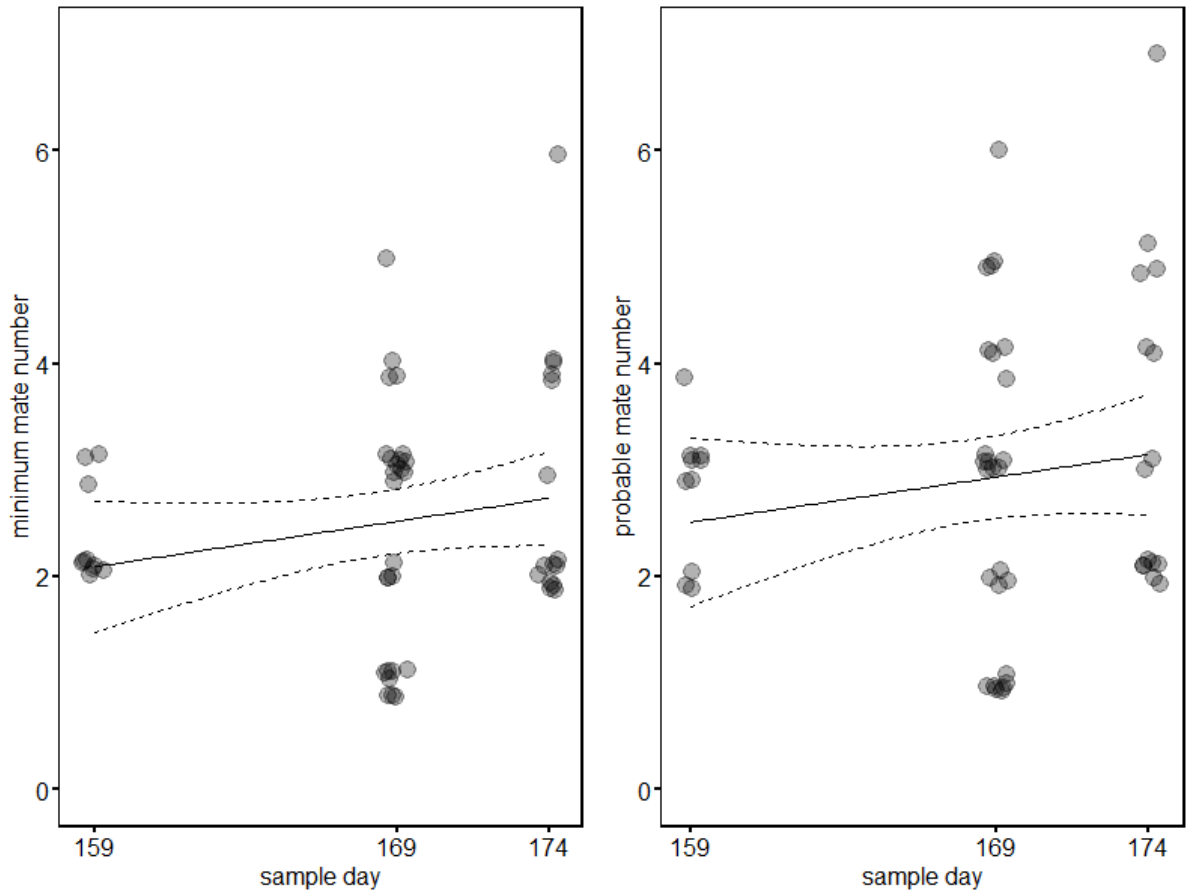

Figure 4.2. Minimum mate number and probable mate number against sampling day. Plotted line shows the prediction from a linear model, while dashed lines indicate 95% confidence intervals. A small amount of noise was added to both the x and y variables after analysis to better visualize overlapping points.

Lifetime mating success and female ornamentation

To test for positive directional sexual selection on female ornamentation in *R. longicauda*, we fitted generalised linear models with two estimates of mate number (minimum and probable) as the response, and hind leg scale area, thorax scutellum height (as a measure of body size) and sampling day as predictors. The summary of the generalised linear models used to predict both minimum mate number and probable mate number are given in table 4.1. If there were

positive directional selection on leg ornamentation, we would expect to find a positive partial effect of hind leg scale area on mate number after accounting for the partial effects of thorax scutellum height and sampling day. We found hind leg scale area had a small positive effect on both minimum mate number and probable mate number, however these effects were not significant (table 4.1; figure 4.3a &4.3b). The results of both minimum mate number and probable mate number were similar, with hind leg scale area having no significant effect on either mate estimate. Thorax scutellum height did not have a significant effect on either mate number estimate (table 4.1) suggesting that larger females were not gaining higher mating success. To account for females acquiring mates throughout the flight period, we also included sampling day as a continuous predictor in the model. Our decision to fit day as a continuous variable rather than factor did not influence the results of the model (results not shown). Although mate number was higher in the later sampling days, it was not a significant predictor of minimum or probable mate number (table 4.1).

Table 4.1. Summary of Generalised Linear Model (GLM) with Poisson family error structure showing the effect of scutellum length, hind leg scale area and sampling day on two estimates of female mate number from stored sperm.

	Minimum mate number				Probable mate number			
Coefficients	Est.	SE	*Z* value	*P* value	Est.	SE	*Z* value	*P* value
Intercept	-2.211	2.928	-0.755	0.450	-1.609	2.696	-0.597	0.551
Scutellum length	-0.012	0.109	-0.113	0.910	0.001	0.101	0.013	0.989
√Hind leg scale area	0.053	0.111	0.475	0.635	0.051	0.103	0.496	0.620
Day	0.019	0.017	1.069	0.285	0.016	0.016	0.994	0.320

Est., estimate; SE, standard error

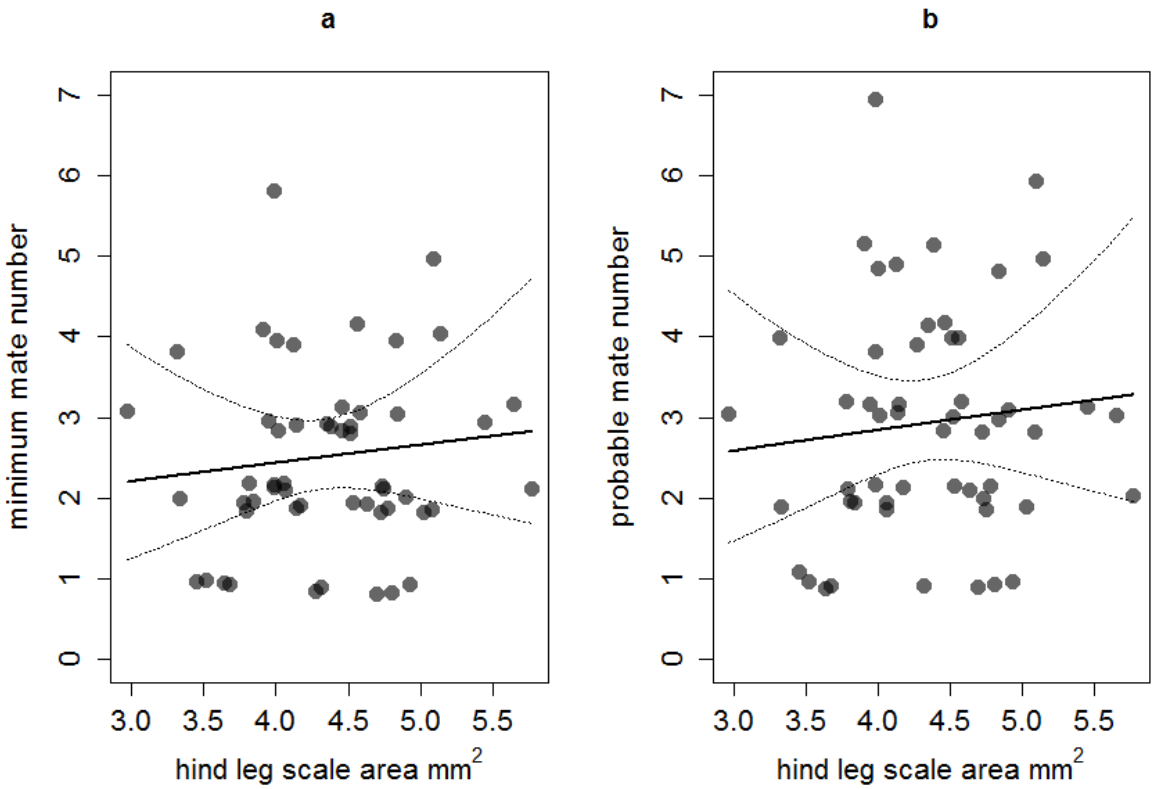

Figure 4.3. Minimum mating number (a) and probable mate number (b) as a function of hind leg scale area (mm^2). Plotted line shows partial effect of hind leg scale area on mate number. A small amount of noise was added to the response variables after analysis to facilitate visualizing overlapping points.

To check the effect of other morphological or reproductive traits on both estimates of female mate number, we constructed a maximal model (Table 4.2) and attempted to simplify the model by sequentially removing nonsignificant predictors. We were unable to include wing length or tibia length in the maximal model as these caused variance inflation due to collinearity with other predictors. Model simplification using AIC indicated a null minimal model; we did not find any morphological (wet mass, thorax scutellum length, hind leg femora length, hind leg scale area) or fecundity traits (mean egg length and egg number) to be associated with either estimate of mate number.

Table 4.2. Summary of the maximal generalized linear model for number of males detected in storage as a function of mean egg length, egg number, wet mass, thorax scutellum length, hind leg femora length, square root hind leg scale area, and day in female long-tailed dance flies.

	Minimum mate estimate				Probable mate estimate			
Coefficients	Est.	SE	Z value	*P* value	Est.	SE	Z value	*P* value
Intercept	-1.663	3.066	-0.542	0.588	-1.202	2.791	-0.431	0.667
mean egg length	-0.047	0.147	-0.321	0.748	-0.048	0.136	-0.353	0.724
egg number	-0.003	0.005	-0.552	0.581	-0.004	0.005	-0.893	0.372
wet mass	0.149	0.218	0.683	0.495	0.096	0.202	0.475	0.634
thorax scutellum length	-0.024	0.137	-0.176	0.860	0.020	0.127	0.155	0.877
hind leg femora length	-0.029	0.187	-0.157	0.875	-0.024	0.175	-0.139	0.889
√hind leg scale area	-0.008	0.200	-0.038	0.970	0.012	0.185	0.062	0.950
day	0.015	0.018	0.845	0.398	0.014	0.017	0.820	0.412

Est., estimate; SE, standard error

Quantifying the direct benefits of nuptial feeding

To quantify the potentially direct benefits of feeding on nuptial gifts, we regressed both the number and size of the fraction of eggs that had not yet been oviposited at the time of death on liftime mating success. If females were directly benefitting from nuptial feeding, we might expect to see a positive effect of lifetime mating success on fecundity measures. We constructed two linear models to predict developing egg size, and each model included mate number and sample day but differed in the body size measure we included. The first linear model included wing length as a measure of body size, as well as probable mate number and day as predictor vaiables, and the coefficients of the linear model which is summarised in Table 4.3. The second linear model included thorax scutellum length and a measure of body size, as well probable mate number and day as predictor variables, and the results of this model are summarised in Table 4.4. Using different measures of body size in the linear models predicting egg number did not qualitatively affect the results of the analysis.

Table 4.3. Results of the linear model quantifying the effect of wing length, probable mate number and sampling day on egg number in female long-tailed dance flies.

coefficients	Est.	SE	*T* value	*P* value
Intercept	45.698	6.253	7.309	<0.001
wing length	6.864	2.820	2.434	0.019
probable mate number	-0.469	2.049	-0.229	0.820
day 169	21.041	7.430	2.832	0.007
day 174	11.758	7.976	1.474	0.148

Est., estimate; SE, standard error

We found larger females had more eggs, with the number of developing eggs being significantly positively associated with both measures of body size: wing length (table 4.3; figure 4.4) and thorax scutellum length (table 4.4). However, egg number was not significantly associated with the number of mates a female had acquired (table 4.3; table 4.4).

Egg number was associated with sampling day: females of average size (mean wing length) and mating success (mean probable mate) sampled on day 169 had 21 more eggs than those sampled on day 159 (table 4.3; figure 4.5). The egg number of females sampled on day 174 did not differ significantly from females sampled on day 159.

Table 4.4. Results of the Linear Model quantifying the effect of thorax scutellum length, probabilistic mate number and sampling day on egg number in female long-tailed dance flies

coefficients	Est.	SE	*T* value	*P* value
Intercept	50.210	5.725	8.771	<0.001
thorax scutellum length	7.910	2.649	2.986	0.005
probable mate number	-1.299	1.860	-0.698	0.488
day 169	15.288	6.779	2.255	0.029
day 174	9.121	7.472	1.221	0.228

Est., estimate; SE, standard error

To quantify the effect of nuptial feeding on developing egg size, we regressed mean egg length on mate number (table 4.5). We included wing length as a predictor in the model to account for body size. Mean egg length was not associated with female mate number or wing length (table 4.5). To account for sampling females at different times during the flight period, we also included sample day as a predictor in the model; it was the only significant predictor (table 4.5; figure 4.6). Females sampled on day 169 had significantly larger eggs than those sampled on day 159 or day 174.

Table 4.5. Results of the Linear Model quantifying the effect of wing length, probable mate number and sampling day on mean egg length in female long-tailed dance flies

coefficients	Est.	SE	*T* value	*P* value
Intercept	0.281	0.061	4.647	<0.001
wing length	0.015	0.027	0.566	0.574
probable mate number	0.014	0.020	0.708	0.483
day 169	0.186	0.072	2.589	0.013
day 174	0.023	0.077	0.297	0.768

Est., estimate; SE, standard error

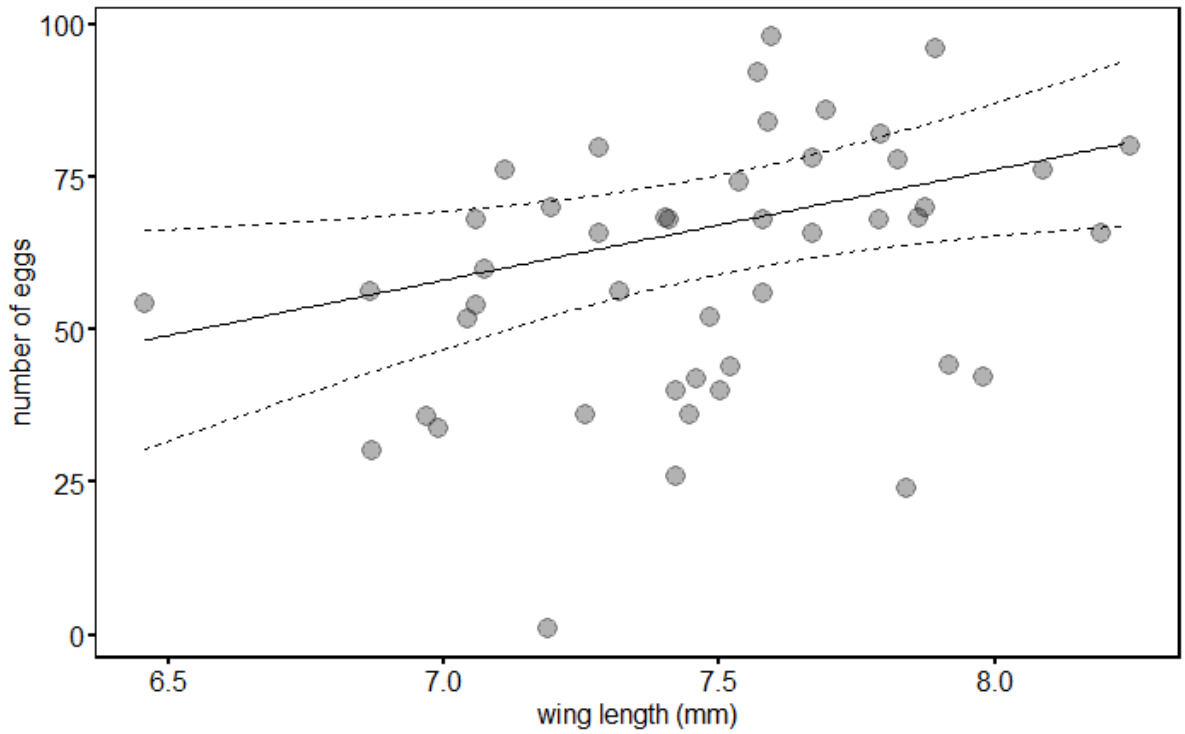

Figure 4.4. Partial effect of wing length on the number of eggs counted, for mean probable mate number and sampling day 169 in a female long-tailed dance fly. Dashed lines represent 95% confidence intervals.

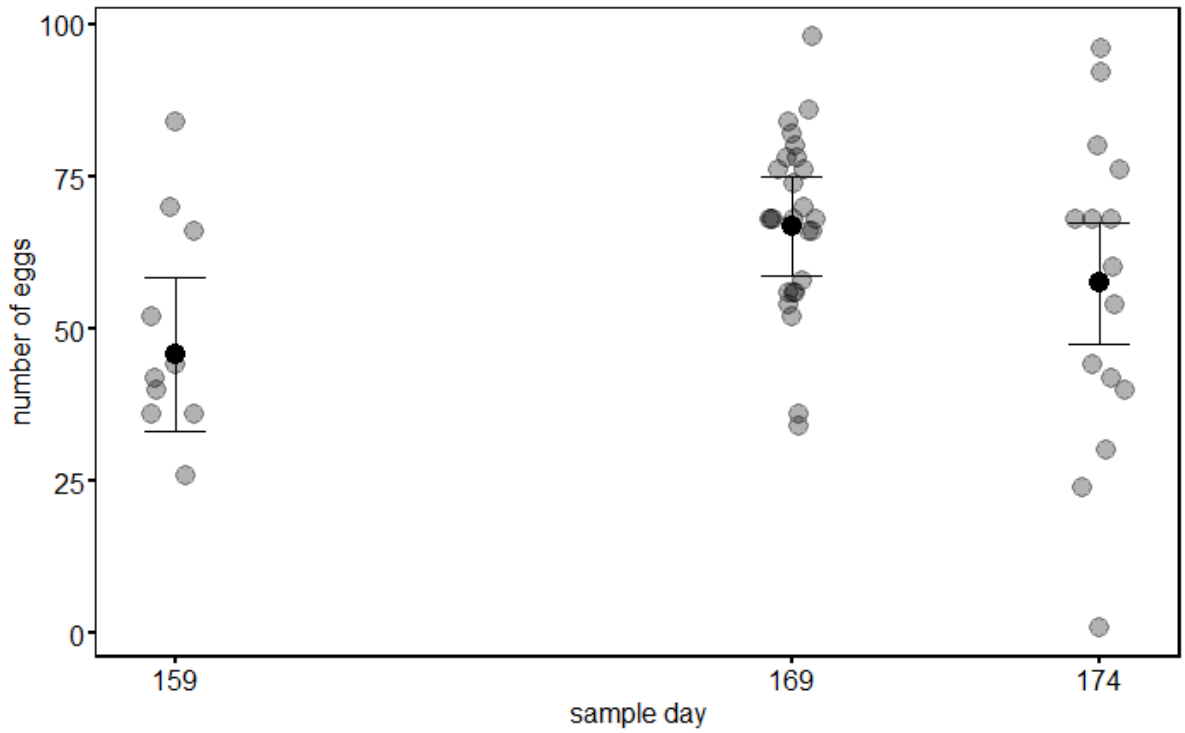

Figure 4.5. The number of eggs on each sampling occasion (day of year) during the flight period. Grey points are raw values. Black points are the partial effects of each sample day on mean egg length, for females of average mating success and body size. Error bars represent 95% confidence intervals. A small amount of noise was added to sample day after analysis for ease of viewing overlapping points.

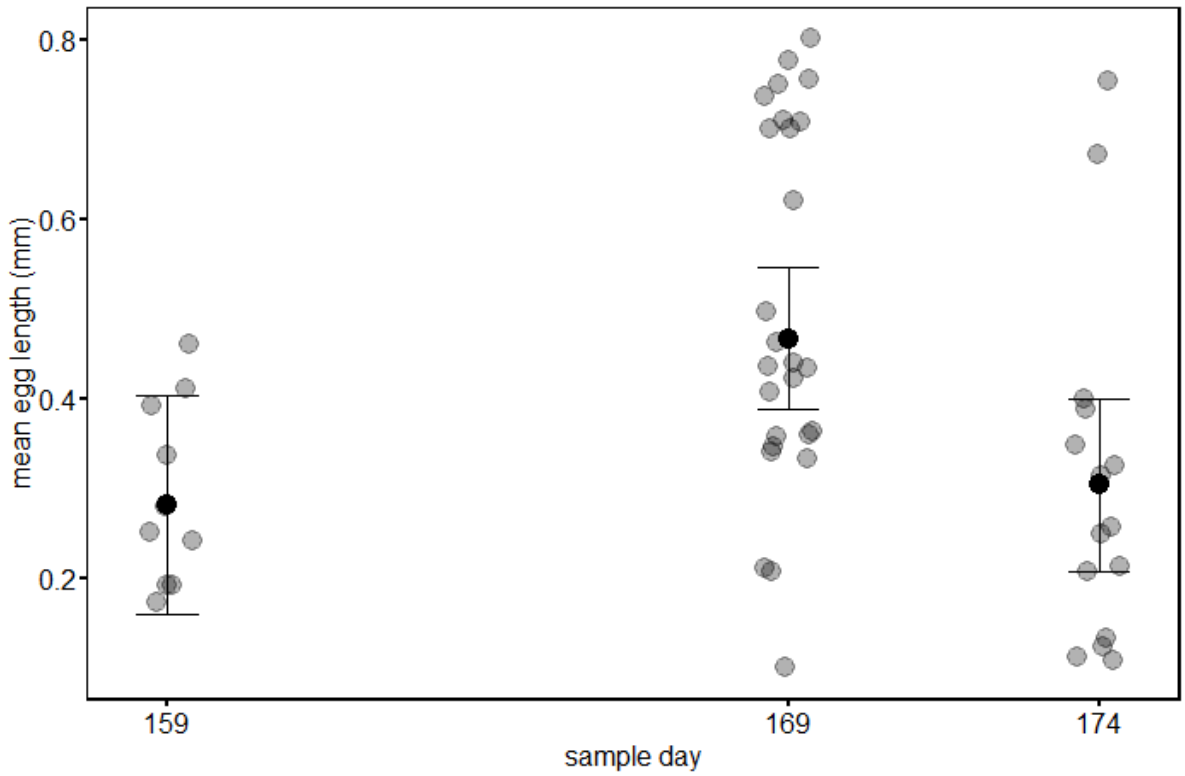

Figure 4.6 Mean egg length of female on each sampling occasion (day of year) during the flight period. Grey points are raw values. Black points are the partial effects of each sample day on mean egg length, for females of average mating success and body size. Error bars represent 95% confidence intervals. A small amount of noise was added to sample day after analysis to facilitate viewing overlapping points.

4.4. Discussion

Wild polyandry rates in the long-tailed dance fly

In this study, we assessed natural polyandry rates of the long-tailed dance fly, *Rhamphomyia longicauda*. The presence of sperm indicated that all sampled females had mated at least once, which is slightly higher than the previously reported 93% for this species (Wheeler *et al.*, 2012). It is possible that we did not sample mating swarms early enough in the swarming period to capture newly eclosed females that would not yet have mated. Nevertheless, it is clear that nearly all swarming females are already carrying stored sperm, which has important implications for the sperm competition levels faced by their mates (see also Chapter 6).

We genotyped stored sperm in wild-caught females at 13 microsatellite loci, and used two methods to estimate polyandry rates in females. First, we used allele counts to make a minimum mate estimate; second, we used population allele frequencies to assess the most probable mate number. The probable mate number is more likely to accurately reflect the rate of polyandry, but our models were qualitatively identical when using either estimate in our analyses. Both probable and minimum mate numbers are probably underestimates of the true polyandry rate for a few reasons, including the fact that we captured females before they finished mating. Measuring polyandry rates in the wild is problematic as it is difficult to document all matings. For example, assessing polyandry rates through behavioural observations is extremely labour intensive, and can be misleading because not all pairings result in successful insemination. In addition, the displacement of previously stored sperm could result in a failure to detect prior mates with our method.

Previous authors have used dissection to document polyandry rates in several systems, , including the green-veined white butterfly *Pieris napi* (Wedell *et al.*, 2002), and in bushcrickets (Vahed, 1998). Such methods are possible in those groups because each male transfers a spermatophore that retains some of its individual structure within the female, such that investigators can count a separate "spermatodose" for each prior mate. Dance flies unfortunately do not transfer any discrete and long-lasting spermatophores, so we were unable to employ this method.

In common with many previous studies of insects, we found a high incidence of polyandry in wild long-tailed dance flies, with 85% of females having mated at least twice. Although it has been predicted that females of this species should mate multiply, because of the likely direct benefits provided by nuptial gifts (Cumming, 1994), this is the first study to quantify the extent of multiple mating. *R. longicauda* females mated with a minimum average of 2.5 males (range 1-6 males) and a slightly higher probable average of 2.8 males (range 1-7 males). These estimates are broadly similar to the few previously reported estimates of polyandry rates from some other wild insects, including green-veined white butterflies *Pieris napi* (mean = 2.1, range = 1-5) (Wedell *et al.*, 2002); wild field crickets *Gryllus bimaculatus*, *Teleogryllus oceanicus*, and *T. commodus* (mean = 2.7-5.1, range = 2-7) (Bretman & Tregenza, 2005; Simmons & Beveridge, 2010); katydids *Requena verticalis* (mean = 1.6-2.8, range = 1-4) (Simmons *et al.*, 2007); swordtail crickets *Laupala cersina* (mean = 3.6, range = 1-6) (Turnell & Shaw, 2015); two-spot ladybirds *Adalia bipunctata* (mean = 2.5-3.7, range = 1-6) (Haddrill *et al.*, 2008); and yellow dung flies *Scathophaga stercoraria* (mean = 3.3, range = 1-11) (Demont *et al.*, 2011). The estimates are also notably smaller than some other taxa that do not appear to have extraordinarily strong sexual selection on females (at least not

strong enough to favour the evolution of sexually dimorphic female traits), including decorated crickets (Sakaluk, 1987) and scaly crickets (Andrade & Mason, 2000).

We did not find statistical support for a change in mating frequency across the mating season, but our data were consistent with a modest increase in mating frequency of females in later sampling days (Table 3.1). It is obvious that individual females will acquire mates gradually, but the population need not necessarily reflect individual levels of cumulative mating, depending on the distribution of eclosion dates and the behaviour of females following mating. We tested for an effect of sampling date because we predicted that females sampled later in the season were more likely to be older and therefore have accumulated more matings by the time that they were caught. The fact that we did not detect a strong trend in mating rates across the season may be because females continued to emerge throughout the season, and so we were still catching young females even at the end of our sampling period. Alternatively, it may be that females who mate several times early on disappear from the mating swarms, either to oviposit or because they perish after doing so. The lack of significant association between mate number and sampling date also shows that males had a choice of females with different mating histories throughout the flight period.

Sexual selection on female-specific ornamentation

Life history theory predicts that extravagant ornaments should be under strong directional sexual selection, even at equilibrium. We did not find any evidence to support positive directional sexual selection on pennate leg scale area in dance flies. Our findings confirm that the well-documented benefits of larger sexual traits for initially attracting males (Funk & Tallamy, 2000; Murray, 2015) do not appear to result in higher mating success for females with larger ornaments (see also Wheeler et al, 2012). Males may therefore be initially

attracted to larger females, but decline to mate upon closer inspection of other traits that indicate the stage of egg development. The stage of egg development may be important, for example, if there is last male sperm precedence (a form of non-random utilization of sperm where the last male to mate is likely to gain the highest proportion of paternity, which is common in insects) (Bonduriansky, 2001). If last male precedence is occurring, males may prefer females with fully mature eggs, which are close to oviposition. There is as yet no compelling evidence of the factors that regulate male choice following the initial attraction stage. Behavioural studies of the interactions between males and females during pair formation could do much to enlighten our understanding of what mediates pairing success in this species.

The lack of strong directional selection on female ornaments in *R. longicauda* contrasts sharply with findings from another dance fly species that also features female ornaments. In *R. tarsata*, a close relative of *R. longicauda*, there is evidence of escalating sexual selection on female pennate scales, such that the most heavily adorned females are more likely to win contests for males (LeBas *et al.*, 2003). This inconsistency across taxa may reflect the evolutionary instability of male preferences for ornamental traits, as predicted by theory and explained in the introduction. In the next chapter, I explore patterns of sexual selection on female ornaments in a further two species of dance fly in an attempt to generalize expectations for selection on female ornaments across species.

Benefits of mating

We considered two developing egg measures that we thought may be influenced by mating success and body size, egg number (a measure of fecundity) and mean egg length (a measure of egg development stage). On average, females with larger body size, measured using

thorax scutlleum height (table 3.4) and wing length (table 3.5) had more eggs than smaller females, a result consistent with studies on another ornamented dance fly species, *R. tarsata* (LeBas *et al.*, 2003). A positive association between the number of mates and egg development was expected as females are presumed to benefit directly from mating (Cumming, 1994), but there was no significant effect of mating frequency on female egg number. We also did not find significant associations between mean egg length and mating frequency (table 3.6), for which there are several possible explanations. Females need to mature their eggs before they can be fertilised, and one possibility is that feeding bouts (and the matings that enable them) have been too recent for the protein from nuptial gifts to be converted to eggs. Another possibility is that we may have caught some females after they had already laid eggs. Both these possibilites affirm that mean egg length as a measure of benefits is an imperfect measure of fitness (albeit the best measure we have in the absence of an ability to culture flies in the laboratory). Finally, as noted above, we may have underestimated mate number, which may limit our ability to detect an association between egg traits and mate number.

Females tended to vary in their stage of egg development across sampling dates, but there was also substantial variation in egg stage on each sampling date, suggesting that males could choose between females that differed sharply in the maturity of their egg clutch. We did not find any females with fully developed eggs on our first sampling date, suggesting that females egg development is a gradual process that requires a substantial fraction of the flight period to complete. Mean egg length increased significantly between the first two sampling days, then decreased on the final sampling day, which is probably because some females later in the season have already deposited eggs. The extent to which females can develop multiple clutches within a single season before dying remains unclear, but the fact that females with

underdeveloped eggs were captured within a mating swarms at the latest date suggests that such females compete for mates even after oviposition.

In summary, we assessed polyandry rates in wild female dance flies of the species *R. longicauda* using microsatellite genotyping of stored sperm. We found evidence that the vast majority of females mated more than once, and that polyandry rates were broadly similar to many estimates reported in other wild insect populations, and not unusually large as might be expected based on the exorbitance of female ornaments in the focal species. Our results did not reveal any support for the prediction that females with the largest sexual ornaments (pennate leg scales) would achieve higher mating success, and therefore call into question the argument that female ornaments are maintained in a similar manner as those of males. If, in contrast, sexual selection on female ornaments is quite distinct from that on male ornaments, that may help explain the general rarity of female adornments. What remains, of course, is to clarify the special conditions that favour female ornaments (especially in the absence of strong sexual selection to maintain them) in species with ornamented females like dance flies.

Chapter 5

Quantifying sexual selection on female ornaments in three species of dance fly

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Abstract

Sexual ornaments should be usually be under directional sexual selection, via increased mating success, even at evolutionary equilibrium. Furthermore, more extravagant sexual traits should theoretically experience stronger sexual selection, since the most extravagant traits might reasonably require more resources to construct and maintain, and therefore impose fitness costs on other aspects of organismal performance apart from mating success. We test the hypothesis that female ornament expression covaries positively with selection on mating success across species by measuring the strength of sexual selection in three species of dance fly that differ continuously in their expression of female ornamentation. We used molecular microsatellite markers to assess female mating success by amplifying the stored sperm of wild flies. We then used this measure of polyandry to estimate the direction and magnitude of sexual selection on female leg traits (which include the variable sexual ornaments in this group) in each of the three species. In spite of our prediction, we found no evidence for strong directional sexual selection acting on leg traits in any of the species. We did find that polyandry rates varied across species, but the species with female-specific ornaments were not consistently the most polyandrous, and the opportunity for sexual selection was similar across species. These findings imply that sexual selection operates rather distinctly on female ornaments in dance flies than is does in most taxa with elaborate male adornments.

5.1. Introduction

Sexual selection is responsible for the evolution of secondary sexual traits, usually in males (Darwin, 1871). The evolution of secondary sexual traits should depend upon the mating system of a given species (Andersson & Iwasa, 1996): sexual selection on males should be strongest in polygynous systems and sexual selection on females should be strongest in polyandrous systems. The correlated evolution of mating system and display traits has been observed in plants (Goodwillie *et al.*, 2010) and animals (Andersson & Iwasa, 1996). The evolution of exaggerated sexual traits, such as ornaments, is more likely to occur when variation in mating success, or the 'opportunity for sexual selection', is high. Sexual ornaments should be under directional sexual selection, via increased mating success, even at equilibrium (Rowe & Houle, 1996). The evolution of more extravagant sexual traits should require stronger sexual selection, since the most extravagant traits should also be the most costly in terms of the resources needed to maintain them (Rowe & Houle, 1996) and the potential trade-offs with other traits (Stearns, 1989) The prediction that sexual selection should be strongest on the most elaborate traits has not been extensively tested or supported in empirical studies. Meanwhile, most estimates of sexual selection on exaggerated ornaments have been conducted in males, with studies quantifying sexual selection on female ornaments exceedingly rare.

The rarity of female ornaments

Polyandry is common across taxa (Jennions & Petrie, 2000) and yet examples of exaggerated female-specific ornaments are scarce (Amundsen, 2000; Funk & Tallamy, 2000; Amundsen & Forsgren, 2001). Since sexual ornaments require mate choice to evolve, one possible explanation for their rarity in females is that investing in ornaments comes at a cost to fecundity, and this trade-off undermines any presumed benefits of male preference for the

trait (Fitzpatrick *et al.*, 1995). Theory suggests that male choice for ornaments can still occur despite these apparent trade-offs, but that male preference for female sexual ornaments is likely to be complex (Chenoweth *et al.*, 2006). When male choice is observed, the female traits under selection usually indicate fecundity, for example if males prefer larger females, or other correlates of reproductive value, such as when males prefer virgins (Bonduriansky, 2001). Females in some systems gain direct benefits from mating, such as through nuptial feeding, which could help to compensate for the fecundity cost of investing in ornaments (Vahed, 1998). However, while mating success could improve female fecundity in such systems, it is unclear what would sustain selection on male preferences; males who prefer the most elaborately adorned females might face more intense levels of sperm competition by virtue of the high levels of mating success achieved by their chosen mates.

Empidinae dance flies (Diptera: Empididae) display remarkable sexual diversity, and the evolution of exaggerated female ornaments has occurred multiple times throughout the lineage (Murray, 2015). Female ornaments in this system have been described as deceptive, and could function to obscure the stage of egg development from males (Funk & Tallamy, 2000). Males of the species *Rhamphomyia longicauda,* when given a choice, have been shown to preferentially approach larger females, suggesting that female ornaments are under directional sexual selection (Funk & Tallamy, 2000). However, this preference does not seem to straightforwardly lead to higher mating success for more ornamented females in this species (Wheeler *et al.*, 2012); instead ornament expression seems to covary weakly with mating success. The evidence for directional selection on female ornaments in dance flies is not consistent across species. In *R. tarsata*, female ornaments are under escalating sexual selection, and ornament expression covaries with egg number and size, suggesting either that ornaments honestly signal female fecundity, or at least that the high mating success achieved

by ornamented females can lead to higher levels of fecundity (LeBas *et al.*, 2003). In order to clarify whether the patterns in *R. longicauda* and *R. tarsata* are aberrations or the norm, we need further studies of the covariance between ornament expression and mating success in other dance fly species.

Our aim was to compare the strength of selection for mating success (as measured by investigating ejaculates in female sperm storage organs) on female ornament expression in several dance fly species that differed in female ornament expression. We caught wild females of three species of dance flies. In *Rhamphomyia longicauda*, females possess pennate leg scales on all three pairs of legs, as well as inflatable abdominal sacs that are displayed during mating swarms (we investigated the association between mating success and ornament expression in this species in chapter 4). In *Empis aestiva*, females lack eversible abdominal sacs, but appear to have independently evolved pennate leg scales, which are not as extensive as in *R. longicauda* and only expressed in the two posterior pairs of legs. In *E. tessellata*, females do not possess any discernable female-specific ornaments (see figure 5.1). Despite this marked variation in morphology, all three species exhibit similar mating habits. Prior to the formation of mating, males secure nuptial gifts in the form of prey items such as flying insects. In all species, both sexes then congregate in mating swarms during which males and females pair, the first stage of which is transfer of the nuptial gift from the male to the female. Mating then follows, and lasts for as long as the female feeds on the nuptial gift. After the female has fed, the pair separate. Males then go in search of another nuptial gift and re-enter the mating swarm, while females can rejoin the swarm immediately. We compared polyandry rates and the magnitude of sexual selection on ornamentation of these three species to test the hypothesis that ornament expression covaries positively with selection on mating success across species.

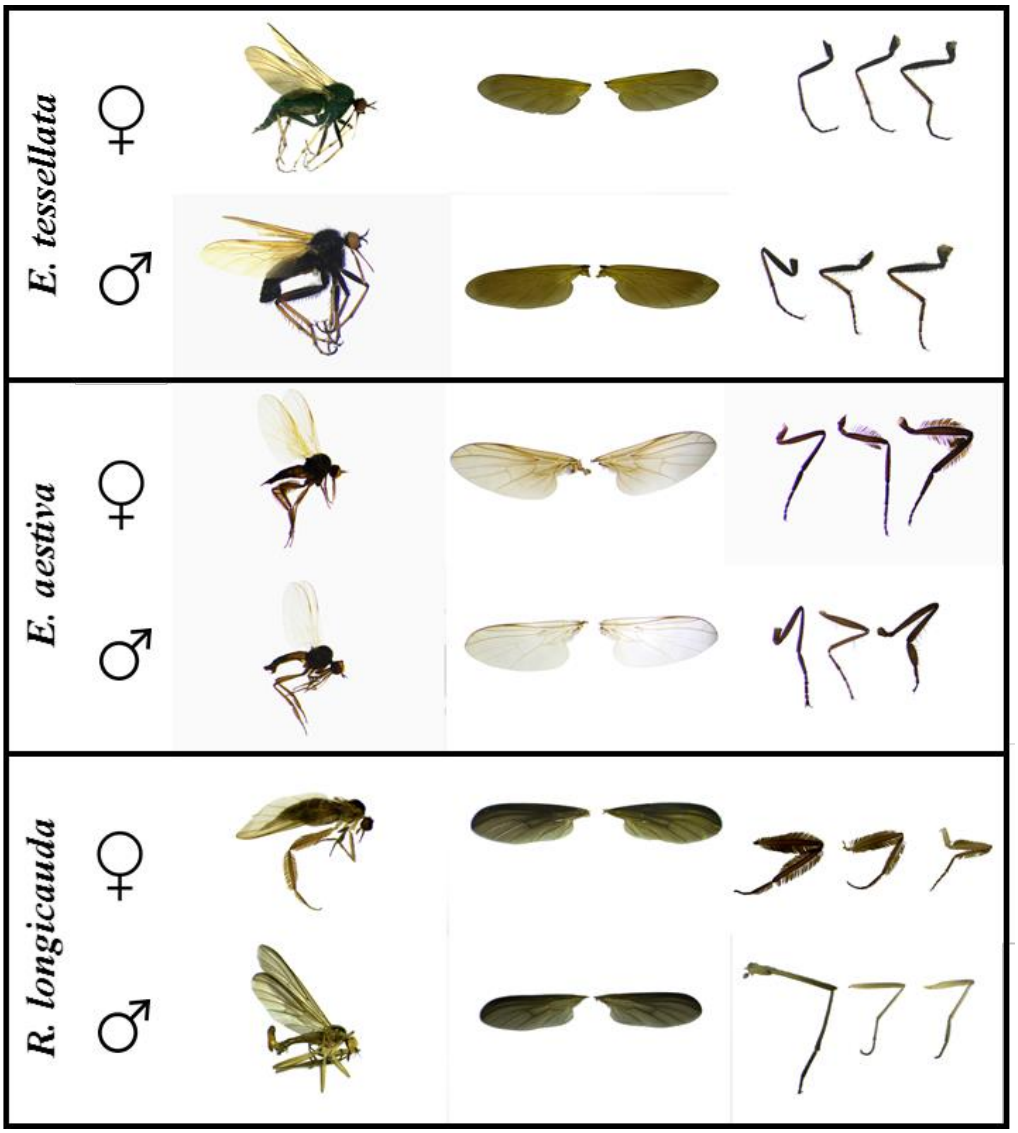

Figure 5.1. Sexual dimorphism in *Empis tessellata* (top panel), *Empis aestiva* (middle panel) and *Rhamphomyia longicauda* (bottom panel). Both females and males are shown in full, as are their disembodied wings, and front, mid and hind legs. Photos by Frederick Hunter.

5.2. Methods

Sample collection

We collected adult females from three species of Empidinae dance fly of two genera of the subfamily Empidinae (Diptera; Empididae). When possible we sampled adult females over the middle to later days of their adult flight period, to collect females that had completed the majority of their matings. We were limited with the number of samples of *E. tessellata* so included females from earlier dates in our analysis. Adult *R. longicauda* swarm from late May to end of June (Funk & Tallamy, 2000). We sampled 53 adult female *R. longicauda* from a population on the banks of the Credit River, Ontario, Canada (GPS coordinates: 43°41'11.00"N, 0.79°55'34.00"W) in June 2012; these are the same individuals as measured in chapter 4. In this chapter, we use these data again to compare patterns of sexual selection in the highly ornamented *R. longicauda* with the two other species to test the prediction that sexual selection should be stronger in species with more exaggerated ornaments. In 2012, the *E. aestiva* flight period ranged from 12th June to 18 July. In July 2012 we collected 32 *E. aestiva* at the Scottish Centre for Ecology and the Natural Environment (SCENE), Scotland (GPS coordinates: 56°09'06.35"N, 004°38'36.20"W). We collected 22 *E. tessellata*, from mating swarms, sweep netting vegetation or using Malaise traps during May and June of 2011 and 2015 at the following three locations: SCENE, Dollar (49°86'00.75N, 07°43'36.05"W) and near Lapanouse-de-Cernon, France (43°59'24.00"N, 3°05'24.00"E). The flight period of *E. tessellata* in Scotland ranged from 20th May to 29th. Females were either frozen after capture at -20°C, or if caught using a Malaise trap they were stored in 70% ethanol prior to dissection.

Morphological measurements

We photographed individuals through a dissecting microscope (Leica MZ 12 5), with camera (Panasonic Lumix DMC-G10) attachment. We used the digital imaging program Image J (Rashband, 2008) to take measurements of the wing length, thorax scutellum length, and hind leg scale area (total area of the hind femora and tibia in all three species and this included scales in *R. longicauda* and *E. aestiva*). Despite the absence of pennate leg scales in *E. tessellata*, we included leg area as a measure of "female ornamentation" in this species, since leg area is the target of sexual selection in many dance flies, and pennate scales have evolved several times independently (Murray, 2015). Leg scales can be placed alongside the abdomen to mimic high levels of fecundity and robust or adorned legs might conceivably be effective in convincing males that a female was gravid. As in Chapter 4, we did not measure abdominal sacs of *R. longicauda* since they deflated following capture; in addition, we wanted to compare directly across species, and only *R. longicauda* possess abdominal sacs. If possible, both right and left body parts were measured and a mean of the two values taken. When one of the body parts was damaged we used a single measure of the undamaged side.

Spermathecal dissection

To isolate stored ejaculates for DNA extraction we dissected all females to remove their spermatheca and followed the spermathecal dissection protocol previously described in chapter 4 (based on Tripet et al. 2001 and Bussière et al. 2010). This protocol involves dehydrating the spermatheca and contents in 100% ethanol overnight, so when the spermatheca is ruptured the spermathecal contents can be removed in a single pellet avoiding any female tissue. Sperm pellets were transferred to 180µL of buffer solution (ATL buffer from QIamp DNA Micro Kit, Qiagen) and then stored at -20°C until DNA extraction.

DNA extraction, amplification and analysis

We extracted DNA from sperm pellets using QIAamp DNA Micro Kits, Qiagen. We followed the protocol outlined in Bussière *et al.* (2010), including the addition of 1µl carrier RNA to 200µL buffer AL and eluting using the smallest volume of elution buffer AE recommended (30µL). To increase DNA yield from sperm, we also added 12 µL DTT to each sample then lysed overnight at 56°C.

To allow us to check for any female tissue contamination we also extracted DNA from the head of each female previously stored in 100% ethanol using an ammonium acetate method based on Bruford et al. (1992). We ground the samples using a pestle before digesting overnight at 56°C. As DNA yield was low, we dissolved the purified DNA product in 50µL of low EDTA TE buffer.

We genotyped all females and their stored sperm using microsatellite markers described in chapter 3. We used 14 markers for *R. longicauda*, seven markers for *E. tessellata* and six marker for *E. aestiva*. The species did not have identical levels of allelic diversity for these markers, unfortunately, but we reasoned that there might be enough genetic diversity to reveal differences in spite of the potentially different resolving power of the loci across species. The number of possible alleles at each locus placed an upper limit on the minimum mate number that could be detected; for *R.longicauda* this was 15 males, while for *E. aestiva* this was three males and for *E. tessellata* this was six males. To reduce the number of reactions markers were multiplexed when possible.

The PCR reaction volumes were 2µl, with 1µl (air-dried) DNA from female head tissue samples and 2µL (air-dried) DNA from spermathecal samples, 1µl primer mix (forward and

reverse fluoro-labelled primers at 0.2 mM) and 1µl Qiagen Mulitplex Master Mix. We amplified products under the following PCR conditions: for *R. longicauda*, 95 °C for 15 min, followed by 44 cycles of 94 °C for 30 s, 56 °C for 90 s, 72 °C for 90 s and finally 72 °C for 10 min; for *E. tessellata* and *E. aestiva,* 95 °C for 15 min, followed by 44 cycles of 94 °C for 30 s, 57 °C for 90 s, 72 °C for 90 s and finally 72 °C for 10 min. We genotyped the resulting PCR products on an ABI 3730 48-well capillary DNA Analyser using GeneScan ROX 500 size standard, and scored the alleles using GENEMAPPER v3.7 software.

We used the genotype data to derive two separate estimates of mating history: a minimum estimate using a count of alleles and a less conservative estimate that exploited information on allele frequencies. The technique for determining these estimates is taken from Bretman & Tregenza (2005) and described in detail in chapter 4.

Statistical analysis

We performed all statistical analyses in R version 3.2.1 (R Development Core team, 2015).

Polyandry rates among dance flies

To determine how the polyandry rates of *E. aestiva* and *E. tessellata* compare to *R. longicauda*, we constructed a generalised linear model with a Poisson error structure and a log link function. We used two estimates of mate number as response variables in separate models: the minimum mate number and the probable mate number, with species fitted as the only predictor variable, as a factor with three levels.

Measuring the opportunity for sexual selection

Since evolutionary change is the product of trait variation and selection upon that trait (Falconer & Mackay, 1996), we calculated the opportunity for sexual selection, I_s, for females of each species, which measures the within-sex variation in mating success across species. We first calculated the variance in lifetime mating success, using the probable mate number estimate. We used the probable mate number estimate rather than the minimum estimate since I_s is meant to reflect the maximal strength of premating sexual selection (Jones, 2009) and our probable estimate was the less conservative, but more likely, measure. We then divided the variance for each species by the squared mean mating success (squared mean probable mate estimate).

Temporal variation on polyandry rates

We sampled dance flies mutiple times thoughout their adult flight period. To quantify the change in female mate number across the flight period, we first standardized the sampling dates within each species. In order to standardize the flight period, we redefined the date of capture as a proportion of the flight period for each species. The flight period for each species was designated as follows: for *R. longicauda*, 25th May to 1st July (Funk & Tallamy, 2000); for *E. aestiva*, 12th June to 18th June, based on continuous Malaise trap sampling at SCENE; and for *E. tessellata*, 17th May to 21st June, based on Malaise trap sampling in Dollar. We then calculated the capture date as a proportion of the known flight period. We constructed generalised linear models with minimum mate number and probable mate number as the response variables in separate models, and sampling day as a proportion of flight period as the sole continuous predictor.

Quantifying associations between lifetime mating success and ornamentation

Ensuring predictor variables are on a common scale improves their interpretability as regression coefficients (Schielzeth, 2010). To ensure all numeric predictor were the same dimension, we square root transformed the variable hind leg scale area to match the dimensionality of the other morphological trait measures prior to scaling and centring. We also ensured the numeric predictors thorax scutellum length, wing length and √hing leg area were standardized on a common scale by first centring (subtracting the mean), and then scaling (dividing by 2 standard deviations) each variable.

We constructed generalised linear models to quantify associations between hind leg area and our two estimates of mating success, minimum mate number and probable mate number, for each species. We included a body size index as a continuous predictor in each model. For *R. longicauda* and *E. tessellata* we used thorax scutellum length as a body size index, and for *E. aestiva* we used wing length. The different choice of body size index for each species was designed to reduce the potential for variance inflation: wing length was strongly collinear with our hing leg area measures in *R. longicauda* and *E. tessellata*, but it was preferred to scutellum length for the smallest species, *E. aestiva*, because it is a larger structure that is measured with less error. We also included sampling day as a proportion swarming period in each model.

Quantifying linear selection gradients

We also quantified the strength of directional selection by computing standardized linear sexual selection gradients (β), on hind leg area. These selection gradients are directly

analogous to sexual selection gradients in males, although the association between mate number and fitness may be more complex in females. This may be especially true in species such as dance flies in which the role of nuptial gifts for promoting fecundity is still unclear. To calculate the standardized linear selection gradients for each species we regressed relative mating success (probable mate number/mean probable mate number) on standardized trait measures, by centering and scaling (as described above) for each species. For *R. longicauda* and *E. tessellata*, the trait measures we included were thorax scutellum length and hind leg area. For *E. aestiva* the trait measures we included were wing length and hind leg area. We also included sampling day as a proportion of the swarming period in each model as above.

5.3. Results

Wild polyandry rates of three differently adorned dance fly species

We sampled adult female dance flies of three species that differed in their expression of female specific ornamentation. All 53 *Rhamphomyia longicauda* and 32 *Empis aestiva* females sampled had mated at least once, as they had sperm present in their spermathecae. Of the 22 *E. tessellata* adult females we sampled, only two had not mated. The *R. longicauda* females analysed here are the same individuals analysed in chapter 4, where we found no evidence for directional sexual selection on leg traits. In this chapter, these data are used again to compare patterns of sexual selection with *E. aestiva* and *E. tessellata*, which have markedly different expression of female sexual ornaments.

Using microsatellite genotyping, we estimated that at least 85% of *R. longicauda*, 25% of *E. aestiva* females and 86% of *E. tessellata* had mated more than once. The average number of mates for adult female *R. longicauda* was 2.49 ± 0.19 (mean ± SE) using our minimum estimate (range 1-6), and 2.91 ± 0.17 (range 1-7) using our probable mate estimate (figure 5.2 & table 5.1; chapter 4). For *E. aestiva* our minimum estimate was 1.125 ± 0.17 (mean ± SE; range 1-2), and our probable estimate was 1.312 ± 0.15 (range 1-4) (figure 5.2 & table 5.1). Female *E. tessellata* mated 1.5 ± 0.24 (mean ± SE) times on average according to our minimum estimate (range 0-3), or 2.55 ± 0.20 times using our probable mate estimate (range 0-5) (figure 5.2 & table 5.1). All of these polyandry rates were lower than the limit of minimum detectable males (based on the available allelic diversity) of 15 males, 3 males and 6 males for *R. longicauda*, *E. aestiva* and *E. tessellata*, respectively.

These polyandry rates differed statistically across species, but the taxa that were statistically distinguishable depended on whether we considered a minimum or probable mate estimate. Using our most conservative estimate of minimum mate number, our models estimated that on average *R. longicauda* had, on average, approximately 0.8 more mates than both *E. aestiva* and *E. tessellata*, which were themselves indistinguishable (figure 5.2 & table 5.1). In contrast, our probable mate number model estimate was not significantly different between *E. tessellata* and *R. longicauda*, but suggested both species mated significantly more that *E. aestiva* (by 0.7 and 0.8 mates on average respectively; figure 5.2 & table 5.1).

Opportunity for sexual selection

To compare the within-sex variation in mating success among dance fly species, we calculated the opportunity for sexual selection (I_S). The variance in probable mate number across species was as follows: *R. longicauda* = 1.972; *Empis aestiva* = 0.415; *E. tessellata* = 1.498. We estimated the opportunity for sexual selection, I_s as follows: *R. longicauda* = 0.234; *E. aestiva* = 0.241; *E. tessellata* = 0.231.

Temporal variation in polyandry rates

We sampled *R. longicauda* and *E. aestiva* midway to late into their flight period, and although we found a trend for a small positive effect of date within the flight period on mate number, in both *R. longicauda* and *E. aestiva* this effect was not significant (table 5.2 & figure 5.3). We sampled *E. tessellata* slightly earlier in the flight period than the other two species, and here there was no trend nor any statistical support for an effect of date on mate number (table 5.2 & figure 5.3). These findings did not depend on whether we modelled minimum or probable mate number (see table 5.2). We recorded two female *E. tessellata* that

had not mated, and these were not from our earliest sampling dates for this species but occurred approximately one quarter of the way through the flight season (figure 5.3).

Does ornament expression covary with levels of polyandry?

We constructed separate generalised linear models for each species to quantify associations between hind leg area and our two estimate of mating success, minimum mate number and probable mate number. We predicted that leg area would be positively associated with mate acquisition in all three species. As previously reported in chapter 4, in *R. longicauda* the partial effect of hind leg area on minimum mate number was 0.058 (±0.121 SE), and on probable mate number the effect was 0.056 (±0.112 SE). Neither of these estimates was significantly different from zero (table 5.3; figure 5.4). In *E. aestiva* the partial effect of hind leg area on minimum mate number was -0.009 (±0.222 SE), and on probable mate number the effect was 0.016 (±0.206 SE). Once again, neither of these estimates was significantly different from zero (table 5.3; figure 5.4). In *E. tessellata* the partial effect of hind leg area on minimum mate number was 0.129 (±0.233 SE), and on probable mate number the effect was 0.085 (±0.180 SE), with neither estimate significantly different from zero (table 5.3; figure 5.4). As *E. tessellata* samples came from three different populations, we also tested models with population as three-level categorical variable, and a model with an interaction between population and hindleg area. Neither the inclusion of the main effect of population (ΔAIC=+3.54) nor the interaction between population and hind leg area (ΔAIC=+4.94) improved model fit. We also quantified the strength of directional selection by computing standardized sexual selection gradients (β), on hind leg area. The magnitude of the standardized linear selection gradients (β) for hind leg area in adult female dance flies were as follows: $\beta = 0.051$ for *R. longicauda*; $\beta = 0.091$ for E.tessellata; $\beta = -0.019$. As with the

unstandardized measures, the standardized linear selection gradients did not differ significantly from zero in any of the three species we tested (table 5.3).

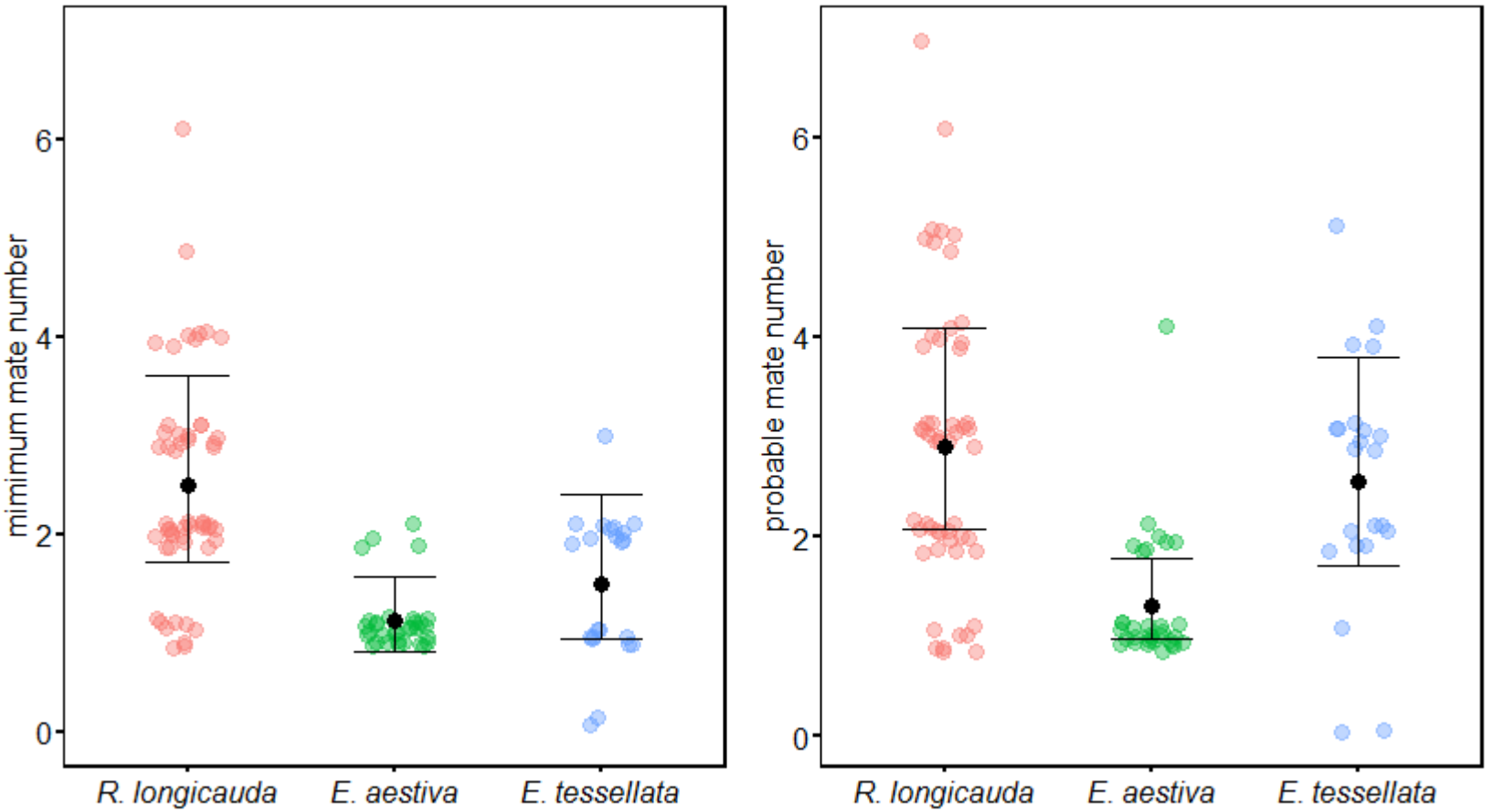

Figure 5.2. Minimum mate number (left panel) and probable mate number (right panel) for three species of dance fly, *Rhamphomyia longicauda* (pink), *Empis aestiva* (green) and *E. tessellata* (blue). Coloured points show raw values; black points show model predictions for each species; and error bars indicate 95% confidence intervals. Small random deviations have been added to point coordinates to avoid overlapping points.

Table 5.1. Coefficients of a generalised linear model predicting both minimum and probable mate number for three species of dance fly, *Rhamphomyia longicauda* (N = 53), *Empis aestiva* (N=32), and *E. tessellata* (N= 22).

	minimum mate number				probable mate number			
Coefficients	Est.	S.E.	Z value	P value	Est.	S.E.	Z value	P value
Intercept	0.913	0.087	10.484	<0.001	1.067	0.081	13.237	<0.001
E. aestiva	-0.795	0.188	-4.227	<0.001	-0.795	0.174	-4.565	<0.001
E. tessellata	-0.507	0.195	-2.605	0.009	-0.132	0.156	-0.848	0.396

Est., estimate; S.E., standard error

Table 5.2. Coefficients of generalised linear models predicting probable mate number from the proportion of flight period sampled for three species of dance fly, *Rhamphomyia longicauda, Empis aestiva* and *E. tessellata*.

		minimum mate number				probable mate number			
Species	Coefficients	Est.	S.E.	Z value	P value	Est.	S.E.	Z value	P value
R. longicauda	Intercept	0.491	0.422	1.164	0.244	0.711	0.387	1.838	0.066
	proportion of flight period	0.68	0.658	1.033	0.302	0.575	0.605	0.949	0.343
E. aestiva	Intercept	-0.323	0.676	-0.478	0.633	-0.324	0.638	-0.508	0.611
	proportion of flight period	0.656	0.96	0.683	0.495	0.882	0.898	0.982	0.326
E. tessellata	Intercept	0.295	0.387	0.761	0.447	0.943	0.299	3.149	0.002
	proportion of flight period	0.495	1.525	0.325	0.745	-0.039	1.217	-0.032	0.975

Est., estimate; S.E., standard error

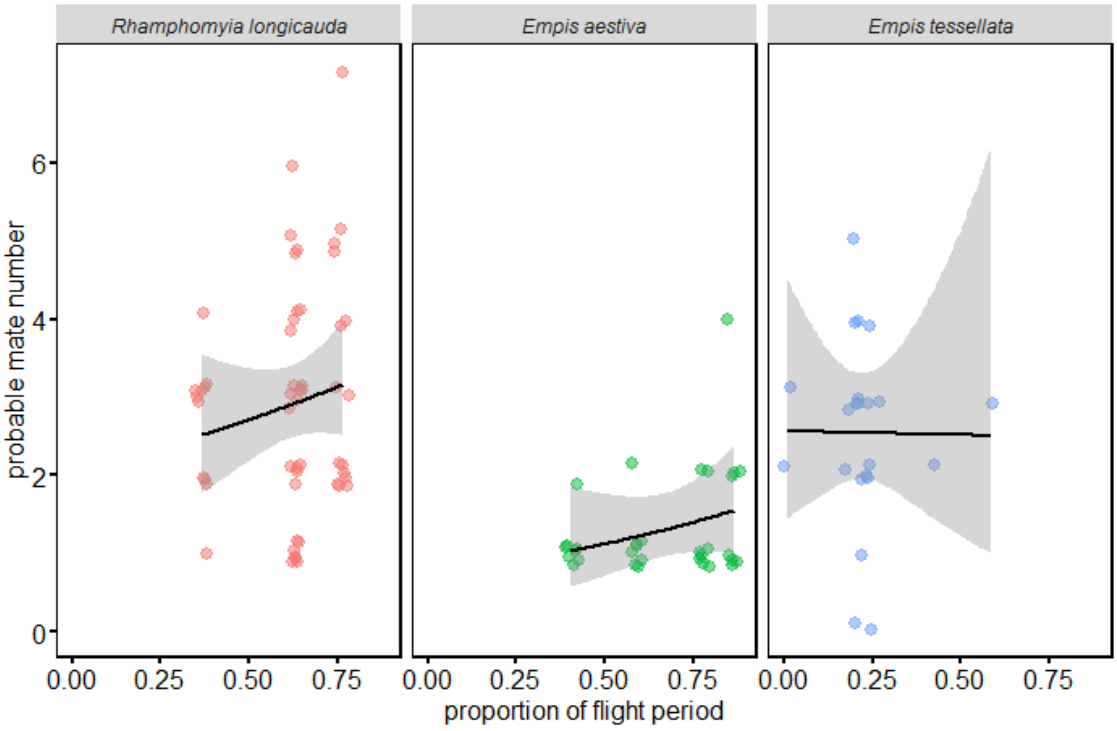

Figure 5.3. Association between probable mate number and sampling day as a proportion flight period for three species of dance fly, *Rhamphomyia longicauda* (pink), *Empis aestiva* (green) and *E. tessellata* (blue). Points show raw data, lines show the model predictions, and shaded areas show 95% confidence intervals. Small random deviations have been added to point coordinates to avoid overlapping points.

Table 5.3. Results of a generalised linear model with Poisson family error structure of mate number predicted by the proportion of the swarming period a sample was taken, hind leg area and a body size index (scutellum length or wing length) in *Rhamphomyia longicauda*, *Empis aestiva* and *E. tessellata*.

		minimum mate number				probable mate number				β (probable mate number)			
Species	Coefficients	Est.	S.E.	Z value	P value	Est.	S.E.	Z value	P value	Est.	S.E.	Z value	P value
R. longicauda	Intercept	0.469	0.423	1.108	0.268	0.686	0.389	1.763	0.078	0.641	0.308	2.085	0.042
	proportion of flight period	0.704	0.659	1.069	0.285	0.603	0.607	0.994	0.320	0.584	0.489	1.195	0.238
	√hind leg area	0.058	0.121	0.475	0.635	0.056	0.112	0.496	0.620	0.051	0.085	0.600	0.551
	scutellum length	-0.012	0.109	-0.113	0.910	0.001	0.101	0.013	0.989	0.001	0.084	0.016	0.987
E. aestiva	Intercept	-0.311	0.7	-0.445	0.656	-0.304	0.662	-0.459	0.646	0.459	0.341	1.345	0.189
	proportion of flight period	0.637	0.999	0.638	0.524	0.847	0.938	0.903	0.366	0.818	0.498	1.641	0.112
	√hind leg area	-0.009	0.222	-0.042	0.966	-0.016	0.206	-0.075	0.94	-0.019	0.112	-0.171	0.866
	wing length	-0.033	0.214	-0.152	0.879	-0.066	0.198	-0.332	0.74	-0.066	0.109	-0.609	0.548
E. tessellata	Intercept	0.386	0.382	1.009	0.313	0.991	0.298	3.329	0.001	1.061	0.196	5.413	<0.001
	proportion of flight period	0.370	1.471	0.251	0.802	0.016	1.173	0.014	0.989	0.015	0.779	0.020	0.984
	√hind leg area	0.129	0.233	0.555	0.579	0.085	0.180	0.472	0.637	0.091	0.117	0.779	0.448
	scutellum length	-0.006	0.230	-0.028	0.978	-0.046	0.178	-0.258	0.797	-0.049	0.115	-0.425	0.677

Est., estimate; S.E., standard error; β, standardized linear selection gradient

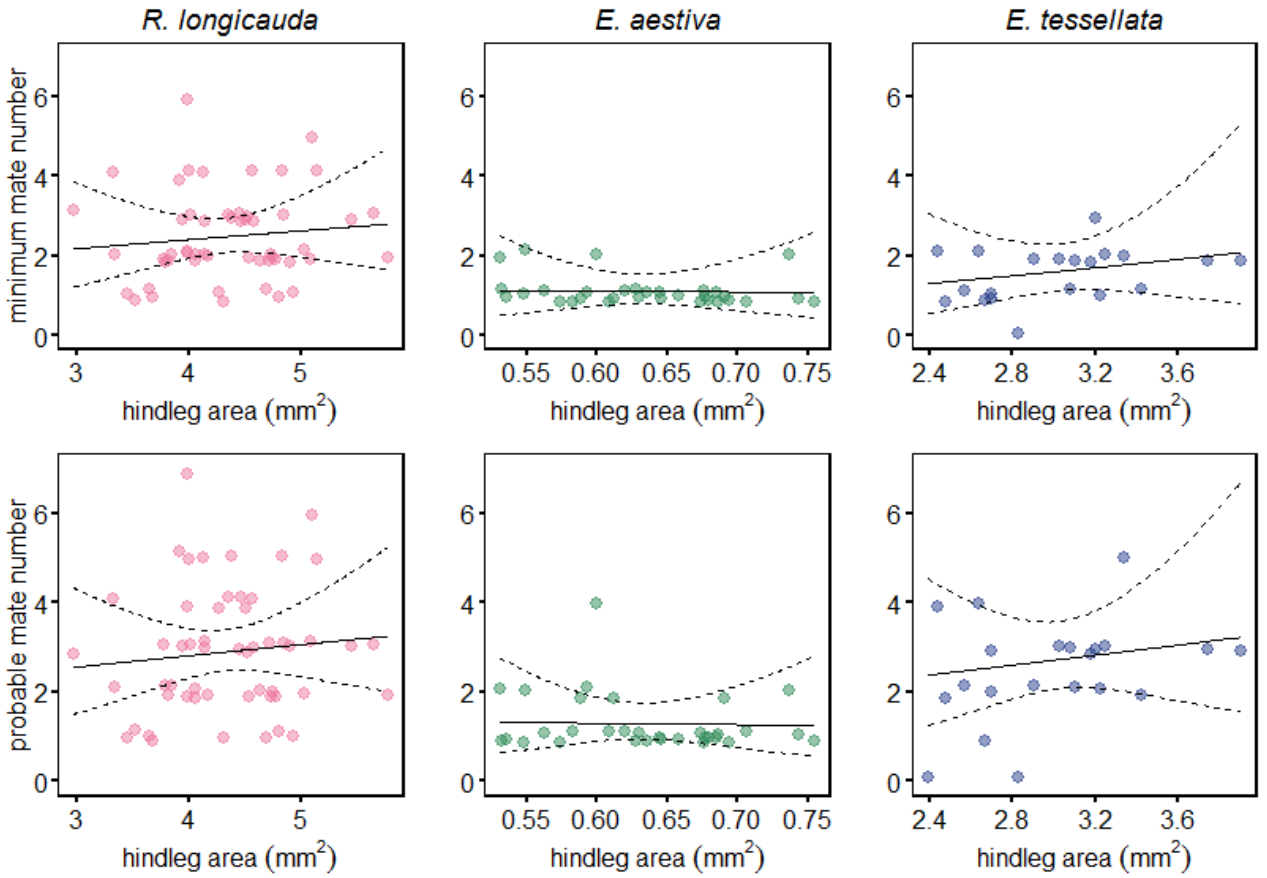

Figure 5.4. Minimum mate number (top panel) and probable mate number (bottom panel) for three species of dance fly, *Rhamphomyia longicauda* (pink), *Empis aestiva* (green) and *E. tessellata* (blue). Coloured points show raw values; black lines show model predictions for each species (tables 5.3-5.5); and dashed lines indicate 95% confidence intervals. Small random deviations have been added to point coordinates to avoid overlapping points.

5.4. Discussion

In this study we tested the hypothesis that ornament expression covaries positively with the strength of sexual selection across species. Life history theory predicts that sexual ornaments should be under directional sexual selection, via increased mating success, even at equilibrium (Rowe & Houle 1996). The evolution of extravagant sexual traits should require stronger sexual selection, since the most extravagant traits should also be the most costly in terms of the resources needed to maintain them. We estimated the direction and magnitude of sexual selection on female ornaments in three species of dance fly, and found no evidence for strong directional sexual selection acting on leg traits in any of the species. We did find polyandry rates to vary across species, but the species with female-specific ornaments were not always the most polyandrous, and the opportunity for sexual selection was similar across species. We discuss these results below and then consider possible alternative explanations.

Polyandry rates across species

Almost all females had mated once, and the majority of female *R. longicauda* and *E. tessellata* (85% and 86% respectively) had mated more than once, which means that males are highly likely to face sperm competition when mating. We only sampled two females that had not mated, both of which were *E. tessellata* females.

In chapter 4 we estimated mating success in *R. longicauda*, which suggested that levels of polyandry in this species are not extraordinary among insects, even though this species exhibits exaggerated female ornamentation. In this chapter, we compared polyandry rates and sexual selection in *R. longicauda* to that in other closely related dance fly species (*E. aestiva* and *E. tessellata*) that differed in their expression of ornamentation. Because mating system is usually associated with the degree of sexual dimorphism across taxa, one might expect *a*

priori that polyandry rates would be higher in species that are more sexually dimorphic. However, we found that *R. longicauda* females have similar levels of polyandry to *E. tessellata*, a species with no female ornamentation. Meanwhile, polyandry rates in these two species were higher than polyandry rates in *E. aestiva* (figure 5.2), a species with intermediate expression of female ornaments.

Opportunity for sexual selection

There is little consensus as to the best method of quantifying sexual selection when comparing across species or sexes (Arnold & Duvall, 1994; Shuster & Wade, 2003; Jones, 2009; Klug *et al.*, 2010; Krakauer *et al.*, 2011; Fritzsche & Arnqvist, 2013). When comparing across species, trait-based measures of sexual selection such as standardized selection gradients and variance-based measures of sexual selection such as the opportunity for sexual selection, have been found to correspond (Fritzsche & Arnqvist, 2013), and this may be due to the opportunity of sexual selection placing an upper limit on selection (Shuster & Wade, 2003). As I_S measures the potential of sexual selection, rather than the actual strength of sexual selection, it has been suggested as a useful summary statistic in describing mating systems to describe the distribution of matings in a population (Arnold & Duvall, 1994; Shuster & Wade, 2003; Jones, 2009; Krakauer *et al.*, 2011). We calculated the opportunity for sexual selection, using variances in mating success. This may be termed the opportunity for *precopulatory* sexual selection and does not include *postcopulatory* processes. Hence, it has been advocated that the total opportunity for sexual selection, encompassing both pre- and post-copulatory processes, should be applied (Evans & Garcia-Gonzalez, 2016). It is less clear how post-mating episodes of selection affect females (e.g females are not subject to sperm competition or cryptic female choice post-mating) but considering variances in reproductive success as well as mating success may be important, especially in systems

where females benefit directly through nuptial feeding such as dance flies. We were, however, unable to measure reproductive success in this study since females were captured before oviposition.

We found I_S was similar across species: *R. longicauda* = 0.234; *E. aestiva* = 0.241; *E. tessellata* = 0.231. This similarity in I_S across species suggests that each species has a similar potential for sexual selection. Our estimates of the opportunity for sexual selection are at the lower end of the range of values of I_S values estimated in other natural studies. For example in males, values of I_S ranging from 0.25 to 2.03 were reported in a selection of highly polyandrous species (Jones *et al.*, 2001) and values of I_S ranging from 0.16 to 8.99 were reported in taxa including insects, fish, amphibians, birds and mammals (Wade & Shulter, 2004; Tatarenkov *et al.*, 2008). We could not find any studies reporting I_S for wild females. One laboratory study did report I_S in females to be lower than males in convential mating systems and similar to males in unconventional mating systems: I_S for females ranged from approximately 0.09 in conventional sytems to 0.33 in "sex role reversed" systems (Fritzsche & Arnqvist, 2013). Our estimates of I_S are therefore similar to the existing measures of I_S in females, and suggest that even in unconventional mating systems, females tend to have a lower potential for sexual selection than males.

Changes with flight period

We expected mate numbers to increase throughout the flight period, as females are likely to accumulate mates throughout their adult lives. Indeed, probable mate number did tend to increase throughout the flight period for *R. longicauda* and *E. aestiva* females, but this effect was not statistically significant. As discussed previously for *R. longicauda* in chapter 4, an accumulation of mates at the individual level may not be reflected at the population level

because females vary in both emergence date and mating success, which could lead to substantial variation in mate numbers throughout the flight period. Most *E. tessellata* samples were taken over a couple of days in the first half of the flight period. There was reasonable variation in *E. tessellata* female mate number at this time, but it is possible that we did not sample a high enough proportion of the flight period to detect an accumulation of matings in females.

The considerable variation in mating success and lack of association between mate number and date may have important implications for male mate choice. Depending on the prevailing sperm priority pattern in these systems (see chapter 6), the mating history and mating potential of females will undoubtedly affect male choice. For example, the lack of a population-wide trend for accumulating mate number in females means that there are females with a range of mating histories (and therefore possibly also sperm competition intensities and stages of ovarian development) from which a male could choose throughout the flight period we sampled.

Sexual selection on female ornaments

We found no evidence of significant directional sexual selection on hind leg area via mating success in any species despite their wide range of expression of female ornamentation. A lack of directional sexual selection was robust across both minimum and probable mate number estimates (table 5.3 & figure 5.4) and overall showed that females with larger leg areas did not achieve increased mating success. A previous study that attempted to measure sexual selection on female ornaments in dance flies did find evidence of directional sexual selection (LeBas *et al.*, 2004). A lack of pattern of measures of sexual selection with ornament expression across dance fly species could suggest that sexual selection on female ornaments

may be unstable. Our estimates of standardized selection gradients, β, ranged from -0.019 in *E. aestiva* to 0.091 in *E. tessellate* (table 5.3). These estimates are much lower than median estimates of β in the wild, which have been around 0.15 (Endler, 1986; Kingsolver *et al.*, 2001). Our standardized selection gradients are especially low when compared to sexual selection estimates specifically, where the median standardized gradient is 0.18 (mean = 0.25) (Hoekstra *et al.*, 2001).

Below we discuss several possible alternative explanations for our failure to detect significant selection on ornaments, including technical limitations of molecular markers, low power imposed by inadequate sample sizes, incorrect premises supporting the prediction, imperfect measures of fitness, and unusual features of mating systems featuring strong male choice and female ornamentation.

Technical limitations of the molecular markers

The total number of possible alleles at an individual locus place an upper limit on the conservative minimum mate number estimate for each female, which is half the allele number of the most polymorphic marker. For *R. longicauda* this upper limit was 15 males, and our estimates of polyandry rates were well below this limit (the highest minimum mate estimate was six). In *R. longicauda* at least, our estimate of polyandry was not limited by the diversity of our markers. For *E. tessellata*, the markers we used were less polymorphic than those for *R. longicauda*, and the upper limit for minimum mate estimate was six for this species. The minimum mate estimate for *E. tessellata* was 3 males, again well within the upper limit imposed by the number of possible alleles. For *E. aestiva* we had fewer and less polymorphic markers, and the upper limit imposed on the minimum mate estimate was three males. The highest minimum mate estimate for *E. aestiva* was two males, still below the upper limit

imposed by the possible number of alleles. Although we sometimes approached the theoretical upper limit, the fact that the minimum mate estimates based on allele counting were not saturated in any species makes it unlikely that our estimates of polyandry are constrained by marker sensitivity.

Notwithstanding the fact that our estimates never met the upper limit of the resolution of our markers, it is still likely that all our estimates of lifetime mating success are conservative, because we only amplified the sperm that was present in the spermatheca at the time we caught a female. It is possible that some sperm had already been used by females to fertilise eggs, or displaced by rival males prior to our sampling. Many females may also have mated again in her lifetime had we not killed them. Previous studies have all acknowledged this conservative feature of genotyping sperm to estimate mate number (Bretman & Tregenza 2005; Bussière et al. 2010; Demont et al. 2011; Hall et al. 2010; Turnell & Shaw 2015b; Turnell & Shaw 2015a).

Constraints due to sample sizes

Our sample sizes varied per species (N =53 in *R. longicauda*; N = 32 in *E. aestiva*; N = 32 in *E. tessellata*), and, especially for the less well sampled species, the low sample sizes could conceivably have reduced our power to detect directional selection. However, the coefficients in our models were nonsignificant primarily because they were small rather than because we had no confidence in the estimates, and the visualizations of selection illustrated in figure 5.4 do not suggest that power was the primary reason we failed to detect an effect. Instead, our data suggest that the true effects are likely to be modest, even for the two species in which there is a trend supporting weak directional sexual selection on *R. longicauda* and *E. tessellata*.

The prediction is wrong

The prediction that sexual selection should be strongest on the most elaborate traits is based on life history theory. Life history theory predicts that sexual ornaments should be under directional sexual selection, via increased mating success, even at equilibrium (Rowe & Houle 1996) and that the more extravagant sexual traits should require stronger sexual selection, since the most extravagant traits should also be the most costly in terms of the resources needed to maintain them. This prediction is based on patterns we see in males and it is not clear if we should expect it in females. The life history consequences of investing in costly secondary sexual traits are quite different for males and females (Houslay & Bussière, 2012), as females are likely to suffer a fecundity cost of investing in ornaments (Fitzpatrick *et al.*, 1995). This constraint on ornament evolution may help explain the general rarity of female sexual ornaments.

Mating success may not fully reflect fitness

We measured mating success as a measure of fitness, rather than reproductive success. This decision is justified by the expected role of sexual ornaments in acquiring mates. However, females with similar mating success may vary in their reproductive success, for example if it is not strictly mate number but mate quality that is the focus of female contests. In such a scenario, each mating may not be worth the same. As females benefit directly from mating through the procurement of nutritious prey-item nuptial gifts, it is possible that highly ornamented females receive larger gifts. Thus, the primary benefit accorded to more ornate females could be higher-quality nuptial gifts rather than more matings per se. Those advantages would leave no signature in our assessments of mate number inferred from analysing sperm genotypes and we were unable to test for variation in gift quality in this study.

Antagonistic coevolution and sperm competition

The lack of discernible directional selection on ornamentation in female dance flies may point to antagonistic selection. Ornament evolution is mediated through mate preference for a trait, and maintaining selection on the preferences requires a benefit to the choosy male for mating with highly adorned females. As noted in the introduction, however, this form of mate choice seems unstable when it involves polyandrous females that store sperm: any trait that promotes female attractiveness potentially undermines the benefit accrued by male mates, because more attractive and polyandrous females might provide smaller paternity shares to each of their many mates than less attractive and less frequently mated rivals. The degree to which male choice might be eroded in such a system may depend on the conditions under which sperm competition takes place. For example, if last male precedence occurs, as is common in insects, then male preferences could be primarily directed towards detecting females that will soon oviposit (in order to minimize the risk of their ejaculate being displaced by a subsequent mate). It is not clear to what extent ornaments might complement or interfere with detecting ovarian maturation. However, sclerotized traits in insects are all more or less fixed at eclosion, which makes them incapable of changing to reflect maturing oocytes (assuming the latter develop after adulthood). In the next chapter, I extract more information from the microsatellite analyses described in this chapter in order to study the conditions under which sperm competition takes place.

Conclusions

In this study, we found no evidence for directional sexual selection on female ornamentation in three species of dance flies that differed in their expression of female ornamentation. Leg traits are often the subject of sexual selection across the dance fly family, but we found that female dance flies with larger hind leg area did not achieve higher lifetime mating success.

Although there are several potential explanations, the most likely is that current selection is not strong in any of the species we studied, and does not seem to increase along with ornament expression. This result contrasts sharply with a well-justified prediction, supported by life history theory, that elaborate ornaments should be under strong directional sexual selection.

Chapter 6

The role of polyandry in the conditions of sperm competition

Elizabeth J. Herridge and Luc F. Bussière.

Abstract

In systems where females mate multiply and store sperm of multiple males, the benefits of male choice will be mediated by sperm competition. To reduce sperm competition intensity, males may deploy offence or defence strategies. Studying sperm competition in wild populations is difficult since it takes place within the bodies of females and the contributions of males cannot be detected. In wild caught females both a female's mating history and the genotypes of her mates are usually unknown. We develop methods that allow us to study patterns in sperm storage in wild caught females dance flies in spite of these constraints. We collected wild females of three species of dance fly and used microsatellite markers to assess the proportional DNA representation of males in stored sperm in the female spermatheca. We investigated how the skew in male representation in mixed sperm stores in females of the highly-ornamented species *R. longicauda* changed with varying levels of polyandry and conducted a permutation test of our data against a simulated null distribution representing a "fair raffle", where sperm representation is nearly equal among males (albeit subject to natural levels of sampling variation). Our results show that sperm stores were dominated by a single male in *R. longicauda*, and that this skew was largely independent of the number of rival males' sperm present in the spermatheca. The proportional representation of the single "winning male" differed significantly from the null distribution expected under a "fair raffle", even after allowing for random variation among males. These results are consistent with the use of "sperm offence strategies" by males in this system, and mirror patterns of sperm storage in other species in which the last mate displaces the sperm of preceding rival males.

6.1. Introduction

Polyandry is a mating system in which females mate with multiple males and is common across animal taxa (Jennions & Petrie, 2000). In species where females mate multiply, male mating success does not necessarily lead to reproductive success, because postcopulatory sexual selection can favour one of several mated males at the expense of rivals. Postcopulatory sexual selection can involve two non-mutually exclusive mechanisms: sperm competition (Parker, 1970), which consists of intrasexual contests between the sperm of different males to fertilize ova; and cryptic female choice (Eberhard, 1996), in which females favour the ejaculates of certain males over others.

The intensity of postcopulatory sexual selection should increase if females store sperm from several mates prior to fertilizing eggs (Parker, 1970; Parker *et al.*, 1996). Among many insects, females store sperm within a specialized sperm storage organ, the spermatheca, and fertilization is typically delayed until the moment when eggs descend the common oviduct just prior to oviposition. Because females can store sperm from multiple males, the spermatheca increases the opportunity for sperm competition by prolonging the residency and viability of sperm within the reproductive tract. In response, males of some species seek to reduce the intensity of sperm competition. There are two main strategies that seek to reduce sperm competition: traits that help to displace the ejaculates of prior males (often referred to as "sperm offence" characters, since they tend to involve active interactions with rival ejaculates), or traits that seek to prevent a male's own displacement by future males (known as "sperm defence" traits, because these primarily forestall displacement of the focal male's ejaculate) (Boorman & Parker, 1976).

Empirical evidence supports a wide range of sperm offence traits including sperm flushing, sperm removal, sperm incapacitation and sperm stratification (Waage, 1979, 1984; Parker & Simmons, 1991; Price *et al.*, 1999; Simmons, 2001). Both the number of rival males and the proportion of sperm displaced by the last male can influence the intensity of sperm competition experienced. If all previously stored sperm is displaced, there is no sperm competition and extreme last male precedence (Pischedda & Rice, 2012), while if sperm displacement is incomplete, the potential for sperm competition remains, the intensity of which depends on the degree of displacement. For example, if the final male displaces 60% of the previously stored sperm, he faces more intense sperm competition than a final male displacing 80% of the stored sperm. Sperm defence traits, in turn, can include mechanisms such as mate guarding and sperm plugs (Parker, 1972; Simmons & Siva-Jothy, 1998; Simmons, 2001) and function to improve fertilisation success. In systems featuring strong sperm offence traits, the last male to mate typically gains most of the paternity; while in systems featuring strong defence the converse occurs. Nevertheless, any one species can feature both kinds of adaptations.

The presence of sperm offence or defence is undoubtedly shaped to a large degree by the particular natural history and physiology of the species in question. For example, the morphology of the female reproductive tract might constrain the degree to which sperm plugs are effective, while the structure of the spermatheca may affect the degree to which sperm can mix as opposed to remaining stratified in storage. Small, spherical, inelastic spermatheca are more likely to facilitate sperm displacement (Simmons *et al.*, 1999), while tubular spermathecae or larger, more elastic spermatheca make sperm mixing more likely (Vardell & Brower, 1978; Simmons, 1986). A further strategy males can employ to reduce the intensity of sperm competition is to avoid mating with highly polyandrous females, recognizing that

they provide a lower paternity return compared to virgin females or females with lower matedness. For example, male bushcrickets *Requena verticalis* preferred young unmated females both in the lab and the wild (Simmons *et al.*, 1994). Male mate choice is an adaptation that can arise through sperm competition, as selection should favour the number of fertilisations and not just the number of matings.

In species with ornamented females, sperm competition could therefore impose a limit on ornament attractiveness, because the most attractive females may also present the highest levels of sperm competition. Sperm competition can therefore interfere with ornament evolution: if ornaments succeed in making females attractive, then males who are attracted to those females may receive smaller paternity shares thanks to heightened sperm competition. If, by contrast, ornaments do not lead to higher mating rates, their value to females is unclear.

Consistent with this apparently instability, female ornaments in Empidinae dance flies (Diptera: Empididae) have evolved (and perhaps disappeared) multiple times within the group, suggesting a high level of evolutionary lability (Cumming, 1994; Murray, 2015). In these flies, adult females appear to acquire all of the dietary protein from nutritious nuptial gifts provided by males during mating. In some species, contests for these gifts has led to elaborate ornaments. Presumably, any fecundity costs associated with investing in these sexual ornaments (Fitzpatrick *et al.*, 1995) is more than offset by the direct benefits of nuptial feeding. However, the adaptive explanation for male attraction to ornaments is unclear: there is evidence that preferences for female ornamentation vary substantially across species in the Empidinae subfamily, suggesting an instability in mate choice functions. In the species *Rhamphomyia longicauda*, female ornaments could be deceptive and mask a female's stage of egg development, since although males are attracted to females with larger ornaments

(Funk & Tallamy, 2000; Murray, 2015), females with larger ornaments are not more likely to be observed mating (Wheeler *et al.*, 2012). In a closely related species, *R. tarsata*, females with larger ornaments were more likely to be paired with a male (LeBas *et al.*, 2003). This finding may be counter-intuitive, given that females with high mating success will present males with more intense sperm competition and a lower share of paternities. Preliminary work on spermathecal morphology of dance flies suggest that some sperm displacement is likely, as females' spermatheca appear spherical and sclerotized (personal observation). If strong last male precedence is occurring males should choose the most gravid females, a strategy which may be exploited by females by disguising egg development stage using deceptive ornaments. Assessing the conditions of sperm competition in this system is crucial to determining the role of post-copulatory sexual selection on male mate choice, and how this may shape female ornament evolution.

In this study we consider the sperm competition conditions under different levels of polyandry in a wild population of three species of dance fly: *Rhamphomyia longicauda*, *Empis aestiva*, *and E. tessellata*. Dance flies (Diptera: Empidinae: Empididae) are polyandrous and store sperm from multiple males (chapter 4 & 5), creating high potential for sperm competition among males. We used differences in allele signal intensities to quantify the association between the proportion of sperm stored from a winning male (the male with the largest proportion of sperm) and number of rival males with which a female has mated. Specifically, using allelic information from genotyped sperm stores, we compare observations of skew in competing male DNA concentrations to predictions generated by alternative sperm competition processes, including the 'fair raffle', high first male priority and high last male priority. Patterns in how the proportion of sperm in storage organs changes with respect to the number of rival males may be suggestive of whether first or last male

precedence is occurring in this system. Under a 'fair raffle', each male's contribution to the stored sperm would on average be inversely proportional to the number of mates. If last male precedence predominates, we would expect a single male to achieve more insemination success that rivals. Furthermore, the proportion of sperm belonging to the largest sperm contributor should be relatively insensitive to mate number. This is because most mechanisms of sperm offence serve to displace sperm of rival males. In contrast, if first male precedence predominates, the proportion of sperm from the largest contributor should be more sensitive to mate number, since every succeeding male should conceivably take a small share from the preceding rivals. This is because most mechanisms of sperm defence serve to prevent displacement of a male's own sperm by that of future males.

6.2. Methods

Sample collection and dissections

We sampled 105 female dance flies from three species, *Rhamphomyia longicauda*, *Empis aestiva* and *E. tessellata*. All *R. longicauda* females were collected from mating swarms located in Glen William, Ontario, Canada (43°41'11"N, 79°55'34"W) during June 2012. All *E. aestiva* females were collected from mating swarms located near the Scottish Centre for Ecology and the Natural Environment (SCENE), Scotland (56°09'06.35"N, 004°38'36.20"W) during July 2012. The *E. tessellata* females were collected from mating swarms (N = 9), sweep netting vegetation (N = 1) or using Malaise traps (N = 12) during May and June of 2009, 2011 and 2015 at the following three locations: SCENE, Dollar (49°86'00.752N, 007°43'36.05"W) and near Lapanouse-de-Cernon, France (43°59'24"N, 3°05'24"E). Females were either frozen after capture at -20°C, or if caught using a Malaise trap, they were stored in 70% ethanol prior to dissection.

To isolate stored ejaculates we dissected all females to remove their spermatheca, which we then stored overnight in 100% ethanol to desiccate any sperm stored inside, allowing the contents to coagulate into a single sperm pellet for easy removal (Tripet et al. 2001). The following day we ruptured the spermatheca and removed the sperm pellet, avoiding any female tissue (Bussière et al. 2010). Sperm pellets were transferred to 180µL of buffer solution (ATL buffer from QIamp DNA Micro Kit, Qiagen) and then stored at -20°C until DNA extraction.

DNA extraction, PCR amplification, and genotyping

We extracted DNA from sperm pellets using QIAamp DNA Micro Kits, Qiagen. We followed the protocol outlined in Bussière et al. (2010), including the addition of 1µl carrier RNA to 200µL buffer AL and eluting using the smallest volume of elution buffer AE recommended (30µL). To increase DNA yield from sperm, we also added 12 µL DTT to each sample then lysed overnight at 56°C.

To allow us to check for any female tissue contamination, we also extracted DNA from the head of each female previously stored in 100% ethanol using an ammonium acetate method based on Bruford *et al.* (1992). We ground the samples using a pestle before digesting overnight at 56°C. As DNA yield was low, we dissolved the purified DNA product in 50µL of low EDTA TE buffer.

We genotyped all females and their stored sperm using microsatellite markers described in chapter 3. We used 14 markers for *R. longicauda*, seven markers for *E. tessellata* and six markers for *E. aestiva*. To reduce the number of reactions, markers were multiplexed when possible as detailed in chapter 3.

The PCR reaction volumes were 2µl, with 1µl (air-dried) DNA from female head tissue samples and 2µL (air-dried) DNA from spermathecal samples, 1µl primer mix (forward and reverse fluoro-labelled primers at 0.2 mM) and 1µl Qiagen Mulitplex Master Mix. We amplified products under the following PCR conditions: for *R. longicauda*, 95 °C for 15 min, followed by 44 cycles of 94 °C for 30 s, 56 °C for 90 s, 72 °C for 90 s and finally 72 °C for 10 min; for *E. tessellata* and *E. aestiva*, 95 °C for 15 min, followed by 44 cycles of 94 °C for 30 s, 57 °C for 90 s, 72 °C for 90 s and finally 72 °C for 10 min. We genotyped the resulting PCR products on an ABI 3730 48-well capillary DNA Analyser using GeneScan ROX 500 size standard, and scored the alleles using GENEMAPPER v3.7 software.

Quantification of insemination success

The response variable in our results is the proportion of sperm in storage assigned to the most successful male, which we term S_W for the currently "winning" male. The implication of this notation is that although we do not know the genotypes of any males, much less the order in which they mated, we can nevertheless make inferences about these based on the relative contributions of individual alleles to the total amplified DNA. Allele signal intensities are known to provide accurate estimates of DNA concentration (Bussière *et al.* 2010), so we used the relative signal intensity of alleles to determine the relative concentration of different sperm genotypes at each locus of study.

We used two mate number estimates: the minimum mate number and the probable mate number (previously reported in chapter 5) to allow us to find "informative arrays". The minimum mate number is a conservative estimate of mate number and is based on allele counting, which relies on the microsatellite markers used to be highly polymorphic with low frequencies of allelic dropout. The minimum mate number probably represents an

underestimate of the true mate number, as asserted in chapters 4 and 5. The probable mate number estimate uses population allele frequencies to calculate the most probable mate number given the genotypes observed. The probable mate estimate relies on allele frequency data from the population studied and therefore sufficient individuals need to be sampled to gain an accurate estimate of allele frequency. Both methods of estimating mate number are described in detail in section 4.2.

The first step involved in selecting "informative arrays" of genotyped sperm from the samples amplified in chapters 3 and 4. An array was considered informative if it satisfied three conditions: 1) it suggested females had mated more than once; 2) the minimum and probable estimates were either identical or differed by only 1 (which suggested that most alleles were "private", and therefore that we could use allelic intensity to make inferences about male insemination success); and 3) the allele(s) belonging to the most successful male could be distinguished from those of rivals with confidence.

Both allele peak height and area are measures of allele signal intensity, but as alleles with very high intensities have the tendency to "spread out" during electrophoresis, we used the area under each allele peak as allele signal intensity in our analyses. We used allele signal intensity to assign individual allele peaks with the highest signal to S_W males (figure 6.1). The female in the top panel of figure 6.1 fulfils all three conditions for an informative array. First, the female has mated more than once, since there are three male alleles present. Second, the minimum and probable mate number estimates are the same, in this case, two. Third, since there are three alleles, one male must be a homozygote. Since the left-hand and central alleles match in size, it is likely that the right-hand peak represents a single homozygous male. In this instance, we divided the height or the homozygote peak by two: if the higher

peak was more than twice as high as the heterozygote peaks (as it is in this case), S_W was assigned to the homozygote; if the higher peak was less than double the height of the heterozygote peaks, S_W was assigned to the heterozygote male. The female in the second panel of figure 6.2 also fulfils all three conditions for an informative array. The female has mated more than once, since there are four male alleles. The minimum and probable mate numbers are both two. Since there are four alleles, the males must be two heterozygotes. In this instance, the heterozygote with the highest matching peaks was assigned S_W. When matching allele peaks for heterozygote S_W males, the smaller of two peaks had to be at least 61% of the magnitude of the larger peak (Gilder *et al.* 2011), although the vast majority of heterozygote peaks were much more similar in height than this (see Results). We then calculated the relative signal intensity of S_W males as the proportion of the total signal intensity (sum of allele peak areas) of alleles in an array.

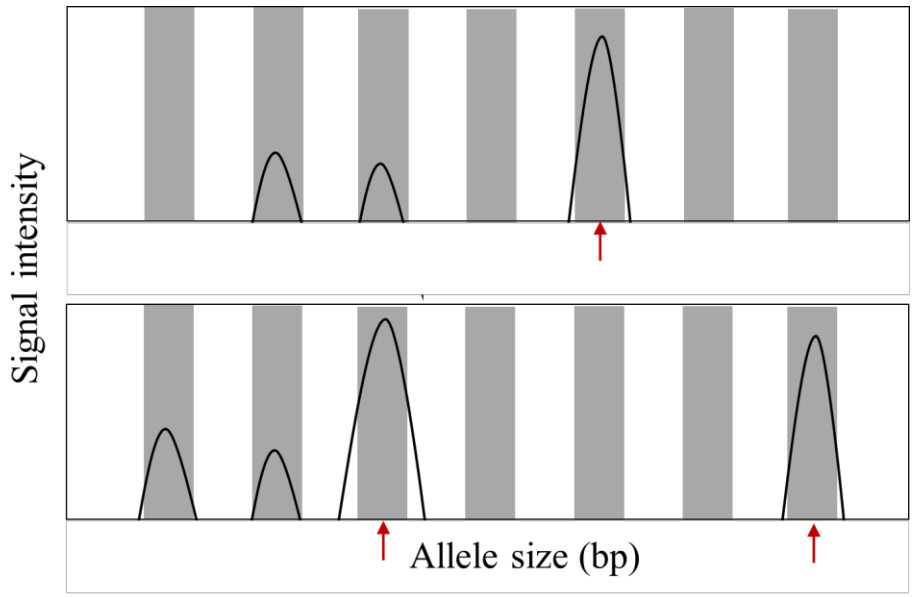

Figure 6.1. Cartoon electropherogram to illustrate how we assigned alleles to S_W males from DNA extracted from a pool of sperm from two males in each panel. Note that because the minimum and probable mate numbers are similar, it is likely that individual alleles are private (see methods). Consequently, each peak represents either half (for heterozygotes) or all (for homozygotes) of the contribution a male makes to sperm in storage, which can be quantified by the area of the peak. Red arrows indicate allele peaks assigned to S_W males in each case. The S_W male in the top panel is probably a homozygote at this locus and has one informative allele (because the highest intensity peak has no "partner" peak of similar size, as would be expected for a heterozygote), whereas the S_W male in the bottom panel is probably a heterozygote, and has two informative alleles.

Because the number of informative arrays was too small for both *E. tesselata* and *E. aestiva*, we focussed on the species *R. longicauda* in our analysis of whether the winning male's insemination success changed with accumulating mate number. As outlined in the Introduction, if insemination success of the winning males is relatively insensitive to the number of rival males, that suggest offensive sperm competition strategies are predominating, whereas a substantial decline in insemination success with increasing rival mate number would suggest either a fair raffle or defensive strategies. We used a linear model to predict the proportion of winning male sperm from the number of rival males, which we modelled as

factorial predictor to account for any nonlinear effects. As the estimates we used were based on different loci, we also included locus as a predictor in the model.

Modelling the distribution of S_W assuming a fair raffle

We used the expectations given a fair raffle as a null model against which to test our findings of S_W. However, our null expectation was not simply an equal share for all males. Instead, we wanted to recognize that males would differ from one another in their precise insemination success even in the absence of sperm displacement or stratification mechanisms. Thanks to such errors along with the fact that we always focussed on the most successful male, equal paternity shares were a theoretical minimum value in our analysis that was likely to be exceeded even if only by chance. For example, we could never have observed a male contributing less than 50% to a twice-mated female because we always focussed on the more successful inseminator. Even the two alleles of a single individual do not have exactly the same peak intensity, which can be explained by allele size or sampling error exaggerated by low starting DNA concentrations. We wanted to test whether the variation observable between alleles of heterozygotes (where the clear expectation is of equal contribution to both allele intensities) could have produced estimates as far above the null as those we observed in our arrays. To do this we simulated three normal distributions where the mean was either 0.5, 0.333 or 0.25, the mean expected under an ideal "fair raffle". For each of these means we set the variance in the distribution equal to the observed variation we found between heterozygote S_W alleles. For example, to estimate the null distribution of proportion S_W when one rival male was present, we simulated a normal distribution with a mean of 0.5 (such that each male contributed half of the stored sperm) and a standard deviation of 0.1222 (equal to the standard deviation for paired S_W allele peaks in our observed data). We then restricted these simulated data to values of greater than 0.5 to mimic our sampling procedure of

focussing only on winning males. For each iteration of our resampling procedure, we computed a mean S_W based on 19 samples (equal to the number of samples in our study), and resampled from the distribution 999 times to produce a null distribution of expected means. We then added our observed mean to these 999 trials, and used twice the value of its rank among the 1000 observations as the two-tailed probability that our observed mean could have arisen under a "noisy" ideal lottery scenario. We repeated this simulation procedure to produce null distributions for S_W plus two rival males, using an expected mean of 0.333 (each of the three males contributing a third of the stored sperm), and for S_W plus three rival males using an expected mean of 0.25 (each of the four males contributing a quarter of the stored sperm).

6.3. Results

In chapter 5 we genotyped the sperm stored in 105 females of three dance fly species, *Rhamphomyia longicauda*, *Empis aestiva* and *E. tessellata*. Seventy-two females had mated with more than one male. From these polyandrous females, we excluded 13 because the minimum mate estimate differed by more than 1 from the probable mate number. We then further restricted our data to 32 females that provided informative arrays from genotyped mixed stored sperm. We were very conservative in our selection of informative arrays to be confident that the allele(s) belonging to the most successful male could be distinguished from those of rivals. The number of informative arrays across species were as follows: 19 *R. longicauda*, 3 *E. aestiva*, and 10 *E. tessellate* (figure 6.3).

The contribution of S_W to stored sperm in all three species ranged from 0.261 to 0.904 with a mean of 0.714 (median = 0.73) (figure 6.2). On average, the smaller allele peak assigned to

heterozygote S_W males had an intensity of 0.814 ± 0.052 (mean ± SE) relative to the larger peak (range from 0.985 to 0.649).

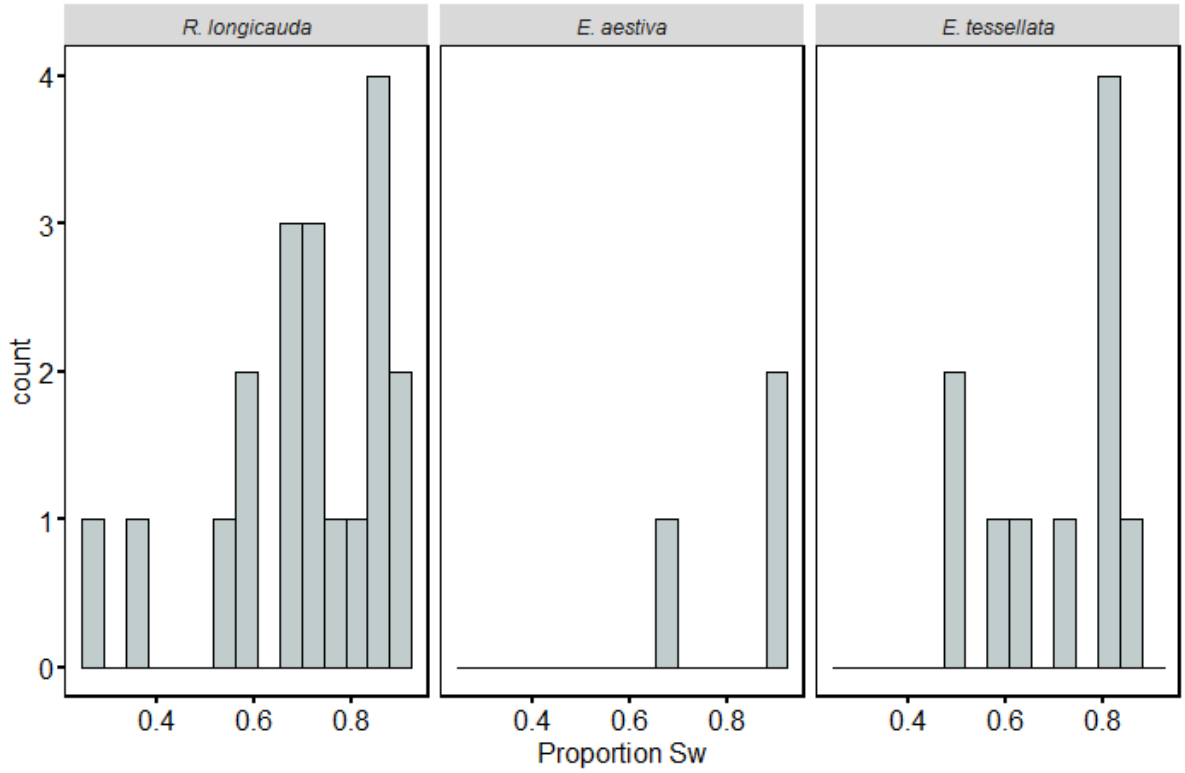

Figure 6.2. Frequency histograms of the proportion of S_W sperm in spermatheca for all three dance fly species.

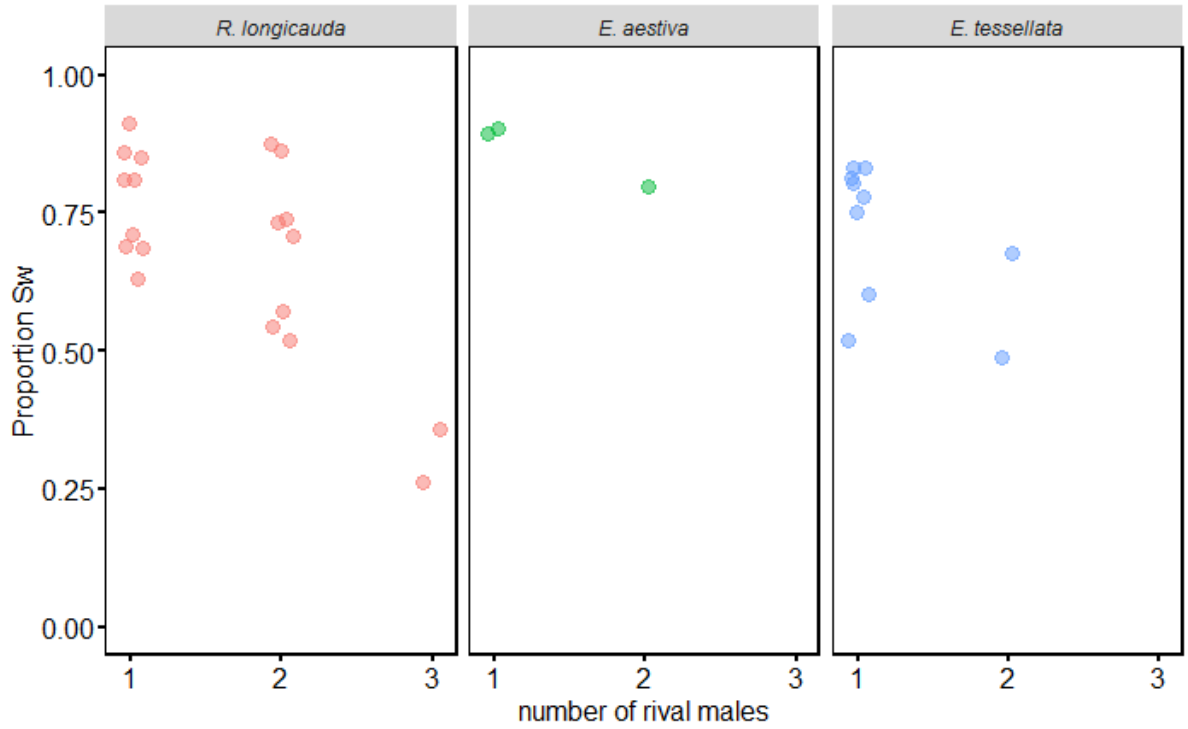

Figure 6.3. Proportion of S_W sperm in a female's spermatheca plotted against the number of rival males present for three species of dance fly: *Rhamphomyia longicauda* (pink), *Empis aestiva* (green), and *E. tessellata* (blue).

How does the proportion of S_W sperm change with the accumulating mate number?

To determine whether the proportional S_W sperm changed with accumulating mate number we focussed on the species *R.longicauda*, the only species for which we had an appreciable sample at more than one mate number. We used a linear model to predict the proportion of S_W sperm from the number of rival males (table 6.1).

The proportion of S_W sperm when one rival was present in the spermatheca was estimated at 0.753 (95% CI: 0.624 - 0.88) (figure 6.4). The mean proportion of S_W sperm when two rival males were present in the spermatheca did not differ significantly from the value when only one rival was present (table 6.1; figure 6.4). When three rival males were present, however,

this proportion was significantly reduced, although this conclusion is based on only two informative arrays of amplified sperm featuring 3 rivals (table 6.1; figure 6.4). Our model produced qualitatively consistent results when we excluded four estimates for which we were less confident about the number of rivals present in the spermatheca (table 6.2, which is based on the remaining 15 individuals). For these four females, the heights of two allele peaks suggested the presence of another rival that was undetected in the analyses from Chapter 4. For example if there were four allele peaks present in an array, and both minimum mate and probable mate estimates suggested the female had mated with two males, then the assumption would be that both males present were heterozygote. However, if the two larger allele peaks, which would normally belong to a heterozygote S_W male, were of sharply different areas (e.g., we found the smaller of the peaks had a height less than 0.61 of the magnitude of the larger peak), it was likely that another male was present in the mixed sperm store.

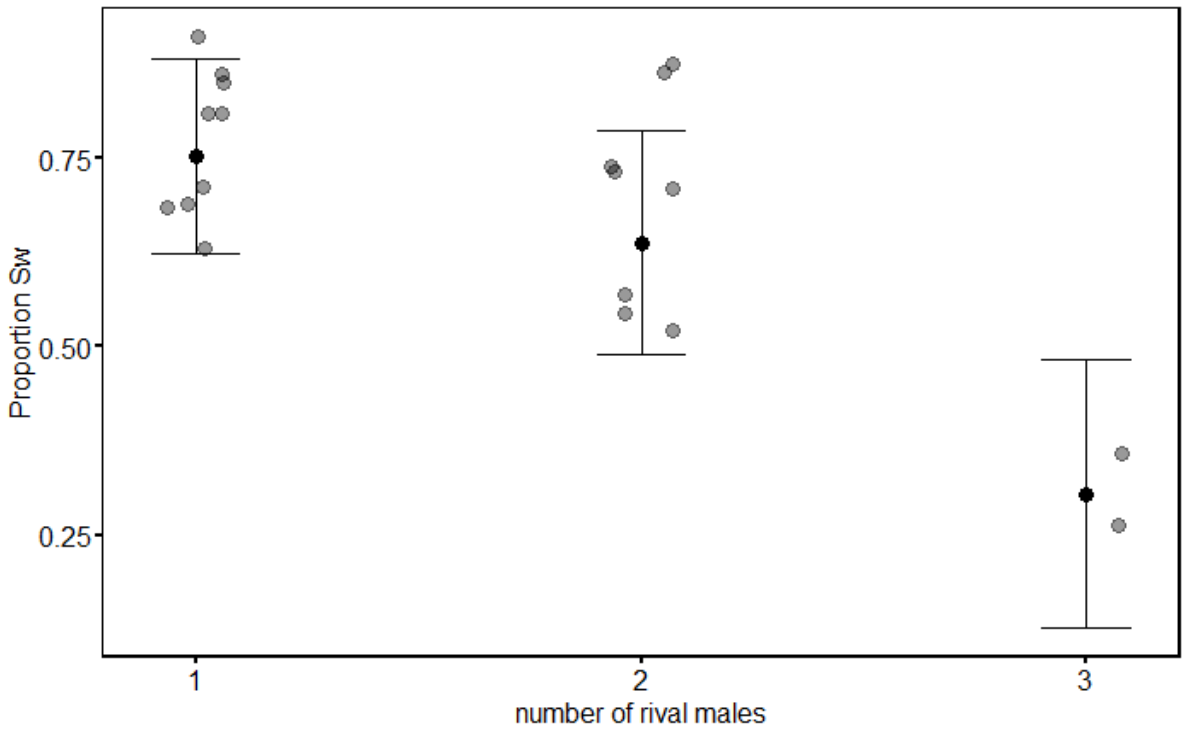

Figure 6.4. Proportion of S_W sperm against number of rival males in the spermatheca of female *Rhamphomyia longicauda*. Grey points are raw data, black points are the model estimates of the partial effect of the number of rival males on the proportion of sperm for locus RL1BWMXW. Error bars indicate the 95% confidence intervals.

Table 6.1. Results of a linear model predicting proportion S_W from the number of rival males and loci.

term	estimate	std.error	*T* value	*P* value
Intercept	0.904	0.115	7.864	<0.001
2 rival males	-0.115	0.074	-1.551	0.147
3 rival males	-0.449	0.101	-4.458	<0.001
Locus RL1AXKU5	-0.117	0.134	-0.875	0.399
Locus RL1B5KYR	-0.026	0.152	-0.170	0.868
Locus RL1BWMXW	-0.150	0.129	-1.163	0.268
Locus RL1CUOWO	-0.113	0.133	-0.855	0.409

Table 6.2. Results of a linear model predicting proportion S_W from the number of rival males and loci after removal of four females.

term	estimate	std.error	*T* value	*P* value
Intercept	0.904	0.126	7.183	<0.001
2 rival males	-0.106	0.095	-1.115	0.297
3 rival males	-0.465	0.112	-4.167	<0.01
Locus RL1AXKU5	-0.141	0.148	-0.950	0.370
Locus RL1B5KYR	-0.065	0.202	-0.324	0.755
Locus RL1BWMXW	-0.134	0.143	-0.941	0.374
Locus RL1CUOWO	-0.113	0.145	-0.781	0.457

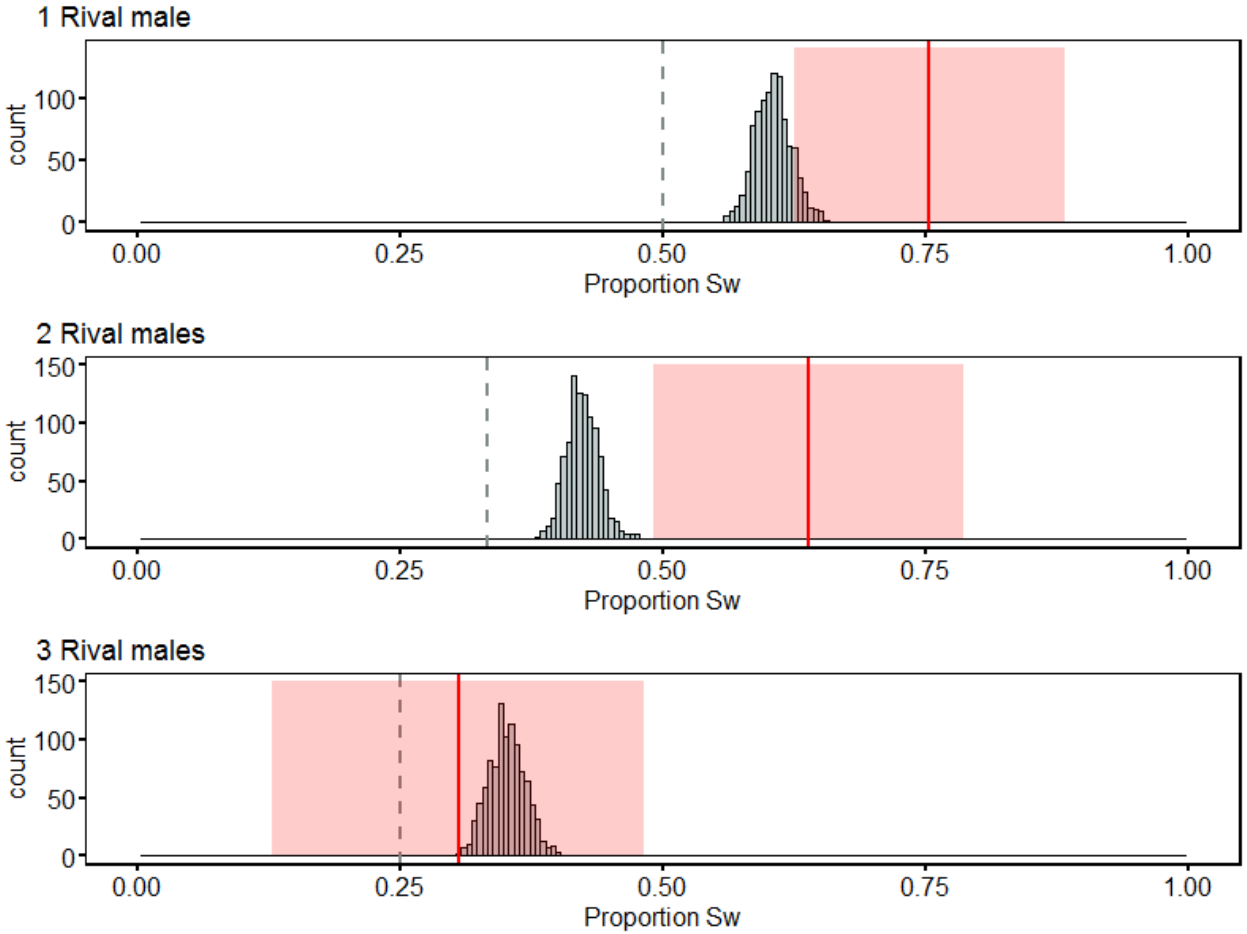

Figure 6.5. The red line indicates the model estimates of the observed proportion S_W, and the shaded red area indicates 95% confidence intervals around the model estimate. Dashed grey lines indicate the theoretical minimum proportion S_W under a "fair raffle" scenario. The frequency histogram illustrates the distribution of mean S_W assuming equal mixing of sperm for 1, 2 and 3 rival males, but incorporating noise from unequal amplification of alleles.

How does the proportion S_W compare to a "fair raffle"?

We simulated data to produce three null distributions of the proportion of S_W expected in "fair raffle" scenarios with either one, two or three rivals males (assuming only measurement error in allelic intensities of the magnitude we found within genotypes; see figure 6.5). The proportion of S_W were significantly higher than expected under this null model of the "noisy fair raffle" model when there were one or two rival males present ($P = 0.001$). For situations where three rivals were present, the estimated proportion S_W was significantly lower than the null expectation of the "fair raffle" ($P = 0.002$). However, as noted above, our estimate of S_W when three rival males were present is based on only two observations, and the 95% confidence intervals around this estimate overlapped with both the null distribution and the theoretical minimum (figure 6.5).

6.4. Discussion

In most species, studying sperm competition in the wild is a difficult task: the mechanisms involved are usually concealed within females, and the evidence of distinct male contributions is often ephemeral and cryptic. Some previous laboratory studies that amplified stored sperm within insects have clarified some of the conditions of sperm competition, but the genotypes of competing males and complete mating histories of females were known with certainty in those cases (Bussière *et al.*, 2010; Hall *et al.*, 2010). In wild caught females we usually cannot know either a female's mating history or the genotypes of her mates. We sought to develop methods that allow us to study patterns in sperm storage in wild caught females dance flies in spite of these constraints. Our approach exploited the fact that allelic intensities should be proportional to the representation of sperm genotypes in storage, and that divergent

models of sperm competition processes predict distinct patterns of sensitivity for the most successful male to increasing numbers of rival males.

We amplified sperm stores in an attempt to make inferences about the context for sperm competition in three dance fly species: *Rhamphomyia longicauda*, *Empis aestiva* and *E. tessellata*. To do this we focussed on a strict subset of samples in order to ascribe alleles to inferred male genotypes with some confidence. Using this subset of informative arrays, we found patterns consistent with a single sperm competitor, which we termed the currently 'winning male' (S_W, to reflect our uncertainty about that male's place in the sequence of mates), appearing to dominate the proportion of sperm stored in females (figure 6.3). We then focused on one species, *Rhamphomyia longicauda*, to formally assess how the skew in DNA concentrations of the S_W sperm changed with varying number of rival sperm competitors present. We found that the proportion of S_W sperm stored in females was similar when there was only one or two rival males (table 6.1; figure 6.4). When three rival males were present the proportion of S_W sperm was significantly lower (table 6.1; figure 6.4), however this finding is based on only two observations. In our discussion, we begin by carefully considering the assumptions and mechanics of our newly developed approach, before proceeding to discuss the broader implications of our findings for dance fly mating systems and the study of post-copulatory sexual selection in general.

Methodological considerations

In this chapter we used amplified sperm stores to make inferences about the context for sperm competition in the wild using DNA from males for which we did not know the genotypes. To do this successfully we excluded many genotype arrays of mixed sperm

DNA which we considered uninformative about the process of sperm competition. We justify this exclusion on the basis of uncertainty concerning male genotypes and female mating history, which cannot be known with certainty for wild females. Nevertheless, proceeding in this way dramatically reduced the sample size, and prevented us from formally analysing our findings across species as we had originally intended; instead we focussed our statistical assessment of how rival number affected S_W on one species, *Rhamphomyia longicauda*, for which we had a reasonable number of informative arrays. We consider the implications of each condition for the quality of our inferences below.

The first condition was that the array had to suggest a female had mated more than once. To satisfy this condition we used two mate number estimates, minimum mate number and probable mate number (reported and described in more detail in chapter 4 & 5). These mate number estimates use allele counting (minimum mate number) as well as the frequency of an allele in the population (probable mate number), and arrays satisfied this condition if either estimate was greater than 1. We used this condition because sperm competition only occurs in polyandrous females, but it is possible that some excluded females had mated more than once, but with males of identical genotypes (especially likely for low allelic diversity loci). Alternatively, we would also have missed females in which sperm priority or displacement is complete (such that one of the males has no detectable contribution to sperm stores). In either case, we expect a downward bias in our estimate of the average contribution of the most successful male's sperm; our estimate that S_W is 0.70 is therefore more accurately an estimate of S_W within females with multiple detectable male genotypes in storage.

A second criterion for including a sperm array was that the minimum and probable mate number estimates from chapters 4 & 5 either be identical or differ by only 1. This second condition was intended to improve our confidence that most alleles were "private", which allows us to use allelic intensity to make inferences about male insemination success (obviously, sperm of the same genotype that originate from different males cannot be distinguished using our molecular approach). We do not think that restricting arrays to satisfy this condition should have caused any bias in our results. However, one remote possibility is that by restricting the arrays in this way, we overrepresented the contributions of S_W males with rare alleles. If by chance these rare alleles were associated with any traits involved in sexual selection, that might skew our assessment of the average performance of males in the whole population. Because the majority of our markers were in Hardy-Weinberg equilibrium (chapter 3), and because we further did not find any effects of loci in our results (table 6.1 & 6.2), there is no compelling reason to suspect that selection is affecting our estimates in either direction.

We then considered the allelic intensities of those arrays that passed the first two conditions to assess whether the remaining arrays satisfied condition three, that allele(s) belonging to the most successful male could be distinguished from those of rivals with confidence. During this stage we assigned one or two alleles to an individual male that was currently the most successful, or "winning", which we termed S_W. As we had already restricted the arrays to those where the probable mate number was similar to the minimum mate estimate, it was likely that most alleles were "private". Therefore if the number of alleles present in array was an odd number, we knew there was likely to be a homozygote present in the array. Knowing this helped us to assign either a single allele peak or two allele peaks to a S_W male. When matching allele peaks for heterozygote S_W

males, we used a rule that the smaller of two peaks had to be at least 61% of the magnitude of the larger peak (a cutoff based on the observations of Gilder *et al.* 2011). The vast majority of heterozygote peaks were much more similar in intensity than this, confirming for most females that a single male was likely to account for the top pair of alleles. However, this process also highlighted four instances in which the allele intensities could not easily be grouped into matched pairs, and therefore suggested the presence of an additional male that had not been revealed by either the minimum or probable mate estimates. The findings confirm our suspicion that both minimum and probable mate counts underestimate polyandry. Furthermore, the rate at which we encountered this situation suggests a lower limit for the bias in these measures: since four out of 19 of our females fell in this category, we can infer that at least a fifth of our mate number estimates in chapters 4 and 5 were too conservative. Future studies of polyandry in wild females could similarly use allele matching methods to infer the genotypes of mates, and gauge the degree of underestimation in mate numbers.

We were conservative in our selection of informative arrays, but such restrictions are necessary to be confident that the allele(s) belonging to the most successful male could be distinguished from those of rivals. Our approach could consequently be used in other studies assessing contributions to stored sperm, but in most systems authors will need large sample sizes of females to achieve enough statistical power to draw any conclusions.

Are males using sperm offence or sperm defence strategies?

The relative stability of the proportion of S_W sperm when comparing males facing one and two rival situations is consistent with expectations for a system where sperm

offence strategies predominate (figure 6.4 & table 6.1). For example, if the last male displaced much of the previously stored sperm, his success in doing so should not depend substantially on the number of prior mates. In contrast, strategies to defend against subsequent rivals might gradually be eroded by following males, which would lead to a gradually declining rate of success as a function of rival number. Although patterns of paternity observed in offspring can be determined by many factors, factors such as sperm displacement predicts last male precedence to be high (Simmons, 2001).

Furthermore, the proportion of S_W sperm in *R. longicauda* females, which was approximately 0.7 on average, is similar to that seen for the last male to mate (S_2) in yellow dung flies, which exhibit last male precedence (Parker & Simmons, 1991; Gwynne *et al.*, 2007). Last male precedence brought about through sperm displacement is common in insects. Studies determining the mechanisms of sperm displacement are not common but it is likely to be a process that involves the volume of sperm transferred to flush out any residing sperm. For example, the tree cricket *Trujalia hibinonis* flushes out any previous male's sperm by ejaculating into the anterior portion of the spermatheca (Ono *et al.*, 1989).

Sperm offence strategies such as sperm flushing can be facilitated by the physical characteristics of a female's spermatheca. The spermatheca of *R. longicauda* is spherical and sclerotized (personal observation), which contrasts sharply with the relatively elastic spermathecae found in many other species in which sperm competition has been studied, including *Teleogryllus commodus* crickets (Hall *et al.*, 2008). The spherical and inelastic nature of the *R. longicauda* spermatheca makes sperm flushing a plausible mechanisms affecting sperm competition in this species.

Studies assessing the relative contributions of individual males to sperm stores are scarce (Bussière *et al.*, 2010; Hall *et al.*, 2010; Manier *et al.*, 2013), and generally conducted in a laboratory where the mating history of a female is known. Most mechanisms generating non-random paternity are usually inferred from studies assessing the proportion of offspring sired by the second of two mates, or "P2". In such studies, a high P2 indicates last male precedence. However, even in these studies considering a simple situation where a female mates with two males of known genotypes, we still find remarkable variation in paternity patterns: 44% of species show P2 bias, 43% of insect species show mixed sperm utilization, and 12% show P1 bias (Simmons, 2001).

Sperm competition involves several different episodes in the mating sequence, and can be mediated by processes occurring in each of many stages, such as during sperm transfer, storage, or use at the time of fertilisation. We focussed on stored sperm, which is likely to reflect processes in the first two stages, but obviously ignores any effects of contests to reach unfertilized ova. The degree to which such contests can also be affected by mate order and duration remain unknown.

Although we were unable to formally analyse sperm storage in *E. aestiva* and *E. tessellata*, the sperm storage patterns in the few arrays that were informative appeared similar to *R. longicauda* (figure 6.3), with a high proportion of S_W sperm in females where SW was competing with one or two other males. These trends are consistent with offence strategies.

How does S_W compare to expectations for an ideal lottery or a noisy fair raffle?

The proportions of S_W sperm when one, two or three rival males were present were always higher than the expectation of an ideal lottery, but this was inevitable given our sampling procedure. We simulated a "noisy fair raffle" null model to test whether the high proportion of S_W sperm was an artefact of our sampling procedure. The "fair raffle" model developed by Parker *et al*. (1990) works on the idea that if males contribute equally to a sperm store, and there are no other factors affecting paternity (such as displacement or the competitive ability of each male's sperm) then each male will get an equal share of fertilisations. For example, if two males mate with a female and contribute equally to the sperm store, each male would sire 50% of the offspring. The fair raffle is a useful and simple null model against which to compare our data on contributions of males to stored sperm. All of our observations suggested that males did not contribute to sperm stores in proportion to the number of makes, suggesting our data did not meet the expectations of a "fair raffle".

We based our "noisy fair raffle" on Parker's fair raffle concept, but incorporated some expected variation between males that is simply due to errors in amplifying DNA. Because we always focussed on the most successful genotype and could not verify this male's place in the sequence of mates), it is inevitable that our estimates should have exceeded the theoretical minima suggested by the fair raffle: any small differences in amplification would necessarily tip one male above the other and cause S_W to exceed null expectations by chance. Consequently we used our own observations of variation in allelic intensity within a male to model a "noisy fair raffle" in which the upward bias away from the theoretical minimum was expected purely through uncertainty in allelic

quantity, rather than any underlying process that skewed sperm storage to one male over rivals. The resampling of null expectations under a noisy raffle confirmed that our observations were not merely a consequence of amplification error. For example, the mean of the "noisy fair raffle" null distribution for S_W plus one rival male present (total of two males) was ~0.6 (figure 6.5).

We found the proportion of S_W were significantly higher than expected under this null model of the "noisy fair raffle" model when there were one or two rival males present (figure 6.5). When three rivals were present, the estimated proportion S_W was significantly lower than the observations for both one or two rivals as well as the null expectation from the "noisy fair raffle". Our confidence in the S_W estimate for 3 rivals (4 males) is low as we only had two observations where a female had mated with four males in this analysis. Clearly, a more robust sample would help boost confidence in this curious finding. If it is an accurate estimate, one possible explanation for the sharp drop in S_W compared to the other two levels of sperm competition intensity is that the mate numbers in this group are serious underestimates. If a few females mate many times but we cannot reliably detect more than a few genotypes, then the null expectations we used for this group are too high. Despite our efforts to extract tiny amounts of DNA using forensic extraction kits, we might have underestimated mate number if the DNA of some males in the sperm stores was not initially extracted due to low concentrations of starting tissue. If there was a real shift in the proportions of S_W sperm between 2 and 3 rival males in sperm competition then it may suggest that mate order effect may disappear after a female mates with two males. For example, in the harlequin beetle-riding pseudoscorpion last male sperm precedence patterns break down when a female mates with three males (Zeh & Zeh, 1994). This would suggest that we

cannot extrapolate the pattern seen when females mate with two or three males to females mating with more than three males.

Implications for male choice

The conditions under which sperm competition occurs is likely to have implications for male mate choice. For example, if sperm defence predominates, one might expect strong selection for males to avoid previously or recently mated females in favour of virgins. Such a scenario would make the evolution of female ornaments highly unlikely, because any trait that improves attractiveness necessarily erodes the reproductive value of its bearer as she accumulates mates. By contrast, if sperm offence predominates, males can displace the sperm of rivals even in highly polyandrous females. In such circumstances, of course, most of the selection on males would be to find females who are unlikely to mate again prior to oviposition. In this scenario, we can imagine that females might exploit male preferences for cues of ovarian maturity, and any ornament that seduced choosy males searching for gravid females might evolve in spite of a poor relationship with reproductive value.

Conclusions

In summary, we developed an approach that uses amplified sperm stores to make inferences about the conditions of sperm competition in wild females, in spite of not knowing a female's mating history or any of the male genotypes. Our approach involved careful selecting informative arrays of sperm alleles, which unfortunately constrained our sample size. However such restrictions are inevitable to be confident that the allele(s) belonging to the most successful male can be distinguished from those of rivals. We exploited our newly developed method in three species of dance fly,

assessing the representation in mixed sperm stores of the winning male, S_W, in 32 females. We further studied how this representation declines as a function of the number of rivals in *R. longicauda*. We found evidence that stored sperm was dominated by a single male in all species. In *R. longicauda*, this skew was relatively insensitive to the number of rival males' sperm present in the spermatheca. These results are consistent with strong sperm offence strategies, and with patterns of sperm storage seen in other species where males at least partially displace the sperm of rival males.

Chapter 7

General discussion

In this thesis, I have assessed the rate of polyandry and the conditions of sperm competition, and explored the role these phenomena might play in sexual selection among dance flies (Diptera: Empididae: Empidinae). I this final chapter, I summarise the main findings of the preceding chapters below, before drawing some general conclusions about how this thesis contributes to explaining diversity in sexually dimorphic traits and other expensive life history characters.

In Chapter 2, I reviewed the causes of sexual differences in mating and parental sex roles, including parental investment. I then went on to describe some as yet unanswered questions around the processes controlling diversity in sexual behaviour, including the extent to which differences in parental care are a primary cause of the general sex role syndromes (i.e., the fact that males can avoid care may be what allows them to maximize fitness by mating repeatedly), or rather mainly a consequence of differences in sexual selection (i.e., anisogamy fundamentally sets the stage for mating differences in post-natal care, which emerge primarily thanks to sex differences in selection on mating success). Finally, I suggested that we could study how sexual selection covaries with sexual trait expression in species with unusual mating systems to help these

questions, because such systems can feature atypical costs and benefits that allow tests of life history theory. In the remaining empirical chapters, I studied one such group of unusual organisms: dance flies of the subfamily Empidinae that feature remarkable variation in the presence and expression of female sexual ornaments.

Documenting polyandry rates is difficult in wild systems, and this is especially true for flying insects, like dance flies which have large population sizes. This difficulty stems from the fact that tracking individual females and using behavioural observations to record each mating event is not feasible. In chapter 3, I developed microsatellite markers for several species so that we might estimate mating rates in females by genotyping DNA extracted from the mixed sperm stores of wild females. Initially, we designed markers with the potential for high cross-species utility by first searching unenriched sequence data of seven dance fly species for conserved regions. Unfortunately, there were very few conserved regions from which we could design primers, and the few we did design were not polymorphic. We then sequenced a further four species, this time enriching to increase the yield of sequences containing repeat regions, which we hoped would provide a higher potential for developing markers with cross species utility. Sadly, attempts to develop markers using enriched libraries failed twice, producing spurious results that were not polymorphic. We did however successfully develop markers separately for three dance fly species, *Rhamphomyia longicauda*, *Empis aestiva* and *E. tessellata* using unenriched sequence data.

High levels of multiple mating by females had been predicted for *R. longicauda* (Funk & Tallamy, 2000), a species with multiple elaborate female ornaments, but had never been documented. In chapter 4, I documented wild polyandry rates in this system and

found them to be similar to many insect species in which female ornaments have not evolved. Life-history theory suggests that costly ornamental traits should be under strong directional sexual selection, since without strong selection, the costs of maintaining ornaments might outweigh the benefits (Stearns, 1989). To test the prediction that ornamental traits would be under strong selection, I measured the association between ornament expression and mating success, and found no significant evidence that females with larger ornaments mate more frequently. There was a small, but not significant association between ornament expression and mating success. This finding was consistent with previous cross-sectional analyses of *R. longicauda* females, and confirmed the puzzling observation of, at best, weak selection in spite of highly exaggerated ornamental traits. As females are thought to directly benefit from mating through nuptial feeding, I further measured the effect of mating success on the number and size of developing eggs remaining inside the females when caught. Once again, I did not find an association between the frequency of mates and either the number or size of the eggs still present. Although we would expect mate number to have a positive effect on egg development for an individual female (because each mating should provide some nutrition to the female), this effect might not be discernable at the population level for two reasons. First, some sampled females may have already laid eggs; second, the benefit of some matings might not yet be evident in ovarian development (because of the lag between taking a meal and maturing the eggs).

Although we found no evidence to support strong selection on ornaments, we did find a strong association between overall body size and egg size. As ornament expression covaries with body size, ornaments could theoretically still signal a female's potential fecundity. Such covariance has been posited as the basis for concluding "signal

honesty" in other species with sexual ornaments. However, whether these traits actually signal reproductive value is more complicated, because females in this species, like many insects, store sperm. Consequently, the value of a female to her mate depends not only on her total fecundity, but on his chances of siring individual offspring. In *R. longicauda*, the majority of females had mated multiply, which suggests that males typically face a minimum level of sperm competition. Therefore, even if a male chooses a female with high potential fecundity, he may only sire a small share of her offspring. The degree to which ornaments actually reflect reproductive value to males is therefore unclear. To the extent that ornaments are effective in attracting more males (an open question given the weak association in chapter 4 between ornament expression and mating success), then males might actually benefit from avoiding the most heavily ornamented females. Such conflicting pressures on male choice (on one hand, favouring signals of high fecundity, while on the other disfavouring signals of high mating frequency) could lead to dynamic changes over time in selection on male mating preferences.

In fact, the lack of directional sexual selection on female ornamentation found in chapter 4 is not consistent across all ornamented dance fly species. In *R. tarsata*, female ornamentation appears to be under escalating sexual selection, with heavily adorned females attracting many more mates than rivals with smaller ornaments (LeBas *et al.*, 2003). Even within *R. longicauda,* selection on ornaments is not consistent over all episodes of the mating sequence. Males are initially attracted to larger female silhouettes (Funk & Tallamy, 2000), and when given the choice, males prefer silhouettes of females with larger ornaments (and especially larger abdomens) (Murray 2015). However, the most ornamented females are not more likely to be found in a

mating pair (Wheeler et al., 2012), which suggests that initial attractiveness of a female does not always result in a mating (see further discussion below).

Increasing evidence of sexual conflict and the lack of consistency in the nature of selection in dance flies suggested that male choice for ornamented females in dance flies is perhaps more dynamic and unstable than we initially predicted. To comparatively test the hypothesis that more exaggerated sexual ornaments are under stronger directional selection, in chapter 5 we compared measures of sexual selection on females in several dance fly species with varying levels of female ornamentation.

As discussed above, the most extravagant secondary sexual traits should be under stronger sexual selection, and it is generally assumed that the most sexually-dimorphic species are also those with highest variation in mating success for the more competitive sex (Arnold & Duvall, 1994; Shuster & Wade, 2003; Jones, 2009; Krakauer *et al.*, 2011). To test the prediction that sexual selection should be strongest on the most elaborate traits, I compared standardized selection gradients on female leg traits in the highly ornamented *R.longicauda* (chapter 4) to those for two other species: *Empis aestiva*, in which females have independently evolved pennate leg scales; and *E. tessellata*, where females do not possess any discernible female-specific ornaments. Overall I found no evidence for directional sexual selection in any species, despite the wide range in expression of female ornamentation. It is possible that female fitness does not covary straightforwardly with mating success, and that each mating is not equal in terms of fitness gains: for example, males may provide larger gifts to the most ornate females. Alternatively, our lack of evidence for selection could, in principle, be linked to highly ornamented females obtaining indirect genetic benefits from mating

multiply. Females could mate multiply to gain enough nutritious gifts, but beyond a threshold may not need to remate unless there are indirect benefits such as 'good genes', enabling them to produce sons that outcompete other males in sperm competition. If there were some form of assortative mating, such that the males with the best gifts and most competitive sperm preferred the most ornamented females, this could even lead to Fisherian 'runaway' processes, with females producing attractive, highly ornamented, daughters and sons attracted to ornamented females. As such, the most highly ornamented females would not gain more matings; they would simply be mating with males of higher fitness. However, if our main findings (that selection is not strong in any of the species we studied and the strength of selection does not increase along with ornament expression) are true, they contrast sharply with the well-justified prediction that sexual selection should be strongest on the most elaborate traits.

Antagonistic coevolution between deceptive females and discerning males

One possible explanation for our results is that the nature of selection on both sexes is more complex than in classic scenarios of honest male signals and female choice. Understanding this explanation requires a careful consideration of the conflicting interests of males and females within mating swarms. In dance flies, females have apparently lost the ability to hunt, and so rely on males for prey items that provide females with the resources to mature their eggs. Females appear to be using mating as a chance to forage: all of our sampled females (and nearly all samples collected from mating swarms in other studies; see Wheeler *et al*., 2012) already contain sperm, and so do not strictly need gametes from males within mating swarms. Instead, their heightened sexual receptivity (leading in many cases to overt contests for access to

males) is explainable by competition for food resources. Each female would like to acquire more mating simply because each mating is accompanied by another meal. Males, by contrast, must approach mating swarms with more care than is usual for swarming flies that do not feature nuptial gift transfer. This is because each mating requires a nuptial gift resource that is almost certainly lost when the male passes it to his female during coupling. Obtaining nuptial gifts requires time and effort, and undoubtedly contributes to the female biases in mating swarms of many dance fly species. Consequently, given a superabundance of females at the mating swarm, a male should choose carefully among his willing partners and find one who is likely to afford him the best possible reproductive return.

There are at least three factors that choosy males might evaluate when considering possible female suitors: a female's overall fecundity, the intensity of sperm competition a male currently faces within that female, and the probability that the female will mate again following the current copulation. It is plausible that male preferences for females with large and swollen abdomens initially evolved to help discern highly fecund and gravid females (i.e., those females of likely high reproductive value). Faced with such discerning males, and under pressure to obtain proteinaceous gifts, females may have resorted to behavioural and structural traits that enhanced their apparent abdominal size, including the careful positioning of legs alongside the abdomen in swarming flight (see Fig. 3 in chapter 1), the accessorizing of legs with flattened scales that further exaggerate size, and even the eversion of abdominal membranes as achieved by swallowing air, as observed in *R. longicauda*. In turn, males might conceivably have faced pressure to resist these seductive characters, and directly perceive the female fecundity in spite of the disguising ornaments that females used to promote their mating

chances. The fact that large females in *R. longicauda* are initially attractive (Funk & Tallamy, 2000; Murray, 2015) but nevertheless do not have higher pairing success (Wheeler *et al.*, 2012) supports this interpretation by suggesting a mechanism during pair formation that allows males to reject initially attractive mates after closer inspection.

The conflict between hungry females and discerning males goes beyond the detection of total reproductive value, of course. Males are primarily interested in the share of that value that each male can win. The circumstances that determine the outcome of sperm competition were completely unknown in dance flies, but in chapter 6 we exploited information from our amplified sperm stores to discern some of the parameters that mediate contests for sperm storage and use. We developed a new method for assessing the skew in male DNA representation within female sperm stores without knowing a female's mating history or the genotypes of her mates. We found evidence that stored sperm was dominated by a single male in *R. longicauda* and that this skew seemed largely independent of the number of rival males' sperm present in the spermatheca. These results suggest that sperm offence strategies are employed by males in this system, and are consistent with patterns of sperm storage seen in other species where males at least partially displace the sperm of rival males. Sperm storage patterns in this species therefore suggest that one male, likely the last male to mate with a female, will sire the majority of offspring, and that therefore last male precedence is operating. The conditions under which sperm competition occurs have clear implications for male mate choice, because last male priority sets the stage for male preferences that focus heavily on ovarian maturity (as opposed to fecundity *per se*). This distinction is important and relevant to the presumed honesty of sexual signals among dance flies.

Although sexual traits, like all morphological characters, tend to covary positively with other life history functions (including fecundity), a male is undoubtedly better served by finding the most gravid rather than the most fecund female. The former provides him with a better chance to be the last mate, with all the advantages in sperm priority that position affords, whereas the latter may well mate again, and, as a consequence afford the male a relatively small fraction of her higher overall fecundity. This tension between signals of overall body size (which ornaments are because all of them are fixed in maximum size at eclosion) and signals of ovarian maturation (which changes throughout adult development) may be crucial for the low stability of the male mate choice system among dance flies, and may help explain our otherwise puzzling failure to find strong sexual selection on exaggerated ornamental traits.

Sexual selection on female ornaments

Notwithstanding the role that antagonistic forces facing male and female dance flies may play, the observation of variation in sexual trait expression remains unexplained. Throughout my thesis, I have suggested that at best weak sexual selection is acting on female ornamentation in dance flies. This lack of significant directional sexual selection on female ornaments is very different from the strong directional sexual selection we tend to find on male ornamental traits - a relationship that is predicted by life history theory. As discussed in chapter 2, the best measures of the strength of sexual selection are a matter of debate (Arnold & Duvall, 1994; Jones, 2009; Klug *et al.*, 2010; Krakauer *et al.*, 2011; Kokko *et al.*, 2012; Fritzsche & Arnqvist, 2013). The controversy in how to measure sexual selection is especially true when trying to compare strengths across sexes or species. Measures such as the standardized Bateman gradient (the regression slope of relative reproductive success on relative mating

success, which describes the strength of selection on mating rate (Jones, 2009) or the Jones Index (the maximum intensity of precopulatory sexual selection on a trait (Jones, 2009) which incorporates the opportunity for sexual selection and the Bateman gradient have been suggested to be good measures of sexual selection when comparing across sexes and species (Fritzsche & Arnqvist, 2013; Henshaw *et al.*, 2016).

Knowing the reproductive consequences of mating for both sexes appears to be important in determining the strength of sexual selection in dance flies. However, measuring reproductive success is difficult in small, mobile, wild animals like dance flies. It would be informative to compare the fitness consequences of mating in terms of the reproductive success achieved by both males and females across multiple dance fly species that vary in their degree of ornamentation. In practice, however, obtaining such measures is difficult in wild flies, as we have yet to successful keep them in the lab. In chapter 5, I found that the opportunity for sexual selection, which measures the relative variation in mating success, was similar among the three species. However, if the reproductive benefit of each mating varied between species, the more important differences between species would have only been evident in the Bateman gradient. For example, perhaps the distinct levels of ornamentation among taxa could be explained by the degree to which females rely upon nuptial feeding to mature their eggs, which would be positively associated with the unit increase in reproductive success per mating. I did consider egg size and number as possible indexes of reproductive fitness in chapter 4, but these traits are imperfect estimators of fitness, as we could only measure the eggs remaining inside the female when caught. Hence, any recent nuptial feeding may not have materialised as increased egg development at the point at which

we captured the female, or the eggs themselves may not have resulted in viable offspring.

We found that the general prediction that ornament expression should covary with the intensity of sexual selection was not supported in female dance flies. This lack of detectable sexual selection on ornaments may be due to a conflict between the sexes, brought about in part by the conditions of sperm competition. As last male sperm precedence is likely, male preferences for gravid females is favoured by selection, and females may have evolved deceptive ornaments to exploit this preference to gain the benefits of nuptial gifts. The conflict between selection acting on hungry females and discerning males reveals a rich and complex interaction between the sexes. Coupled with new findings about the nature of sperm competition, my results may help explain the dynamic nature of selection on female ornaments as well as underscore the fact sexual selection on females is not the mirror image of selection on males.

Literature cited

Ah-King, M. & Ahnesjö, I. 2013. The "sex role" concept: an overview and evaluation. *Evol. Biol.* **40**: 461–470.

Alonzo, S. & Klug, H. 2012. Paternity, maternity, and parental care. In: *The Evolution of Parental Care* (N. J. Royle et al., eds), pp. 189–205. Oxford University Press, Oxford.

Amundsen, T. 2000. Why are female birds ornamented? *Trends Ecol. Evol.* **15**: 149–155.

Amundsen, T. & Forsgren, E. 2001. Male mate choice selects for female coloration in a fish. *Proc. Natl. Acad. Sci. U. S. A.* **98**: 13155–60.

Andersson, M. 1982. Sexual selection, natural selection and quality advertisement. *Biol. J. Linn. Soc.* **17**: 375–393.

Andersson, M. & Iwasa, Y. 1996. Sexual selection. *Trends Ecol. Evol.* **11**: 53–58.

Andersson, M.B. 1994. *Sexual Selection*. Princeton University Press.

Andrade, M.C.B. & Mason, A.C. 2000. Male condition, female choice, and extreme variation in repeated mating in a scaly cricket, *Ornebius aperta* (Orthoptera: Gryllidae: Mogoplistinae). *J. Insect Behav.* **13**: 483–497.

Arnold, S.J. & Duvall, D. 1994. Animal mating systems: a synthesis based on selection theory. *Am. Nat.* **143**: 317–348.

Arnqvist, G. 1989. Multiple mating in a water strider: mutual benefits or intersexual conflict? *Anim. Behav.* **38**: 749–756.

Arnqvist, G., Jones, T.M. & Elgar, M.A. 2006. Sex-role reversed nuptial feeding reduces male kleptoparasitism of females in Zeus bugs (Heteroptera; Veliidae). *Biol. Lett.* **2**: 491–3.

Arnqvist, G. & Nilsson, T. 2000. The evolution of polyandry: multiple mating and

female fitness in insects. *Anim. Behav.* **60**: 145–164.

Bateman, A.J. 1948. Intra-sexual selection in *Drosophila*. *Heredity (Edinb).* **2**: 349–68.

Berglund, A. 1994. The operational sex ratio influences choosiness in a pipefish. *Behav. Ecol.* **5**: 254–258.

Berglund, A. 2013. Why are sexually selected weapons almost absent in females? *Curr. Zool.* **59**: 564–568.

Berglund, A., Rosenqvist, G. & Bernet, P. 1997. Ornamentation predicts reproductive success in female pipefish. *Behav. Ecol. Sociobiol.* **40**: 145–150.

Bonduriansky, R. 2001. The evolution of male mate choice in insects: a synthesis of ideas and evidence. *Biol. Rev. Camb. Philos. Soc.* **76**: 305–39.

Boorman, E. & Parker, G.A. 1976. Sperm (ejaculate) competition in *Drosophila melanogaster*, and the reproductive value of females to males in relation to female age and mating status. *Ecol. Entomol.* **1**: 145–155.

Borg, A.A., Forsgren, E. & Magnhagen, C. 2002. Plastic sex-roles in the common goby - the effect of nest availability. *Oikos* **98**: 105–115.

Bretman, A. & Tregenza, T. 2005. Measuring polyandry in wild populations: a case study using promiscuous crickets. *Mol. Ecol.* **14**: 2169–79.

Bruford, M.W., Hanotte, O., Brookfield, J.F. & Burke, T. 1992. Multilocus and single-locus DNA fingerprinting. In: *Molecular Genetic Analysis of Populations: A Practical Approach.* (A. R. Hoelzel, ed), pp. 225–269. IRL Press, Oxford.

Burnham, K.P. & Anderson, D.R. 2002. *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*. Springer.

Bussière, L.F., Demont, M., Pemberton, J.M., Hall, M.D. & Ward, P.I. 2010. The assessment of insemination success in yellow dung flies using competitive PCR. *Mol. Ecol. Resour.* **10**: 292–303.

Chapman, T., Liddle, L.F., Kalb, J.M., Wolfner, M.F. & Partridge, L. 1995. Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature* **373**: 241–4.

Chenoweth, S.F., Doughty, P. & Kokko, H. 2006. Can non-directional male mating preferences facilitate honest female ornamentation? *Ecol. Lett.* **9**: 179–84.

Clutton-Brock, T. 2009. Sexual selection in females. *Anim. Behav.* **77**: 3–11.

Clutton-Brock, T. 2007. Sexual selection in males and females. *Science* **318**: 1882–5.

Clutton-Brock, T.H. 1991. *The Evolution of Parental Care.* Princeton University Press.

Clutton-Brock, T.H. & Huchard, E. 2013. Social competition and selection in males and females. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **368**: 20130074.

Collin, J. 1961. *British Flies: Empididae*. Cambridge Univ. Press, Cambridge, UK.

Crudgington, H.S. & Siva-Jothy, M.T. 2000. Genital damage, kicking and early death. *Nature* **407**: 855–6.

Cumming, J. 1994. Sexual selection and the evolution of dance fly mating systems (Diptera: Empididae; Empidinae). *Can. Entomol.* **126**: 907–920.

Curtsinger, J.W. 1991. Sperm competition and the evolution of multiple mating. *Am. Nat.* **138**: 93–102.

Darwin, C. 1859. *On the Origin of Species by Means of Natural Selection*. John Murray, London.

Darwin, C. 1871. *The Descent of Man, and Selection in Relation to Sex*. John Murray, London.

Demont, M., Buser, C.C., Martin, O.Y. & Bussière, L.F. 2011. Natural levels of polyandry: differential sperm storage and temporal changes in sperm competition intensity in wild yellow dung flies. *Funct. Ecol.* **25**: 1079–1090.

Downes, J.A. 1970. The feeding and mating behaviour of the specialized Empidinae

(Diptera); observations on four species of *Rhamphomyia* in the high Arctic and a general discussion. *Can. Entomol.* **102**: 769–791.

Eberhard, W.G. 1996. *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton University Press.

Emlen, D.J. 2008. The evolution of animal weapons. *Annu. Rev. Ecol. Evol. Syst.* **39**: 387–413.

Emlen, S.T. & Oring, L.W. 1977. Ecology, sexual selection, and the evolution of mating systems. *Science (80-.).* **197**: 215–23.

Emlen, S.T. & Wrege, P.H. 2004. Division of labour in parental care behaviour of a sex-role-reversed shorebird, the wattled jacana. *Anim. Behav.* **68**: 847–855.

Enders, M. 1993. The effect of male size and operational sex ratio on male mating success in the common spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae). *Anim. Behav.* **46**: 835–846.

Endler, J.A. 1986. *Natural Selection in the Wild*. Princeton University Press.

Evans, J.P. & Garcia-Gonzalez, F. 2016. The total opportunity for sexual selection and the integration of pre- and post-mating episodes of sexual selection in a complex world. *J. Evol. Biol.* **29**: 2338–2361.

Falconer, D.S. & Mackay, T.F.C. 1996. *Introduction to Quantitative Genetics*. Longman.

Fedorka, K.M. & Mousseau, T.A. 2002. Nuptial gifts and the evolution of male body size. *Evolution (N. Y).* **56**: 590–596.

Fisher, R. 1930. *The Genetical Theory of Natural Selection*. Clarendon Press.

Fitzpatrick, S., Berglund, A. & Rosenqvist, G. 1995. Ornaments or offspring: costs to reproductive success restrict sexual selection processes. *Biol. J. Linn. Soc.* **55**: 251–260.

Fritzsche, K. & Arnqvist, G. 2013. Homage to Bateman: sex roles predict sex differences in sexual selection. *Evolution* **67**: 1926–36.

Funk, D. & Tallamy, D. 2000. Courtship role reversal and deceptive signals in the long-tailed dance fly, *Rhamphomyia longicauda*. *Anim. Behav.* **59**: 411–421.

García-González, F. & Simmons, L.W. 2007. Paternal indirect genetic effects on offspring viability and the benefits of polyandry. *Curr. Biol.* **17**: 32–36.

García-González, F. & Simmons, L.W. 2005. The evolution of polyandry: intrinsic sire effects contribute to embryo viability. *J. Evol. Biol.* **18**: 1097–103.

Gardner, M.G., Fitch, A.J., Bertozzi, T. & Lowe, A.J. 2011. Rise of the machines - recommendations for ecologists when using next generation sequencing for microsatellite development. *Mol. Ecol. Resour.* **11**: 1093–1101.

Gilder, J.R., Inman, K., Shields, W. & Krane, D.E. 2011. Magnitude-dependent variation in peak height balance at heterozygous STR loci. *Int. J. Legal Med.* **125**: 87–94.

González, A., Rossini, C., Eisner, M. & Eisner, T. 1999. Sexually transmitted chemical defense in a moth (*Utetheisa ornatrix*). *Proc. Natl. Acad. Sci. U. S. A.* **96**: 5570–4.

Goodwillie, C., Sargent, R.D., Eckert, C.G., Elle, E., Geber, M.A., Johnston, M.O., *et al.* 2010. Correlated evolution of mating system and floral display traits in flowering plants and its implications for the distribution of mating system variation. *New Phytol.* **185**: 311–321.

Guo, S.W. & Thompson, E.A. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* **48**: 361.

Gwynne, D. 2001. *Katydids and Bush-crickets: Reproductive Behavior and Evolution of the Tettigoniidae*. Cornell University Press.

Gwynne, D., Bussiere, L. & Ivy, T. 2007. Female ornaments hinder escape from spider

webs in a role-reversed swarming dance fly. *Anim. Behav.* **73**: 1077–1082.

Gwynne, D.T. 1988. Courtship feeding in katydids benefits the mating male's offspring. *Behav. Ecol. Sociobiol.* **23**: 373–377.

Gwynne, D.T. & Bussière, L.T. 2002. Female mating swarms increase predation risk in a "role-reversed" dance fly (Diptera: Empididae: *Rhamphomyia longicauda* Loew). *Behaviour* **139**: 1425–1430.

Gwynne, D.T. & Simmons, L.W. 1990. Experimental reversal of courtship roles in an insect. *Nature* **346**: 172–174.

Haddrill, P.R., Shuker, D.M., Amos, W., Majerus, M.E.N. & Mayes, S. 2008. Female multiple mating in wild and laboratory populations of the two-spot ladybird, *Adalia bipunctata*. *Mol. Ecol.* **17**: 3189–3197.

Hall, M.D., Bussière, L.F., Demont, M., Ward, P.I. & Brooks, R.C. 2010. Competitive PCR reveals the complexity of postcopulatory sexual selection in *Teleogryllus commodus*. *Mol. Ecol.* **19**: 610–619.

Hall, M.D., Bussière, L.F., Hunt, J. & Brooks, R. 2008. Experimental evidence that sexual conflict influences the opportunity, form and intensity of sexual selection. *Evolution (N. Y).* **62**: 2305–2315.

Henshaw, J.M., Kahn, A.T. & Fritzsche, K. 2016. A rigorous comparison of sexual selection indexes via simulations of diverse mating systems. *Proc. Natl. Acad. Sci. U. S. A.* **113**: E300-8.

Hoekstra, H.E., Hoekstra, J.M., Berrigan, D., Vignieri, S.N., Hoang, A., Hill, C.E., *et al.* 2001. Strength and tempo of directional selection in the wild. *Proc. Natl. Acad. Sci.* **98**: 9157–9160.

Houde, A.E. 2001. Sex roles, ornaments, and evolutionary explanation. *Proc. Natl. Acad. Sci. U. S. A.* **98**: 12857–9.

Houslay, T.M. & Bussière, L.F. 2012. Sexual selection and life history allocation. In: *eLS*. John Wiley & Sons, Ltd, Chichester, UK.

Howard, R.D. 1978. The influence of male-defended oviposition sites on early embryo mortality in bullfrogs. *Ecology* **59**: 789–798.

Iyengar, V.K. & Eisner, T. 2004. Male indifference to female traits in an arctiid moth (*Utetheisa ornatrix*). *Ecol. Entomol.* **29**: 281–284.

Jennions, M.D., Kokko, H. & Klug, H. 2012. The opportunity to be misled in studies of sexual selection. *J. Evol. Biol.* **25**: 591–8.

Jennions, M.D. & Petrie, M. 1997. Variation in mate choice and mating preferences: a review of causes and consequences. *Biol. Rev. Camb. Philos. Soc.* **72**: 283–327.

Jennions, M.D. & Petrie, M. 2000. Why do females mate multiply? A review of the genetic benefits. *Biol. Rev. Camb. Philos. Soc.* **75**: 21–64.

Jiggins, F.M., Hurst, G.D. & Majerus, M.E. 2000. Sex-ratio-distorting *Wolbachia* causes sex-role reversal in its butterfly host. *Proc. Biol. Sci.* **267**: 69–73.

Jirotkul, M. 1999. Operational sex ratio influences female preference and male-male competition in guppies. *Anim. Behav.* **58**: 287–294.

Jones, A.G. 2009. On the opportunity for sexual selection, the Bateman gradient and the maximum intensity of sexual selection. *Evolution* **63**: 1673–84.

Jones, A.G., Walker, D. & Avise, J.C. 2001. Genetic evidence for extreme polyandry and extraordinary sex-role reversal in a pipefish. *Proc. R. Soc. B Biol. Sci.* **268**: 2531–5.

Jones, I. & Hunter, F. 1999. Experimental evidence for mutual inter- and intrasexual selection favouring a crested auklet ornament. *Anim. Behav.* **57**: 521–528.

Jones, T.M., Quinnell, R.J. & Balmford, A. 1998. Fisherian flies: benefits of female choice in a lekking sandfly. *Proc. R. Soc. B Biol. Sci.* **265**: 1651–1657.

Keller, L. & Reeve, H.K. 1995. Why do females mate with multiple males? The sexually selected sperm hypothesis. *Adv. Study Behav.* **24**: 291–315.

Kessel, E.L. 1955. The mating activities of balloon flies. *Syst. Zool.* **4**: 97–104.

Kingsolver, J.G., Hoekstra, H.E., Hoekstra, J.M., Berrigan, D., Vignieri, S.N., Hill, C.E., *et al.* 2001. The strength of phenotypic selection in natural populations. *Am. Nat.* **157**: 245–261.

Klug, H., Heuschele, J., Jennions, M.D. & Kokko, H. 2010. The mismeasurement of sexual selection. *J. Evol. Biol.* **23**: 447–62.

Kokko, H. & Jennions, M. 2003. It takes two to tango. *Trends Ecol. Evol.* **18**: 103–104.

Kokko, H., Klug, H. & Jennions, M.D. 2014. Mating systems. In: *The Evolution of Insect Mating Systems* (D. Shuker & L. W. Simmons, eds), pp. 42–58. Oxford University Press.

Kokko, H., Klug, H. & Jennions, M.D. 2012. Unifying cornerstones of sexual selection: operational sex ratio, Bateman gradient and the scope for competitive investment. *Ecol. Lett.* **15**: 1340–51.

Kokko & Jennions. 2008. Parental investment, sexual selection and sex ratios. *J. Evol. Biol.* **21**: 919–48.

Krakauer, A.H., Webster, M.S., Duval, E.H., Jones, A.G. & Shuster, S.M. 2011. The opportunity for sexual selection: not mismeasured, just misunderstood. *J. Evol. Biol.* **24**: 2064–71.

Kvarnemo, C. 2006. Evolution and maintenance of male care: is increased paternity a neglected benefit of care? *Behav. Ecol.* **17**: 144–148.

LeBas, N.R., Hockham, L.R. & Ritchie, M.G. 2003. Nonlinear and correlational sexual selection on "honest" female ornamentation. *Proc. R. Soc. B Biol. Sci.* **270**: 2159–65.

LeBas, N.R., Hockham, L.R. & Ritchie, M.G. 2004. Sexual selection in the gift-giving dance fly Rhamphomyia sulcata, favors small males carrying small gifts. *Evolution (N. Y).* **58**: 1763.

Manier, M.K., Lüpold, S., Belote, J.M., Starmer, W.T., Berben, K.S., Ala-Honkola, O., *et al.* 2013. Postcopulatory sexual selection generates speciation phenotypes in *Drosophila*. *Curr. Biol.* **23**: 1853–1862.

Marshall, T.C., Slate, J., Kruuk, L.E.B. & Pemberton, J.M. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. *Mol. Ecol.* **7**: 639–655.

McNamara, K.B., van Lieshout, E. & Simmons, L.W. 2014. A test of the sexy-sperm and good-sperm hypotheses for the evolution of polyandry. *Behav. Ecol.* **25**: 989–995.

Meglécz, E., Anderson, S.J., Bourguet, D., Butcher, R., Caldas, A., Cassel-Lundhagen, A., *et al.* 2007. Microsatellite flanking region similarities among different loci within insect species. *Insect Mol. Biol.* **16**: 175–185.

Møller, A.P. & Birkhead, T.R. 1993. Certainty of paternity covaries with paternal care in birds. *Behav. Ecol. Sociobiol.* **33**: 261–268.

Murray, R.L. 2015. The ecology and evolution of female-specifc ornamention in the dance flies (Diptera: Empidinae). University of Stirling.

Neff, B.D. & Gross, M.R. 2001. Dynamic adjustment of parental care in response to perceived paternity. *Proc. Biol. Sci.* **268**: 1559–65.

Ono, T., Siva-Jothy, M.T. & Kato, A. 1989. Removal and subsequent ingestion of rivals' semen during copulation in a tree cricket. *Physiol. Entomol.* **14**: 195–202.

Owens, I.P.F., Burke, T. & Thompson, D.B.A. 1994. Extraordinary sex roles in the Eurasian dotterel: female mating arenas, female-female competition, and female

mate choice. *Am. Nat.* **144**: 76–100.

Parker, G.A. 1972. Reproductive behaviour of *Sepsis cynipsea* (L.) (Diptera: Sepsidae). I. A preliminary analysis of the reproductive strategy and its associated behaviour patterns. *Behaviour* **41**: 172–206.

Parker, G.A. 1970. Sperm competition and its evolutionary consequences in the insects. *Biol. Rev.* **45**: 525–567.

Parker, G.A., Ball, M.A., Stockley, P. & Gage, M.J.G. 1996. Sperm competition games: individual assessment of sperm competition intensity by group spawners. *Proc. R. Soc. London B Biol. Sci.* **263**: 1291–1297.

Parker, G.A. & Simmons, L.W. 1991. A model of constant random sperm displacement during mating: evidence from Scatophaga. *Proc. R. Soc. B Biol. Sci.* **246**: 107–15.

Parker, G.A., Simmons, L.W. & Kirk, H. 1990. Analysing sperm competition data: simple models for predicting mechanisms. *Behav. Ecol. Sociobiol.* **27**: 55–65.

Petrie, M. 1983. Female moorhens compete for small fat males. *Science* **220**: 413–5.

Pischedda, A. & Rice, W.R. 2012. Partitioning sexual selection into its mating success and fertilization success components. *Proc. Natl. Acad. Sci. U. S. A.* **109**: 2049–53.

Pleszczynska, W.K. 1978. Microgeographic prediction of polygyny in the lark bunting. *Science (80-.).* **201**: 935–7.

Preston-Mafham, K.G. 1999. Courtship and mating in *Empis (Xanthempis) trigramma* Meig., *E. tessellata* F. and *E. (Polyblepharis) opaca* F. (Diptera: Empididae) and the possible implications of "cheating" behaviour. *J. Zool.* **247**: 239–246.

Price, C.S.C., Dyer, K.A. & Coyne, J.A. 1999. Sperm competition between *Drosophila* males involves both displacement and incapacitation. *Nature* **400**: 449–452.

Prokuda, A.Y. & Roff, D.A. 2014. The quantitative genetics of sexually selected traits, preferred traits and preference: a review and analysis of the data. *J. Evol. Biol.* **27**:

2283–96.

Reynolds, J.D., Colwell, M.A. & Cooke, F. 1986. Sexual selection and spring arrival times of red-necked and Wilson's phalaropes. *Behav. Ecol. Sociobiol.* **18**: 303–310.

Rowe, L. & Houle, D. 1996. The lek paradox and the capture of genetic variance by condition dependent traits. *Proc. R. Soc. B Biol. Sci.* **263**: 1415–1421.

Rozen, S. & Skaletsky, H. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* **132**: 365–86.

Sakaluk, S.K. 1987. Reproductive behaviour of the decorated cricket, *Gryllodes supplicans* (Orthoptera: Gryllidae): calling schedules, spatial distribution, and mating. *Behaviour* **100**: 202–225.

Schielzeth, H. 2010. Simple means to improve the interpretability of regression coefficients. *Methods Ecol. Evol.* **1**: 103–113.

Searcy, W.A. 1979. Female choice of mates: a general model for birds and its application to red-winged blackbirds (Agelaius phoeniceus). *Am. Nat.* **114**: 77–100.

Shuker, D.M. 2010. Sexual selection: endless forms or tangled bank? *Anim. Behav.* **79**: e11–e17.

Shuster, S.M. & Wade, M.J. 2003. *Mating Systems and Strategies*. Princeton University Press.

Simmons, L.W. 1986. Female choice in the field cricket *Gryllus bimaculatus* (De Geer). *Anim. Behav.* **34**: 1463–1470.

Simmons, L.W. 2001. *Sperm Competition and Its Evolutionary Consequences in the Insects*. Princeton University Press.

Simmons, L.W. & Beveridge, M. 2010. The strength of postcopulatory sexual selection

within natural populations of field crickets. *Behav. Ecol.* **21**: 1179–1185.

Simmons, L.W., Beveridge, M. & Kennington, W.J. 2007. Polyandry in the wild: temporal changes in female mating frequency and sperm competition intensity in natural populations of the tettigoniid *Requena verticalis*. *Mol. Ecol.* **16**: 4613–23.

Simmons, L.W. & Emlen, D.J. 2008. No fecundity cost of female secondary sexual trait expression in the horned beetle *Onthophagus sagittarius*. *J. Evol. Biol.* **21**: 1227–35.

Simmons, L.W., Llorens, T., Schinzig, M., Hosken, D. & Craig, M. 1994. Sperm competition selects for male mate choice and protandry in the bushcricket, *Requena verticalis* (Orthoptera: Tettigoniidae). *Anim. Behav.* **47**: 117–122.

Simmons, L.W., Parker, G.A. & Stockley, P. 1999. Sperm displacement in the yellow dung fly, *Scatophaga stercoraria*: an investigation of male and female processes females of many insect species receive sperm from more. *Am. Nat* **153**: 302–314.

Simmons, L.W. & Siva-Jothy, M.T. 1998. Sperm competition in insects: mechanisms and the potential for selection. In: *Sperm competition and sexual selection* (T. Birkhead & A. Møller, eds), pp. 341–434. ACADEMIC PRESS LTD, San Diego, California.

Slatyer, R.A., Mautz, B.S., Backwell, P.R.Y. & Jennions, M.D. 2012. Estimating genetic benefits of polyandry from experimental studies: a meta-analysis. *Biol. Rev. Camb. Philos. Soc.* **87**: 1–33.

Smiseth, P.T. 2014. Parental care. In: *The Evolution of Insect Mating Systems* (D. Shuker & L. W. Simmons, eds), pp. 221–241. Oxford University Press.

Smith, R.L. 1979. Paternity assurance and altered roles in the mating behaviour of a giant water bug, *Abedus herberti* (heteroptera: Belostomatidae). *Anim. Behav.* **27**: 716–725.

South, S.H. & Arnqvist, G. 2008. Evidence of monandry in a mosquito (*Sabethes cyaneus*) with elaborate ornaments in both sexes. *J. Insect Behav.* **21**: 451–459.

South, S.H., Steiner, D. & Arnqvist, G. 2009. Male mating costs in a polygynous mosquito with ornaments expressed in both sexes. *Proc. Biol. Sci.* **276**: 3671–8.

Stearns, S.C. 1989. Trade-offs in life-history evolution. *Funct. Ecol.* **3**: 259.

Svensson, B. & Petersson, E. 2000. Swarm site fidelity in the sex role-reversed dance fly *Empis borealis*. *J. Insect Behav.* **13**: 785–796.

Tallamy, D. 2000. Sexual selection and the evolution of exclusive paternal care in arthropods. *Anim. Behav.* **60**: 559–567.

Tatarenkov, A., Healey, C.I.M., Grether, G.F. & Avise, J.C. 2008. Pronounced reproductive skew in a natural population of green swordtails, *Xiphophorus helleri*. *Mol. Ecol.* **17**: 4522–34.

Tobias, J.A., Montgomerie, R. & Lyon, B.E. 2012. The evolution of female ornaments and weaponry: social selection, sexual selection and ecological competition. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **367**: 2274–93.

Tripet, F., Touré, Y.T., Taylor, C.E., Norris, D.E., Dolo, G. & Lanzaro, G.C. 2001. DNA analysis of transferred sperm reveals significant levels of gene flow between molecular forms of *Anopheles gambiae*. *Mol. Ecol.* **10**: 1725–1732.

Trivers, R.L. 1972. Parental investment and sexual selection. In: *Sexual Selection and the Descent of Man* (B. Campbell, ed), pp. 136–179. Aldine.

Turnell, B.R. & Shaw, K.L. 2015. Polyandry and postcopulatory sexual selection in a wild population. *Mol. Ecol.* **24**: 6278–6288.

Vahed, K. 2007. Comparative evidence for a cost to males of manipulating females in bushcrickets. *Behav. Ecol.* **18**: 499–506.

Vahed, K. 1998. The function of nuptial feeding in insects: a review of empirical

studies. *Biol. Rev.* **73**: 43–78. Cambridge University Press.

Vardell, H. & Brower, J. 1978. Sperm precedence in *Tribolium confusum* (Coleoptera: Tenebrionidae). *J. Kansas Entomol. Soc.* **51**: 187–190.

Vincent, C.M. & Gwynne, D.T. 2014. Sex-biased immunity is driven by relative differences in reproductive investment. *Proc. R. Soc. B Biol. Sci.* **281**: 20140333.

Waage, J.K. 1979. Dual function of the damselfly penis: sperm removal and transfer. *Science* **203**: 916–8.

Waage, J.K. 1984. Sperm competition and the evolution of Odonate mating systems. In: *Sperm Competition and the Evolution of Animal Mating Systems.* (R. L. Smith, ed), pp. 251–290. Academic Press Ltd, London.

Wade, M. 1979. Sexual selection and variance in reproductive success. *Am. Nat.* **114**: 742–747.

Wade, M.J. & Shulter, S.M. 2004. Sexual selection: harem size and the variance in male reproductive success. *Am. Nat.* **164**: E83-9.

Watson, P.J. 1991. Multiple paternity as genetic bet-hedging in female sierra dome spiders, *Linyphia litigiosa* (Linyphiidae). *Anim. Behav.* **41**: 343–360.

Wedell, N., Wiklund, C. & Cook, P.A. 2002. Monandry and polyandry as alternative lifestyles in a butterfly. *Behav. Ecol.* **13**: 450–455.

West-Eberhard, M. 1979. Sexual selection, social competition, and evolution. *Proc. Am. Philos. Soc.* **123**: 222–234.

Wheeler, J., Gwynne, D.T. & Bussière, L.F. 2012. Stabilizing sexual selection for female ornaments in a dance fly. *J. Evol. Biol.* **25**: 1233–42.

Williams, G.C. 1975. *Sex and Evolution.* Princeton University Press.

Yasui, Y. 1997. A “good sperm” model can explain the evolution of costly multiple mating by females. *Am. Nat.* **149**: 573–584.

Yasui, Y. 1998. The “genetic benefits” of female multiple mating reconsidered. *Trends Ecol. Evol.* **13**: 246–250.

Zeh, J.A. & Zeh, D.W. 1994. Last-male sperm precedence breaks down when females mate with three males. *Proc. R. Soc. London B Biol. Sci.* **257**: 287–292.

Zeh, J.A. & Zeh, D.W. 1996. The evolution of polyandry I: intragenomic conflict and genetic incompatibility. *Proc. R. Soc. London B Biol. Sci.* **263**: 1711–1717.

Zeh, J.A. & Zeh, D.W. 1997. The evolution of polyandry II: post–copulatory defenses against genetic incompatibility. *Proc. R. Soc. London B Biol. Sci.* **264**: 69–75.