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Sexual conflict in three arthropod species: experimental evolution approach

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Summary

Sexual conflict occurs due to conflicting reproductive interests of males and females and is prevalent among sexually reproducing organisms. Two forms of sexual conflict can be identified: interlocus sexual conflict and intralocus sexual conflict. Owing to the numerous potential evolutionary consequences of sexual conflict, studying sexual conflict can be considered imperative in the field of evolutionary biology. In this dissertation, various aspects of sexual conflict in three arthropod species were studied employing experimental evolution approach.

Seminal fluid proteins such as sex peptide (SP) in *Drosophila melanogaster* can mediate interlocus sexual conflict by increasing fitness of males while simultaneously decreasing fitness of females, which can lead to sexually antagonistic coevolution. The sex peptide-sex peptide receptor (SPR) system was utilized to investigate the evolutionary response of males from artificial selection lines in which females lacked expression of SPR; this may have influenced the intersexual arms race. The expression levels of SP and another related seminal protein Dup99B (which can also bind SPR) were investigated using RT-qPCR. In contrast to predictions, a significant increase in SP expression was found in males from artificial selection lines compared to control males, but no difference in Dup99B expression was observed. The results indicate that genetic manipulations in females which influence the intersexual arms race can lead to a fast response in males. The results also point towards the possible presence of additional sex peptide receptors other than SPR.

Although interlocus sexual conflict has been demonstrated extensively in *D. melanogaster*, there is no definitive evidence for it in *D. simulans*, a closely related sister species. However, experimental evolution could perturb equilibrium of adaptations and

counter-adaptations, thus increasing the power to detect sexual conflict. Previous work with *D. simulans* populations evolved under altered mating systems and rearing temperatures showed that both elevated polyandry and increased temperature resulted in increased male sexual competitiveness. Here it was tested whether evolution of increased competitiveness would in turn lead to increased mating costs to females in terms of decreased longevity and/or productivity. Neither the mating system nor the evolution temperature of males showed any effect on the longevity of females. However, there was significant interaction between male mating system and evolution temperature in their effect on female productivity: females mated with males evolving under polyandry at elevated temperature showed the highest productivity. Thus, contrary to predictions of IRSC the results of this study demonstrated that mating with males that evolved higher reproductive fitness is advantageous for females.

Bulb mite species *Rhizoglyphus robini* presents an intriguing model system to study sexual conflict, owing to two male phenotypes with alternative reproductive tactics: aggressive fighters and benign scramblers. The final experiment investigated the genetic basis of intralocus sexual conflict associated with increased sexual dimorphism of fighters in *R. robini*. Artificial selection in combination with high-throughput transcriptome sequencing was used to compare gene expression patterns of males and females from replicate lines selected for increased proportion of fighters (F-lines) or scramblers (S-lines). Differential gene expression analysis identified 438 genes showing significant expression difference between F-line and S-line males. The results indicated that selection on a more sexually dimorphic fighter male morph entails an increase in expression of a significantly higher number of genes compared to selection on a relatively feminized scrambler morph, thus increasing the potential for intralocus sexual conflict. Genes overexpressed in F-line males than in S-line males also had higher mean expression levels

in F-line females than in S-line females, whereas the reverse was observed for the S-male biased genes. Nine candidate genes underlying the intralocus sexual conflict were identified, one of which may be involved in energy metabolism. The results were consistent with the hypothesis that correlated changes in gene expression patterns in males and females underlie the elevated intralocus sexual conflict.

General Introduction

Under anisogamy, males and females typically fulfill distinct reproductive roles, and frequently experience contrasting selection pressures, which can lead to a sexual conflict (Trivers 1972, Dawkins 1976, Parker 1979). Sexual conflict is prevalent among sexual species, as demonstrated by several empirical studies in the last few decades (Arnqvist and Rowe 2005). Sexual conflict can be further differentiated into two forms: Interlocus sexual conflict and intralocus sexual conflict.

Sexual conflict can occur over the outcome of interaction between males and females, such as mating rate, copulation duration, fertilization, remating behaviour and parental investment (Arnqvist and Rowe 2005). Typically individuals of one sex evolve traits that improve their own reproductive fitness, which may negatively affect the fitness of the other sex. Since such a conflict is mediated by phenotypes assumed to be encoded by different loci in the two sexes, it is termed as interlocus sexual conflict (IRSC) (Reviewed in Arnqvist and Rowe 2005). IRSC can potentially result in an arms race between the two sexes with a chain of adaptations in one sex, often males, and counter-adaptations in the other, usually females, leading to sexually antagonistic coevolution (Parker 1979, Holland and Rice 1998). For example, coevolution of male grasping and female anti-grasping structures has been detected in water striders (Arnqvist and Rowe 2002) and diving beetles (Bergsten *et al.* 2001), and coevolution of harmful male genitalia and reinforced female tissue has been demonstrated in seed beetles (Rönn *et al.* 2007) and bed-bugs (reviewed in Siva-Jothy 2006).

Sexual conflict can also occur over shared phenotypic traits in males and females whose expression is controlled by shared gene loci, hence termed intralocus sexual conflict (IASC). Such conflict arises when the optimal phenotypes for the shared morphological,

physiological and behavioural traits do not coincide in the two sexes. However, the shared genetic architecture and the resultant high intersexual genetic correlation (r_{mf}) constrain males and females from reaching their respective optima independently (Chippindale *et al.* 2001, Bonduriansky and Chenoweth 2009). An evolutionary tug-of-war ensues over trait expression where selection on one sex can result in displacement of the other sex from its fitness optimum (Lande 1980). Alleles for shared traits thus lead to sexually antagonistic effects on fitness in the two sexes. For example, in wild red deer *Cervus elaphus*, high fitness males sire low-fitness daughters (Foerster *et al.* 2007). Intralocus sexual conflict has also been demonstrated in humans over traits such as height (Stulp *et al.* 2010) and pelvis size (LaVelle 1995, Rice and Chippindale 2001). Larger hip width benefits women allowing safer childbirth, the males on the other hand only suffer the cost of less efficient locomotion, thus leading to an intralocus conflict.

Interlocus and intralocus conflict may also show some interactions (reviewed in Pennell and Morrow 2013). IRSC may result in selection on a shared trait to cause IASC. For example, selection on high mating frequency leads to higher male fitness but often imposes relatively greater costs on females (Thornhill and Alcock 2001) resulting in IRSC. This may lead to positive selection on females to evolve mating resistance in order to reduce the effects of male harm. If the genes underlying resistance traits show high intersexual genetic correlations, it can potentially result in IASC (Pennell and Morrow 2013). Secondly, if IASC over such resistance traits is unresolved, they may remain below the female-specific optimum and counter-adaptations in response to the IRSC may not evolve, thus impeding the escalation of IRSC. Recently, Innocenti and Morrow (2010) suggested a potential link between IASC and IRSC in *Drosophila melanogaster*. Sex-limited tissues such as male accessory glands and female sperm storage organs can mediate sexually antagonistic coevolution that results from IRSC (Chapman *et al.* 1995, Pitnick *et*

al. 2009). However, these tissues were also found to be enriched in candidate sexually antagonistic loci that contribute to negative genetic correlation for adult fitness i.e. IASC (Innocenti and Morrow 2010). Lastly, resolution of IASC in a way that allows males and females to reach their sex-specific optima may result in an exaggerated male trait that increases male fitness but reduces the female fitness due to harmful interactions (Pennell and Morrow 2013). For example, several sperm traits are expressed solely in males by means of gene duplication (Wyman *et al.* 2012), which may have evolved to resolve IASC through sex-specific gene expression. The sperm-related genes also show signatures of rapid evolution under strong positive selection (Swanson and Vacquier 2002), which might be a result of the intersexual arms race due to IRSC.

Interlocus sexual conflict and intralocus sexual conflict have been described to have distinct evolutionary consequences. IRSC has direct consequences for evolution of mating strategies and sexual selection (Arnqvist and Rowe 2005) and it is also central to evolution of parental care (Trivers 1972). IRSC can lead to a coevolutionary arms race between the sexes (Rice and Holland 1997, Holland and Rice 1998), resulting in rapid evolution of male and female reproductive characters (Gavrilets *et al.* 2001, Hosken et al. 2001). Therefore IRSC is hypothesized to generate reproductive isolation between allopatric populations and eventually speciation (Parker and Partridge 1998, Rice 1998, Gavrilets 2000, Arnqvist and Rowe 2005).

Intralocus sexual conflict (IASC) has also been suggested to contribute to speciation, albeit through a different mechanism of coevolution between sexually antagonistic genes and sex limited genes (Rice and Chippindale 2002). Further, IASC is hypothesized to be involved in several evolutionary processes (reviewed in van Doorn 2009, Bonduriansky and Chenoweth 2009, Pennell and Morrow 2013) such as evolution of sex chromosomes (Bull 1983, Rice 1987, Charlesworth 1991, Mank *et al.* 2014), evolution

of sex determination (Rice 1986, Kraak and Pen 2002, Van Doorn and Kirkpatrick 2007) and sex ratio allocation (Alonzo and Sinervo 2007). IASC potentially also plays a role in regulation of gene expression (Ellegren and Parsch 2007), genomic imprinting (Day and Bonduriansky 2004, Patten and Haig 2008), sexual selection (Brommer *et al.* 2007, Pischedda and Chippindale 2006) and ageing (Vieira *et al.* 2000, Bonduriansky *et al.* 2008). Recent studies have indicated that IASC may be important for maintenance of genetic variation (Rice 1984, Rostant *et al.* 2015) and maintenance of alternative reproductive tactics (ARTs) (Plesnar-Bielak *et al.* 2014).

Acceptance of sexual conflict as a potentially significant evolutionary force is a new paradigm in recent years (Tregenza *et al.* 2006) and study of sexual conflict has gained increasing importance. Over the years, sexual conflict and its consequences have been demonstrated in several organisms using a variety of approaches (reviewed in Chapman *et al.* 2003a). Life-history studies in combination with genetic manipulations can give important insights into costs and benefits of mating for males and females. In *Drosophila melanogaster*, this approach demonstrated IRSC over mating frequency (Bateman 1948, Fowler and Partridge 1989, Partridge and Fowler 1990) and cost to females mediated by male accessory gland proteins (Chapman *et al.* 1995, Chapman *et al.* 2003b, Wigby and Chapman 2005) as well as IASC (Chippindale *et al.* 2001). An important line of evidence in detecting sexual conflict comes from comparative studies between species, which have helped reveal coevolution between sexes in water striders (Arnqvist and Rowe 2002a, b) and rapid evolution of reproductive proteins in *Drosophila* as well as in mammals (Begun *et al.* 2000, Swanson and Vacquier 2001, Swanson *et al.* 2001a, Swanson *et al.* 2001b), predicted to be consequences of IRSC.

Experimental approaches involving artificial selection and experimental evolution have also proven significantly useful in study of sexual conflict. It is a research framework

that allows for the study of evolutionary processes by means of cross-generational changes occurring in experimental populations as a result of conditions imposed or traits selected by the investigator (Kawecki *et al.* 2012, Fuller *et al.* 2005, Swallow and Garland 2005, Fry *et al.* 2003). The use of control populations that provide a baseline for comparison with experimental populations, and the ability to replicate an experiment under identical conditions which allows for differentiation between stochastic and deterministic effects (Schlötterer *et al.* 2015) make this approach a powerful tool to study evolutionary processes. Selection experiments may be distinguished into two types: laboratory natural selection and artificial selection (Fry *et al.* 2003, Fuller *et al.* 2005). Laboratory natural selection allows the investigator to vary the environment of a laboratory-maintained population in a regulated way, in comparison with an unaltered control population. On the other hand, artificial selection allows the investigator to directly control selection on a particular trait (or traits) and then make comparisons with either a non-selected population or populations selected in the opposite direction (reviewed in Fuller *et al.* 2005, Swallow and Garland 2005).

Experimental evolution has been previously employed to address a broad range of questions in the field of evolutionary biology such as studying adaptation to specific environments, evolutionary trade-offs and constraints, estimating population genetic parameters, as well as testing evolutionary theories such as sexual selection and sexual conflict (Fry *et al.* 2003, Fuller *et al.* 2005, Edward *et al.* 2010, Kawecki *et al.* 2012). One of the first experiments to demonstrate sexual antagonism using experimental evolution was in *D. melanogaster* (Rice 1996), where females prevented from coevolving with males resulted in increased male fitness and reduced female lifespan. Since then, selection experiments that manipulated mating systems or adult sex ratio have proven particularly instrumental in detecting male harm to females and female resistance to harm in multiple

species, including *D. melanogaster* (Holland and Rice 1999, Wigby and Chapman 2004, Stewart *et al.* 2005), dung fly *Sepsis cynipsea* (Hosken *et al.* 2001, Martin and Hosken 2003a) and bulb mite *Rhizoglyphus robini* (Tilszer *et al.* 2006). Experimental evolution under high male-male competition or no male-male competition in *C. elegans* also demonstrated rapid evolution of male-induced collateral harm to mates (Palopoli *et al.* 2015). Experimental evolution with enforced monogamy and polyandry was employed to demonstrate evolution of reproductive isolation through sexual conflict in *Sepsis cynipsea* (Martin and Hosken 2003b, Hosken *et al.* 2009). However, a similar approach in *D. melanogaster* (Wigby and Chapman 2006), *D. pseudoobscura* (Bacigalupe *et al.* 2007), *Tribolium castaneum* (Michalczyk 2008), *Callosobruchus maculatus* (Gay *et al.* 2009) and *R. robini* (Plesnar-Bielak et al. 2013) failed to find evidence for reproductive isolation though sexual conflict. Intralocus sexual conflict was demonstrated using artificial selection on alternative male phenotypes in *R.robini* (Plesnar-Bielak *et al.* 2014) and broad-horned flour beetles *Gnatocerus cornutus* (Harano *et al.* 2010). Combining experimental evolution with sequencing technologies also provides a powerful approach to investigate the response to selection. Theory suggests that under relaxed selection on males, genes that show sex-biased expression due to sexually antagonistic selection should evolve female-like gene expression. Transcriptional profiling of *D. melanogaster* populations experimentally evolved under enforced monogamy or polygamy supported this prediction as monogamous males showed feminized gene expression compared to polygamous males (Hollis *et al.* 2014).

In the present dissertation, the experimental evolution approach was utilized to study various aspects of sexual conflict in three species of arthropods: *Drosophila melanogaster*, *Drosophila simulans*, and *Rhizoglyphus robini*. The three species share features such as short generation time, easy rearing and easy manipulation of mating

system which are advantageous for experimental evolution. With an additional virtue of ease of genetic manipulation, *D. melanogaster* has been a widely used system for investigating various aspects of sexual conflict (Bateman 1948, Manning 1963, Fowler and Partridge 1989, Partridge and Fowler 1990, Rice 1992, Rice 1996, Holland and Rice 1999, Civetta and Clark 2000, Chippindale *et al.* 2001, Wigby and Chapman 2004, Stewart *et al.* 2005, Wigby and Chapman 2006, Innocenti and Morrow 2010, Hesketh *et al.* 2013, Hollis *et al.* 2014, Rostant *et al.* 2015).

Seminal proteins in *D. melanogaster* are involved in interlocus sexual conflict (Fowler and Partridge 1989, Chapman *et al.* 1995, Chapman *et al.* 2001, Lung *et al.* 2002, Wigby and Chapman 2005, Arnqvist and Rowe 2005) and in particular the accessory gland protein sex peptide (SP) is described as a classic phenotype of sexual antagonism (Wigby and Chapman 2005, Arnqvist and Rowe 2005; but see Perry and Rowe 2014). Costs of sexually antagonistic traits will be important for determining their dynamics with the evolving resistance traits, yet little is known about these dynamics. In absence of the receptor via which most of postmating responses of SP in females are triggered, males evolving with such receptorless females may be expected to reduce the expression of *SP*. Expression levels of another related seminal peptide (Dup99B) could also be expected to change in these males. This was examined in **Chapter 1** by comparing artificial selection lines of *D. melanogaster* that lacked expression of the sex peptide receptor and control populations that expressed the receptor. The expression levels of peptides from the sex peptide family in males from selection lines were compared with those in males from control populations using RT-qPCR. The evolutionary response of males to experimental evolution in absence of the receptor can potentially give insights into sexually antagonistic coevolution.

D. simulans, a sister species of *D. melanogaster*, has been previously used to study aspects like cost of mating and intersexual genetic correlation (Civetta *et al.* 2005, Taylor *et al.* 2008a, Taylor *et al.* 2008b, Duffy *et al.* 2014). Despite similar biology to D. melanogaster, IRSC has not been demonstrated in this species. However, this may be because adaptation in one sex will often be balanced by a counter-adaptation in another sex and thus the outcome of such antagonistic interactions remains unchanged (Rice 1996, Chapman and Partridge 1996, Rice 2000, Arnqvist and Rowe 2002). However, experimental evolution may be used to alter such equilibrium of adaptations and counter-adaptations which could increase the power to detect IRSC. In **Chapter 2**, experimental evolution under altered mating system (enforced monogamy or elevated polyandry) was utilized to this aim, in combination with ancestral (lower) or novel (higher) rearing temperatures. Previous work showed that evolution under both elevated polyandry and increased temperature resulted in increased male sexual competitiveness. Males subjected to experimental evolution for more than 65 generations were used with non-coevolved test females to investigate whether evolution of increased competitiveness would in turn lead to increased mating costs to females in terms of decreased longevity and/or productivity.

In bulb mite *R. robini*, sexual conflict has been previously demonstrated (Kolodziejczyk and Radwan 2003, Konior *et al.* 2006, Tilszer *et al.* 2006, Plesnar-Bielak *et al.* 2013, Plesnar-Bielak *et al.* 2014) and it presents an interesting model system to study sexual conflict because of the complex sexual competition, owing to two male phenotypes with alternative reproductive tactics. In order to understand why intralocus sexual conflict persists, sexually antagonistic genes need to be identified, yet very little is known about the genetic basis of IASC, apart from in D. melanogaster. Plesnar-Bielak *et al.* (2014) had demonstrated IASC in *R. robini* lines artificially selected for increased proportion of fighter males or scrambler males. In **Chapter 3**, genetic basis of this IASC was

investigated by studying differences in gene expression between the artificial selection lines with the help of high throughput sequencing. Specifically, the study tested the hypothesis that correlated changes in gene expression patterns in males and females in response to selection may have caused the sexual conflict and aimed to identify the candidate genes underlying IASC.

Chapter 1

Transcriptional changes in seminal proteins of *D. melanogaster* populations evolving in the absence of sex peptide receptor

Abstract

Interlocus sexual conflict occurs over the outcome of male-female interactions such as mating rate and parental investment. Seminal fluid proteins such as sex peptide (SP) in *Drosophila melanogaster* can mediate sexual conflict by increasing fitness of males while simultaneously decreasing fitness of females, which could potentially lead to sexually antagonistic coevolution. SP binds to a specific sex peptide receptor (SPR) located in the reproductive tract and nervous system of females. In this study, the peptide- receptor system was utilized to investigate the evolutionary response of males from populations (S) in which females lacked SPR expression. It was predicted that S males may be selected to decrease investment in SP depending on the cost-benefit ratio, but may compensate by increased expression of related seminal proteins, such as Ductus ejaculatorius peptide 99B (Dup99B). RT-qPCR was used to investigate the expression levels of *SP* and *Dup99B*; a significant increase in *SP* expression was found in S males compared to control males, but no difference in *Dup99B* expression was observed. Although the results did not support the predictions based on cost of expression, they suggest presence of other receptors that bind SP and Dup99B, which warrant further research.

1.1 Introduction

In sexually reproducing species, males and females pursue divergent evolutionary interests in order to maximize their respective reproductive output, which leads to sexual conflict (Trivers 1972, Dawkins 1976, Parker 1979, Arnqvist and Rowe 2005). Interlocus sexual conflict, mediated by distinct loci in males and females, occurs over the outcome of male-female interactions such as mating rate, parental investment or remating behavior, such that the optimal outcome is different for the two sexes (Rice and Holland 1997, Arnqvist and Rowe 2005). Male seminal fluid proteins in *Drosophila melanogaster*, which influence reproductive physiology and behavior of mated females to male benefit, have been identified as candidates mediating interlocus sexual conflict (Chapman *et al.* 1993, Chapman *et al.* 1995) and have been extensively studied (reviewed in Chapman 2001).

In *D. melanogaster*, as in numerous other internally fertilizing species, males transfer seminal fluid to the female reproductive tract during copulation. Apart from sperm, the seminal fluid contains accessory gland proteins (Acps), synthesized in and secreted from the paired accessory glands, as well as proteins from seminal vesicles, ejaculatory duct, ejaculatory bulb and testes. More than 100 Acps have been identified in *D. melanogaster* to date (Ravi Ram and Wolfner 2007). The Acps play a variety of important roles in sperm transfer, sperm storage, egg production, egg laying, female remating rate and mating plug formation (reviewed in Wolfner 1997, Chapman 2001, Gillott 2003). Although these functions collectively serve to improve the male's chances of siring a significant proportion of a female's offspring, they also adversely affect the female's fitness (Fowler and Partridge 1989, Chapman *et al.* 1995, Chapman *et al.* 2001, Lung *et al.* 2002, Wigby and Chapman 2005), thus leading to interlocus sexual conflict (Arnqvist and Rowe 2005).

The most extensively studied Acp, sex peptide (SP or Acp70A), elicits a wide array of post-mating responses in females (Kubli 2003, Ram and Wolfner 2007). Two important post-mating effects of SP are reduced female receptivity to remating and increased rates of oogenesis and oviposition (Chen *et al.* 1988, Aigaki *et al.* 1991, Soller *et al.* 1997, Chapman *et al.* 2003b, Liu and Kubli 2003). In addition, SP aids in release of stored sperm (Avila et al 2010), mediates sperm competition (Clark *et al.* 1995, Harshman and Prout 1994) and influences female feeding patterns (Carvalho *et al.* 2006, Ribeiro and Dickson 2010, Cognigni *et al.* 2011) and sleep patterns (Isaac *et al.* 2010). However, SP is also responsible for the reduction in fitness, lifetime reproductive success and survival of mated females (Fowler and Partridge 1989, Chapman *et al.* 1995, Moshitzky *et al.* 1996, Wolfner 1997, Wigby and Chapman 2005), although this effect is dependent on female diet (Fricke *et al.* 2009). In contrast, males benefit from SP transfer in terms of absolute reproductive success (Fricke *et al.* 2009). Such sexually antagonistic effects make SP one of the chief seminal proteins mediating sexual conflict in *D. melanogaster* (Arnqvist and Rowe 2005, Wigby and Chapman 2005).

Another seminal fluid protein from the sex peptide gene family, ductus ejaculatorius peptide 99B (Dup99B) expressed in the male ejaculatory duct, also elicits post mating responses of reduced receptivity to remating and increased oviposition in females, but only on a minor scale compared to SP (Saudan *et al.* 2002, Rexhepaj *et al.* 2003, Ding *et al.* 2003, Kubli 2003). *SP* (located on the left arm of chromosome III) and *Dup99B* (located on the right arm of chromosome III) belong to the *SP* gene family and are believed to have arisen by gene duplication, based on the strong similarity exhibited by the signal sequences of their precursors and the carboxy-terminal regions of their peptides (Rexhepaj *et al.* 2003).

The carboxy-terminal end of SP protein is indispensable for induction of the post-mating responses of increased oviposition and remating inhibition in females (Schmidt et al. 1993, Ding *et al.* 2003), and binds to a high affinity G-protein coupled receptor (sex peptide receptor, SPR), conserved in many insect species (Ottiger *et al.* 2000, Kubli 2008, Yapici *et al.* 2008). It is not known whether a specific receptor for Dup99B exists in *D. melanogaster*, but Dup99B can bind SPR *in vitro*, although with a lower affinity than SP (Ottiger *et al.* 2000, Yapici *et al.* 2008). In *D. melanogaster*, *SPR* gene is located on the X chromosome and is broadly expressed in the female genital tract tissues and the nervous system (Yapici et al. 2008, Kubli 2008). SP passes from the reproductive tract into the haemolymph (Pilpel *et al.* 2008) and ultimately targets the nervous system of the female, specifically a small subset of internal sensory neurons that innervate female uterus and oviduct and project to the central nervous system (CNS) (Yapici *et al.* 2008, Häsemeyer *et al.* 2009, Yang *et al.* 2009, Rezaval *et al.* 2012, Haussmann et al. 2013).

Thus, apart from the nervous system targets expressing *SPR*, the endocrine organ corpus allatum is suggested to be an additional target with unidentified receptors for SP (Moshitzky *et al.* 1996, Soller *et al.* 1999). The amino-terminal of SP protein, which binds to sperm in ejaculates transferred by males, can activate corpus allatum stimulating juvenile hormone III-bisepoxide (JHB_3) biosynthesis in females (Moshitzky *et al.* 1996). JHB_3 induces vitellogenesis and progression of oocytes in the ovary in sexually mature females (Soller *et al.* 1997, Soller *et al.* 1999). Although JHB_3 is not involved in the regulation of oviposition or receptivity (Soller *et al.* 1999), at least a part of SP's post-mating response of increased fecundity, i.e. oogenesis, is modulated through JHB_3 synthesis.

Sex peptide is one of the male seminal proteins involved in interlocus sexual conflict (IRSC) in *D. melanogaster* (Arnqvist and Rowe 2005, Wigby and Chapman 2005).

This could potentially lead to sexually antagonistic coevolution (Parker 1979, Holland and Rice 1998), where females would counter-adapt to the male-derived harmful post-mating responses, e.g. by modulating receptors for the sexually antagonistic SP (Poels *et al.* 2010). The dynamics of sexual conflict are suggested to be cyclical, with male adaptations to manipulate female physiology followed by female counter-adaptations (Rice 1996). Such adaptations and counter-adaptations are likely to be costly for males and females to maintain and thus impose fitness load on the population (Rice 1992, Holland and Rice 1999). Insights into the nature of such costs, and hence dynamics of sexual conflict, may be gained by manipulating genetic pathways known to be involved in sexual conflict. In the present study, the sexual conflict in *D. melanogaster* mediated by the interaction between SP and SPR was employed to investigate evolutionary response of males evolving with genetically engineered females devoid of SPR, thus preventing females from responding to male SP. The response of males would depend upon the cost-benefit ratio of expressing SP in absence of SPR.

Assuming that post-mating responses would not be initiated by C-terminal region in absence of SPR, males coevolving with receptorless females may evolve lower *SP* expression, considering that it would be costly owing to energy costs of gene expression (Stoebel *et al.* 2009, Lang *et al.* 2009, Novick and Weiner 1957; Andrews and Hegeman 1976; Dykhuizen and Davies 1980; Koch 1983). On the other hand, function of the N-terminal domain of SP may be sufficient to prevent a decrease in its expression if the fitness benefits imparted to males through JHB_3 synthesis in females are higher than the expression costs. Expression of *Dup99B* was also investigated: if Dup99B acts solely by binding to SPR, a decline in expression levels would be expected, but if alternative pathways are used, no change in expression levels or a compensatory increase would be

expected. To this aim, we investigated the expression of both *SP* and *Dup99B* genes using RT-qPCR.

1.2 Materials and methods

1.2.1 Experimental populations

The experimental and control populations of *D. melanogaster*, with 4 replicate lines per treatment, were derived at the University of Oxford (see Table 1.1 for summary). The control W population was an outbred, lab-adapted population ($white^{Dahomey}$) wild-type except for a white mutation (w^{1118}) which gives the flies white eyes. On the other hand, the experimental S population flies lacked expression of *SPR* due to a deletion mutation and possessed red eyes due to a transgene that served as a marker for deletion.

The control W population was generated by backcrossing a loss of function allele w^{1118} for the X-linked *white* gene (located on the X chromosome) into a wild-type Dahomey background (Broughton 2005), leading to a white eye colour. The experimental S population was derived by backcrossing the deletion mutation *Df(1)Exel6234* (Yapici *et al.* 2008, Dean *et al.* 2012), which covers the entire X-linked *SPR* gene, into the control W population for 5 generations. The mutation also carries a $white^{+}$ transgene, which provides a partial rescue of w^{1118} mutation. Thus, in a w^{1118} background, male hemizygote and female homozygote carriers of *Df(1)Exel6234* possess red eyes, whilst heterozygote females possess orange eyes, which facilitate tracking the SPR deficiency. Four replicates each were set up for the experimental (S1-S4) and control (W1-W4) populations. (See Dean *et al.* 2012 for details on experimental and control populations).

The lines were maintained for 25 generations as follows. Each generation began with 100 adult males and 100 adult females, which were allowed to interact in 4.5 L cages for 9 days. Each chamber contained three bottles of food media (50 mL) with granules of live yeast on the surface. Bottles were replaced on days 4 and 7. Females were then allowed to oviposit onto petri dishes containing an agar-grape juice medium, from which

first instar larvae were transferred to vials containing standard food, at a density of 100 larvae/7.5 mL food. Adults were collected from these vials to start the next generation. From generations 25-45, the populations were maintained by transferring adults to new food every two weeks at an uncontrolled sex ratio and density. From generations 0-45, flies were maintained on sugar-yeast-molasses medium in plastic bottles or vials at 25°C on a 12:12 h light: dark cycle.

Table 1.1: Summary description of the experimental populations including their expression (or lack of expression) of sex peptide receptor (SPR)

Population	***Treatment***	***SPR expression***
W	Coevolution between males and females bearing wild-type *SPR* and the w^{1118} allele	*SPR* expressed
S	Coevolution between males and females bearing the *SPR* deficiency and the w^{+} transgene	No *SPR* expressed

After generation 45, the populations were maintained at University of Exeter as follows. To avoid high larval density, each replicate line of S and W populations was divided among 10 vials with food medium, with 3 males and 3 females per vial. Virgin progeny were collected over 2 days using CO2 anaesthesia and housed in sex-specific vials. For each replicate line, progeny from all 10 vials were pooled and 30 males and 30 females were used to start the next generation. Three virgin males and three virgin females were housed per vial for a two-day oviposition period, after which they were removed and the offspring were allowed to develop for 10-11 days. From generation 46-55, flies were maintained on Jazz-mix food medium (Fisher Scientific) in plastic vials at 25°C on a 12:12 h light: dark cycle.

1.2.2 Gene expression of sex peptides

To prepare the flies for measurement of *SP* and *Dup99B* gene expression, all replicate lines were standardized for larval density at generation 55. For each replicate line, we paired 3-4 day old virgin males and females, with one pair per vial in each of 15 vials, and allowed them to interact for 24 hours. Males were then removed and females were permitted to oviposit on agar-apple juice plates. To standardize larval density, eggs were transferred to vials containing food medium (40 eggs per vial, 5 vials per replicate line). Upon emergence, adult flies were collected and separated by sex under CO_2 anesthesia at 8 hour intervals to ensure virginity. Males were housed individually for 5 days.

Six-day old virgin males from each replicate line were dissected and their abdomens stored individually in RNA*later* reagent (Sigma-Aldrich) at 4°C for 24 hours and then at -80°C for approximately 30 days. For RT-qPCR, four samples from each replicate line were used (total number of samples = 32).

To prepare samples for RNA extraction, abdominal tissue from virgin males was frozen using liquid nitrogen and homogenized using microcentrifuge pestles. Total RNA was extracted using a Purelink RNA mini kit (Ambion) using ethanol and 2-mercaptoethanol (Ambion) in addition to the kit reagents. On-column DNase treatment was used (Purelink DNase I) during the extraction. RNA was eluted with RNA storage solution (Ambion) and stored at -80° C. The RNA yield (quantified with Qubit 2.0 Fluorometer, Invitrogen) ranged between 3-6 µg/ml.

RT-qPCR assays were set up manually using Brilliant III Ultra-Fast SYBR Green QRT-PCR Master Mix (Stratagene, Agilent Technologies) on an Applied Biosystems 7500 Fast Real-Time PCR system. Amplification reactions were performed in 20 µl total volume with 2 µl of RNA and 0.1 µM of each primer, in 96-well optical plates (MicroAmp,

Applied Biosystems, #N8010560) sealed with Optical 8-Cap Strips (MicroAmp, Applied Biosystems, #4323032) under the following sequential conditions: incubation at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

For the two target genes (*SP and Dup99B*) and the reference gene (*RpL32*), 4 biological replicates per line (i.e. 4 individuals) and 3 technical replicates per sample were used. Relative standard curves for the gene transcripts were generated with serial dilutions of RNA (i.e., 1, 1/5, 1/25, 1/125, 1/625). Stock RNA used for the relative standard curves was extracted from whole males pooled from the four replicate W (control) lines. For the calibrator sample, RNA from abdominal tissues of 15 males each from the four replicate W (control) lines was pooled and diluted 10 times. Triplicate reactions for calibrator, no-template control (NTC) and no-reverse transcriptase (no-RT) control were used on each PCR plate.

Primers for *SP*, *Dup99B* and *RpL32*, were designed using NCBI Primer-BLAST (Supplemental table 1.5.1) and manufactured by Genomed, Poland. The primers spanned exon–intron boundaries (i.e., each primer had sequences from two exons).

Raw data were obtained from the Sequence Detection Systems Software v1.3 as mean values and standard deviations across technical replicates of the target and reference genes. Raw data were normalized using the relative standard curve method (Guide to performing relative quantitation of gene expression using real-time quantitative PCR, Applied Biosystems). For each biological replicate and for the calibrator sample on each plate, the mean quantity of *SP* or *Dup99B* was normalized to the mean quantity of *RpL32*). The fold difference between a treatment sample and the calibrator was calculated as the normalized value of the sample divided by the normalized value of the calibrator.

1.2.3 Statistical analysis

To examine the effects of experimental evolution treatment on expression levels of *SP* and *Dup99B* in males from S lines and W lines, mixed-effect models were fitted using the lme4 package (Bates *et al.* 2014) and lmerTest package (Kuznetsova *et al.* 2014) with R version 3.1.2 (R core team 2014). The mean fold difference values for a line were log-transformed to meet the assumptions of a linear model. Experimental evolution treatment was included as a fixed factor and replicate line as a random factor nested within treatment. For SP, inclusion of replicate line was considered unwarranted by the lmer model (variance=0, Bates 2010).

1.3 Results

It was found that *SP* expression in experimentally evolved S males was significantly higher compared to control W males ($F_{1, 30} = 4.2$, $p = 0.048$) (Figure 1.1).

In contrast, there was no significant difference in Dup99B expression (F1, 6 = 0.5, p = 0.49) (Figure 1.2). The characteristics of calibration curves for RT-qPCR are given in supplemental table 1.5.2).

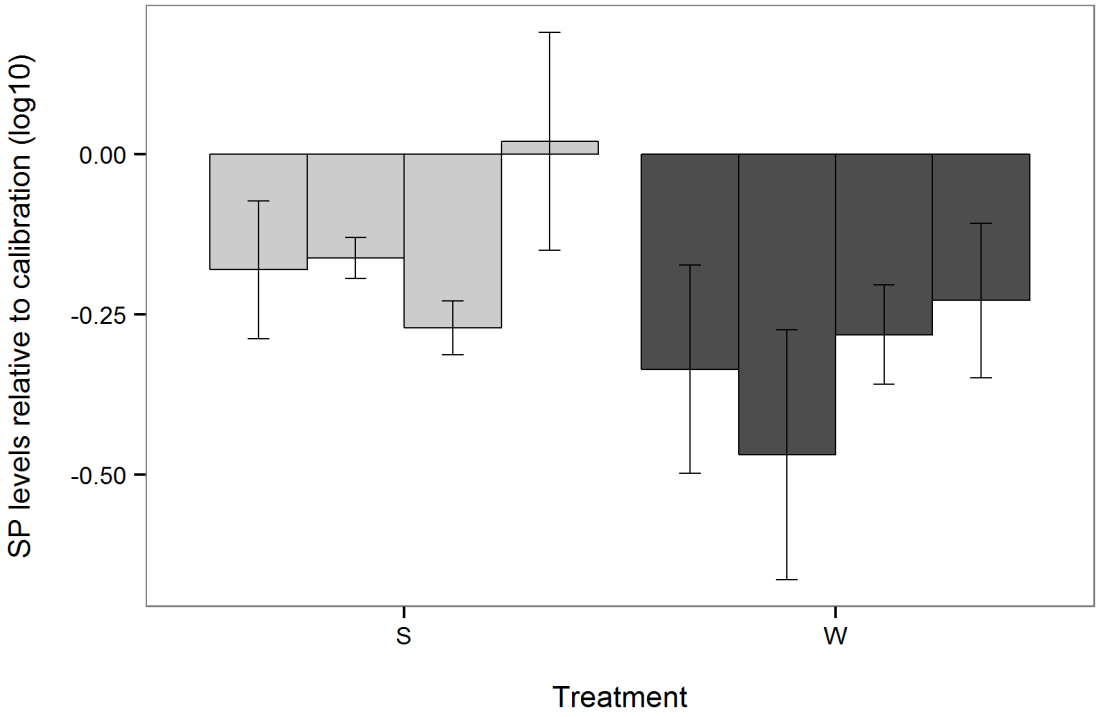

Figure 1.1: Mean levels of expression of SP in four S lines (coevolving with *SPR*- females) and four W (control) lines ($F_{1, 30} = 4.2324$, $p = 0.0484$). Error bars represent +/- 2SE.

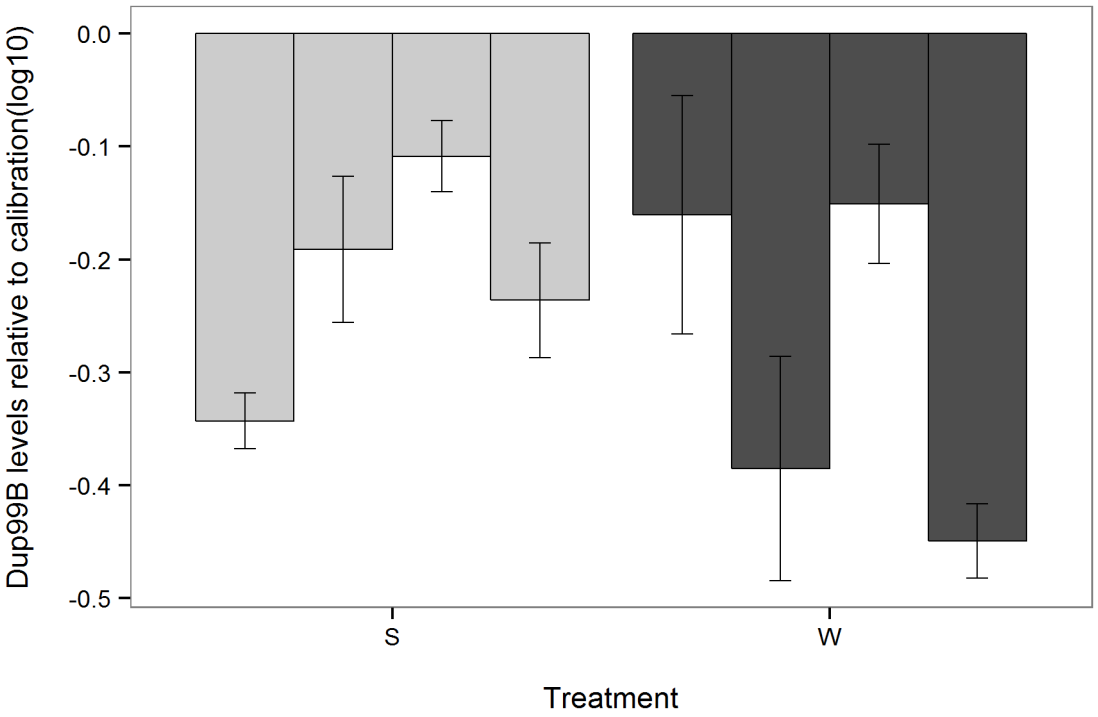

Figure 1.2: Mean levels of expression of Dup99B in four S lines (coevolving with *SPR*- females) and four W (control) lines ($F_{1,6}$ = 0.53927, p = 0.4904). Error bars represent +/- 2SE.

1.4 Discussion

It was predicted that *SP* gene expression in males coevolving with females devoid of *SPR* (S lines) would either be lowered or be unchanged, depending on the benefit-cost ratio of expressing SP in absence of SPR. This was not what was observed, but there are several potential explanations for why *SP* expression may have increased in these males.

The increased *SP* expression in S males may be explained owing to other pathways, in addition to SPR, via which SPs can influence female physiology, e.g. the yet unidentified receptors in corpus allatum that bind the amino-terminal domain of SP protein. Positive selection on the amino-terminal end of SP might arise to compensate for the lack of oviposition stimulation from the carboxy-terminal end of SP in S populations, because the amino-terminal end induces juvenile hormone (JH) synthesis in adult females and stimulates oogenesis (Moshitzky et al. 1996, Soller *et al.* 1997, Soller *et al.* 1999), see also Bontonou et al. 2015). This hypothesis is consistent with the lack of increase in Dup99B expression, as Dup99B is homologous with SP only at the carboxy-terminal end. However, JHB_3 synthesis in corpus allatum induced by SP influences only oogenesis and does not induce mating latency (Soller *et al.* 1997, Soller *et al.* 1999). Interestingly, a recent study showed that the amino-terminal of SP downregulates pheromone and hydrocarbon (CHC) production in females through JH synthesis, although it is not known whether this affects the mating latency of females (Bontonou *et al.* 2015).

Another study has recently suggested that SPR might not be the only receptor mediating the sex peptide response in females and multiple pathways might exist through which SP can trigger the postmating behavioural switch (Haussmann *et al.* 2013). The study demonstrated that SP can trigger the postmating responses in females in absence of SPR, but only when ectopically expressed in neurons, and not when introduced to female genital tract during mating. However, higher amounts of SP and longer time are needed to

initiate the postmating behavior via the alternative pathways (Haussmann *et al.* 2013). This would explain why males coevolving with females devoid of SPR (i.e. S line males) evolved increased SP levels when compared to males from W lines, contrary to expectations. The upregulation of such a pathway may work as compensatory means for decreased post-mating response in females that lacked SPR. On the other hand, there was no significant difference between Dup99B expression levels in S lines and control W lines. This suggests that loss of (or decline in) SP functionality due to deletion of SPR in females is not compensated for by elevating expression of Dup99B. It is known that Dup99B can bind SPR *in vitro* (Ottiger 2000) but it is unclear if a specific receptor for Dup99B exists. If Dup99B acts solely through SPR, no compensatory pathway might have been available for this seminal peptide in the absence of SPR.

Alternatively, *SP* gene expression as measured in males might not represent the amount of SP transferred to females in ejaculate during mating. Indeed, a recent study found no correlation between the two (Smith *et al.* 2012). Increased *SP* expression in males may instead represent increased amount of male SP stores, and might indicate that these males have increased capacity to mate many times, even if SP protein transfer at each mating is constant or decreases. However, there is no evidence for this, as the size of accessory glands, capacity to sustain ejaculate transfer over multiple matings, or sperm defense did not differ between S and W males (Perry *et al.*, in prep.).

Another possibility is that the gene expression levels may not correlate with the protein abundance of SP protein (Vogel *et al.* 2010, Schwanhäusser *et al.* 2011). If the process of translation of mRNA to protein is costlier than gene expression, selection may not act on gene expression, but instead protein abundance may be lowered. This was not investigated in the present study; however, it may explain the finding of decreased mating

latency in wild-type females mated with S males compared to those mated with W males (Perry *et al.*, in prep.).

Although the results did not support predictions for *SP* and *Dup99B* expression in absence of SPR based on cost of gene expression, it may be worth investigating the difference between SP protein abundance in S and W males in addition to expression levels. Future research should also investigate potential benefits to males of increased SP expression when the females lack SPR. The results indicate that components of male genomes respond fast to mutations in females that affect the intersexual arms race and also reiterate the possibility that SPR may not be the sole receptor for SP and the antagonistic postmating effects of SP may be brought about through alternate pathways in the nervous system and/or endocrine system, which warrants further research. Overall, the research highlights the use of genetic manipulation and experimental evolution in the study of seminal proteins mediating sexual conflict in *D. melanogaster*.

1.5 Supplementary information

Supplemental table 1.5.1: Primer characteristics for the target genes and the reference gene

Primer Characteristics	***SP(Acp70A)***	***Dup99B***	***RpL32***
NCBI accession for mRNA	NM_079333.2	NM_206582.2	NM_079843.4, NM_170461.3, NM_170460.2, NM_001144655.3
Forward primer sequence	TGGGAATGGC CGTGGAATAG	CAGAAGGATCGT GAGAAGTGGTGC	TGCTAAGCTGT CGCACAAATG G
Reverse primer sequence	CGGCACCACT TATCACGAGG	TTCGGCATCTGCC ACCGAGGTA	TGCGCTTGTTC GATCCGTAAC
Location of primer	Reverse primer spans two exons	Forward primer spans two exons	Forward primer spans two exons
Primer Specificity screen (BLAST)	Not highly specific to the input template Larger product (681 bp) on potentially unintended template.	Specific to the input template	Specific to the input template.
Amplicon length	74 bp	64 bp	113 bp
Splice variants targeted	No splice variants	Of the 2 splice variants, the primers targeted transcript variant A.	all four splice variants A, B, C and D

Supplemental table 1.5.2: Characteristics of calibration curves for SP, Dup99B and RpL32 (Mean across 8 plates)

Characteristics	***SP (Acp70A)***	***Dup99B***	***RpL32***
Mean slope	-3.064	-3.21	-3.51
Mean y-intercept	54.83	61.25	66.14
Mean efficiency %	112.05	105	92.84
Mean r^2	0.983	0.987	0.98

Chapter 2

Effect of experimental evolution of *D. simulans* males under altered mating system and temperature on harm to females

Abstract

Interlocus sexual conflict (IRSC) often results in higher fitness in one sex at the cost of the fitness of the opposite sex. Male induced harm to females as a result of IRSC can be investigated using experimental evolution selecting for increased/decreased male sexual competitiveness. We utilized experimental lines which evolved under altered mating systems (increased/decreased polyandry) and standard or increased temperature in a fully factorial design. Previous work showed that evolution under both elevated polyandry and increased temperature resulted in increased male sexual competitiveness. Here it was tested whether evolution of increased competitiveness would in turn lead to increased mating costs to females in terms of decreased longevity and/or productivity. Neither the mating system nor the evolution temperature of males showed any effect on the longevity of females. However, there was significant interaction between male mating system and evolution temperature in their effect on female productivity: females mated with males evolving under polyandry at elevated temperature showed the highest productivity. Thus, contrary to predictions of IRSC, the results of this study demonstrated that mating with males that evolved higher reproductive fitness is advantageous for females.

2.1 Introduction

Sexual reproduction is far from being a cooperative harmonious venture between males and females; rather, a conflict between sexes often occurs as they pursue divergent evolutionary interests in order to maximize their respective reproductive output (Trivers 1972, Dawkins 1976, Parker 1979). Such conflicts may occur over the outcome of male-female interactions such as mating frequency, relative parental investment, fertilization and female remating rate, leading to different optimal outcomes for the two sexes (Arnqvist and Rowe 2005). For example, when a female remates, it leads to sperm competition and possible loss of paternity for the male that previously mated with her, leading to sexual conflict over the female remating rate. In order to prevent the female from mating again, males evolve some traits that eventually increase their own fitness but negatively impact the female fitness (Parker 1979, Rice 1996). In turn, the females may evolve counter-adaptations in order to reduce the costs imposed by the harmful male traits, thus leading to sexually antagonistic coevolution between males and females (Holland and Rice 1998). Because in the course of such antagonistic coevolution, male and female adaptations occur due to alleles at different loci, this process is called interlocus sexual conflict (IRSC), to differentiate it from intralocus conflict over an optimal value of a trait coexpressed in both sexes (Rice and Chippindale 2001).

IRSC can lead to increased fitness in one sex, typically the male, and lowered fitness in the other (Rice and Holland 1997). For example, spiked genitalia of male *Callosobruchus maculatus* puncture the female genitalia, and females prevented from terminating copulation suffer more damage (Crudgington and Siva-Jothy 2000). Male bed bugs possess specialized genitalia to inseminate females by piercing body wall of females, which leads to reduced longevity and reproductive success in females (Stutt and Siva-Jothy 2001). In dung flies *Sepsis cynipsea*, where male intromittent organ damages the female

reproductive tract, copulation negatively impacts female lifespan (Blanckenhorn *et al.* 2002) and female reproductive success (Martin *et al.* 2003). Male induced harm via 'toxic' seminal proteins has been demonstrated in beetles (Gems and Riddle, 1996), *C. elegans* (Das *et al.* 1980) and *D. melanogaster* (Fowler and Partridge 1989, Chapman *et al.* 1995, Wigby and Chapman 2005). In *D. melanogaster*, multiple mating can lead to lowered lifetime reproductive success (LRS) and reduced lifespan (Fowler and Partridge 1989), which was shown to be mediated by the seminal proteins such as Acp70A (sex peptide) and Acp62F (Chapman *et al.* 1995, Wigby and Chapman 2005, Lung *et al.* 2002). Additionally, it has been shown that when *D. melanogaster* females were prevented from coevolving with males, male fitness increased at the cost of female fitness (Rice 1996).

Nevertheless, there are other studies that demonstrated no longevity cost of mating in females of several insect species (reviewed in Chapman *et al.* 1998, Kotiaho and Simmons 2003). This may be because sexual conflict is typically difficult to detect, since adaptation in one sex will often be balanced by a counter-adaptation in another sex and thus the outcome of such antagonistic interactions remains unchanged (Rice 1996, Chapman and Partridge 1996, Rice 2000, Arnqvist and Rowe 2002). However, alterations in the equilibrium of adaptations and counter-adaptations may increase the power for sexual conflict to be detected, for example by comparing naturally promiscuous populations evolving under altered mating systems of increased/decreased polyandry. For example, monogamy enforced on naturally promiscuous mating system eliminates the opportunity for both precopulatory (direct male-male competition and female choice) as well as postcopulatory (sperm competition and cryptic female choice) sexual selection. As a result, the evolutionary interests of males and females are expected to become congruent and thus selection may result in reduced harmfulness of males evolving under enforced monogamy as compared to those evolving under polyandry (Holland and Rice 1999,

Pitnick et al. 2001, Martin and Hosken 2003; but see Holland 2002, Wigby and Chapman 2004). In contrast, enforcing elevated polyandry leads to increased competition between males and increases probability of female remating, and thus should select for any traits in males which increase their reproductive fitness, even if such adaptations is costly to females. For example, in *D. pseudoobscura*, elimination and elevation of sexual selection by manipulating adult sex ratio led to variation in the males' ability to suppress female remating (Crudgington *et al.* 2005).

In this study, it was tested whether evolution of increased male competitiveness under altered mating systems and temperatures would in turn lead to increased mating costs to females in terms of decreased longevity and/or productivity in *Drosophila simulans.* In *D. simulans*, a sister species of *D. melanogaster*, no unambiguous evidence for the cost of mating in females has been found. While multiple mating led to an increased LRS, longevity costs were seen in females continually housed with males, but not in females intermittently exposed to males or once mated females housed with virgin females (Taylor *et al.* 2008b). However, a comparison of females mated once, twice or thrice showed that residual longevity (in days since the first mating) was unaffected by number of matings (Taylor *et al*. 2008b). Replicate *D. simulans* populations were experimentally evolved either under monogamy, which should select for decreased male harm, or under elevated polyandry at a highly male-biased sex ratio, which should have the opposite effect. Previous work with these populations (Duffy *et al*., in prep.) showed that after 55 generations of experimental evolution, in a competitive fitness assay with test males, males evolving under elevated polyandry were better competitors than males evolving under monogamy. In these experiments, for the purpose of testing alignment of sexual selection with natural selection, the effect of evolution temperature on male sexual fitness was also tested, by evolving replicates of monogamy and polyandry populations at either an

ancestral temperature (25°C) or a novel temperature (27°C). It was seen that males evolving at an elevated temperature of 27°C performed better in the competitive fitness assay than those evolving at 25°C (Duffy *et al*., in prep.). In the present study, it was investigated whether this increased competitiveness resulted in increased male harm to females. To avoid confounding effects of female evolution under these conditions, non-coevolved test females from a base population were used. It was tested whether females that mated with males evolved under elevated polyandry and higher temperature showed increased cost of mating in terms of longevity and productivity than those mated with males from monogamy lines and lower temperature.

2.2 Materials and methods

2.2.1 Base population and selection lines

Focal males used for the study came from replicate selection populations of *D. simulans*, evolved at ancestral or elevated temperatures under either monogamous or polyandrous mating system. The test females used for the experiment were taken from the base population, from which the selection lines were derived.

The base population was established using twenty isofemale lines, collected from the wild in 2004, and obtained from the Centre of Environmental Stress and Adaptation Research, La Trobe University, Australia. These isofemale lines were used to establish several laboratory stock populations at CLES, university of Exeter, UK. As the intracellular parasite *Wolbachia* can adversely affect fitness in D. simulans, for example reduced fecundity (Hoffmann *et al.*, 1990), decreased male fertility (Snook *et al.*, 2000) and reduced sperm competitiveness (Champion de Crespigny & Wedell, 2006), to avoid any possible confounding fitness effects, a *Wolbachia* free base population was established. Females from three laboratory stock populations were cured of *Wolbachia* using a wide-spectrum antibiotic tetracycline hydrochloride (Hoffman et al., 1986) and the offspring from these *Wolbachia* cured females were pooled together to establish the base population. The flies were housed in a large population cage (ca. 800-1000 flies) with overlapping generations and free mate choice and maintained at 25°C and 12:12 hour light: dark cycle, on oatmeal based food medium (10 g agar, 85 g sugar, 60 g oats, 20 g yeast, 1.67 g Methyl parahydroxy benzoate (antifungal), 0.625 g Benzoic acid (antibacterial), 5.8 ml Propionic acid (antibacterial), 1000 ml deionized water).

Selection lines utilized in the study to examine the effect of evolution temperature and mating system on harm to females had been derived from the base population and

experimentally evolved for more than 65 generations, and were originally established for an experiment to test effect of sexual selection and natural selection on male and female fitness (Duffy *et al.* in prep.). The selection lines were maintained using a fully factorial design of ancestral (25° C) or novel (27° C) environment with monogamous (-SS, no sexual selection) or polyandrous (+SS, with sexual selection) mating system. The 16 selection lines (four replicate populations for each treatment combination) were maintained on oatmeal based food medium described above, at their respective evolution temperature (25° C or 27° C) and 12:12 hour light: dark cycle.

2.2.2 Selection protocol

The opportunity for sexual selection was eliminated with enforced monogamy (-SS) and elevated with polyandry (+SS) (Crudgington *et al.*, 2005). For lines selected for polyandrous mating system (+SS), four males were housed with a single female per vial (50ml; 32 x 68mm). Although naturally promiscuous, *D. simulans* females are likely to remate a maximum of two times, despite repeated opportunities for more (Taylor *et al.*, 2008), therefore a male-biased sex ratio of 4 males per female in the +SS lines, should present conditions of elevated polyandry for females of this species, increasing the opportunity for both precopulatory and postcopulatory sexual selection. On the other hand, a female was housed with a single male per vial in the lines selected for monogamous mating system (-SS), which eliminated the opportunity for precopulatory and postcopulatory sexual selection (Andersson 1994, Holland and Rice 1999, Pitnick *et al.* 2001). Every generation, the +SS lines utilized 60 vials/population (240 males and 60 females per population), whereas the –SS lines consisted of 64 vials/population with a male-female pair each (64 males and 64 females per population). The difference in fly numbers approximately equalized effective population size (N_e) between the +SS and –SS mating regimes (Sharma *et al.* 2012). The protocol that was followed for over 65

generations of experimental evolution (Figure 2.1) is described below: Males and females from a particular line were housed together (following their mating regime) in interaction vials for 6 days, after which males were discarded, and for every replicate line, females were transferred to 4 tubs (1000 ml; 100 x 135mm) with excess food media. Females were removed after 48 hours of oviposition, and after 9-10 days of development, virgin offspring were collected over 2 days using light CO_2 anesthesia. Offspring were separated by sex using light microscopy and housed in sex specific vials before being used to establish the next generation of experimental evolution.

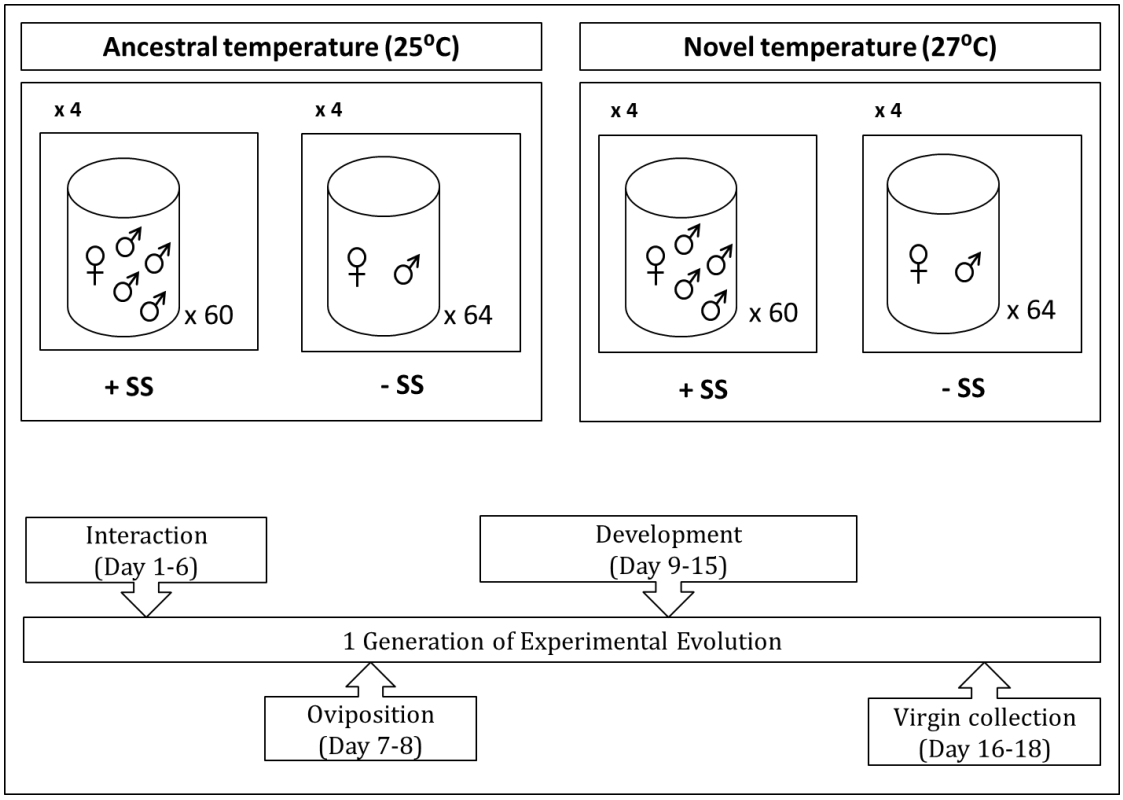

Figure 2.1: The design and protocol for experimental evolution: Four replicate lines each were established under conditions of either enforced monogamy (-SS) or polyandry (+SS) at ancestral temperature (25^0C) and novel temperature (27^0C). The lower section of the figure depicts a single generation of experimental selection. At the start of every generation, virgin males and females from +SS and –SS lines were housed in individual vials (according to their respective mating regime) and allowed to interact for six days after which females were transferred to new vials and allowed to oviposit for 48 hours. After 48 hours, they were removed and offspring were allowed to develop for 9-10 days. Virgin offspring collected on days 16, 17 and 18 were then used to start the next generation.

2.2.3 Longevity and productivity assay

To investigate whether increased male competitiveness under increased SS and elevated temperature is associated with the harm inflicted on females as a result of mating, two measures of female fitness were used, longevity and productivity. Female fitness assays were performed at 25°C, which is the temperature to which test females were adapted. The competitive fitness of males evolving at novel temperature (27°C) was higher than that of males evolving at ancestral temperature (25°C) and this difference was greater at test temperature of 25°C than at 27°C (Duffy *et al.* in prep.). This ensured that a test temperature of 25°C for the longevity and productivity assay gave no advantage to males evolved at this temperature.

Before the assay, all experimental populations were subjected to standardizing conditions for a single generation to eliminate any environmental and maternal effects. During the standardizing generation, all populations were housed at an intermediate temperature of 26℃ and an intermediate male to female ratio (2 males per female) following the same experimental protocol as above (Figure 2.1). Following this generation, emerging virgin adults were collected every 12 hours, separated and housed by sex with an excess of culture medium for 4 days before experiment.

At the same time as the standardizing generation for the selection lines, vials with excess food medium were placed in the base population cage for 48 hours to collect virgin test females. After 9-10 days of development, virgin females were collected from these vials every 12 hours and housed in groups of no more than 10 in vials with food ad libitum.

All males and females used were 4 day old at the time of the assay to ensure sexual maturity. To measure the longevity and productivity of test females, virgin males from each replicate population were paired with virgin test females (15 pairs per replicate

population) and housed together for 7 days, which emulates the interaction period in the selection protocol used for experimental evolution (Figure 1). Pairs of males and females were aspirated into the vials (45 ml; 35mm x 95mm) with excess food medium. After 3 days, the pairs were transferred to vials with fresh food medium, 4 days after which the males were discarded and females were transferred to new set of vials. The females were then transferred to fresh laying vials after 3 days, after 4 days and then every 5 days until vials ceased to contain any developing larvae (Figure 2.2). Females were checked daily for death and female longevity was recorded in days since eclosion. 5 females that escaped during the course of experiment were excluded from the longevity analysis. Many of the food vials exhibited a layer of slime, which could affect the longevity of females so it was accounted for in the analysis of longevity data.

The number of eclosed flies in a vial was scored seven days after the first eclosion was recorded. This ensured that almost all progeny are accounted for without including any grandchildren (Taylor *et al.* 2008a, Sharma *et al.* 2010). Female productivity was recorded for a period of 19 days (i.e. progeny count was summed for all the vials in this period in which a particular female laid eggs). Data from 5 females that escaped during the course of experiment and 34 females (out of 235) that had slime in the vials during this period was excluded from productivity analysis.

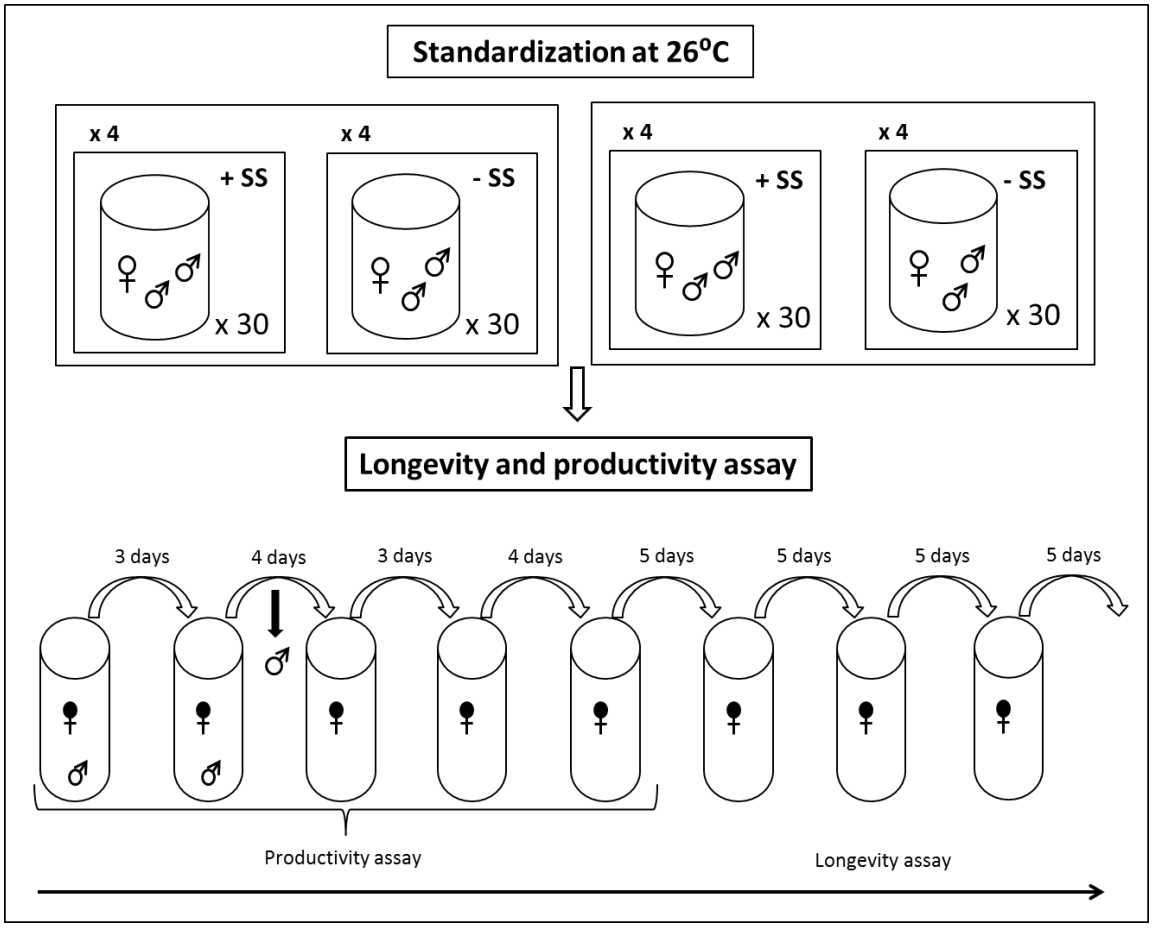

Figure 2.2: Standardization followed by Longevity and Productivity assay: During the standardizing generation, all populations were housed at an intermediate temperature of 26℃ and an intermediate male to female ratio (2 males per female). For the productivity assay, test females (black filled symbols) were housed with males from selection lines for 7 days, after which males were discarded and test females were moved to new laying vials after every few days. Productivity equaled summed offspring count over the first 19 days. Longevity assay continued until the last test female in the assay died.

2.2.4 Statistical analysis

Since the response variables Longevity and Productivity are count data and did not show a normal distribution, generalized linear mixed modeling (glmm) in R was used (version 3.1.3, R Core Team, 2015). To account for overdispersion in the data, glmmadmb function from the package 'glmmADMB' (version 0.8.1, Skaug *et al.* 2006, Bolker *et al.* 2012) was used with negative binomial distribution (Bolker *et al.* 2010, Zuur *et al.* 2009).

To analyze the effect of mating system (-SS or +SS) and evolution temperature (25℃ or 27℃) of males on the longevity of test females, a two way interaction of 'Mating system' and 'Temperature' was included and 'Day of Slime onset' was also included as a fixed covariate to account for the occurrence of slime in food vials. Line was included as a random factor nested in both Mating system and Temperature. Interaction terms were removed when non-significant ($p > 0.1$).

To analyze how productivity of test females was affected, an interaction term of 'Mating system' and 'Temperature' and a covariate 'Longevity' were included. Line was included as a random factor nested in both Mating system and Temperature.

2.3 Results

Interaction term of Mating system and temperature was removed from the longevity model, being non-significant (p >0.1). In the reduced model, the main effects mating system or evolution temperature did not show any significant effect on longevity of females (Figure 2.3, Table 2.1). In spite of presence of slime in many laying vials, the day of slime onset did not affect female longevity significantly (Table 2.1).

Table 2.1: The glmmADMB reduced model testing effects of mating system and evolution at elevated temperature on female longevity.

Source	***Z***	***P***
Mating system	-0.77	0.442
Evolution temperature	1.01	0.311
Day of slime onset	-1.77	0.076

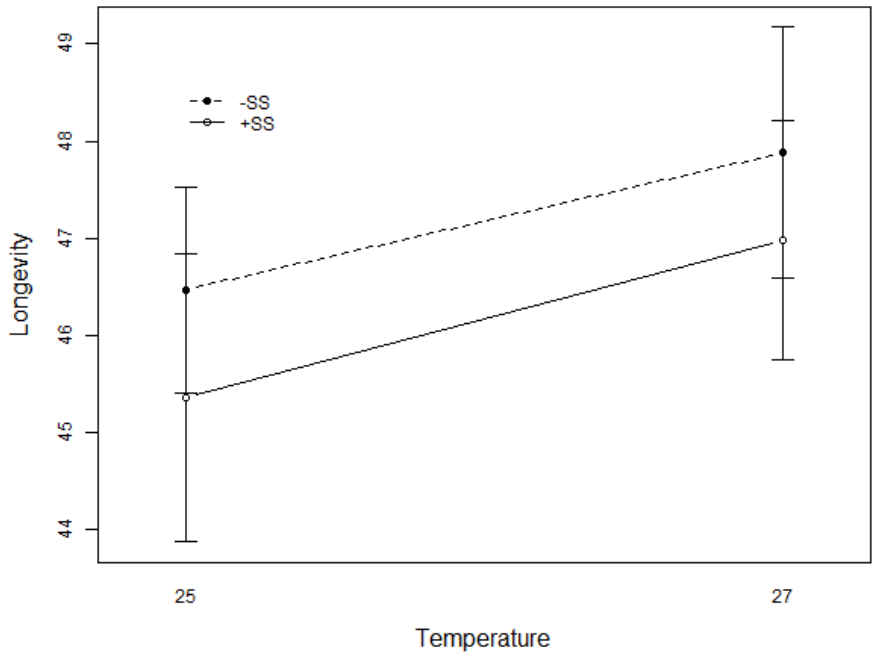

Figure 2.3: Mean longevity, measured in days since eclosion, of test females mated with males which have evolved under two mating regimes (+SS, -SS) at the ancestral (25^0C) and novel temperatures (27^0C). Dashed line depicts -SS lines and solid line depicts +SS lines ± s.e.

There was a significant effect of interaction between mating system and rearing temperature (Figure 2.4, Table 2.2). Test females mated with males from +SS lines evolved at 27^0C showed higher productivity, which resulted in a significant mating system by evolution temperature interaction. Longevity also had a significant effect on the test female productivity, as could be expected.

Table 2.2: The glmmADMB model testing effects of mating system, evolution at elevated temperature and longevity on female productivity. * indicates significant P values.

Source	***Z***	***P***
Mating system	-0.80	0.423
Evolution temperature	-0.47	0.637
Longevity	4.29	< 0.001*
Mating system x Evolution temperature	2.29	0.022*

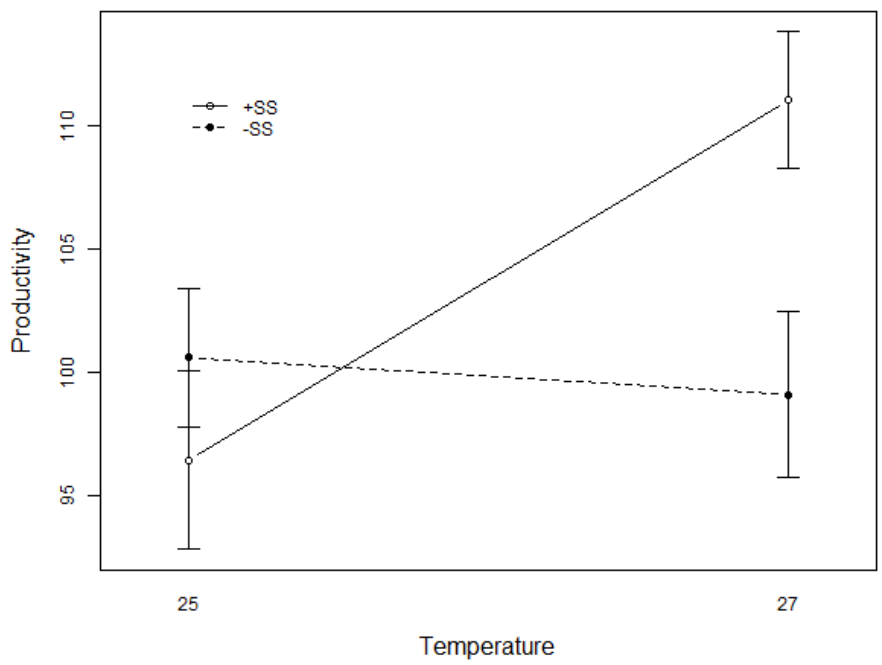

Figure 2.4: Mean productivity of test females mated with males evolving under two mating regimes (+SS, -SS) at the ancestral (25^0C) and novel temperatures (27^0C). Dashed line depicts -SS lines and solid line depicts +SS lines ± s.e.

2.4 Discussion

The study tested whether increased male sexual competitiveness is associated with increased harm inflicted on females. Contrary to predictions derived from sexual conflict theory, there was no difference in the longevity of females mated with less competitive males (evolved under monogamy or ancestral temperature) and more competitive ones (evolved under elevated polyandry or increased temperature). These results in *D. simulans* differ from previous studies in *D. melanogaster* that demonstrated that male induced harm (in terms of female longevity) was reduced when males were reared at high temperature (Cohet and David 1976).

However, experimental evolution of males did affect productivity of females as the females mated with males evolving under polyandry at elevated temperature showed the highest productivity. Increased female productivity is one of the expected outcomes of IRSC, as males mating with the female are selected to maximize current female reproduction (and thus their reproductive success) even at the cost of her reproductive lifespan (because they will most likely not be related to future female progeny) (reviewed in Arnqvist and Nilsson 2000). Previous studies in *D. subobscura* (Maynard Smith 1958), *D. melanogaster* (Partridge *et al.* 1987) and Mediterranean fruitfly *Ceratitis capitata* (Chapman *et al.* 1998) have shown that egg production imposes a cost on female longevity independently of other costs. However, the results from the present study showed that in spite of increased productivity in females mated with males from +SS lines at 27° C, longevity of these females did not differ from other females with lower productivity. Thus, the results are not consistent with IRSC as an explanation for increased female productivity.

Previous studies in *D. simulans* (Taylor *et al.* 2008b) also did not provide explicit evidence for IRSC in this species. Multiple mating led to reduced longevity in female *D.*

simulans when females were continually housed with males, but not when intermittently exposed to males, suggesting that in this species, the longevity costs may be due to male courtship and harassment, rather than to mating *per se*. (Taylor *et al.* 2008b). In the present study, females were housed with only a single male during the assay but had opportunity to remate during the 7 day period. The males evolving under elevated polyandry might have evolved a higher courtship rate, and thus a higher degree of harassment, than the males evolving under monogamy, as has been previously demonstrated in *D. melanogaster* (Holland and Rice 1999, Wigby and Chapman 2004) and *D. pseudoobscura* (Crudgington *et al.* 2010). Thus, irrespective of whether experimental evolution resulted in increased male investment in seminal fluids/proteins or harassment rate, males which evolved higher mating success (i.e. those evolving under male-biased sex ratio and increased temperature) could be expected to be more harmful to females. However, this was not the case, which indicates that adaptation of *D. simulans* males to increased sperm competition did not have harmful effect on females, supporting earlier conclusion based on equilibrium populations (Taylor *et al*. 2008b).

Previous studies in *D. melanogaster* also suggest that evolution of males under monogamy and polyandry (Holland 2002) or altered sex ratio (Wigby and Chapman 2004) do not always result in differences in the ability of males to harm test females in terms of fecundity and lifespan. In *D. pseudoobscura*, female survival was not influenced by the mating system treatment of her partner (Crudgington *et al.* 2010). Females continuously housed with males evolving under elevated opportunities for female promiscuity produced fewer total progeny, but a relatively greater number of progeny early in their lives, than females housed with males evolving under obligate monogamy (Crudgington *et al.* 2010). These results suggest that IRSC may not always be an imminent consequence of polyandry. Although polyandry holds the potential for interlocus sexual conflict over

female mating rate, it has been suggested that sexual conflict over the mating rate may sometimes be lower in polyandrous species, because females are mating at a rate that is closer to the male optimum (Holman and Kokko 2013). Similarly, sexual conflict over mating rate in populations evolving under elevated polyandry for over 60 generations may be lower than expected which may explain why these males did not evolve increased harmfulness to their mates.

The fact that females mating with males evolving under elevated polyandry at novel temperature showed the highest productivity is intriguing, especially that under this combination of features males also achieved the highest reproductive success (Duffy *et al.* in prep). Interestingly, also, a comparison of female fitness across the selection lines showed that females evolving under elevated polyandry had higher productivity than females evolving under monogamy when tested at ancestral (25° C) or novel temperature (27° C). Particularly, females evolving under elevated polyandry at elevated temperature of 27° C showed the highest productivity when tested at ancestral temperature (25° C) (Duffy *et al*. in prep.). This unexpected interaction may indicate that the increased productivity of females evolved under elevated polyandry lines at 27° C may not be only due to adaptation of these females, but also as a result of mating with the males from those lines. The increased productivity coupled with the unaffected longevity of test females suggests that mating with males which evolved superior reproductive competitiveness is overall beneficial to females. In *D. simulans*, male attractiveness is associated with higher fertilization success during sperm competitiveness (Hosken *et al.* 2008) and therefore the higher productivity of the females mated with such males could be explained based on differential allocation hypothesis i.e. females may show higher reproductive investment when breeding with more attractive males (Burley 1986, Burley 1988, Sheldon 2000). Alternatively, high quality males may provide sperm of higher quality or in higher quantity

(hence the higher sperm competitiveness) which may affect fecundity if females are sperm limited.

Previously, studies in *D. melanogaster* have shown that mating with larger males (of higher quality) can impose cost of mating to females in terms of reduced antibacterial immune defense (Imroze and Prasad 2011). Thus, although this study could not detect costs of mating with high fitness males in terms of longevity, it is possible that the females experience costs in the form of other traits, e.g. post-mating suppression of immunity and susceptibility to diseases (reviewed in Lawniczak *et al.* 2006).

To conclude, the results from the analysis of fitness components longevity and productivity of females mating with experimentally evolved males contradicted the predictions of increased male competitiveness leading to increased male inflicted harm to females. The study therefore could not demonstrate the occurrence of interlocus sexual conflict in *D. simulans*, which echo findings from previous studies. However, the results suggested an intriguing interaction between reproductive fitness of males and females evolving under elevated polyandry.

Chapter 3

Genetic basis of intralocus sexual conflict in bulb mites *Rhizoglyphus robini*

Abstract

In bulb mite *Rhizoglyphus robini*, two male phenotypes with alternative reproductive tactics (ARTs) and differing degrees of sexual dimorphism coexist: aggressive fighters and benign scramblers. The present study investigated the genetic basis of intralocus sexual conflict (IASC) in this species by comparing gene expression patterns of males and females from replicate lines selected for increased proportion of fighters (F-lines) or scramblers (S-lines) for more than 60 generations. In particular, we tested the hypothesis that elevation of IASC observed in F-lines is due to correlated changes in gene expression patterns in males and females.

Differential gene expression analysis between directions of selection identified 438 genes differentially expressed (FDR < 0.05) among F-line males and S-line males, majority of which (269) had increased expression in F-lines. The latter group of genes was enriched for several gene ontology categories associated with energy metabolism. Of the 438 genes differentially expressed among F-line males and S-line males, nine genes showed significant differential expression (FDR < 0.05) between females from F-lines and S-lines. Conserved protein domains were identified in three of these nine candidate genes for IASC. Gene ontology categories associated with these domains suggested involvement of one of them in energy metabolism. Consistent with the hypothesis, the F-male biased genes had higher mean expression levels in F-line females than in S-line females, and the reverse was observed for the S-male biased genes.

Our results showed that selection for a more sexually dimorphic male morph entails a correlated change in gene expression in females. Gene ontology analysis suggested that at the level of gene expression, the response to selection on male morph reflects increased energy demands

associated with armoured phenotype and aggressive behaviour of fighter males. Expression level of these genes in females apparently is not decoupled from that in males, thus increasing the potential for IASC.

3.1 Introduction

Sexes often have distinct phenotypic optima for many traits that are expressed in both males and females. If these sex-specific optima cannot be achieved simultaneously due to the constraint of a shared genome, intralocus sexual conflict (IASC) results, characterized by negative fitness correlation between the sexes (Lande 1980). Although the extent to which IASC occurs in natural populations is still debated, it has been observed in multiple species across several taxonomic groups (reviewed in Boduriansky and Chenoweth 2009, van Doorn 2009). IASC has been described to have potential consequences for various evolutionary processes such as speciation, evolution of sex chromosomes, sex determination, regulation of gene expression, sexual selection, sex allocation and aging (reviewed in Boduriansky and Chenoweth 2009, van Doorn 2009). Recently it was also suggested that IASC may facilitate the maintenance of genetically determined alternative reproductive tactics (ARTs) (Plesnar-Bielak *et al*. 2014).

Species that exhibit ARTs provide an excellent system to study IASC as they show discontinuous male phenotypes with differing degrees of sexual dimorphism. Typically, the male types that exhibit a dominant and aggressive phenotype are more sexually dimorphic when compared to females. Comparative evidence shows that sexually-selected dimorphism is associated with elevated IASC (Cox and Calsbeek *et al*. 2009). Occurrence of ARTs makes it possible to investigate this association more directly, by manipulating the degree of IASC through changing frequencies of ARTs within populations. Indeed, in horned beetles (Harano *et al*. 2010) and bulb mites (Plesnar-Bielak *et al*. 2014) artificial selection for armored and aggressive morphs resulted in decreased female fitness, confirming that sexually selected dimorphism can act as a driver of sexual conflict.

Elevated sexual conflict should select for various mechanisms of its resolution (reviewed in Stewart *et al.* 2010), including gene duplication and subfunctionalization

(Wyman *et al*. 2012), sex-dependent regulation of gene expression (McIntyre *et al*. 2006) or genomic imprinting (Day and Bonduriansky 2004). Studies of ARTs in turkeys (Pointer *et al*. 2013), horned beetles (Snell-Rood *et al*. 2010) and bulb-mites (Stuglik *et al*. 2014) have shown that higher phenotypic dimorphism is indeed correlated with higher magnitude of sex bias in gene expression. Yet, elevated IASC associated with more sexually dimorphic male phenotypes (Plesnar-Bielak *et al*. 2014, Harano *et al.* 2010) suggests that sex bias does not fully resolve sexual conflict. This is likely to result from constraints resulting from males and females sharing most of their genomes. Such constraints were inferred by Griffin *et al*. (2013), who documented that evolution of sex-bias in gene expression among Drosophilids can be predicted by intersexual correlations of gene expression within species. Thus, while sex bias apparently evolved for some genes expressed in armored morphs (Snell-Rood *et al*. 2010, Stuglik *et al*. 2014), many other genes might be constrained, so that the sex bias cannot evolve. Based on this reasoning, it was predicted that genes which changed expression in males in response to selection on male morphs would undergo a correlated expression change in females.

This prediction was tested in the present study in the bulb mite, *R. robini*. As in several other species within mite family Acaridae (Radwan 2009), two male morphs are observed in *R. robini*: aggressive fighters and benign scramblers. The fighter males possess a heavily sclerotized, thickened and sharply terminated third pair of legs used during male-male competition to stab other males, whereas the scrambler males have unmodified legs, similar to those in females. While fighters outcompete scramblers in direct competition for mates (Radwan and Klimas 2001), Plesnar-Bielak *et al.* (2014) hypothesized that scramblers are still maintained in populations because IASC leads to lower fitness of daughters of fighter males. The present study took advantage of the replicate lines selected by Plesnar-Bielak *et al*. (2014) for fighter males (F-lines) and scrambler males (S-lines) to

compare how they differ in gene expression patterns using RNAseq. It was hypothesized that increased IASC in F-lines might be a result of correlated changes in gene expression in males and females as a response to selection on male morph. The first aim here was to identify genes significantly differentiated between males from F-lines and S-lines. The second aim was to test the prediction that expression of these genes will also be significantly different in females from respective selection lines. The final aim was identification of candidate genes underlying IASC associated with increased sexual dimorphism in the bulb mite, i.e. those genes which changed expression in the same direction in both sexes in response to selection on fighter morph.

3.2 Materials and methods

3.2.1 Samples and sequencing

Samples came from lines selected for increased proportions of either fighter males (F-lines) or scrambler males (S-lines) in four replicates in each direction, as described in detail in Plesnar-Bielak *et al*. 2014. After about 40 generations, all lines maintained > 90% of the desired morph (Plesnar-Bielak *et al*. 2014).

At generation 96, tritonymphs (last larval stage of mites) were isolated from the selection lines (300 per line), adults were collected as they emerged and separated by sex and morph. For each replicate line of the two selection regimes, RNA was extracted from adult females and males mated once. Each sample contained approximately 100 individuals of the same sex and age, therefore expression levels reported in this study can be treated as means over many individuals of the same sex. Samples were collected in a randomized order. RNA was also extracted from pooled samples of females, fighter male morphs and scrambler male morphs from the stock population (100 individuals pooled for each sex and morph), which was used for transcriptome assembly. RNA extractions were done using RNAzol®RT kit (Chomczynski *et al*. 2010). RNA samples were purified using DNA-free™ kit (Ambion) to eliminate DNA contamination. Quality of extracted RNA was checked using Agilent 2100 Bioanalyzer System. Library and RNA-Sequence samples were prepared using NEXTflex™ Rapid Illumina RNA-Seq library prep kit (Bioo Scientific) and Illumina sequencing was performed at the medical university of Warsaw producing single end (SE) 100 bp reads.

Quality assessment for the resulting data was done using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) prior to genome/transcriptome assemblies and analysis. Reads with low quality bases or short read length were removed from subsequent analyses using Skewer (Jiang *et al*. 2014). Although the bulb mite

genome has been sequenced and assembled (Konczal *et al.* in prep), its annotation is still in progress. Therefore a comprehensive transcriptome database was built using genome-guided and de novo transcriptome assembly with PASA (Program to assemble spliced alignments) (http://pasapipeline.github.io/#A_ComprehensiveTranscriptome).

3.2.2 Assembly and gene models

De-novo assembly of transcriptome was carried out using reads from selection lines (377.7 mln single end reads) and unselected populations (137.3 mln paired-end reads) sequenced earlier (Stuglik *et al.*, 2014, data deposited in BioProject portal (PRJNA213807)). Transcripts were reconstructed with Trinity transcriptome assembler (Grabherr *et al.*, 2011; release 2014-07-17) with an extra parameter, --PasaFly, which reduced the number of reported isoforms. Additionally, a genome guided assembly of transcriptome was made with Trinity (http://trinityrnaseq.github.io/#genome_guided), using draft genome consisting of 6599 scaffolds with N50 of 345 kb and half of the genome assembled in 174 largest scaffolds (Konczal *et al.* in prep.). Reads were first aligned to the draft genome with gsnap (Genomic Short-read Nucleotide Alignment Program) (Wu and Nacu 2010) and separated according to putative loci followed by de novo transcriptome assembly at each genome-defined locus. Transcripts expressed at very low levels are likely to be artefacts (Bhargava *et al.* 2014); therefore transcripts below 0.1 FPKM were removed from the assemblies before building the comprehensive transcriptome database.

Using the de-novo and genome-guided transcriptome assemblies as input, a comprehensive transcriptome database was generated within PASA (http://pasapipeline.github.io/#A_ComprehensiveTranscriptome), a program that infers gene models for assembled transcripts. The program identifies clusters of aligned transcripts that are likely to represent transcribed genes and the longest transcript from each cluster is selected as a representative of gene model. Protein coding regions for each

transcript are inferred by TransDecoder, software bundled with PASA. The comprehensive transcriptome database, containing coding as well as non-coding gene models, was used as the reference for further analysis.

3.2.3 Gene expression analysis

Gene expression analysis was performed with the pipeline included in Trinity. For each sample, reads were aligned to the reference comprehensive transcriptome database with Bowtie (Langmead *et al*. 2009), followed by estimation of transcript abundance using RSEM (Li and Dewey 2011). Next, edgeR (Robinson *et al*. 2010) was used to identify and analyze differentially expressed genes in pairwise comparisons between males and between females from the fighter and scrambler selection regimes, treating the replicate lines within each regime as biological replicates. A false discovery rate (FDR) of 0.05 was used to identify genes with significant differential expression between F-lines and S-lines. Differential gene expression analysis between females from F-lines and S-lines was also done for the group of genes that showed a significant differential expression between F-line and S-line males.

The difference in mean expression levels of male biased genes in females from fighter and scrambler selection regimes was also tested. If selection on male morph caused correlated expression changes in females, F-male-biased genes would show higher mean expression in F-line females than in S-line females, and vice versa for S-male-biased genes. Means over four replicate lines per selection regime for log-transformed values of gene expression in females were analysed with paired Wilcoxon signed rank test (stats package, version 3.1.3, R core team 2015); the non-parametric test was used due to non-normality of distribution.

3.2.4 Functional annotation, gene ontology and enrichment analysis

For the genes that exhibited significant fighter or scrambler specific expression bias in both males and females, gene ontology (GO) analyses were performed using Blast2GO (ver.3.0; Conesa *et al.* 2005). A command-line BLAST+ blastx (Camacho *et al.* 2008) was performed for the gene model reference transcriptome using the Swissprot database on NCBI FTP site (ftp://ftp.ncbi.nlm.nih.gov/blast/db/, version 25/04/2015) and the results were imported into Blast2GO (Version 3.0, Conesa *et al*. 2005), where the mapping, functional annotation and enrichment analyses were done using default settings (except for FDR < 0.1 for enrichment analysis). For the genes differentially expressed in F-line and S-line females, NCBI conserved domain search (Marchler-Bauer and Bryant 2004) was performed using the database CDD v3. 14- 47363 PSSMs (Marchler-Bauer *et al*. 2014) and the GO categories for these domains were identified with EMBL-EBI InterPro (Mitchell *et al*. 2014).

3.3 Results

Among the total of 54,475 gene models (henceforth referred to as genes for simplicity), 10,821 were identified as protein coding. Of the 13,508 genes showing more than two-fold expression difference between males from F lines and S lines, 438 genes showed significant differential gene expression at FDR < 0.05, majority of which (269) had an increased expression in the F-line males (Odds ratio = 1.6, P < 0.001, Fisher's exact test). Out of these 438 genes significantly biased between F-line males and S-line males, 9 genes showed significant differential expression (FDR < 0.05) between females from F-lines and S-lines. The 4 genes overexpressed in F-line females were also F- line biased in males, and similarly the 5 genes found to be S-female-biased were also S-male-biased.

Consistent with our hypothesis, expression patterns in females changed in response to selection on male morph. Mean expression level of F-male biased genes was significantly higher in F-line females than in S-line females (V = 30082, n= 268, p-value < 0.001, Wilcoxon signed rank test). Similarly, mean expression level of S-male-biased genes was higher in S-line females than in F-line females (V = 4920, n=168, p-value = 0.003, Wilcoxon signed rank test) (Figure 3.1).

Taking into account all 54,475 genes expressed in females, , differential gene expression analysis between directions of selection identified only 2 genes (out of 10,625 genes with more than two-fold expression difference between F and S regimes) with a significant expression difference (at FDR < 0.05). The gene showing significantly higher expression in F-line females was also among the 269 genes with elevated expression F-line males. Similarly, the gene with S-female-biased expression in females showed a significant S-line bias in males. These two genes were also present in the group of 9 genes from the analysis above.

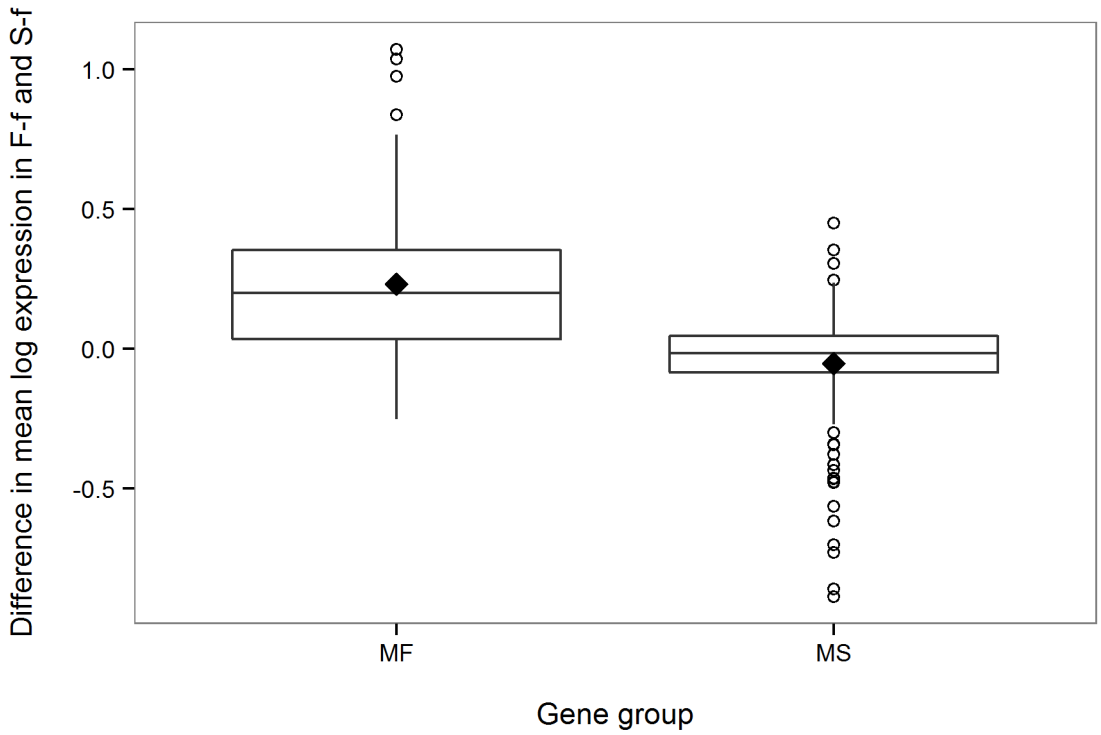

Figure 3.1: Box plot for difference in expression between F-line females (F-f) and S-line females (S-f) for F-male-biased genes (MF) and S-male-biased genes (MS). Y-axis depicts difference in mean over replicate lines for log transformed expression values in F-line and S-line females. Diamonds represent means.

The enrichment analysis showed that the S-male-biased genes were neither over nor underrepresented for any gene ontology categories. However, F-male-biased genes were overrepresented in several gene ontology categories (Table 3.1); for example, hydrolase activity, ATPase regulator activity, ATPase activator activity and lipid metabolic process. Also, some F-male-biased genes were overrepresented in the gene ontology category Hsp70 protein binding.

Genes biased among F-line and S-line males that also showed significant expression bias between F-line and S-line females were not annotated, but conserved domains could be identified for 3 out of the 9 genes, and GO categories for these could be identified (Table 3.2). One of the F-female biased genes showed a conserved domain (Oxidored_q3 super family NADH-ubiquinone/plastoquinone oxidoreductase chain 6)

involved in oxidation-reduction process, while the other showed a conserved domain (DDE superfamily endonuclease/transposase) associated with DNA cleavage followed by strand transfer reaction. One of the S-female biased genes showed conserved domains associated with PMT_2 super family, specifically the enzyme dolichyl-phosphate-mannose-protein mannosyltransferase involved in protein glycosylation.

Table 3.1: Enrichment analysis of F-male-biased genes: The gene ontology terms overrepresented (FDR < 0.1) belonged to one of the three categories: Cellular component (C), Biological process (P) and Molecular function (F). The column FDR shows corrected p-value by False Discovery Rate control.

GO-ID	***Term***	***Category***	***FDR***
GO:0005576	extracellular region	C	0.054
GO:0070389	chaperone cofactor-dependent protein refolding	P	0.054
GO:0016787	hydrolase activity	F	0.054
GO:0090084	negative regulation of inclusion body assembly	P	0.054
GO:0051085	chaperone mediated protein folding requiring cofactor	P	0.054
GO:0001671	ATPase activator activity	F	0.054
GO:0090083	regulation of inclusion body assembly	P	0.054
GO:0030544	Hsp70 protein binding	F	0.054
GO:0060590	ATPase regulator activity	F	0.054
GO:0070841	inclusion body assembly	P	0.055
GO:0042026	protein refolding	P	0.098
GO:0006629	lipid metabolic process	P	0.099

Table 3.2: Conserved domains and GO categories identified for F-female biased and S-female biased genes. F-line female biased (F-f), S-line female biased (S-f), Cellular component (C), Biological process (P) and Molecular function (F).

Bias	***Conserved domain***	***GO-ID***	***Term***	***Category***
F-f	Oxidored_q3 super family	GO:0055114	oxidation-reduction process	P
		GO:0008137	NADH dehydrogenase (ubiquinone) activity	F
F-f	DDE_Tnp_4	GO:0003676	nucleic acid binding	F
S-f	PMT_2 super family	GO:0004169	dolichyl-phosphate-mannose-protein mannosyltransferase activity	F

3.4 Discussion

The results showed that divergent selection on male phenotype in a species with alternative male morphs causes changes in patterns of gene expression in both males and females. Selection for fighters involved an increased expression of much higher number of genes in males than selection for scramblers (the difference was 1.5 fold at FDR < 0.05).

Some of these differentially expressed genes might reflect physiological differences between morphs rather than genetic differences fixed between the lines. However, the fact that these genes also showed a correlated difference in expression between F-line and S-line females indicates that at least some of them reflect response to selection in terms of changes in allele frequencies of regulatory sequences or trans-acting genes. The fact that higher number of genes showed increased expression in fighters highlights the higher potential for IASC associated with this morph.

In order to understand why sexual conflict persists in populations, sexually antagonistic genes need to be identified, yet it has been done in only few studies. Gene ontology analysis indicated that F-male biased genes were overrepresented in categories that corresponded to catalytic activity and metabolic processes associated with energy metabolism, such as ATP activator and regulator activity and lipid metabolic process. This may be associated with the higher energy needs of fighter males since they engage in frequent and long fights with other males. Interestingly, in a study of sex-biased genes in unselected population of bulb mites Stuglik *et al*. (2014) found that many sex-biased genes, especially those with high expression in fighters, are also related to energy metabolism. This indicates that genes associated with higher energy demands of fighters are sexually antagonistic, and bias in expression is a way of resolution of this conflict (McIntyre *et al*. 2006, Stewart *et al*. 2010). However, Plesnar-Bielak *et al*. (2014) suggested that if such resolution cannot be achieved for all metabolic genes, some of these

genes may underlie increased intralocus sexual conflict associated with selection favouring more sexually dimorphic fighter morph. The fact that genes with increased expression in males from F-lines had increased expression in females from these lines (as compared to females from S-lines) is consistent with this hypothesis. The nine male-biased genes which were also biased in females from the same selection regimes can be considered strong candidates for loci underlying sexual conflict. Although not all nine could be annotated, conserved domains could be identified for three of these genes such that biological functions may be inferred for them. One of the F-line biased genes showed conserved domains for NADH dehydrogenase enzyme involved in the oxidation-reduction process, while the other for a DNA endonuclease/transposase enzyme. One of the genes overexpressed in both males and females from S-line showed conserved domains for a glycosyltransferase enzyme involved in protein modification. The non-specific nature of functions associated with these genes may indicate that these genes are expressed in many tissues, rather than being tissue-specific, which is similar to results from a previous study in *D. melanogaster* (Innocenti and Morrow 2010). The study had demonstrated that sexually antagonistic genes were highly expressed in most tissues, not only in sex-limited reproductive tissues but also in neural tissues and tissues associated with metabolism, nutrient uptake and transport, such as crop, midgut, hindgut fat body and heart. Many of these sexually antagonistic genes were also associated with diverse categories of metabolic processes (Innocenti and Morrow 2010).

Apart from changes in female expression profiles resulting from correlated response to changes in male expression profiles, there might be changes in expression of other genes responsible for adjusting female physiology to direct effects of changed proportion of male morphs. However, the transcriptome-wide analysis of female revealed only two genes with showed significantly differentiated expression between S and F-line

males at FDR < 0.05, and both these genes were also biased in males from respective lines. This does not necessarily imply that changes in female expression profiles other than those resulting from correlated response to selection on males did not occur; because our power to detect them at FDR < 0.05 was lower (the first analysis involved 438 genes differentially expressed in males, whereas the other involved 54,475 genes from the comprehensive transcriptome reference database).

Overall, the results were consistent with the hypothesis that the higher IASC in lines selected for the more sexually dimorphic male phenotype is attributable to correlated changes in gene expression patterns in males and females in response to selection. Nine candidate genes underlying the conflict were identified with at least one of them associated with metabolic activity. With improved annotation of the *R. robini* genome in the future, the functions of these genes may be inferred with more certitude which would facilitate further elucidation of the genetic basis of IASC in this species.

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Streszczenie

Konflikt międzypłciowy jest wynikiem sprzecznych interesów rozrodczych między płciami i występuje powszechnie u organizmów rozmnażających się płciowo. Wyróżnia się dwie formy konfliktu międzypłciowego: konflikt między loci i konflikt wewnątrz locus. Ze względu na konsekwencje ewolucyjne konfliktu międzypłciowego, badania nad nim są istotną częścią współczesnej biologii ewolucyjnej. W niniejszej rozprawie badano, wykorzystując techniki ewolucji eksperymentalnej, różne aspekty konfliktu międzypłciowego u trzech gatunków stawonogów.

Białka płynu nasiennego, takie jak sex peptide (SP) u *Drosophila melanogaster* są zaangażowane w konflikt między loci, powodując wzrost dostosowania samców i obniżenie dostosowania samic, co może prowadzić do antagonistycznej ewolucji między płciami. System sex peptide-sex peptide receptor (SPR) wykorzystano do zbadania odpowiedzi ewolucyjnej samców z linii selekcyjnych, w których samice nie wykazują ekspresji SPR. Taka sytuacja może wpływać na dynamikę „wyścigu zbrojeń" między płciami. Poziom ekspresji genu *SP* oraz innego genu kodującego pokrewne białko płynu nasiennego, Dup99B (również wiąże się do SPR) badano wykorzystując RT-qPCR. Niezgodnie z przewidywaniami, stwierdzono istotny wzrost ekspresji *SP* u samców z linii selekcyjnych w porównaniu z samcami z linii kontrolnych, natomiast nie stwierdzono różnic w ekspresji *Dup99B*. Wyniki te wskazują, iż manipulacje genetyczne przeprowadzone u samic, wpływające na przebieg międzypłciowego "wyścigu zbrojeń", mogą powodować szybką odpowiedź ewolucyjną u samców. Wyniki wskazują również na możliwość występowania dodatkowych receptorów dla SP, innych niż SPR.

Pomimo, iż występowanie konfliktu między loci wykazano przekonująco u *D. melanogaster*, nie stwierdzono go dotychczas u *D. simulans*, blisko spokrewnionego

gatunku siostrzanego. Jednak w pod wpływem ewolucji eksperymentalnej może ulec zaburzeniu równowaga między adaptacjami a kontradaptacjami w zakresie konfliktu międzypłciowego, stwarzając potencjał dla jego wykrycia. Wcześniejsze badania nad populacjami *D. simulans* ewoluującymi w warunkach zmienionych systemów kojarzeń i temperatury pokazały, że zarówno zwiększony stopień poliandrii jak i podwyższona temperatura otoczenia podnosiły konkurencyjność samców. W niniejszej pracy przetestowano czy ewolucja zwiększonej konkurencyjności doprowadzi do podwyższenia kosztów kojarzenia u samic, przejawiających się obniżoną długością życia lub płodnością. Nie stwierdzono wpływu systemu kojarzenia ani temperatury, w których ewoluowały samce, na długość życia samic. Stwierdzono jednak istotną interakcję między systemem kojarzenia a temperaturą we wpływie na płodność samic: samice kojarzone z samcami, które ewoluowały w warunkach podwyższonej poliandrii i podniesionej temperatury wykazywały najwyższą płodność. Tak więc, niezgodnie z przewidywaniami przy założeniu występowania konfliktu między loci, wyniki tych badań wskazują że kojarzenie z samcami o wyższej konkurencyjności są korzystne dla samic.

Rozkruszek hiacyntowy (*Rhizoglyphus robini*) jest interesującym systemem do badań nad konfliktem międzypłciowym, ze względu na występowanie dwóch fenotypów samców, wykazujących alternatywne strategie rozrodcze: samców walczących i niewalczących. W ostatniej części rozprawy badano genetyczne podstawy konfliktu międzypłciowego wewnątrz locus związanego z silniej zaznaczonym dymorfizmem płciowym samców walczących u *R. robini*. Wykorzystano dobór sztuczny oraz wysokoprzepustowe sekwencjonowanie transkryptomów aby porównać ekspresję genów samców i samic z linii selekcjonowanych na zwiększony udział samców walczących (F) i niewalczących (S); w każdym kierunku selekcjonowano cztery linie. Zidentyfikowano 438 genów wykazujących statystycznie istotne zróżnicowanie poziomu ekspresji między

samcami z linii F i S. Stwierdzono, iż dobór na zwiększenie częstości bardziej dymorficznych samców walczących powoduje zwiększenie poziomu ekspresji istotnie większej liczby genów niż dobór na zwiększenie częstości samców niewalczących; zwiększa to prawdopodobieństwo konfliktu wewnątrz locus. Geny o wyższej ekspresji u samców z linii F miały także średnio wyższą ekspresję u samic z linii F w porównaniu z samicami z linii S, podczas gdy odwrotny związek zaobserwowano dla genów o zwiększonej ekspresji u samców z linii S. Zidentyfikowano 9 genów kandydackich, które leżeć u podstaw konfliktu wewnątrz locus, jeden z nich jest prawdopodobnie zaangażowany w metabolizm energetyczny. Wyniki otrzymane w tej części były zgodne z hipotezą, która mówi, iż skorelowane zmiany w ekspresji genów u samców i samic leżą u podłoża nasilonego konfliktu wewnątrz locus.

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