Female aggression in flies



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Abstract

Competition among individuals for access to resources crucial for survival and reproduction is ubiquitous across the animal kingdom. However, most research has focused on male-male competition over access to mates. Female-female competition (hereon "female competition") over resources vital for reproduction, such as food to provision offspring, nesting sites, or social dominance, has been largely neglected. In this thesis, I aim to contribute to the growing body of literature examining female competition by investigating female competition over food in two species of fly.

I start by assessing the role of an exaggerated secondary sexual trait (eyespan) as a signal in female competition in the stalk-eyed fly *Teleopsis dalmanni*. Sexual selection theory argues that females only possess exaggerated traits as a result of correlation with males and that they play no functional role. I find that eyespan is an honest indicator of condition but does not appear to function as a signal in either male or female intrasexual encounters, as previously argued.

Next, I consider the effects of mating on female aggression in the fruit fly *Drosophila melanogaster*. I find that mating doubles the amount of time females spend fighting over food. The ability to produce eggs is not required for this effect, but the receipt of sperm is essential. I also find that the magnitude of the effects of mating is determined by a female's developmental environment – mating significantly increases a female's chance of winning if she is raised at high larval density, but has no effect on her chances of winning if she is raised at low larval density. Finally, I find suggestions that variation in males and their ejaculates could alter levels of post-mating female aggression, suggesting that post-mating female aggression could potentially be under sexual conflict.

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Declaration and author contributions

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Chapter 2: Condition, not eyespan, predicts contest outcome in female stalk-eyed flies, *Teleopsis dalmanni*

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E.B designed and ran the experiment. E.B and C.V scored the behavioural data. E.B analysed the data, and drafted and revised the paper. S.W, N.S and J.A.T assisted in designing the experiment, interpreting the data, and revised the paper.

Chapter 3: Sperm stimulates female aggression

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Chapter 4: The role of male condition in mating-induced female aggression

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E.B and S.W conceived of the project. E.B and D.B performed the behavioural experiments, collected flies for cVA analysis, and scored the behavioural data. D.B watched the majority of the videos, with training from E.B. E.B analysed the data and

wrote the manuscript. S.W and N.S assisted in interpreting the data, and revised the paper.

Additional contribution: Zhanru Yu at the Nuffield Department of Medicine performed the gas chromatography – mass spectrometry.

Chapter 5: Developmental environment and mating status mediate outcomes of female competition

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Note on references

To ensure consistency throughout this thesis, I use the referencing style from the journal *Evolution*. Although in print, each of these articles uses a different reference style and has a separate reference list, to save space and avoid duplication, there is a single reference list at the end of this thesis.

Chapter 1: General Introduction

In evolutionary biology, female competition with other females (hereafter 'female competition') has traditionally been viewed as a rare behaviour with few fitness consequences (Hrdy and Williams 1983). Research on intrasexual competition has therefore focused mainly on male-male competition over mates, ignoring female competition (Andersson 1994; Rosvall 2011d; Stockley and Bro-Jørgensen 2011; Tobias et al. 2012; Clutton-Brock and Huchard 2013a; Stockley and Campbell 2013). This focus on male intrasexual competition has led to a serious deficit in our knowledge of how one sex competes. Imposing analytical frameworks developed solely to study male aggression and competitive interactions does not allow us to adequately understand needs and trade-offs that are specific to females, and that are essential for determining the nature of female competition. Studying female competition is crucial for understanding the selective forces acting on females, as well as the phenotypic and genotypic traits associated with such selection (Stockley and Campbell 2013). There is now a growing realization that female competition can have serious ramifications for individual fitness, and have broader effects on group formation and dispersal patterns (Clutton-Brock and Huchard 2013b). Empirical and theoretical work focusing solely on female aggression are sorely needed to fill this gap (Cant and Young 2013; Clutton-Brock and Huchard 2013b; Stockley and Campbell 2013). This thesis aims to help address this gap by investigating the proximate causes of female aggression in two species of fly.

Female competition and fitness

Females compete over resources that are vital for survival and reproduction, such as food, nesting sites, and social dominance, which can result in a dramatic variation in fitness among females in a variety of taxa (Eggert et al. 2008; Watson and Simmons 2010; Stockley and Bro-Jørgensen 2011; Clutton-Brock and Huchard 2013b; Stockley and Campbell 2013). Females that are more successful in intrasexual competition often have higher reproductive success, greater access to food or higher quality mates, and experience reduced rates of predation and infanticide (Clutton-Brock et al. 1998; Young et al. 2006; Eggert et al. 2008; Kahlenberg et al. 2008; Cain and Ketterson 2012). In mammals, in particular, social dominance (often acquired through competitive interactions) results in greater access to higher quality food and mates (vervet monkeys: Whitten 1983), as well as reduced risks of predation (long-tailed macaques: van Noordwijk and van Schaik 1987; chacma baboons: Ron et al. 1996), and infanticide (dwarf mongoose: Clutton-Brock et al. 1998; meerkat: Young et al. 2006). In insects, female burying beetles fight over access to small vertebrate carcasses, which are used to attract mates and provision offspring (Pukowski 1933). Females that are more successful in intrasexual competition kill their competitors' larvae, and raise significantly more offspring than unsuccessful females (Eggert et al. 2008; Eggert and Müller 2011). Female eclectus parrots compete over access to nesting hollows, where females that succeed in intrasexual competition acquire drier hollows, more males competing to mate with them, and have higher reproductive success (Heinsohn 2008).

In addition to the individual consequences of female competition, there may be knock-on effects that influence the ecology and evolution of a species (Clutton-Brock and

Huchard 2013b). Rates of female competition may play a role in determining group size and formation, altering operational sex ratios and the potential for sexual selection in both females and males (Clutton-Brock and Huchard 2013b). With our increasing understanding that female aggression can have serious effects on fitness, it is important to rigorously examine how females compete, as this may differ significantly from their male counterparts (Tobias et al. 2012; Clutton-Brock and Huchard 2013a; Rosvall 2013b). Given the striking differences in how males and females secure reproductive success in other areas, such as procuring matings and parental care, theory and empirical evidence suggest that there will be clear differences in how males and females compete aggressively (Draud 2004; Tobias et al. 2012; Clutton-Brock and Huchard 2013a).

Sex differences in aggression

Males and females acquire fitness in very different ways, suggesting that the sexes will also differ substantially in the costs and benefits of engaging in intrasexual competition (Bateman 1948; Trivers 1972; Andersson 1994). Sexual selection theory predicts that differences in potential reproductive rates between sexes will lead to the sex with the highest potential reproductive rate competing over access to mates (Trivers 1972). In the majority of studied species, this prediction has been shown to be at least partially accurate, with more obvious male intrasexual competition than female competition (Andersson 1994). However, sexual selection theory has predominantly focused on the number of matings and offspring an individual can secure – measurements which more closely fit how males secure reproductive success, but which fail to fully capture variation in female fitness (Hrdy and Williams 1983;

West-Eberhard 1983; Tobias et al. 2012). The popularity of the sexual selection framework over the last decades is one reason that female competition has been relatively neglected (Tobias et al. 2012; Stockley and Campbell 2013). Incorporating competition over resources less directly linked to reproduction, such as competition for nesting sites or paternal care, allows us to examine in much more detail how female competition influences survival and reproductive success (West-Eberhard 1983; Tobias et al. 2012).

Reproduction and female aggression

One major way in which males and females differ, and the primary focus of this thesis, is in the relationship between reproduction and aggression (Stockley and Campbell 2013). Females competing over resources involved in reproduction will value such resources differently depending on their reproductive status or stage in the reproductive cycle (McDonough 1994; Bebié and McElligott 2006). There are clear temporal associations between reproductive stages and levels of female aggression across a wide range of taxa (McDonough 1994; Palanza et al. 1996; Bowler et al. 2002; Bebié and McElligott 2006; Robinson and Kruuk 2007; Huchard and Cowlishaw 2011; Seebacher et al. 2013). Prior to mating, females compete for access to mates, in both vertebrates (e.g. topi antelope: Bro-Jorgensen 2002, red deer: Bebié and McElligott 2006), and invertebrates (e.g. Mediterranean fruit flies: Papadopoulos et al. 2009, katydids: Gwynne and Bailey 1999), with correlations between levels of aggression and rates of competitive interactions correlated with reproductive stage (e.g. oestrous in red deer :Bebié and McElligott 2006), or mating status (e.g. virgins are more aggressive

than mated females in Mediterranean fruit flies and katydids: Gwynne and Bailey 1999; Papadopoulos et al. 2009).

Alternatively, females can compete after mating over resources important for their offspring, such as food, social rank or spatial position within a group (Whitten 1983; Kraaijeveld 2004; Kahlenberg et al. 2008). In vertebrates, in particular, females display increased aggression after giving birth to protect their offspring from infanticidal conspecifics or predators (Robinson and Kruuk 2007; refs in Rosvall 2011d; Stockley and Bro-Jørgensen 2011; Clutton-Brock and Huchard 2013b). In taxa with high levels of maternal care, such as gestation and/or lactation, females display regular reproductive cycles, with energy and nutrient demands varying greatly across time (Seebacher et al. 2013). These changing energy demands lead to the prediction that pregnant females will need to acquire more nutrients after mating and becoming pregnant to meet their increasing energy demands (Barton Browne 1995). To secure these nutrients, females will need to compete with other females for access to food, with more successful females receiving more food. This increased competition should therefore lead to pregnant females displaying more aggression than non-pregnant females to perform better in female competition and secure more resources. Pregnant females are more aggressive than non-pregnant females in numerous vertebrate species, including meerkats, mice, mongooses, mosquitofish, bank voles, and armadillos (McDonough 1994; Palanza et al. 1996; Clutton-Brock et al. 1998; Kapusta and Marchlewska-Koj 1998; Young et al. 2006; Seebacher et al. 2013). However, only two studies (on prairie voles: Bowler et al. 2002; and bank voles: Kapusta and Marchlewska-Koj 1998) tested whether pregnancy is required for an increase in aggression. Both found that females

only needed to be housed with males but not mate with them to display the same increase in aggression, suggesting the link between pregnancy, energy demands, and aggression is less clear-cut than proposed (Kapusta and Marchlewska-Koj 1998; Bowler et al. 2002). While studies discuss the role of maternal aggression in offspring defence as a general reason for the increase in aggression after mating, few studies have attempted to explicate the proximate mechanisms of such an increase. Investigating the link between mating, offspring production, and female aggression is therefore of critical importance when attempting to understand female competition, and is one of the main topics of this thesis.

Males in species where females do have reproductive cycles tend not to experience the same temporal variation in reproductive state and aggression as females. Instead, male aggression and competition is often determined by the reproductive stage of females – e.g. the majority of male competition in red deer occurs during the breeding season, when females are physiologically able to reproduce (Clutton-Brock et al. 1979). This greater temporal and individual variation in reproductive state and accompanying physiological changes in females with high levels of maternal investment suggests that we need to develop predictions explicitly focusing on how reproduction and female competition interact (Cant and Young 2013).

The close association between reproduction and aggression may result in trade-offs between investing in offspring, and investing in costly competitive traits and behaviours (Fitzpatrick et al. 1995; Clutton-Brock 2007; Rosvall 2013a; Stockley and Campbell 2013). Males may also face similar trade-offs, as producing gametes and ejaculate components can be costly (Wedell et al. 2002). However, as females usually

invest much more heavily in their offspring they will face a heavier fecundity cost from investing in competitive traits (Fitzpatrick et al. 1995). For example, it has been argued that females less commonly develop exaggerated secondary sexual traits; when they do, these are often much less exaggerated than males as resources invested in growing and maintaining such traits are not available for reproduction, thereby reducing a female's fecundity (Fitzpatrick et al. 1995). Investigating the female-specific costs and benefits of secondary sexual traits and their role in female competition has been an increasing area of interest for biologists, with extensive research now being performed in birds (reviewed in Amundsen 2000; Tobias et al. 2012), mammals (reviewed in Stockley and Bro-Jørgensen 2011; Clutton-Brock and Huchard 2013a,b), and insects (Al-khairulla et al. 2003; Watson and Simmons 2010). Studying whether females use exaggerated secondary sexual traits in female competition helps us to better understand the selective pressures operating on female ornamentation, as well as allowing us to investigate potential constraints on the evolution of such traits. Such fecundity costs may also impose evolutionary constraints on the frequency and intensity of behaviours such as female aggression. Engaging in physical encounters can be energetically demanding, resulting in potential trade-offs within females between investing resources in aggressive behaviours and reproduction (Seebacher et al. 2013). Additionally, in species with maternal care, the risk of injury from engaging in contests could have a direct influence on their ability to protect and provision their offspring (Robinson and Kruuk 2007; Clutton-Brock and Huchard 2013b). The risks and costs of engaging in competition may therefore be much greater for females than for males, requiring detailed studies on the dangers involved in participating in female competition.

These potential trade-offs in females between reproduction and competitive traits may be mediated by physiological mechanisms that reduce females' ability to invest in competitive ability (Rosvall 2013b). Understanding these proximate mechanisms can help us to identify potential trade-offs and constraints on phenotypic plasticity in females (Rosvall 2013b). Testosterone is associated with female aggression in numerous vertebrate species (Rosvall 2013b), such as hyenas (Yalcinkaya et al. 1993; Dloniak et al. 2006), cichlids (Desjardins et al. 2006), dunnocks (Langmore et al. 2002) and lemurs (Drea 2007). High levels of testosterone can also reduce fertility or maternal care, resulting in serious costs for females (Parker 1979; Dloniak et al. 2006; Rosvall 2011c; Gerlach and Ketterson 2013). However, other than in extremely well-studied species (e.g. rats; Albert et al. 1991, dark-eyed juncos; Cain and Ketterson 2012, 2013,Rosvall 2013a), we know little about the proximate mechanisms governing female aggression. In particular, we know little of the proximate mechanisms linking reproduction and aggression, which are of considerable interest when considering potential constraints on the evolution of female aggressive behaviour.

The costs and benefits of engaging in female competition, as well as physiological constraints, will influence the nature, frequency and intensity of such contests. The predicted higher costs and lower benefits for females that could arise from engaging in aggressive encounters suggest that females should engage in fewer physical aggressive encounters than males, or that when they do engage, females should retreat at lower levels of injury than males (Clutton-Brock and Huchard 2013a). However, there is mixed evidence for whether female encounters end with less serious injuries inflicted (Pie 1998; Fowler-Finn and Hebets 2006; Elias et al. 2010; MacCormick et al. 2012).

Female contests are often less ritualized than male contests, reducing the ability for females to accurately judge the fighting ability of their competitor through stereotyped behaviour (Enquist and Leimar 1987; Elias et al. 2010). Females instead often use less risky strategies of competition, such as threatening physical attacks (Cant and Young 2013), reproductive suppression (Young et al. 2006; Huchard and Cowlishaw 2011), expulsion (Clutton-Brock et al. 1998), and infanticide (Chek and Robertson 1991; Eggert and Müller 2011). Studying the sex-specific causes and nature of female competition is therefore of crucial importance to establish potential fitness effects.

Female aggression in insects

While the majority of research on intrasexual competition has focused on males, the research that has been conducted on female competition and aggression has been primarily directed towards vertebrates, particularly mammals and birds, both taxa which have heavy parental investment in offspring (Stockley and Bro-Jørgensen 2011; Tobias et al. 2012; Clutton-Brock and Huchard 2013b). However, much less research on female competition has been conducted in invertebrates. Even in the model organism, *D. melanogaster*, there are to date, to the best of my knowledge, only 3 studies examining female aggression in any detail (Ueda and Kidokoro 2002; Nilsen et al. 2004; Edwards et al. 2006), compared to hundreds addressing male aggression (e.g. Hoffmann 1987; Dierick and Greenspan 2006, 2007; Cabral et al. 2008; Hoyer et al. 2008; Rezával et al. 2012; Asahina et al. 2014). This is a serious research gap as invertebrates, particularly insects, play significant ecological and economic roles, acting as pollinators, prey, and pests (Department for Environment 2014).

Understanding more about the behavioural ecology and its evolutionary effects in insects is therefore a worthwhile goal in its own right. In addition, studying invertebrates, particularly model organisms, has led to important advances in our understanding across a wide variety of fields, not just in sexual selection, but also genetics, developmental biology, and neurobiology (Bellen et al. 2010; Jennings 2011).

The work that has been conducted on female competition in insects falls into three main themes:

- Mutual ornamentation – whether females use their secondary sexual traits in similar ways to males in intrasexual competition (Al-khairulla et al. 2003; Tibbetts 2008; Watson and Simmons 2010)
- Sex differences in aggressive behaviours – whether the sexes use different behaviours in aggressive interactions, and what determines the winner of intrasexual contests (Adamo and Hoy 1995; Pie 1998; Shelly 1999; Fowler-Finn and Hebets 2006; Elias et al. 2010)
- Effects on fitness – whether females that are more successful in female competition have higher reproductive success (Eggert et al. 2008; Watson and Simmons 2010).

However, few studies have explored the effects of reproduction on female competition in invertebrates. Those that have show conflicting results – virgin females fight for longer than mated females in the Mediterranean fruit fly (Papadopoulos et al. 2009), while mated females fight for longer than virgin females in the model organism, *Drosophila melanogaster* (Nilsen et al. 2004). The fact that so little has been done on the relationship between reproduction and aggression in female invertebrates raises

questions as to how reproduction and aggression will be related in species which do not have gestation or lactation, and rarely have parental care. It seems possible that the predictions made about reproduction and aggression, such as mating leading to an increase in aggression due to increased energy demands, will be less applicable to invertebrates. For example, there is no fecundity cost to females from producing an exaggerated secondary sexual trait in dung beetles (Simmons and Emlen 2008), as has been predicted in species with heavy maternal investment (Fitzpatrick et al. 1995). One of the main aims of this thesis is to investigate the relationship between reproduction, particularly mating and egg production, on female aggression in an insect model.

To better understand how reproduction and female aggression interact, it is necessary to study the proximate mechanisms of both suites of behaviour. The proximate mechanisms of female aggression in invertebrates remain seriously understudied, despite vast tracts of work on the proximate mechanisms of male aggression. Research identifying the neurocircuitry and hormonal networks of male aggression in *D. melanogaster* has given us deep insight into the functioning of highly conserved neuropeptides, such as serotonin, that are relevant for a broad range of taxa (Dierick and Greenspan 2007; Miczek et al. 2007; Heiming et al. 2013; Alekseyenko et al. 2014). It seems likely that dissecting the proximate mechanisms of female competition in model organisms, such as *D. melanogaster*, could provide us with valuable insights into how female aggression is regulated and stimulated. The existing genetic and molecular tools available in such species are extremely well-developed, enabling an easier study of female competition than is possible in most vertebrate species (e.g.

manipulating egg production status - Oliver et al. 1987). Given the short generation times of most insects, it is also possible to investigate the fitness effects of female competition in more manageable time periods, rather than relying on long-term, multi-generational datasets that take decades to generate for wild vertebrate species (Clutton-Brock and Huchard 2013a), making insects excellent study systems for the study of female competition.

Study systems

Stalk-eyed fly (Teleopsis dalmanni)

Stalk-eyed flies (Diptera: Diopsidae) are increasingly a model system for the study of sexual selection and exaggerated secondary sexual traits (de la Motte and Burkhardt 1983; Wilkinson and Dodson 1997; Cotton et al. 2004a, 2006; Rogers et al. 2008). There are over 200 described species of stalk-eyed fly, mainly spread throughout the tropics of Asia and Africa (Feijen 1998). The Diopsidae are characterised by the elongation of the head into long stalks and are the only group of hypercephalic flies in which both males and females possess this exaggerated head morphology (de la Motte and Burkhardt 1983; Baker and Wilkinson 2001). The relative size of eye stalks, as well as size differences between males and females, vary dramatically across species (Feijen 1998; Wilkinson and Taper 1999; Baker and Wilkinson 2001). In numerous species, males compete for access to females, with contest outcome determined by the size of their eye stalks relative to body length (Burkhardt and de la Motte 1983; Panhuis and Wilkinson 1999). Females also prefer more ornamented males (i.e. males with longer eye stalks) both in the wild, (Burkhardt and de la Motte 1988) and in controlled preference experiments in the lab (Hingle et al. 2001a,b).

Males also prefer females with large eye stalks as eye span is a reliable indicator of fecundity (Cotton et al. 2014). Females have also been observed to fight (de la Motte and Burkhardt 1983; Al-khairulla et al. 2003), leaving open the possibility that female eye span may function in a similar way to male eye span in intrasexual competition.

Fruit fly (Drosophila melanogaster)

Drosophila melanogaster provides a malleable and well-studied system in which to investigate female competition. Male aggression and competition has been well documented (reviewed in Zwarts et al. 2012), but female intrasexual interactions have so far received less attention (but see Ueda and Kidokoro 2002; Nilsen et al. 2004). Both male and female aggressive behaviours have been categorised and described ethologically (Hoffmann 1987; Chen et al. 2002; Nilsen et al. 2004). Male and female intrasexual interactions differ both in the behaviours used and the motivations for fighting (Hoffmann 1987; Ueda and Kidokoro 2002; Nilsen et al. 2004; Edwards et al. 2006). Females are more likely to fight over high-value food resources, while males compete for access to mates and control of territories (Nilsen et al. 2004; Edwards et al. 2006). Using a model organism allows a much closer investigation of the proximate mechanisms involved in regulating female aggression. Much work has been done on the genetics of aggression in fruit flies, including work on the gene *fruitless*, which has been shown to play a role in male courtship (Rideout et al. 2007) and regulate sex-specific aggression (Vrontou et al. 2006). Work on chemical signals (e.g. serotonin: Johnson et al. 2009 and octopamine: Miczek et al. 2007), signalling pathways (Kuo et al. 2012) and neurobiology (Rezával et al. 2012) has also provided a much clearer understanding of the proximate mechanisms of sex-specific behaviour in *Drosophila*

(Zwarts et al. 2012). The primary aim of these studies, however, is to describe how such systems function, rather than their evolutionary significance. By combining the powerful molecular and genetic tools available for *D. melanogaster* with an evolutionary approach, focusing on behaviour; I aim to address important questions about the evolution of female competition in *D. melanogaster*.

Thesis overview

In my thesis, I will contribute to developing this new field of research on female competition that challenges our assumptions about sex roles and differences by focusing squarely on females, the sex long ignored by evolutionary biologists (Hrdy and Williams 1983). I study contest dynamics in two species of fly in which male-male competition is well-characterised but female competition has been relatively neglected. Across my chapters, I investigate how and why females compete over a limited resource essential for survival and reproduction – food.

In Chapter 2, I examine the role of an exaggerated secondary sexual trait (eye span) in female competition in the stalk-eyed fly *Teleopsis dalmanni*. I find that condition determines outcomes of female contests but, contrary to predictions, eye span does not appear to play a role in determining female-female contests. I also reanalyse classic data on male-male contests and find that eye span does not function as a signal in male intrasexual competition either, demonstrating similarities in the selective pressures faced by males and females in this species.

In Chapter 3, I examine how mating increases female aggression in the fruit fly *Drosophila melanogaster*. I find that mating doubles the amount of time females spend fighting over food. Contrary to predictions, this increase is not due to elevated

energetic demands as a result of egg production. I then test the role of various male ejaculate components and find that sperm is required for the full post-mating increase in female aggression. I further find that the seminal fluid protein, 'sex peptide', which is involved in many crucial post-mating responses, induces a partial increase in female aggression after mating.

In Chapter 4, I study how male condition can influence levels of mating-induced female aggression in *D. melanogaster*. I find that manipulating male feeding status may result in changes in rates of post-mating female aggression, suggesting variation in males can induce variation in levels of female aggression.

In Chapter 5, I investigate how larval density and mating status interact to determine contest dynamics and outcomes in female *D. melanogaster*. I find that larval density primarily determines contest outcome, but that there is a developmental environment-dependent effect of mating. In contrast, I find that contest duration is mainly determined by mating status. I then discuss how resource holding potential and resource payoff value can interact to influence contest dynamics in female competition.

Chapter 2: Condition, not eyespan, predicts contest outcome in female stalk-eyed flies, *Teleopsis dalmanni*

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Abstract

In contests among males, body condition is often the key determinant of a successful outcome, with fighting ability signalled by so-called armaments, that is, exaggerated, condition-dependent traits. However, it is not known whether condition and exaggerated traits function in the same way in females. Here, we manipulated adult condition by varying larval nutrition in the stalk-eyed fly, *Teleopsis dalmanni*, a species in which eyespan is exaggerated in both sexes, and we measured the outcome of contests between females of similar or different body condition and relative eyespan. We found that females in higher condition, with both larger bodies and eyespan, won a higher proportion of encounters when competing against rivals of lower condition. However, when females were of equal condition, neither eyespan nor body length had an effect on the outcome of a contest. An analysis of previously published data revealed a similar pattern in males: individuals with large relative eyespan did not win significantly more encounters when competing with individuals of a similar body size. Contrary to expectations, and to previous findings in males, there was no clear effect of differences in body size or eyespan affecting contest duration in females. Taken together, our findings suggest that although eyespan can provide an honest indicator of condition, large eyespans provide no additional benefit

to either sex in intrasexual aggressive encounters; body size is instead the most important factor.

Introduction

Exaggerated ornamental traits in males are widely viewed as the most conspicuous product of sexual selection (Darwin 1871; Andersson 1994). These secondary sexual characters are used to attract mates and often play a role in mediating competition with rivals over mating opportunities (Andersson 1994). The majority of studies on exaggerated traits have focused on males, neglecting the wide variety of taxa in which females also possess similar traits ("mutual ornamentation": Kraaijeveld *et al.*, 2007; Tobias *et al.*, 2012). The existence of exaggerated traits in females has traditionally been attributed to a genetic correlation between the sexes, where sexual selection favouring male trait expression results in a correlated response in females (Darwin 1871; Lande 1980). Theory (Lande 1980) and empirical work (Price 1996; Wilkinson 2009; Potti and Canal 2011) have shown that genetic correlations between the sexes exist in certain situations. However, recent studies indicate that female exaggerated traits can also be under strong selection (e.g. Watson & Simmons, 2010; Rosvall, 2011; Mahr *et al.*, 2012). A crucial next step is to establish the form of this selection, particularly whether it is similar to, or different from, that acting on males (Tobias et al. 2012).

Studies investigating adaptive explanations for female ornamentation tend to focus on male mate choice and often neglect other explanations, such as female competition over ecological or social resources (Kraaijeveld *et al.*, 2007; but see Watson & Simmons, 2010; Midamegbe *et al.*, 2011). Nonetheless, access to these resources is a key factor for female fitness, suggesting that traits that increase resource acquisition ability are

potential targets for selection (Berglund et al. 1993). In "classical" sexually selected mating systems, males often fight over and monopolize access to females, resulting in dramatic differences between winners and losers. Females, on the other hand, often compete over access to resources which are more likely to increase survival or fecundity or be used to rear offspring, such as favourable nesting sites (Rosvall 2008) or food to provision offspring (Robinson and Kruuk 2007; Watson and Simmons 2010). Winners in female competition may be able to acquire larger shares of such resources, but are unlikely to be able to monopolize them, perhaps leading to incremental increases in fitness over their competitors rather than the "winner takes all" results in males (Clutton-Brock 2009).

Condition, defined as "the pool from which resources are allocated" (Rowe and Houle 1996), plays a key role in male intrasexual competition, where proxies of condition such as body size or sexually selected trait size are often key predictors of contest outcome and duration (McCann 1981; Dugatkin and Biederman 1991). The role of condition in female competition is, however, much less clear. In some species, individuals in higher condition are more directly successful in female competition or acquire higher social status (Petrie 1988; Griggio et al. 2010; Watson and Simmons 2010; Cain and Ketterson 2012), but in others, condition appears to play little or no role in deciding intrasexual encounters (Dugatkin and Biederman 1991; Draud 2004; Elias et al. 2010). If condition is important in determining the outcome of female competition over resources which influence fitness, we would expect that traits that signal an individual's condition or competitive ability to be used in intrasexual contests (Berglund et al. 1993). We would also expect that the ability to determine a rival's

size or fighting ability will not only affect contest outcome but also the duration of intrasexual contests (Parker 1974; Maynard Smith and Parker 1976). Contest duration should be determined by differences in resource holding potential ("RHP": Parker, 1974) between competitors - the larger the difference, the shorter the contest (Parker 1974). Traits which enable the quick recognition of RHP or competitor ability should help to minimize costs by shortening duration or preventing individuals from participating in contests they have no chance of winning (Parker 1974). One implication of these hypotheses is that exaggerated traits in females may often be "armaments," which are used as weapons or signals of dominance in intrasexual contests, rather than "ornaments," which are used to attract mates. We follow the previous literature in using this definition of "armament" to include signals (rather than just weapons), as it allows us to draw useful parallels with "ornaments" (Berglund et al. 1996).

While single traits functioning as both ornament and armament are widespread in males (Berglund et al. 1996), the situation in females is less clear (Tobias et al. 2011). The question of whether exaggerated traits could function as female armaments and mediate female-female competition has received little attention (Berglund et al. 1996; Griggio et al. 2010; Kekäläinen et al. 2010), in part because exaggerated traits and their fitness consequences are less easily quantified in females (Tobias et al. 2011). Female competition can be difficult to study because few contests are decisively resolved, leading to unclear dominance patterns, as well as less dramatic escalation of behaviours than in males (Nilsen et al. 2004; Clutton-Brock and Huchard 2013a). Exaggerated condition-dependent traits in females have also often been neglected in studies of intrasexual competition because they fail to show the same levels of

exaggeration and heightened condition dependence as their male homologs (Kraaijeveld et al. 2007). Females are argued to experience a trade-off between fecundity and trait expression, which hampers the evolution of trait exaggeration (Fitzpatrick et al. 1995). This lack of exaggeration has been used to justify a lack of research interest into the potential signalling qualities of female traits (Amundsen 2000). However, although female traits are, in general, not as exaggerated as male traits, this is no reason why they cannot function as honest, condition-dependent signals. In fact, the proposed trade-off between fecundity and trait expression may be the very mechanism which ensures honesty in female traits (Simmons and Emlen 2008).

To explore the role of condition and female traits in intrasexual competition, we investigated the function of an exaggerated trait in the stalk-eyed fly, *Teleopsis dalmanni*. In *T. dalmanni*, males possess large sexually selected eyestalk ornaments and females possess smaller but nonetheless exaggerated eyestalks of unknown function (de la Motte and Burkhardt 1983; Baker and Wilkinson 2001; Al-khairulla et al. 2003). It has recently been suggested that there is male mate choice for females with longer eyestalks, suggesting that there are selective pressures acting on female eyespan (Cotton et al. 2014). Individuals vary in eyestalk length, measured as the span across both eyestalks, and it is easily quantifiable in both sexes. To test the importance of condition and eyespan, we experimentally manipulated female larval nutrition to generate variation in condition, and hence, body size and eyespan, and conducted intraspecific contests over food, measuring the outcome of contests in terms of their duration and proportion of encounters won. We used body length as our proxy for condition (David et al. 1998; Cotton et al. 2004a). As eyespan is also used as a

measure of condition in *T. dalmanni*, we aimed to evaluate whether eyespan solely functions as a measure of body length or whether it can provide additional information about the condition of an individual (David et al. 1998; Cotton et al. 2004a). It has been suggested that eyestalks are more sensitive to condition than body size traits and may therefore provide additional, or more accurate, information during rival assessment in intrasexual competition (Wilkinson and Dodson 1997; Panhuis and Wilkinson 1999). To test this, we investigated whether eyespan explained any additional variance in contest outcome after controlling for larval diet treatment and body size. Furthermore, to evaluate similarities between the sexes in the use of exaggerated traits in intraspecific competition, we reanalysed data from a classic study on male competition in *T. dalmanni* using analogous methods (Panhuis and Wilkinson 1999). Panhuis and Wilkinson used randomly selected individuals, representing the variance available in a laboratory population but did not manipulate condition in their experiment. While larval diet treatment is the main cause of variation in condition in females in our experiment, pre-existing standing variation in body size of unmanipulated males from a laboratory population is used as an index of condition in Panhuis and Wilkinson (1999), meaning that male body size is not identical to a larval diet treatment. The differences in experimental design, and evaluation of condition, mean that although we cannot therefore directly compare our results, qualitative comparisons should highlight any major differences between the sexes in how they use exaggerated traits in intrasexual competition.

If eyespan is a condition-dependent signal of quality mediating intrasexual competition in female *T. dalmanni,* we predicted the following:

1. Flies in higher condition (i.e., those from less restricted larval diet treatments) will have larger eyespans.
2. Flies in higher condition will win a higher proportion of agonistic encounters than flies in lower condition.
3. As the difference in condition between competitors increases, contest duration will decrease.
4. When flies are matched for condition, individuals with larger eyespan relative to their body condition will win a higher proportion of encounters.

We also hypothesized that prediction 4 would also hold true for all males from Panhuis and Wilkinson (1999).

Methods

Study species

Teleopsis dalmanni is a sexually dimorphic stalk-eyed fly found in Southeast Asia (de la Motte and Burkhardt 1983; Swallow et al. 2005). The eyestalks of males are generally longer, thinner and flatter than those of females and can be up to one and a half times male body length (Swallow et al. 2005; Worthington et al. 2012). Male eye-span is highly condition dependent, increasing dramatically with increased larval nutrition and body size (David et al. 1998; Cotton et al. 2004a); female eyespan is also condition dependent, though to a lesser degree (David et al. 1998; Cotton et al. 2004a). Previous studies showed that males with larger eyespan relative to their body length are more likely to win in male-male competition (Panhuis & Wilkinson, 1999; but see Brandt & Swallow, 2009), and females prefer to roost and mate with males with larger relative eyespan (Burkhardt and de la Motte 1988; Hingle et al.

2001a). Females also engage in physical contests generally over food, both in the wild and in the laboratory (Burkhardt and de la Motte 1983; Al-khairulla et al. 2003). Although female contests resemble those of males, where individuals line up face-to-face and strike each other with their forelegs (Panhuis and Wilkinson 1999), it is unknown whether condition plays any role in determining contest outcome, and whether eyespan explains any further variation in contest outcome (Al-khairulla et al. 2003).

Fly rearing

Flies used were from the laboratory population of *T. dalmanni* founded in 1993 with wild-captured individuals from Gombak, Malaysia (Cotton et al. 2004a; Rogers et al. 2006). We kept all flies in cages at 25 °C with 70% humidity on a 12:12 h cycle (light: dark). Population size has been kept high (>200 individuals) to minimize inbreeding (Cotton et al. 2004a; Rogers et al. 2006). The flies were fed ad libitum with blended sweet corn, which was replaced twice a week. All experiments were conducted in 2013, with initial treatments (block 1) replicated later in the year (block 2).

Manipulation of condition and eyespan

Larval nutrient availability determines adult body size and eyespan and has been established as a determinant of condition in *T. dalmanni* (David et al. 1998). In block 1, batches of 20 eggs were collected from population cages and placed in Petri dishes lined with damp cotton wool (the same quantity for each treatment). Following thresholds proposed by David et al. (1998), we generated differences in female eyespan by assigning eggs randomly to one of three diet manipulations: 0.015 g (restricted), 0.03 g (medium), and 0.06 g (fully fed) corn per egg. In block 2, we transferred batches

of 15 eggs, each randomly assigned to one of the same three provisioning treatments. Previous studies have shown that individuals raised on more restricted larval diets (e.g., flies on the restricted treatment vs. flies on the medium treatment) eclose into smaller adults which have shorter eyestalks than flies from less restricted larval diet treatments (e.g., flies on the fully fed treatment are on a less restricted diet than those on the medium treatment) (David et al. 1998; Cotton et al. 2004a). Flies took approximately 3 weeks to eclose. One week after eclosion, we separated flies according to sex and dietary treatment to ensure virginity. Flies were then housed in same-sex and same-treatment group cages (10–20 individuals per 2 L cage in block 1 and 40–60 per 10 L population cage in block 2, resulting in similar population densities across treatment blocks). In total, 526 females were used in this experiment.

Female contests

When females reached sexual maturity (4 weeks after eclosion), we anaesthetized flies by placing them on ice and took photographs of all individuals lying on their thoracic spines at 7.5_9 magnification using a Canon EOS 600D SLR camera mounted on a Leica M80 microscope. We then used the program ImageJ (Rasband 1997) to measure eyespan and body length following landmarks used by Wilkinson (1993).

Flies were removed from group cages 24 h before being used in a contest and placed in separate Petri dishes containing damp cotton wool but no food. Depriving flies of food was to increase their motivation to fight and increase our chances of observing contests, as flies were fighting over food (blended sweet corn) in the contest. In block 1, we placed a dot of acrylic paint (red or yellow) on females'

thoraxes 24 h before they were used in a contest (Al-khairulla et al. 2003). Unfortunately, the flies removed the paint used to mark them, so we used body length to distinguish individuals. We used ImageJ to measure individuals from still images taken from each contest video and matched these to the measurements taken under the microscope, enabling us to identify individual flies. In block 2, to aid identification of individual flies, a small part of one wing (left or right) was cut close to the tip of the wing before flies were placed in their individual Petri dishes (Chenoweth et al. 2007). Flies do not appear to use their wings during fights, although there is the potential for flies to use the wings for balance (Al-khairulla et al. 2003). Half (50%) of the flies in each treatment had their left wing clipped; the rest had their right wing clipped. Flies were randomly assigned to treatments, but were always matched against a fly which had the opposite wing clipped.

There were six fight treatments (all possible combinations), based on larval dietary manipulations: fully fed versus fully fed (FF), fully fed versus medium (FM), fully fed versus restricted (FR), medium versus medium (MM), medium versus restricted (MR), and restricted versus restricted (RR). “F” females tended to develop into the largest individuals, “R” the smallest, and “M” intermediate. Females from each group were randomly assigned to a fight treatment on the day of the contest but were never matched with a female from the same cage. There were between three and five cages for each treatment in each block. The contest arena consisted of a Petri dish, with one damp cotton wool pad as lining and a small dab of blended corn in the centre. Contests were held over 2 h following lights-on with females matched for age. Flies were aspirated into the arena at the same time and allowed

5 min to acclimatize. The arena was then filmed for 20 min. Each fly was used in only one contest.

Scoring contests

Contest videos were analysed using JWatcher (http://www.jwatcher.ucla.edu/; Blumenstein *et al.*, 2006). We scored four variables to quantify the intensity and result of contests: the number of encounters, the duration of each encounter, the fly initiating each encounter, and the contest outcome. For each individual, there were three possible results for each encounter: win, loss, or no result. The winner of each interaction was scored as the fly that did not retreat or turn away first (Panhuis and Wilkinson 1999; Al-khairulla et al. 2003). If it was unclear which fly turned away first, the encounter was scored as a "no result." Videos were scored blind with respect to the female treatment, although in size-mismatched treatments, there was no way to conceal obvious size differences.

Statistical analysis

Generalized linear models (GLMs) with a quasibinomial error distribution were used to test the effects of larval diet treatment, body length, and eyespan on the "proportion of encounters won" and "contest duration." A quasibinomial distribution was used to adjust for overdispersion. To allow for nonindependence of individuals from the same dyad, dispersion was calculated using the number of dyads to calculate degrees of freedom, rather than the number of individuals, and doubling the sum of the residuals from the fitted model (McCullagh and Nelder 1989). "Proportion of encounters won" was used as the primary response variable and was calculated as the number of encounters won by the focal individual divided by the total number of

decided encounters (where there was a winner and loser). To test predictions related to contest duration, and to ensure model residuals were normally distributed, the natural log of total fight duration and the natural log of mean fight duration were used as response variables in general linear models. Block was originally included as a fixed effect in all models, but had no significant effects so was removed and is not included in the models reported here. Data on male–male competition came from a previous study on the role of eyespan in male stalk-eyed flies, including *T. dalmanni* (Panhuis and Wilkinson 1999). Males and females were analysed in separate models as the data came from different experiments. The first set of female analyses focused solely on differences between larval diet treatments, without including body length or eyespan as factors. This allowed us to test our prediction that flies from less restricted larval treatments would win more encounters.

To control for differences in diet treatment and body size between competitors in a second set of models, treatments were reclassified into “matched” and “unmatched” body size treatments. Treatments were “matched” by pairing females with an individual from the same larval diet treatment, so that both individuals were similar in body size (range of differences: 0.002–1.397 mm). We investigated the importance of investment in eyespan relative to body length by analysing only the “matched” treatments, including larval diet and body length as covariates in the analysis, thus allowing us to evaluate the effect of eyespan after taking into consideration the effect of larval diet treatment and body length (Cotton et al. 2009). For males, where there was no larval diet manipulation, we used all data from Panhuis and Wilkinson

(1999), so only body length and eyespan were included in the model. All data were analysed in R version 2.15.2 (R Core Team 2012).

Results

Prediction 1: Flies in higher condition (i.e. those from less restricted larval diet treatments) will have larger eyespans

In support of this prediction, we found that larval diet treatment had a significant effect on both eyespan and body length (eyespan: $F_{2,522} = 723.5$, $P < 0.0001$ and body length: $F_{2,522} = 636.9$, $P < 0.0001$; Inset Fig. 1). Females from the fully fed treatment had the largest eye-spans (5.75 ± 0.01 mm) and body lengths (6.69 ± 0.02), while flies from the restricted treatment had the smallest (ES: 4.32 ± 0.04, BL: 5.34 ± 0.04). Flies from the medium treatment were intermediate in size (ES: 5.25 ± 0.03, BL: 6.23 ± 0.03). In addition, there was a significant positive correlation between female eyespan and body length across all treatments ($F_{1,522} = 5727$, $P < 0.0001$, eyespan = body length - 0.978, $R^2 = 0.92$; Fig. 1).

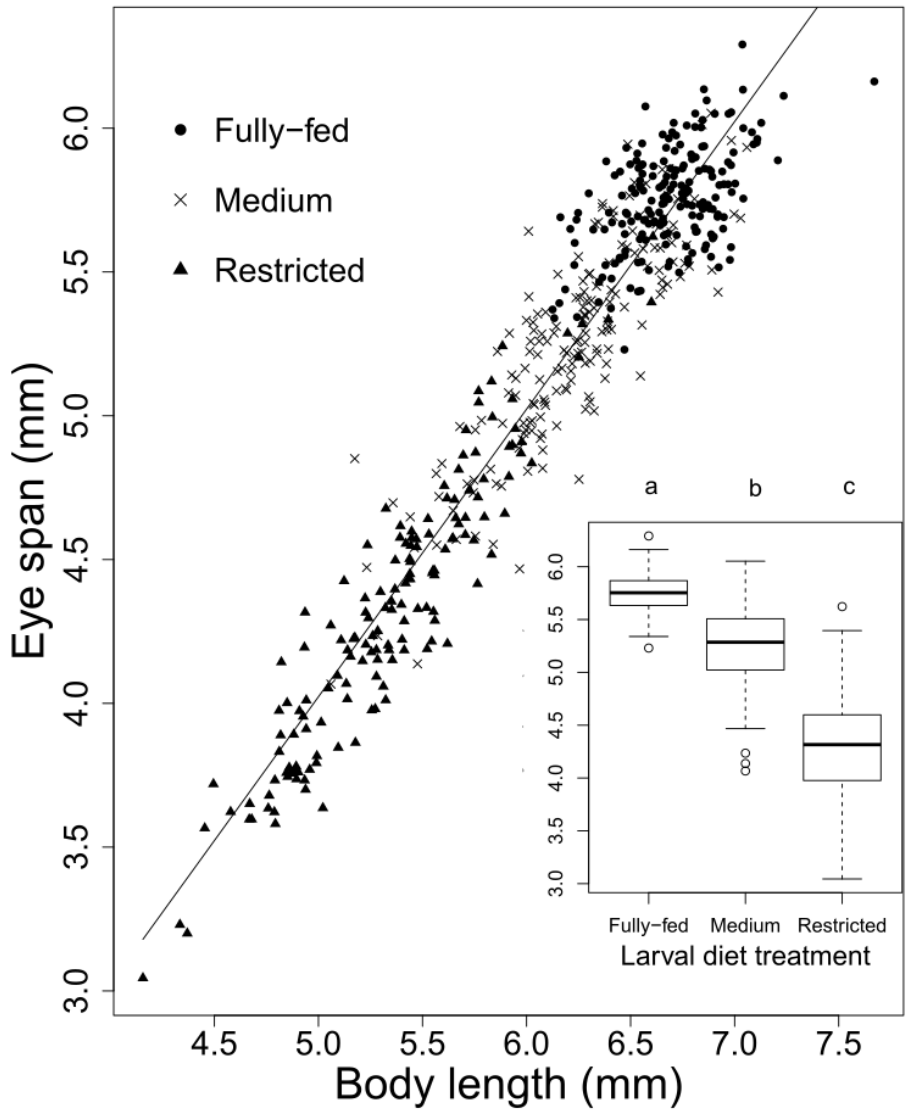


Figure 1: Relationship between eyespan and body length in female *T. dalmanni*

Circles = fully fed diet treatment (n = 193), crosses = medium larval diet treatment (n = 178), and triangles = restricted larval diet treatment (n = 154). The linear regression line is shown (eyespan = 0.999 x body length – 0.97, R2=0.92). The inset boxplot reflects the differences in mean between the treatments, with post-hoc Tukey tests indicated by letter subscripts. Different letters indicate significant differences between treatments.

Prediction 2: Flies in higher condition will win a higher proportion of agonistic encounters than flies in lower condition

In support of this prediction, we found that diet treatment of the focal fly had a significant effect on the proportion of encounters won, with females of higher condition winning a greater proportion of encounters ($X^2_{2,287}$ = 12.12, P = 0.028; Fig. 2). Competitor diet treatment also had a significant effect on the proportion of encounters won with focal individuals winning a higher proportion of encounters when competing against individuals from more restricted diet treatments ($X^2_{2,285}$ = 20.07, P = 0.002). However, there was no significant interaction ($X^2_{4,281}$ = 0.57, P = 0.99). In other words, flies from less restricted diet treatments (i.e., fully fed and medium) won a higher proportion of encounters than females from more restricted diet treatments (i.e., restricted).

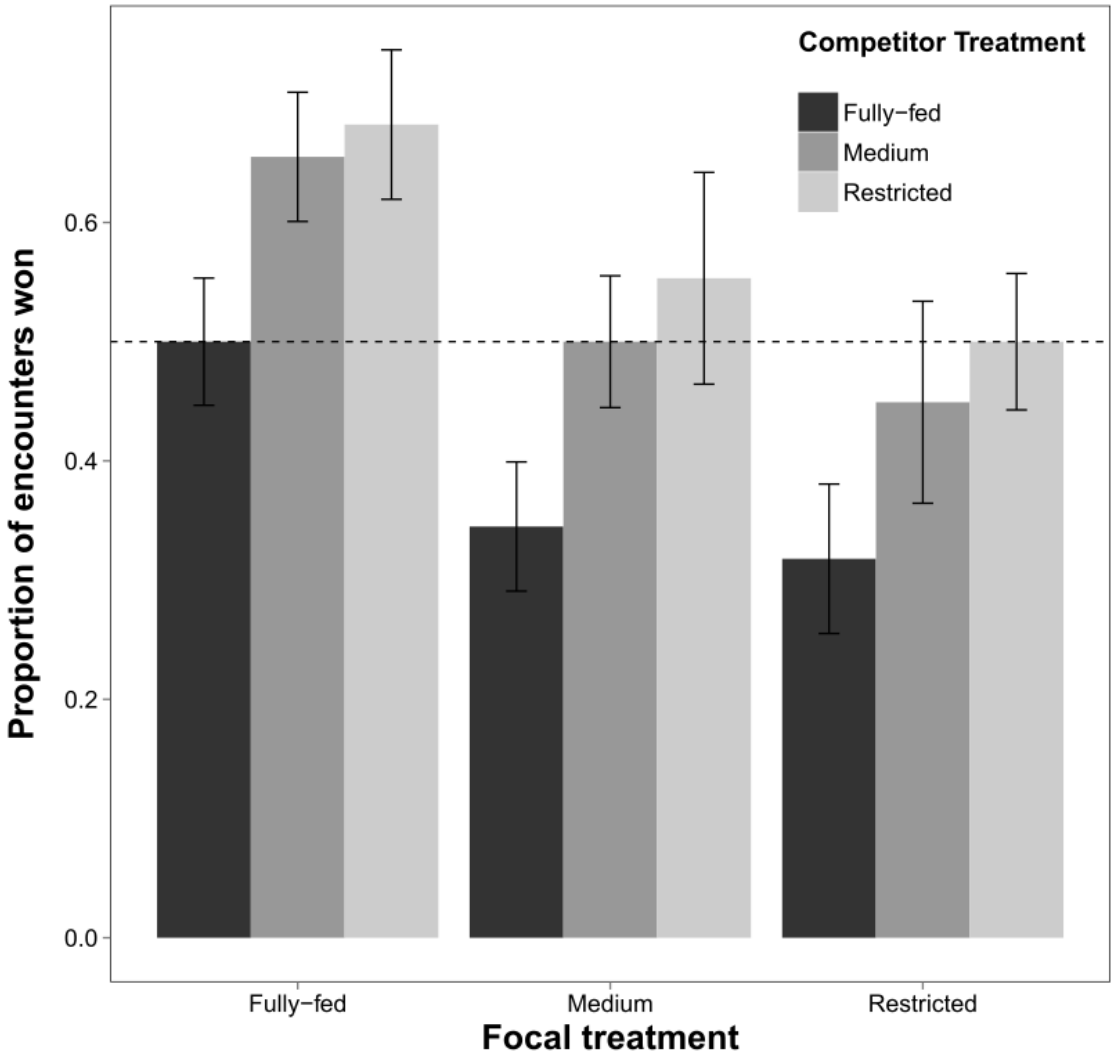


Figure 2: Effect of diet treatment on proportion of encounters won in females

Individuals from less-restricted diet treatments won a higher proportion of encounters when competing against individuals from more-restricted larval diet treatments. Columns represent means, with error bars indicating standard errors. The dotted horizontal line indicates a proportion of 0.5 – when the focal fly wins the same number of fights as its competitor.

Prediction 3: As the difference in condition between competitors increases, contest duration will decrease

We did not find support for this prediction. Specifically, we found that diet treatment of the focal fly had a significant effect on total contest duration: females of higher body condition fought for longer ($X^2_{2;259}$ = 208,525, P = 0.038; Fig. 3). However, there was no significant effect of competitor diet treatment ($X^2_{2;257}$ = 23,249, P = 0.694) or the interaction between focal and competitor treatment on contest duration ($X^2_{4;253}$ = 108,310, P = 0.492). When focusing on instances where flies fought individuals from the same larval diet treatment, there was a significant difference in total contest duration between treatments, where females from the "fully fed" treatment fought for longest, followed by those from the "restricted" treatment, with females from the "medium" treatment fighting for the least amount of time ($F_{2,252}$ = 3.59, P = 0.029). However, the only significant difference between treatments in post hoc tests was between "medium" and "restricted" treatments (Tukey multiple comparison: restricted – medium – t = 2.67, P = 0.022), where females from the "restricted" fought for longer than those from the "medium" treatment.

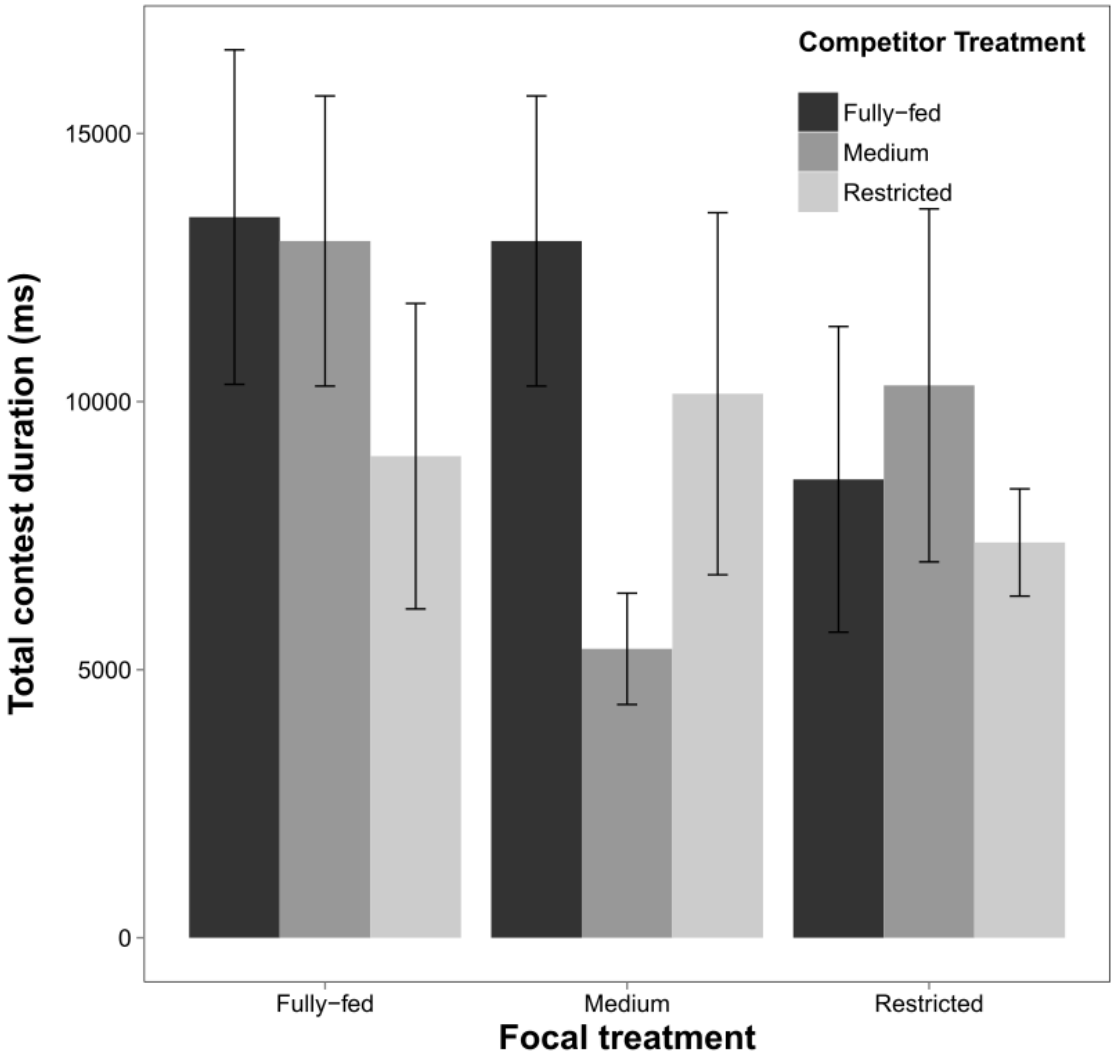


Figure 3: Effect of diet treatment on total contest duration in females

There was no discernible pattern due to larval diet treatment in total contest duration in females. Columns represent means, with error bars indicating standard errors.

Prediction 4: When flies are matched for condition, individuals with larger eyespan relative to their body condition will win a higher proportion of encounters in:

Females

We found no support for this prediction in females. There was no significant effect of larval diet treatment ($X^2_{1;133} = 0$, P = 0.99), body length ($X^2_{1;132} = 0.53$, P = 0.576), or eyespan after controlling for body length on the proportion of encounters won ($X^2_{1;131} = 0.25$, P = 0.7; Fig. S1.a). Furthermore, there was also no significant interaction between diet treatment and body size ($X^2_{1;130} = 3.56$, P = 0.149) or between diet treatment and eyespan ($X^2_{1;129} = 0.57$, P = 0.564). In other words, when a female faced a competitor of the same level of condition, possessing larger eyestalks relative to body length did not increase the proportion of encounters an individual won.

Males

We also found no support for this prediction in males, contrary to previous findings. Specifically, we found that while there was a significant effect of body length on the proportion of encounters won ($X^2_{1;54} = 51.9$, P = 0.009), with larger individuals winning a higher proportion of encounters, there was no effect of eyespan after controlling for body length ($X^2_{1;53} = 1.02$, P = 0.714; Fig. S1.b). In other words, males with longer eyestalks relative to body length did not win a higher proportion of encounters.

Discussion

In this study, we found that eyespan is strongly correlated with body length (our proxy for condition) in female *T. dalmanni,* suggesting there is the potential for eyespan to function as an armament in intrasexual competition. However, body length rather than eyespan was the key determinant of contest outcome, with larger females winning a higher proportion of encounters than those in lower condition. However, contrary to our predictions and previous findings in males, body length appeared to play no role in determining contest duration. Eyespan relative to body length was not associated with success in aggressive interactions when fighting similarly sized same-sex rivals, in either males or females.

Eyespan is closely linked to body length (i.e., it is condition dependent) in both male and female *T. dalmanni* (David et al. 1998; Cotton et al. 2004a). As contest outcome is determined by body length, it seems logical that condition is the underlying factor determining contest outcome. If condition is important in determining contest outcome, it is possible that eyespan functions as an armament to signal body size and/or fighting ability. In the wild, *T. dalmanni* form linear aggregations on long, thin root hairs at dusk (de la Motte and Burkhardt 1983). When both males and females fight, they orient themselves face-to-face, with their eyestalks parallel to each other (Panhuis and Wilkinson 1999; Al-khairulla et al. 2003). This would make it difficult to judge body size directly, but allows the easy assessment of eyespan. This method of fighting, which appears to be closely linked to the narrow, vertical nature of their aggregation environment (Burkhardt and de la Motte 1983; de la Motte and Burkhardt 1983), could lead females to use eyespan as

the main means by which to assess body size and fighting ability of their opponent. Detectable differences in eye-span may indicate differences in condition and/or fighting ability that could influence the outcome of a contest.

Once we controlled for body length, we found that eye-span did not significantly influence contest outcome. This does not mean that eyespan does not function as an armament, but merely that after body length is controlled for, eyespan explains no more variance in contest outcome. By controlling for body length, we hoped to test whether eyespan merely functions as a measure of body length or whether it represents a "wider range of condition factors that act independently of body size" (Cotton et al. 2004a). For neither females nor males does eyespan appear to reflect additional factors of condition that influence intrasexual competition. In other words, when competing against an individual of the same body length, having more exaggerated eyestalks (which should indicate higher condition) does not appear to give an individual an advantage. This is what we would expect if there was a trade-off between eyestalk length and other fitness traits, such as fecundity. The prediction would be that females with traits exaggerated beyond what is necessary to adequately signal condition should have reduced fitness, because resources invested in "extra" eyestalk length could have been spent on increasing body size, and hence increasing fecundity (Fitzpatrick et al. 1995). This trade-off may not just restrict the mean eyestalk length of the population but also prevent cheating by smaller individuals (Simmons and Emlen 2008). If individuals overly invest in eyestalks to win a higher proportion of encounters, they will be able to produce fewer offspring than those that invest at the "right" level (Chenoweth et al. 2007).

Eyestalks in stalk-eyed flies have been used as a prime example of intrasexual competition leading to extreme trait exaggeration in evolutionary textbooks, but our results do not support this interpretation. As long as eye-span accurately reflects body size, it should be able to function as an armament, regardless of the level of trait exaggeration. Male eyespan is under sexual selection by means of female mate choice, as females prefer males with longer eyestalks (Burkhardt and de la Motte 1988). Therefore, it is possible that male eyespan has become more exaggerated due to female choice and has remained an accurate signal of condition to be used in male competition, but it seems unlikely to have been selected for its function in male intrasexual competition ("ornament-armament" hypothesis: Small *et al.*, 2009).

To more rigorously examine whether *T. dalmanni* actually use eyespan as a signal in intrasexual competition, we would need to uncouple eyespan and body length, which is experimentally challenging. In one study where eyespan relative to body length was artificially selected for, flies selected for increased relative eyespan also showed an increase in body size, demonstrating the difficulty in trying to decouple eyespan and body length (Wilkinson 2009). In our current study, we were able to manipulate body length (and therefore condition), but not alter the relationship between body length and eye-span. Further work manipulating eyestalk length through altering imaginal discs during development, or manually transferring eyestalks, to create large flies with small eye-spans and vice versa, may give a better indication of whether and how *T. dalmanni* use eyespan as a signal of condition in intrasexual competition (Warren and Smith 2007; Brandt and Swallow 2009). As condition can be affected by both larval and adult diet, it would be informative in

future studies to manipulate adult diet and larval diet, giving a better understanding of what role condition plays in determining the outcomes of intrasexual competition.

The trend that flies of larger body size, that is, those with higher resource holding potential ("RHP": Parker, 1974), win more fights concurs with previous findings in males of the same species (Panhuis and Wilkinson 1999; Brandt and Swallow 2009), as well as with females in other species (Petrie 1988; Watson and Simmons 2010; Crowhurst et al. 2012). Contest duration, however, did not show the expected trend. There was no indication that differences in size between competitors affected contest duration. According to theory, competitors that are extremely different in size (or any other indicator of RHP) should be able to quickly determine who is larger and more likely to win a fight, resulting in smaller individuals giving up sooner against a larger opponent to minimize costs (Parker 1974; Maynard Smith and Parker 1976; Hammerstein and Parker 1982). A fight between a smaller individual and a larger individual should therefore take less time than a contest between two similarly sized individuals. This theory assumes that there is some method of assessing a rival and that individuals are able to judge the relative strength of the two competitors (Fawcett and Mowles 2013). Our finding that contest duration is not affected by relative size differences between female competitors suggests that there may be no rival assessment in female *T. dalmanni*. Individuals may instead "decide" how long they compete for based on their own energy reserves and the perceived resource payoff value ("RPV": Draud, 2004) of the contested resource. It has been suggested that in male *T. dalmanni*, contest duration is determined by loser body size rather than the difference in size between the two competitors (Brandt and Swallow 2009). If duration is determined by

losers reaching an internal threshold sooner and retiring from the contest, condition is still the primary determinant of contest outcome, but is functioning in a more indirect way than what has been envisaged under mutual assessment theory (Taylor and Elwood 2003; Fawcett and Mowles 2013). If condition acted this way in females (i.e., individuals persisted until they reached their internal energy threshold), we would have expected to see flies in higher condition fighting for longer against flies in similar condition and contests decreasing in duration as the condition of competitors decreased (Taylor and Elwood 2003). We did observe flies from the "fully fed" larval diet treatment fighting for longer against opponents matched for condition, but "medium" treatment flies fought for less time than "restricted" treatment flies. "Restricted" females fighting for longer than "medium" females could indicate that "restricted" flies have an increased threshold for fight length because they are so short of resources that they must continue to fight despite their lack of energy reserves.

A second possible explanation for this lack of expected trend in contest duration is that individuals may value the contest resource differently, leading to different levels of motivation and willingness to compete (Bishop et al. 1978; Enquist and Leimar 1987). The individual that values the resource more should be willing to expend more energy and/or to risk more to secure the resource (Draud 2004). Females may be more variable in their resource valuation than males, due to characteristics that change over time, such as mating status, fertility, and age (Clutton-Brock and Huchard 2013b). Both the costs and benefits of engaging in competition may therefore change for an individual female over time (Bowler et al. 2002; Papadopoulos et al. 2009; Seebacher et al. 2013). It seems unlikely that there was a great deal of variation between females in perceived resource

value in our experiment, however, as females were all virgins of the same age and underwent the same period of food deprivation before their trials. It is, however, possible that this starvation period affected females from different treatments differently - females in lower condition may have been more strongly affected than those in higher condition with the internal resources to withstand a period without food. An increased desire for food in the lower condition flies could lead them to fight for longer against larger opponents than they would have if they valued the resource equally. Despite their increased motivation, however, these lower condition flies may still have been unable to overcome the size disparity between the competitors, leading to our observation of flies in better condition winning a higher proportion of encounters.

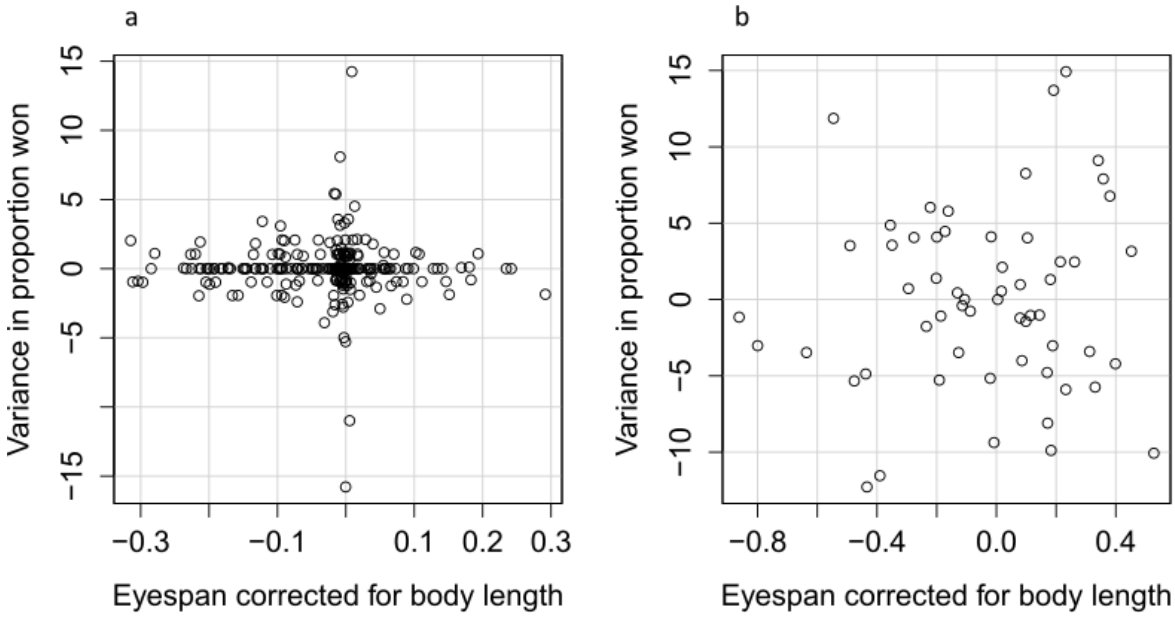
Conclusion

Female *T. dalmanni* fight over food, and these contests are primarily determined by condition. Eyespan may function as an honest indicator of condition, but having larger eyestalks relative to body length does not appear to provide any additional predictive power for the outcome of intrasexual competition. The same appears to be true in males, despite the large differences in trait exaggeration between the sexes. It is possible that female exaggerated traits serve as armaments in deterring rivals from initiating intrasexual contests over ecological resources, but having higher relative eyespan confers no competitive advantage during such contests. Further work on female competition and the use of condition-dependent traits can help us to better understand the adaptive value of exaggerated traits in females and explain the understudied phenomenon of mutual ornamentation.

Acknowledgments

We thank Tami Panhuis and Gerald Wilkinson for making their data available for analysis and Kevin Fowler and Nadine Chapman for providing stocks for the original fly culture. This research was supported by funding from The Royal Society (to Nathalie Seddon), NERC, and the BBSRC (Fellowships to Stuart Wigby) as well as the Rhodes Trust, the Royal Entomological Society and St John's College, Oxford (to Eleanor Bath).

Supplementary Information



Supplementary Figure S1: The effect of eyespan controlled for body length on proportion of encounters won

The unexplained residuals of proportion of encounters won regressed on body size (i.e. the variance in proportion won not explained by body size) plotted against the residuals of eyespan regressed on body size (i.e. the variance in eyespan not explained by body size) in a) females matched for body size and b) all males from Panhuis & Wilkinson 1999. There was no significant effect of eyespan after being corrected for body length on the proportion of encounters an individual won.

Chapter 3: Sperm stimulates female aggression

Note: A version of this chapter has been submitted to Nature Communications as Eleanor Bath, Samuel Bowden[a], Carla Peters, Anjali Reddy, Joseph A. Tobias, Evan Easton-Calabria, Nathalie Seddon, Stephen F. Goodwin, Stuart Wigby. 'Sperm stimulates female aggression'

See 'Author Contributions' for full details.

Abstract

Female aggression increases after mating in a wide range of taxa. There have been few tests of how this increase in aggression is stimulated. In this article we propose two non-mutually exclusive hypotheses – that increased post-mating aggression reflects elevated resource demands during reproduction, and that female aggression is stimulated by components of the male ejaculate. Here we experimentally test whether female aggression in *Drosophila melanogaster* is elevated innately by female reproduction, or alternatively whether female aggression is stimulated by males during copulation. Contrary to expectations, we show that post-mating increases in female aggression are decoupled from costs of egg-production in females, and instead directly induced by sperm and an associated seminal fluid protein in the male ejaculate. We conclude that male ejaculates have a direct influence on aggressive behaviours in their recipient, which can in turn shape the competitive environment encountered by other females in the population. By highlighting the importance of the male ejaculate in regulating female aggression, our results identify a mechanism with potentially broad relevance across taxa.

Introduction

Aggressive behaviours are widespread in nature, yet most research to date has focused on males. A spate of recent studies has shown that aggression in females can also have important fitness consequences, for example resulting in territorial displacements, as well as death or injury to individuals or their offspring (Watson and Simmons 2010; Eggert and Müller 2011; Rosvall 2011c; Cain and Ketterson 2012). Some of the most influential fitness effects may be associated with reproduction, particularly as female intrasexual aggressive behaviour (hereon "female aggression") increases after mating in many species (McDonough 1994; Palanza et al. 1996; Bowler et al. 2002; Robinson and Kruuk 2007; Seebacher et al. 2013). This pattern of post-mating female aggression typically occurs in parallel with other dramatic changes in female behaviour and physiology during this period, including sexual receptivity, feeding rates, and hormone levels (Moshitzky et al. 1996; Barnes et al. 2008; Stockley and Campbell 2013; Cunningham et al. 2014). In vertebrate taxa, this increase in aggression has been linked to protecting offspring from infanticide and predation (Robinson and Kruuk 2007; Rosvall 2011d; Stockley and Bro-Jørgensen 2011; Clutton-Brock and Huchard 2013b). In addition, increased levels of female aggression during reproduction should be related to increased resource demands due to offspring production (Robinson and Kruuk 2007), which induce females to compete for access to resources such as food, nesting sites, and breeding territories (Tobias et al. 2012; Clutton-Brock and Huchard 2013b). However, the link between costs of reproduction and female aggression remains to be tested experimentally, and the proximate causes of post-mating alterations in female aggression remain unknown.

We propose that one possible mechanism linking costs of reproduction and post-mating increases in female aggression is that signals of nutrient depletion are received by the female as offspring are produced, leading to feeding and aggressive behaviours necessary to address that deficit (Barton Browne 1995). Under this hypothesis, egg production results in a deficit of critical nutrients, such as protein or salt (Walker et al. 2015), which then elicits an appetite for those depleted nutrients through a negative feedback loop (Barton Browne 1995). In comparison with virgin females, mated females have increased energetic demands, and thus require more, and different, nutrients (Barton Browne 1995; Wheeler 1996; Walker et al. 2015). The elevated value of these resources to mated females will theoretically lead to an increase in motivation to compete over them (Stockley and Bro-Jørgensen 2011; Clutton-Brock and Huchard 2013a). This 'demand-mediated' nutrient-uptake mechanism is a possible mechanism by which the increased energetic demands of reproduction could be translated into increased aggression.

A proximate alternative to 'demand-mediated' post-mating female aggression is stimulation by components of the male ejaculate transferred to the female during mating. Male ejaculates could either act as a cue for females to optimally modify their behaviour, or as a mechanism by which males modify female behaviour for their own gain, potentially generating conflict between the sexes (Arnqvist and Rowe 2005). In many species, components of the male ejaculate, such as sperm and seminal fluid proteins (sfps), mediate other female post-mating responses (PMRs), including ovulation, egg laying rates, receptivity to remating, immune changes and feeding patterns (Avila et al. 2011; Rodríguez-Martínez et al. 2011; Ratto et al. 2012; Sirot et al.

2015). Although the physiological causes of female aggression have received some empirical attention (McDonough 1994; Cain and Ketterson 2012; Rosvall 2013a; Tibbetts et al. 2013), the question of whether male ejaculates influence female-female aggression remains unexplored.

In female fruit flies, *Drosophila melanogaster,* many PMRs are induced by sfps, such as the 'sex peptide' (SP). SP binds to sperm and is the molecular basis of the "sperm effect", which results in a variety of PMRs, including increased egg-laying (Liu and Kubli 2003), decreased receptivity to remating (Liu and Kubli 2003), increased rates of feeding (Carvalho et al. 2006), and altered sleeping patterns (Isaac et al. 2010). Females that do not receive SP during mating show greatly reduced PMRs and behave similarly to virgins (Manning 1962; Liu and Kubli 2003). SP signals through the female sex peptide receptor (SPR; Yapici et al. 2008; Dean et al. 2012; Rezával et al. 2012) to mediate SP-induced egg-laying and remating PMRs; mated females that lack the SPR, or mates of males that lack SP, behave similarly to virgins in terms of fecundity and refractoriness (Yapici et al. 2008). SP and the SPR are therefore prime candidates for regulating any female aggression PMRs in *D. melanogaster*.

In this study, we investigated the effects of mating on female aggression in *D. melanogaster*, and tested whether any changes in behaviour were associated with either egg production or the male ejaculate. In the wild, this species aggregates on rotting fruit, where limited food and oviposition resources result in larval and adult competition (Wertheim et al. 2006). Thus, differential reproductive success could result from variation in female ability to aggressively acquire and defend food and oviposition resources. Our experiments involved placing age-matched pairs of female

D. melanogaster in a contest arena (consisting of a food cap with a drop of yeast paste) and then quantifying the total number of aggressive encounters and the duration of any fights during the subsequent 30 minutes. In the first set of experiments, each pair consisted of either two females that had mated once (24hrs previously), or two virgins, to test the prediction that mated females fight for longer than virgin females.

To examine whether any increase in female aggression is 'demand-mediated' (Barton Browne 1995; Sgrò and Partridge 1999), we then compared the post-mating responses of fertile females with those of ovo^{D1} females, who have arrested oogenesis and cannot produce eggs (Oliver et al. 1987). Costs of egg production are lacking in ovo^{D1} females (Sgrò and Partridge 1999), and thus our experiments test whether elevated female aggression is linked to the energetic demands of egg production pathway signalled downstream of vitellogenesis (egg yolk formation). If egg production is an essential pre-requisite for an increase in female aggression, the difference in post-mating aggression between mated and virgin ovo^{D1} females should be significantly reduced compared to controls. To explore the role of fighting motivation and ability on resource uptake, we also quantified the time spent feeding by females in different treatments, and the influence of contest outcomes on feeding behaviour.

Finally, to investigate the role of different components of the male ejaculate, we tested whether the receipt of sperm, or SP, or the possession of SPR, were required for females to exhibit post-mating changes in aggression. If increases in female aggression are mediated by male ejaculate signals we predicted that females that do

not receive sperm or SP, or females that lack the SPR, would display reduced or virgin-like levels of aggression after mating.

Methods

Fly stocks and culture

All flies were kept and experiments conducted at 25°C on a 12:12 light: dark cycle. We used the Dahomey genetic background as our stock population, and genetic mutations (see Supplementary Methods 4) were backcrossed into this background for >5 generations where appropriate.

Experimental Design

3d post-eclosion virgin females were marked with acrylic paint (red or yellow) on the thorax to allow individual identification(Nilsen et al. 2004), and returned to individual vials. 24hrs later, females in the "mated" treatments were placed individually with one male, and a single copulation was directly observed. After each female mated exactly once, males were discarded and all females (i.e. both mated and virgins) were individually transferred to fresh vials, again containing standard media and no live yeast.

The following morning (5d post-eclosion), females were individually placed in vials containing damp cotton wool and no food for 2hrs(Edwards et al. 2006) after which pairs of females were simultaneously aspirated from these vials into the contest arena. The arena was a single well of a twelve-well plate, containing an Eppendorf tube cap filled with standard fly food media and a ~2 μL drop of yeast paste, providing a limited resource to compete over(Bradley and Simmons 1997). Females were either both mated, both virgin, or – to test the relative competitive ability of mated vs virgin females – one of each. Females were allowed 5 minutes to acclimatise, and then behaviour was recorded for 30 mins using Toshiba Camileo X400 HD video cameras.

Behavioural analysis: Videos were scored blind to treatment. Fighting behaviours (head-butt, leg "fencing" (later excluded), shove, retreat (Nilsen et al. 2004)) and feeding behaviours were recorded. We recorded the number and duration of encounters, the identity of females initiating each encounter, the outcome of each encounter (i.e. win, lose , draw), and time spent feeding, using the program JWatcher + Video (Blumenstein et al. 2006).

Statistical analysis: We used generalized linear models (GLMs) with a Gamma error distribution as our response variables were continuous and most closely fit a Gamma distribution to test the effects of mating status, egg production status, and various ejaculate components on total contest and feeding duration. For feeding analyses, we additionally included proportion of encounters won as an explanatory variable. Because Gamma error distributions use a logistic function, we added 1 to all scores of total contest duration prior to transformation to include replicates with scores of 0. We tested for outliers using the Grubbs outlier test and excluded outliers below a threshold of P = 0.001. We excluded one outlier in the egg production experiment in the fertile mated treatment. Winsorizing the data did not qualitatively alter the results (S5). To test the relative fighting ability of mated and virgin females, we replicated the MV treatment once but not the MM and VV treatments. We replicated the SP experiment, finding that experimental series (block) had no effect on analyses (contest duration: $Dev_{1, 126} = 1.225$, P = 0.131). To allow for non-independence of individuals, we used each dyad as the unit of replication, rather than individual flies. For MV treatments, we randomly chose one focal individual from each dyad and analysed the

proportion of encounters won using a GLM with a quasibinomial distribution. All models were run in R version 3.0 (R Core Team 2012).

Results

Mating elevates female aggression

We found that aggression was significantly elevated by mating: interactions between two mated females (MM) involved a greater number of aggressive encounters ($X^2_{1, 248}$ = 296.61, P < 0.001), and a longer total duration of such encounters ($Dev_{1, 240}$ = 26.01, P < 0.001), than interactions between two virgins (VV) (total duration of mated pairs relative to virgins indicated in Fig. 1). We next paired mated females with virgin females (MV) to test relative fighting ability. We found no evidence that mating status influenced who won or lost aggressive encounters ($X^2_{1, 26}$ = 2.59, P = 0.41; Fig. 2.a). Moreover, there was no significant difference in contest duration between MM and MV pairs (MM vs. MV: $q_{2, 20}$ = 0.81, P = 0.58) whereas both MM and MV pairs fought more than VV pairs (MM vs VV: $q_{3, 24}$ = 4.81, P = 0.006; MV vs VV: $q_{2, 24}$ = 3.69, P = 0.015; overall model: $Dev_{2, 74}$ = 11.36, P = 0.004; Fig. 2.b). Thus, the presence of one mated female is sufficient to elevate the overall aggression level of a dyad, but mated females do not then win significantly more aggressive encounters.

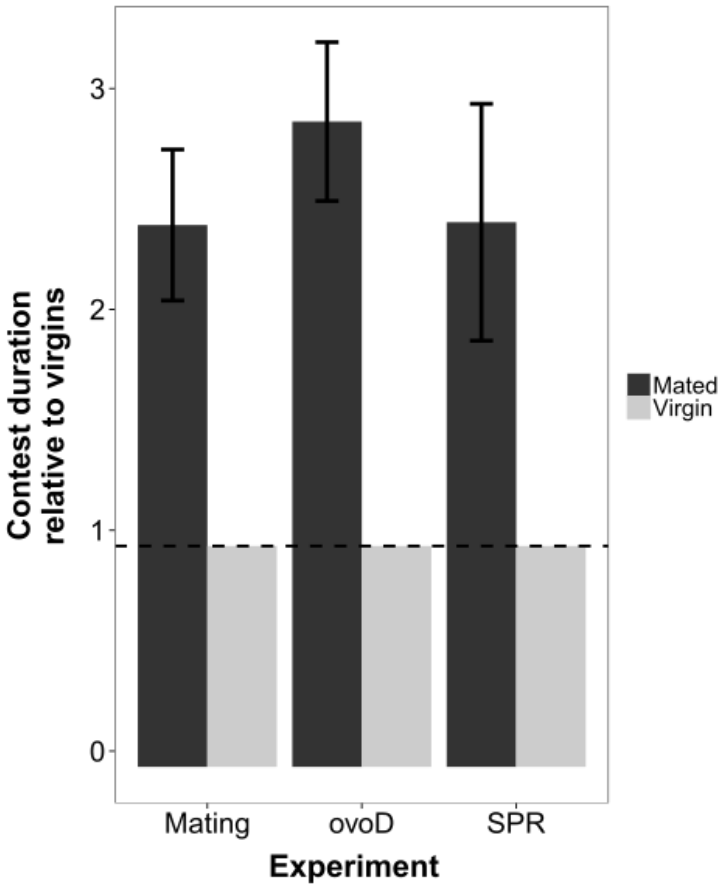


Figure 1:

Mating stimulates increased aggression in females. Three replicate experiments are shown (N = 25-35 pairs per treatment). Pairs of females were either both virgins, or both mated to a standard wild-type male. Data are standardised by dividing by virgin control means within each replicate (i.e. so virgins = 1).

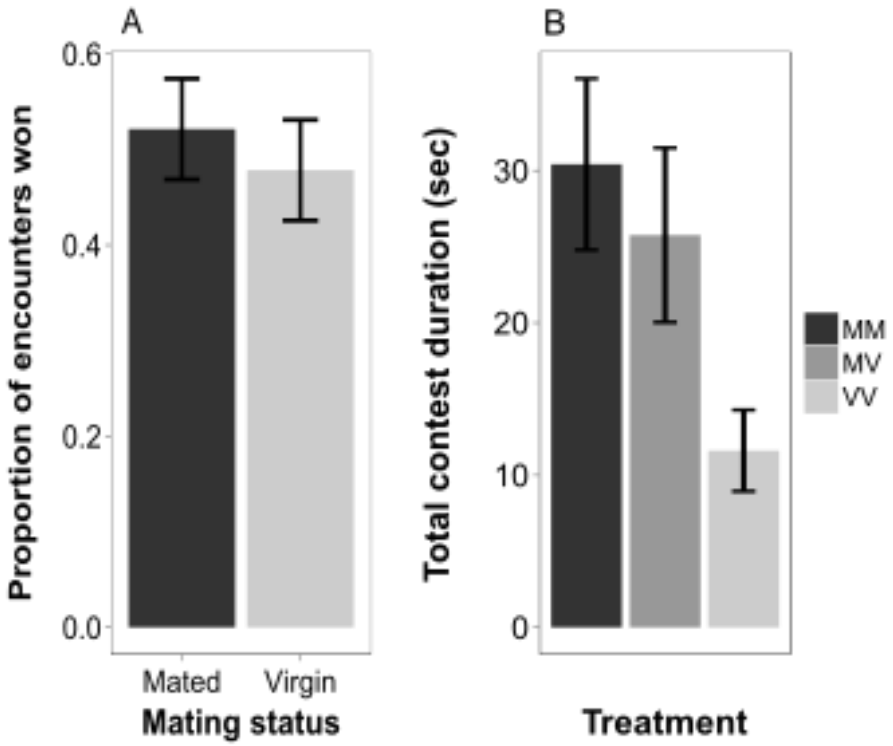


Figure 2:

Mated females fight for longer than virgins but do not win more fights. (a) Encounters won by mated females = black bar (n = 45) and virgin females = grey bar (n = 45). (b) Contest duration in mated, virgin and mixed mating-status female dyads. MM = two mated females (N = 29), MV = one mated female and one virgin female (N = 22), VV = two virgin females (N = 26). Model estimate means and standard errors are shown.

Effects of egg production and male ejaculate components on female aggression

We found that mating-induced increases in aggression were not significantly different between *ovo*^{D1} females and controls (mating status, $Dev_{1, 126} = 17.32$, $P < 0.001$; *ovo*^{D1} vs control, $Dev_{1, 125} = 1.27$, $P = 0.12$; interaction, $Dev_{1, 124} = 0.25$, $P = 0.49$; Fig. 3.a). Thus, post-mating increases in female aggression are not restricted to females able to

produce eggs, and are thus unlikely to be driven by the energetic demands of egg production.

Mates of sterile *son-of-tudor* males (hereon, "*tudor* males"*)*, who transfer sfps but no sperm to females (Boswell and Mahowald 1985), did not differ from virgins in levels of post-mating aggression, and were significantly less aggressive than control mated females (overall model: $Dev_{2, 68} = 14.32$, $P < 0.001$; control *vs*. virgin: $q_{3, 24} = 6.8$, $P < 0.001$; control *vs.* mates of *tudor*: $q_{2, 24} = 5.3$, $P = 0.001$; virgin *vs*. mates of *tudor*: $q_{2, 24} = 1.45$, $P = 0.32$; Fig. 3.b). Sperm is therefore required for the mating-induced increase in female aggression.

Aggression in females mated to SP^0 males (who did not receive SP from their mates) was significantly higher than in virgin females ($q_{2, 30} = 3.4$, $P = 0.023$) and significantly lower than in mates of *SP+* males ($q_{2, 30} = 3.05$, $P= 0.04$; overall model $Dev_{2, 127} = 11.26$, $P < 0.001$; Fig. 3.c). These results suggest that SP contributes only partially to the female aggression PMR, and that SP cannot fully explain the sperm effect on aggression. *SPR*- females did not significantly differ from wild-type females in their aggression response to mating (mating status, $Dev_{1, 101} = 11.53$, $P < 0.001$; interaction mating status x *SPR*, $Dev_{1, 99} = 0.05$, $P = 0.78$; Fig. 3.d). Moreover, *SPR*- females fought for longer than wild-type females overall, irrespective of their mating status ($Dev_{1, 100} = 12.71$, $P < 0.001$).

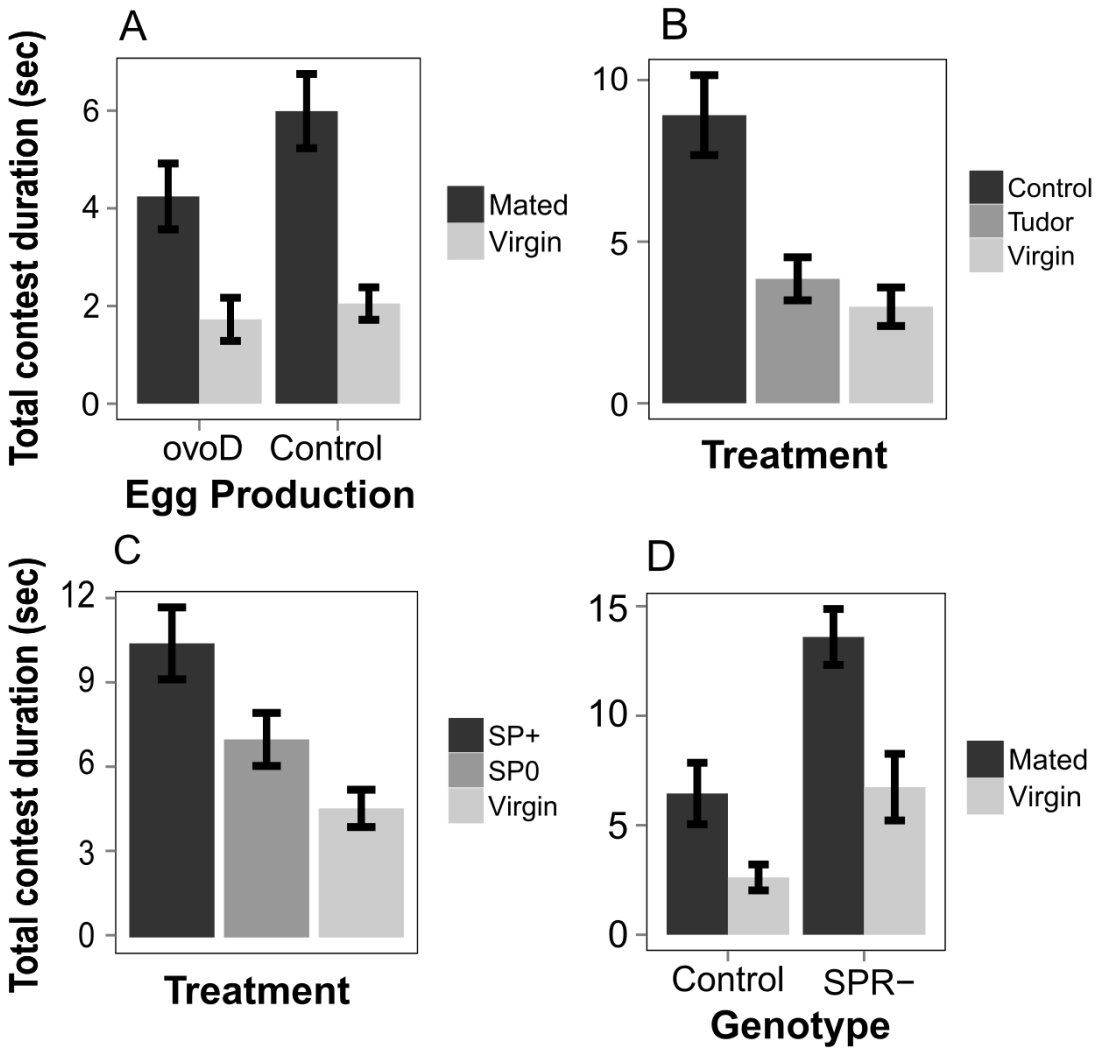


Figure 3:

Effects of male ejaculate components, and female egg-production and sex peptide receptor on female contest duration. a) Contest duration of mated *ovo*^{D1} females (sterile) and control females. N pairs: Mated *ovo*^{D1} = 33, Virgin *ovo*^{D1} = 31, Mated wild-type = 31, Virgin wild-type = 33. b) Effect of sperm transfer on female contest duration. N pairs: Control = 26, *tudor* = 25, Virgin = 25. The experiment was carried out in two blocks, but results are pooled in this figure. c) Effect of sex peptide transfer on female contest duration. N pairs: *SP+* = 39, *SP*^{0} = 42, Virgin = 49. d) Effect of SPR on female contest duration. N pairs: Mated wild-type = 25, Virgin wild-type = 24, Mated

SPR– = 29, Virgin *SPR*– = 25. Bars represent means, while error bars indicate standard errors.

Feeding behaviour responses to mating, egg production

As expected, wild-type mated females spent significantly more time feeding than virgin females ($Dev_{1, 153}$ = 1.61, P = 0.017; Supplementary Figure 1). In the ovo^{D1} experiment, there was a marginally non-significant trend for higher feeding in mated females ($Dev_{1, 102}$ = 1.128, P = 0.056; Fig. 4.a) but no significant effect of the ovo^{D1} mutation ($Dev_{1, 101}$ = 0.15, P = 0.49) or interaction ($Dev_{1, 99}$ = 0.31, P = 0.97). Virgin females and mates of spermless males did not differ in feeding ($q_{2, 24}$ = 0.05, P = 0.97), but both fed less than controls (control vs spermless: $q_{3, 25}$ = 4.67, P = 0.008; control vs virgin: $q_{2, 24}$ = 4.62, P = 0.003; Fig. 4.b). There was a non-significant trend for reduced feeding in mates of SP^0 females compared to controls ($q_{2, 35}$ = 2.502, P = 0.086) and mates of both *SP+* and SP^0 females fed for longer than virgins (*SP+* vs virgin, $q_{3, 35}$ = 6.05, P = 0.0004; SP^0 vs virgin; $q_{2, 37}$ = 3.53, P = 0.02; Fig. 4.c). In the SPR experiment, deletion of the female SPR did not significantly affect feeding duration ($Dev_{1, 100}$ = 0.14, P = 0.55), and there was no significant effect of mating status ($Dev_{1, 101}$ = 0.88, P = 0.13; Fig. 4.d), or interaction ($Dev_{1, 99}$ = 0.16, P = 0.52). The proportion of encounters an individual won was not associated with feeding duration in any experiment (Dev < 0.61 P > 0.13; see Supplementary Figure 1 and Supplementary results 2).

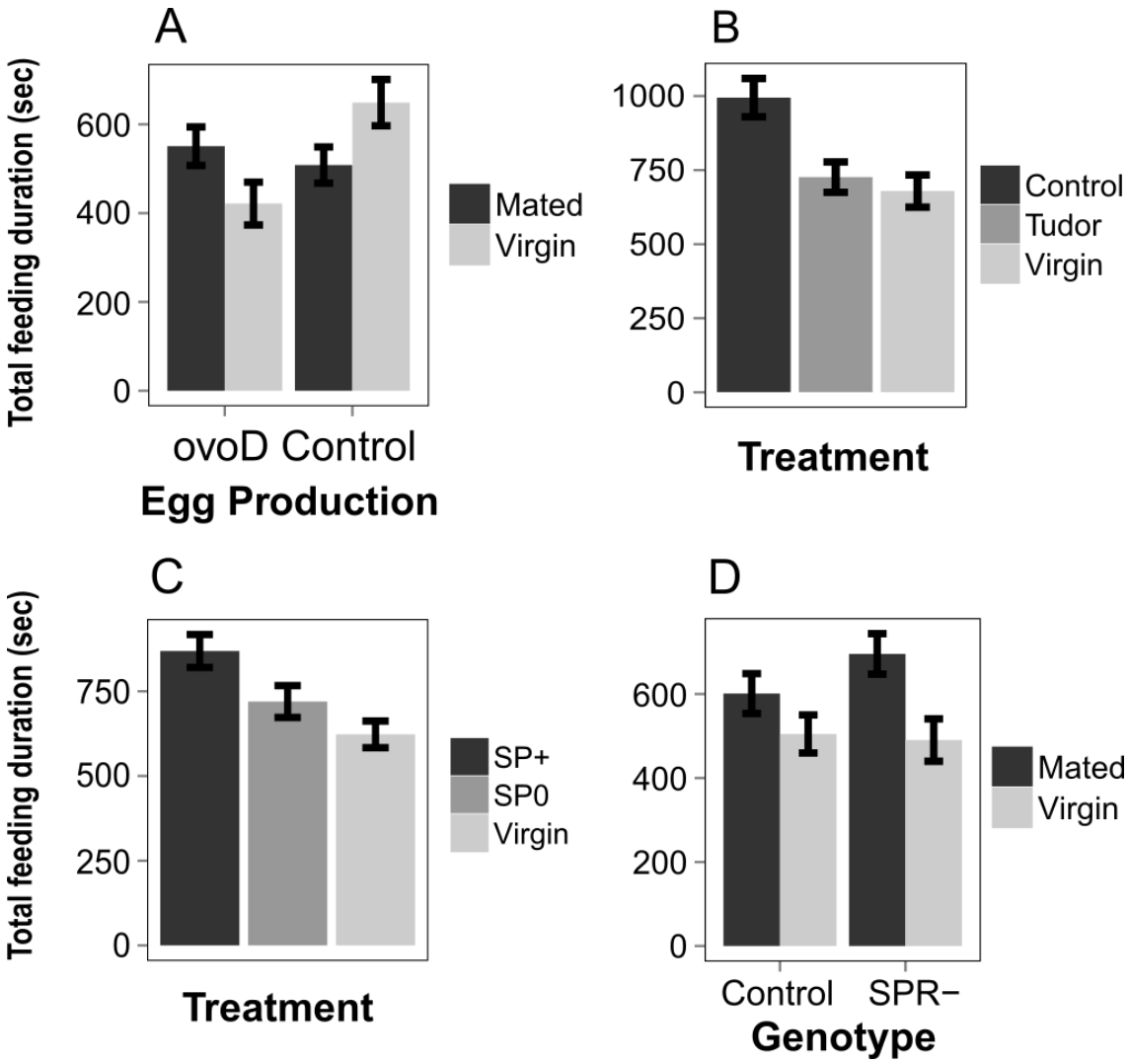


Figure 4:

Effects of male ejaculate components, and female egg-production and the sex peptide receptor on female feeding duration. a) Effect of egg production ability on female feeding duration. N females: Mated ovo^{D1} = 66, Virgin ovo^{D1} = 62, Mated wild-type = 62, Virgin wild-type = 66. b) Effect of sperm transfer on female feeding duration. N females: Control = 52, *tudor* = 50, Virgin = 52. c) Effect of sex peptide transfer on female feeding duration. N females: *SP+* = 78, SP^0 = 84, Virgin = 98. d) Effect of sex peptide receptor presence or absence on female feeding duration. N females: Mated wild-type = 50, Virgin wild-type = 48, Mated *SPR-* = 56, Virgin *SPR-* = 50. Bars represent means, while error bars indicate standard errors.

Discussion

The results show that mated female *D. melanogaster* fight more than twice as long as virgins, and that an increase in aggression requires the receipt of sperm in the ejaculate, but not the receipt of sex peptide (SP), the presence of the female sex peptide receptor (SPR), or the ability to produce eggs. However, SP appears to be required for the full female aggression PMR. Our analyses also indicate that mated females do not win a higher proportion of fights than virgins, but that the presence of a single mated female is sufficient to elevate aggression in a dyad. Moreover, although mated females tend to feed for longer than virgin females, there is no association between feeding rate and the proportion of fights females win.

The finding that mating elevates female aggression is consistent with a previous study (Nilsen et al. 2004) which found a small (21%) but significant increase in the duration of aggression in female *D. melanogaster* that had been housed with males for three days, as compared to virgins. This pattern also fits into a broader trend of mating leading to increased female aggression across taxa, e.g. gestating and lactating mammals and fish display higher levels of aggression towards conspecifics (Clutton-Brock et al. 1998; Bowler et al. 2002; Robinson and Kruuk 2007; Seebacher et al. 2013).

Our data do not support the 'demand-mediated' hypothesis that nutrient deficiencies caused by egg production stimulate female aggression after mating. Sterile ovo^{D1} females displayed the same rate of increase in aggression after mating as wild-type females, despite lacking the costs of egg production (Sgrò and Partridge 1999), while females that lacked the sex peptide receptor (*SPR*-) fought for longer than wild-type

females despite producing fewer eggs (Supplementary Figure 3). These results suggest that egg production and aggression can be decoupled and may be modulated by different pathways. In addition, sexual receptivity and female aggression are likely decoupled. We found that females that do not receive SP, or lack the SPR, display significant increases in aggression, but previous studies show that SP or SPR lacking females do not show the dramatic reduction in receptivity of wild-type females (Liu and Kubli 2003; Yapici et al. 2008).

The finding that SP has a partial effect on aggression, and is required for the full female aggression PMR, whereas the SPR is not required, suggests that SP might act through alternative receptors (Avila et al. 2011; Sirot et al. 2015) to boost aggression. Alternatively, SP may be required for the functioning of other aggression-stimulating sfps. Moreover, the increased overall aggression levels of *SPR*- females were unexpected, and could indicate that the SPR regulates aggression independently of mating status, analogous to its role in regulating sleep in both sexes (Oh et al. 2014). Alternatively, female aggression might be influenced by one or more of the other 4 genes covered by the deletion that removes *SPR* (see methods). Nonetheless, our results show that the post-mating increase in female aggression does not require SP signalling through the SPR, leaving open the possibility that females might detect sperm in their storage organs (Avila et al. 2011; Lee et al. 2015) to trigger aggression, or that sperm might function as a vehicle for other – as yet unidentified – aggression-stimulating sfps (Xue and Noll 2000; Liu and Kubli 2003).

Previous research has shown that mated females sleep less than virgin females, particularly during the day (Isaac et al. 2010), raising the possibility that differences in

siesta sleep could contribute to differences in fighting behaviour. However, mates of SP^0 males exhibit similar levels of sleep to virgins (Isaac et al. 2010) but display a significantly higher level of fighting behaviour. In addition, there is little difference in sleep behaviour between mated and virgin *SPR*- females (Oh et al. 2014), but we detected a significant increase in female aggression in mated *SPR*- females. Thus, SP-induced changes in female sleep patterns cannot explain the post-mating increases in female aggression seen in our experiments.

There has been remarkable progress in identifying the molecular stimuli and neuronal circuits that control male aggression in Drosophila (reviewed in Zwarts et al. 2012)). However, the genetic and neuronal bases of aggression are sexually dimorphic, and the downstream mechanisms that regulate female aggression remain unclear. Potential neuromodulator candidates for mediating female aggression include serotonin and octopamine, both of which modulate aggression in male *D. melanogaster* (Dierick and Greenspan 2007; Hoyer et al. 2008; Zhou et al. 2008; Johnson et al. 2009; Alekseyenko et al. 2014; Andrews et al. 2014), while octopamine plays a similar role in female burying beetles (Cunningham et al. 2014); and juvenile hormone, which is released by SP to increase oocyte progression (Moshitzky et al. 1996) and is linked to increased aggression in other species (Scott 2006; Tibbetts et al. 2013). Furthermore, neuronal circuits that specify other sexually dimorphic behaviours, such as *fruitless* and *doublesex* (Vrontou et al. 2006; Kubli and Bopp 2012; Zwarts et al. 2012), are possible candidates for activating aggression. Exploring the role of these factors in the aggression PMR – and female aggression in general – represents a key challenge for the future.

The fitness implications of female aggression in *D. melanogaster* are currently unknown. Our experiments reveal that mated females fight for longer than virgins, although we found no evidence that they are better fighters (i.e. mated females do not win a higher proportion of encounters). In addition, females that won more fights did not spend more time feeding, suggesting that increased aggression may not function in increasing access to a food source. However, it remains possible that the longer duration of contests involving mated females reflect an increased motivation to fight that translates into improved access to food in wild populations. For example, it is possible that feeding duration might not accurately represent consumption if food intake rate is not constant (Wong et al. 2009). Moreover, in natural situations, losing an aggressive encounter might lead to dispersal away from a food patch, and thus the persistent dyadic encounters in our fight arenas might not represent adaptive outcomes in nature. An alternative possibility is that increased post-mating female aggression is not beneficial to females, but instead an area of sexual conflict. Sfps (including SP) can, under certain conditions, lower female lifetime survival and reproductive output (Chapman et al. 1995; Wigby and Chapman 2005; Fricke et al. 2010), and ejaculate-stimulated female aggression could contribute to these costs. The net fitness costs and benefits of female aggression, and the role of males in determining this, remains an area ripe for exploration.

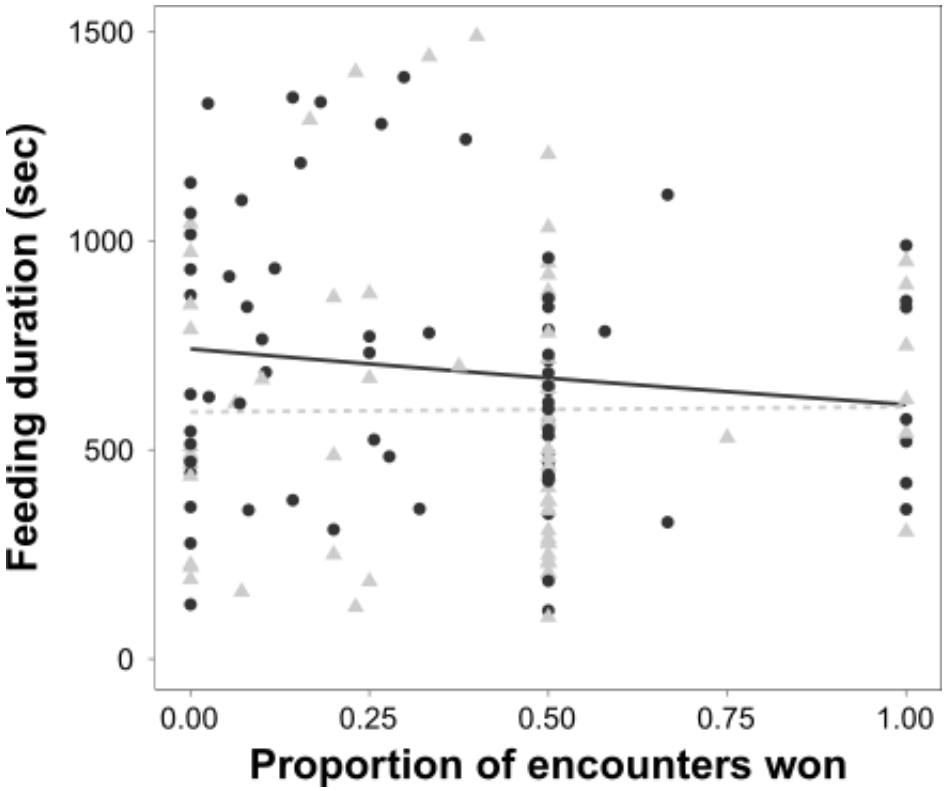
Conclusion

Our results challenge the traditional assumption that female aggression after mating results from the increased energetic demands of reproduction. Instead, we have shown that female aggression in *D. melanogaster* is stimulated directly by male ejaculates, with downstream indirect effects on other females, whereby post-mating aggression affects the wider female competitive environment. The role of males in influencing female aggressive behaviour raises the possibility of conflict between the sexes over female aggression. Our results have potential implications for a wide range of sexually reproducing animals. For example, some broadly conserved mammalian ejaculate components, such as ovulation-inducing factor, can influence changes in female physiology, implying that similar sfps could also shape variation in female aggression across broad taxonomic scales. Future research should focus on determining the generality of ejaculate-stimulated female aggression, identifying the neuronal mechanisms producing these effects in females, and understanding the fitness implications for both females and their competitors.

Acknowledgements

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Supplementary Information



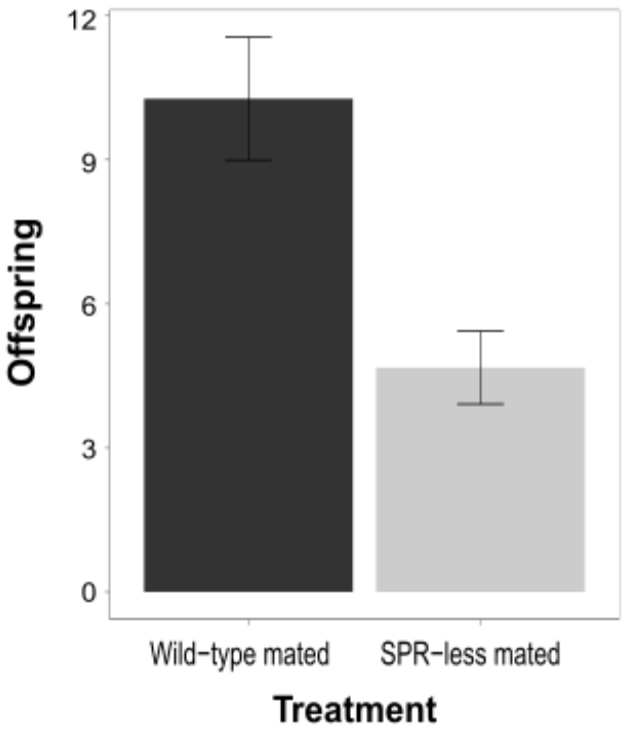
Supplementary Figure 1:

Relationship between proportion of fights won and feeding behaviour in mated and virgin wild-type flies. MM = black solid line and filled circles (N = 86) and VV = grey dashed line, filled triangles (N = 83). The model prediction is represented by the line across the graph (Proportion won: $Dev_{1, 152} = 0.08$, P = 0.6); proportion won * mating status: $Dev_{1, 149} = 0.08$, P = 0.6).

Supplementary results 2: Additional feeding behaviour results

Proportion of encounters won was not significantly associated with feeding duration in any experiment (*ovo*^{D1} experiment: $Dev_{1, 100} = 0.0006$, P = 0.97; *tudor* experiment:

$Dev_{1, 69} = 0.61$, P = 0.13; SP experiment: $Dev_{1, 112} = 0.09$, P = 0.53; SPR experiment: $Dev_{1, 99} = 0.16$, P = 0.52).



Supplementary Figure 2:

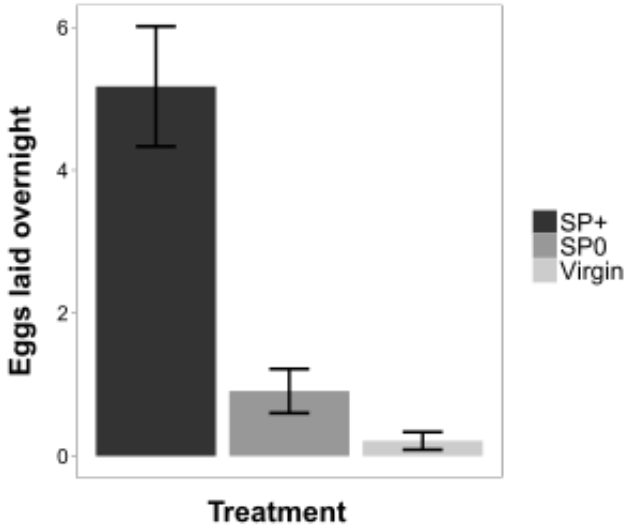
Experimental females were kept in individual vials overnight (see 'Methods'). Offspring that emerged from these vials were frozen and counted 14 days later. As only mated females produced offspring, only data from the wild-type mated (N = 50) and SPR-less mated (N = 56) treatments are presented. Bars represent means, while error bars indicate standard errors.

Supplementary results 3: Sex peptide receptor experiment

Offspring analysis

There was a significant effect of treatment on the number of offspring a female produced (GLM: $X^2_{1, 101} = 111.22$, P < 0.001; Supp. Fig. 3). Wild-type females produced

significantly more offspring than *SPR-* females. These results indicate that the *SPR-* mutant performed as expected.



Supplementary Figure 3:

Eggs counted from overnight vials in block 2 of SP experiment. Black bar indicates females mated to *SP+* males (N = 46), dark grey bars indicate females mated to SP^0 males (N = 56), and light grey bars indicate virgin females (N = 62). Bars represent means, while error bars indicate standard errors.

Supplementary Information 4: Supplementary Methods

Production of wild-type females: In all experiments, we used wild-type Dahomey females (Wigby and Chapman 2004), which were reared in bottles at standardized larval density (Clancy and Kennington 2001). Virgins were collected within 8hrs of eclosion, and sexes were housed separately (females individually) in vials containing standard fly food media (Lewis 1960), with no live yeast. For the mated vs. virgin

experiment and the ovo^{D1} experiment, Dahomey males were used as the mating partners of experimental females.

Production of ovoD1 (sterile) females: To produce sterile ovo^{D1} females, we crossed males carrying the dominant ovo^{D1} mutation to Dahomey virgin females (Barnes et al. 2008). Sterile ovo^{D1} females have ovaries that degenerate prior to S5, meaning that females cannot produce eggs (Oliver et al. 1987). Regular Dahomey females, from the stock into which the ovo^{D1} flies were crossed, were used as controls. We tested the efficacy of the mutation by counting offspring collected from overnight vials (see 'Treatment of experimental females'). No ovo^{D1} females produced offspring.

Production of SP0 males: To produce males that did not produce sex peptide (SP^0) and their controls (*SP+*), we used stocks created by Liu & Kubli (Liu and Kubli 2003). SP^0 males carry one non-functional SP gene, and a deletion (Δ130) which removes SP (Liu and Kubli 2003). The *SP+* control males are genetically matched, but show wild-type SP expression. The stocks had been previously backcrossed into our Dahomey stock. To verify the phenotype of SP^0 and *SP+*, we counted offspring from a subset of female overnight vials (see 'Treatment of experimental females'). Females mated to *SP+* males produced more eggs and offspring than females mated to SP^0 males, confirming that our mutants were acting in the expected way (i.e. SP^0 males were not transferring SP; S6). The SP experiment was carried out over two blocks.

Production of sex peptide receptor-less females: To produce females that lack the sex peptide receptor, we used the genetic deficiency *Df(1)Exel6234* (Yapici et al. 2008). This deficiency consists of a deletion, covering the sex peptide receptor gene, and four other genes of unknown function. These lines were backcrossed into a $white^{Dahomey}$

background (for 5 generations in 2012 (Dean et al. 2012)), and thereafter kept in stock cages (>200 individuals in each) to ensure that all females were of the same genetic background. Females which were homozygous for the deletion did not respond to SP, in that they did not display reduced receptivity after remating, nor did they show elevated levels of egg production (Supplementary Figure 2) (Yapici et al. 2008).

Production of sperm-less males: To produce males that did not produce sperm, we used *tudor* (Boswell and Mahowald 1985). *tudor* is a maternal effect mutation which prevents germ plasm assembly. Sons of homozygous *tudor* females have no germline and so do not produce sperm (Boswell and Mahowald 1985; Xue and Noll 2000). We collected the male offspring of homozygous *tudor* females mated to wild-type Dahomey males as our spermless males. Males of the same genetic background, but with mothers that did not possess the *tudor* mutation were used as controls.

Supplementary results 5: ovoD experiment

Outlier analysis

We identified one outlier in the wild-type mated treatment using the Grubbs outlier test ($G = 6.15$, $P < 0.0001$). We performed two analyses – one with winsorized data and one with the outlier excluded. In the winsorized analysis, mating status had a significant effect on contest duration ($Dev_{1, 127} = 13.413$, $P < 0.001$), egg production capability was marginally non-significant ($Dev_{1, 126} = 1.364$, $P = 0.069$), and there was no interaction between egg production capability and mating status ($Dev_{1, 125} = 0.124$, $P = 0.583$).

When the outlier was excluded, mating status had a significant effect on contest duration ($Dev_{1, 126} = 17.319$, $P < 0.001$; Fig. 3.a), and there was no interaction between

egg production and mating status ($Dev_{1, 124}$ = 0.2466, P = 0.494), or effect of egg production capability on contest duration ($Dev_{1, 125}$ = 1.274, P = 0.120). We excluded the outlier in our final analysis even though the results did not qualitatively change as it seemed that even when winsorized, the one outlier was still exerting undue leverage on the results.

Supplementary results 6: Sex peptide experiment

Egg production data

Data was analysed separately for the two blocks as we counted surviving offspring in block 1 and eggs in block 2.

Block 1

As no offspring emerged from the virgin treatments, we analysed SP^0 and *SP+* treatments only. The data was analysed using a GLM with a quasipoisson distribution. There was a significant difference between treatments in the number of offspring produced ($X^2_{1, 109}$ = 82.917, P = 0.009). As expected, females mated to *SP+* males produced more offspring than females mated to SP^0 males (*SP+*: 20.04 ± 1.89; SP^0: 13.04 ± 1.82).

Block 2

There was a significant difference between treatments in the number of eggs produced ($X^2_{2, 160}$ = 360.16, P < 0.001; S3). There was a significant difference between females mated to *SP+* males and both females mated to SP^0 males and virgins (*SP+* vs. SP^0: $q_{2, 46}$ = 11.089, P < 0.001; *SP+* vs. V: $q_{3, 46}$ = 9.711, P < 0.001). There was no significant difference between SP^0 treatment females and virgins ($q_{2, 56}$ = 1.677, P = 0.241).Results from both blocks confirm that the SP^0 male mutants performed as expected.

Chapter 4: The role of male feeding status in mating-induced female aggression

Abstract

In many taxa, mating dramatically changes female behaviour and physiology. One of these behavioural changes is an increase in female aggression after mating. In previous work, we discovered that components of the male ejaculate can directly stimulate female aggression, suggesting that variation in males could lead to variation in levels of post-mating female aggression. Here I tested the effect of male adult feeding status on levels of post-mating female aggression in the fruit fly *Drosophila melanogaster*. Males who are 'starved' for their adult lives produce reduced levels of a vital ejaculate component – the sex pheromone *cis*-vaccenyl acetate (cVA) – which reduces courtship of male rivals, and may play a role in reducing female receptivity after mating. Starved males mated for shorter durations and transferred reduced levels of cVA to females, suggesting a possible overall reduction in ejaculate size. There was evidence that male feeding status influenced levels of aggression over food in their mates. Mates of fed males fought for significantly longer than virgin females, while females mated to starved males displayed intermediate levels of aggression. These results suggest that variation in male condition could affect levels of mating-induced aggression in their mates, raising the possibility that males could alter the female competitive environment through their ejaculates.

Introduction

Across taxa, such as insects, mammals, and fish, mating alters female behaviour and physiology (Palanza et al. 1996; Kapusta and Marchlewska-Koj 1998; Nilsen et al. 2004; Seebacher et al. 2013). Many of these post-mating changes, such as alterations in offspring production or remating rates, can have important effects on female fitness traits, ranging from increasing reproductive success to reducing lifespan (Wigby and Chapman 2005; Rogina et al. 2007; Seebacher et al. 2013). Often, changes in female behaviour after mating are mediated by components of the male ejaculate, such as sperm or seminal fluid proteins (Sfps) (Avila et al. 2011; Rodríguez-Martínez et al. 2011; Ratto et al. 2012; Oh et al. 2014; Sirot et al. 2015). However, the composition of male ejaculates is likely to vary between males, depending on social and ecological factors, such as male condition, mating history or perceived levels of sperm competition (Bretman et al. 2010; Perry and Rowe 2010; Perry et al. 2013; Tennant et al. 2014; Lane et al. 2015; Ramm et al. 2015). Additionally, males in some species can tailor their ejaculate in response to female states, including condition or mating status (Wedell et al. 2002; Lüpold et al. 2011; Sirot et al. 2011; Jarrige et al. 2015), and variation in male ejaculate traits can influence the strength and direction of female post-mating responses (Rowe et al. 1996; Bretman et al. 2009; Fricke et al. 2010; Papanastasiou et al. 2013; Perry et al. 2013).

One important response to mating across a wide range of taxa is an increase in female aggression. Female aggression increases dramatically after mating in mammals, birds, fish, and insects (McDonough 1994; Palanza et al. 1996; Bowler et al. 2002; Robinson and Kruuk 2007; Seebacher et al. 2013). In theory, this increase in aggression could be

driven by the greater energy demands of reproduction. As females produce offspring, they require more resources, such as food or nesting sites, and therefore have a greater motivation to compete over such resources, leading to elevated levels of aggression (McDonough 1994; Robinson and Kruuk 2007). Male ejaculates can also stimulate increased aggression, separately from offspring production (Bowler et al. 2002; Bath et al. *in review*).

Although the link between mating and an increase in female aggression is reasonably well-established in vertebrate taxa (e.g. in mice: Palanza et al. 1996; bank voles: Kapusta 1998; Kapusta and Marchlewska-Koj 1998; mosquitofish: Seebacher et al. 2013; and meerkats: Young et al. 2006), there has been little work evaluating how variation in male condition, or other male traits, could influence rates of post-mating female aggression. Variation in male body size, genotype, and competitive ability can affect aspects of female fitness, including offspring production, remating rate and immune responses (Pitnick 1991; Pitnick and Garcia-Gonzalez 2002; Wedell 2006; Imroze and Prasad 2011; Kiyose et al. 2015). In *Drosophila melanogaster*, for example, large males inflict more harm on their mates, and induce stronger immune responses than small males (Pitnick and Garcia-Gonzalez 2002; Imroze and Prasad 2011). Variation in male body size, or other traits that influence female post-mating responses, could also result in variation in levels of post-mating female aggression either through variation in male ejaculates, or due to differences in female investment in offspring as a result of male variation.

To test whether male traits influence levels of mating-induced female aggression, I manipulated adult feeding in male fruit flies, *Drosophila melanogaster*. I chose to

manipulate feeding status because it is likely to alter overall ejaculate composition and size (Bissoondath and Wiklund 1996; Zarazaga et al. 2009). Evidence suggests that non-sperm components of the ejaculate are costly for males to produce and replenish, with seminal fluid proteins becoming depleted over subsequent matings, and taking up to three days to be replaced (Hihara 1981; Sirot et al. 2009), so altering feeding regime should affect non-sperm components to a greater extent than sperm. Manipulating male feeding status has been shown to result in changes in the levels of the male sex pheromone *cis*-vaccenyl acetate (cVA), which is produced in the male ejaculatory bulb and transferred to females during mating (Ejima et al. 2007; Lebreton et al. 2014). Females do not synthesise cVA and only receive it from males during copulation, making it easy to quantify levels of cVA transferred from males (Ejima et al. 2007). cVA may therefore serve as a good indicator of whether manipulating feeding regimes affects the ejaculate as a whole – i.e. the less cVA transferred, the smaller the overall ejaculate.

In this study, I tested the hypothesis that male feeding status would influence post-mating female aggression. I predicted that male feeding status would influence the male ejaculate, and consequently female post-mating behaviour. I predicted that fed males should transfer large, cVA rich, ejaculates, and induce females to fight more than starved males (that transfer substantially less cVA and potentially smaller ejaculates), and both types of mated female should fight for longer than virgin females. In addition, if females are fighting over access to food to enable higher rates of egg production, we would expect females mated to fed males to feed for longer than females mated to starved males, and both types of mated female to feed for

longer than virgin females. Finally, if females are competing over access to food, we expect females that win a higher proportion of these encounters to have greater access to food and therefore spend more time feeding than females that are less successful in intrasexual competition.

Methods

Fly stocks and culture

We used the Dahomey wild-type stock, which was first collected in Benin, Africa in 1970 (Partridge and Farquhar 1983). Flies have been maintained in large, outbred populations in cages with overlapping generations. All flies were cultured and experiments conducted at 25°C on a 12:12 light: dark cycle in a non-humidified room. Except where stated, adult flies were kept on standard Lewis medium (Lewis 1960), with no access to live yeast.

Experimental flies were grown at controlled larval density of ~5 larvae per mL of food (~200 larvae in a 75 mL plastic bottle containing ~ 45 mL of food) (Clancy and Kennington 2001). Flies were collected within 7hr of eclosion to ensure virginity. Females were then kept in individual vials with standard fly medium, but no live yeast.

Experimental Design

There were three treatments in this experiment:

1. Females mated to ‘fed’ males
2. Females mated to ‘starved’ males
3. Virgin females

Males were kept in groups of 15-20 for three days prior to mating (kept in their diet treatments from eclosion). Males from the 'fed' treatment were kept in vials with standard fly medium, while males in the 'starved' treatments were kept in vials containing only damp cotton wool, which provides no nutritional value.

3d post-eclosion virgin females were marked with acrylic paint (red or yellow) on the thorax to allow individual identification (Nilsen et al. 2004), and returned to individual vials. 24hrs later, females in the first two treatments (mated to 'fed' males and mated to 'starved' males) were placed individually with one male, and a single copulation was directly observed over the course of four hours from artificial dawn. After each female mated exactly once, males were discarded and all females (i.e. both mated and virgins) were individually transferred to fresh vials, again containing standard media and no live yeast. A subset of females (Total N = 15) were used for chemical analysis of cVA transfer (see 'Chemical analysis' section below).

The following morning (5d post-eclosion), females were removed from vials, which were retained for egg counts. Females were then individually placed in vials containing damp cotton wool and no food for 2hrs (Edwards et al. 2006) after which pairs of females were simultaneously aspirated from these vials into the contest arena. The arena was a single well of a twelve-well plate, containing an Eppendorf tube cap filled with standard fly food media and a ~2 µL drop of yeast paste, providing a limited resource to compete over (Bradley and Simmons 1997). Females were either both mated to a fed male, both mated to a starved male, or both virgin. Females were allowed 5mins to acclimatise, and then behaviour was recorded for 30mins using Toshiba Camileo X400 HD video cameras.

Behavioural analysis

Videos were scored with the observer blind to treatment. Fighting behaviours (head-butt, leg "fencing" (later excluded), shove, retreat (Nilsen et al. 2004) and feeding behaviours were recorded. We recorded the number and duration of encounters, which female initiated each encounter, the outcome of each encounter (i.e. win, lose , draw), and time spent feeding, using the program JWatcher + Video (Blumenstein et al. 2006).

Chemical analysis

We tested whether our male feeding treatments affected the levels of cVA transferred to females during mating. An hour after mating, a subset of individual females were dropped into a 1.5 mL plastic eppendorf tube containing 100 µL hexane to extract compounds present on the cuticle. Females were then left in the hexane for 24 hours, while being stored at -80°C. These extracts were then analysed on a gas chromatograph and mass spectrometer (GC-MS), following procedures outlined in Lebreton et al. (2014).

Statistical analysis

We analysed latency to mate using a Cox proportional hazards model, which included the latency of each female to mate. As 7 females did not mate, we censored the data (by adding an additional column to account for whether or not a female mated). We analysed the data using the R package 'survival' (Therneau and Grambsch 2000). To analyse mating duration, we used a two sample t-test. To analyse cVA transfer, we compared whether the amount of cVA transferred (measured as total cVA transferred (measured in millimoles)) differed between treatments using a non-parametric

Kruskal-Wallis test as we had a small sample size and were unable to transform our data to meet the assumptions of any parametric models. We used an ANOVA to test whether egg production differed between treatments.

We used generalized linear models (GLMs) with a Gamma error distribution to test the effects of treatment on total contest and feeding duration. For feeding analyses we additionally included proportion of encounters won as an explanatory variable. As Gamma error distributions use a logistic link function, we added 1 to all scores of the response variable prior to transformation to include replicates with scores of 0. We tested for outliers using a Grubbs test. One significant outlier was detected in the virgin treatment in contest duration. To address this, we winsorized the data by replacing this data point with the next most extreme value in the virgin treatment (Quinn and Keough 2002). This made a significant difference to the results (Model without winsorizing: $Dev_{2, 84} = 5.06$, $P = 0.15$; Model with winsorizing: $Dev_{2, 84} = 7.8$, $P = 0.02$). To avoid non-independence of data points, we used dyad as the unit of replication for analyses of contest and feeding duration, rather than individual flies. All analyses were conducted in R version 3.0 (R Core Team 2012). All multiple comparisons were conducted using a Tukey test from the R package 'multcomp' (Hothorn et al. 2008).

Results

Mating traits

Latency to mate

Females showed no significant difference in latency to mate between fed or starved males ($X^2_1 = 0.75$, P = 0.388; Fig. 1).

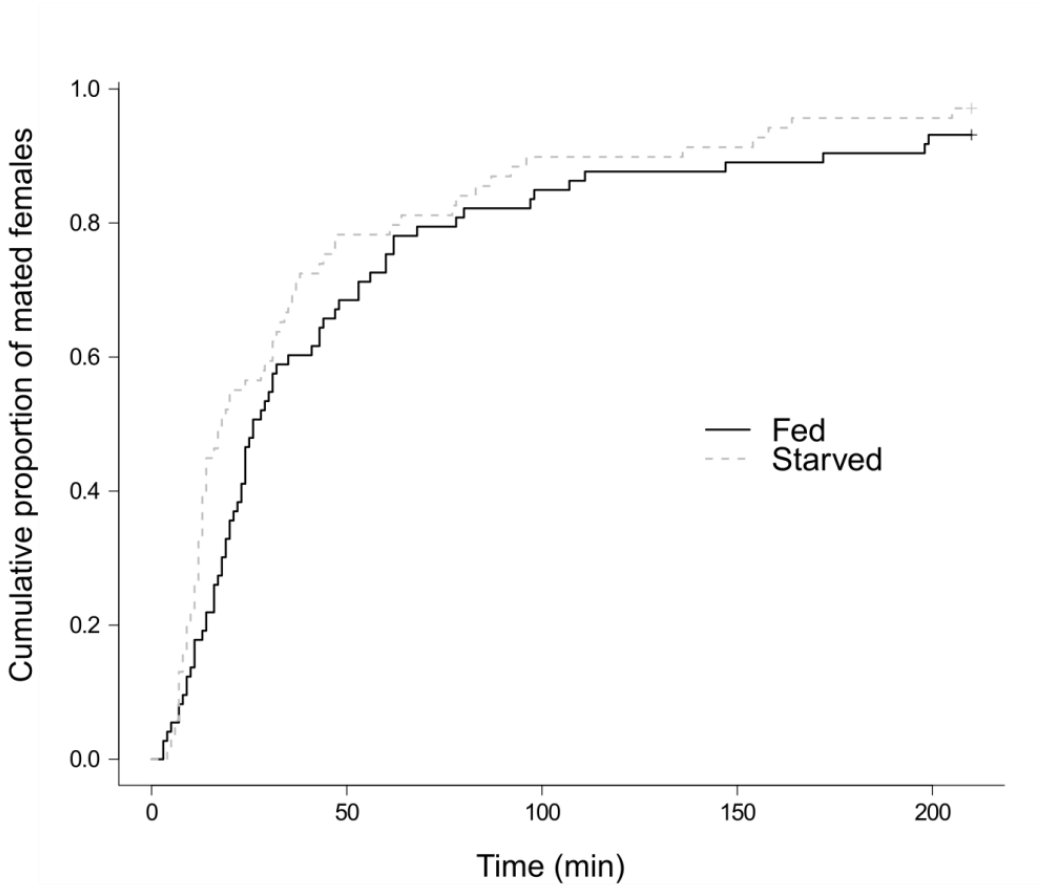


Figure 1: Cumulative proportion of females that mate over time.

There was no significant difference between treatments in time until mating or the proportion of females that mated (Cox proportional hazards: $X^2_1 = 0.75$, P = 0.388). N individuals: Females mated to fed males = 68/73 (mated/total), Females mated to starved males = 67/69. + demonstrates time where observations stopped (not all flies mated within 4 hours).

Mating duration

There was a significant difference in mating duration between the two mating treatments (t = 4.37, P < 0.001; Fig. 2). Females mated to fed males mated for longer than females mated to starved males.

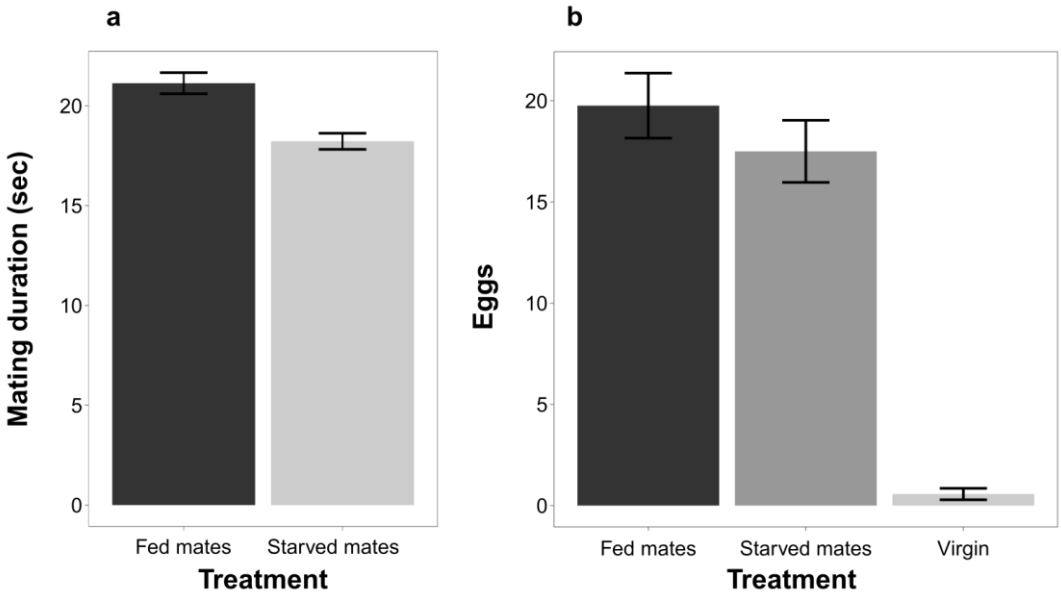


Figure 2: Mating duration and egg production by treatment

a) Females mated to fed males mated for longer than females mated to starved males. N individuals: Females mated to fed males = 62, Females mated to starved males = 54. Bars = means ± SE.

b) Females mated to fed and starved males laid more eggs than virgins, but there was no significant difference between the two mated treatments. N females: Mated to fed males (black bar) = 62, Mated to starved males (dark grey) = 54, virgin (light grey) = 58. Bars = means ±SE.

cVA transfer

There was a significant effect of treatment on the amount of cVA found on the cuticles of females (Kruskal-Wallis: $X^2_2 = 8.569$, $P = 0.014$; Fig. 3) whereby mates of 'fed' males had more cVA than mates of 'starved' males (Non-parametric multiple comparison, 'fed' vs. 'starved': $X^2 = -0.786$, $P < 0.001$). We did not detect any cVA on any virgin females.

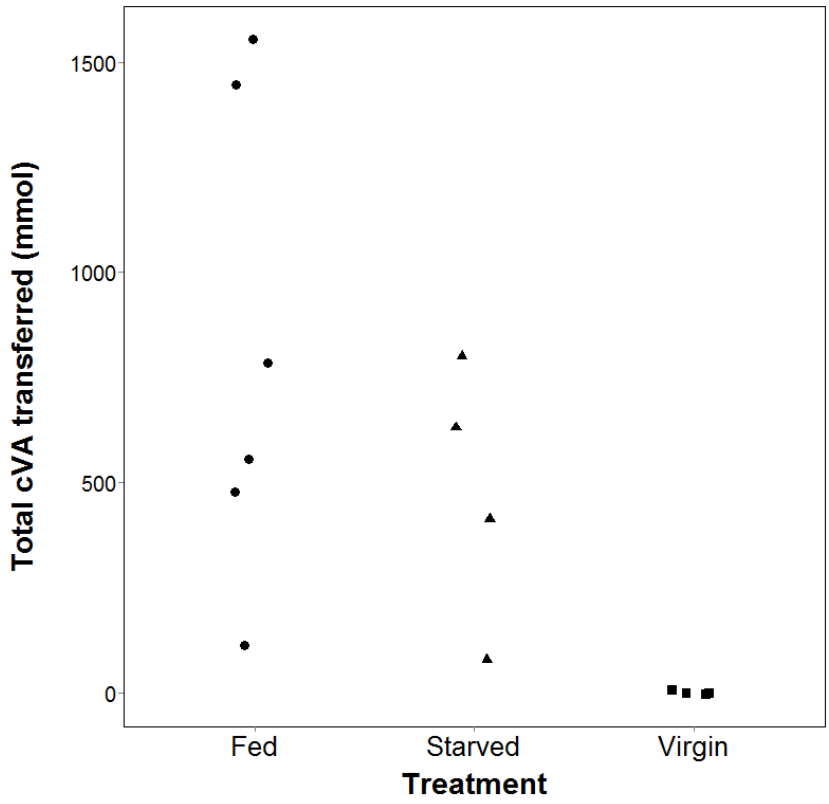


Figure 3: Females mated to fed males had more cVA than females mated to starved females or virgin females

N females: Mated to fed males = 6, Mated to starved males = 4, Virgin = 6.

Egg production

There was a significant difference between treatments in egg production ($F_{2, 171}$ = 65.63, P < 0.001; Fig. 2.b). Females mated to 'fed' and 'starved' males did not lay a significantly different number of eggs (Tukey: t = -1.23, P = 0.44), but both laid significantly more eggs than virgin females ('fed' vs. virgins: t = -10.61, P < 0.001; 'starved' vs. virgins: t = -9.04, P < 0.001).

Feeding duration

There was no significant effect of treatment (GLM: $Dev_{2, 79}$ = 0.75, P = 0.217; Fig. 4), proportion of encounters won ($Dev_{1, 81}$ = 0.21, P = 0.259), or an interaction between the two ($Dev_{2, 77}$ = 0.41, P = 0.436) on feeding duration.

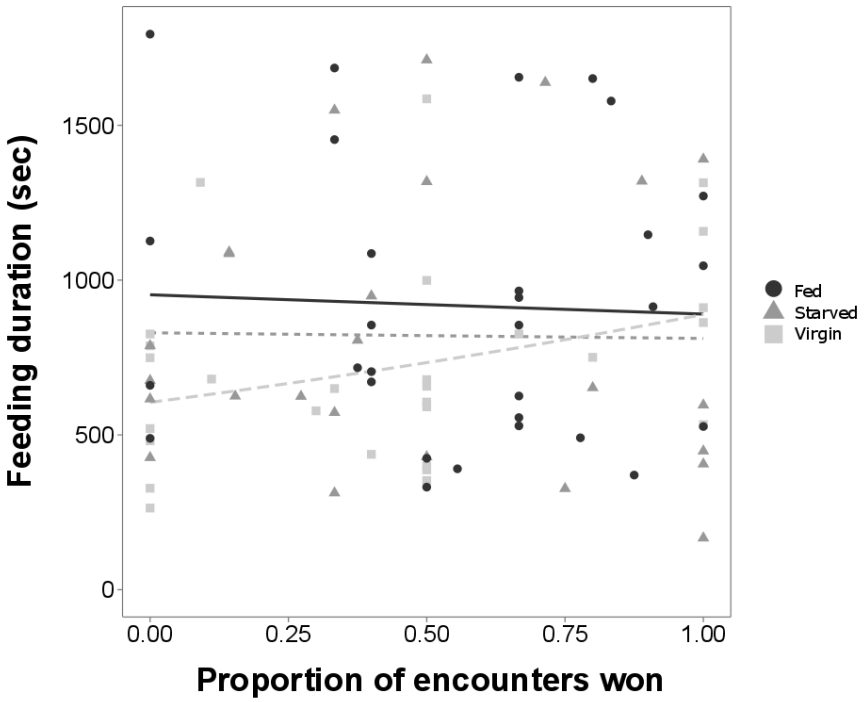


Figure 4: Feeding duration showed no relationship with proportion of encounters won by an individual

There was no difference between treatments in total feeding duration, nor was there any effect of the proportion of encounters a female won on how much time they spent feeding. N focal females: Mated with fed males (black line, filled circles) = 31, Mated to starved males (dark grey dotted line, triangles) = 27, Virgin females (light grey dashed line, squares) = 29. Lines represent model predictions. Points represent actual data points.

Contest duration

There was a significant difference between treatments in contest duration ($Dev_{2, 84}$ = 7.8, P = 0.02; Fig. 5). Females mated to fed males fought for significantly longer than virgin females (Tukey: Z = -2.83, P = 0.013). Females mated to starved males were intermediate between the other two treatments but were not significantly different from females mated to fed males (Z = -1.27, P = 0.41), or virgin females (Z = -1.48, P = 0.3).

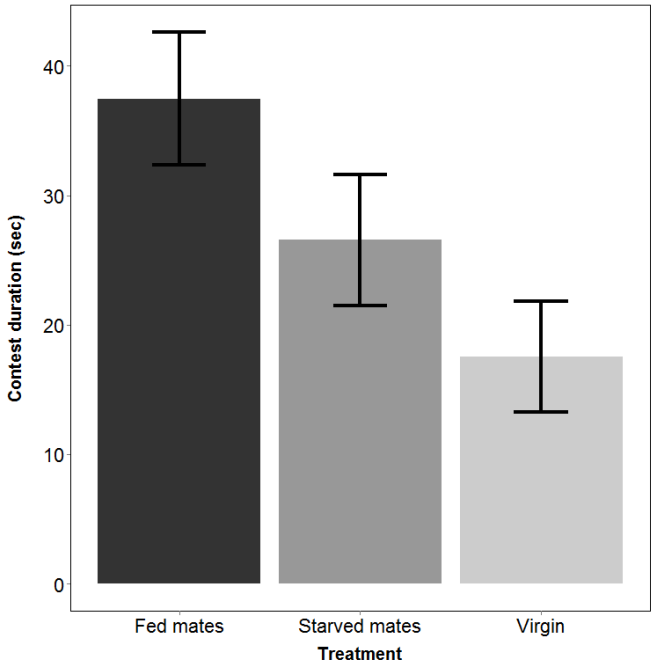


Figure 5: Mating treatment has no significant effect on contest duration

There was no significant difference across treatments in contest duration, but there was a significant difference between females mated to fed males (black bars) and virgins (light grey bars). N pairs: Mated to fed males = 31, Mated to starved males = 27, Virgin females = 29. Bars = mean ±SE

Discussion

In this study, I tested whether male feeding status influences levels of post-mating female aggression. First, male feeding status appears to have altered the male ejaculate with females mated to fed males having more cVA present on their cuticle, and mating for longer, than females mated to starved males. In addition, there was no difference in egg production between females mated to fed and starved males, but both produced significantly more eggs than virgin females. Females mated to fed males fought for significantly longer than virgin females, but females mated to starved males were not significantly different from virgin females or females mated to fed males, providing inconclusive evidence for an effect of male feeding status on post-mating female aggression. I also found that, contrary to our predictions, there was no difference in feeding duration between treatments, nor was there an effect of the proportion of encounters won on feeding duration. Taken together, these results provide some evidence that male feeding status could influence post-mating female aggression in *D. melanogaster.*

Manipulating male feeding status had strong effects on male behaviour and ejaculates. Starved males reduced their mating duration and the amount of cVA they transferred to females (Lebreton et al. 2014). If cVA is an accurate indicator of male ejaculate size, starved males also transferred less ejaculate to females than fed males. Copulation duration does not correlate with sperm transfer in *D. melanogaster* (Gilchrist and Partridge 2000; Lüpold et al. 2011), but is related to the transfer of accessory gland products, such as seminal fluid proteins (sfps) (Gilchrist and Partridge 2000; Wigby et al. 2009; Sirot et al. 2011). Differences in mating duration between fed and starved

males may indicate that fed males spent longer transferring a larger ejaculate to females. Despite these differences in mating duration and ejaculate transfer, there were no differences between mates of fed and starved males in egg production or feeding duration, with the possibility of a difference in contest duration.

The fact that there was no difference between females mated to fed and starved males in terms of egg production could be due to a number of confounding factors. First, there may be no significant difference in ejaculate quantity or quality between fed and starved males. Wigby et al. (2015) found that large males did not transfer a larger quantity of sfps than small males, despite producing more. Smaller males appeared to transfer a larger proportion of their stored sfps, resulting in equal absolute amounts of sfps received by females (Wigby et al. 2015). A similar situation may occur here, where fed males may be able to produce more ejaculate but starved males transfer a greater proportion of their stored ejaculate to remove any difference in ejaculate quantity over one mating. Alternatively, there may be differences in the composition of ejaculates between starved and fed males. Males are able to plastically tailor their ejaculate and mating duration to adjust for anticipated sperm competition, and various aspects of female condition, such as age or mating status (Perry and Rowe 2010; Lüpold et al. 2011; Sirot et al. 2011). This can be done with the ejaculate as a whole, or in a protein-specific manner (Sirot et al. 2011; Perry et al. 2013). Starved males may have altered their investment in different ejaculate components to focus on fecundity and mating-inducing responses, rather than other post-mating responses, such as reducing sexual receptivity.

My results for contest duration do not conclusively show whether male feeding status influences female post-mating aggression. Although mates of starved males were intermediate between females mated to fed males and virgin females, it is unclear whether this is due to a lack of power to detect differences in the experiment, or whether mates of starved males are actually intermediate between the other two treatments. If mates of starved males are intermediate between females mated to fed males and virgin females, it is possible that variation in male feeding status results in variation in levels of female aggression. This intermediate phenotype resembles the pattern observed for the seminal fluid protein sex peptide (SP), where females that received SP from their mates displayed a full increase in aggression, but those that received an entire ejaculate except for SP only showed a partial increase (Bath et al. *in review*). A partial increase in mates of starved males suggests that starved males transfer some, but not enough ejaculate components necessary for an increase in post-mating female aggression.

Interestingly, there was no effect of male feeding treatment on latency to mate. This suggests that females do not display a preference for fed or starved males before mating (Partridge and Farquhar 1983). Females bias matings towards large males (Partridge and Farquhar 1983; Partridge et al. 1987; Pitnick 1991), even though this may prove costly to females over their lifespan (Friberg and Arnqvist 2003). As there was no difference in egg production between mates of fed and starved males, it is possible that there is no reason for females to display a preference for fed males, unlike their preference for large males. It is also possible that females may be unable to distinguish male feeding status prior to courtship or copulation, as adult body size is

determined by larval diet in *D. melanogaster*, rather than adult food intake (Clancy and Kennington 2001). Females may be able to detect differences in ejaculate transfer during mating, resulting in females attempting to shorten mating duration. However, mating duration appears to be primarily under male control in *D. melanogaster*, as males alter mating duration in response to the presence of rivals (Bretman et al. 2013), the mating status of females (Friberg 2006; Lüpold et al. 2011), and is heritable in males, but not in females (Gromko 1987, 1989). It therefore seems unlikely that the difference in mating duration between the fed and starved treatments is a result of female choice. It is much more likely that fed males mate for longer than starved males because they transfer larger ejaculates or because they can force females to mate for longer.

As in previous studies (Chapters 3 & 5), I found no association between the proportion of encounters an individual won and the amount of time they spent feeding. This is contrary to our expectations that females fight to increase their access to food and that this access is determined by the outcomes of female-female encounters. It is possible that my experimental design prevented losers from dispersing as they would in more natural conditions, forcing them to keep engaging. However, in male contests in similar experimental set-ups, dominant males were able to successfully exclude competitors from spending time on the contested food cap (Nilsen et al. 2004). Alternatively, female aggression after mating may not be an adaptive response. It could be a by-product of potent male ejaculates inducing other PMRs, such as increased egg production and reduced receptivity, or the result of sexual conflict (Liu and Kubli 2003; Wigby and Chapman 2005). Males inducing increased aggression in

their mates after mating may reap an immediate benefit if more aggressive females acquire more food or better oviposition sites, but this may come at a cost to females' lifetime reproductive success (Wigby and Chapman 2005; Fricke et al. 2013).

Conclusion

I found that male feeding status could influence post-mating female aggression in *D. melanogaster*. This finding has potential implications for other taxa, where variation in male condition and ejaculate composition may also influence post-mating responses in females, such as female aggression. Incorporating male condition into studies on female aggression could help to explain a great deal of variation in female-female aggression, which has previously been unexplained under the current framework of demand-mediated female aggression.

Chapter 5: Developmental environment and mating status interact to mediate female competition in Drosophila melanogaster

Abstract

Females compete with each other (hereon "female competition") over access to resources that are crucial for reproduction and survival. Factors in both the developmental and adult environment can potentially influence female competition in adulthood but interactions between the two remain largely unexplored. In this study, we used female *Drosophila melanogaster* to test how the developmental environment, which can influence traits such as body size, and mating status, which has a strong influence on aggression, affect contests. As expected, females raised at low larval densities developed into larger, more fecund adults than females raised at high larval densities, and overall won a higher proportion of competitive encounters. However, there was a developmental environment-dependent effect of mating on contest outcome, whereby mating improved the fighting success of females raised at high density (small females) but had no effect on low density (large) females. Contest duration was higher for mated females than for virgin females – which is consistent with previous studies – but this effect was exacerbated in females raised at high larval density. Our results suggest that individuals who experience competitive, resource-limited, rearing conditions are more sensitive to adult reproductive status, highlighting the importance of the developmental environment in mediating adult behaviour. Our results suggest the importance of both juvenile and adult environments in female competition, and the value of taking their interaction into account when studying this phenomenon.

Introduction

Females compete over access to vital resources for reproduction, such as food, nesting sites, and social dominance (Eggert et al. 2008; Heinsohn 2008; Wilson et al. 2013; Grønstøl et al. 2014). Female-female competition (hereafter 'female competition') is widespread across taxa, with important consequences for reproductive success and survival (Heinsohn 2008; Watson and Simmons 2010; Cain and Ketterson 2012; Nelson-Flower et al. 2013). Moreover, female competition can also strongly influence aspects of ecology and evolution, regulating group formation and group size, as well as affecting dispersal (McDonough 1994; Clutton-Brock and Huchard 2013a; Nelson-Flower et al. 2013). However, the majority of research on intra-sexual competition has focused on male-male competition over mates (Bateman 1948; Pitnick 1991; Andersson 1994; Pitnick and Garcia-Gonzalez 2002). As a result, we know little about the underlying causes of female competition, and how these differ from those shaping aggression in males (Rosvall 2013b).

The developmental environment could play a major role in influencing females' willingness to engage in – and ability to succeed in – adult competitive encounters. Developmental environments can vary significantly in a number of aspects, including nutrient availability and population density (Etges and Heed 1987; Kupferberg et al. 1994), and this can lead to variation in a number of factors that influence adult competitive ability, such as adult body size (Clancy and Kennington 2001), investment in pre- and post-copulatory traits (Cotton et al. 2004a,b; Sentinella et al. 2013; Wigby et al. 2015), as well as readiness to face adult competition (Gage 1995; McNamara et al. 2010). Body size in particular often plays a major role in intrasexual competition, as

it acts as a proxy for 'resource holding potential' ('RHP': Parker 1974). An individual's RHP is determined by their condition – individuals in higher condition have higher RHP (Parker 1974). If RHP is the primary determinant of contest outcome, individuals with higher RHP should win more contests than individuals with lower RHP (Parker 1974; Maynard Smith and Parker 1976) – e.g. larger individuals will win more often than smaller individuals (McCann 1981). As RHP is hard to quantify directly, proxies such as body size, territory possession, and experience are often used to estimate the effects of RHP on contest outcome (Parker 1974; Hammerstein and Parker 1982). In situations where body size is heavily influenced by the developmental environment, such as in holometabolous insects, where adult body size is fixed at eclosion and is determined by the amount of food consumed in the larval stage (Boggs 1981), developmental environment can be an important factor in determining RHP and how individuals perform in adult intrasexual competition.

The developmental environment can also influence how individuals value a resource as adults – i.e. the perceived 'resource payoff value' ('RPV'; Enquist and Leimar 1987). Individuals that face nutrient restriction during development may consequently value access to food more highly as adults than individuals that were not restricted during development (Boggs 1981). RPV theory suggests that individuals that value the contested resource more highly are more likely to initiate, escalate, and win contests (Enquist and Leimar 1987). The developmental environment could therefore play an important role in determining the motivation of females to engage in competition.

The developmental environment might act as a signal of the intensity of future competition an individual may face – i.e. an individual that encounters many

competitors at the larval stage may also expect to face many competitors in the adult stage (Gage 1995; McNamara et al. 2010). For example, larval population density is a strong indicator of future sperm competition for males in a number of insect species, and can significantly alter male investment in reproductive traits (Gage 1995; He and Miyata 1997; Katsuki et al. 2013). It is therefore possible that larval density may also signal future female competition, resulting in differences in investment in competitive traits in females. However this has not yet been tested directly.

One final area in which developmental environment could influence female competition is in how females receive and respond to male ejaculates. We have previously shown that mating significantly increases female aggression in *Drosophila* melanogaster, and does so by the action of sperm and seminal fluid proteins (Bath et al. *in review*). However, the developmental environment can strongly affect what ejaculates females receive, and how they respond to them. In *D. melanogaster*, males transfer larger ejaculates to females raised at low larval densities (which have a larger body size), but females raised at high larval densities exhibit stronger post-mating responses to male ejaculates (Wigby et al. 2015). If the developmental environment influences what type of ejaculate females receive and how they respond to them, it could have subsequent effects for the strength of post-mating aggression that females display. The interaction between developmental environment and ejaculate receipt on female competition had not been previously tested before this study.

In this study, we aimed to evaluate how larval density and its interaction with mating status determine the outcome and dynamics of female competition in the fruit fly *Drosophila melanogaster.* The hypotheses and predictions tested are as follows:

Predictions

Contest outcome

1. If contests are determined exclusively by RHP, females raised at low larval density (large) will win a higher proportion of encounters than females raised at high larval density (small), and there will be no effect of mating status (Fig. 1.a). When females from the same larval treatment fight each other there should be no significant difference in RHP between the two individuals, leading to a more even distribution of encounters won (i.e. proportion of encounters won will be around 0.5).

However, if RHP does not determine contest outcome, there are a number of possible predictions:

2. Mated females will win more fights than virgin females, regardless of larval density, because they value the resource more highly ('RPV') (see Fig. 1.b; Enquist and Leimar 1987).
3. High density females will win more fights than low density females, because they value the resource more highly, or because they are more primed to fight due to experiencing higher levels of larval competition (Gage 1995).
4. There will be an interaction between mating status and larval density, where mated females raised at high density will be the most successful in competition, with virgins from low density the least successful.

Contest duration

Given our previous results (Bath et al. *in review*), we predicted that contest duration would follow one of two patterns:

5. Mating status of the two competitors will determine contest duration, whereby pairs with two mated females will fight for longest, followed by pairs with one mated and one virgin female, followed finally by pairs with two virgin females (Fig. 1.c). Larval density will have no effect on contest duration.
6. Alternatively, if both mating status and larval density treatment affect contest duration, we predicted that high density mated females fighting against each other would fight for the longest, followed by pairs of low density mated females and pairs of high density virgin females, with pairs of low density virgin females with the shortest contest duration (Fig. 1.d).

Relationship between contest outcome and duration

7. Both RHP and RPV theory predict that contest outcome and duration should show a quadratic relationship, where contests that are most closely contested show an even distribution of wins and the longest contest duration (Enquist and Leimar 1987). Contests that are less closely contested should show an uneven distribution of wins between the two competitors (i.e. one individual wins the majority of the encounters and proportion of encounters won = 1 or 0), and should last for the least amount of time.

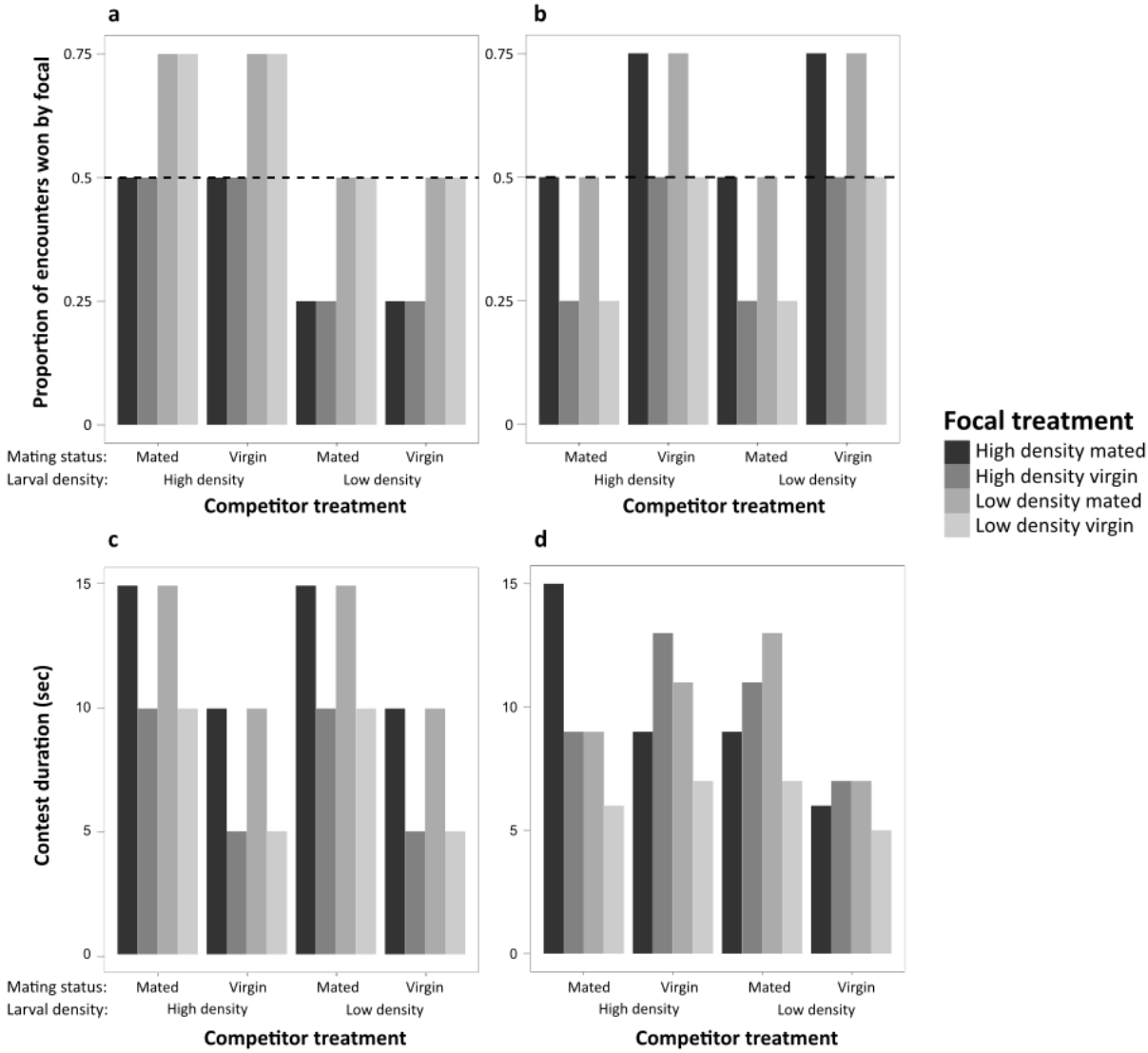


Figure 1: Predictions for contest outcome and contest duration based on larval density and mating status

This figure was constructed using hypothetical data to illustrate the predictions outlined above. The panels are as follows:

a) Proportion of encounters won predicted by RHP theory (prediction 1)

b) Proportion of encounters won predicted by mating status (prediction 2),

c) Contest duration predicted for each treatment if contest duration is determined solely by mating status of the two competitors (prediction 5).

d) Contest duration predicted for each treatment if contest duration is influenced by both mating status and larval density (prediction 6).

Methods

Fly stocks and culture

We used the Dahomey wild-type stock, which was first collected in Benin, Africa in 1970. Flies have been maintained in large, outbred populations in cages with overlapping generations (Partridge and Farquhar 1983). All flies were kept and experiments conducted at 25°C on a 12:12 light: dark cycle in a non-humidified room. Adult flies were kept on standard fly medium (Lewis 1960), with no access to live yeast except where stated.

Larval diet manipulation

Larval density was manipulated to generate females of different adult body size. Larval density and nutrition affect adult body size in *D. melanogaster* (Clancy and Kennington 2001), which has subsequent effects on female fecundity and attractiveness (Long et al. 2009; Lüpold et al. 2011; Wigby et al. 2015), as well as male success in sexual competition (Partridge and Farquhar 1983; Partridge et al. 1987; Lefranc and Bundgaard 2000; Bangham et al. 2002; Pitnick and Garcia-Gonzalez 2002). Manipulating larval density is a widely used technique, which changes food available to each larva while keeping food composition constant (Pitnick 1991; Lefranc and Bundgaard 2000; Pitnick and Garcia-Gonzalez 2002; Byrne and Rice 2006; Lüpold et al. 2011; Wigby et al. 2015).

We controlled egg density following methods described in Clancy and Kennington (2001). We had two treatments: high density = ~70 larvae per mL of standard fly food (~400 larvae in a 34 mL plastic vial containing 6 mL of food), and low density = ~5 larvae per mL of food (~200 larvae in a 75 mL plastic bottle containing ~ 45 mL of

food). To check that our larval density manipulations worked to alter adult body size, we froze a subset of females directly after recording their behaviour. We then defrosted them and weighed them.

Experimental Design

Flies were collected within 7hr of eclosion using ice anaesthesia to ensure virginity. Females were then kept in individual vials with standard fly medium (Lewis 1960), but no live yeast. Males were kept in groups of 15-20 individuals in vials with *ad libitum* live yeast. We used males from the low density treatment as mates for all females in 'mated' treatments.

3d post-eclosion virgin females were marked with acrylic paint (red or yellow) on the thorax to allow individual identification (Nilsen et al. 2004), and returned to individual vials. After 24hrs, females in the 'mated' treatments were placed individually with one male, in which the pair was observed until a single mating occurred. Females that did not mate within 5hrs after lights on were discarded. After each female mated exactly once, males were immediately discarded and all females (i.e. both mated and virgins) were individually transferred to fresh vials with standard fly media and no live yeast. We kept females in these vials overnight, and we counted eggs in each vial to determine differences in egg production due to mating and larval density treatment.

The following morning (5d post-eclosion), females were individually placed in vials containing damp cotton wool and no food for 2hrs (Edwards et al. 2006) after which pairs of females were simultaneously aspirated from these vials into the contest arena. The arena was a single well of a twelve-well plate, containing an Eppendorf tube cap filled with standard fly food media and a ~2 µL drop of yeast paste, providing a limited

resource for females to compete over (Bradley and Simmons 1997). All ten possible combinations of larval density and mating status for the two competitors were tested (e.g. High mated vs. high mated, high mated vs. high virgin, high mated vs. low mated, high mated vs. low virgin etc.). After introducing females to the contest arena, the fighting pair was allowed to acclimatize for 5 min, after which their behaviour was recorded for 30 min using Toshiba Camileo X400 HD video cameras.

Behavioural analysis

Videos were scored blind to treatment, although it was impossible to conceal obvious differences in size due to larval density treatment. Fighting behaviours (head-butt, leg "fencing", shove, retreat (Nilsen et al. 2004) and feeding behaviours were recorded. We recorded the number and duration of encounters, which female initiated each encounter, the outcome of each encounter (i.e. win, lose, draw), and time spent feeding, using the program JWatcher + Video (Blumenstein et al. 2006).

Statistical analysis

We used generalized linear models (GLMs) with a Gamma error distribution to test the effects of larval density and mating status on body mass, attacks initiated, total feeding duration and total contest duration, as these response variables were continuous and most closely fit a Gamma distribution. For feeding analyses we additionally included proportion of encounters won as an explanatory variable. As Gamma error distributions use a logistic link function, we added 1 to all scores of total contest duration prior to transformation to include replicates with scores of 0. To avoid pseudoreplication, we used dyad as the unit of replication, not individual flies, for analyses of contest and feeding duration.

To analyse egg production, we used a GLM with a quasipoisson distribution, as our data was count data and overdispersed, which compromises the use of a Poisson model. We also calculated the "proportion of encounters won" as the number of encounters won by the focal individual divided by the total number of decided encounters (where there was a winner and loser). We tested the effects of focal larval density treatment, focal mating status, competitor larval density treatment, and competitor mating status on the "proportion of encounters won" by fitting a GLM with a quasibinomial error distribution, which accounted for the overdispersion of the data. For these analyses, we incorporated data from all individuals. We controlled for nonindependence of the data points of individuals from the same dyad by calculating the dispersion and the degrees of freedom of the model using the number of dyads (rather than the number of individuals), and doubling the sum of the residuals from the fitted model (McCullagh and Nelder 1989). To conduct multiple comparisons, we used a Tukey test from the package 'multcomp' (Hothorn et al. 2008).

In contest duration, we detected one outlier in the HVHV treatment using the Grubbs outlier test. To address this, we winsorized the data by replacing the outlier with the next most extreme value within the HVHV treatment (Quinn and Keough 2002). To test the relationship between proportion of encounters won and contest duration, we used a GLM with a Gamma error distribution, with contest duration as the response variable, and proportion of encounters won, proportion of encounters won^2 and treatment as explanatory variables. We included proportion of encounters won^2 to test for a non-linear relationship between contest duration and proportion won. All analyses were conducted in R version 3.0 (R Core Team 2012).

Results

Body mass

As predicted, adult females from the low density larval treatment weighed significantly more than females from the high density larval treatment (Low density: 1.27g ± 0.01; High density: 0.72g ± 0.01; $Dev_{1, 245} = 20.116$, $P < 0.001$; Fig. 2.a). There was no effect on body mass of mating status ($Dev_{1, 246} = 0.002$, $P = 0.79$), or an interaction between mating status and larval density treatment ($Dev_{1, 244} = 0.003$, $P = 0.91$).

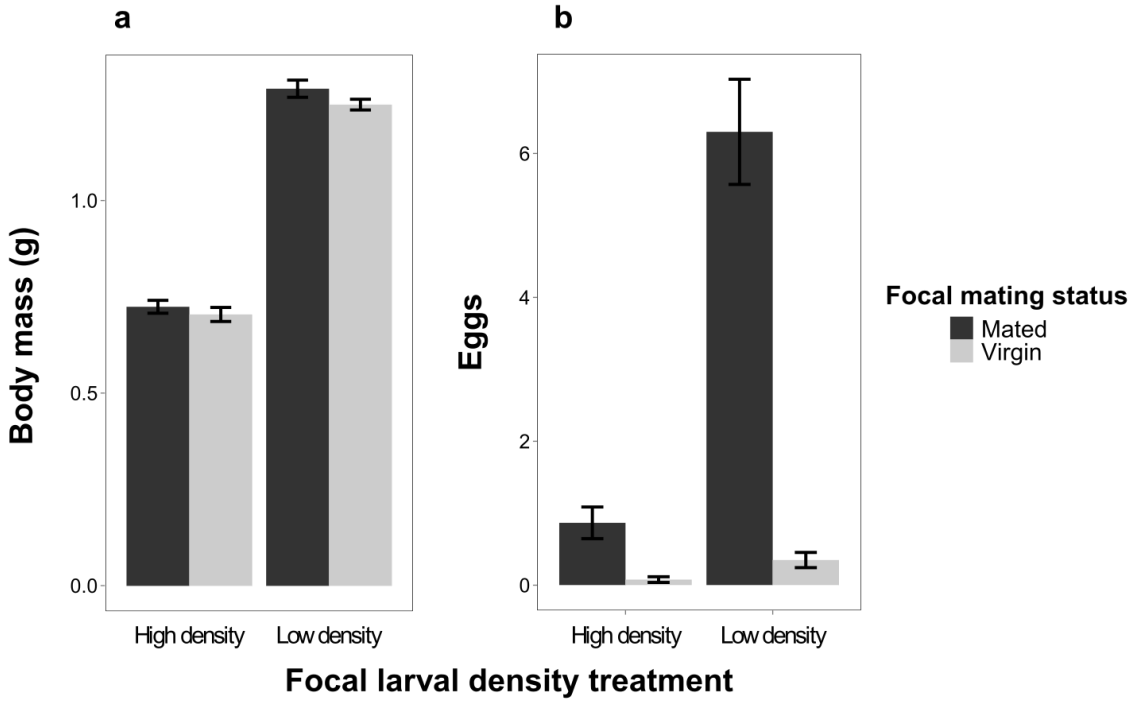


Figure 2: Body mass and egg production

a) Low density females were significantly heavier than females raised at high density. Sample sizes: HM = 59, HV = 66, LM = 66, LV = 57.

b) Low density females and mated females produced more eggs than high density and virgin females. Sample sizes (no. of females laying eggs): HM = 110, HV = 107, LM = 107, LV = 118.

Egg production

Mated females laid significantly more eggs than virgin females the day before being used in contests, regardless of larval density ($Dev_{1, 440}$ = 784.1, P < 0.001; Fig 2.b). Females from the low density larval treatment produced significantly more eggs (Low mated: 6.3 ± 0.73; Low virgin: 0.35 ± 0.11) than females from the high density treatment (High mated: 0.87 ± 0.22; High virgin: 0.07 ± 0.04; $Dev_{1, 439}$ = 529.39, P < 0.001; Fig. 2.b). There was no interaction between mating status and larval density ($Dev_{1, 438}$ = 1.19, P = 0.64).

Feeding duration

Mated focal females fed for significantly longer than virgin females ($Dev_{1, 220}$ = 2.07, P = 0.007; Fig. 3) and high density females fed for longer than low density females ($Dev_{1, 219}$ = 2.332, P = 0.004). There was no interaction between focal larval density and mating status ($Dev_{1, 216}$ = 0.001, P = 0.95). There was no effect of competitor mating status or larval density, or any interactions between focal and competitor larval density or mating status (Table S1.a). In summary, the time individuals spent feeding was solely determined by their own individual mating status and larval density treatment, and was not affected by any aspect of their competitor. In addition, there was no significant effect of the proportion of encounters an individual won based on the amount of time they spent feeding ($Dev_{1, 211}$ = 0.229, P = 0.347, Table S1.b).

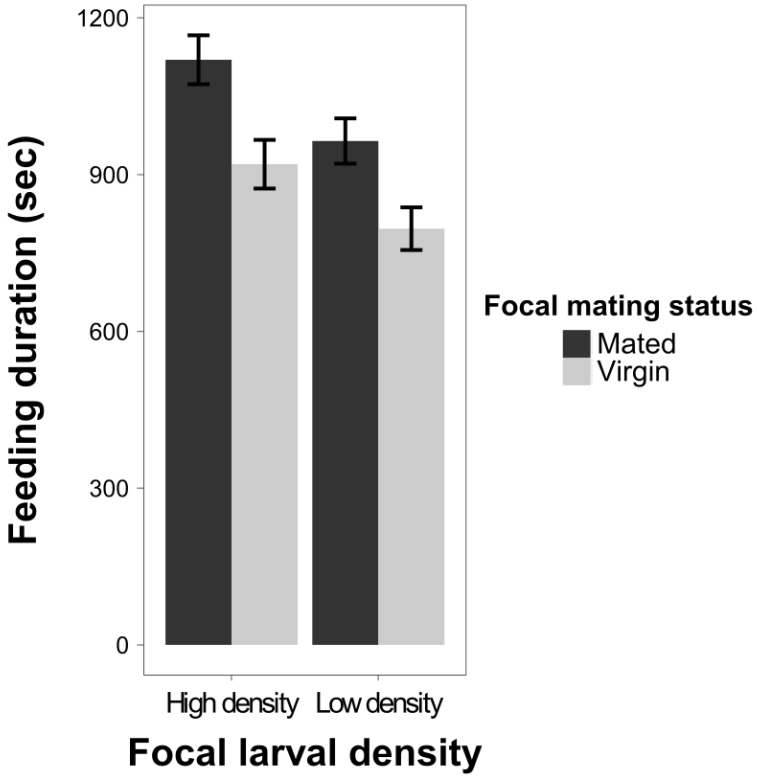


Figure 3: Mated females and high density females spent more time feeding

Sample sizes: HM = 108, HV =119, LM = 110, LV = 107

Initiating contests

We found significant effects of focal mating status ($Dev_{1, 220}$ = 46.399, P < 0.001), larval density ($Dev_{1, 219}$ = 67.77, P < 0.001), and their interaction ($Dev_{1, 216}$ = 19.369, P = 0.028; Fig. 4) on which fly initiated contests – i.e. low density females initiated more attacks than high density females, as did mated females of both treatments. However, the effect of mating status was more pronounced in high density females, where mated females initiated a much higher proportion of encounters than virgins (Fig 4). There was also a significant effect of competitor larval density on the number of attacks launched by the focal individual ($Dev_{1, 218}$ = 163.475, P < 0.001), where focal females attacked more if their competitor was raised at high larval density. There was also a

marginally non-significant three-way interaction between focal mating status, focal larval density, and competitor larval density ($Dev_{1, 210}$ = 13.54, P = 0.067; Table S2), which supports the previous results that all focal individuals initiated more attacks against high density competitors, but that the nature of this increase weakly differed according to focal mating status and larval density.

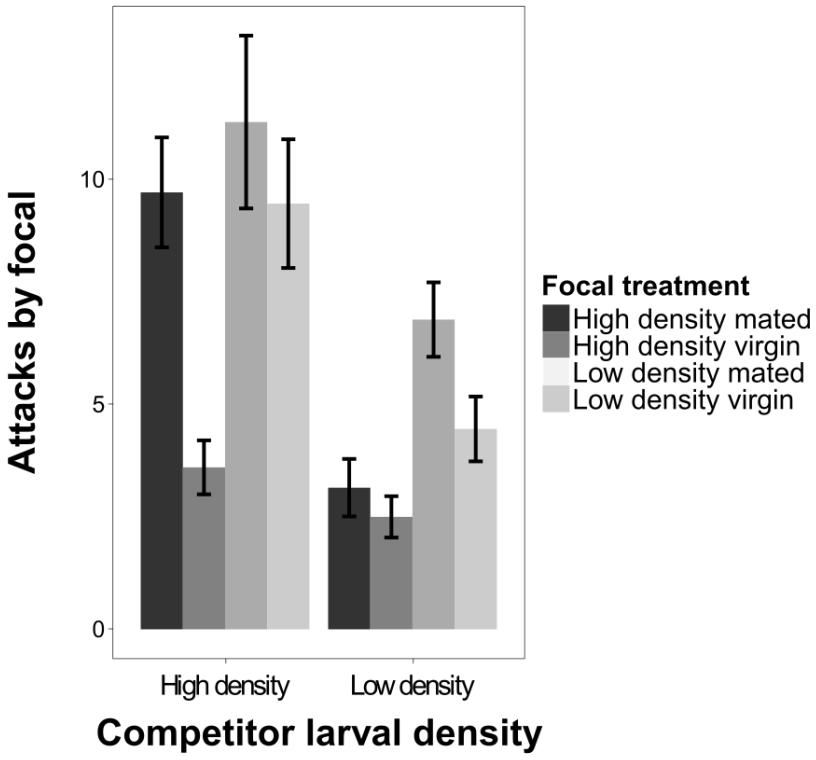


Figure 4: Attack rates of mated and virgin females from low or high density developmental environments.

Competitor mating status is not shown as it was not significant in the model, while competitor larval density was. Sample sizes: HM vs H: 28, HV vs H: 18, LM vs H: 27, LV vs H: 35, HM vs L: 35, HV vs L: 28, LM vs L: 18, LV vs L: 33.

Proportion of encounters won

There was a significant three-way interaction between focal mating status, competitor mating status, and competitor larval density ($Dev_{1, 216}$ = 16.778, P = 0.008; Fig. 5), where focal mating status had no effect when females faced low density females, or high density mated females. When facing virgin high density opponents, however, mated focals won a higher proportion of encounters than virgin focals. This effect of mating status appears to have been driven primarily by high density mated females experiencing greater success than high density virgins against both high and low density virgin females; this effect was absent for low density females (Fig. 5). There was also an interaction between focal larval density and competitor mating status ($Dev_{1, 222}$ = 11.175, P = 0.031; Table S3), where low density focals won a greater proportion of encounters than high density focals against all competitors, but this difference was greater when facing mated competitors – i.e. low density females won more fights against mated females than against virgin females, while high density females won more fights against virgin females than against mated females.

Focal female larval density and mating status had a significant effect on the proportion of encounters an individual won (Focal larval density: $Dev_{1, 227}$ = 170.09, P < 0.001; Focal mating status: $Dev_{1, 228}$ = 11.833, P = 0.026; Fig. 5). Low density females won a greater proportion of fights than high density females (supporting prediction 1), while mated females won a higher proportion of fights than virgins (supporting prediction 2). Competitor larval density also had a significant effect ($Dev_{1, 225}$ = 226.976, P < 0.001), whereby females won a higher proportion of encounters when their competitor was raised at high density (supporting prediction 1).

In summary, females from the low density larval treatment won a higher proportion of encounters, independently of their mating status, compared to females from the high density larval treatment, providing strong support for prediction 1. There was, however, a significant effect of mating status on females from the high density larval treatment, where mated females from the high density larval treatment won a higher proportion of encounters than virgin females from the high density treatment – i.e. there was a developmental environment-dependent effect of mating on the proportion of encounters won by females.

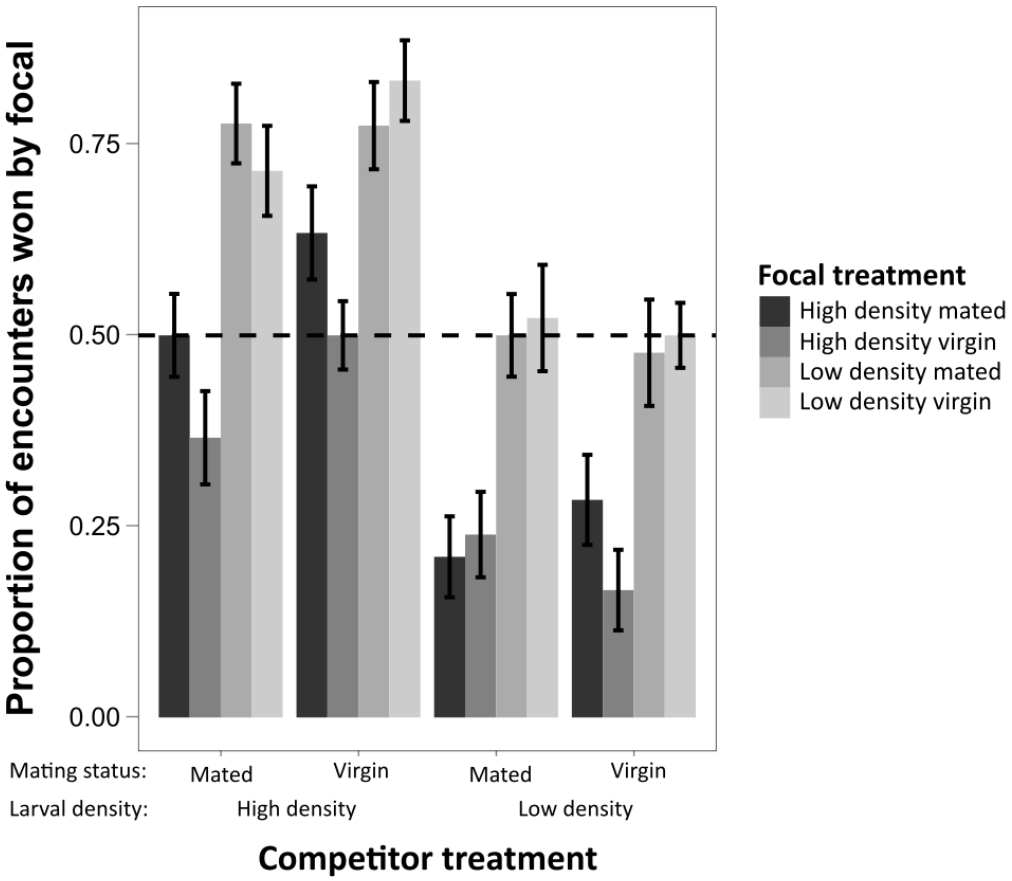


Figure 5: Proportion of encounters won by focal female treatment split by competitor female mating status and larval density treatment

Different coloured bars represent different focal treatments (i.e. combined mating status and larval density treatment). All combinations of focal and competitor are shown, so some treatments are the reciprocals of others – e.g. High density mated focal vs. High density virgin competitor is the exact reciprocal of High density virgin focal vs. High density mated competitor. Sample sizes (no. of pairs of females): HMHM: 22, HVHM: 20, HVHV: 21, LMHM: 22, LMHV: 23, LMLM: 20, LVHM: 24, LVHV: 22, LVLM: 23, LVLV: 25.

Contest duration

There was a significant three-way interaction between focal mating status, focal larval density, and competitor larval density ($Dev_{1, 210}$ = 2.81, P = 0.018; Fig. 6): there was a greater difference between high density virgin focals and high density mated focals when facing a high density competitor than when facing a low density competitor. The difference between low density virgin focals and low density mated focals did not change depending on the larval density of their competitor. There were significant effects of focal mating status ($Dev_{1, 220}$ = 8.7, P < 0.001), and competitor mating status ($Dev_{1, 217}$ = 5.03, P = 0.001) on contest duration, where mated females fought for longer than virgin females (supporting prediction 5). No other factors or interactions were significant (refuting prediction 6; Table S4.a).

When treatments were classified as having two mated females ('MM'), two virgins ('VV') or one of each ('MV'), there was a significant effect on contest duration ($Dev_{2, 219}$ = 14.46, P < 0.001; Fig. 6). MM contests were significantly longer than both MV (Tukey: Z = -3.62, P < 0.001), and VV contests (Z = -5.16, P < 0.001), but there was no difference between MV and VV contests (Z = -1.91, P = 0.135; Table S4.b).

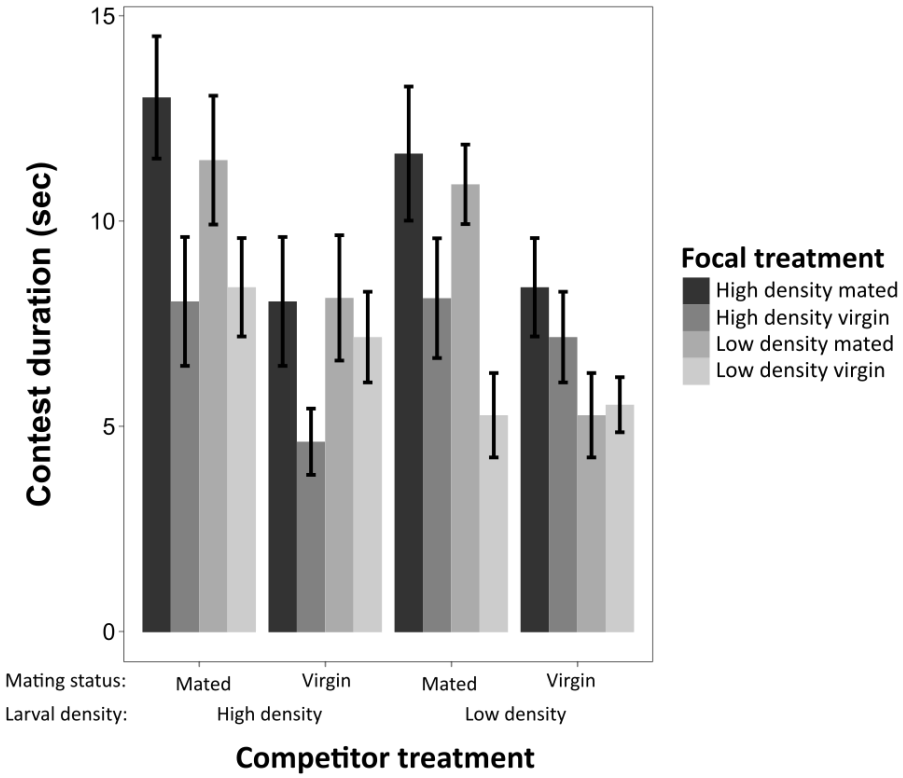


Figure 6: Contest duration by focal female treatment split by competitor female mating status and larval density treatment

Different coloured bars represent different focal treatments (i.e. combined mating status and larval density treatment). All combinations of focal and competitor are shown, so some treatments are the reciprocals of others – e.g. High density mated focal vs. High density virgin competitor is the exact reciprocal of High density virgin focal vs. High density mated competitor. Sample sizes (no. of pairs of females): HMHM: 22, HVHM: 20, HVHV: 21, LMHM: 22, LMHV: 23, LMLM: 20, LVHM: 24, LVHV: 22, LVLM: 23, LVLV: 25.

Relationship between proportion of encounters won and contest duration

There was a significant effect of the square of proportion of encounters won (included to test the quadratic nature of the relationship: $Dev_{1, 210} = 25.07$, $P < 0.01$; Fig. 7), but no effect of proportion of encounters won on contest duration ($Dev_{1, 211} = 0.28$, $P = 0.4$) – there was a non-linear relationship between proportion of encounters won and contest duration (supporting prediction 7). Contests lasted for longest when proportion of encounters won was 0 or 1 - i.e. when the majority of encounters were won by the same fly. Contests lasted the shortest amount of time when the proportion of encounters was around 0.5, where neither fly dominated the contests (opposite to prediction 7). There was also an effect of combined larval density and mating status treatment on contest duration, confirming our previous results (see "Contest duration" results: $Dev_{9, 201} = 14.79$, $P < 0.01$). There was no interaction between proportion of encounters won squared and treatment ($Dev_{9, 192} = 1.9$, $P = 0.85$), i.e. the pattern observed in Fig. 7 was similar in all ten treatments.

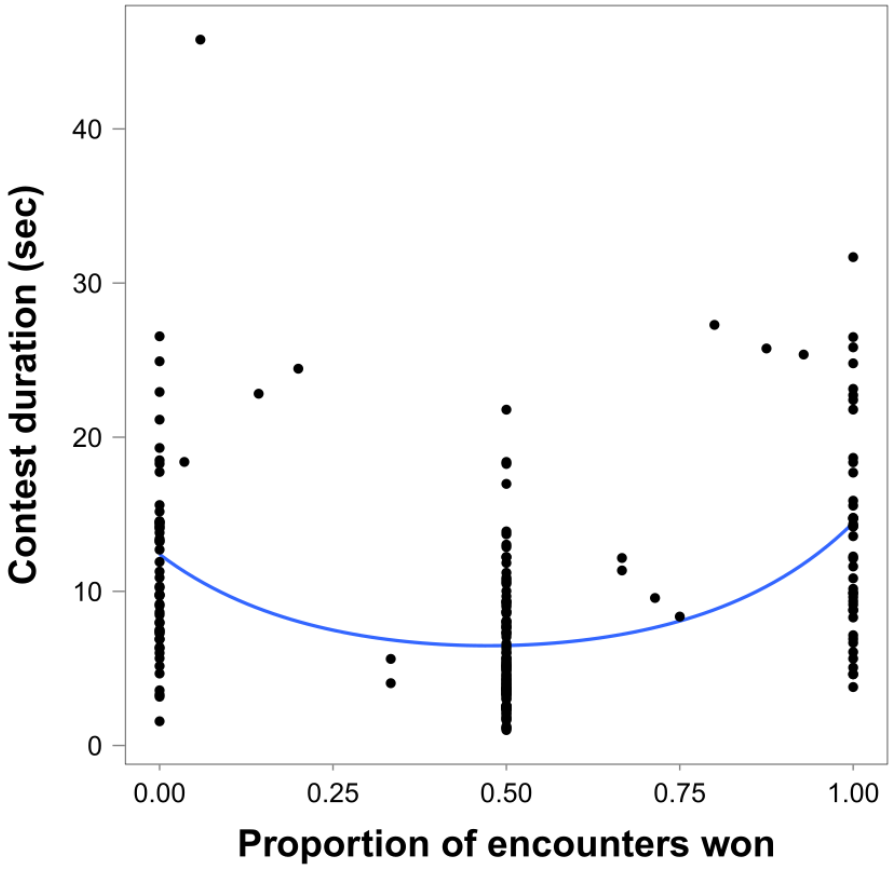


Figure 7: Flies spent more time fighting when one fly won a high proportion of encounters

Contrary to predictions, females spent less time fighting when the proportion of encounters won was more evenly spread between the two flies – i.e. when the proportion of encounters won was around 0.5. Contest duration was longer when one female won a large proportion of encounters – i.e. proportion of encounters won was 0 or 1. Sample size = 222 individual females from all 10 treatments, with one female chosen randomly from each dyad.

1 **Table 1: Relationship between predictions and results**

	Predicted results	**Key results**	**Supported**
Contest outcome			
1. Contest outcome determined by RHP	Low density flies win No effect of mating status	Low density flies won a higher proportion overall No effect of mating status in low density flies	Yes
Contest outcome determined by RPV			
2. RPV determined only by mating status	Mated females win, no effect of larval density	Mated flies won a higher proportion than virgin flies	Partial
3. RPV determined only by larval density	High density females win, no effect of mating status	Low density flies won a higher proportion overall No effect of mating status in low density flies	No – opposite of predicted
4. RPV determined by both mating status and larval density	High density mated females win Low density virgins least successful	High density mated flies won higher proportion than high density virgin Low density flies won a higher proportion than high density with no effect of mating status	Partial
Contest duration			
5. RPV determined only by mating status	Pairs with mated females will fight for longest No effect of larval density	Pairs with mated flies fought for longest, followed by MV pairs and VV pairs No effect of larval density	Yes
6. RPV determined by both mating status and larval density	Pairs with mated high density females will fight for longest Followed by pairs with low density mated and high density virgins Pairs with low density virgins will fight for the least time	Pairs with mated flies fought for longest, followed by MV pairs and VV pairs No effect of larval density	No – only support for mating status
Relationship between duration and outcome			
7. As contests are more lopsided, they should be shorter and one individual should win the majority of encounters	As proportion of encounters nears 1 or 0, contests should be shorter As proportion of encounters nears 0.5, contests should be longer	As proportion of encounters nears 1 or 0, contests are longer As proportion of encounters nears 0.5, contests are shorter	No – opposite of predicted

Discussion

We tested whether the developmental and adult environments interacted to influence female competition in *D. melanogaster*. We predicted that larval density and mating status would influence both the outcome and duration of female contests. We found that contests were primarily decided by larval density in female-female aggressive interactions (prediction 1), but that contest duration was primarily determined by mating status (prediction 5). Females raised at low larval density won a higher proportion of encounters and initiated more attacks than females raised at high larval density, providing support for our prediction 1 that RHP determines contest outcome. However, mating status had a significant effect on females raised at high density, where mated females raised at high density initiated attacks more, won a higher proportion of encounters, and fought for significantly longer than virgin high density females, providing evidence for an interaction between larval density and mating status on female competition (prediction 4).

RHP and contest outcome

The fact that females raised at low density were much larger than females raised at high density could play a substantial role in explaining our results. The outcome of contests was primarily determined by larval density without any effect of mating. Larger females therefore won more often than smaller females, meaning contest outcome predominantly followed the predictions made by RHP theory (prediction 1: compare Fig. 1.a with Fig. 5).

Body size has a major effect on male contests in *D. melanogaster*, with larger males consistently preventing smaller males from being present in their territory (Partridge

and Farquhar 1983; Hoffmann 1987). This suggests that RHP determines contest outcome in both male and female *D. melanogaster*. However, males usually establish a dominance hierarchy, while females do not (Nilsen et al. 2004). Winning females do not prevent losing females from accessing the food resource – there was no correlation between the proportion of encounters an individual won and feeding time, which we would expect if females fought to gain access to food. The lack of relationship between proportion of encounters won and feeding duration suggests that, unlike males, females may be unable or unwilling to exclude other females from the food source making the benefits of winning in female competition much less clear. As discussed in earlier work, it is possible that female aggression is under sexual conflict, where females receive few benefits from exhibiting aggressive behaviours (Bath et al. *in review*). Alternatively, females may instead be competing over access to oviposition sites, as females oviposit in the same rotting fruit that they eat (Richmond and Gerking 1979).

Females raised in high larval density environments faced higher levels of competition as larvae, which may affect their levels of aggression as adults (Gage 1995; Marks et al. 2005; McNamara et al. 2010). Where larval density predicts adult population density, individuals can prepare for the competitive reproductive environment they face during development by adjusting to cues in the larval environment (Gage 1995; He and Miyata 1997; McNamara et al. 2010; Katsuki et al. 2013). Female *D. melanogaster* kept in large social groups as adults are less aggressive than females kept individually, suggesting social experience can suppress aggression (Ueda and Kidokoro 2002). However, it is unclear whether experiencing higher levels of larval competition also

results in reduced levels of aggression in adult females, or whether it has the opposite effect – priming females raised at high density to invest more heavily in competitive traits so they can succeed in a more competitive adult environment. However, females raised at high density did not initiate more attacks than those raised at low density, indicating that they did not seek to engage in more encounters. There was also no effect of larval density treatment on contest duration, which suggests that it is unlikely that larval density affects willingness to participate in encounters. Females raised at high density did not win a higher proportion of encounters than females raised at low density, which suggests that even if they were investing more in competitive traits, they were not more successful against larger females raised in a less competitive larval environment.

Developmental environment-dependent effect of mating

Interestingly, we observed a developmental environment-dependent effect of mating on the proportion of encounters females won – mated females raised at high density were more likely to attack and win than virgin females raised at high density, but mating status had no effect on females raised at low density. This partially met our prediction 4, that high density mated females would be more likely to win encounters, but we did not see the expected effects in low density females. There also appeared to be a larger increase in the time spent fighting between high density virgin and mated females than between mated and virgin low density females (compare HVHV vs. HMHM and LVLV vs. LMLM in Fig. 5). Although mating status did have an effect on high density females' chance of winning a contest, it primarily did so when females faced individuals from the same larval treatment. These results suggest that

differences in developmental environment are difficult to overcome for female *D. melanogaster* – low density females are too difficult to beat for high density females, despite potentially having greater motivation, probably due to the dramatic differences in body size (see Fig 1). The fact that all individuals attacked more when their competitor was raised at high density supports this – females were more likely to initiate encounters when they faced a smaller individual and therefore had a higher chance of winning.

There are a variety of reasons which may explain a developmental environment-dependent effect of mating on female aggression. First, females raised at high larval density may be more in need of nutrients to enable reproduction due to having reduced food availability during larval development. Since adult female egg production is significantly influenced by protein availability, live yeast becomes a valuable source of protein for female fruit flies (Bradley and Simmons 1997). Therefore, females from a high larval density environment may have more limited reserves of protein (and other nutrients) required for egg production and may be more motivated to compete for access to these nutrients than females from low larval density environments. High density mated females produced substantially fewer eggs overnight than low density mated females (Figure 2.b), which could be due to them possessing a smaller number of mature eggs, as well as the lack of appropriate nutrients to develop eggs (Lefranc and Bundgaard 2000; Pitnick and Garcia-Gonzalez 2002; Byrne and Rice 2006; Lüpold et al. 2011). Interestingly, mated females and females from the high larval density treatment ate more than virgins and females from low larval density treatments, respectively, suggesting that both mating activity as well as high larval density increase

the value of the food to females (Fig. 3). High density females may value food more highly as they have smaller bodies (see results), and are potentially less able to withstand periods of starvation (Baldal et al. 2005 but see Zwaan et al. 1991). However, low density mated females fought for longer than low density virgin females as in Bath et al. (in review), suggesting that there is still a motivation to acquire more resources even in the less restricted larval density treatment.

The second potential reason for a developmental environment-dependent effect of mating on female aggression is that it may also reflect differences in females' receipt and response to male ejaculate components. Males transfer smaller ejaculates to smaller females, but not in direct proportion to their body weight, in terms of both sperm and non-sperm components (Lüpold et al. 2011; Wigby et al. 2015). High density females may get a relatively larger ejaculate from males during mating, resulting in high density females receiving a larger proportion of aggression-stimulating ejaculate components (Bath et al. *in review*). Sperm has been shown to be crucial for inducing a post-mating increase in female aggression (Bath et al. *in review*), so differences in the amount of sperm received by low and high density females has serious potential to affect their relative levels of aggression. Similarly, the male seminal protein sex peptide (Kubli, 2003, Chen et al., 1988, Chapman et al., 2003) contributes to the stimulation of mating-induced female aggression, so differences in sex peptide transfer could have similar effects on levels of female aggression. Larval density can therefore potentially mediate female competition through influencing how much male ejaculate females receive, and how they respond to it physiologically.

Contest duration is determined by mating status (RPV)

Contest duration predominantly corresponded with our prediction that mating status alone would influence contest duration (prediction 3, compare Fig. 1.c with Fig. 6). These results agree with previous results (Bath et al. *in review*) that contest duration is primarily determined by mating status, presumably as a result of females valuing food more after mating (Carvalho et al. 2006; Barnes et al. 2008). The fact that the dramatic increase in contest duration in high density females depended on the larval density treatment of their competitor suggests a certain plasticity in female aggressive behaviours, rather than it being merely an automatic response. Contest duration followed a very similar pattern to that of attacks, where females initiated more attacks against individuals from high larval density, which suggests that females may both initiate more attacks and fight for longer in situations where they perceive they have a higher chance of winning, and that high density mated females are particularly responsive to such changes in their chances of success.

The relationship between contest duration and proportion of encounters won was the opposite of what we expected (prediction 7). We expected that contests would be longest when females were more closely matched in RHP or RPV, i.e. when the proportion of encounters won by each individual was around 0.5 (Enquist and Leimar 1987). However, we found that contests were longest when one female won a majority of encounters (Fig. 7). This could be due to a high number of draws in our dataset – many contests ended without a clear winner and loser. It is possible that these contests ended quickly because neither fly was motivated enough to continue until they won.

Future research

Mating induces an increase in female aggression across taxa, including species with high levels of parental care, such as mammals, birds, and fish (McDonough 1994; Palanza et al. 1996; Clutton-Brock et al. 1998; Kapusta and Marchlewska-Koj 1998; Bowler et al. 2002; Seebacher et al. 2013). It seems likely that similar relationships between dietary restriction during development and mating status could exist in species with gestation or prolonged periods of parental care, with an offspring's potential aggressive responsiveness to mating determined by their mother's maternal provisioning (Cain and Ketterson 2013). Maternal effects, such as overall resource provisioning, or prenatal exposure to androgens, can strongly influence a female's developmental environment and ability to compete in adulthood (Dloniak et al. 2006). If female aggression is adaptive, females may alter their offspring's developmental environment to favour enhanced competitive success in their daughters, as appears to occur in spotted hyenas (Dloniak et al. 2006). Although research is now being conducted on evaluating the effects of developmental environment on female competition in vertebrates, few studies have examined how the adult environment can mediate or exacerbate the influence of the developmental environment. More broadly, the interaction between the developmental and adult environments could also influence other key components of female fitness and behaviour, such as reproduction or mate choice. Investigating how developmental influences interact with aspects of the adult environment, such as social status, mating status, or food availability, could provide us with a more detailed picture of the nature of female competition and the potential selection pressures that act on females at different stages of life.

Conclusion

Larval density strongly influences adult female-female competition in *D. melanogaster*. Moreover, larval density also showed a strong interaction with mating status, where individuals raised at different larval densities demonstrated different strengths of response to mating in their interactions with other females. While work on female competition has investigated the role of mating on female aggression, few studies have considered the interaction between mating and other potentially important factors, such as developmental environment or social status. The results from this experiment suggest that evaluating both developmental environment and aspects of reproduction could provide useful insights into understanding the dynamics and outcomes of female-female competition in a broad range of taxa. Expanding such research to include species with gestation, or prolonged maternal care, would help to increase our understanding of the overall lifetime influences on female aggression.

Supplementary Tables

* = results marginally non-significant (P=0.05), *** = results significant at P<0.05. Text for significant results also highlighted in red.

Table S1: Feeding duration generalized linear model results

Response variable	Explanatory variable	Deviance	d.f.	P
a. Feeding duration	Focal mating status	2.072	1	0.007***
	Focal condition	2.332	1	0.004***
	Competitor mating status	0.633	1	0.138
	Competitor condition	0.424	1	0.224
	Focal mating status * Focal condition	0.001	1	0.947
	Focal mating status * Competitor mating status	0.234	1	0.367
	Focal condition * Competitor mating status	0.022	1	0.784
	Focal mating status * Competitor condition	0.127	1	0.506
	Focal condition * Competitor condition	0.003	1	0.919
	Competitor mating status * Competitor condition	0.007	1	0.876
	Focal mating status * Focal condition * Competitor mating status	0.029	1	0.75
	Focal mating status * Focal condition * Competitor condition	0.169	1	0.444
	Focal mating status * Competitor condition * Competitor mating status	0.508	1	0.187
	Focal condition * Competitor condition * Competitor mating status	0.002	1	0.935
	Focal condition * Focal mating status * Competitor condition * Competitor mating status	0.029	1	0.75
b. Feeding duration	Proportion of encounters won	0.229	1	0.347
	Focal combined treatment (condition and mating status)	5.66	3	<0.001***
	Competitor combined treatment (condition and mating status)	0.439	3	0.638
	Proportion of encounters won * Focal treatment	0.37	3	0.791
	Proportion of encounters won * Competitor treatment	0.471	3	0.611
	Focal treatment * Competitor treatment	0.945	9	0.933
	Proportion of encounters won * Focal treatment * Competitor treatment	2.101	9	0.523

Table S2: Initiating encounters generalized linear model results

Response variable	Explanatory variable	Deviance	d.f.	P
Initiating encounters (attacks)	Focal mating status	46.399	1	<0.001***
	Focal condition	67.77	1	<0.001***
	Competitor mating status	8.333	1	0.15
	Competitor condition	163.475	1	<0.001***
	Focal mating status * Focal condition	19.369	1	0.028***
	Focal mating status * Competitor mating status	0.143	1	0.85
	Focal condition * Competitor mating status	8.52	1	0.146
	Focal mating status * Competitor condition	0.282	1	0.791
	Focal condition * Competitor condition	5.227	1	0.254
	Competitor mating status * Competitor condition	0.588	1	0.702
	Focal mating status * Focal condition * Competitor mating status	5.471	1	0.244
	Focal mating status * Focal condition * Competitor condition	13.54	1	0.067*
	Focal mating status * Competitor condition * Competitor mating status	7.278	1	0.179
	Focal condition * Competitor condition * Competitor mating status	0.387	1	0.757
	Focal condition * Focal mating status * Competitor condition * Competitor mating status	0.176	1	0.834

Table S3: Proportion of encounters won generalized linear model results

Response variable	Explanatory variable	Deviance	d.f.	P
Proportion of encounters won	Focal mating status	11.833	1	0.026***
	Focal condition	170.09	1	<0.001***
	Competitor mating status	8.151	1	0.065
	Competitor condition	226.976	1	< 0.001***
	Focal mating status * Focal condition	6.849	1	0.091
	Focal mating status * Competitor mating status	0.035	1	0.904
	Focal condition * Competitor mating status	11.175	1	0.031***
	Focal mating status * Competitor condition	1.858	1	0.379
	Focal condition * Competitor condition	0.023	1	0.922
	Competitor mating status * Competitor condition	4.44	1	0.174
	Focal mating status * Focal condition * Competitor mating status	3.565	1	0.223
	Focal mating status * Focal condition * Competitor condition	2.001	1	0.361
	Focal mating status * Competitor condition * Competitor mating status	16.778	1	0.008***
	Focal condition * Competitor condition * Competitor mating status	0.472	1	0.658
	Focal condition * Focal mating status * Competitor condition * Competitor mating status	0	1	1

Table S4: Contest duration generalized linear model results

Response variable	Explanatory variable	Deviance	d.f.	P
a. Contest duration	Focal mating status	8.698	1	<0.001***
	Focal condition	0.035	1	0.792
	Competitor mating status	5.025	1	0.001***
	Competitor condition	1.43	1	0.09
	Focal mating status * Focal condition	0.7361	1	0.224
	Focal mating status * Competitor mating status	0.704	1	0.235
	Focal condition * Competitor mating status	0.943	1	0.169
	Focal mating status * Competitor condition	1.56	1	0.077
	Focal condition * Competitor condition	1.12	1	0.134
	Competitor mating status * Competitor condition	0.0001	1	0.99
	Focal mating status * Focal condition * Competitor mating status	0.28	1	0.453
	Focal mating status * Focal condition * Competitor condition	2.811	1	0.018***
	Focal mating status * Competitor condition * Competitor mating status	1.045	1	0.148
	Focal condition * Competitor condition * Competitor mating status	0.575	1	0.283
	Focal condition * Focal mating status * Competitor condition * Competitor mating status	0.005	1	0.922
b. Contest duration	Mating status of both females	14.458	2	<0.001***

Chapter 6: General Discussion

Summary

In Chapter 2, I showed that female stalk-eyed flies raised on less restricted larval diets grew into larger flies, with larger eyestalks, and that they won a higher proportion of encounters than females raised on more restricted larval diets. However, contrary to our expectations, eyestalks did not provide information in addition to body size to indicate whether a female was more likely to win an encounter. After reanalysing data on male contests and morphology, I found a similar result – male eyestalks did not provide any additional information on an individual's competitive ability. I concluded that eyestalks are an honest indicator of condition, but do not appear to provide any additional benefit in intrasexual contests in either sex.

In Chapter 3, I showed that mating doubles the amount of time females spend fighting over food in the fruit fly, *Drosophila melanogaster*. This mating-induced increase in female aggression did not require females to produce eggs, but instead required the presence of sperm. A seminal fluid protein, sex peptide, also appears to play a significant role in stimulating female aggression after mating, although it does not induce aggression using the same pathway as it does to induce other post-mating responses.

In Chapter 4, I showed that there is the potential for variation among males to influence mating-induced female aggression. Although I found no significant difference in contest duration between females mated to fed and starved males, there is evidence that males in different diet treatments mated for significantly different amounts of time and transferred different ejaculates.

In Chapter 5, I showed that females raised at low larval densities won a higher proportion of encounters than females raised at high larval densities. There was however, a developmental environment-dependent effect of mating on contest outcome – mating increased the proportion of encounters won by high density females but had no effect on the likelihood of winning in low density females. Contest duration, on the other hand, was primarily determined by mating status of the two individuals, with no real effect of larval density on contest duration.

Future directions

Exaggerated traits and female competition

In the last ten years, there has been a vast increase in the importance placed on studying the evolution of exaggerated traits in females (reviewed in Kraaijeveld et al. 2007; Clutton-Brock 2009; Tobias et al. 2012; Stockley et al. 2013). There has also been a corresponding increase in studies investigating the fitness benefits of female intrasexual competition, with or without exaggerated traits being involved (Watson and Simmons 2010; Rosvall 2011b; Clutton-Brock and Huchard 2013a; Stockley and Campbell 2013; Cain and Rosvall 2014). There is now a much broader understanding that female competition can produce selection pressures strong enough to lead to the development of exaggerated traits. However, the majority of the research conducted on female competition and exaggerated traits has been conducted on visual and acoustic signals in birds (Amundsen 2000; Tobias et al. 2012), and other vertebrate taxa (Kraaijeveld et al. 2007; Tobias et al. 2012; Stockley and Campbell 2013). Expanding the research to focus on other types of signalling (e.g. olfactory, chemical cues: Stockley et al. 2013), and incorporate more invertebrate taxa is critically important for the future study of exaggerated traits in females. Invertebrates, and

particularly insects, make for an exciting new taxa into which to expand this research, due to enormous diversity in morphology, mating systems, and signalling modalities (Stork 1988; Cocroft and Rodríguez 2005; Kaupp 2010). It is also generally easier to manipulate trait size and expression in invertebrates, particularly insects, and evaluate the corresponding results on female competition and broader fitness effects.

Ejaculate-stimulated aggression

A key next step in this area of research is to investigate how widespread a phenomenon ejaculate-induced female aggression is. Sperm and seminal fluid proteins (Sfps) are found across a broad range of taxa, which raises the possibility that ejaculate-induced female aggression could occur in other species. One ejaculate component of potential broad relevance is 'ovulation-inducing factor' (OIF), which is present in the seminal fluid of numerous mammalian species, from mice to camels to pigs (Bogle et al. 2011). OIF induces ovulation in species of induced ovulators, such as rabbits, llamas and koalas, as well as hastens the onset of ovulation in spontaneous ovulators, such as rodents, cattle and humans (Salas et al. 2006; Ratto et al. 2012). OIF has recently been identified as the protein ß- nerve growth factor, which is also involved in the development of neurons (ß-NGF: Ratto et al. 2012). As a key modulator of mammalian reproduction, ß-NGF has the potential to indirectly influence post-mating female aggression through its effects on the timing of reproduction. ß-NGF could also play a more direct role in mediating post-mating female aggression, as it regulates hormones associated with both reproduction and aggression. In human ovarian cells, ß-NGF stimulates the secretion of estradiol (Salas et al. 2006), a hormone which has been shown to play a key role in modulating female aggression in rats (Albert et al. 1991). Estradiol has also been associated with increased aggression in

various mammals and lizards (Kapusta 1998; Woodley et al. 2000; Ross and French 2011). The links between male ejaculate components and hormones associated with female aggression have not yet been studied in species other than *D. melanogaster*. However, there are hormones present in other species that have been identified as being involved in both reproduction and aggression, which suggests that they could function in some form of ejaculate-induced modulation of female aggression.

In species with high levels of maternal care, such as mammals (gestation and lactation) or birds (incubation and offspring provisioning), we may expect female aggression over food to be linked to successful fertilisation, rather than solely to mating status or ejaculate transfer. Gestation, lactation, and parental care are extremely energetically costly, so acquiring resources in female competition can have important effects on female and offspring fitness (Watson and Simmons 2010; Rosvall 2011c; Cain and Ketterson 2013). Females are predicted to display greater behavioural flexibility than males – i.e. they are more likely to employ aggressive behaviours only in particular circumstances, rather than at all times, and should therefore be more responsive to cues about what levels of aggression are adaptive (Rosvall 2013b; Stockley and Campbell 2013). If increasing aggressive or competitive behaviours is costly for females, and the primary reason for displaying such costly behaviour is to acquire nutrients for their offspring, then mating (and the ejaculate) alone may be inadequate to stimulate female aggression. In species where there is sperm limitation, such as stalk-eyed flies, or where fertilisation is not assured by mating, it is possible that receipt of male ejaculates would be insufficient to stimulate female aggression. In these cases, we may expect female aggression to increase only after females actually

achieve fertilization, or perhaps that female aggression only begins after a certain point in offspring development when the likelihood of producing an offspring is high. One example is that of marsupials, where early investment in offspring is limited and increases only when environmental conditions are adequate for offspring provisioning (Low 1978). It seems unlikely that ejaculates alone would induce female aggression in such a system as offspring survival is so uncertain at the time of mating.

However, it is also possible that in species with maternal care, there could be even greater selection for an ejaculate-induced increase in aggression than in *D. melanogaster*. Male ejaculate components may act as a reliable cue for females to identify a change in reproductive status and impending need to acquire more resources for reproduction –i.e. it may be more beneficial to respond directly to the ejaculate rather than waiting for reproduction to actually begin before changing behaviour. Bank vole females (*Clethrionomys glareolus*) require olfactory cues indicating males are present to initiate higher levels of aggressive behaviour, indicating a cue other than pregnancy is used by females to stimulate increased aggression (Kapusta and Marchlewska-Koj 1998). Similarly, in prairie voles (*Microtus ochrogaster*), females only need to cohabitate with males to display the same increase in aggression as from mating (Bowler et al. 2002). These examples suggest that cues that indicate potential imminent reproduction can influence female aggression, without the corresponding investment in reproduction. Components of the ejaculate are likely candidates to act as such a cue as they are tightly associated with mating and fertilisation events. When ejaculates are detected, females increase their investment in competitive behaviours due to the cue of imminent reproduction, or because

ejaculates directly stimulate responses in females. Females that are able to respond quickly and efficiently to such a cue may have an advantage in female competition. One could imagine this functioning in a situation where females breed synchronously or during a defined breeding season, such as in species of mammals (e.g. topi antelope; Bro-Jorgensen 2002), birds (egg. European starlings; Smith 2004), and lizards (e.g. striped plateau lizards; Weiss et al. 2009). Females in synchronous breeding species that are faster to respond to mating and begin competing for resources may procure higher quality territories, more food, or higher social status. Being able to compete as soon as fertilisation could possibly have occurred, rather than waiting for fertilisation to definitely have occurred could provide a serious advantage in acquiring resources to ensure successful offspring production.

As there may be both costs and benefits to responding quickly to male ejaculates, there may be variability in how females are able/'choose' to respond to the signal of potential fertilization from male ejaculates, such as the greater response of high density females to mating in Chapter 5. There are many axes of variation among females other than larval density and mating status that could be involved in modulating responses to mating-induced aggression. Factors such as social status, age, armament presence or size, and social and environmental conditions, may also affect how females respond to ejaculates that induce aggressive behaviours. In systems with dominance hierarchies, for instance, subordinate females may face higher costs from exhibiting mating-induced aggressive behaviours because of their inability to successfully compete with dominant females (Nelson-Flower et al. 2013). Dominant females will often suppress reproduction in subordinate individuals physiologically, or

by aggressively harassing or evicting them from the group, in cooperative mammals, such as meerkats and mongooses (Clutton-Brock et al. 1998; Young et al. 2006), in birds (Nelson-Flower et al. 2013), and in insects (Ratnieks et al. 2006). Research that evaluates variation in female aggressive responses to mating is therefore of great interest in better understanding whether, and how, an ejaculate-induced increase in aggression could function in more complex social systems. Manipulating mating status for individuals of different social ranks and investigating the resulting levels and targets of female aggression by those mated females is one potential way to test this experimentally.

Another potential source of variation in ejaculate-induced female aggression is in males themselves. Males that are able to induce increased aggression in their mates may benefit from their mate's increased aggression towards other females if it helps her succeed in acquiring more resources for their offspring. Males in a variety of species strategically allocate their ejaculates, based on female mating or social status, as well as the risk of sperm competition, suggesting at least one possible source of variation in male ejaculates (Nicholls et al. 2001; Pizzari et al. 2003; Wigby et al. 2009). Other than strategic allocation by males, females may also be able to distinguish aspects of male quality from their ejaculate and alter their investment in offspring accordingly (Eberhard 1996). My results in Chapter 4 suggest (though do not provide strong evidence) that variation in males could alter the strength of post-mating aggression in females due to differences in their ejaculates. It would be particularly interesting to extend this work to species with more complex social systems, and across different mating systems. Establishing whether and which ejaculates stimulate

aggression in a wider range of species is a crucial first step to investigating how ejaculates could be differentially used by males to induce aggression in females. In species where one male mates exclusively with multiple females, for example, inclusive fitness theory might suggest there are fewer benefits for the dominant male to induce competition amongst his mates (Hamilton 1963, 1964a,b). For subordinate or sneaker males that acquire fewer matings, however, it may be beneficial to induce higher levels of aggression in their mates as they are less likely to compete with other mates of that male.

Burying beetles (*Nicrophorus vespilloides*) are one potential system in which this could be tested. Female burying beetles undergo high-intensity battles over small vertebrate carcasses (an essential breeding resource), with the winning female excluding the loser from the carcass, severely reducing the subordinate's ability to produce young (Pukowski 1933). As a result, dominant females produce more offspring than subordinates, meaning winning or losing these fights has dramatic effects on female reproductive success (Eggert et al. 2008). Males also compete with other males to control and mate on carcasses (Pukowski 1933). However, males can also mate without controlling a carcass, attracting females by emitting pheromones away from carcasses (Eggert 1992). Testing whether the aggression levels of females differ after mating with pheromone-emitting males or carcass-controlling males would be a first step towards establishing that male mating tactic could influence female aggression. It would then be possible to use proteomics to investigate differences in the ejaculates between the two types of males to identify potential causes of any differences in female behaviour. Ultimately, this would then be able to be combined through using a

gene-editing technique, such as CRISPR, to manipulate specific ejaculate components to identify which components are responsible for inducing an increase in female aggression (Findlay et al. 2014). The development of tools such as CRISPR, proteomics, and fast, affordable sequencing technologies, means that such research no longer has to be confined to traditional model organisms (Findlay et al. 2014; Valcu and Kempenaers 2014).

Fitness effects of mating-induced aggression

Ascertaining what effects mating-induced female aggression has on fitness is a crucial direction for future research, as it helps us to determine under what conditions mating-induced female aggression is beneficial for females, or whether it is a trait undergoing sexual conflict. If mating-induced female aggression is stimulated by male ejaculate components, this raises the possibility that female aggression could be under sexual conflict under certain situations. Males may benefit from stimulating high levels of aggression in their mate directly after mating to increase the fitness of their offspring, even if this results in long-term costs for the female, in terms of survival or future reproductive potential. In addition, given the energetic costs and risk of injury to females from engaging in competitive interactions in some species, it seems likely that the effects of female aggression will also vary in relation to individual internal factors, such as diet or age. For example, diet and age change whether the transfer of sex peptide is beneficial or detrimental to female *D. melanogaster*, and some of these effects may be due to the effects of female aggression (Fricke et al. 2010, 2013). As female aggression is a behaviour in which the costs and benefits are heavily dependent on other individuals in the vicinity, it seems likely that changing the social environment will also alter the fitness effects of mating-induced female aggression.

Being in a densely populated location may make maintaining high levels of aggression too costly or unfeasible due to the large number of potential competitors and inability to monopolise a resource. In a less densely populated environment, however, increasing aggression may be beneficial because of a more manageable number of competitors and the ability to successfully defend resources. I found no evidence of benefits accruing to females that performed better in female competition in any of my experiments, but my experiments were not designed to directly test the fitness effects of aggression. Being able to directly manipulate female aggression (see 'Proximate causes of female aggression in *D. melanogaster*') would allow us to properly determine the fitness effects of female aggression in response to different biotic and abiotic environments. Future studies investigating interactions between multiple individuals, in group situations, or in mixed species groups, will also help us to better understand the fitness effects of mating-induced female aggression.

Identifying fitness effects is more difficult in females than in males, as females invest in both the quantity and quality of their offspring. Studies that have investigated the effects of female aggression on fitness have produced mixed results (Packer et al. 1995; Kraaijeveld 2004; Heinsohn 2008; Watson and Simmons 2010; Eggert and Müller 2011; Wilson et al. 2013). In tree swallows (*Tachcineta bicolor*) females will kill nestlings of other females, showing a strong benefit from being aggressive (i.e. aggressive females kill less aggressive females' chicks but protect their own, resulting in differences in offspring survival due to aggressiveness: Chek and Robertson 1991). However, when all females raised chicks, more aggressive females had reduced hatching success and produced offspring of lower quality (Rosvall 2011c, 2013a).

Similarly, in dark-eyed juncos (*Junco hyemalis*) more aggressive females produced smaller eggs and lighter chicks in years where resources were plentiful, but produced larger eggs and had greater nest success in years with more limited resources (Cain and Ketterson 2012, 2013). The different fitness components measured in these studies indicate that evaluating fitness effects of female aggression requires a broader approach than studying offspring number alone, as well as multigenerational studies to fully examine the effects of levels of female aggression on offspring reproductive success and survival (Clutton-Brock and Huchard 2013b). Conducting multigenerational studies is much more feasible in short-lived species, such as *D. melanogaster*, than in wild, long-lived vertebrate species, suggesting a possible study system to begin establishing the fitness effects of ejaculate-induced female aggression.

Proximate causes of female aggression in *D. melanogaster*

Identifying the proximate causes of female aggression in *D. melanogaster* is fundamental for our understanding of female aggression across species for a variety of reasons. First, as it is impossible to directly manipulate aggression without knowledge of the proximate causes of female aggression, it is currently difficult to estimate the fitness effects of female aggression in any species. In male *D. melanogaster*, where the neural circuitry of aggression is well-characterised, it is possible to directly manipulate levels of aggression and thus establish the fitness effects of male aggression (although this has not yet been done; Dierick and Greenspan 2007; Wang et al. 2008; Asahina et al. 2014). However, the female aggression circuitry has not yet been identified. Further study of the proximate causes of mating-induced female aggression may assist us in

identifying the neural and hormonal circuitry and thus manipulate female aggression directly to examine its fitness effects.

Second, due to evolutionarily conserved genetic pathways found in insects, birds, and mammals, our understanding of the proximate mechanisms governing female aggression in *D. melanogaster* is likely to be broadly generalizable, meaning that establishing the proximate mechanisms of mating-induced female aggression in *D. melanogaster* also has significant potential to elucidate the proximate causes of female aggression in other organisms. As *D. melanogaster* is a well-established model organism, many molecular and genetic techniques exist to enable us to target specific components of the male ejaculate and female reproductive physiology (e.g. knocking down specific Sfps and female receptors: Liu and Kubli 2003; Yapici et al. 2008). One major step would be to identify how the sperm effect on female aggression is achieved – i.e. is female aggression stimulated by females detecting sperm in their storage organs, or through the action of sperm-associated seminal fluid proteins (Sfps)? There are a variety of potential candidate Sfps and hormones that could be involved in stimulating post-mating aggression: Sfps that bind to sperm and are responsible for the long-term 'sperm effect', including sex peptide (Ram and Wolfner 2009; Kubli and Bopp 2012), juvenile hormone (stimulates oogenesis and is triggered by the receipt of sex peptide: Heifetz et al. 2000), and serotonin (plays a key role in regulating male aggression (Hoyer et al. 2008; Johnson et al. 2009). Tools exist in *D. melanogaster* to test each of these candidates separately, making it an achievable short-term goal to establish whether any are identified in post-mating female aggression.

Example for establishing the proximate mechanisms: sex peptide

The first potential candidate to identify how ejaculates induce female aggression in *D. melanogaster* is through investigating the sex peptide. As discussed in Chapter 3, sex peptide is a small Sfp, which induces important changes in female behaviour and physiology (Liu and Kubli 2003; Kubli and Bopp 2012). Sex peptide is composed of three main sections (see Figure 1):

1. The C-terminal end, which binds to the sex peptide receptor to induce the receptivity and egg laying responses (Yapici et al. 2008; Rezával et al. 2012);
2. The hydroproxoline motif, which induces an immune response (Domanitskaya et al. 2007);
3. The N-terminal end, which stimulates juvenile hormone production in the corpora allata (the insect equivalent of endocrine glands) (Moshitzky et al. 1996; Fan et al. 2000).

As well as reducing remating receptivity and increasing egg laying, sex peptide also induces a partial increase in female aggression after mating (Chapter 3). However, it does not use the sex peptide receptor pathway to do so, despite the fact that this is the main pathway through which post-mating responses are initiated by sex peptide (Yapici et al. 2008; Häsemeyer et al. 2009; Kubli and Bopp 2012; Rezával et al. 2012). The most logical candidate for inducing female aggression is therefore the N-terminal end of the sex peptide. This end stimulates juvenile hormone production in the corpora allata, although the mechanism of this stimulation is unknown (Moshitzky et al. 1996; Fan et al. 2000). Juvenile hormone facilitates vitellogenesis (the incorporation of yolk proteins into eggs), resulting in the production of more mature eggs in mated females, thus playing a crucial role in reproduction (Soller et al. 1999). Juvenile

hormone titres (the amount of juvenile hormone in the haemolymph) have also been linked to aggression in both males and females in other insect species, such as burying beetles, paper wasps, and cockroaches (Scott 2006; Kou et al. 2009; Tibbetts et al. 2013; Tibbetts and Crocker 2014). It is therefore a prime candidate to be involved in modulating a post-mating increase in female aggression in *D. melanogaster*.

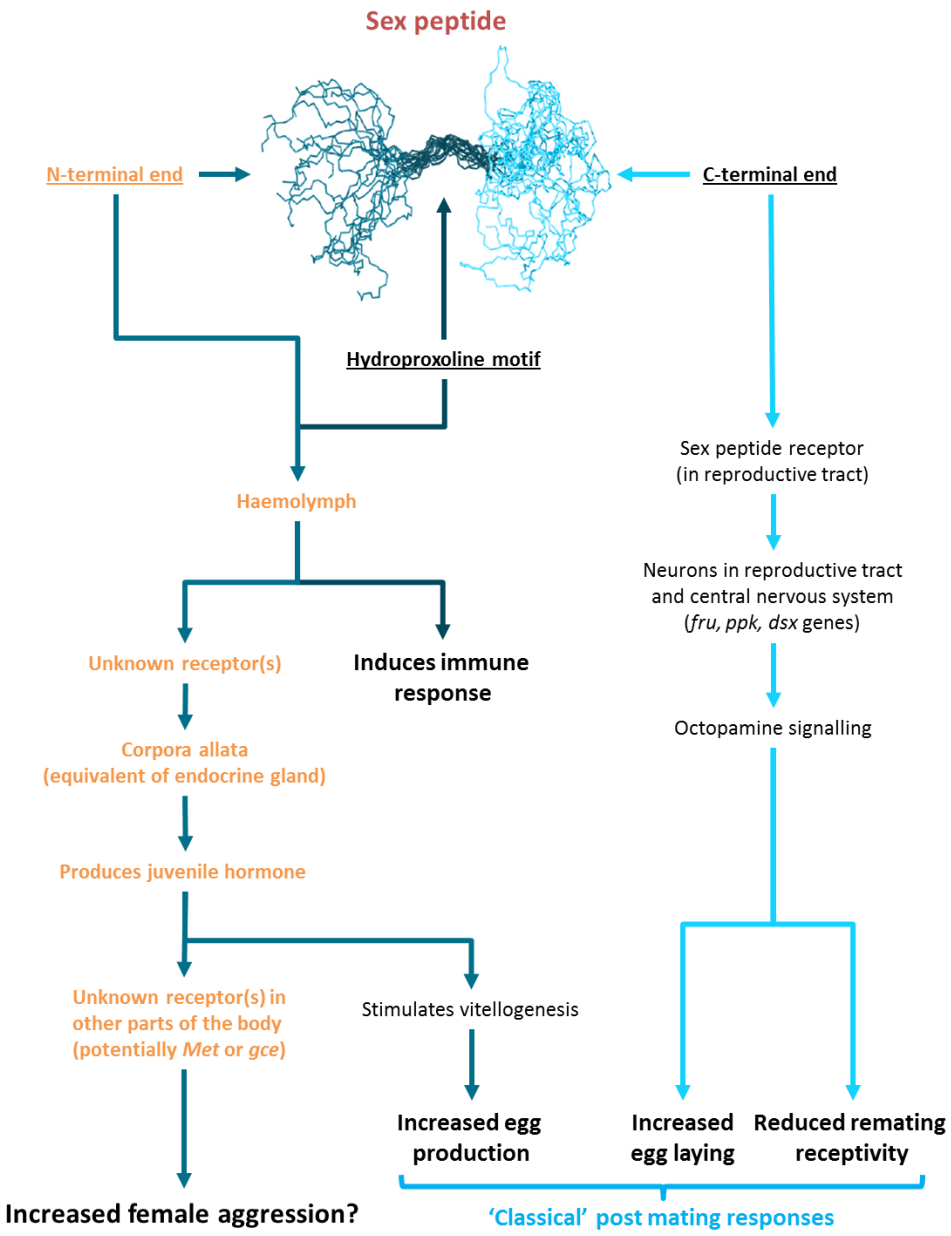


Figure 1: Dissecting how sex peptide induces an increase in female aggression

Sex peptide induces three 'classical' post-mating responses through known pathways, but how sex peptide induces an increase in female aggression is currently unknown. I aim to investigate the N-terminal pathway as one potential way in which sex peptide could induce an increase in female aggression (all the stages in **orange**). It is possible to manipulate the binding of the N-terminal end of sex peptide, restrict the production of juvenile hormone in the corpora allata, and artificially regulate the levels of juvenile hormone present in the female to identify whether these factors influence rates of female aggression.

It is possible to manipulate levels of juvenile hormone directly, through injection with a juvenile hormone analogue – methoprene (Scott 2006; Tibbetts et al. 2013). It should also be possible to investigate potential juvenile hormone receptors throughout the body, such as those encoded by *Methoprene-tolerant* and *gce* genes (Jindra et al. 2013; Marchal et al. 2014; Reiff et al. 2015), to investigate how juvenile hormone induces the complex behavioural responses associated with aggression. Juvenile hormone has also been shown to have condition-dependent effects on behaviour and fecundity (Tibbetts and Izzo 2009), thus allowing further examination of the developmental-environment effects we observed in Chapter 5.

Establishing the pathways through which ejaculates stimulate female aggression can also help us identify how these mechanisms interact with other female behaviours, such as offspring production or remating receptivity. Studies in birds and mammals suggest that elevating testosterone increases success in female competition, but imposes serious costs and constraints on other female behaviours (Rosvall 2013a). Juvenile hormone in insects is involved both in reproduction and aggression, suggesting such constraints may also exist in insects (Tibbetts and Izzo 2009). Investigating these physiological constraints could provide insights into the prevalence and intensity of female competition across species, as well as potential trade-offs with other life-history traits.

Conclusion

Female-female competition is a complex set of behaviours, with vast potential to influence individual fitness and the ecology and evolution of species. In my thesis, I aimed to shed light on new aspects of female competition, such as the proximate causes of mating-induced female aggression, and the role of the developmental environment in contest outcomes and dynamics. Despite being neglected for decades, female competition is an area well worthy of study and greater research focus. Understanding both the proximate and ultimate causes of female aggression will help us to more fully understand the selective pressures of intrasexual competition in females.

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