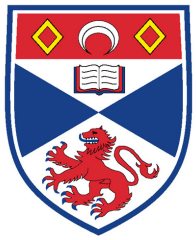
Evolutionary genetics and genomics of the female side of sexual interactions in *Drosophila*

ELINA IMMONEN



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I, Elina Immonen, hereby certify that this thesis, which is approximately 43,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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Abstract

Sexual interactions play an important role in generating sexual selection and antagonistic co-evolution. These forces can shape differences between the sexes, but also have the potential to generate population divergence and contribute to speciation. The aim in this thesis was to provide new insights into the genes involved in different stages of female sexual interactions, using *Drosophila* as a model system. In chapter 2 I tested whether a candidate gene (*period)* that influences species-specific rhythmic characteristics in male courtship song in *D. melanogaster* also has a pleiotropic effect on female song preference. Using mutant and transgenic strains I found support for this. In chapter 3 I examined further how females respond to the song at the level of gene expression, using microarrays. Expression profiles revealed modest changes in transcripts abundance overall, which were dominated by antennal olfactory genes, neuropeptide encoding genes and immunity genes. Many of these have previously been found to respond to mating. In chapter 4 I therefore studied further two of these genes, *TurandotM* and *TurandotC* and their role in female post-mating fitness. Using RNA interference I found that knocking down these genes influenced immediate fecundity. In chapter 5 I focused on analysing post-mating gene expression patterns in relation to sexual selection in *D. pseudoobscura* using microarrays. I explored the consequences of experimental variation in female promiscuity on gene expression divergence as a whole, and in response to mating. I found large-scale expression divergence between monandrous and polyandrous females after 100 generations of experimental evolution. Experimental polyandry increased the expression of genes that show female-biased expression in wild-type individuals and decreased male-biased gene expression. Females experiencing no sexual selection showed the opposite pattern. Out of the genes affected by mating, the majority showed increased expression in polyandrous compared to monandrous females, with enrichment e.g. in oogenesis-related genes.

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My parents Tuulikki and Eero sparked my interest for the natural world in our countless cabin, bird watching and hiking trips, and mom also in the classroom. Thank you for your support throughout (and making sure I've been regularly stocked with Finnish chocolate and magazines!). Thanks for my brothers Lauri and Juhani who both visited me here: there is nothing like a good discussion with you two.

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Contents

CHAPTER 1

General Introduction

Sexual selection and the origins of sex-specific traits

"If not for sex, much of what is flamboyant and beautiful in nature would not exist. Plants would not bloom. Birds would not sing. Deer would not sprout antlers. Hearts would not beat so fast."

– Dr. Tatiana's Sex Advice To All Creation –

Inspired by such beauty, biologists have been fascinated by sexually dimorphic traits (i.e. traits that differ between the sexes) and explored their evolutionary origins since Darwin's seminal work (1871). Sexual dimorphism is widespread across the animal kingdom and includes anatomical and physiological characteristics involved directly in sexual reproduction (primary sex traits), morphological and behavioural traits that contribute to their possessor's reproductive success (secondary sex traits), and ecological characteristics that differ between the sexes when they occupy partly different niches. Sexually dimorphic characteristics arise despite virtually identical DNA sequences between the sexes, apart from a few genes on the heterogametic sex chromosomes (Y in XY systems, W in ZW systems) in species with genetic sex determination. Mechanistically, the differences must therefore largely result from the way genes are used: sex-specifically regulated gene expression (WILLIAMS *et al.* 2008).

The difference in size or morphology of male and female gametes (i.e. anisogamy, whereby females produce larger ova and males smaller spermatozoa) sets the stage for the evolution of sex differences. The unbalanced primary parental investment into the gametes generates competition for more numerous male sperm to fertilize less abundant eggs (PARKER *et al.* 1972). However, it is a combination of several factors that influence the degree to which animals evolve sexual dimorphism,

beyond the initial differences in gamete size and primary sex traits. Such factors include how much each sex invests into parental care beyond the gametes, and how much into finding mates. These in turn depend on the costs and benefits involved in these strategies for each sex (BATEMAN 1948; CLUTTON-BROCK and PARKER 1992; EMLEN and ORING 1977; MAYNARD SMITH 1977; TRIVERS 1972). In the majority of animal species the sexes try to maximise their reproductive success by using different strategies, whereby males are the more competitive sex and females provide most of the care, but there are several examples of species with sex-role reversal (EENS and PINXTEN 2000).

Females are often the limiting factor for male reproductive success, which not only creates competition among males but also opportunities for females to be choosy. Darwin recognized the possibility that many elaborate traits in males do not function in male-male interactions, but have evolved because females prefer to mate with males carrying elaborate ornaments (DARWIN 1871). The active role of females in driving male evolution remained controversial until the recent decades, which have seen a proliferation of studies that show that female preferences exist (ANDERSSON and SIMMONS 2006). In **chapters 2** and **3** I examine female preference for one male trait: courtship song in *Drosophila melanogaster*.

How females choose their mates impacts directly on their own fitness. Evolution of mate choice is based either on direct selection acting on preference that gives survival or fecundity advantage to its bearer (including models of 'direct benefits' and 'sensory bias', but also choice based on resistance to male harm) (ANDERSSON 1994; FULLER *et al.* 2005; GAVRILETS *et al.* 2001; PRICE *et al.* 1993), or on indirect selection on a preference that becomes genetically correlated with directly selected male traits (models of 'Indicator mechanisms' and 'Fisherian sexy sons')

(LANDE 1981; ZAHAVI 1975; ZAHAVI 1977). Such indirect selection can arise if the mate choice results in higher reproductive success of sons or superior genetic quality of both male and female offspring. In addition, rather than females favouring any particular male traits, they may choose a mate based on genetic compatibility (see e.g. ANDERSSON and SIMMONS 2006 for a review). Research has proven each of these mutually compatible mechanisms possible, however there is a debate over their relative importance.

The role of polyandry in shaping the sexes

Evolution of female mating rate lies at the heart of understanding the role of females in male mating success and subsequent selection acting on both sexes (consequences of polyandry on female gene expression is the topic of **chapter 5**). Theory predicts that female reproductive success is maximized by the number of viable eggs produced, and often only one or few matings is enough to transfer an adequate amount of sperm to females to fertilize all of her eggs (BATEMAN 1948). Despite this, empirical studies have shown that polyandry (i.e. female multiple mating with different males) is common in nature and can be explained by the benefits females gain from multiple matings that increase their lifetime reproductive success compared to monandry (ARNQVIST and NILSSON 2000). Polyandrous females have multiple opportunities to benefit from pre-mating choice. Receiving sperm from several males also allows the potential for females to selectively fertilize their eggs, and this cryptic post-copulatory mate choice can benefit females in an analogous way to premating choice, including indirect genetic benefits (JENNIONS and PETRIE 2000; SLATYER *et al.* 2011). Benefits of polyandry also include inbreeding avoidance (FIRMAN and SIMMONS 2008; MICHALCZYK *et al.* 2011; TREGENZA and WEDELL 2002) and in species where males

give nuptial gifts, receipt of multiple edible gifts increases not only female fecundity but also survival (ARNQVIST and NILSSON 2000). Other aspects of sexual interactions are likely to benefit females. In addition to sperm, males transfer seminal fluid molecules into the females, and many of these products increase female egg production rate and fertility (HEIFETZ *et al.* 2001; WOLFNER 2009). Post-mating molecular interactions in regards to female fecundity are topics included in **chapters 4** and **5**.

Polyandry also generates post-copulatory sexual selection on males in the form of sperm competition, which selects for male traits that enhance their fertilization success (analogous to pre-copulatory selection on traits that increase male mating success) (BIRKHEAD and MØLLER 1998). Such traits include male seminal products in *Drosophila* that manipulate female reproductive behaviour to the advantage of male, often at the expense of the female. Male seminal products are essential for sperm storage (KALB *et al.* 1993; NEUBAUM and WOLFNER 1999; TRAM and WOLFNER 1999) and thus involved in sperm competition (CHAPMAN *et al.* 2000; CLARK *et al.* 1995), but can be toxic to females (LUNG *et al.* 2002) contributing to the decrease in female lifespan (CHAPMAN *et al.* 1995; FOWLER and PARTIGRIDGE 1989). They also increase female refractory period (CHAPMAN *et al.* 1995), and manipulate egg-laying rate in a potentially suboptimal manner (CHAPMAN *et al.* 2001; CHAPMAN *et al.* 1995). Another example is structures in male genitals that may help the males to prolong copulation duration but also contribute to increase in female mortality, such as spines in the male aedeagus in the bean weevil *Callosbruchus maculatus* that rupture the female reproductive tract (CRUDGINGTON and SIVA-JOTHY 2000). Evolution of female mating rate thus needs to be viewed in terms of selection that arises from the relative costs and benefits of mating to the female. These can vary

between closely related species (see e.g. TAYLOR *et al.* 2009), but also within species depending on environmental factors such as diet (FRICKE *et al.* 2010).

Sexually antagonistic selection between and within loci

Sexual conflict can arise from differences between the sexes in optimal mating frequency, fertilization, relative parental effort, female remating behaviour and reproductive rate. Both sexes are expected to evolve adaptations that bias the outcome of the conflict to their own advantage, generating sexually antagonistic coevolution (CHAPMAN *et al.* 2003). Many morphological, physiological and behavioural traits are selected to increase male fitness, and in order to reduce susceptibility to male manipulation females are predicted to evolve counteradaptations (e.g. GAVRILETS *et al.* 2001), thereby generating cycles of evolutionary change between the sexes (HOLLAND and RICE 1998).

Sexually antagonistic coevolution involves sex-specific traits encoded by different genes, and thus the underlying selective force has been named inter-locus conflict. However, this is not the only type of conflict between the sexes. Differences in the reproductive strategies also lead to selection favouring different trait optima for males and females. Whenever such opposing selection acts on a sexually homologous trait encoded by shared loci it is predicted to generate an intralocus conflict and subsequently sexually antagonistic selection on the expression of a such trait (LANDE 1980). A resolution for intralocus conflict is provided by the evolution of sex-specific gene regulation, because a lower expression of a gene in the sex that suffers the cost can lead to a lower expression of the harmful trait. However, most genes are not sex limited in expression, and it has been suggested that intralocus conflict may be common (BONDURIANSKY and CHENOWETH 2009).

Sexual selection and conflict as engines of divergence

Sexual selection and conflict have potentially strong influences on speciation. Speciation in sexually reproducing organisms involves a splitting of one species into two due to the accumulation of genetic incompatibilities that act as barriers to gene flow, causing populations to become reproductively isolated from another (definition for the Biological Species concept) (MAYR 1942; COYNE and ORR 1998; NOOR and FEDER 2006; WU and TING 2004). Isolating barriers can occur by several mechanisms that act at either pre- or post-mating stages of potential interbreeding (COYNE and ORR 2004).

Sexual selection is predisposed to generate reproductive isolation because of its direct effect on traits involved in sexual signalling (PANHUIS *et al.* 2001; RITCHIE 2007). Traits involved in sexual communication often show rapid divergence between closely related species, such as courtship song in crickets and birds. However, the ease by which sexual signalling systems may diverge depends on their underlying genetics. Divergence in sexual signals is constrained by the need to maintain coordination between signals and their reception. Sexual selection models demonstrate that such coordination and subsequent diversification can be achieved by male-female coevolution that generates assortative mating through linkage disequilibrium (Fisherian run-away process; LANDE 1981). However, pleiotropic effect of shared loci on both signal and preference, or a tight physical linkage between separate loci, are considered potentially more efficient mechanisms to cause rapid divergence. This is because they also prevent recombination and thus ensure that coordination between the signalling components is maintained even if hybridisation occurs during divergence (BUTLIN and RITCHIE 1989). Moreover, if assortative mating is caused by a pleiotropic effect, a novel mutation that arises in one population

might automatically produce a change in both traits and therefore pre-mating isolation between populations ('One-allele' model of speciation, FELSENSTEIN 1981). Despite pleiotropy being predicted to be a powerful mechanism generating divergence, its commonness is unknown and currently there are only few examples of such effects on sexual signals and preferences (SHAW *et al.* 2011). Pleiotropic effect on female preference is a topic addressed in **chapter 2.**

Inter-locus sexual conflict and 'chase-away selection' has a potential to influence speciation through its effect on female resistance to male harm, and has been suggested to be important force in generating isolation barriers (RICE *et al.* 2005). If females in different populations evolve different resistance traits, males will "chase" them and can themselves separate into two distinctive mating types (GAVRILETS and WAXMAN 2002). Traits involved in molecular interactions between the sexes during and after mating are particularly interesting candidates to influence sperm-egg incompatibilities and therefore post-mating pre-zygotic isolation. Theory suggests that sexually antagonistic selection due to intra-locus conflict, on the other hand, can both impede and increase evolution, however it's role in speciation is still unclear (BONDURIANSKY and CHENOWETH 2009).

Sexual interactions and molecular evolution

Comparing protein coding gene sequences within and between closely related species has shown that the genes mediating sexual reproduction, such as gamete recognition, are generally more divergent than non-reproductive genes across taxa, and show signs of adaptive evolution, (e.g. mammals, sea urchins, plants, gastropods, algae and insects) (SWANSON and VACQUIER 2002). In *D. melanaogaster* an example of such proteins includes Acp26Aa. This accessory gland protein encoding gene is to date the

fastest evolving gene identified in *Drosophila* (TSAUR et al. 1998; TSAUR and WU 1997).

Fast protein coding gene evolution related to sexual interactions has been found in genes that show sexually dimorphic gene expression (ELLEGREN and PARSCH 2007). A large proportion of the genome is sexually dimorphic in expression in a range of taxa. Such genes are referred to as sex-biased and include genes expressed exclusively in one sex (sex-specific expression), as well as those expressed in both sexes but at a higher level in one sex compared to the other (sex-enriched expression) (ELLEGREN and PARSCH 2007). Sex-biased genes are further separated into male- and female-biased genes, depending on which sex shows higher expression levels. Genes with equal expression in both sexes are called un-biased genes. It has been estimated that in adult *D. melanogaster* up to 57% of the genome shows sex-biased expression (RANZ *et al.* 2003), and that the majority of these genes are expressed in reproductive tissues (PARISI *et al.* 2003). However sex-biased expression is also common in somatic tissues; for example over 10,000 genes are sex-biased in mouse somatic tissues (YANG *et al.* 2006).

Many studies report faster coding sequence evolution for male-biased genes compared to female- or un-biased genes, and particularly for those expressed in male reproductive tissues (e.g. *D. melanogaster* versus *D. simulans*, *Caenorhabditis elegans* vs. *C. briggsae*, human vs. mouse, human vs. chimpanzee) or during spermatogenesis (e.g. mouse vs. rat) (reviewed in ELLEGREN and PARSCH 2007). A similar pattern has been found for male-biased genes in a comparison between *D. pseudoobscura, D. persimilis* and *D. pseudoobscura bogotana* (JIANG and MACHADO 2009). However, male-biased genes specific to *D. pseudoobscura* provide an exception when compared to *D. melanogaster* (METTA et al. 2006). Also *D.*

ananassae shows no increased rate of adaptive evolution of male-biased genes compared to *D. melanogaster,* but instead a tendency for increased nonsynonymous substitutions for female-biased genes (MULLER *et al.* 2012). Female-biased genes expressed in bird's brains show faster divergence between chicken and zebra finch (MANK *et al.* 2007). Although some differences in observed patterns may be due to methodological issues (e.g. different techniques and numbers of genes sampled), it is likely that the patterns also vary between species.

Sex-biased genes also show more expression divergence between species (e.g. RANZ *et al.* 2003). There is generally more evidence for higher expression divergence for male-biased genes, for example between *D. melanogaster* and *D. simulans* (RANZ *et al.* 2003). Male-biased genes in testes also show higher expression differences between mammal species, such as humans and chimps (KHAITOVICH *et al.* 2005) and between different mouse species (VOOLSTRA *et al.* 2007), in comparison to somatic tissues. In contrast to these findings, a comparison of sex-biased gene expression in *D. pseudoobscura, D. persimilis* and *D. pseudoobscura bogotana* did not find higher expression differences in male-biased genes compared to un-biased or female-biased genes (JIANG and MACHADO 2009).

The observation that sex-biased genes, and particularly male-biased, show faster rates of adaptive evolution suggests these genes evolve under positive selection due to sexual interactions. The faster evolution of many male-biased genes specific to male reproductive track (testes and accessory glands) suggests a strong role for sperm competition (e.g. WONG 2010). However, molecular evolutionary or comparative expression analyses alone do not provide evidence for the underlying cause of positive selection (ELLEGREN and PARSCH 2007). Also, currently our understanding of the evolution of female-biased genes is still limited and inconclusive, as in general

they have not received the same attention that has been given to male-biased genes. Theory and empirical observations of inter-locus sexual conflict suggest strong selection also on females via antagonistic coevolution (CHAPMAN *et al.* 2003). More work is therefore needed for a better understanding of female-specific molecular evolution, and to provide direct evidence that sexual selection and conflict drive some of the observed patterns (expression divergence of sex-biased genes is the topic in **chapter 5**).

Searching for novel candidate genes

The past decade has seen a proliferation of techniques that have taken studies of sexual interactions from describing phenotypic traits that evolve under sexual selection to identifying their underlying genes. However, the task is by no means an easy one, particularly for traits with complicated phenotypes that are hard to measure. Perhaps partly for this reason the advancement in understanding the genes involved in female-specific traits, such as mating preferences, has been much slower compared to identifying the genetic factors contributing to the development and variation in male traits.

The methods involved in studying the genetic basis of traits (behavioural or other) include those aiming to identify genes that contribute to the manifestation of a focal trait (e.g. a gene that gives rise to a tail), and those that explore the genes involved in variation in a trait (e.g. a gene that influences tail length, which may or may not be involved in trait manifestation). The former methods include mutagenesis and transgenesis, while the latter involves quantitative genetic approaches such as quantitative trait loci (QTL) mapping (ANHOLT and MACKAY 2010). Development of large-scale genomic methods has taken the focus from single genes to whole genomes.

These methods are casually termed ‘genomic approaches’, which refers to sequencing whole genomes, sequencing and analyses of genome-wide gene expression (i.e. transcriptome) patterns using RNA sequencing (RNAseq) and microarrays (also called the ‘transcriptomic approach’). The genomic approach also includes an extension of the QTL technique to identify single nucleotide polymorphisms (SNP) associated with variation in the phenotype of interest (genome-wide association study or GEWAS) (ANHOLT and MACKAY 2010).

Transcriptome profiles, obtained for example with microarrays, can be analysed to detect genes that change expression in response to a physical or social factors of interest. They therefore give us cues about the genes and their networks associated with a manifestation of a trait. This technique can be used to identify gene functions involved in producing a focal trait (i.e. by inferring processes associated with the treatment from gene annotation information), but also discovering new functions for previously un-annotated genes (DRNEVICH *et al.* 2004). The microarray technique can also be powerful in detecting functionally related gene networks that cause only subtle expression changes under controlled environmental stimuli.

Comparing gene expression profiles of different genotypes (e.g. populations) helps to identify genes that have evolved differences in their regulatory mechanisms, which may also contribute to the variation in a phenotypic trait of interest (e.g. MICHALAK *et al.* 2007). Indeed, differentiation in behavioural traits between diverging populations and species is often associated with changes in the time, level and location of gene expression (CARROLL 2005; RANZ and MACHADO 2006). Transcriptome profiles of different genotypes can be associated with variation in coding regions using a method called expression QTL (eQTL) (MACKAY *et al.* 2009).

The microarray technique has been used in a variety of animals to demonstrate changes in transcriptional activation mediating mating related behaviours in females. For example, sexual experience has been shown to modify gene expression in the brain of female hamsters (BRADLEY *et al.* 2005), and perceived attractiveness of the partner also leads to gene expression changes in the female swordtail fish (*Xiphophorus nigrensis*) (CUMMINGS *et al.* 2008). Similarly, *D. melanogaster* females respond to courtship and mating at the gene expression level: thousands of differentially expressed genes have been identified between mated and virgin females (INNOCENTI and MORROW 2009; LAWNICZAK and BEGUN 2004; MACK *et al.* 2006; MCGRAW *et al.* 2008; MCGRAW *et al.* 2004), and a handful between courted and unexposed females (LAWNICZAK and BEGUN 2004). Differences in expression levels can emerge very rapidly after exposure to sexual stimuli (CARNEY 2007), and they can be detectable for at least two hours after sexual interaction has taken place (LAWNICZAK and BEGUN 2004). Microarrays thus provide a useful tool for identifying novel genes expressed in female sexual interactions.

This thesis

In this thesis I have used both transcriptomic and gene manipulation approaches for identifying genes associated with female-specific reproductive traits in *Drosophila*. Below I will introduce these methods, followed by an introduction to the two study species I have used. Finally, I will introduce each of the chapters presented in this thesis.

Microarrays

In **chapters 3** and **5** I have used microarrays as tools to quantify female gene expression in *D. melanogaster* and *D. pseudoobscura*, respectively.

Microarrays utilize the propensity of DNA (and RNA) sequences to form spontaneous bonds with each other when sequences are complementary. A microarray is a surface to which tens of thousands of synthetic oligonucleotide sequences (probes) are attached, and each of the unique probes on an array slide represents a complimentary DNA to the target gene of interest. The target transcripts (RNA) are extracted from the organism, prepared (commonly reverse transcribed into cDNA), labelled with a fluorescent dye and hybridized onto the microarrays. The abundance of each transcript is inferred from the level of fluorescence emitted from each probe, which corresponds to the amount of target transcript bound.

In **chapter 3**, I opted for two-channel long oligonucleotide microarrays (FL003-INDAC). With two-channel arrays, two samples of interest are labelled with different dyes (Cy3 and Cy5), and the relative amount of transcript in the samples is inferred from their competitive hybridisation onto a probe. I used technical replicates with samples labelled with reversed dyes to take into account differential hybridisation properties of the dyes that can otherwise bias the results. The RNA extraction, quality controls, microarray preparation and image scanning steps were performed by FlyChip at the University of Cambridge. The data has been deposited to Gene Expression Omnibus (GEO) under the accession number GSE31190.

In **chapter 5** I used one-colour Agilent custom designed oligonucleotide microarrays for *D. pseudoobscura*. This was the only one available for this species but also facilitated the data-analysis (no need to perform dye swaps). The custom

array was designed by Jiang and Machado (2009). The Liverpool Microarray Facility (University of Liverpool) performed the RNA extractions, quality controls, microarray preparation and image scanning steps. The data from this chapter has been deposited to GEO under accession number GSE35410.

Microarray techniques enjoy a solid and mature statistical framework, one of its advantages over more recently developed RNAseq techniques (MALONE and OLIVER 2011). Among the wide range of options developed by manufacturers and independent institutions, I chose to utilize tools within the BIOCONDUCTOR project (GENTLEMAN *et al.* 2004). This is a suite of open source bioinformatics software that runs within the R environment (RDEVELOPMENTCORETEAM 2011). Exact details of the data pre-processing and downstream statistical analyses are provided in the Materials and Methods of the corresponding chapters.

RNA interference

In **chapters 2** and **4** I have utilized the availability of strains that enable selective gene expression knock-down of target genes of interest. This is made possible by *GAL4-UAS* binary system that can drive RNA interference (RNAi). *GAL4* is a yeast transcription factor that is not normally found in higher eukaryotes. Cell-specific promotors can be constructed to drive *GAL4* that binds to a promotor known as *Upstream Activator Sequence (UAS)*. Target transgenes can be cloned behind the *UAS* sequence, such as reporter genes (e.g. *GFP*) and transgenes that induce RNAi. When flies (either homozygous or heterozygous with a genetic marker) with the *GAL4* transgene are crossed with flies carrying the *UAS* construct, the resulting F1 offspring heterozygous for each will express the target gene of interest in *GAL4* specific cells (DUFFY 2002).

RNA interference is based on the effect double stranded RNA (dsRNA) has on silencing its homologous mRNA. When dsRNA is introduced into a cell it is processed by a ribonuclease (RNase) III enzyme called Dicer into small interfering RNAs (siRNA). These small fragments subsequently direct cleavage of homologous mRNA via an RNA-induced silencing complex (RISC). Because the introduction of dsRNA does not always eliminate the gene expression, but rather substantially reduces it, RNAi is termed a 'knock-down' technique (ANHOLT and MACKAY 2010). In *Drosophila*, RNAi is cell autonomous and can be triggered by the expression of a double-stranded 'hairpin' RNA (hpRNA) from a transgene containing a gene fragment cloned as an inverted repeat (IR). The *GAL4-UAS* binary system can be exploited to selectively drive the expression of the hpRNA, when the inverted repeats are cloned into *UAS* constructs (Figure 1). I obtained strains containing such *UAS-IR* constructs from a genome-wide library of flies carrying RNAi transgenes from Vienna Drosophila RNAi Center (VDRC) (DIETZL *et al.* 2007), and from Bloomington *Drosophila* Stock Centre (flystocks.bio.indiana.edu).

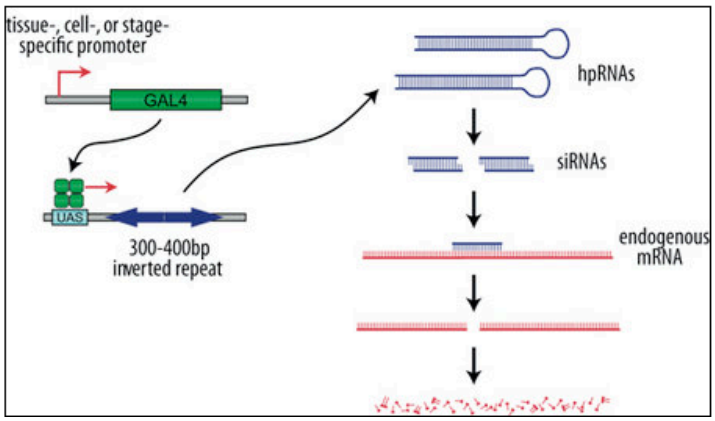

Figure 1. *GAL4/UAS* system drives the expression of a double-stranded hairpin RNA (hpRNA) to induce sequence-specific degradation of the target mRNA (http://stockcenter.vdrc.at/control/rnailibrary).

Playback technique

Two of the data chapters in this thesis (**2** and **3**) take advantage of an acoustic playback technique to stimulate female mating response with a controlled acoustic signal. I have used three types of artificial songs that vary in rhythmic characteristics: a song with a mean IPI and IPI cycle length corresponding to *D. melanogaster*, an arrhythmic song with invariable mean IPI matching to that of a *D. melanogaster,* and a song with a mean IPI and IPI cycle length corresponding to *D. simulans.* The song parameters are presented in the respective chapters and the song synthesis has been described in detail in Ritchie *et al.* (1999).

Study species 1: Drosophila melanogaster

In **chapters 2-4** I have used *Drosophila melanogaster*, which is an insect of the order Diptera, and the best known member of the large *Drosophila* genus. It has been used for genetic analyses for over a century (MORGAN 1910), and is arguably the best studied invertebrate model species. The genetic architecture of this species has been characterised in detail, but also its reproductive behaviour under laboratory conditions is well understood, which makes it an ideal study system.

The *D. melanogaster* genome contains approximately 14,000 genes. The nucleus carries 4 pairs of chromosomes: sex chromosomes (X/Y), 2 autosomes and a 'dot' fourth chromosome with only a few genes. The genome has been fully sequenced (ADAMS *et al.* 2000), and the vast majority of the genes have been annotated and functionally characterised.

Males and females of this species are sexually dimorphic in a range of morphological and behavioural characteristics. Females are larger than males and

show a different abdominal pigmentation pattern, and males carry modified bristles on the tarsal segment of their front legs known as sex combs. Sexes also show difference in cuticular hydrocarbons (CHCs) that act as sexual pheromones. The courtship is multimodal and involves visual, auditory and chemosensory signals (GREENSPAN and FERVEUR 2000). During the courtship male orients towards the females, follows her and taps her abdomen with his forelegs containing the sex combs. Male then performs a courtship song by extending his wing and rapidly vibrating it. Courtship song is followed by genital licking and the male attempts to mount the female, who can accept the male or reject him by jumping away, flicking wings, kicking with front legs or extruding ovipositor. Song and pheromones are the two most important sexual signals that affect male mating success (RYBAK *et al.* 2002; TALYN and DOWSE 2004), and in this thesis I have focused on female acoustic preference for male song (**Chapters 2** and **3**).

Courtship song

Male courtship song consists of two main components; pulse song and sine song (Figure 2) (EWING and BENNETT-CLARK 1968). The sine song produces a humming sound with a frequency between 160 and 170 Hz (MORAN and KYRIACOU 2009). Pulse song consists of repetitive trains of pulses, varying from 2 to 50 pulses per train. Each of these pulses is made up of 1-3 cycles and lasts between 3-8 msec (KYRIACOU and HALL 1982). The pulses are separated by inter-pulse intervals (IPI), which can fluctuate widely within the courtship (KYRIACOU *et al.* 1992). The average length of IPI in *D. melanogaster* is ~35 msec (EWING and BENNETT-CLARK 1968), but commonly varies between 30-40 msec, although IPI's as short as 15 msec and as long as 100 msec may be produced (KYRIACOU *et al.* 1992). The mean IPI oscillates in a

sinusoidal pattern, with a cycle period of 55-60 sec in *melanogaster* (KYRIACOU and HALL 1980; 1986; 1990). Thus, the fly song consists of two rhythmic components - the basic repetitive IPI and the superimposed IPI oscillation - and the sine song. Each species in the *melanogaster* species subgroup has a unique courtship song (EWING and BENNETT-CLARK 1968) that varies particularly in the mean IPI and IPI cycle length (reviewed in KYRIACOU *et al.* 1992; TOMARU *et al.* 2000).

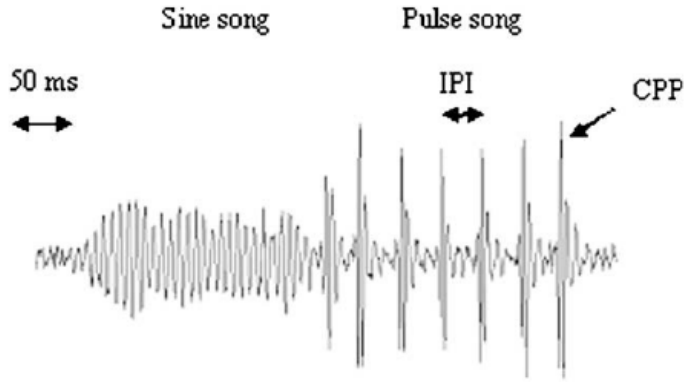

Figure 2. A phrase of *D. melanogaster* song (MORAN and KYRIACOU 2009). Sine song, pulse song, inter-pulse interval (IPI) and cycles per pulse (CPP) are shown.

Song reception

Female flies detect the courtship song with antenna that serve several functions. They form the olfactory organs, gravity sensors and constitute the hearing organs sensitive to the particle velocity component of airborne sound (GOPFERT and ROBERT 2002; KAMIKOUCHI *et al.* 2009; YOROZU *et al.* 2009). The antennae in *Drosophila,* as in most flies, are composed of three segments including (from proximal to distal) the scape, the pedicel and the funiculus, which also carries the elongated and branched feather-like arista (Figure 3). The aristae and the funiculus vibrate sympathetically in response to acoustic stimuli and (analogous to human eardrums) serve the reception of

sound (GOPFERT and ROBERT 2002). Vibrations of this antennal receiver are picked up by the Johnston's organ (JO). JO is a mechanosensory chordotonal organ situated in the pedicel of the antennae and it consists of ~480 primary sensory neurons (KAMIKOUCHI *et al.* 2009). The role of the antennae in hearing has been demonstrated by physiological ablation of antennae and isolation of audition-impairing and -demolishing mutations, which also support the conclusion that auditory reception is critical for sexual receptivity (see e.g. GOPFERT and ROBERT 2002; TAUBER and EBERL 2003 for reviews).

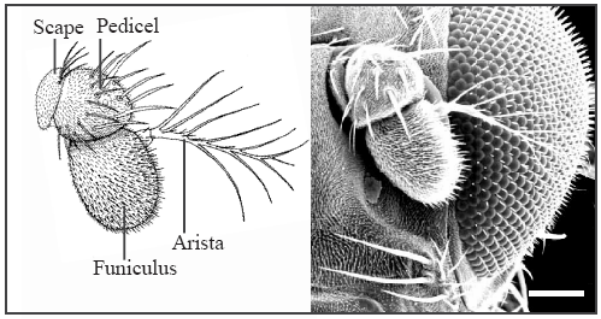

Figure 3. Three segments in *D. melanogaster* antennae. Schematic drawing (left) and scanning electron micrograph image (right) (GOPFERT and ROBERT 2002).

Study species 2: Drosophila pseudoobscura

Drosophila pseudoobscura is used as a study species in **chapter 5**. A member of the *obscura* group, it was the second *Drosophila* species sequenced (RICHARDS *et al.* 2005). *D. pseudoobscura* has five chromosomes: a metacentric X chromosome (with arms named XL and XR), Y chromosome, three large telocentric autosomes (chromosomes 2-4), and a fifth, "dot" autosome. The genome contains nearly 19,000 predicted genes (JIANG and MACHADO 2009).

D. pseudoobscura is a naturally polyandrous species with a similar courtship to *D. melanogaster*. However, the mating system of this species differs in that *D. pseudoobscura* males are sperm heteromorphic. Alongside long sperm used for fertilization ('eusperm'), males produce short 'parasperm', which are not capable of fertilization (SNOOK and KARR 1998), but appear to protect the former from female-mediated sperm death inside female reproductive tract (HOLMAN and SNOOK 2008).

The *D.pseudoobscura* flies used in **chapter 5** were experimentally evolved females from populations that have been reared under either elevated levels of polyandry (E; one female housed together with six males) or monandry (M; a single female and a male housed together), for 100 generations. These selection lines have been described previously in detail (CRUDGINGTON *et al.* 2005) and I will only briefly describe them here. The stock was established from 50 wild-caught females collected in November 2001, and the original baseline population was subsequently divided into four replicate populations, each of which was separated into E and M treatments. The numbers of male-female 'family groups' comprising the M and E lines were 80 and 40, respectively, to ensure monogamy treatment group does not have a lower effective population sizes (Ne), which could increase the effect of random drift. These family sizes cause M to have higher Ne compared to E both in autosomal and X-linked loci, however both of the treatments have Ne higher than 100 and harbour equal genetic diversity (SNOOK *et al.* 2009).

For each non-overlapping generation of maintaining the selection lines, the sexes were allowed to interact for five days, after which they were transferred to fresh vials for another five days to reduce any effect of larval overcrowding. Females were allowed to oviposit freely during this time. The flies that eclosed on the first day were discarded to eliminate selection for early emergence or shorter developmental time

and only the progeny produced in the second set of vials was used for the next generation. Of the flies eclosing on day two and onwards, the sexes were isolated and held until reproductively mature. The progeny from each family per treatment were pooled and the requisite numbers were randomly selected for the next cycle of male-female interactions. This allowed differential reproduction across families to be reflected in the genetic composition of subsequent generations: offspring of more fecund females occur in higher proportion of eclosed progeny, and therefore will have a higher representation among the flies chosen for the next generation. These selection regimes have been effective in producing a number of behavioural and morphological differences in both males and females between the treatment groups (BACIGALUPE *et al.* 2008; CRUDGINGTON *et al.* 2005; CRUDGINGTON *et al.* 2009; CRUDGINGTON *et al.* 2010; SNOOK *et al.* 2005). For more information about this see chapter 5.

Outline and aims of the thesis

The aim of this thesis has been to identify genes and genetic processes in females that are involved in sexual interactions important for female-male coevolution. To achieve this I have studied multiple stages of female reproduction: from pre-mating preference and the transcriptome response to courtship signalling, to post-mating gene expression and the evolution of sexually dimorphic gene expression.

Divergence in female preference and male courtship signal is important for the process of speciation, because it can generate pre-mating isolation through assortative mating. Theory predicts that a pleiotropic effect of a single gene or a tight physical linkage between the genes underlying both male courtship signal and female preference facilitates their divergence by maintaining coordination between the two

(BUTLIN and RITCHIE 1989). In **Chapter 2** of this thesis I focus on the pleiotropic effect of a candidate gene on song preference known to influence species-specific courtship song in *D. melanogaster*.

Little work has been done in identifying genes associated with female courtship response in *Drosophila* (but see e.g. LAWNICZAK and BEGUN 2004), apart from characterising sensory organ receptors and their neural projections (often tested in males) (KAMIKOUCHI *et al.* 2009; KAUPP 2010). In **Chapter 3** I explore the genome-wide female gene expression response to male courtship song in *D. melanogaster*. The findings of this chapter lead to **Chapter 4**, in which I test the effect of two immunity genes on female post-mating fitness. In this chapter I also briefly review the current literature on the function of immunity genes in female reproduction and discuss their potential role in sexually antagonistic molecular interactions, in the light of my findings.

As outlined above in this introduction, female promiscuity causes sexual selection and potential for sexual conflict, something which is predicted to cause intense selection on females. In **Chapter 5** I explore the consequences of experimental mating system variation on female transcriptome divergence using the experimental evolution approach with *D. pseudoobscura*. I focus particularly on the patterns of sexually dimorphic gene expression and how selection has shaped these in females. I also characterise the gene expression response to mating in this species and quantify the effect of male sexual selection history on female post-mating response.

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CHAPTER 2

Does the *period* gene affect female song recognition in *Drosophila melanogaster*?

Abstract

The evolution of sexual signalling systems is integral to the process of speciation, as it can both initiate divergence and discourage gene flow between incipient species. Theory predicts that a physical linkage between genes underlying both signal and preference reduces recombination in the face of occasional hybridisation and hence facilitates divergence. However, currently there are few examples of tight genetic linkage connecting both the emission and preference of sexual signals. The X-linked transcription factor *period (per)* in *Drosophila* encodes the species-specific ultradian cycle in inter-pulse interval (IPI) in the male courtship song, and here I test the hypothesis that the same gene influences female perception of cycle length. I show that wild-type females of *D. melanogaster* are better stimulated by the song model containing a conspecific IPI cycle, whereas null mutant (per^{01}) and transgenic strains with abolished *per* expression in the peripheral nervous system ($per^{01}7.2:2^{(x)}$) are stimulated equally well with both conspecific and heterospecific song models, as well as by a conspecific song with a constant IPI. I test further the potential role of *per* expression in the fly hearing neurons in the Johnston's Organ (JO) for song recognition using *UAS/GAL4* binary system with two independent lines carrying RNA interference targeted to JO neurons. The results from this experiment are inconclusive. In a third experiment I test the hypothesis that sexual isolation due to song recognition varies with circadian mating activity encoded by *per*, and is strongest at the time of peak mating activity of sympatric *D. simulans* which could reduce the risk of inter-specific matings. No support was found for this. The results show that the *period* gene provides a promising but inconclusive example of a pleiotropic influence of a single gene on both song and preference that can facilitate divergence in sexual signaling.

Introduction

Traits involved in sexual communication often differ substantially between sister-species, demonstrating rapid divergence in mating signals and preferences. Differences in sexual signalling systems reduce gene flow between populations and maintain genetic boundaries between closely related species, which makes their evolution central to the process of speciation (PANHUIS *et al.* 2001; RITCHIE 2007). Diversification of sexual signals is constrained, however, by the need to maintain coordination between signals and their reception (BUTLIN and RITCHIE 1989). Indeed, signalling systems often experience stabilizing selection within species whereby extreme signal values are often perceived as less attractive (BROOKS *et al.* 2005) that ensures maintenance of coordination. This enhances assortative mating and thus strengthens reproductive boundaries between species. However, selection against novel variants also reduces the potential for evolutionary divergence for these traits. How do the signalling systems diversify in the face of stabilizing selection? Concerted changes in both signals and preferences can be achieved through genetic covariance between the components, which can occur due to coevolution of genetically independent traits (linkage disequilibrium), due to physical linkage of separate loci or a pleiotropic influence of shared loci (BOAKE 1991; BUTLIN and RITCHIE 1989).

According to coevolutionary models the genetic covariance of independent loci for signal and preference can arise through assortative mating, as offspring inherit genes for both preference and signal generating linkage disequilibrium between the two (KIRKPATRICK 1982; LANDE 1981). Sexual selection theory suggests several mechanisms how assortative mate choice can arise (ANDERSSON and SIMMONS 2006). Speciation models requiring linkage disequilibrium, however, are considered

evolutionarily unstable because of their reliance on the maintenance of strong assortative mating: the genetic covariance can break down with recombination if occasional hybridisations occur (FELSENSTEIN 1981), or if preferences are expressed only under limited conditions (such as nutritional or seasonal variation) (HEBETS *et al.* 2008; HUNT *et al.* 2005; MILNER *et al.* 2010; WOODGATE *et al.* 2010). Tight physical linkage and particularly pleiotropy, on the other hand, protect the genetic association from recombination, therefore buffering differentiated mate recognition systems against the effects of hybridization. Moreover, if the signal and receptor are affected by the same allele of a single locus (known as genetic coupling), a new mutation will immediately add genetic variation to both of the traits and establish genetic covariance (BUTLIN and RITCHIE 1989; FELSENSTEIN 1981). The positive genetic covariance required for the mechanisms of sexual selection that arise due to physically linked or shared loci can therefore facilitate divergence more easily than non-pleiotropic or unlinked loci. This idea has a long history, but still only little empirical evidence (SHAW *et al.* 2011).

The *period (per)* gene in *Drosophila* is a candidate locus with a pleiotropic influence on both male sexual signal and female preference (KYRIACOU *et al.* 1992). *Per* is an X-chromosome linked transcription factor that determines periodicity in a range of rhythmic processes, such as circadian cycles of locomotor activity and adult eclosion (KONOPKA and BENZER 1971). In *D. melanogaster* and *D. simulans per* also determines the length of a species-specific ultradian cycle in the interpulse interval (IPI) of the male courtship song (55-60 sec and 35-40 sec, respectively) (KYRIACOU *et al.* 1990; WHEELER *et al.* 1991). Initially, three mutant strains were discovered that show either a shortened period of rhythmic eclosion and adult activity (per^{short}), a lengthened period in both rhythms (per^{long}), or complete arrhythmicity (per^{0})

(KONOPKA and BENZER 1971). Kyriacou and Hall (1980) demonstrated a pleiotropic effect of the same three *per* mutations on *D. melanogaster* song IPI cycle, either shortening, lengthening or abolishing the oscillations. Species-specific differences in the song cycle were mapped to *per* using genetic transformation experiments (WHEELER *et al.* 1991), whereby inserting the *D. simulans per* allele into an arrhythmic *D. melanogaster* per^{0} strain caused males to produce a song with *simulans*-like characteristics while insertion of *D. melanogaster* per^{+} allele rescued the wild-type song.

Song produced by male wing vibration is important for male mating success, as demonstrated by a number of studies that show reduced success of wingless mute males and its partial recovery by adding playback song (EWING and BENNETT-CLARK 1968; GREENACRE *et al.* 1993; KYRIACOU and HALL 1982; KYRIACOU and HALL 1986; RITCHIE *et al.* 1999; RYBAK *et al.* 2002). The species-specific aspects of the song; IPI and the sinusoidal IPI cycle length, also contribute to species discrimination: synthetic song with the correct combination of mean IPI and cycle length stimulates *D. melanogaster* and *D. simulans* females in a species-specific manner (KYRIACOU and HALL 1980; 1982; 1986; RITCHIE *et al.* 1999).

Because *per* influences multiple temporal traits, it is possible that it could also influence female perception of song rhythm. Greenacre *et al.* (1993) tested this by exposing isogenic females carrying different *per* alleles (per^{short}, per^{long}, per^{0}, per^{+}) to playback songs that corresponded to those produced by different mutant males (short cycle=40s, long cycle=80s, wild type song cycle=55s), in the presence of wingless males. Females did not show preference for 'homoallelic' song types, therefore not supporting a strict genetic coupling hypothesis. However, null mutant females of *per* did not differentiate between any song types (while discriminating against males in

the absence of song), supporting some effect of *per* in female ability to recognize and be stimulated by song.

Greenacre *et al*. (1993) used mutant strains with systemic expression. Here I re-visit the idea that *per* could influence female mating discrimination based on song in *D. melanogaster,* but with the aim of testing further how tissue-specific expression of *per* might affect female behaviour. First I repeated Greenacre *et al*.'s (1993) approach to test whether the systemic lack of *per* expression influences female's ability to differentiate between con- and heterospecific songs using a loss-of-function mutant strain. I then explored the role of *per* expression in the peripheral nervous system using a transgenic strain where per^{+} expression is restricted to certain clock neurons within the central brain (FRISCH *et al.* 1994). This strain carries a 7.2kb per^{+} transgene inserted into per^{01} background. This fragment can robustly rescue the locomotor arrhythmicity of the host strain (FRISCH *et al.* 1994). However, circadian olfactory responses (KRISHNAN *et al.* 1999; LEVINE *et al.* 2002), as well as cyclic pheromone synthesis (KRUPP *et al.* 2008a) are abolished. Antibody staining has demonstrated that in this strain PER expression is restricted only to a subset of lateral neurons of the anterior margins of the optic lobe (FRISCH *et al.* 1994). Thus, *per* expression remains abolished in the peripheral nervous system including the antennal nerve, where *per* expression has been demonstrated in wild-type flies (PLAUTZ *et al.* 1997).

Flies detect courtship song with Johnston Organ (JO) neurons located in the pedicel segment of their antenna (KAMIKOUCHI *et al.* 2009; YOROZU *et al.* 2009). I therefore hypothesized that *per* may influence song recognition via its expression in the antennal neurons. In the second experiment I tested this idea further by using RNA interference (RNAi), which is a powerful technique for inducing targeted

suppression of gene expression in *D. melanogaster* (e.g. DUFFY 2002). I generated two independent lines carrying *per* sense-antisense transgenes, with their expression driven exclusively in the JO using GAL4, and tested whether the lack of expression in the JO neurons interferes with song discrimination.

In addition to directly affecting song recognition, the *period* gene could also influence assortative mating or isolation via its effect on species-specific circadian mating activity rhythms (SAKAI and ISHIDA 2001; TAUBER *et al.* 2003). Sakai and Ishida (2001) showed how the mating frequency of *D. melanogaster* is suppressed at the time when sympatric sister species *D. simulans* frequently mates (at dusk, *zeitgeber time* ZT12) and vice versa. Tauber *et al.* (2003) confirmed a similar pattern between *D. melanogaster* and *D. pseudoobscura*, and using transgenic flies showed that *period* conveys information about the species-specific mating activity rhythm. This study also demonstrated assortative mating for a conspecific *period* allele in the transgenic flies that varied in strength in a circadian way, being highest at dusk.

The mating-activity patterns may be generated by circadian rhythms in pheromone release or responsiveness to auditory signals. Indeed, the *period* gene shows a circadian expression pattern in male pheromone producing coenocyte cells (KRUPP *et al.* 2008b) and also the chemosensory cells in the antennae express clock gene cycles, which generate circadian physiological rhythms in response to odorants (KRISHNAN *et al.* 1999). If the circadian production and perception of sexual signals drive the mating activity patterns, they may also influence the temporal pattern of sexual isolation as a by-product. This kind of temporal mating isolation may be favoured as a strategy against hybridization during the time of day when the activity of sympatric sister species is high. In the third experiment I tested the possibility that the pattern of song preference and thus the relative strength of sexual isolation

changes in a circadian manner in *D. melanogaster*, which I predicted to be highest at dusk.

Materials and Methods

Strains

In experiment 1 I used Oregon-K wild-type (control females and all males), null-mutant per^{01} and a transgenic $per^{01}7.2{:}2^{(x)}$ (kindly provided by Jean-Christophe Billeter). $per^{01}7.2{:}2^{(x)}$ has been described previously (FRISCH *et al.* 1994; KRISHNAN *et al.* 1999; KRUPP *et al.* 2008a; LEVINE *et al.* 2002), but briefly, these flies were generated by inserting a 7.2kb fragment of *per* DNA into per^{01} host strain (insertion in X chromosome). This DNA fragment lacks 5' flanking material, *per's* first (noncoding) exon and most of the 2.3kb first intron. However the remaining sequence rescues *per* expression in a subset of cells in the central nervous system enabling normal circadian locomotor activity rhythm (FRISCH *et al.* 1994).

In the second experiment I generated two independent lines of *per* knockdown flies by crossing females carrying either one of the two RNAi transgenes (*UAS-PER-IR1*, strain no. 31285; *UAS-PER-IR2*, strain no. 31659, both obtained from Bloomington Stock Centre) with males carrying JO neuron specific *GAL4* (*JO-GAL4*, strain no. NPO761, kindly provided by J. Albert). Both of these strains were homozygous viable. I confirmed that the *GAL4* construct drives JO specific expression of a UAS-transgene by assessing the expression of *UAS-GFP* reporter gene (*UAS-mCD8::GFP*, kindly provided by S. Goodwin) driven under *JO-GAL4*, using a fluorescent microscope (Supplementary Figure 1). The parental strains (*UAS-*

IR-PER1, *UAS-IR-PER2*, *JO-GAL4*) and a wild-type Canton-S were used as controls, and Canton-S males were used with all females.

In experiment 3 I used an Oregon-K wild-type strain. Flies were reared in12-h light and dark cycle, in un-crowded cultures at 23°C on a cornmeal-sugar-agar-yeast medium, with added dried baker's yeast. For all three experiments, virgin flies were collected during six hours after eclosion under light anesthesia and housed in same-sex groups of 20 flies or less. When three days old, males (Oregon-K for experiments 1 and 3, Canton-S for experiment 2) had their wings removed to prevent them from producing song, after which they were housed individually in new vials. Flies were used in playback experiments when five days old.

Experiment 1: Effect of the period *gene on song recognition*

In order to test whether the *per* gene influences female song discrimination, I compared the cumulative proportion of mated females from Oregon-K, per^{01} and per^{01}*7.2:2* strains when stimulated with three types of artificial songs or white noise (control). The songs differed only in rhythmic characteristics: conspecific *D. melanogaster*- like song has the mean IPI of ~35ms and IPI cycle of 55s, arrhythmic conspecific song has the mean IPI of ~35ms but no ultradian cycle, while heterospecific *D. simulans*-like song has the mean IPI of ~45ms an IPI cycle length of 40s (song synthesis is described in RITCHIE *et al.* 1999). In each trial 20 virgin females from each strain and wingless Oregon-K males were aspirated into an acoustically transparent mating chamber mounted over a loudspeaker. Song stimuli, or white noise, were played back to flies at a volume of 80dB, and the number of copulating pairs was counted in every minute over a period of 15 minutes. I

conducted 10 trials and thus tested 200 pairs per strain and treatment. The trials were carried out within two hours during the peak mating activity time (ZT5-7, personal observation and SAKAI and ISHIDA 2001) at 24-25^{o}C with playback order randomised.

Experiment 2: Effect of per *expression in JO neurons on song recognition using RNAi*

In the second experiment I tested the effect of the same four treatments (conspecific, heterospecific and arrhythmic songs or white noise, as described above) on the cumulative proportion of mated females from the two *per* knockdown strains (*JO-GAL4/UAS-PER-IR1*; *JO-GAL4/UAS-PER-IR2*) and their parental control strains (*JO-GAL4; UAS-PER-IR1; UAS-PER-IR2),* and Canton-S as a wild-type control (only conspecific and heterospecific songs tested). I conducted 10 replicate trials for both conspecific and heterospecific song treatments for Canton-s, *JO-GAL4; UAS-PER-IR2* and *JO-GAL4/UAS-PER-IR2,* eight replicate trials for these treatments for *UAS-PER-IR* and *JO-GAL4/UAS-PER-IR1,* and five replicates trials for each strain for white noise and arrhythmic song treatments. I followed the same experimental procedures as in the experiment 1, with the exception of extending the treatment duration to 20 minutes. The trials were carried out within four hours (ZT1-5) at 21-24^{o}C with playback order randomised.

Experiment 3: Effect of circadian mating activity on the level of song discrimination

In the third experiment I tested whether the strength of female song discrimination varies with circadian mating activity time, using the Oregon-K strain. 20 virgin females and wingless males were stimulated with either conspecific *D. melanogaster*-like song or with heterospecific *D. simulans*-like song (as described above) for 20 minutes, and the number of mated pairs was counted every two minutes. I observed

matings at the peak activity time (ZT5-7) and at the time of reduced mating activity (ZT10-12) (SAKAI and ISHIDA 2001). For each song treatment and activity time I conducted 10 trials, thus testing 200 pairs per song and time. All trials were carried out at 24-25^{o}C with playback order randomised.

For the experiments 1 and 2 I analysed the mating data in two ways. In the first analysis I assessed the differences in mating frequency between strains and songs, using ANCOVA with binomial error distribution (GLM) where the proportion of mated females after 15 min (out of 20) was used as the response variable. This analysis allows us to test the effect of song and strain on the average mating frequency at the end of the observation time. However, in order to test whether the rate of mating is affected differently, I conducted a second analysis where we tested the effect of song and strain in interaction with time on the mating response. I did this with a mixed effects ANCOVA (GLMM) where the cumulative proportion of mated females (out of 200 per song, or less) was used as the response variable, observation time as a covariant and replicate as a random factor (also date in the experiment 2). In both of the analyses the strain and treatment were fitted as fixed factors and temperature as a covariate.

I analysed the data from experiment 3 otherwise similarly, but this time song type and activity time were fitted as fixed factors in both of the analyses (GLM and GLMM). For all experiments the significance of terms from the GLMM are reported from model comparisons and for the GLMs from the final model's Analysis of Deviance tables after model reductions. Model reductions were performed by excluding non-significant terms one at a time from the full model, starting from the least significant term, and subsequently comparing the reduced model to the previous model using the p-value information. The analyses were done with R version 2.13.0

(RDEVELOPMENTCORETEAM 2011). In the experiment 2, two-sample t-tests were additionally used to compare the mean mating frequencies with conspecific and heterospecific song treatments for Canton-S and *JO-GAL4/UAS-PER-IR2*.

Results

Experiment 1

The first experiment tested the effect of *per* null mutation and *per* 7.2 transgene on female song recognition. There were significant main effects of the strain and song treatment, as well as a significant interaction between the two, in both of the models (Table1, Figure 1). The second model, mixed effects ANCOVA with observation time as a covariate, also showed an effect of strain and song treatment on the cumulative pattern of mating frequency as there was a significant interaction between each of the factors and time. This suggests that the mating speed also differed between the strains and songs (Table1, Figure 2).

Wild-type Oregon-K females mated significantly more when stimulated with a conspecific rhythmic song compared to the other treatments across the observation period (Figure 2a). Null-mutant per^{01} females did not discriminate against heterospecific or arrhythmic songs, however overall the levels of mating were low and did not increase due to the presence of song (Figures 1, 2b). The transgenic per^{01}*7.2:2*$^{(x)}$ females mated significantly more in the presence of song, but did not differentiate between the song treatments (Figures 1, 2c).

Experiment 2

I tested how *per* expression in the antennal auditory organs affects song discrimination using two independent knockdown lines with RNAi targeted to JO neurons. There were significant main effects of both strain and song in both of the models (ANCOVA GLM and GLMM with time as a covariate), however no interaction between the two (Table 1). The model assessing the differences in the cumulative proportion mated over time showed a significant interaction between strain and time, but no interaction with song (ANCOVA GLMM, Table 1).

Wild-type Canton-S mated significantly more when stimulated with the conspecific compared to heterospecific song model (T-test: $t_{(18)}$=1.8, p=0.045; Figures 3, 4a). Overall the *JO-GAL4* strain mated least of all the strains and all the strains mated less in the absence of song compared to acoustic stimulation (Figures 3, 4b-f). However, I did not detect any significant difference between the three song treatments for any of the three parental control strains or for the two knockdown lines (Figures 3, 4b-f), although the knockdown strain JO-GAL4/UAS-PER-IR2 had a tendency for increased mating when stimulated with conspecific compared to heterospecific song (T-test: $t_{(18)}$=1.6, p=0.06; Figure 3, 4f).

Experiment 3

In the third experiment I tested whether the strength of song discrimination depends on the time of day, and thus mating activity. Wild-type females mated significantly more when stimulated with the song model of *D. melanogaster* compared to that of *D. simulans* (Table 1, Figures 5, 6). The time of day also significantly affected the mating activity: flies mated more frequently 5-7 hours after dawn (lights on) than at

dusk (Table 1, Figures 5, 6). However, there was no effect of the time of day upon female discrimination level, as I did not detect any significant interaction between the song type and time of day (Table 1).

Discussion

The results demonstrate the stimulatory effect of the presence of song for female mating behaviour, confirming previous findings (EWING and BENNETT-CLARK 1968; GREENACRE *et al.* 1993; KYRIACOU and HALL 1982; KYRIACOU and HALL 1986; RITCHIE *et al.* 1999; RYBAK *et al.* 2002). Moreover, wild-type females showed a higher mating frequency and speed when stimulated with a conspecific compared to a heterospecific song in all three experiments, which corroborates that song has the ability to contribute to pre-mating isolation in *Drosophila* (KYRIACOU and HALL 1980; 1982; 1986; RITCHIE *et al.* 1999; TOMARU *et al.* 2000). In the first experiment the song with arrhythmic IPI was no more stimulating than a heterospecific song for wild-type females, which demonstrates that the IPI cycle is important for female mating stimulation.

The *period* gene affected female mating behaviour. In experiment one, the lack of discrimination against *D. simulans* song in the null-mutant per^{01} is in agreement with Greenacre *et al.* (1993), who found no difference in female mating frequency depending on the IPI cycle length. The transgenic $per^{01}7.2{:}2^{(x)}$ females, who have normal gene expression in the central nervous system, mated significantly more in the presence of song, but did not differentiate between the song treatments. This finding demonstrates more clearly how the *period* gene can influence song recognition, by apparently abolishing heterospecific song discrimination. This effect

is independent of circadian locomotor behaviour that remains intact in these flies (FRISCH *et al.* 1994).

Previous studies have demonstrated that *per* expression in $per^{01}7.2:2^{(x)}$ strain is largely absent from the peripheral nervous system (FRISCH *et al.* 1994), including the antennal nerve (KRISHNAN *et al.* 1999). Fly hearing organs are located in the third antennal segment, the pedicel (GOPFERT and ROBERT 2001; KAMIKOUCHI *et al.* 2009; TAUBER and EBERL 2003), and thus *per* expression in the antennal neurons could play an important role in sexual discrimination based on female auditory perception of the rhythmic components in the song. Our second experiment aimed to test this possibility more explicitly by abolishing *per* expression exclusively in JO neurons. The results from this experiment are inconclusive: one of the knockdown strains (*UAS-PER-IR1*) showed a lack of wild-type preference, while the other (*UAS-PER-IR2*) showed a tendency to prefer conspecific song. Moreover, the parental control strains did not show a significant preference for conspecific song, and therefore whether there is an effect of *per* RNAi in JO on the song preference or not cannot be determined from these results.

In the third experiment I tested another way by which *per* could influence female song discrimination – through its effect upon circadian mating activity (TAUBER *et al.* 2003). I found that the time of day influenced female mating frequency, which was significantly higher 5-7ZT than at dusk, confirming the pattern observed previously (SAKAI and ISHIDA 2001). However, there was no effect of the time of day upon female song discrimination, as I did not detect any significant interaction between the song type and time of day on female mating frequency. Thus, I found no support for the prediction that sexual isolation due to song recognition

might vary with circadian mating activity, which could reduce the risk of inter-specific matings at the time of peak mating activity of sympatric *D. simulans*.

The results demonstrate the possibility that species-specific features of song and female preference evolve under pleiotropic control of the same gene: lack of *per* expression abolishes both the IPI cycle (KYRIACOU and HALL 1980) and female ability to discriminate males based on the cycle length (GREENACRE *et al.* 1993; this study). The results further demonstrate how *per* expression in the peripheral nervous system appears to be critical for the female song recognition, although a role for JO neurons is unclear. Song cycle and its preference, however, are not genetically coupled in the strictest sense: previous work has shown that females do not mate assortatively with males carrying the same repetitive sequence on the fifth exon of the *per* locus that determines the length of the IPI cycle (RITCHIE and KYRIACOU 1994). This sequence encodes threonine-glycine repeats, and it is included in the 7.2kb fragment inserted into the $per^{01}7.2{:}2^{(x)}$, thus further suggesting that its presence does not rescue song discrimination. Instead, this strain largely lacks the 5' flanking region of *per*, and together with the *per* expression pattern in this strain (FRISCH *et al.* 1994) suggests the importance of regulatory elements on the female mating behaviour through their effect on the patterns of tissue-specific expression.

So far only two studies have demonstrated an effect of a single mutation on both the emission and perception of a trait (SHAW *et al.* 2011). Fukamachi *et al*. (2009) found that a deletion in a gene *somatolactin alpha* reduces skin pigmentation in the Japanese medaka fish, and demonstrated how wild-type males discriminate against these mutant females while mutant males showed assortative preference for them. The second and best-studied example comes from *D. melanogaster,* where cuticular hydrocarbon (CHC) profiles are sexually dimorphic and act as short-range or

contact pheromones in sexual discrimination and sexual isolation (FERVEUR *et al.* 2008). One of the genes encoding the CHC composition is *desat1* (COYNE et al. 1999). Marcillac *et al.* (2005) showed that a transposable element insertion into *desat1* locus reduced sex differences in CHC profile and simultaneously decreased male ability to discriminate between the sexes. A recent study by the group further demonstrated a mechanism for this pleiotropic influence by finding distinct regulatory regions that drive tissue-specific expression of this gene. These separate regulatory sequences targeted the expression in non-neuronal cells for pheromone synthesis and in neuronal cells for pheromone perception (BOUSQUET *et al.* 2012).

The study by Bousquet *et al*. (2012) highlights the need to consider the mechanism of pleiotropy when determining whether a locus indeed has a coupled effect or not. Two major ways have been proposed through which a single gene can have a pleiotropic effect on multiple traits: a gene can either have multiple functions - through alternative splicing, RNA modification, or tissue-/developmental-specific expression - or a gene can maintain the same molecular function but in different pathways (SINGH and SHAW 2012). The separate regulatory regions coding the same *desat1* transcript in different tissues found in Bousquet *et al.* (2012) points to the former mechanism. Such a mechanism will not, however, automatically produce a coupled effect on sexual signal and preference, but should instead have different consequences on the potential for coupling (and therefore the speed and likelihood of divergence) depending on where in the sequence a new mutation arises. If a mutation targets the coding region, it will have a direct consequence on both the trait and preference. However, if the mutation occurs in a distinct regulatory region, it will only affect the expression of one of the traits (STERN and ORGOGOZO 2008). In such a case, co-evolutionary change is required for maintaining synchrony between the signal and

preference. Thus, without identifying the underlying mechanism it is impossible to disentangle the genetic coupling effect from co-evolutionary models, and therefore discovering that a locus has a common effect on male and female traits does not necessarily mean an evolutionarily coupled effect. Nevertheless, even if separate alleles are involved, the pleiotropic consequence of a single gene will facilitate divergence due to lack of recombination, which ensures the co-inheritance of the two traits. The *period* locus is likely to be an example of such pleiotropic gene without strict coupling.

The studies on medaka and *D. melanogaster* provide support for genetic coupling caused by a single mutation, however, whether natural genetic variation exists that could produce a coupled divergence of mating signal and preference is currently unknown. Two recent examples have explored a potential pleiotropy in relevant traits in the wild, and have identified quantitative trait loci (QTL) for both trait and preference that map to the same chromosomal locations using genetic association techniques (KRONFORST *et al.* 2006; SHAW and LESNICK 2009; WILEY *et al.* 2012). However, although trait and preference co-localize within the same region it is not yet known whether the genes underlying the QTLs are the same or if they are tightly linked.

These studies highlight the need to identify an ecologically relevant example where a single locus is known to affect the trait and preference, influencing reproductive isolation. Does the *per*-encoded song-preference inheritance in *Drosophila* have the potential to fulfill these criteria? The experiment on wild-type song preference demonstrates that a heterospecific song cycle is enough to reduce attractiveness of conspecific males, making it potentially an important signal contributing to behavioural isolation (see also RITCHIE *et al.* 1999). In addition to

mutational and transgenic evidence, it is important to demonstrate that the pleiotropic locus also harbours natural genetic variation that affects the trait and preference. The repetitive threonine-glycine sequence in *period* that affects male song, shows natural polymorphism in *D. melanogaster* (COSTA *et al.* 1992), as well as hallmarks of past selection (COSTA and KYRIACOU 1998). However, in order to demonstrate relevant natural variation also occurs in song preference we need to understand better how the *per* locus encodes this trait in females. There are several reasons why this may be particularly hard. The finding that the effect of *per* appears restricted to peripheral tissues suggests that a mechanism for tissue-specific regulation for expression must be in place. Also, *per* is a transcription factor and therefore it is possible that the relevant species-specific variation resides in a downstream component of the pathway expressed in the peripheral tissues rather than in *per* sequence itself. With the molecular tools available for *Drosophila* it will be possible to unravel further the genetic mechanism underlying song preference.

I have thus demonstrated that rhythmic components in male song influence female mating propensity, and that expression of the *period* gene in the peripheral nervous system plays a role in this. *Period* provides a promising example of a pleiotropic influence of a single gene that can facilitate divergence in sexual signaling. *Per* also affects other, naturally selected traits that are likely involved in local adaptations, such as the circadian locomotor rhythm (PETERSEN *et al.* 1988). Variable day length, for example, is likely to cause divergent selection on *per* locus (KYRIACOU *et al.* 2008; MIYATAKE 2002), which may consequently affect also the song and preference. Theory predicts that such effects would be particularly efficient in generating divergence, because if a locus affects local adaptation and is therefore under divergent selection, it will generate reproductive isolation as an automatic by-

product (such traits are sometimes named 'magic traits') (reviewed in SERVEDIO *et al.* 2011). Dissecting further the specific mechanism of how *per* affects song recognition will help to understand the degree to which the same gene encodes the two traits in sexual signaling, what are the other genetic components involved, as well as the influence of other pleiotropic naturally selected traits. Only then can we start to disentangle the different predictions of pleiotropy or genetic coupling and co-evolutionary models.

Tables

Table 1. Model outputs for all three experiments. See the effect sizes in the Figures 1; 3 for experiments 1 and 2, respectively. All GLMM models have replicate trial fitted as a random factor (see Materials and Methods).

Experiment	Response variable	Model	Fixed effects	Deviance	Df	Chisq	P-value
I	Proportion mated	ANCOVA with binomial errors (GLM)	Strain Wild-type Per0 Per0_7.2:2	184.92	2, 117		<0.001
			Song Conspecific Heterospecific Arrhythmic Control	98.01	3, 114		<0.001
			Temperature	3.86	1, 113		0.05
			Strain X Song	17.25	6, 107		0.01
	Cumulative proportion mated	Mixed effect ANCOVA with binomial errors (GLMM)	Strain		2	1438.01	<0.001
			Song		3	1004.52	<0.001
			Temperature		1	36.08	<0.001
			Strain X Song		6	190.46	<0.001
			Song X observation time		3	11.98	<0.001
			Strain X observation time		2	17.96	0.007

II	Proportion mated	ANCOVA with binomial errors (GLM)	Strain Wild-type UAS-PER-IR1 UAS-PER-IR2 JO-Gal4 JO-Gal4/UAS-PER-IR1 JO-Gal4/UAS-PER-IR2	426.58	5, 153	<0.001
			Song Conspecific Heterospecific Arrhythmic Control	147.21	3, 158	<0.001
			Date	8.63	1, 151	0.005
			Temperature			NS
			Strain X Song			NS
	Cumulative proportion mated	Mixed effect ANCOVA with binomial errors (GLMM)	Strain Wild-type UAS-PER-IR1 UAS-PER-IR2 JO-Gal4 JO-Gal4/UAS-PER-IR1 JO-Gal4/UAS-PER-IR2		5	6554.8	<0.001
			Song Conspecific Heterospecific Arrhythmic Control		3	1880.3	<0.001
			Song X observation time		3	1.43	NS
			Strain X observation time		5	47.60	<0.001
III	Proportion mated	ANOVA with binomial errors (GLM)	Song Conspecific Heterospecific	10.25	1, 38		0.001
			Activity time Early Late	33.70	1, 37		<0.001
			Song X Activity time	0.001	1, 36		NS
	Cumulative proportion mated	Mixed effect ANOVA with binomial errors (GLMM)	Song Conspecific Heterospecific		1	110.73	<0.001
			Activity time Early Late		1	272.24	<0.001
			Song X observation time		1	0.96	NS
			Activity time X observation time		1	1.06	NS
			Song X Activity time		1	0.23	NS

Figures

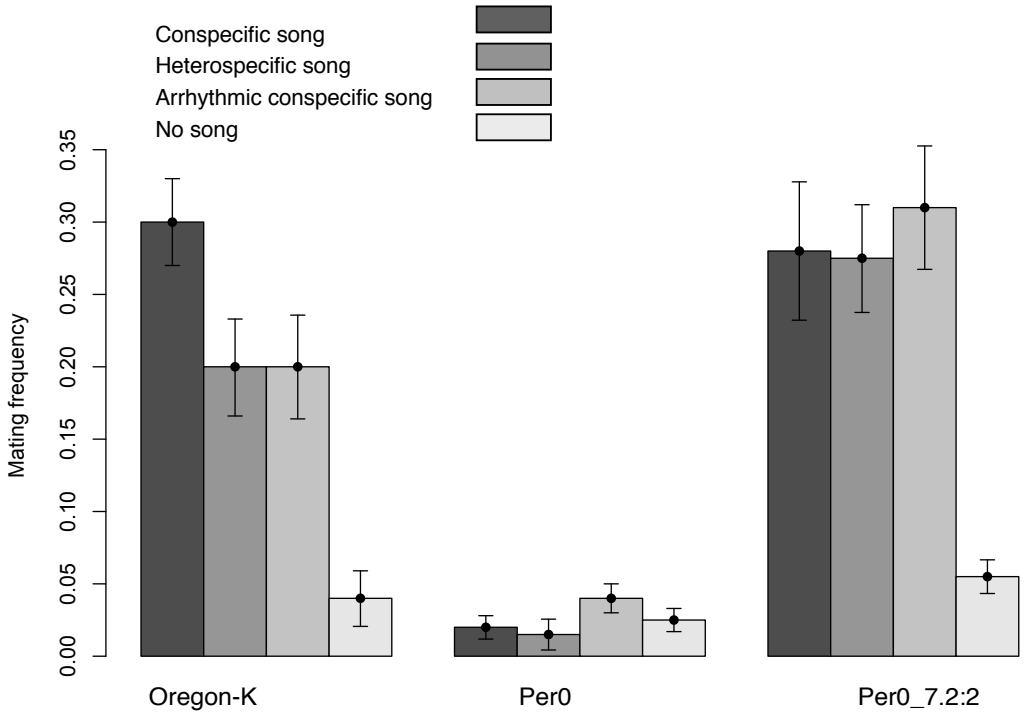
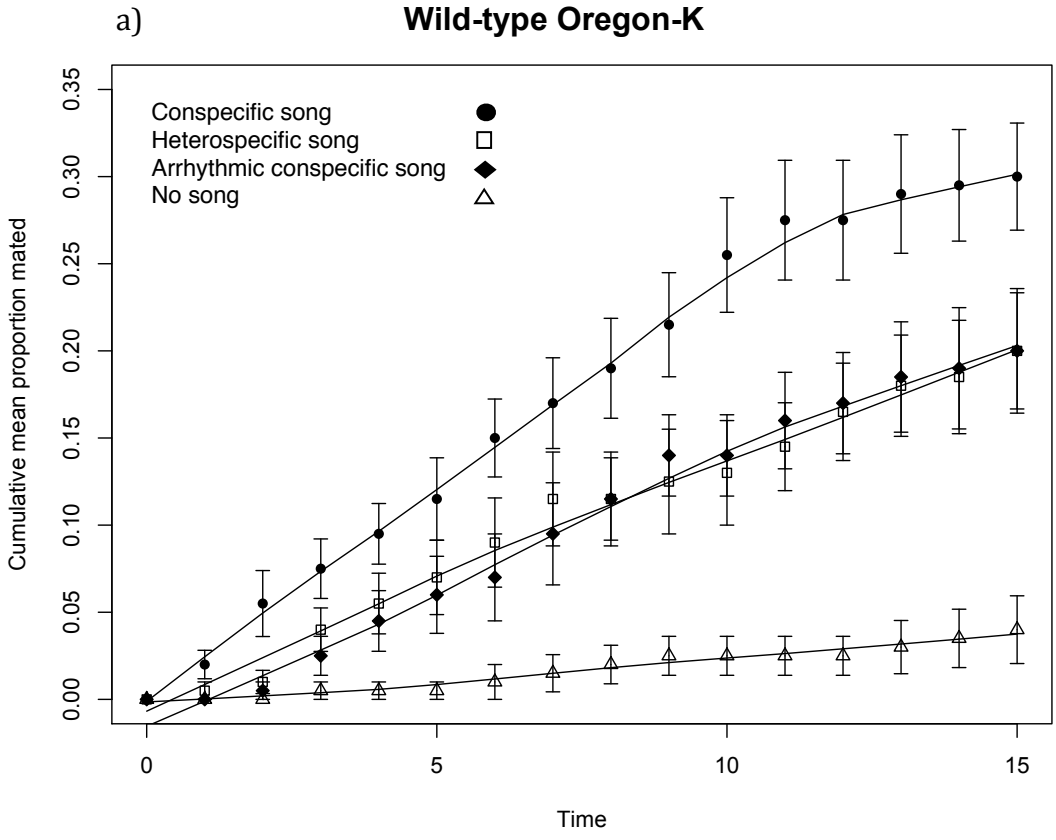
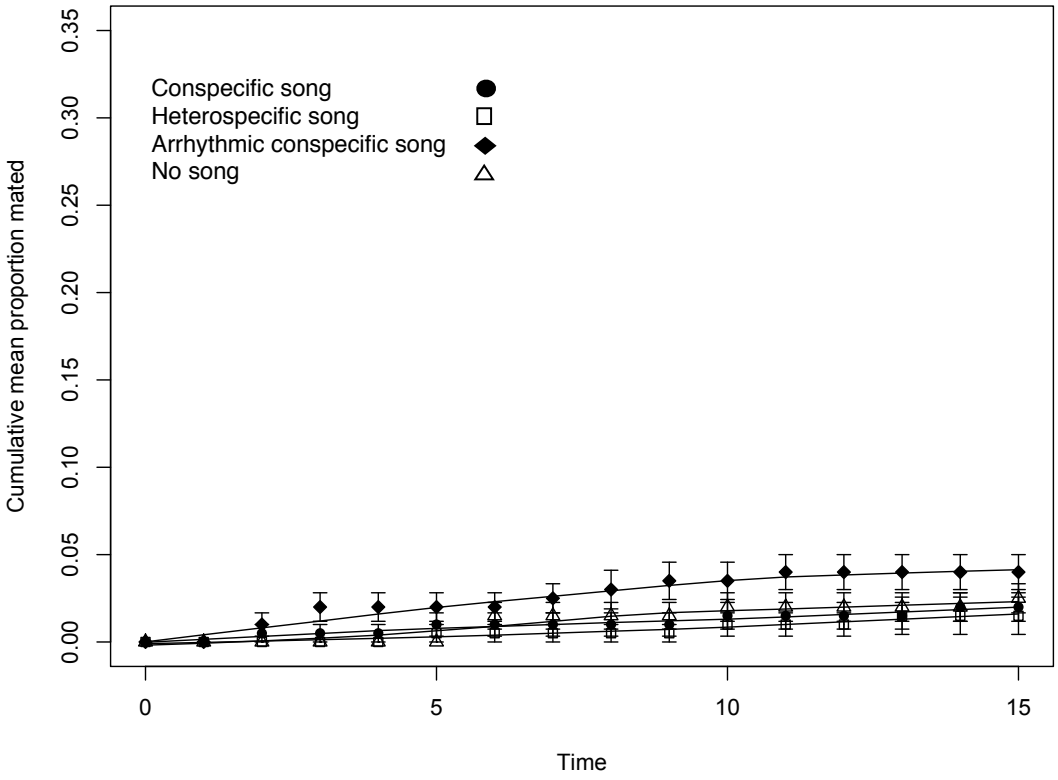


Figure 1. Effect of song treatment on the mating frequency (with standard errors) for each strain (experiment 1).



b) ***per*^{01}**



c) ***per*^{01}*7.2:2***

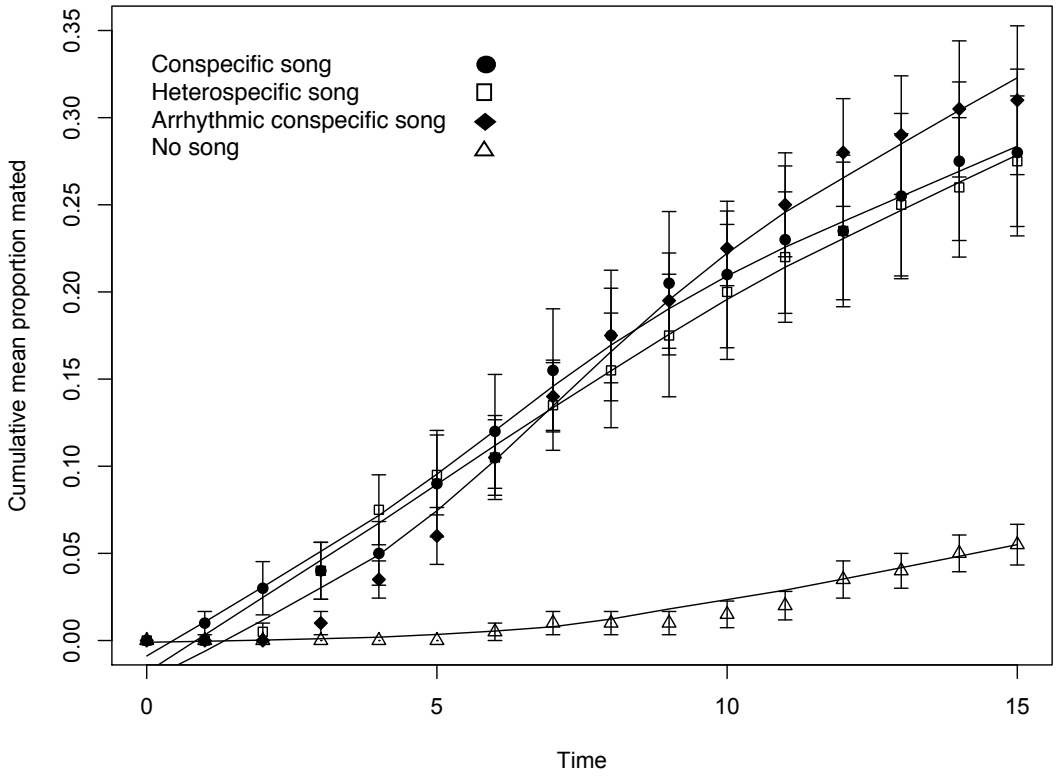


Figure 2. Effect of song treatment on female mating frequency across the observation period for a) a wild-type Oregon-K, b) *per*^{01} knockout strain and c) transgenic *per*^{01}*7.2:2* strain. Means ± 1 SE shown, the fitted line is the loess curve.

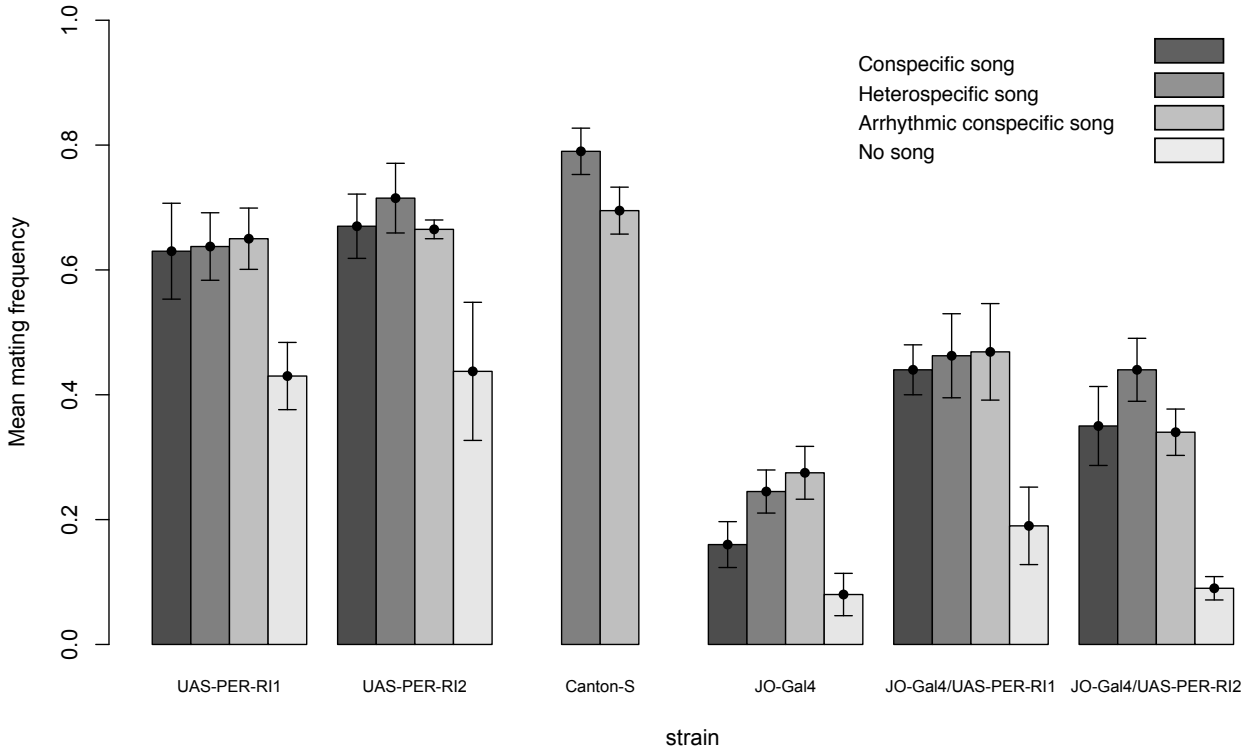
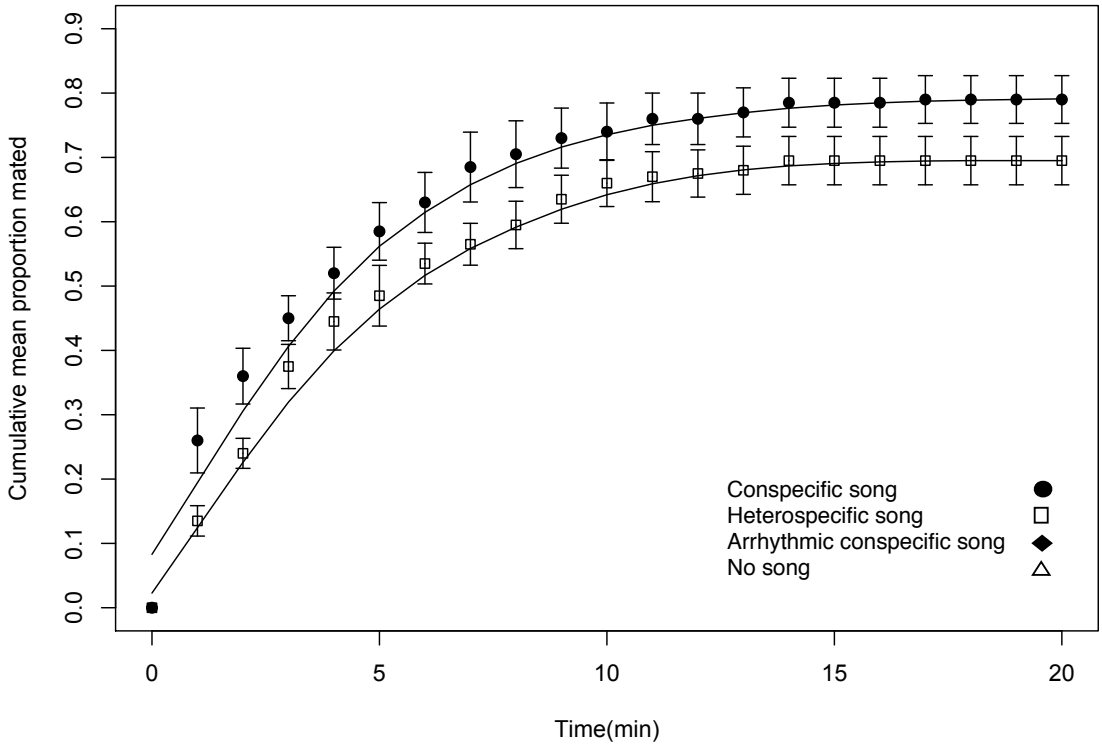
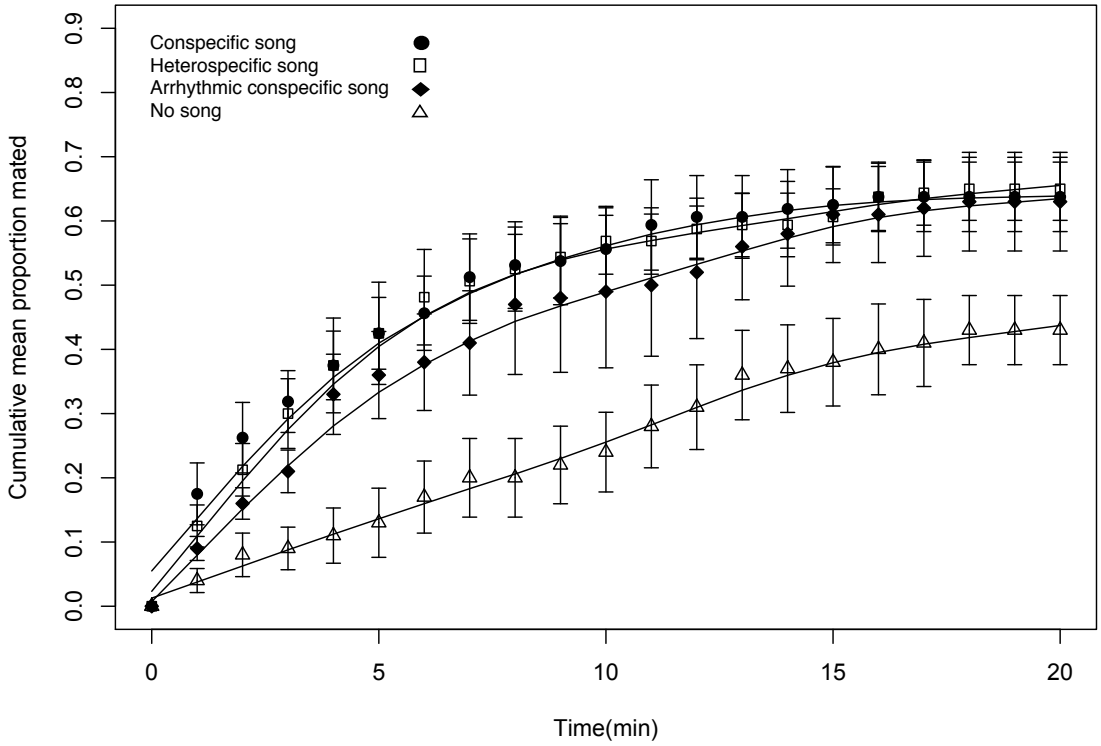


Figure 3. Effect of song treatment on the mating frequency (with standard errors) for each strain (experiment 2).

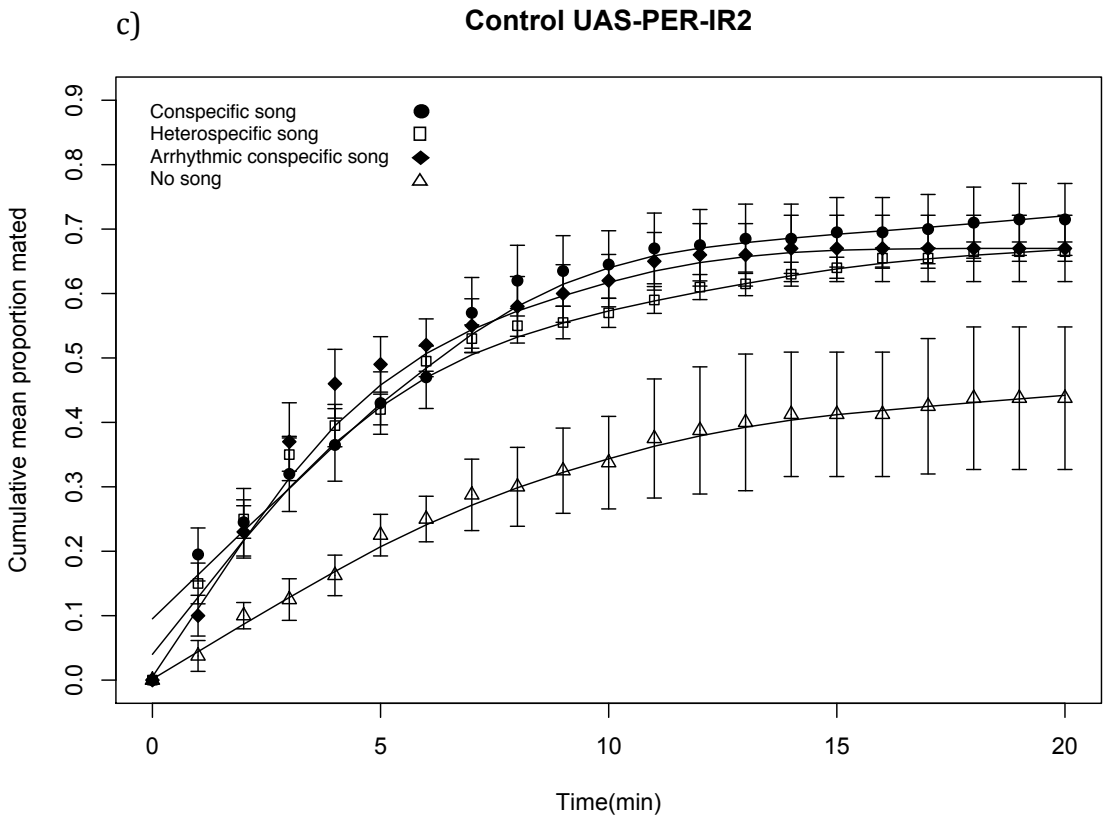
a)


wild-type (Canton-S)
Cumulative mean proportion mated
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
5
10
15
20
Time(min)
Conspecific song
Heterospecific song
Arrhythmic conspecific song
No song

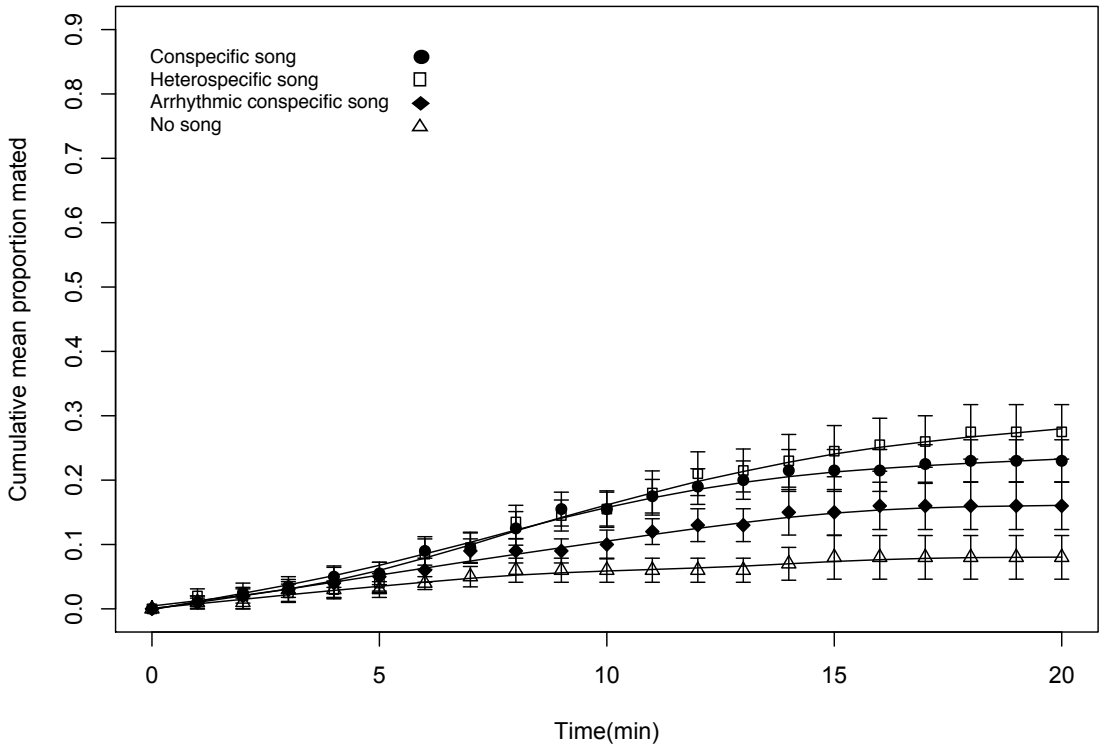
b)


Control UAS-PER-IR1
Cumulative mean proportion mated
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
5
10
15
20
Time(min)
Conspecific song
Heterospecific song
Arrhythmic conspecific song
No song

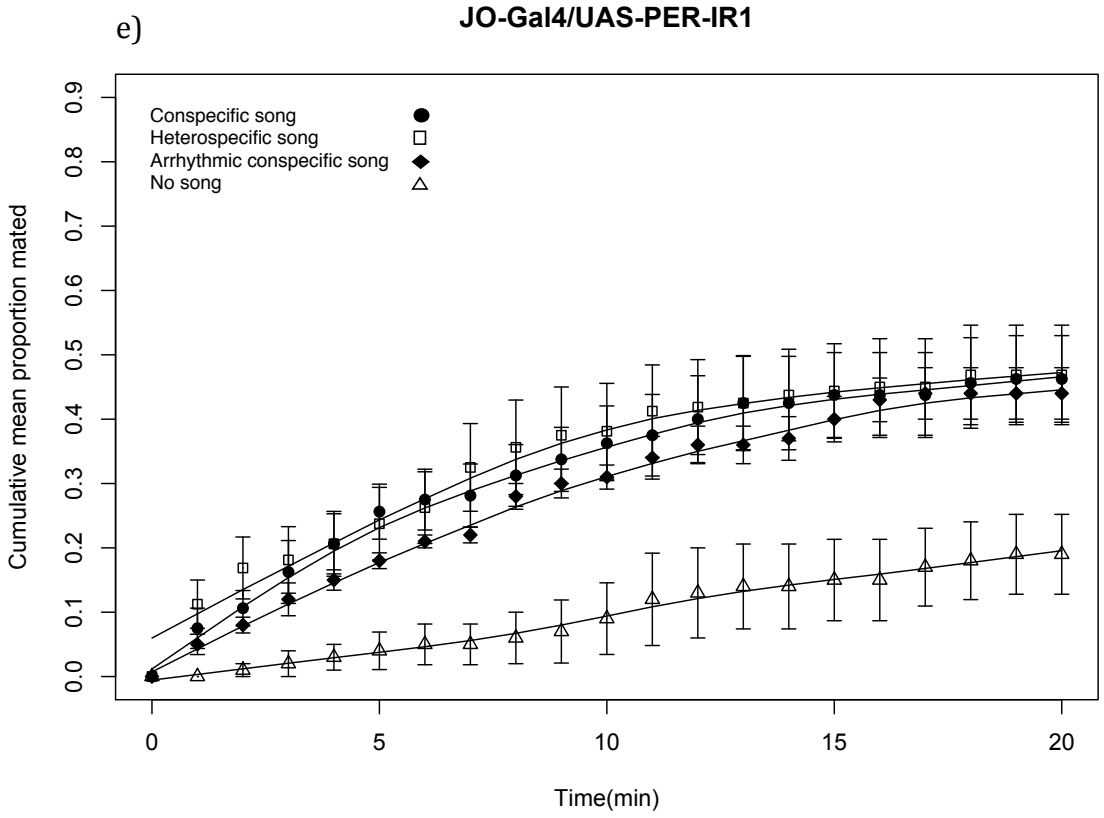
c)


Control UAS-PER-IR2
Conspecific song
Heterospecific song
Arrhythmic conspecific song
No song
Cumulative mean proportion mated
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
5
10
15
20
Time(min)

d)


Control JO-Gal4
Conspecific song
Heterospecific song
Arrhythmic conspecific song
No song
Cumulative mean proportion mated
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
5
10
15
20
Time(min)

e)


JO-Gal4/UAS-PER-IR1
Conspecific song
Heterospecific song
Arrhythmic conspecific song
No song
Cumulative mean proportion mated
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
5
10
15
20
Time(min)

f)

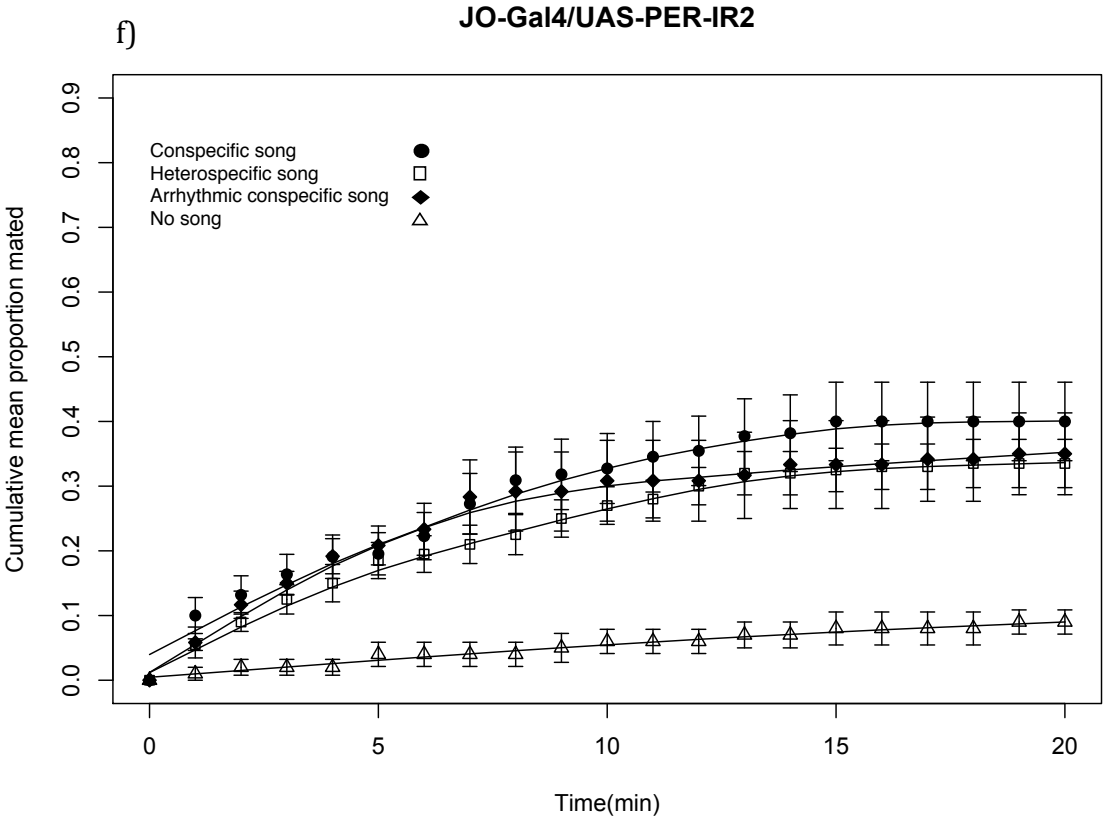

JO-Gal4/UAS-PER-IR2
Conspecific song
Heterospecific song
Arrhythmic conspecific song
No song
Cumulative mean proportion mated
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
5
10
15
20
Time(min)

Figure 4. Effect of song treatment on female mating frequency across the observation period for each strain (as indicated in the figure header). Means ± 1 SE shown, the fitted line is the loess curve.

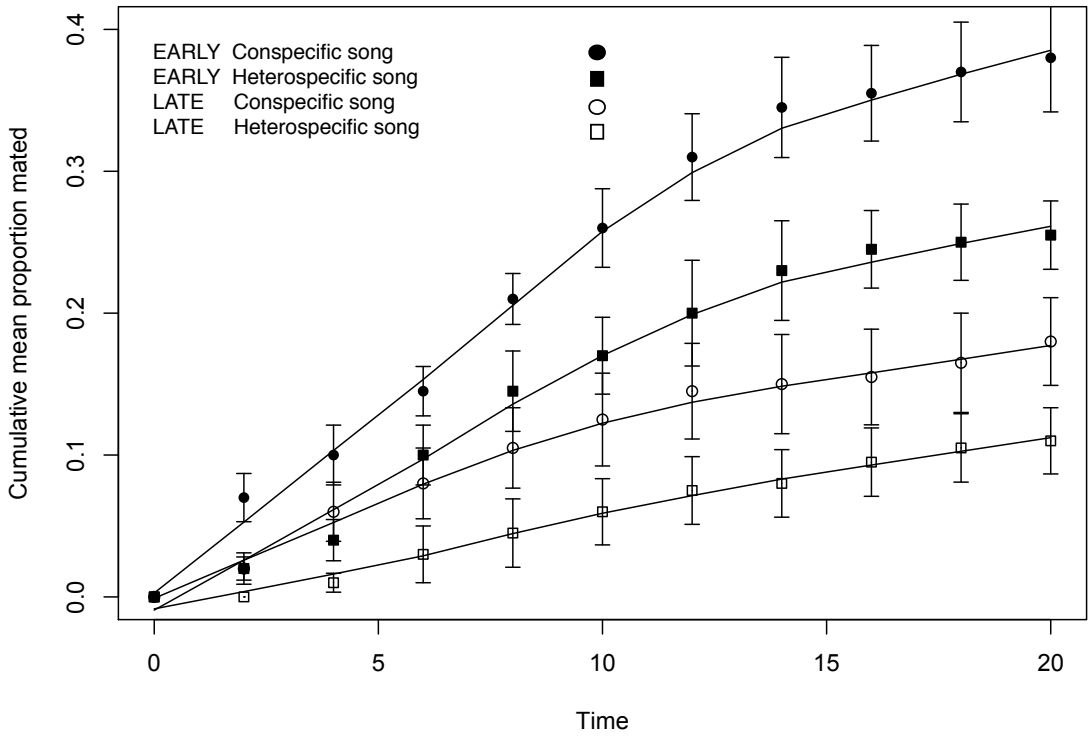


Figure 5. Effect of song treatment and time of day on female mating frequency across the observation period. The fitted line is the loess curve.

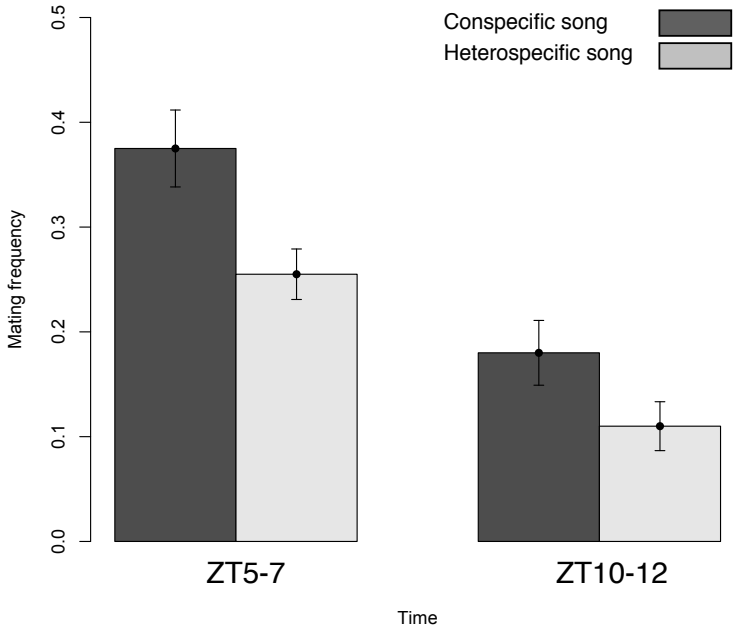
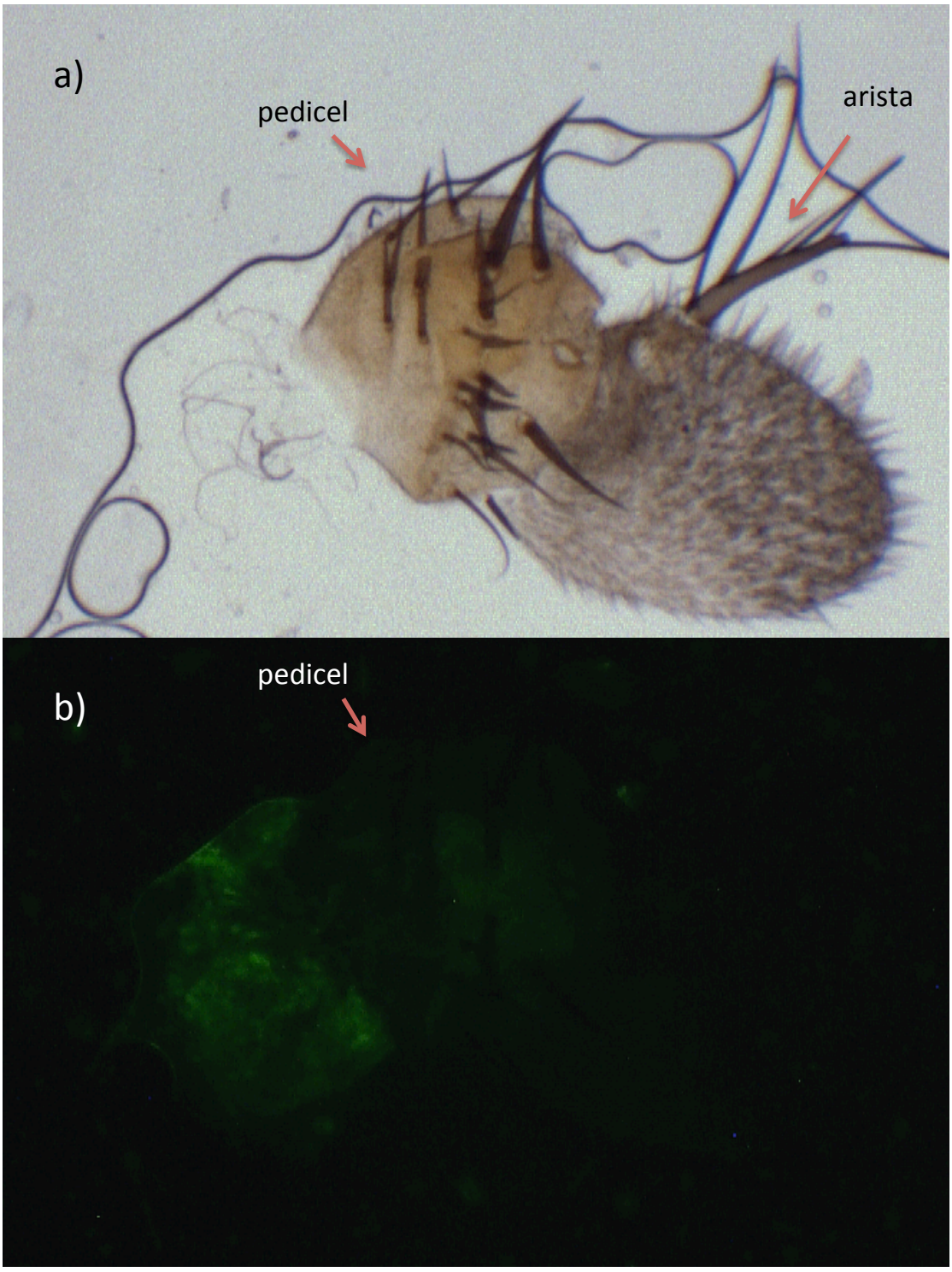


Figure 6. Effect of song on the mean mating frequency (standard errors shown) for each time point (ZT= *zeitgeber time*, 0=dawn).



Supplementary Figure 1. Fluorescence microscopy image of antennae of a *JO-GAL4/ UAS-mCD8::GFP* female shows GFP (green fluorescent protein) expression in JO neurons.

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CHAPTER 3

The genomic response to courtship song stimulation in female *Drosophila melanogaster*

Abstract

Courtship behaviour involves a complex exchange of signals and responses. These are usually studied at the phenotypic level, and genetic or transcriptional responses to courtship are still poorly understood. Here I examine the gene expression changes in *D. melanogaster* females in response to one of the key male courtship signals in mate recognition, song produced by male wing vibration (chapter 1, Figure 2). Using long-oligonucleotide microarrays, I identified several genes that responded differentially to the presence or absence of acoustic courtship stimulus. These changes were modest in both the number of genes involved and fold-changes, but notably dominated by antennal signalling genes involved in olfaction as well as neuropeptides and immune response genes. Secondly, I compared the expression patterns of females stimulated with synthetic song typical of either conspecific or heterospecific (*D. simulans*) males. In this case also antennal olfactory signalling and innate immunity genes were enriched among the differentially expressed genes. I confirmed and investigated the time course of expression differences of two identified immunity genes using real-time quantitative PCR (qPCR). These results provide novel insight into specific molecular changes in females in response to courtship song stimulation. These may be involved in both signal perception and interpretation and some may anticipate molecular interactions that occur between the sexes after mating.

Introduction

Sexual reproduction often involves complex interactions between males and females extending from pre-mating courtship signalling to post-mating molecular interactions. Recently some of the physiological and neuronal changes associated with the

reception of sexually important signals have been identified both in vertebrates and invertebrates including *Drosophila* (GENTNER *et al.* 2001; HOKE *et al.* 2005; MURTHY 2010; SOCKMAN *et al.* 2002). Progress has been made also in identifying transcriptional changes associated with social interactions in *Drosophila,* particularly in males (CARNEY 2007; ELLIS and CARNEY 2009; ELLIS and CARNEY 2011). However, the genes involved in female responses to male signals still remain largely unknown, and very few studies have attempted to identify transcriptional changes involved in pre-mating responses to stimulation using a genome wide analysis of gene expression. One such study (LAWNICZAK and BEGUN 2004) assessed gene expression of *D. melanogaster* females 24h after they had been courted by and rejected males, while another (CUMMINGS *et al.* 2008) focused on expression changes in females in response to visual cues of attractive males in swordtail fish (*Xiphophorus nigrensis*).

Although the molecular responses to courtship signals are *a priori* expected to include genes involved in mating preference, an intriguing and previously un-explored possibility is that courtship may also induce molecular and physiological changes in females in anticipation of mating. Recent studies of female *Drosophila* have identified male-induced molecular changes associated with a response to sperm and accessory gland proteins (INNOCENTI and MORROW 2009; MCGRAW *et al.* 2008; MCGRAW *et al.* 2004). Do changes start to occur during courtship, in anticipation of mating?

D. melanogaster is an ideal species for studying genes involved in behaviour, because it has a long history as a model organism in genetic studies and a well annotated genome, and also its courtship behavior is well understood. Courtship in *D. melanogaster* involves visual, acoustic, olfactory and tactile signals (GREENSPAN and FERVEUR 2000). Courtship song, produced by male wing vibration, is perhaps the

most important courtship signal influencing male mating success (RYBAK *et al.* 2002) and in the chapter 2 I confirmed song preference in our laboratory strain of *D. melanogaster* flies using synthetic song. Song is detected with a modified antennal receiver, which transfers air vibrations to the hearing neurons (GOPFERT and ROBERT 2002; GOPFERT and ROBERT 2003). In many *Drosophila* species song consists of two main components; pulse song and sine song (EWING and BENNETT-CLARK 1968). Pulse song includes repetitive trains of pulses and their inter-pulse intervals (IPI) as well as a distinctive rhythm in IPI (KYRIACOU and HALL 1980; 1982; 1986; KYRIACOU *et al.* 1990) and contributes to inter-specific mate discrimination (BENNET-CLARK and EWING 1969; KYRIACOU and HALL 1982; 1986; RITCHIE *et al.* 1999). However, apart from general hearing genes (EBERL *et al.* 1997; GONG *et al.* 2004; KAMIKOUCHI *et al.* 2009), very little is known about the genetic basis of female response to song, especially in comparison to song production.

In this study I used *Drosophila melanogaster* to trace transcriptomic changes that occur in females upon hearing male song in order to identify the molecular components involved in female response to acoustic stimulation as well as to study the species-specificity in this response. Gene expression changes were studied in response to the presence and attractiveness of an acoustic courtship stimulus without exposing the females to courting males, i.e. by excluding the confounding effects of other male traits upon female gene expression. A study on the swordtail fish (CUMMINGS *et al.* 2008) is the only similar attempt to identify gene expression responses to a sexually important courtship signal in isolation.

Material and Methods

Flies

An isogenic wild-type *D. melanogaster* strain (Oregon-K) was used for all experiments. Flies were reared in un-crowded cultures at 23°C with a 12-h light-dark cycle on a cornmeal-sugar-agar-yeast medium, seeded with dried baker's yeast.

Microarrays

Behavioural playback experiments to obtain transcriptome profiles were carried out by aspirating 40 five-day old virgin females at a time into a chamber mounted on a loudspeaker. No males were introduced in order to isolate the effect of auditory signal perception from other male signals. Conspecific and heterospecific songs (described in chapter 2), as well as white noise as a control, were played back to females for 15 minutes during peak mating activity time (ZT5-7, *chapter 2*). After the trials, females were removed from the chamber by anesthetising with CO_2, snap-frozen with liquid nitrogen and stored in -70°C. 120 female heads from three playback-trials were randomly pooled to form each sample per treatment, with a total of four biological replicate samples prepared for the control and heterospecific song stimulation treatments and eight for the conspecific song stimulation treatment. Heads were removed individually from the frozen flies to minimize the loss of antennal segments and with the aid of liquid nitrogen to prevent thawing. Total-RNA was extracted using TRIzol, reverse transcribed into double-stranded cDNA and samples labelled with either Cy3 or Cy5 using Klenow polymerase. Samples were hybridised into two-channel long oligonucleotide microarrays (FL003-INDAC) (see www.flychip.org.uk

for protocols). Four arrays were probed with control and conspecific song stimulus groups, three with conspecific and heterospecific song groups. Three of the arrays had reverse labelling to account for dye-bias in hybridisation efficiency. Scanning of the arrays was performed with GenePix, spot finding and quantification with Dapple (BUHLER *et al.* 2000). FlyChip at the University of Cambridge performed sample processing, array hybridisation, image scanning and quality controls. Data are deposited at NCBI (ref number GEO GSE31190).

The following packages within Bioconductor in R were used for the data pre-processing and analysis (GENTLEMAN *et al.* 2004; RDEVELOPMENTCORETEAM 2011). Raw intensity values were normalised within the limma package (SMYTH 2004; SMYTH 2005) using loess for within- and quantile for between array normalisation (SMYTH and SPEED 2003). Genefilter -package was used for non-specific filtering. I also used expression information from FlyAtlas (CHINTAPALLI *et al.* 2007) to eliminate probes not expressed in the head. Differential expression was tested using limma, where firstly a linear model was fitted for each gene and the coefficients estimated using least squares. Secondly, using the ebayes function, empirical Bayes approximation was applied to the coefficients to moderate the standard errors of the fold changes and to replace the variance parameter with a posterior variance estimated from the data. This results in more stable inference and improved power to detect differential expression (SMYTH 2004; SMYTH 2005). The p-values associated with the moderated t-statistics were adjusted using a false discovery rate (FDR) to control for multiple testing (BENJAMINI and HOCHBERG 1995).

Preliminary analyses suggested a mis-labelling had occurred with some of the arrays. Cluster analysis using Euclidean distance was used to confirm the direction of the labelling for the control to song contrast, by comparing the gene expression

profiles of this contrast (whole probeset), labelled in two different ways, to that of conspecific – heterospecific song contrast, under the assumption that the conspecific song treatment group shows a highly similar profile whether compared to silence-control or heterospecific song (Supplementary Figure 1). Cluster analysis confirmed the same labelling direction for conspecific song for each contrast group and qPCR analysis on two chosen genes from the microarrays further confirmed to this.

One of the main aims of this study was to explore whether any *a priori* defined functional sets of genes are over-represented among the genes showing differences in expression due to song treatments. For this, I took advantage of predefined gene sets from several databases, including GO, KEGG and INTERPRO, and tested overrepresentation of functional terms with genes clustered into groups that share similar significantly enriched biological functions, as implemented in DAVID (Gene Functional Classification tool) (DENNIS *et al.* 2003; HUANG *et al.* 2009). This approach not only reveals the major biological themes associated with the genes under study, but also the groups of genes that are likely to be co-regulated based on their functional similarity. Over-representation of terms associated with a gene list was assessed by calculating a moderated Fisher's Exact p-value for each functional annotation (called EASE score in DAVID, FDR estimated to control for multiple testing). Genes were clustered together based on their degree of similarity for enriched functional annotations (using 'Fuzzy' clustering algorithm as implemented in DAVID), and clusters ranked by calculating Enrichment Score (-log transformed geometric mean of the modified Fisher's Exact p-values of all the annotations participating in the given cluster). The clusters with most significantly enriched annotations (Enrichment Score >1.3) (DENNIS *et al.* 2003; HUANG *et al.* 2009) are reported in the main text and in the Table 2. Additional genes not included in the

clusters are reported when participating in the enrichment of functional terms (Table 2). Focusing only on the genes showing largest expression differences with a stringent FDR may fail to capture the biological mechanisms involved in expression changes, especially when the changes are small (MOOTHA *et al.* 2003). I therefore defined the lists of genes included in the gene functional enrichment analyses using a gene-specific p-value cut-off 0.05 (FALCON and GENTLEMAN 2008). The rationale behind less stringent criteria is that smaller fold changes which cannot pass the threshold of multiple testing adjustment can be biologically significant, which can be revealed through identification of functionally related groups of genes (MOOTHA *et al.* 2003). The background list of genes used to assess the enrichment of those with particular annotation included all head-specific probes used in the analyses of differential expression, as this is more conservative than using a whole genome as a background (for example, the relative proportion of genes with neuronal annotation will be higher in head-specific genes than in the whole genome, thus making the threshold for significant enrichment more stringent).

Real-time quantitative PCR

I chose two candidate genes, *TotM* and *TotC*, from the contrast of conspecific and heterospecific songs for validation due their statistical significance and effect size. Six biological replicates per treatment were obtained by independent sample collections following the same experimental procedures as before. However, in addition to exposing the females to the song treatments and control for 15 minutes, another set of flies were exposed for only five minutes in order to test whether expression differences occur quickly after stimulation. For each of the six replicates per treatment and time point, 40 heads were pooled. Total-RNA was extracted with Ambion Micro-

kit, and treated with TURBO DNase following manufacturer's instructions. RNA quality checks were performed with a NanoDrop and gel electrophoresis. Reverse transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad) primed with oligo(dT) and random hexamers. cDNA concentrations were measured with Cary 50 Bio UV-visible spectrofluorometer (Varion) and standardised to the lowest concentration.

One-step real-time qPCR was carried out with iQ cycler (Bio-Rad) using SYBR Green Supermix (Bio-Rad) in 25µl reactions, following the manufacturer's protocol. Two reference genes, *RpS8* and and *Act5C* were used as positive controls to normalise the expression levels of the candidate genes, based on their similar expression profiles between the treatment groups. Primers were designed with NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (*TotM* forward CGTCACAGAAAAACAGCGCC, reverse GCGTGTGTTCAAGTCCGGTT; *TotC* forward CAACGACGCCGAATCGAAGA, reverse TTCAGGGGACAACGTGGGAG; *RpS8* forward TTTTGACACGAGGTGCTGTG, reverse ACTCGAACTTGCGCTTCTTG; *Act5c* forward GGAAGCAGCAGCGAAAGTGC, reverse TGTGCAGGTGGTTCCGCTCT). Standard curves were produced to assess the dynamic range and primer efficiencies, and qPCR conditions were subsequently optimised to yield equal 100% efficiencies for all of the primers. Melting curves were used to ensure the amplification specificity of primers. Three technical replicates were amplified for all of the samples with each gene, and calibration samples were used to assess inter-assay variability. The relative quantification method ($2^{-\Delta\Delta C}{}_t$) was used to normalise the mean gene expression values (Cycle time, C_t) to the geometric mean of the two internal reference genes and to calculate the fold changes between samples (LIVAK and SCHMITTGEN 2001;

VANDESOMPELE *et al.* 2002; WILLEMS *et al.* 2008). The significance of the differences between treatments was assessed by calculating the 95% confidence intervals as well as with Kruskal-Wallis non-parametric test (WONG and MEDRANO 2005). The standardised relative fold changes were calculated in Microsoft Excel 2008 and the significance test in R version 2.9.1 (RDEVELOPMENTCORETEAM 2011).

Results

The transcriptome response to courtship song

In order to detect differences in gene expression associated with hearing a song, I compared conspecific song –stimulated and control females. There were 412 differentially expressed genes, of which 41 were significant with a 5% FDR (Table 1). To identify gene expression changes associated with song discrimination for attractive versus non-attractive songs, I compared the expression profile of conspecific song –stimulated females to that of heterospecific song –stimulated females. This contrast revealed 222 differentially expressed genes, of which two, *TotM* and *TotC*, were significant after correction for multiple testing with a strict 5% FDR (Table 1). That more differences in gene expression were detected between song and no-song than between the two songs is consistent with the results of behavioural experiment, which show that heterospecific song is still stimulatory to females, but less so.

To assess which biological processes are associated with the song responses I performed functional enrichment analysis (DENNIS *et al.* 2003; HUANG *et al.* 2009) on the genes that differed in expression in each of the stimulus comparison (with gene-specific p-value <0.05), and identified several clusters of genes that share similar, significantly enriched annotation terms (Table 2). Hearing conspecific song was

significantly associated with a cluster of six genes that all share functions in signaling, odorant/pheromone binding and cognition (Enrichment score = 2.39). A second significant gene cluster for this contrast contained four genes, all involved in signaling, hormone and neuropeptide activity (Enrichment score = 2.11), and a third cluster five immune response genes (Enrichment score = 1.69). Preference for species-specific song was significantly associated with two clusters of genes with shared annotations. The first cluster included five genes (four from the *Turandot* gene family), which are involved in humoral immunity and stress response as secreted signal peptides (Enrichment score=2.59). The second group was similar to the first cluster identified in comparison between song and control: here four genes shared functions in signaling, cognition and odorant/pheromone binding (Enrichment score=2.32). It is worth noting that both of the comparisons involved significant enrichment of seven antennal genes, the first comparison *Os-C, Os-E, Pbprp3, a5, Or83b, Pbprp1, Pbprp5* (fold enrichment=8.0, FDR= 7.4E-04), and the second one *Os-C, Os-E, Pbprp3, a5, Or43a, Pbprp4, a10* (fold enrichment=15.3, FDR=1.1E-05). See Table 2 for the genes and all of the functional annotations included in the clusters.

Effect of song stimulation duration upon Turandot *gene expression*

I chose to test the expression of two genes, *TotM* and *TotC,* with qPCR, based on their fold change and significant expression changes in the experiments with conspecific and heterospecific song. The expression of these two genes that belong to the family of *Turandot* genes was tested after stimulating the females with either of the two songs or white noise for 5min and 15 min in order to examine the time course of expression variation.

5 min stimulation did not induce significant changes in expression levels between any of the acoustic treatments for either of the genes (Figure 1; *TotM*: χ^2=2.9, df=2, p-value=0.2; *TotC*: χ^2=0.01, d.f.=2, p-value=0.9). However, after 15min of song stimulation, both *TotM* and *TotC* were significantly up-regulated with conspecific song compared to heterospecific song, and *TotC* compared to the control (Figure 1; *TotM*: χ^2=9.6, df=2, p-value=0.008; *TotC*: χ^2=11.38, d.f.=2, p-value=0.003).

Discussion

I have examined gene expression changes associated with the presence and attractiveness of male courtship song in *D. melanogaster*. Song plays a key role in stimulating female mating and females are more stimulated by homospecific song (chapter 2), and genes involved in this species-specific response may be under sexual selection and contribute to sexual isolation.

Signaling and olfactory genes respond to song

Hearing a song in isolation from any other male traits resulted in relatively modest differences in gene expression, however, responses in distinct groups of genes were identified. Song induced changes in genes that function in signaling, and interestingly, many of them are expressed in the antennae, which are the *Drosophila* hearing organs (EBERL 1999). Antennal genes were enriched 7 and 15 times more among those differentially expressed between song and control and between conspecific and heterospecific songs, respectively. Because changes were more pronounced between the song types this cannot simply be due to a general response to acoustic stimulation. Recently it has been shown that antennae are actively tuned to the frequencies within

homospecific song (RIABININA *et al.* 2011). Four of the antennal genes are shared between the two comparisons including *Os-C, Os-E, Pbprp3, a5*. Although effect sizes are small, the significant enrichment of the antennal genes suggests that modest changes can be biologically meaningful. It should also be appreciated that our RNA preparations were from whole heads, so tissue-specific expression changes in antennal neurons are probably considerably greater. I did not detect any previously identified genes involved in hearing in either of the comparisons (EBERL *et al.* 1997; GONG *et al.* 2004; KAMIKOUCHI *et al.* 2009).

The signaling genes responding to song include a significant enrichment of genes (*Pdf, crz, hug, tk*) with functions in neuropeptide signaling pathways and hormone activity (Table 2). The neuropeptide *Pdf* regulates signaling in neurons involved in a variety of circadian rhythmic behaviours, in a species-specific way (BAHN *et al.* 2009), and its regulation responds to selection for increased or decreased mating latency (MACKAY *et al.* 2005). *Crz* has recently been implicated in sex-specific stress-related behaviours (ZHAO *et al.* 2010), and is linked with the regulation of dopamine (ZHAO *et al.* 2010), which modulates female sexual receptivity (NECKAMEYER 1998). These neuropeptides may therefore participate in the perception of the rhythmic conspecific pulse song and downstream signaling modulating arousal (ANDRETIC *et al.* 2005).

Interestingly, song stimulation in both experiments evoked expression changes in genes involved in chemical communication (nearly all of the antennal genes, see Table 2), which cannot be induced by olfaction differences in the present study as females only heard song. These include odorant receptor gene *Or49a* and the co-receptor gene *Or83b* (both differentially expressed between song and control) as well as several odorant-binding protein coding genes, most of which are also involved in

binding pheromones (for example *Pbprp3* and *Os*-E). Olfactory genes found in this study including *Pbprp3*, *Os-C*, *Pbprp5* and *Obp99c*, also respond to mating (MCGRAW *et al.* 2004). Why are there subtle changes in so many genes involved in olfactory signaling? The simultaneous activation of the olfactory system when hearing conspecific song could enhance the sensitivity of pheromone detection during courtship. *Drosophila* sensory neurons responsible for odorant detection cover the surface of the 3rd antennal segment (funiculus), including trichoid sensillae implicated in the recognition of the pheromone 11-cis-vaccenyl acetate (KAUPP 2010). Perhaps mechanical vibrations of the antennal arista connected to the funiculus during song stimulation also influences the expression of other loci expressed in the antennae. Alternatively, the antennal genes may have pleiotropic effects involved in other kinds of signal transmission.

Immune response to song

Hearing an attractive, conspecific song induced expression changes in genes involved in immunity and stress response. While some were downregulated (*Attacin-A (Att-A)* and *–C* (*Att-C*)*, Diptericin B* (*DptB*), *Drosomycin* (*Drs*) and *Immune induced molecule 18* (IM18)) compared to the control, four out of eight members from *Turandot* family (*TotA, TotC, TotX, TotM*) as well as *Immune induced molecule 4* (*IM4*) were up-regulated compared to the heterospecific song. Two *Turandot* genes, *TotC* and *TotM,* were examined more closely with real-time qPCR. For both of the genes, significant changes in gene expression were only detected after 15min of stimulation with attractive, conspecific, song compared to the heterospecific song, and for *Tot-C* also compared to the control. Up-regulation within 15 minutes of the start of courtship may be sufficient to have *Turandot* genes expressed prior to mating.

However, the difference between a biologically meaningful level of expression and what can be significantly detected by qPCR, is unknown.

Many immunity genes are involved in female reproduction in *D. melanogaster*. Also *D. melanogaster* males show expression differences in immunity related genes when courting females (ELLIS and CARNEY 2009), however the function of these changes are not yet known. Interestingly, long-term exposure to acoustic signals has been linked with increased immunity in field crickets (*Teleogryllus oceanicus*) (BAILEY *et al.* 2011), thus an increased probability of mating influences immune function in a variety of organisms. Two of the *Turandot* genes, *TotA* and *TotC,* show sex-specific expression: they are up-regulated in female heads relative to males, and are regulated downstream from the sex-specific pre-mRNA splicing factor *transformer (tra)* (GOLDMAN and ARBEITMAN 2007) which, together with *doublesex* (RIDEOUT *et al.* 2010), controls the sex determination cascade. All the *Turandot* genes observed in this study (*TotM*, *-C, -X* and *–A*) show similar expression patterns across tissues: they are enriched in the female spermatheca, head, heart, adult carcass and fat body (FlyAtlas, CHINTAPALLI *et al.* 2007), and are probably secreted into the hemolymph (EKENGREN and HULTMARK 2001). *Drs* also shows sex-biased fat-body specific expression (PARISI *et al.* 2004). Fat body in the head is involved in expression of many genes that mediate sexual differentiation (FUJII and AMREIN 2002), as well as male responses to mating (ELLIS and CARNEY 2010). Immunity genes are differentially expressed in females when mating (INNOCENTI and MORROW 2009; LAWNICZAK and BEGUN 2004; MCGRAW *et al.* 2008; MCGRAW *et al.* 2004; PENG *et al.* 2005) and nearly all the immunity genes identified in the present study are up-regulated in mated females (INNOCENTI and MORROW 2009). McGraw et al. (2004) demonstrated induced expression of *TotM* and *Att-C* by male sperm and *Att-A*

by Acps and in particular *sex peptide* (SP) (see also DOMANITSKAYA *et al.* 2007; PENG *et al.* 2005). Interestingly, a recent study identified increased expression in *TotC* and *–A* in the brains of mated females (DALTON *et al.* 2010). My results suggest that some of these changes, including increased expression of many *Turandot* genes, begin before copulation. However, other mating-induced immunity genes show decreased expression in response to courtship stimulation. *Att-A* and *-C,* as well as *DptB* are up-regulated in the female abdomen after mating but not in the head tissues (DOMANITSKAYA *et al.* 2007). It is therefore possible that their down-regulation in the head prior to mating represents a location shift in transcriptional activity and resource allocation. Several non-exclusive explanations have been suggested for the mating-related immune response in females (LAWNICZAK and BEGUN 2004; MCGRAW *et al.* 2008; MCGRAW *et al.* 2004; PENG *et al.* 2005), including protection from septic injury (SIVA-JOTHY 2009) and antagonistic male molecules (DOMANITSKAYA *et al.* 2007; INNOCENTI and MORROW 2009). Both *TotM* and *TotC* are amongst the fastest evolving immunity genes between *D. melanogaster* and *D. simulans* (OBBARD *et al.* 2009), and also their regulation has diverged between females of the two species (GRAZE *et al.* 2009). Perhaps the asymmetrical selection that arises from sexual conflict over components of female fitness may have contributed to the sexual dimorphism in the expression patterns of these genes, as well as their divergence between the species.

Previous studies on mated females have suggested that many proteins required for reproduction may be produced during premating reproductive maturation (MACK *et al.* 2006; MCGRAW *et al.* 2004). Here I have identified similar transcriptional changes in response to song as are seen in post-mated females, including *Turandot* and other immunity and olfactory genes. Another intriguing gene showing increased

expression in response to song stimulation is *Glucose dehydrogenase* (*Gld* Table 1), which codes for a protein that facilitates sperm storage in mated females (IIDA and CAVENER 2004). My findings thus suggest that the transcription changes thought to occur in response to mating may begin during courtship and may represent an adaptive preparation for mating, including anticipation of sexually antagonistic post-mating interactions with male molecules or increased risk of pathogen infection. Indeed, these findings are in line with the recent suggestion that increased immunity prior to mating may be a common female strategy in insects (SIVA-JOTHY 2009). That some expression changes depend upon the species-specific nature of song could result from a more stimulatory effect of conspecific song or, perhaps more intriguingly, an influence of a female 'decision' to mate during courtship. The connection between mate recognition and the downstream effects makes the molecules involved a powerful target for studies of evolutionary divergence and provide a starting point for characterizing the genetic pathways activated during courtship stimulation and how they are linked with the adaptive responses to mating. These will provide insights into key evolutionary processes ranging from species recognition, sexual selection and conflict to speciation.

Tables

Table 1. Differentially expressed genes from the two sets of microarrays, with FDR <5%. FC (fold change) > 0 indicates up-regulation with conspecific song.

Contrast	Gene	FC	p-value	FDR adj. p-value
Conspecific – heterospecific song	TotM	2.44	3.62E-08	1.7E-05
	TotC	1.88	1.85E-06	4.0E-04
Conspecific song - control	CG12726	2.13	6.48E-35	2.9E-31
	CG14645	1.70	5.75E-18	1.3E-14
	Cdep	1.57	1.92E-13	2.8E-10
	CG10332	-1.45	1.2E-09	1.3E-06
	CG6188	-1.44	3.6E-09	3.2E-06
	ems	1.42	1.3E-08	9.4E-06
	Or83b	1.37	2.7E-08	1.7E-05
	CG31678	1.37	2.6E-07	1.4E-04
	mRpS26	1.36	2.9E-07	1.4E-04
	CG4230	1.36	6.0E-07	2.7E-04
	DptB	-1.35	9.8E-07	3.9E-04
	CG18542	1.34	1.8E-06	6.6E-04
	CG32533	1.32	6.4E-06	2.0E-03
	CG8600	1.32	7.3E-06	2.0E-03
	CG13607	1.32	7.3E-06	2.0E-03
	trn	1.32	7.5E-06	2.0E-03
	qkr58E-3	1.32	7.9E-06	2.0E-03
	AttA	-1.31	1.0E-05	2.4E-03
	Gld	1.30	1.7E-05	3.9E-03
	X11Lbeta	1.30	1.8E-05	3.9E-03
	skf	1.29	2.9E-05	6.2E-03
	CG10635	1.29	3.2E-05	6.3E-03
	Thd1	1.29	3.3E-05	6.3E-03
	CG10889	1.28	4.8E-05	8.5E-03
	CG14630	-1.28	4.7E-05	8.5E-03
	mthl8	1.27	9.6E-05	1.6E-02
	CG7861	1.27	1.1E-05	1.7E-02
	Cys	1.27	1.1E-05	1.7E-02
	Oseg1	1.27	1.1E-05	1.7E-02
	CG10962	-1.27	1.2E-05	1.7E-02
	Pink1	1.26	1.3E-05	1.8E-02
	Cyp6a14	1.26	1.3E-05	1.8E-02
	CG31708	1.26	1.4E-05	1.9E-02
	mbl	1.26	1.7E-05	2.2E-02
	CG11093	1.25	2.3E-05	2.9E-02
	GstE9	-1.25	2.5E-05	3.0E-02
	CG5966	1.25	2.9E-05	3.5E-02
	nkd	1.25	3.3E-05	3.9E-02
	Atg5	1.24	3.7E-05	4.1E-02
	CG4678	1.24	4.0E-05	4.4E-02
	DIP1	1.24	4.4E-05	4.7E-02

Table 2. Gene Functional Classification Analysis (DAVID) reveals clusters of genes that share functional annotations. Clusters highlighted with dark grey and solid line (on left) are for the comparison of conspecific vs. heterospecific song stimulation (experiment 2), while clusters with light grey and dashed line (on right) are for song vs. control (experiment 1). The genes without shading (white) are included due to their participation in the significant enrichment of individual annotation terms shown here (this table is also provided on the accompanying CD for electronic viewing).

Gene name	**TotM** CG14027	**TotC** CG31508	**TotA** CG31509	**TotX** CG31193	**IM4** CG15231	**a10** CG6642	**Pbpp4** CG1176	**Os-E** CG11421	**Pbpp3** G11421	**Obp99c** CG7584	**Obp19a** CG11748-RB	**Pbpp1** G10436	**Pbpp5** CG6641	**a5** CG5430	**Or83b** CG10609	**Os-C** CG3250	**pdf** CG6496	**hug** CG6371	**Tk** CG14734	**Crz** CG3302	**IM18** CG10332	**DptB** CG10794	**Drs** CG10810	**AttC** CG4740	**Att+A** CG10146
Experiment	2	2	2	2	2	2	2	1/2	1/2	1	1	1	1	1/2	1	1/2	1	1	1	1	1	1	1	1	1
log(FC)	1.3	0.9	0.8	0.4	0.4	0.4	0.4	0.3/ 0.4	0.2/ 0.4	-0.21	0.2	0.2	0.2	0.2/ 0.3	0.5	0.3/ 0.5	0.18	0.22	0.20	0.2	-0.54	-0.43	-0.26	-0.3	-0.39
p-value	<0.001	<0.001	<0.001	0.01	0.03	0.02	0.02	0.007/ 0.01	0.02/ 0.01	0.02	0.03	0.03	0.04	0.04/ 0.02	<0.001	0.002/ 0.007	0.04	0.01	0.02	0.03	<0.001	<0.001	<0.003	<0.001	<0.001
Adj. p-value (FDR)	<0.001	<0.001	0.14	0.8	0.9	0.9	0.9	0.3/ 0.8	0.4/0.8	0.4	0.5	0.5	0.5	0.9/ 0.5	<0.001	0.14/0.8	0.5	0.4	0.5	0.5	<0.001	<0.001	0.16	0.06	0.002
IPR005521: **Attacin, C-terminal region**																						X		X	X
GO:0019730/0019731: **Antibacterial humoral response**																						X	X	X	X
GO:0042742: **Defence response to bacterium**																						X	X	X	X
Go:0009617: **Response to bacterium**																						X	X	X	X
GO:0006959: **Humoral immune response**																						X	X	X	X
IPR010825: **Stress-inducible humoral factor Turandot**	X	X	X	X																					
SP-PIR-Keyword: **Innate immunity**	X	X	X	X	X																X			X	X
SP-PIR-Keyword: **Immune response**	X	X	X	X	X																X			X	X
GO:0006955: **Immune response**	X	X	X	X	X																X	X	X	X	X
GO:0006952: **Defence response**	X	X	X	X	X																X	X	X	X	X
GO:0045087: **Innate immune response**	X	X	X	X	X																X		X	X	X
GO:0009408: **Response to heat**	X	X	X	X																					
GO:0006979: **Response to oxidative stress**		X	X	X																					
GO:0009266: **Response to temperature stimulus**	X	X	X	X																					
GO:0009628: **Response to abiotic stimulus**	X	X	X	X																					
SP-PIR-Keyword: **Signal**	X	X	X	X	X	X	X	X/X	X/X		X	X	X	X/X		X/X	X	X	X	X	X		X	X	X
SP-PIR-Keyword: **Signal peptide**	X	X	X	X	X	X	X	X/X	X/X		X	X	X			X/X	X	X	X	X	X		X	X	X
SP-PIR-Keyword: **Secreted**	X	X	X	X	X	X	X	X/X	X/X		X	X	X	X/X			X	X	X	X	X		X	X	X
GO:0005576: **Extracellular region**	X	X	X	X	X	X	X	X/X	X/X		X	X	X	X/X			X	X	X	X	X	X	X	X	X
GO:0044421: **Extracellular region part**	X	X	X	X	X			X/X	X/X													X		X	X
GO: 0005615: **Extracellular space**	X	X	X	X	X		X	X/X	X/X													X		X	X
IPR006170: **Pheromone/general odorant binding protein, PBP/GOBP**							X	X/X	X/X	X	X	X	X												
IPR006625: **Insect pheromone/odorant binding protein PhBP**							X	X/X	X/X	X	X	X	X												
GO:0007606: **Sensory perception of chemical stimulus**						X	X	X/X	X/X	X	X	X	X		X										
GO: 0007600: **Sensory perception**						X	X	X/X	X/X	X	X	X	X		X										
GO: 0050890: **Cognition**						X	X	X/X	X/X	X	X	X	X		X										
GO: 0005550: **Pheromone binding**						X	X	X/X	X/X			X	X			X/X									
GO: 0005549: **Odorant binding**						X	X	X/X	X/X	X	X	X	X		X	X/X									
GO: 0050877: **Neurological system process**						X	X	X/X	X/X	X	X	X	X												
SP-PIR-Keyword: **Disulfide bond**							X	X/X	X/X		X	X	X												
Up-TISSUE: **Expressed in antennae**						X	X	X/X	X/X			X	X	X/X	X	X/X									
SP-PIR-Keyword: **Neuropeptide**																	X	X	X	X					
GO:0007610: **Behavior**																	X	X	X	X					
GO:0005184: **Neuropeptide hormone activity**																	X	X	X	X					
GO:0007218: **Neuropeptide signalling pathway**																	X	X	X	X					
GO:0005179: **Hormone activity**																	X	X	X	X					
GO:0007186: **G-protein coupled receptor protein signalling pathway**															X		X	X	X	X					
GO:0007166: **Cell surface receptor linked signal transduction**																	X	X	X	X					
Enrichment score D.mel song – control											**2.39**								**2.11**				**1.69**		
Enrichment score D. mel – D. sim song			**2.59**					**2.32**																	

Figures

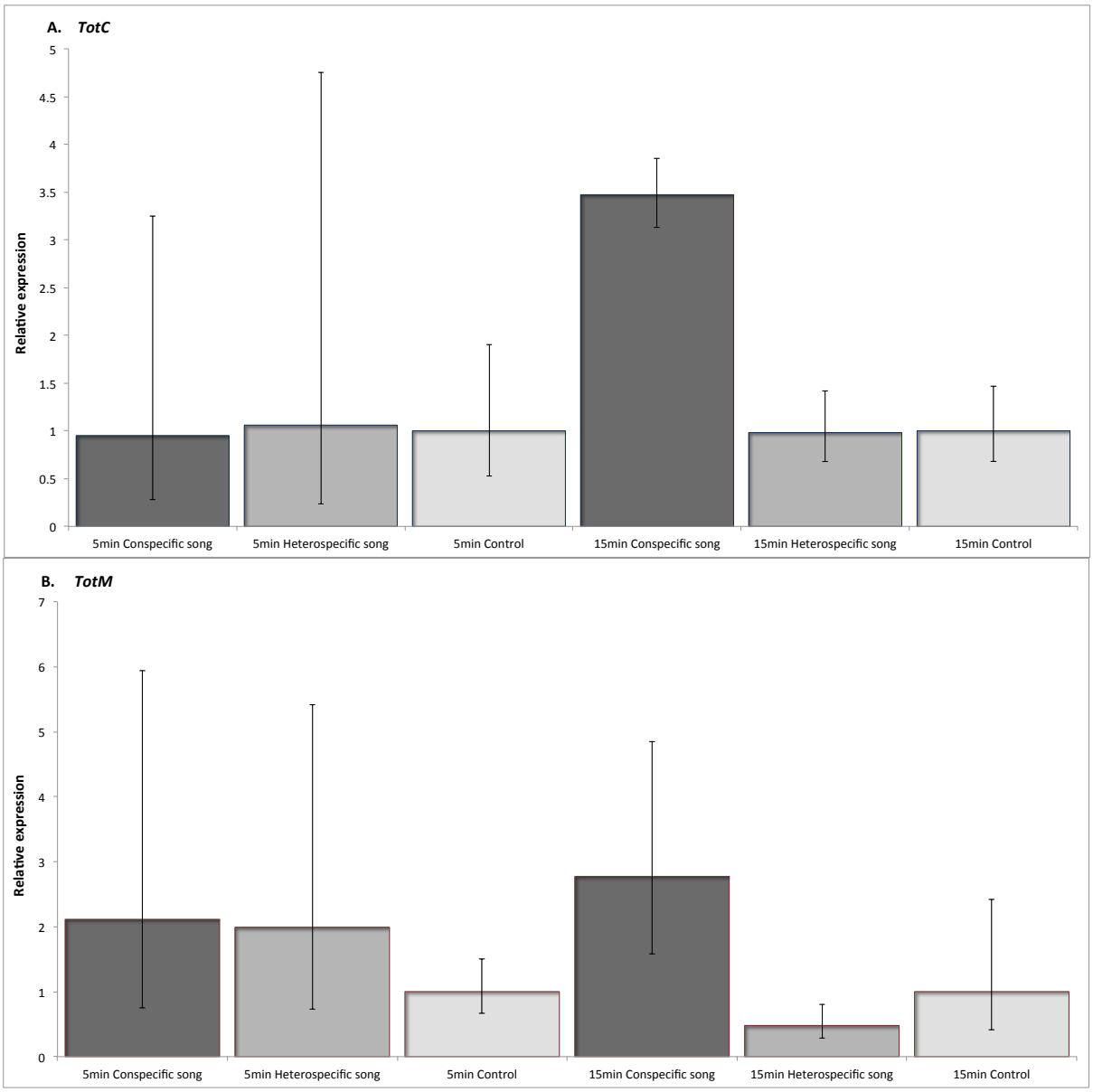
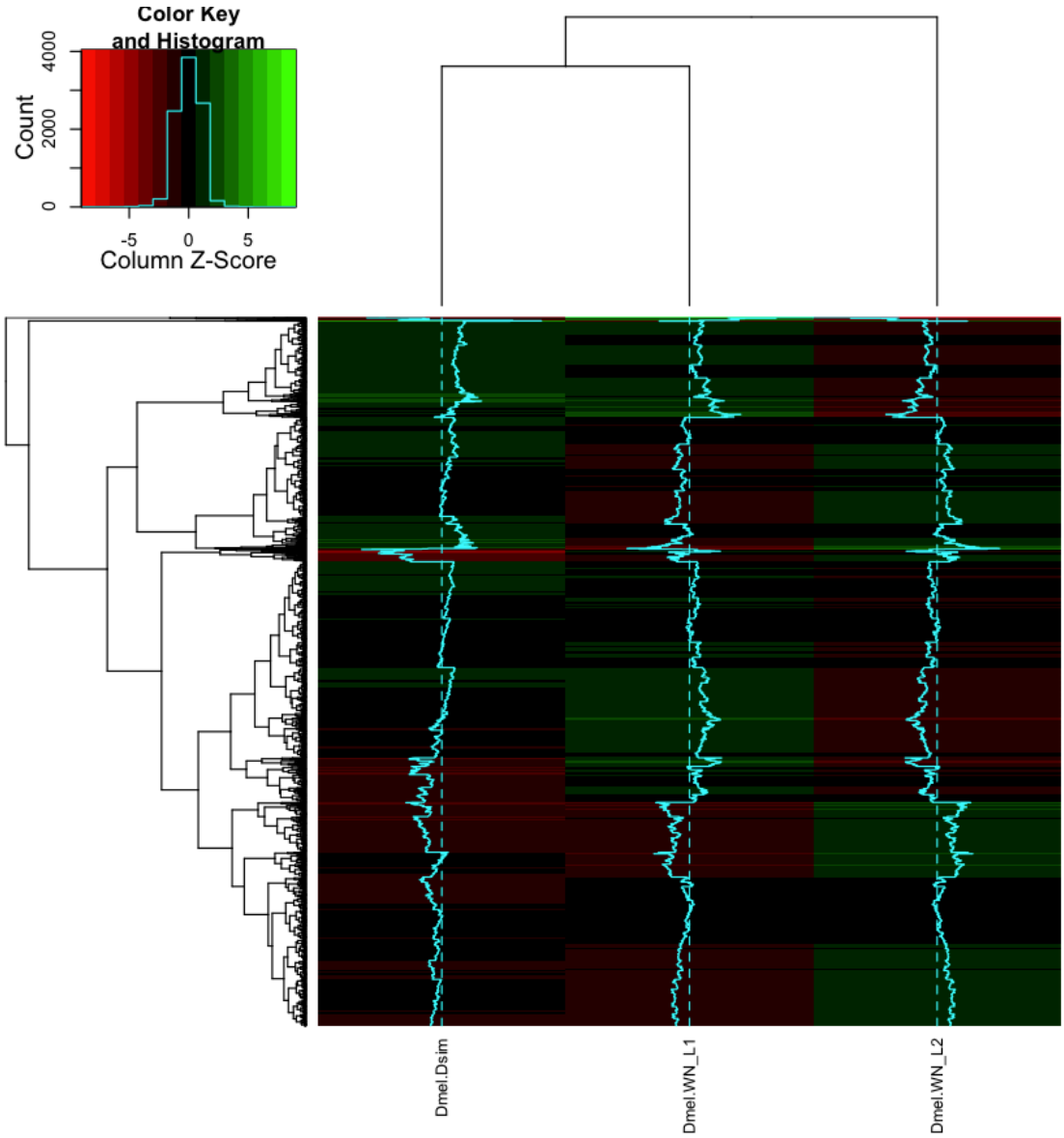


Figure 1. Transcript abundance after five and fifteen minute song stimulation for A) *TotC* and B) *TotM*. 95% Confidence intervals shown for mean relative expression values.



Supplementary Figure 1. Due to uncertainty about the labeling direction for the arrays with samples conspecific song (Dmel) and white noise control (WN), the labeling direction (i.e. which group was labeled with Cy3/Cy5) was confirmed with hierarchical cluster analysis (using Euclidean distance), shown here with the Heatmap image (the arrays are presented as columns and genes as rows). The set of arrays with Dmel-WN samples were assigned two different labeling directions (L1 and L2) and the correct labelling was inferred from the pattern of which group clusters together with the arrays containing samples conspecific and heterospecific song (Dsim). This is based on the assumption that the conspecific song stimulation should result in overall similar expression profile across the probes (whether compared to white noise or heterospecific song).

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CHAPTER 4

What is the role of immunity genes in female reproduction?

A short review and testing the effect of *Turandot* gene knockdown upon female fecundity

Abstract

Over the past three decades research across taxa has demonstrated the involvement of the immune system in reproduction. Mating can either induce or suppress female immunity, and the first part of this chapter discusses the current hypotheses for these phenomena. Traditionally, activation of the female immune system has been viewed as a direct response to the presence of pathogens or as an adaptation against the risk of pathogen infection due to genital contact or seminal fluid transfer ('pre-emptive strike' hypothesis), particularly in species with traumatic insemination. More recently it has been suggested that the induced immune response may arise due to male seminal compounds evolving under sperm competition being recognized as alien substances to female body. This kind of 'immunogenic male' effect could be costly to females if the immune response trades off with resource allocation for reproduction, and thus sexually antagonistic selection may play a role in the evolution of female immunity. In species with male induced suppression of immunity, females may face an increased risk of infection, which suggests another source of conflict to females. However, the immune response to male molecules may also be directly favoured in females. Chapter 3 showed how some immunity related genes respond to attractive song independent of receiving male molecules. In the second part of this chapter I test the role of two of these genes, *Turandot M* and *C,* in female fecundity by using RNA interference with the *GAL4/ UAS* binary system. I find that knocking down the expression of each of these genes increases the egg-laying rate during the first 24h, but not beyond. These findings are against the 'pre-emptive strike against pathogens hypothesis', which would predict the opposite effect. My results suggest instead that

the products of these genes may interact with male seminal fluid proteins known to manipulate female egg-laying rate, often with a detriment to female survival and (possibly) optimal reproductive rate. I discuss a new possibility that at least some immunity related genes involved in female reproduction might be involved in mitigating such reproductive costs.

Introduction

Traits involved in sexual reproduction represent a complex array of adaptations due to the evolutionary interplay between the sexes over the control of fertilization and fecundity. Some of the evolutionary processes involved arise from mutual interests for both sexes, such as natural selection for adaptations to produce offspring in the first place. Others arise due to differences in optimal reproductive rates between the sexes, generating intersexual competition and conflicting evolutionary interests between the sexes (CHAPMAN *et al.* 2003a). Reproductive traits involved in internal insemination such as male genitalia, sperm and seminal fluid together with female reproductive organs and proteins, are at the core of such evolutionary processes.

One potential outcome of sexual interactions is the involvement of the immune defence system. Several mutually non-exclusive theories have been put forward to explain this and in the first part of this chapter I will discuss some of the most compelling hypotheses based on evidence from both mammals and insects, although with a focus on *Drosophila melanogaster*. Additional hypotheses not covered here that are less relevant to *Drosophila* can be found, for example, in a recent review by Morrow and Innocenti (2011). In the second part of this chapter I

will describe an experiment on exploring the effect of two immunity genes, *Turandot C* and *M*, on female post-mating fecundity.

I) Mating-related immunity reactions: a short review

Over the past three decades research across taxa has demonstrated that the immune system is involved in reproduction, through the effects on male autoimmunity and the induction of immune response as well as immunosuppression in females. However, evolutionary explanations for these have caught the interest of biologists only relatively recently (see LAWNICZAK *et al.* 2007; MORROW and INNOCENTI 2011; POIANI 2006; SIVA-JOTHY 2009). Immune reactions are largely generated by interactions between components in the male ejaculate and female reproductive system. Alongside spermatozoa, males transfer seminal fluid into females, produced by accessory glands, the ejaculatory duct and ejaculatory bulbs. Seminal fluid plays an important role in fertilisation (e.g. through sperm capacitation, facilitation of spermatozoa movement, nourishing spermatozoa, sperm storage as well as stimulation of female oogenesis and oviposition) and in sperm competition (e.g. through the formation of mating plugs, modification of spermatozoa speed, reduction of female receptivity to future matings, and allospermicidal functions) (reviewed in POIANI 2006). Increased immune response in females is widely documented in mammals (ALEXANDER and ANDERSON 1987), where both sperm and seminal fluid cause an inflammation of the reproductive tract in mated females initiated by expression changes in cytokine and chemokine genes (O'LEARY *et al.* 2004; ROBERTSON *et al.* 1996; SHARKEY *et al.* 2007). In *D. melanogaster* there is also strong evidence that male seminal fluid, and sperm to some extent, cause an increased expression of many immunity related genes in mated females (DOMANITSKAYA *et al.* 2007; INNOCENTI

and MORROW 2009; LAWNICZAK and BEGUN 2004; MACK *et al.* 2006; MCGRAW *et al.* 2008; MCGRAW *et al.* 2004). However, immunosuppression by substances in male seminal fluid is also commonly documented in both mammals (ALEXANDER and ANDERSON 1987; POIANI 2006) and insects (LAWNICZAK *et al.* 2007), including down-regulation of some immunity-related genes (MCGRAW *et al.* 2008; MCGRAW *et al.* 2004). In the following I will discuss alternative but mutually non-exclusive hypotheses for why the female immune system responds to mating.

Immune response to mating due to pathogens

It is well known that pathogens can be transferred from one partner to another during mating, particularly in vertebrates (POIANI 2006). Some immune responses seen in females are likely to be induced by infective pathogens, or by septic injury. For instance, injury caused by spikes and spurs in male genitalia is likely to elicit immune response in species where traumatic insemination exists (CRUDGINGTON and SIVA-JOTHY 2000; KAMIMURA 2007; REZAC 2009) and the opportunity for infection is higher for species that live in microbe-rich environments, such as bed bugs (REINHARDT *et al.* 2005). However, in insects the risk of receiving sexually transmitted diseases via genital contact or seminal fluid transfer appears to be low in general (KNELL and WEBBERLEY 2004).

'Pre-emptive strike' to potential infection

In most systems, the post-mating immune response is directly related to receiving male ejaculate itself, rather than pathogens, which has prompted several researchers to suggest that the response could be a mutual adaptation of both sexes to pre-empt a risk of potential infection if pathogens are likely to be transferred or if mating is

physically damaging (CASTELLA *et al.* 2009; DOMANITSKAYA *et al.* 2007; FEDORKA *et al.* 2007; GENDRIN *et al.* 2009; MCGRAW *et al.* 2008; PENG *et al.* 2005). Morrow and Innocenti (2011) argue, however, that the 'pre-emptive strike' hypothesis is insufficient from an evolutionary point of view as a universal cause for a number of reasons. Firstly, potential pathogen transfer and septic injury happen simultaneously with male ejaculate transfer, which makes it difficult to see why male-induced effects would be more efficient than a direct response to the presence of pathogens or injury. Secondly, if the risk of pathogen transfer is not high, the unnecessary employment of the female immune system should not be a favourable strategy, as mounting an immune response is costly (MCKEAN *et al.* 2008). Moreover, the expression responses of immunity-related genes to pathogens and to mating appears to be quantitatively and qualitatively different, which casts doubts on why only a part of the machinery would become activated upon mating if the mating-related immunity genes function in pathogen defence (MORROW and INNOCENTI 2011). In *D. melanogaster* there is currently no evidence that increased transcript abundance of immunity genes translate into a better immune defence in general (LAWNICZAK *et al.* 2007), and mating does not appear to increase the female ability to fight against bacterial infection (MCKEAN and NUNNEY 2005). However in a cricket *Gryllus texensis* mating does enhance parasite resistance (SHOEMAKER *et al.* 2006).

Immune attack on male ejaculate as a by-product

A third explanation for a postmating immune response is that the female system recognizes the male spermatozoa coated by seminal fluid proteins as foreign bodies (i.e. antigens), which elicits the immune reaction (e.g. BIRKHEAD *et al.* 1993; POIANI 2002; POIANI 2006). Indeed, in humans several antigens have been identified in the

seminal fluid that are able to elicit an immune reaction (ALEXANDER and ANDERSON 1987). In this light the induction of female immune defence could be viewed as an indirect by-product of male ejaculate (MORROW *et al.* 2003). In most animal species females mate multiply which is expected to create a strong selection on male seminal fluid components due to sperm competition (BIRKHEAD and PIZZARI 2002). The rapid evolution observed in these molecules (SWANSON and VACQUIER 2002; WAGSTAFF and BEGUN 2007) may consequently counter female adaptation to them, thus re-generating the induction of immune reaction time and again. The consequence of this reaction to males is that the female immune system can reduce male fertilization ability. In humans for example this occurs through lymphocyte attack against the spermatozoa (ALEXANDER and ANDERSON 1987). This in turn should generate a counter selection on males to suppress the female immune response leading to an evolutionary arms race between the sexes. Indeed, male seminal fluid in *D. melanogaster* contains several protease inhibiting molecules that potentially protect male sperm from spermicidal attack (CHAPMAN 2001), and down-regulation of immunity genes upon mating has also been observed (LAWNICZAK and BEGUN 2004; MCGRAW *et al.* 2008; MCGRAW *et al.* 2004). In humans a long list of immunosuppressive seminal products have been identified, and their effects are well characterised (ALEXANDER and ANDERSON 1987). There is also evidence for immunosuppression in the cricket *Allonemobius socius*: Fedorka and Zuk (2005) demonstrated that female polyandry was associated with a decrease in macroparasitic defence (measured as encapsulating capacity), suggesting a role of seminal diversity received in female immune system suppression. Immunosuppression upon mating has also been observed in female mealworm beetles (*Tenebrio molitor)* (ROLFF and SIVA-

JOTHY 2002) and in Japanese calopterygid damselflies (*Matrona basilaris japonica*) (SIVA-JOTHY *et al.* 1998).

Adaptive attack on male ejaculate components

From an evolutionary perspective, there are some problems with the idea that female immunity molecules would attack male seminal products as a by-product of recognizing them as alien antigens. Immune defences are costly in terms of energy and resources, and can trade-off with life-history traits associated with reproduction (e.g. MCKEAN *et al.* 2008). Given such costs, why would selection maintain such a strategy of reacting to 'false alarms'? One possibility is that females are constrained due to the need to be inseminated. Induced immunity may, however, also have an adaptive function in females. Lawniczak *et al.* (2007) suggest that polyandrous females' immune system could attack male sperm in order to assess male quality: only the sperm that can withstand or escape from the attack will successfully become stored. Such a mechanism of cryptic female choice could also explain why it is beneficial for males to suppress the female attack. This could be favoured through indirect selection for sons with their fathers' ability for female immunosuppression that increases their chances for fertilisation.

In males, many functional aspects of seminal fluid molecules that influence sperm competition (AVILA *et al.* 2011; CHAPMAN 2001; POIANI 2006) can potentially have adverse side effects on females. Male seminal fluid molecules manipulate aspects of female reproduction and reduce female longevity in some species (AVILA *et al.* 2011). For example in *D. melanogaster,* the seminal fluid protein Sex Peptide (SP) modulates female refractory period, feeding, sleep behaviours, and together with at least Ovulin and CG11864, stimulate egg production (CARVALHO *et al.* 2006;

CHAPMAN *et al.* 2003b; HEIFETZ *et al.* 2000; HEIFETZ *et al.* 2001; ISAAC *et al.* 2010; LIU and KUBLI 2003). In the female abdomen SP elicits transcription of several antimicrobial peptides via the Toll and IMD pathways (PENG *et al.* 2005). SP, together with protease inhibitors Acp62F and CG8137 and the peptide CG10433, are toxic to females when expressed ectopically (LUNG *et al.* 2002; MUELLER *et al.* 2007), and the reduction in female longevity has been directly associated with SP (WIGBY and CHAPMAN 2005). Male seminal fluid proteins also contribute to a mating plug as a way of reducing the female ability to be inseminated by subsequent males (AVILA *et al.* 2011).

Such negative effects are expected to trigger counter selection in females, potentially inducing a co-evolutionary arms race between the sexes (HOLLAND and RICE 1998). However, thus far little is known about the female molecules that interact with male seminal fluid products. The only exception is the G-protein-coupled receptor of SP, called sex peptide receptor (SPR) (YAPICI *et al.* 2008). Perhaps the role of some of the immunity genes that change expression upon mating is to participate in the interaction with male seminal molecules. They may be actively deployed by the female to play a role in ameliorating the adverse fitness effects, for example as counter-adaptations to toxic effects of seminal fluid molecules, and/or to gain female control over fertilization in cryptic female choice (e.g. by selective sperm killing, influencing sperm storage or breaking down the mating plug with proteolysis). Alternatively they may play other roles in female reproductive events that have nothing to do with defence (against males or pathogens). Viewing female immune responses to male molecules as a mere consequence of naturally selected response to alien substances as a by-product of sperm competition (MORROW *et al.* 2003; MORROW and INNOCENTI 2011; POIANI 2006) is perhaps too narrow, as it suggests a

passive role for females in regulating these genes. Males are part of the socio-sexual environments of females, and therefore the immune responses to mating could represent active female adaptations to these environments.

Conclusions

Given the diversity of immune reactions to mating, it is likely that also their selective explanations are complex and differ between the species. Several factors play a role in contributing to the patterns but are likely to do so in different ways depending on the species: the likelihood of sexually transmitted pathogen infection, the importance of life-history trade-offs and the cost of immune defence, as well as the likelihood of sperm competition. These will be affected by ecological factors as well as the mating system of a species. It is clear that currently there is no consensus on which of these factors is most important.

In order to explore between alternative but mutually non-exclusive hypotheses it is important to identify the functions of the molecules involved. In *D. melanogaster* much is already known about the male seminal fluid proteins and other compounds (AVILA *et al.* 2011; CHAPMAN and DAVIES 2004). However knowledge of the functions of the female molecular counterparts is severely lacking, apart from SPR (YAPICI *et al.* 2008) and identifying genes, and their functional annotations, that change expression in response to mating (DOMANITSKAYA *et al.* 2007; INNOCENTI and MORROW 2009; LAWNICZAK and BEGUN 2004; MACK *et al.* 2006; MCGRAW *et al.* 2008; MCGRAW *et al.* 2004). Thus, it is important to identify the functions of the immunity genes involved in female mating response.

II) Testing the effect of Turandot M and C immunity genes upon female fecundity

Turandot (Tot) M and *C* belong to a gene family of eight members (*TotA, B, C, E, F, M, X, Z*) which have previously been implicated in *Drosophila* immune and stress responses. Transcription of both *TotM* and *TotC* respond to bacterial infection (EKENGREN and HULTMARK 2001) and to septic injury (AGAISSE *et al.* 2003; BRUN *et al.* 2006) in adult *D. melanogaster*. TotA has been demonstrated to be an extracellular protein, which is exported into the hemolymph and is therefore likely indicated to play a role in humoral response to various environmental stressors (EKENGREN *et al.* 2001). Proteins encoded by the other members of the gene family, including TotM and TotC, all contain N-terminal signal peptide sequences, suggesting they are similarly likely to enter the hemolymph (EKENGREN and HULTMARK 2001).

TotM and *TotC* have been implicated in a mating-related gene expression response in *D. melanogaster* females. By comparing the expression profiles of females mated to normal males and to males that lacked sperm, or both sperm and accessory gland proteins (Acps), McGraw *et al.* (2004) found that *TotM* was up-regulated when females received sperm compared to when they did not. Similarly, Innocenti & Morrow (2009, Supplementary Table 1) observed increased expression of *TotM* in doubly mated females compared to virgins, while up-regulation of *ToC* has been demonstrated in the brains of mated females (DALTON *et al.* 2010).

Mating, however, is not the only context linking *TotM* and *C* expression to female sexual interactions with males. In chapter 3 I explored gene expression changes in females stimulated with attractive conspecific song, non-attractive heterospecific courtship song or white noise. I observed increased expression levels in both *TotM* and *TotC* when females were stimulated with attractive song compared to

heterospecific song. *TotC* expression levels were also higher with conspecific song compared to white noise. This suggests that expression changes in these genes are not only a result of mating with males: females can actively induce their expression independent of male sperm or other substances (IMMONEN and RITCHIE 2012). This is in contrast with previous suggestions that the increased expression of female immunity genes occurs as a direct response to the presence of male ejaculate products ('immunogenic male hypothesis') or pathogens (MORROW and INNOCENTI 2011). It is, however, compatible with the 'pre-emptive strike' hypothesis (SIVA-JOTHY 2009): if for example mating induced septic injury was common in *D. melanogaster*, females could anticipate this by up-regulating *Tot* genes prior to mating. Alternatively, these genes could play a role in interacting with male seminal fluid molecules to ameliorate reproductive costs associated with the receipt of male seminal fluid molecules.

To understand the evolutionary function that of these genes in females we need to identify their precise role. As a first step in doing this, I generated females that have reduced expression of either *TotM* or *TotC* using RNA interference (RNAi). I hypothesized that if an increase in pre-copulatory transcription levels of these genes is important for female fitness in terms of 'preparing' the female reproductive system for mating, there should be consequences of suppressing these genes on immediate post-mating fecundity. I therefore tested the effect of reduced expression on fecundity during the first five days after mating, using GAL4/UAS binary system (DUFFY 2002). I predicted that if Tot proteins are involved in the 'pre-emptive strike' against e.g. septic injury, their reduction should reduce fecundity. However, an increase in fecundity with *Tot* expression knocked down would support the idea that these gene products interact with male seminal fluid molecules to regulate immediate female fecundity.

Materials and Methods

Fly strains

RNAi strains were obtained from the Vienna Drosophila RNAi Centre (*w;UAS-TotC-IR,* transformant ID 106379; *w;UAS-TotM-IR*, ID 106726). More information on how the RNAi strains were created can be found in Dietzl *et al.* (2007). I used a *GAL4* strain with ubiquitous expression under the *Actin5c* promotor (*yw; Act5c-GAL4/CyO*, obtained from E. Rosato). To generate the experimental RNAi F1 females with reduced expression, I crossed females carrying the *Act5C-GAL4* driver with males carrying either inverted repeats for *TotM* or for *TotC*. *Act5C-GAL4* driver in the parental strain is balanced over *CyO* dominant marker that produces curly wings, and therefore the F1 flies were screened for straight wings to confirm the presence of *GAL4* allele. As a control for maternal genotype, I used F1 females from a backcross between *Act5C-GAL4* females and *w1118* males (the genetic background strain), and as a control for the paternal genotype I used F1 females from a backcross between *w1118* females and either *w;UAS-TotC-IR* or *w;UAS-TotM-IR* males. In this way each of our control strains were heterozygotes for the transgenic loci similar to the knockdown strains. All stocks were housed on a 12h light-dark cycle at 24°C, on a standard agar-sugar-yeast medium supplemented with dried baker's yeast. Virgin flies were collected under light CO_2 anaesthesia within four hours of eclosion. Five-day old virgin F1 females from each of the crosses were used for the experiments.

Fecundity assays

I assessed the effect of *TotC* and *TotM* knockdown on female fecundity in the following three intervals: eggs produced during the first 24h, offspring eclosed during

24-72h (1-3 days) and during 72-120h (3-5 days) after mating. Females from the experimental and control strains were individually paired with *w1118* virgin males and left for up to three hours to ensure a successful mating (N>26 for each female strain). After all matings were finished, females were transferred into vials containing medium with charcoal and left to oviposit for 24h, after which they were removed and eggs counted using a dissecting microscope. Females were subsequently individually transferred into fresh vials with standard medium, and left to oviposit for 48h. They were then transferred to a new vial and again left to oviposit for 48h. Eclosed offspring from these two sets of vials were counted, corresponding to fecundity at 1-3 and 3-5 days after mating. The sample sizes obtained are indicated in Table1.

Statistical analyses

Non-parametric Kruskal-Wallis and Wilcoxon rank sum tests were used for the analyses, as the data could not be transformed to meet the assumptions of normality and homoscedasticity. I tested the effect of strain on the number of eggs laid during the first 24h after mating, as well as on the number of offspring eclosed after 1-3 and 3-5 days after mating. Data analyses were done using R v. 2.13.0 (RDevelopmentCoreTeam 2011) (URL: http://www.R-project.org).

Semi-quantitative PCR

To validate a reduction in gene expression, I estimated the relative quantities of *TotC* and *TotM* mRNA levels in the whole bodies of 24-h post-mated females each of the five strains using semi-quantitative PCR. Total RNA was extracted from 20 females per strain using Qiagen RNeasy Mini –kit, following manufacturer's protocol. Samples were treated with TURBO DNase (Ambion) to eliminate any genomic DNA

contamination. For cDNA synthesis I used iScript^{TM} cDNA Synthesis Kit (Bio-Rad). Ribosomal gene *RpS8* was used as a control gene. Primers were designed with Primer-BLAST (NCBI) (*TotM* forward CGTCACAGAAAAACAGCGCC, reverse GCGTGTGTTCAAGTCCGGTT; *TotC* forward CAACGACGCCGAATCGAAGA, reverse TTCAGGGGACAACGTGGGAG; *RpS8* forward TTTTGACACGAGGTGCTGTG, reverse ACTCGAACTTGCGCTTCTTG). PCR was performed (simultaneously for all the genes) for 40 cycles, and the products were visualized on an Ethidium Bromide gel every five cycles (of cycles 20-40) (Supplementary Figure 1).

Results

There were significant differences between the strains in the number of eggs laid during the first 24h after mating (K-W χ^2_4=24.53, p<0.0001). When both of the strains with reduced expression of *TotM* or *TotC* were compared to the three control strains, they showed a significant difference in the number of eggs (W=3240, p<0.0001, Figure 1). A similar pattern for both of the RNAi strains was confirmed when each of these strains were individually compared to their control strains in planned *post hoc* tests: *Act5c-GAL4/UAS-TotC-IR* females laid significantly higher number of eggs compared to both maternal (*Act5c-GAL4*, W=640, p<0.0001) and paternal genotypes (*UAS-TotC-IR*, W=515, p<0.02). This was also the case for *Act5c-GAL4/UAS-TotM-IR* that showed increased number of eggs when compared to the maternal (*Act5c-GAL4*, W=645, p<0.0001), and paternal (*UAS-TotM-IR*, W=478, p=0.02) strains (Table 1, Figure 2).

Significant differences among the strains were also observed in the number of eclosed offspring 1-3 (K-W $\chi^2=49.84_{(4)}$, $p<0.0001$, Figure 3) and 3-5 (K-W $\chi^2=23.0_{(4)}$, $p=0.0001$, Figure 4) days after mating. However, this was solely due to the lower number of offspring in the *Act5c-GAL4* control strain, as there were no significant differences between the remaining strains when *Act5c-GAL4* was excluded from the analysis for either of the test periods (1-3d: K-W $\chi^2=5.9_{(3)}$, $p=0.11$; 3-5d: K-W $\chi^2=53.7_{(3)}$, $p=0.29$).

Discussion

My results demonstrate that *TotM* and *TotC* influence female oviposition rate during the first 24h after mating: females with either of these genes knocked down showed an increased number of eggs laid compared to their corresponding control strains. After the first day, however, there were no significant differences between the RNAi females and their controls (apart from *Act5c-GAL4*). The increase in fecundity is incompatible with the 'pre-emptive strike' against pathogens hypothesis, which is predicted to result in the opposite effect. It is therefore unlikely that the *TotM* and *–C* gene products are involved in immunity related functions in female mating response.

It is intriguing to find an effect of both *TotM* and *TotC* on immediate egg laying rates. Egg laying in *Drosophila* is regulated by both female- and male-derived molecules (WOLFNER 2009) and results from a sequence of events, starting with oogenesis where germline stem cell divisions within ovarioles give rise to oocytes. Mature oocytes are subsequently released from the ovaries and passed through the oviducts into the uterus where they become fertilized and before being oviposited onto the substratum (CUEVAS 2005). However, because suppression of *TotM* and *TotC*

does not disrupt the egg laying, but on the contrary increases it, this suggests that they do not play a role in the primary process of egg production. Mating rapidly increases oogenesis, ovulation and oviposition, largely triggered by male-derived Acps. As *TotM* and *TotC* transcription is increased by mating (DALTON *et al.* 2010; INNOCENTI and MORROW 2009; MCGRAW *et al.* 2004), it is therefore more likely that the products of these genes could function in interactions with male molecules, and consequently affect fecundity.

During the first 24h after mating the egg laying is largely triggered by the male Acp ovulin (Acp26Aa) that induces ovulation (HERNDON and WOLFNER 1995), but also influenced by BG642312 (encoded by *CG33943*) (RAM and WOLFNER 2007). The sustained increase in egg laying rate beyond the first 24h requires the presence of stored sperm and is largely dependent on the Acp Sex Peptide (Acp70aA) (LIU and KUBLI 2003), but also involves at least four other Acps (CG1652, CG1656, CG17575 and CG9997) (RAM and WOLFNER 2007). The finding that the effect of *TotM* and *TotC* on egg laying was most pronounced during the first 24h raises the possibility that TotM/C proteins interact with ovulin and BG642312 (or other still un-known peptides). An immediate effect could also explain why females start up-regulating these genes early when becoming stimulated by attractive song before mating (IMMONEN and RITCHIE 2012). Ovulin is a prohormone-like polypeptide that stimulates the release of oocytes from the ovary (HEIFETZ *et al.* 2000). In mated females it is mainly found at the base of the ovary (HEIFETZ *et al.* 2000), however some ovulin also enters the hemolymph of the females by crossing the posterior vaginal wall, suggesting a possible neuroendocrine effect on females (LUNG and WOLFNER 1999). After transfer to the female, ovulin is proteolytically cleaved into

four smaller peptides, which are each capable of inducing egg laying (WOLFNER 2009). Currently the molecular targets for ovulin in females are unknown.

Tot proteins, which have signalling properties, are likely secreted into the hemolymph (EKENGREN *et al.* 2001), which raises the possibility that TotM and TotC may function in detection of male ovulin, or other peptides, in the circulatory system of mated females and therefore act as a mediator of egg laying rate. It has been suggested that some Acps, such as Acp62F that also enters the female circulatory system (LUNG and WOLFNER 1999), could function in regulating proteolysis of other Acps such as ovulin (WOLFNER 2002). Acp62F is a proteolysis inhibitor and toxic to females upon ectopic expression (LUNG *et al.* 2002). It is possible that TotM and TotC play a role in interacting with such peptides to mitigate their negative effects. Proteolysis regulators are one of the biggest classes of peptides present within male seminal fluid (AVILA *et al.* 2011), and most of the Acps enter the hemolymph (LUNG and WOLFNER 1999).

Why wild-type expression levels of *Tot* genes yield lower immediate post-mating fecundity compared to their reduced expression is not immediately clear. Eggs that are released immediately after mating are those that have matured prior to mating, and it appears that ovulin stimulates the oviposition of such eggs during the first 6h after mating while sperm is still being stored (CHAPMAN *et al.* 2001). These eggs are fertilized less efficiently than ones oviposited later (CHAPMAN *et al.* 2001), which has led to a suggestion that ovulin would function in 'clearing' mature eggs in order to stimulate further oogenesis and synchronize egg and sperm release (CHAPMAN *et al.* 2001; WOLFNER 2002). Indeed, oviposition relieves the pre-mating arrest of oogenesis caused by the accumulation of mature, un-ovulated eggs (CUEVAS 2005). It is possible

that the suppression of *TotM/C* induces laying of unfertilized eggs, perhaps in interaction with ovulin.

In polyandrous species such as *D. melanogaster* it is in the interest of the male to stimulate a female to use as much of her resources for the current reproductive event as possible. The best strategy for this would be to coordinate the egg production and sperm storage to maximize fertilization efficiency (CHAPMAN *et al.* 2001). However, it is in the female's interest to regulate her own resource expenditure in a given reproductive event to maximize lifetime fitness, due to the limited amount of energy and material resources available. Therefore it is not immediately clear why the female should accumulate mature oocytes before mating only to dump them unfertilized soon after. Although male ovulin is essential for both sexes to initiate egg laying, some aspects of the molecular interplay may be selected to increase this process beyond the female optimum, thus generating sexual conflict and potential for antagonistic co-evolution (HOLLAND and RICE 1998). *Tot* genes could therefore contribute to the process that regulates female egg laying to optimise her resource allocation. However, when testing if increased oviposition due to ovulin could have a cost to females (by comparing the effect of mating with *ovulin*$^{-}$ and *ovulin*$^{+}$ males) Chapman *et al.* (2001) could not detect any differences in survival, lifetime fecundity or lifetime number of progeny. This suggests that ovulin alone does not accrue costs on females. However, ecologically relevant costs can be influenced by food availability (FRICKE et al. 2010), and such effects can be masked in the laboratory conditions with the food available *ad libitum*. Therefore this idea could be tested in the future by assessing the fecundity of *TotM* and *TotC* RNAi females mated to wild-type males and males that lack *ovulin* expression under female dietary restriction. If Tot proteins interact with ovulin to affect immediate fecundity, we should see a

reduced number of eggs for *TotM/C* RNAi females mated to ovulin$^-$ males compared to wild-type males. To test whether increased egg laying of potentially un-fertilized eggs in the *Tot*$^-$ females carries a cost to female lifetime reproductive success (LRS), and to see if this influenced by *ovulin*, *Tot*$^-$ and wild-type females could be mated to multiple wild-type males and their LRS compared to those mated to multiple ovulin$^-$ males.

The idea that *ovulin* is under selection from sperm competition, and potentially antagonistic selection, is supported by its remarkably rapid adaptive evolution: amino acid divergence of ovulin between *D. melanogaster* and its sister species *D. simulans* is around 15% (AGUADE 1998; AGUADE *et al.* 1992; TSAUR *et al.* 1998; TSAUR and WU 1997), whereas on average sequence divergence is only 1- 2% (ANDOLFATTO 2005; TAMURA *et al.* 2004). Association studies have also found a link between polymorphism in *ovulin* and sperm competitive ability (CLARK *et al.* 1995; FIUMERA *et al.* 2005). Interestingly, *TotM* and *TotC* genes are also among the fastest evolving genes between *D. melanogaster* and *D. simulans,* and although some of the selection is likely to arise from their role in pathogen defence (OBBARD *et al.* 2009), it is also possible that co-evolution with male molecules plays a role.

Another interesting candidate seminal molecule that TotM and TotC could interact with is sex peptide (SP). Both injection of SP (CHEN *et al.* 1988) and ectopic expression (AIGAKI *et al.* 1991) stimulate egg production of virgin females. Similarly to ovulin, sex peptide enters the female circulatory system via the vaginal intima (LUNG and WOLFNER 1999), and it has a wide range of target tissues (KUBLI 2008). When the binding sites for sex peptide were investigated by ectopic expression, the

strongest increase in egg production and reduction in receptivity to re-mating were observed when SP was expressed in the female head (NAKAYAMA *et al.* 1997). In line with this, the sex peptide receptor (SPR) is expressed in the central nervous system, as well in the female reproductive tract (YAPICI *et al.* 2008). So far SPR is the only known target for SP (YAPICI *et al.* 2008). According to FlyAtlas (CHINTAPALLI *et al.* 2007) *TotM* and *TotC* show enriched expression in the fat body in the head, and it is possible that their gene products interact with SP in the head tissue, where at least *TotC* shows increased expression in response to mating (DALTON *et al.* 2010). Both of these genes are also expressed in female spermatheca, the main sperm storage organ, which is another target tissue of SP (KUBLI 2008). Sex peptide can reduce female lifespan (CHAPMAN *et al.* 1996; CHAPMAN *et al.* 1995), thus contributing to the cost of mating (CHAPMAN 1992).

By using RNA interference, I have demonstrated that the humoral immunity genes *TotM* and *TotC* have a role in regulating female egg laying rate during the first day after mating. Together with my previous findings that *TotM* and *TotC* show induced expression in female heads in response to courtship song in the absence of males (Chapter 3) (IMMONEN and RITCHIE 2012), these findings suggest that these genes play an important role in female reproduction and can be actively regulated by the females independently of males or pathogens. Whilst several studies have found a mating-induced response of immunity related genes (MORROW and INNOCENTI 2011), the possibility that at least some of these genes may have pleiotropic roles in females beyond immunity has, to my knowledge, not been previously appreciated. The role of *Turandot* genes in female immediate fecundity points to the possibility that they interact with male Acps, such as ovulin and sex peptide, and may evolve under antagonistic selection. Future work will be able to address this exciting possibility.

Previous studies have suggested that immunity genes associated with mating may involve a conflict that arises due to mating associated costs on females. These may arise because eliciting immune response *per se* is costly due to un-optimal resource allocation (INNOCENTI and MORROW 2009; MORROW and INNOCENTI 2011). Where mating associated immunosuppression occurs, however, the cost could arise because of increased risk of pathogen infection (FEDORKA and ZUK 2005; IMROZE and PRASAD 2011; SIVA-JOTHY *et al.* 1998). If Tot proteins indeed turn out to interact with male seminal molecules to regulate optimal egg laying rate, this adds a new possibility of how immunity genes may be pleiotropically involved in mating associated costs by potentially mitigating them. Identifying further the functional roles of immunity related genes in female mating response is therefore vital for understanding whether and how they are involved in antagonistic selection.

Table 1. Sample sizes and results from the post-mating fecundity measurements for each strain (mean ± standard error, h/d= hours/days after mating).

Strain	Sample size	0-24h eggs	Offspring 1-3d	Offspring 3-5d
Act5c-GAL4/UAS-TotM-IR	26	46.5 ± 3.8	78.6 ± 5.2	70.7± 7.6
Act5c-GAL4/UAS-TotC-IR	26	49.8 ± 4.8	84.5 ± 6.9	54.5 ± 8.1
+/UAS-TotM-IR	28	32.7 ± 5.0	89.0 ± 6.3	73.8 ± 9.0
+/UAS-TotC-IR	29	33.7 ± 5.2	90.0 ± 6.0	73.6 ± 8.8
Act5c-GAL4/+	30	21.5 ± 3.5	29.8 ± 4.1	26.4 ± 4.0

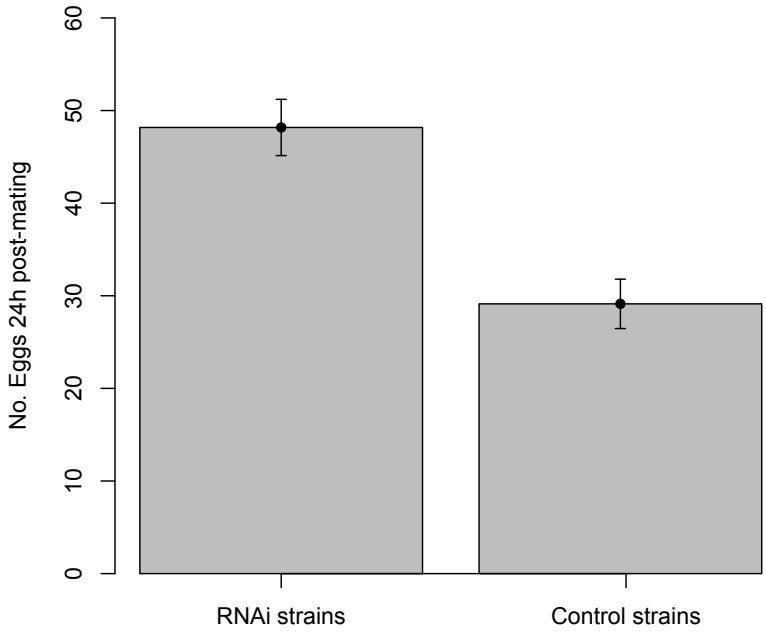


Figure 1. The mean number of eggs laid (with standard errors) during the first 24h after mating by both of the *TotM* and *TotC* knockdown (RNAi) strains and their combined genetic background control strains.

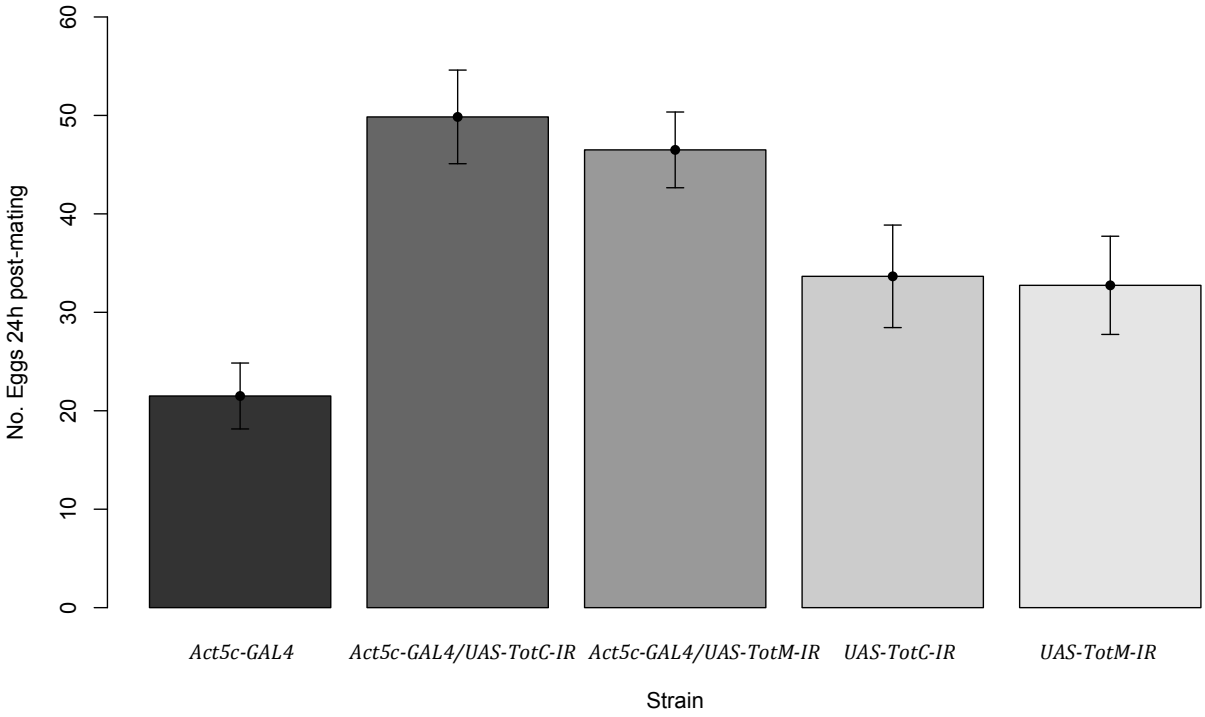


Figure 2. The mean number of eggs laid (with standard errors) during the first 24h after mating by each of the experimental knockdown (Act5c-GAL4/UAS-#-IR) strains and the parental genetic background control strains.

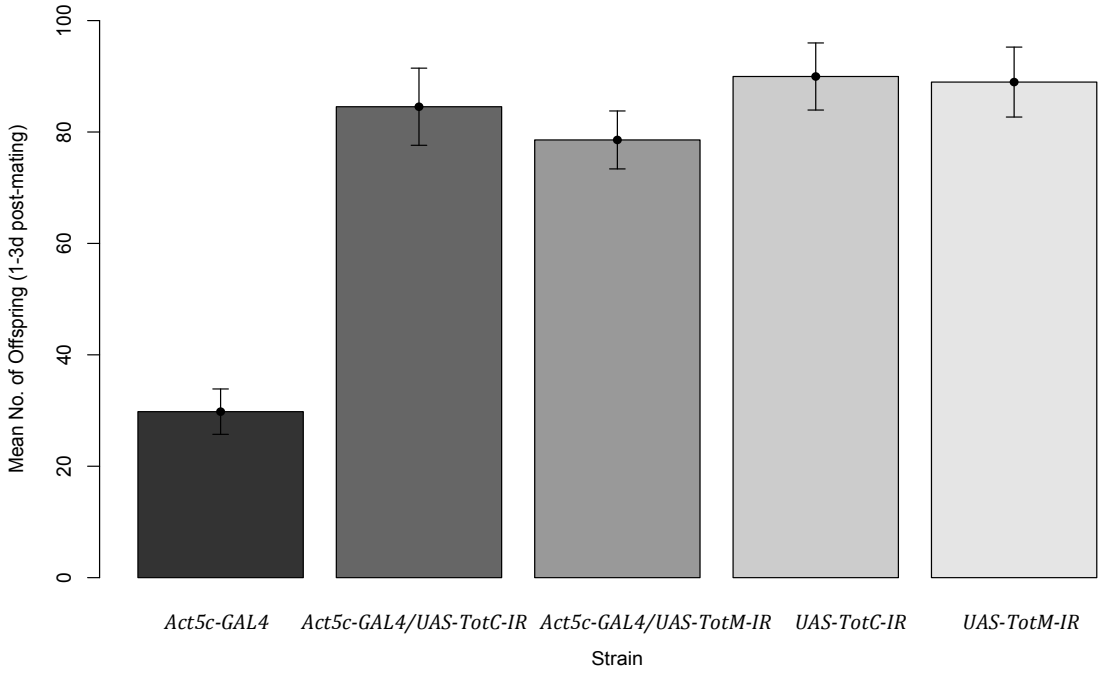


Figure 3. The mean number of eclosed offspring (with standard errors) during 1-3 days (24-72h) after mating by each of the experimental (Act5c-GAL4/UAS-#-IR) strains and the parental genetic background control strains.

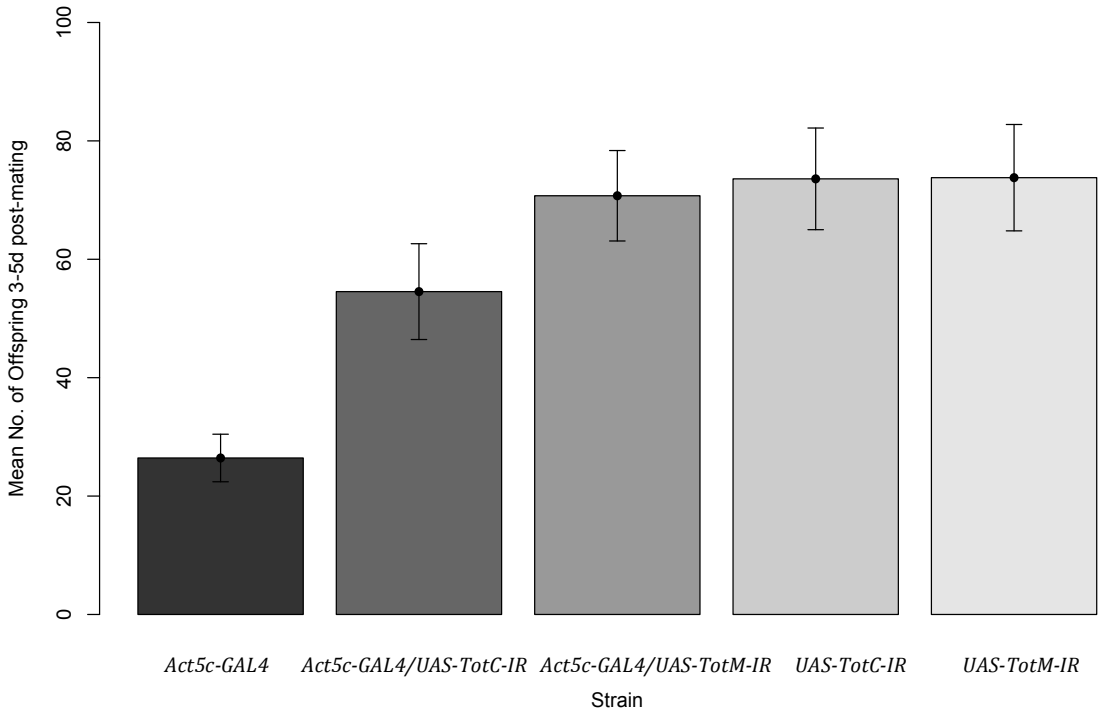
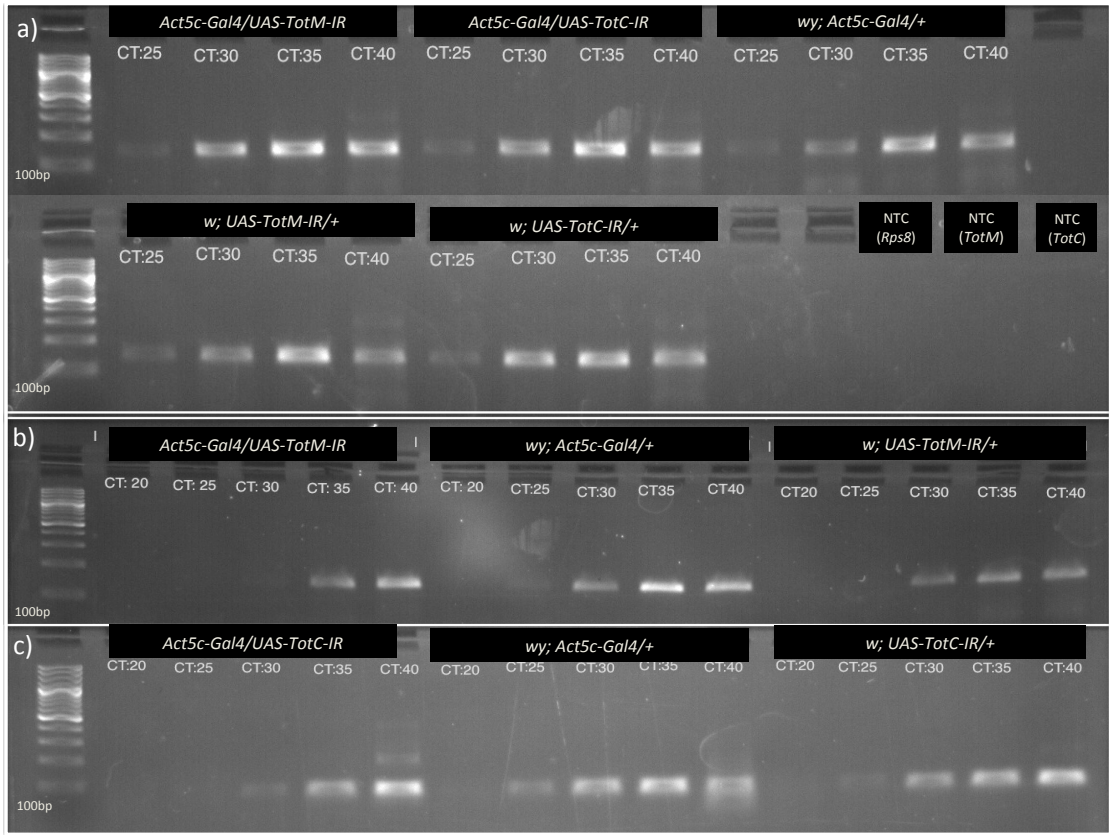


Figure 4. The mean number of eclosed offspring (with standard errors) during 3-5 days (72-120h) after mating by each of the experimental knockdown (Act5c-GAL4/UAS-#-IR) strains and the parental genetic background control strains.



Supplementary Figure 1. Gel electrophoresis image from semi-quantitative PCR (CT = cycle time). A) *RpS8* reference gene amplified equally with all of the samples, B) *TotM* band appears five PCR cycles later in the *Act5c-GAL4/UAS-TotM-IR* knockdown sample compared to either of the control samples, C) *TotC* band appears five PCR cycles later in the *Act5c-GAL4/UAS-TotC-IR* knockdown sample compared to either of the control samples. The no-template negative controls are indicated as NTC in the panel A.

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CHAPTER 5

Experimental polyandry feminizes gene expression

Abstract

Sexual selection has been shown directly to be a major force responsible for the evolution of sexual dimorphism in numerous phenotypic traits, but its role in genetic divergence is indirect. I directly test the role of sexual selection in causing divergence of the transcriptome using replicated experimentally evolved *Drosophila pseudoobscura* females, experiencing either no sexual selection under an enforced monandrous mating system or high sexual selection under an elevated polyandrous mating system, for 100 generations. Using microarrays I compare gene expression of virgin and mated females from the different experimental sexual selection treatments. I show that sexual selection regime affects the expression of up to 43% (N=6,760) of the transcriptome, while mating influences 14% (N=2,220). 77% (N=1,708) of the genes responding to mating have diverged in expression between polyandrous and monandrous females. Sexual selection has predominantly and consistently targeted the expression of genes showing female-biased expression: polyandrous females have increased expression of these genes and concomitantly decreased expression of male-biased genes relative to monandrous females. These female-biased genes are spread across the genome, and not predominantly associated with the X chromosome. Mating status similarly influences the expression of sex-biased genes with polyandrous females showing more pronounced differences than monogamous females. This work provides critical evidence for the direct role of sexual selection in promoting rapid transcriptomic evolution and that sexual selection accentuates sexual dimorphism in gene expression, resulting in increased 'feminization' of the female transcriptome.

Introduction

Sexual selection is one of the most potent sources of accelerated evolution, generating dramatic sexually dimorphic secondary sexual traits that sometimes develop well beyond the limits thought to be optimal under viability selection. Sexual selection can therefore influence the mean fitness of populations and extinction risk (MORROW and PITCHER 2003) but also population divergence and speciation rates when divergence in sexually selected traits reduces gene flow between populations (PANHUIS *et al.* 2001; RITCHIE 2007). However, while the direct role of sexual selection in phenotypic evolution has been studied for some time, its role in molecular evolution is more enigmatic. Males and females share a common genome, apart from a small number of loci located in the heterogametic chromosome, and therefore sex-limited development of a trait results from differential expression of genes that are present in both sexes. Selection for different reproductive strategies of males and females frequently means that the sexes have different phenotypic optima for shared loci, and this difference can generate intra-locus sexual conflict. Theory predicts that this conflict favours the evolution of mechanisms that enable independent expression patterns for each sex for the loci under conflict (LANDE 1980; RICE 1984). Thus, sexual selection is predicted to drive rapid sexual dimorphism of the genome via the evolution of sex-biased expression.

Microarray and sequencing studies have found that a high proportion of genes show sexually dimorphic expression (ELLEGREN and PARSCH 2007). Sex-biased genes, which are usually related to reproduction, show greater rates of coding-sequence and expression divergence among related species compared to un-biased genes (ELLEGREN and PARSCH 2007), particularly for loci expressed at higher levels or

exclusively in males (JIANG and MACHADO 2009; LAWNICZAK *et al.* 2008; ZANGH *et al.* 2004; ZHANG *et al.* 2007). Sexual selection has been invoked as the causal driver of these genetic patterns (ELLEGREN and PARSCH 2007). However, molecular genetic analyses alone cannot directly demonstrate this role and the faster divergence of male-biased genes is not entirely consistent with most sexual selection models, especially antagonistic sexual selection, which predict strong selection on both sexes (GAVRILETS *et al.* 2001; HOLLAND and RICE 1998). One approach to unambiguously determine the action of sexual selection in mediating evolutionary genetic responses is to combine molecular genetic studies with experimental evolution in which replicated populations are allowed to adapt to different sexual selection regimes in the laboratory. Phenotypic and genetic changes in these populations then can be directly associated with the experimental manipulation itself, because these populations start with the same genetic background and are replicated.

I use microarrays and replicated experimental evolution in *Drosophila pseudoobscura* to identify the genes, and their functional roles, that respond to sexual selection in females. I use populations generated by R. R. Snook, where flies have evolved under either obligate monogamy (M) with random mate assignment, which eliminates sexual selection, or elevated polyandry (E), in which one female is housed with six males, which intensifies sexual selection (CRUDGINGTON *et al.* 2005; CRUDGINGTON *et al.* 2009; CRUDGINGTON *et al.* 2010; SNOOK *et al.* 2005), for 100 generations. *D. pseudoobscura* is a naturally promiscuous species, where wild caught females show evidence of mating with 2-3 partners (ANDERSON 1974). Our sexual selection treatment therefore provides females with 2-3 times the natural level of polyandry. The previous work on this system has shown that these treatments are effective; several male and female phenotypic responses to variation in sexual

selection have been documented. Promiscuous E males have faster courtship song (SNOOK *et al.* 2005) and higher courtship frequency (CRUDGINGTON *et al.* 2010). E males have also evolved larger accessory glands (CRUDGINGTON *et al.* 2009), which are organs producing a variety of seminal fluid proteins (Sfps) that influence both male and female fitness after mating (AVILA *et al.* 2011; CHAPMAN *et al.* 1995; WIGBY *et al.* 2009). Sexual conflict occurs as E males, compared to M males, harm M females by reducing the number of her offspring (CRUDGINGTON *et al.* 2010). However, co-evolution with multiple males has benefitted E females who show higher fecundity and offspring hatching success compared to M females when mated to ancestral males (CRUDGINGTON *et al.* 2005).

I capitalize on this well developed system to test the hypothesis that sexual selection (including conflict) generates rapid transcriptome evolution, and focus on the evolutionary genetic response of females to experimental sexual selection. I test the hypothesis that sexual selection increases sexually dimorphic gene expression, and predict that E females will show exaggeration of female-like expression patterns of sex-biased genes compared to M females. Patterns of sex-biased gene expression have been characterised in a wild-type population of this species (JIANG and MACHADO 2009) allowing us to indicate the direction of evolutionary response. Moreover, I examine female gene expression response to mating, because mating involves molecular components of both males and females, and these interactions are a potent source of selection that can act differently on polyandrous and monandrous females. I predict that mating affects sex-biased gene expression by predominantly increasing the expression of female-biased genes because such genes should have important functions in female reproduction. Thus, I also predict that polyandrous and monandrous females will show differences in the expression of sex-biased genes in

response to mating. A further prediction is that the female mating responses are influenced not only by their own genotype but also by their male mates, analogous to genotype by environment interaction.

To test these predictions, I 1) examine divergence in gene expression in response to sexual selection regime and compare these patterns of divergence to wild-type individuals, 2) assess the effect of variation in sexual selection on mating responses by comparing gene expression in virgin and mated females, and 3) determine the extent to which female mating responses are dependent on the male environment.

Materials and Methods

Sexual selection regimes

I used two mating system treatments which varied the opportunity for sexual selection and inter-sexual conflict: obligate monogamy where one male and female are randomly housed together, and elevated promiscuity with one female housed with six males (referred to as the M and E selection regimes, respectively). Mating system treatments were initially established using wild-caught females, and each treatment independently replicated four times, from which two randomly chosen replicate populations per treatment were used in this study. Detailed description of the establishment and maintenance of *D. pseudoobscura* lines can be found in (BACIGALUPE *et al.* 2008; CRUDGINGTON *et al.* 2005; CRUDGINGTON *et al.* 2009).

Experimental setup

I used females that had undergone 100 generations of artificial evolution under the M and E selection regimes. Experimental flies were generated using standard densities of 100 first instar larvae per food vial. Virgin flies were collected and sexed under light CO_2 anaesthesia and used for the experiments 5 days after eclosion. M and E females were exposed to three experimental treatments: virgin, mating with M or mating with E males. The same design was used for each of the two replicate populations. Crosses were performed within replicate populations (e.g. M female * E male from replicate population 1), in order to control for differences due to replicate genetic background variation. Matings were carried out within two hours from ZT0 (i.e. lights on) over three days, with the order randomised. 24h after mating females were anaesthetized with CO_2 and stored in RNALater (Qiagen). Virgins were treated similarly but obviously not mated. Five randomly chosen flies per treatment were pooled to form each sample and stored in RNALater. Three replicate biological samples were prepared for each treatment from each replicate population, resulting in a total of 36 samples (see Supplementary table S6 for design). The mating experiment was carried out at the University of Sheffield, and assisted by R.R. Snook and her lab members.

Microarray data

Sample preparation (including RNA extraction from whole bodies), microarray hybridisation and image scanning were performed by the Liverpool Microarray Facility at the University of Liverpool (see http://www.liv.ac.uk/lmf/protocols.htm for details). Agilent 1-colour custom 4-plex 44K oligonucleotide microarrays were used to hybridise one sample per array. The array platform (GPL15171) was designed by Jiang & Machado (2009), and contains 45,220 spots with positive and negative

controls and oligonucleotide probes representing 18,850 unique gene predictions from the *D. pseudoobscura* genome. Gene annotations were done using the *D. pseudoobscura* genome annotation 2.2 (JIANG and MACHADO 2009). The array also contains *D. persimilis* –specific probes, which were excluded from the present analysis. The microarray data has been submitted to Gene Expression Omnibus with accession number GSE35410.

The data for identifying sex-biased genes was obtained from Jiang & Machado (2009) via the Sebida database (GNAD and PARSCH 2006). I used a false discovery rate (FDR) cutoff 0.0001% (q-value < 0.000001) for identifying sex-biased differentially expressed genes, following the approach of the original article (JIANG and MACHADO 2009). For comparing the gene expression of M and E females to wild-type males and females, I again used the data from Jiang & Machado (2009). Raw data was obtained from C. Machado and pre-processed together with our arrays.

Data pre-processing and statistical analyses

1) Statistical Packages

Packages within BioConductor (GENTLEMAN *et al.* 2004) (URL: http://www.bioconductor.org) in R (version 2.13.0) (RDEVELOPMENTCORETEAM 2011) (URL: http://www.R-project.org) were used for data pre-processing and analyses. Raw intensity values were corrected for background hybridisation using "normexp" with method="mle", and between-array normalization performed using "quantile", as implemented with package 'limma' (SMYTH 2005; SMYTH and SPEED 2003). An average intensity value for annotated replicate probes was calculated with

'genefilter' (GENTLEMAN *et al.* 2011), resulting in a total of 15,734 annotated unique genes to be retained for the analysis.

2) Linear models for identifying differentially expressed genes

To test for differential gene expression, I fitted mixed effects linear models using 'limma' package with empirical Bayes approximation of the standard errors using "eBayes" (SMYTH 2004; SMYTH 2005). The p-values of the moderated t-statistics were adjusted by estimating the FDR to control for multiple testing (BENJAMINI and HOCHBERG 1995). Variation due to hybridisation batch effects was tested using lme4 package, but the estimated variances were extremely small and therefore not considered further.

Firstly, I tested the effect of experimental female sexual selection, mating and their interaction by partitioning the gene expression variation using the following linear mixed effects model:

1) $Y = Fsr * Ms + R + \varepsilon$

where Fsr(female selection regime) and Ms(mating status) were fitted as fixed factors, to estimate their main effects and interaction, and R(replicate population) fitted as a random factor. This model was fitted using all 36 arrays.

Next, I tested the effect of male mate's selection regime on the female gene expression. This second model included only the arrays with mated females to test for main effect of males and female x male interaction:

2) $Y = Fsr * Msr + R + \varepsilon$

where Fsr and Msr(male selection regime) were fitted as fixed and R as a random factor.

With a third set of models I wanted to test how M and E females have diverged from wild-type females (Wt-F) and males (Wt-M) for genes sex-biased in the wild-type. For this, I derived expression differences using models where fly type was fitted as a fixed factor (four groups = M, E, Wt-F, Wt-M), and population origin as a random factor. The following contrasts were performed: M vs. E, M vs. Wt-F, E vs. Wt-F, M vs. Wt-M, E vs. Wt-M. Only virgin M and E females were included into this analysis, and the expression values from these contrasts were used in the next step below.

3) Paired T-tests for comparing differences between M and E females with wild-type flies

In order to examine the extent of differences between experimental females and wild-type females and males, I firstly chose the most differentially expressed genes (FDR < 1%) between M and E virgins that show sex-biased expression in the wild-type flies (N=299 and 266 for female- and male-biased genes, respectively). I then compared the average expression difference of these genes between M and Wt-F/Wt-M to that between E and Wt-F/Wt-M, in order to see which type of experimental female differs most from the wild type. I did these using paired T-tests separately for female- and male-biased genes to test whether the patterns differ.

4) Testing for patterns of sex-bias among differentially expressed genes and their chromosomal distribution

In order to test whether there are disproportionate numbers of sex-biased and un-biased genes among the differentially expressed genes from models 1 and 2, I used

Chi-square tests. The expected numbers were calculated based on the proportions of female-, male and un-biased genes among the genes included into the analysis of differential expression (15, 734), which is close to the number of annotated coding sequences in *D. pseudoobscura* genome (16, 071 in annotation 2.2). Chi-square tests were also used to test whether the proportions of female-/male-biased genes up-regulated in M vs. E, and in mated vs. virgin females, (from model1) were significantly different from the proportions of up-/down-regulated genes observed across all differentially expressed genes. Binomial exact tests were used to test whether the sex-biased differentially expressed genes between M and E females show any disproportionate patterns of chromosome distribution. The expected number of female-/male-biased genes in each chromosome was calculated based on the pattern observed for chromosome distribution of the genes I classed as sex-biased in the *D. pseudoobscura* genome. All tests were performed in R version 2.13.0 (RDEVELOPMENTCORETEAM 2011).

5) Functional Enrichment Analysis

To explore which functional groups of genes have diverged in expression between M and E, and which respond to mating, I used the Gene Ontology (GO) database with levels: "Biological Processes" and "Molecular Functions", the Integrated Documentation Resource for Protein Families, Domains, Regions and Sites (INTERPO) databse, and the Kyoto Encyclopedia of Genes and Genomes (KEGG), as implemented within the Database for Annotation, Visualization and Integrated Discovery (DAVID) (DENNIS *et al.* 2003; HUANG *et al.* 2009). The overrepresentation of functional annotations among the differentially expressed genes was assessed using the Functional Annotation Clustering tool, which not only identifies the enriched terms by calculating a moderated Fisher's Exact p-value (FDR adjusted control for

multiple testing), but also clusters similar functional terms together into non-redundant groups and ranks them according to their average significance. This more easily determines the major biological themes associated with the gene of interest, while also allowing the same genes to participate in different clusters. I applied this to the following lists of genes: 1) female-biased genes up-regulated in E vs. M; 2) male-biased genes up-regulated in M vs. E (Table S1); 3) female-biased genes up-regulated in mated vs. virgin; 4) male-biased genes down-regulated in mated vs. virgin (Table S2); 5) female-biased and 6) male-biased genes differentially expressed between both M vs. E and mated vs. virgin (Table S3); and female-biased 7) and male-biased 8) genes affected by female regime * mating interactions (all from model1) (Table S4). For the differentially expressed genes 9) between females mated to M vs. E males, and 10) those significantly affected by female * male selection regime interactions (both from model2), I tested for the functional enrichment across all the significant genes rather than in respect to their sex-bias, due to their lower numbers, however no significant functional clusters were identified (but see Table S5 for the genes only). All the functional enrichment analyses were done using *D. melanogaster* orthologs (obtained from C. Machado).

Results and Discussion

Expression divergence under sexual selection

Using Agilent one-colour custom microarrays (JIANG and MACHADO 2009) I observed consistent and large-scale divergence in expression patterns of both virgin and mated M and E females, across two replicate experimental populations. 6,760 genes were differentially expressed (DE) (with <5% false discovery rate, FDR), which constitutes

43% of the transcriptome (Figure 1a). Out of these, 4,469 (66% of all DE genes) were genes that show sex-biased expression in wild-type *D. pseudoobscura* (JIANG and MACHADO 2009). Sexual selection altered sex-biased gene expression ($X^2_{(2)} = 425.0$, $p < 0.0001$) targeting pre-dominantly female-biased genes (i.e. genes with higher expression in wild-type females), with a 35% excess of female-biased and a 23% deficit of un-biased genes (Figure 1b). E and M females showed opposing patterns of expression changes with regard to sex-biased genes (Figure 2a-c): a significantly greater proportion of the differentially expressed female-biased genes was up-regulated in E compared to M females ($X^2_{(1)}=647.4$, $p<0.0001$, total gene numbers= 2,428; 477, respectively), while M females had significantly more up-regulated male-biased genes compared to E ($X^2_{(1)}=625.5$, $p<0.0001$, total gene numbers=1111; 453) (Figure 2a-c). This pattern was similar regardless of female mating status (Figure 2b).

The net effect of these changes supports my prediction that the sex-biased gene expression becomes exaggerated in females evolving under higher levels of sexual selection, but in order to determine the direction of change, I tested how M and E females differ in their expression profiles relative to wild-type *D. pseudoobscura* (JIANG and MACHADO 2009). To do this, I first identified the sex-biased genes that showed divergent expression between virgin M and E females (FDR <1%), and quantified the expression difference (log base2 of the fold change, logFC) for these genes between each of the regimes and wild-type females (Wt-F) and males (Wt-M). I then compared the average expression difference of each sexual selection treatment to wild-type across these genes, separately for female- and male-biased genes.

I first tested how different M and E females are from wild-type females in their expression of both female- and male-biased genes. For female-biased genes, M females showed lower expression (average logFC -0.24; i.e. higher expression in Wt-

F) and E female showed slightly higher expression (average logFC of 0.022) compared to Wt females. The difference between the E and M females for female-biased genes is significant in their comparison to the wild-type females ($t_{(298)}$=-9.9, p<0.0001, Figures 3a, 4a). The opposite pattern was found for male-biased genes. M females up-regulated (average logFC of 0.32) and E females down-regulated (average logFC = -0.18) male-biased genes compared to Wt-F, demonstrating similarly significant average differences to Wt-F between the two regimes ($t_{(265)}$=11.3, p<0.0001, Figures 3b, 4b). Thus, monogamous females show more male-like expression patterns when compared to the wild-type females, whereas polyandrous regime females are more similar in their expression of female-biased genes and show even lower expression of male-biased genes than the wild-type females.

I then tested how different M and E females are from one another when compared to wild-type males. As expected, female-biased genes were on average up-regulated in the experimentally evolved females in comparison to males. However, M females showed a less marked difference compared to E females (M-WtM: average logFC=1.89; E – WtM: average logFC = 2.21; $t_{(298)}$=-12.5, p<0.0001, Figures 3a, 4a). For male-biased genes, wild-type males unsurprisingly showed higher expression compared to females from both of the regimes. However, this difference was again less pronounced in monandrous compared to polyandrous females (M-WtM: average logFC =-1.43; E-WtM: average logFC = -1.95; $t_{(265)}$ =12.0, p<0.0001, Figures 3b, 4b).

Overall, the expression profiles of the females that were subject to the high sexual selection regime more closely resemble wild-type females (which are also polyandrous, but to a lesser extent), but with both exaggeration of female-biased, and reduction in male-biased gene expression, whereas monandrous regime females had profiles more similar to wild-type males. These patterns support the prediction that

sexual selection drives dimorphism: the absence of sexual selection decreases sexual-dimorphism in the transcriptome whereas high levels of sexual selection accentuates dimorphism, and specifically 'feminizes' the transcriptome.

Theory predicts that selection should favour female-beneficial alleles which accumulate on the X chromosome (in an XY system), because X-linked loci spend two thirds of their time in females (RICE 1984). In accordance with this, many studies have found enrichment of female-biased genes on the X (ELLEGREN and PARSCH 2007), including in *D. pseudoobscura* (JIANG and MACHADO 2009). I tested whether female-biased genes that show expression divergence in response to sexual selection predominantly reside on the X, but found no support for this (Exact binomial test: ChrX: $p>0.05$). Instead, my results suggest that sexual selection acts evenly on the expression of female-biased genes across all genomic locations (Figure 5). For male-biased genes, however, there was a disproportionate excess of differentially expressed genes on the 2nd and 3rd chromosomes and deficits on the 4th and in the left arm of X chromosome (Exact binomial test: Chr2: $p=0.05$, Chr3: $p=0.04$; Chr4: $p=0.001$; ChrXL: $p=0.01$) (Figure 5).

Sexual selection has been assumed to underlie the evolution of sexual dimorphism in gene expression (ELLEGREN and PARSCH 2007), but this is the first experimental evidence to directly demonstrate this. Recently, microarray studies of expression dimorphism have been criticised for directly comparing males and females, because some of the patterns may arise as an artefact due to dimorphism in body/organ type, size and shape (STEWART *et al.* 2010). Here I compare directly only size and age matched females, thus eliminating any possibility for such a bias. While some of the genes identified as sex-biased in the wild-type flies (JIANG and MACHADO 2009) could potentially be subject to such artefacts, my results provide confirmation

that the loci identified in the present study are likely to be sexually dimorphic and underlie functional traits evolving under sexual selection.

Divergence between females in the expression response to mating

Mating is the main arena in which sexual selection acts through inter-sexual molecular interactions involved in sperm competition and cryptic female choice and thus gene expression responses to mating have been analysed to help understand such post-copulatory sexual interactions (e.g. INNOCENTI and MORROW 2009; LAWNICZAK and BEGUN 2004; MCGRAW *et al.* 2008; MCGRAW *et al.* 2004). Here I test for gene expression changes in response to mating but do so in the experimental context of how this response is influenced by sexual selection. I compared M and E virgin females' expression profiles to those of 24h post-mated M and E females. I found 2,220 differentially expressed genes that respond to mating (FDR < 5%), which corresponds to 14% of all the genes in the analysis (Figure 1a). Expression response to mating of a similar magnitude has been documented in *D. melanogaster* (INNOCENTI and MORROW 2009; LAWNICZAK and BEGUN 2004; MACK *et al.* 2006; MCGRAW *et al.* 2008; MCGRAW *et al.* 2004). 1,428 (64%) of these genes show sex-biased expression (855 female-biased and 573 male-biased genes) with a 21% excess of female-biased and 7% excess of male-biased genes, and 19% deficit of un-biased genes ($X^2_{(2)}$= 69.9, p<0.001) (Figure 6a). Mating has opposite effects on the expression of sex-biased genes: as predicted, mated females significantly up-regulate female-biased genes compared to virgin females (N=472, 383, respectively; $X^2_{(1)}$=11.1, p=0.0009) whereas male-biased genes were significantly down-regulated in mated compared to virgin females (N=158, 415, respectively; $X^2_{(1)}$=110.2, p<0.0001) (Figure 6b).

I examine two classes of sex-biased mating response genes that show differences depending on female selection regime. First, I consider genes that are significant for both main effects of mating and female sexual selection regime, and second, I consider genes that are significant for mating X female selection regime interaction.

I identified 1,708 genes (1,132 sex-biased genes of which 662 were female-biased and 470 male-biased) that responded to mating and differed in expression magnitude between M and E females; this constitutes 77% of all the mating responsive genes (Figure 6c). E females show on average higher expression of the female-biased, and lower expression of male-biased, genes compared to M females (Figure 7a-b), following the same pattern as was observed for all the genes that differ in expression between the regimes (Figure 2a-c).

Functional analysis suggests that the differences in the magnitude of expression between mated M and E females may have important fitness consequences. In *D. pseudoobscura* it takes around 24h to complete sperm storage (SNOOK *et al.* 1994), after which oviposition begins. In line with this I observed increased expression of female-biased genes involved in oogenesis in mated, compared to virgin, females (Table S2, list 3). However, many of these genes involved in oogenesis – with functions including mitosis, meiosis and transcriptional regulation - are more highly up-regulated in mated polyandrous, compared to monandrous, females (Table S3, list 5; Figures 7a; 8). These differentially expressed oogenesis-related genes include a transcription regulator *Notch* (*N, GA28528* in *D. pseudoobscura*) and the major sex-determination transcription factor *Sex Lethal* (*Sxl, GA22653*), which was relatively more repressed in mated polyandrous females (see Figure 8 for predicted protein associations involved). *Sxl* negatively regulates *Notch* transcription in the

ovary, controlling the number of polar cells in follicle cell specification (PENN and SCHEDL 2007). Due to their up-stream role in gene regulatory networks (GEMPE and BEYE 2011; PENALVA and SANCHEZ 2003), transcription factors and their regulators affected by female sexual selection regime are good candidates as direct targets of selection. Observing expression divergence in *Sxl* is intriguing, because it is responsible for the onset of sex-determination and influences the developmental feminization of the whole transcriptome (PENALVA and SANCHEZ 2003). It seems likely that expression differences in *Sxl* contribute to the observed large scale, concerted, divergence in the expression of sex-biased genes. I also identified three other sex-determination genes, *transformer* (*tra, GA28355*) (MCKEOWN *et al.* 1988), which is regulated by *Sxl (PENALVA and SANCHEZ 2003)*, *intersex* (*ix, GA12116*) (CHASE and BAKER 1995), and *hermaphrodite* (*her*, GA18360) (PENALVA and SANCHEZ 2003) as being differentially expressed between polyandrous and monandrous females.

Second, I examined genes that were significant for mating X female selection regime interaction (Figures 1a, 6c). Out of these 958 genes (FDR< 5 %), 67% (N=642) show sex-biased expression in wild-type flies. Average expression profiles across these genes show that mating increases the expression of female-biased genes (N=421) relatively more in polyandrous than in monandrous females, while male-biased genes (N=221) decrease in expression upon mating in polyandrous but increase in monandrous females (Figure 7c-d). The differing response of these sex-biased genes provides an additional 'feminizing effect' on polyandrous females upon mating that does not occur in monandrous females.

Functional analysis indicate that the female-biased genes responding to mating differently between selection regime females are enriched for a variety of cell cycle

processes (Table S4, list 7) whereas the male-biased genes are enriched with those involved in the folate biosynthesis pathway as well as in proteolysis (Table S2, list 8). Genes with proteolytic capacities have been suggested to be important in female sperm storage through their effect on sperm survival by destroying sperm surface proteins and male seminal fluid contains many proteins that regulate female proteolysis (AVILA *et al.* 2011; PENG *et al.* 2005). Females evolving under different mating systems encounter different levels of sperm competition and opportunities for cryptic mate choice, and the mating responses of proteolysis genes in polyandrous and monandrous females might therefore reflect adaptations to differential use or degradation of sperm between the regimes.

Gene by environment interactions: the female mating response relative to male mate

An additional aim of this study was to test the prediction that the selection regime of the male influences the expression response of mated females, either similarly across the females or interacting with female sexual selection history. Many of the post-mating changes that take place in females are triggered by receipt of male sperm and Sfps, such as induction and progression of egg production, regulation of immunity, facilitation of sperm storage and usage, as well as control over the female refractory period to remating (AVILA *et al.* 2011). Many of these processes are implicated among the functional clusters of the differentially expressed genes between polyandrous and monandrous females (Table S1-4), suggesting an important role for male-female molecular interactions in divergence of the female transcriptome. Despite this, there was surprisingly little direct effect of male selection history on the female transcriptome response. I identified significant expression changes (FDR< 5%) in 225 genes that solely depended on the male type, and 152 genes (FDR< 5%)

showing an interaction between female and male selection regimes (Figure 1a). The large-scale differences observed between female types represent adaptations to the females' socio-sexual environments rather than responses to the identity of sexual partners. However, the small male effect detected after 24h of mating does not rule out that interactions with male semen may be more pronounced at an earlier time points post-mating. No significantly enriched functional groups were identified for the genes responding differently to male type. However, I identified eight genes that are involved in female reproduction, which not only were affected by the male mate identity but also differed in expression magnitude between M and E females and responded to mating. These include a follicle cell protein coding gene (Fcp3C, GA17864) and *cappuccino* (*capu, GA26878*), which has roles in oogenesis including chorion shell formation on the egg. Two other egg chorion-coding genes were also among those showing female-male selection regime interactions (*Chorion protein b at 7F, Cp7fb, GA13662; Chorion protein c at 7F, Cp7fc, GA13663*, Table S5). These genes could possibly be part of sperm-egg interactions. A network of all the genes involved in oogenesis that differ in response to mating and have diverged in expression between female regimes (Figure 8) reveals predicted protein associations and how they involve the eight male effect genes.

Evolutionary implications

By comparing gene expression between experimental monandrous and polyandrous females with patterns of sex-biased gene expression in wild-type flies, this study demonstrates that the female transcriptome evolves rapidly under sexual selection. In particular, monandry masculinizes the transcriptome whereas elevated polyandry

leads to a more extreme female-biased pattern of gene expression. I found no evidence that this rapid evolution was biased on the X-chromosome.

What mechanisms are likely to have influenced these patterns? Most changes were observed in the expression of female-biased genes, suggesting a direct role of sexual selection on the regulation of these in females. Mating predominantly increases the expression of female-biased genes, and the majority of genes responsive to mating were also differentially expressed between polyandrous and monandrous females. As mating brings together molecules from each sex, inter-locus interactions are an immediate source of selection acting on females, which are often interpreted as antagonistic in polyandrous mating systems (CHAPMAN 2006). However, polyandry also benefits females, as seen with the increased fecundity of the polyandrous females (CRUDGINGTON *et al.* 2005), as well as in wild-type *D. pseudoobscura* that shows increased offspring productivity and viability without longevity costs (GOWATY *et al.* 2010). The greater expression of oogenesis-related genes in polyandrous females is compatible with this.

Female polyandry exerts differing selection pressures on each sex, potentially inducing antagonistic selection through intra-locus sexual conflict which is expected to be particularly prominent in the lines experiencing elevated levels of polyandry (ELLEGREN and PARSCH 2007). Sex-specific regulation of gene expression is predicted to 'resolve' intra-locus conflict (RICE 1984), and a recent study found that only a fraction of sex-biased genes in wild-type *D. melanogaster* experience intra-locus conflict (INNOCENTI and MORROW 2010). However, because the majority of genes are not sex-limited in expression (STEWART *et al.* 2010), changes in sexual selection intensity could potentially renew conflict, thus favouring further regulatory changes that increase dimorphism in expression. Coupled with direct selection acting

on females, such a mechanism is an excellent candidate contributing to the observed large-scale increase in expression dimorphism seen here for sex-biased genes in the experimental polyandrous females. Reduced sexual selection, on the other hand, may render regulatory elements that maintain gene expression dimorphism between the sexes redundant, which could explain the convergence of the expression patterns seen between experimentally enforced monandrous females and wild-type males (Figure 9).

My analysis of transcriptome divergence under mating system variation in female *D. pseudoobscura* provides a framework for understanding the evolution of sexual dimorphism at the level of gene expression in females. Previous studies comparing the patterns of molecular evolution of sex-biased genes between species have suggested that female-biased genes experience less sexual selection or are more constrained in response due to the observation that they often show lower rates of sequence and expression divergence compared to male-biased genes (ELLEGREN and PARSCH 2007; but see MANK *et al.* 2007; ZHANG *et al.* 2007). However, the rapidity and extent of transcriptome changes observed in the experimentally manipulated females in this study corroborate the predictions of sexual selection theory on females, and demonstrate that even fundamental aspects of reproduction, such as oogenesis, harbour evolvability and can respond to variation in sexual selection.

Tables

Supplementary Tables S1-5 provided as a separate tab delimited Excel file on a disk.

Table S6: Experimental design

Selection regime replicate population	Female selection Regime	Treatment	Male selection regime	Biological replicates
1	M	Virgin		3
2	M	Virgin		3
1	E	Virgin		3
2	E	Virgin		3
1	M	Mated	M	3
2	M	Mated	M	3
1	M	Mated	E	3
2	M	Mated	E	3
1	E	Mated	M	3
2	E	Mated	M	3
1	E	Mated	E	3
2	E	Mated	E	3

Figures

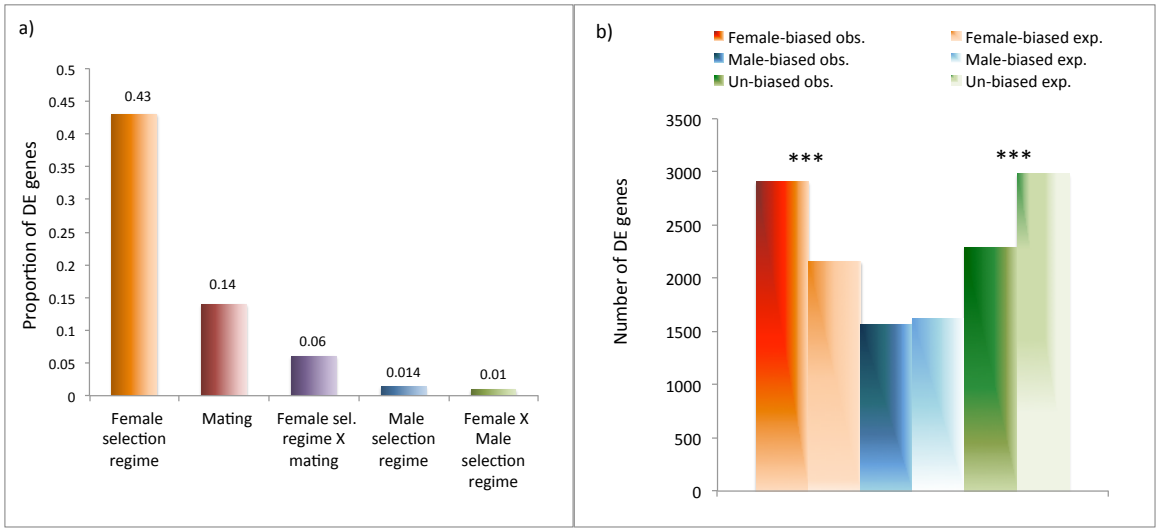


Figure 1: (A) Proportions of genes that are differentially expressed (DE) for each comparison out all the genes analysed. (B) Numbers of observed and expected DE genes affected by female sexual selection regime, separately for sex-biased and un-biased genes (***p<0.001).

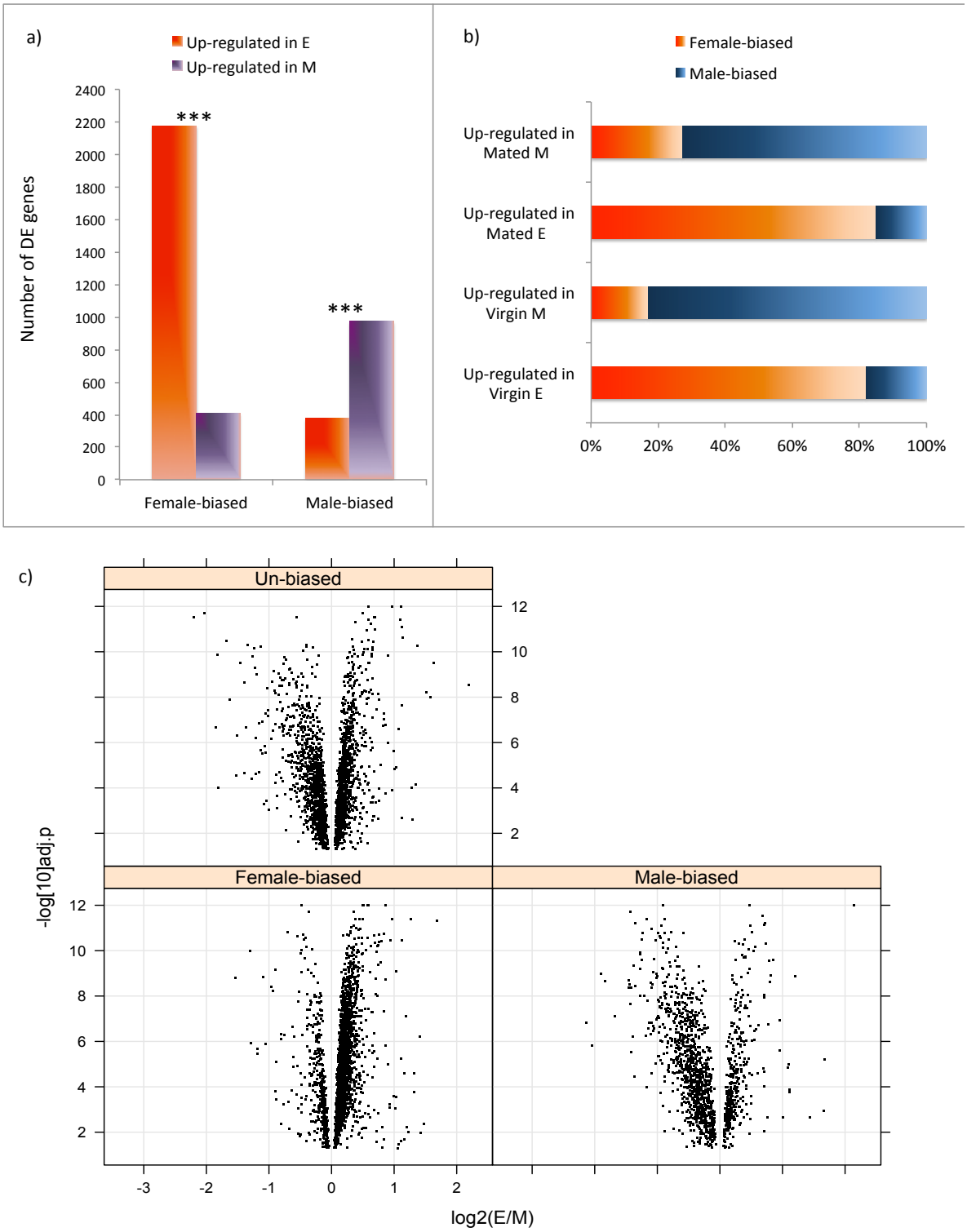
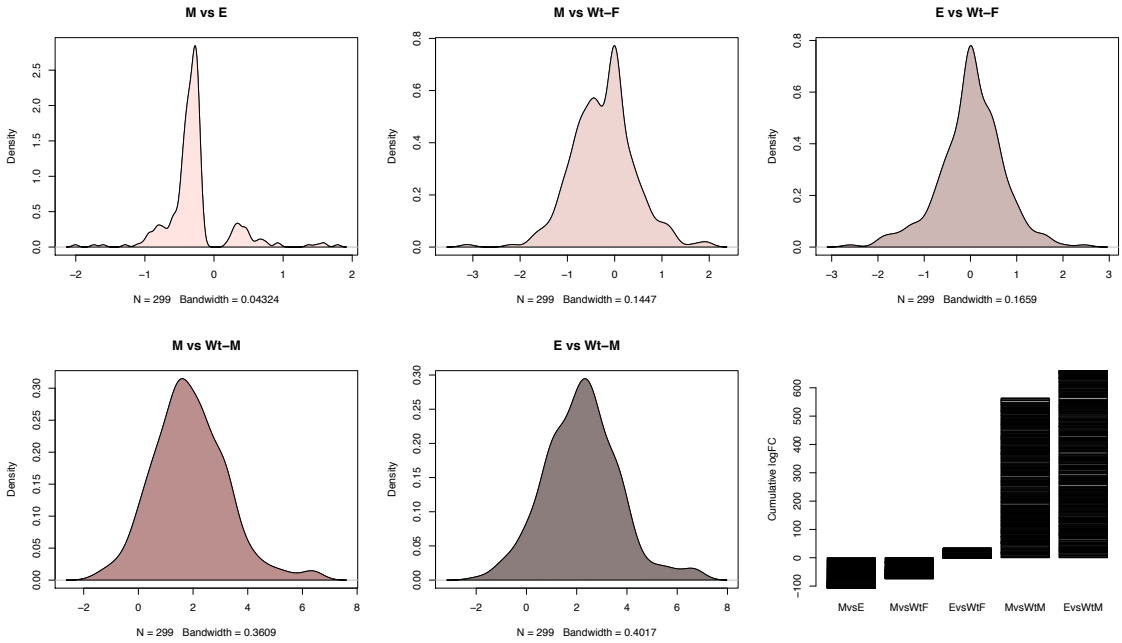


Figure 2. Patterns of gene up-regulation. (A) Numbers of sex-biased differentially expressed (DE) genes affected by the female selection regime that are up-regulated in M and E females (***p<0.001). B) Percentages of sex-biased DE genes that are up-regulated in E and M, for virgins and mated females. (C) The relative expression difference (logFC) of all DE genes between E and M females for un-biased and sex-biased genes, with their FDR adjusted p-value on a reversed log scale (-log10) on the y-axis.

A)


M vs E
M vs Wt–F
E vs Wt–F
M vs Wt–M
E vs Wt–M
Density
N = 299 Bandwidth = 0.04324
N = 299 Bandwidth = 0.1447
N = 299 Bandwidth = 0.1659
N = 299 Bandwidth = 0.3609
N = 299 Bandwidth = 0.4017
Cumulative logFC
MvsE
MvsWtF
EvsWtF
MvsWtM
EvsWtM

B)

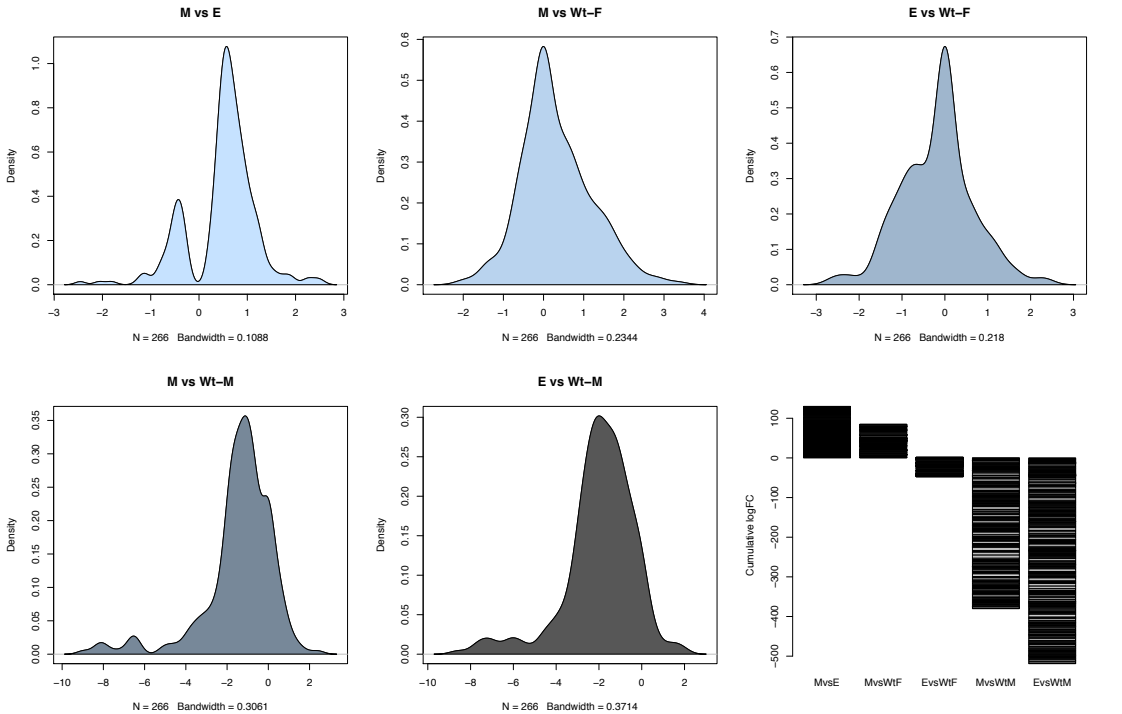
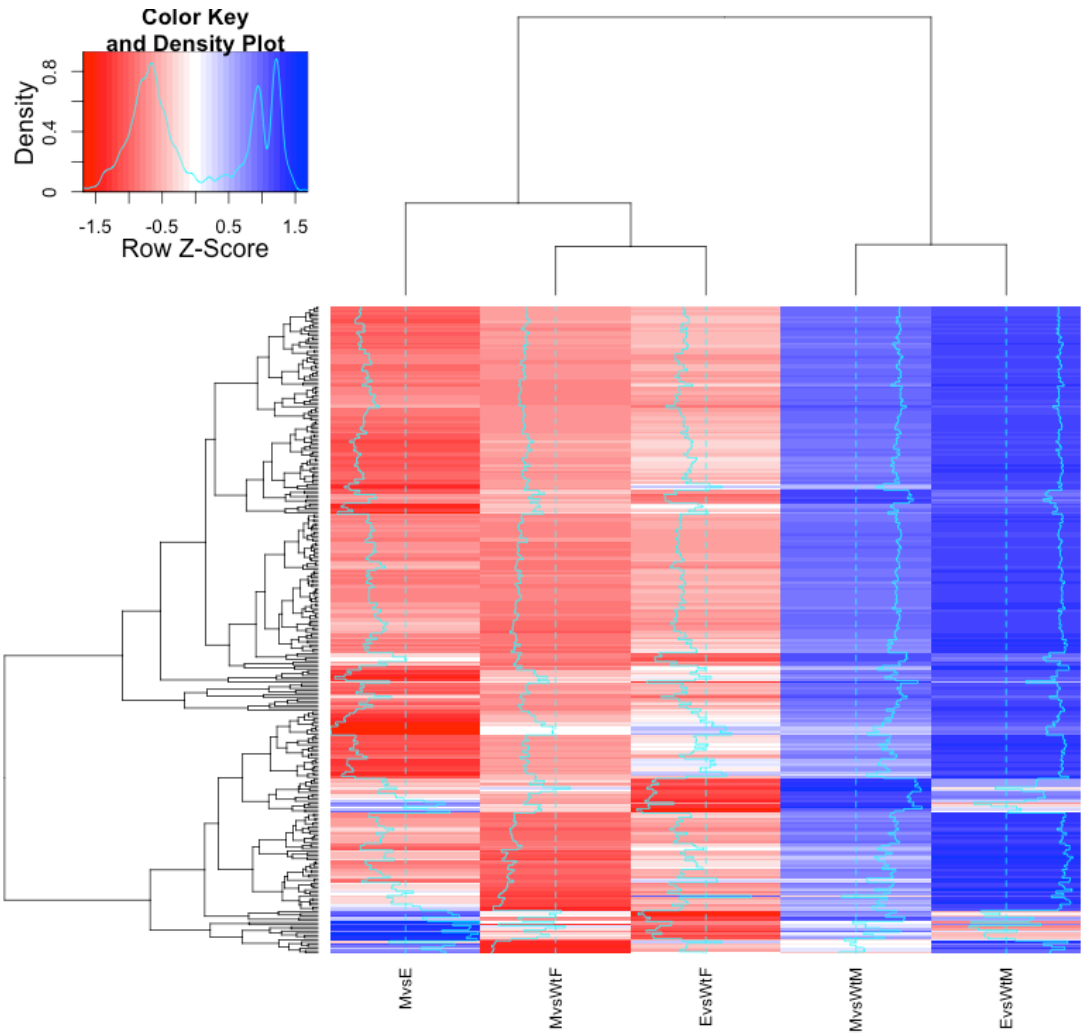

M vs E
M vs Wt–F
E vs Wt–F
M vs Wt–M
E vs Wt–M
Density
N = 266 Bandwidth = 0.1088
N = 266 Bandwidth = 0.2344
N = 266 Bandwidth = 0.218
N = 266 Bandwidth = 0.3061
N = 266 Bandwidth = 0.3714
Cumulative logFC
MvsE
MvsWtF
EvsWtF
MvsWtM
EvsWtM

Figure 3: Density distributions and cumulative barplots of expression differences (logFC) for virgin M vs. E females, M vs. wild-type females and males and E vs. wild-type females and males, for (A) female- and (B) male-biased genes. The focal contrast is indicated in the sub-heading of each plot. Positive values refer to up-regulation in the fly type named first in the contrast (e.g. M in M vs. E).

A)



B)

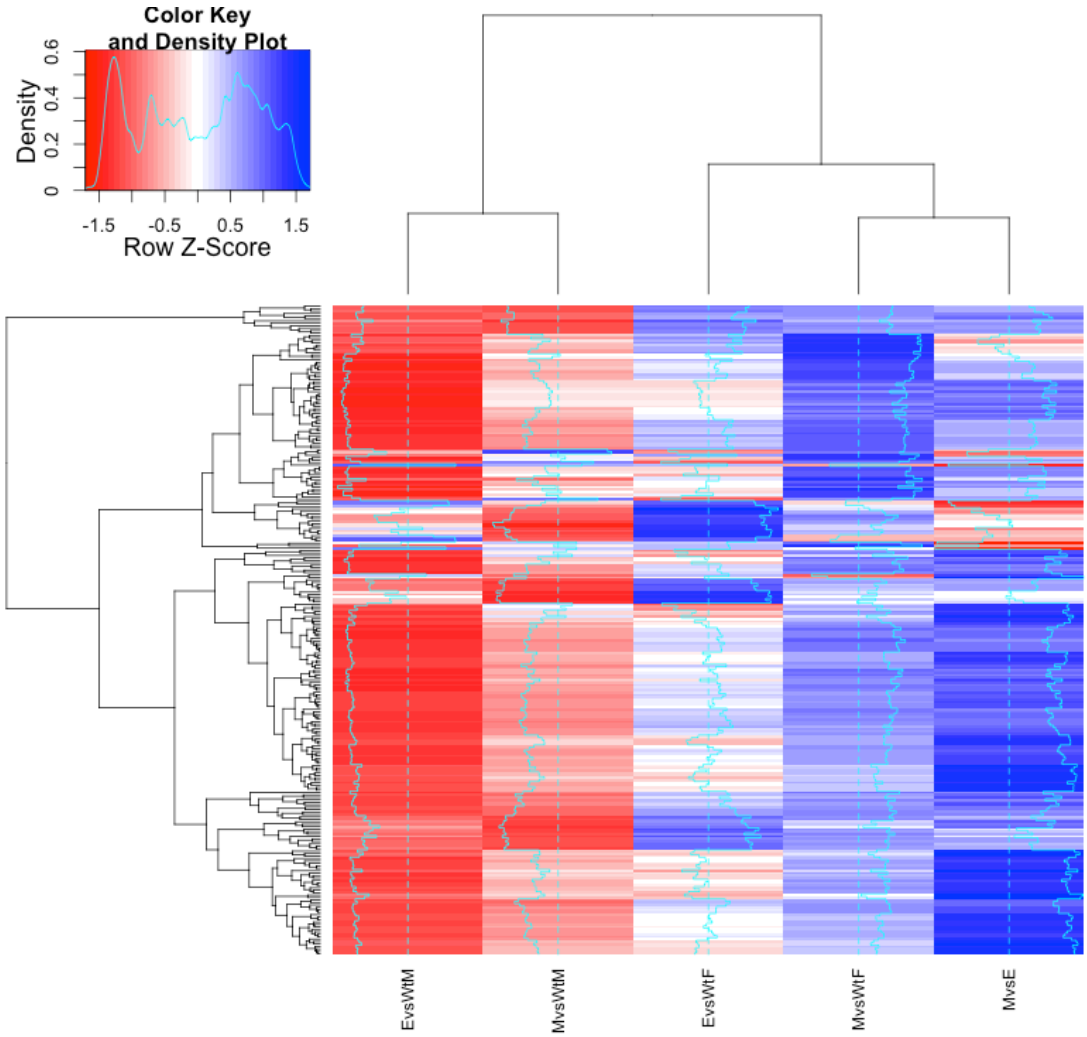


Figure 4: Heatmaps showing expression differences for virgin M vs. E, M vs. wild-type females and males and E vs. wild-type females and males, for (A) female-biased and (B) male-biased genes. The focal contrast is indicated in the column name, where blue (positive values) refers to higher expression in the type named first in the contrast (e.g. M in M vs. E), while red (negative values) refers to higher expression in the type named second. Genes and groups are ordered into clusters using hierarchical clustering.

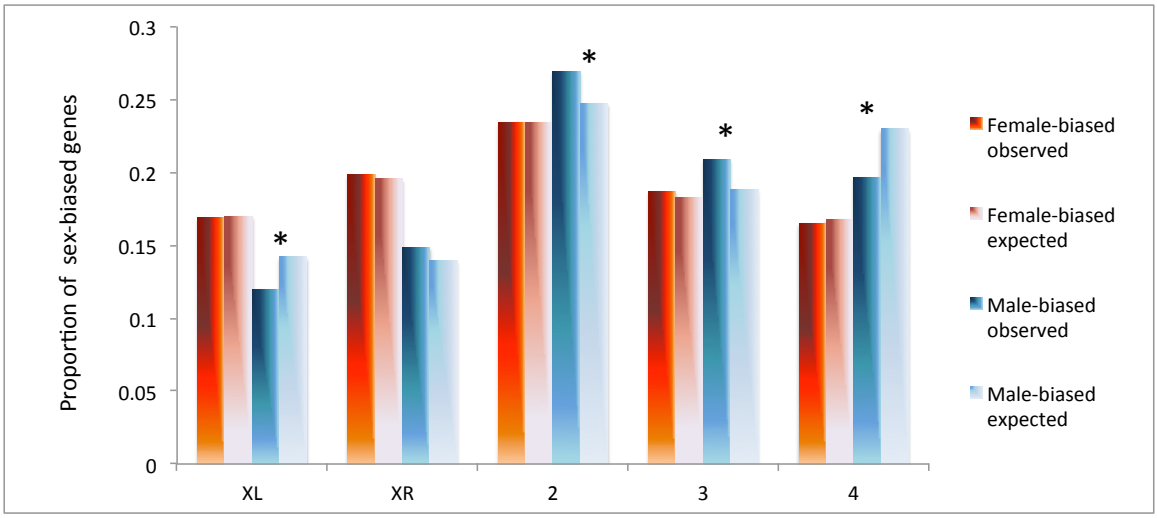


Figure 5: Chromosomal distribution of observed and expected sex-biased differentially expressed genes by female sexual selection regime (* $p<0.05$).

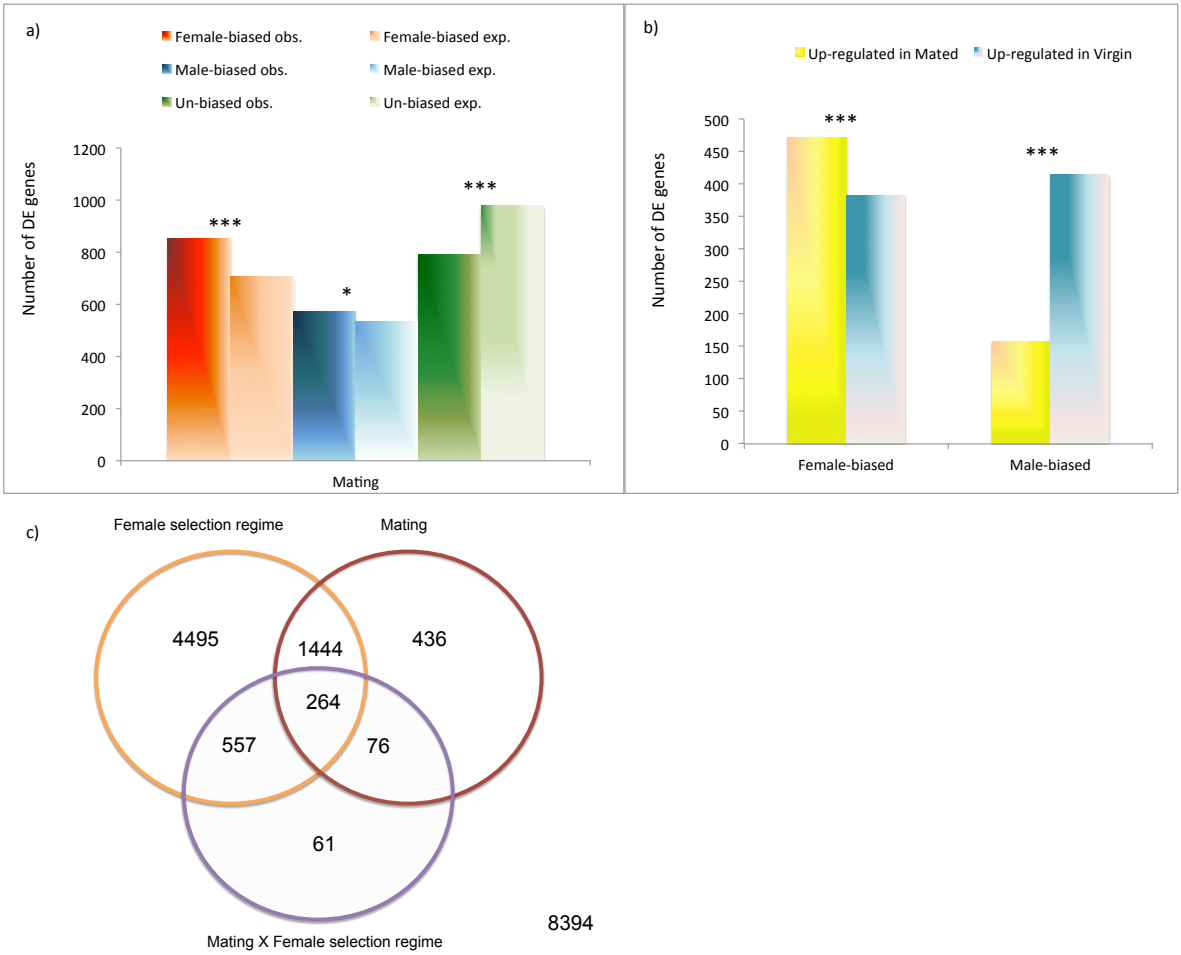


Figure 6. (A) Numbers of observed and expected DE sex-biased and un-biased genes affected by mating status. (B) Numbers of sex-biased DE genes affected by mating that are up-regulated in mated and virgin females. (C) Venn diagram of numbers of all DE genes that are unique or shared between the focal parameters.

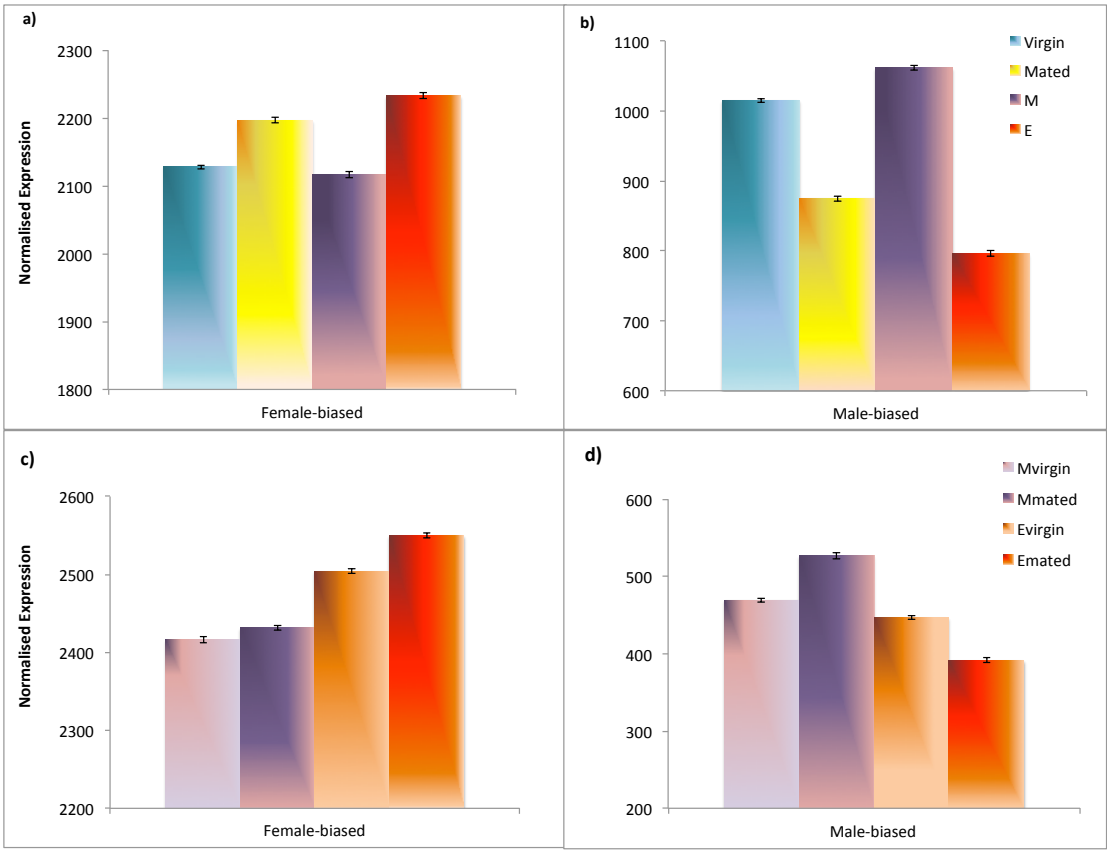


Figure 7: Normalised mean expression profiles (with standard errors) across all the sex-biased genes that are involved in mating response but show divergence in expression depending female selection regime. (A) Female-biased genes that are significant for both mating and female selection regime main effects. (B) Male-biased genes that are significant for both mating and female selection regime main effects. (C) Female-biased genes that are affected by mating X female selection regime interaction. (D) Male-biased genes that are affected by mating X female selection regime interaction.

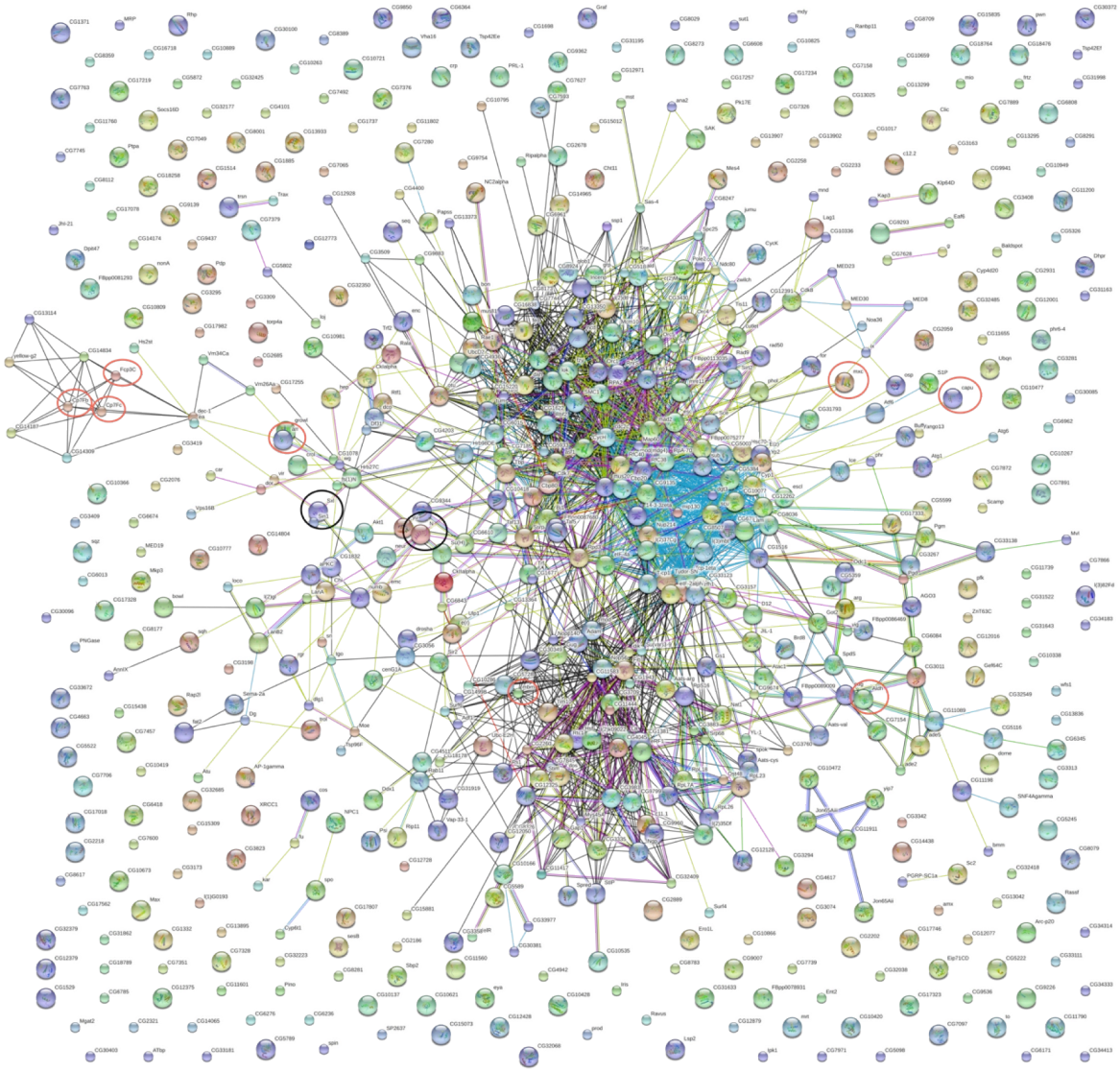


Figure 8: Functional protein association network for the differentially expressed between M and E females that also respond to mating, and are implicated in female gametogenesis related functions, as predicted with STRING (http://string-db.org/) (STRING evidence classes: Neighbourhood, Gene fusion, Co-occurrence, Co-expression, Databases, Text mining). Proteins circled with red also show significant interaction effects with male mating type. *Notch* and *Sex-lethal* are highlighted with a black circle.

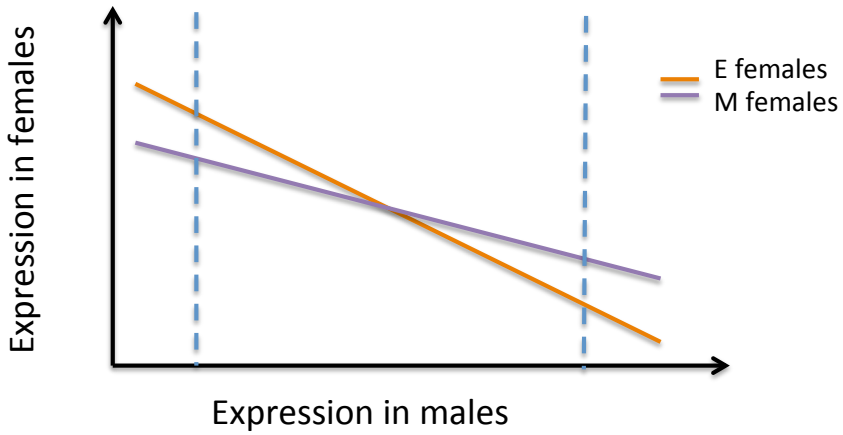


Figure 9: Increased sexual selection coupled with potential intra-locus conflict is expected to cause an increase in negative correlation between the expression levels in females and males in the expression of a sex-biased gene, thus further increasing sexual dimorphism in gene expression. One way of observing a consequence of this on gene expression patterns in females is by exploring the differences between experimental polyandrous (E) and monandrous (M) females for genes identified as most sexually dimorphic.

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CHAPTER 6

General discussion

Synopsis and future directions

Sexual interactions play an important role in generating sexual selection and antagonistic co-evolution, which not only shape differences between the sexes, but also have the potential to generate population divergence and to contribute to speciation (PANHUIS *et al.* 2001; RITCHIE 2007). Recent advances in molecular techniques have made it possible to identify the genes that underlie traits involved in sexual interactions. Such work has mainly focused on male traits and the progress in understanding the genetics of the female side of sexual interactions has been considerably slower. In this thesis I therefore aimed to provide new insights into the genes involved in female sexual interactions. To achieve this I have focused on different stages of female reproduction using *Drosophila* as a model system. I have tested for a pleiotropic effect of a candidate gene on female mate choice based on courtship song (**Chapter 2**) and examined how females respond to this song at the level of gene expression (**Chapter 3**). **Chapter 3** discovered a potential link between the pre- and post-mating stages, which I explored further in **Chapter 4** by testing the effect of two song-responsive genes on female post-mating fitness. Analysis of post-mating gene expression patterns in females was taken further in **Chapter 5**, where I explored the consequences of experimental variation in the female promiscuity on gene expression divergence as a whole, and in regard to female mating response. The results have already been discussed in their respective chapters and therefore I will focus here on summarising the key findings and discuss some questions that remain to be answered and are emerging from this work.

Several studies have shown that song is important in stimulating female mating behaviour (EWING and BENNETT-CLARK 1968; GREENACRE *et al.* 1993;

KYRIACOU and HALL 1982; KYRIACOU and HALL 1986; RITCHIE *et al.* 1999; RYBAK *et al.* 2002) and can contribute to heterospecific mate discrimination and therefore potentially pre-mating isolation in *Drosophila* (KYRIACOU and HALL 1980; 1982; 1986; RITCHIE *et al.* 1999). In chapter 2 I showed that wild-type *D. melanogaster* females are stimulated most by conspecific rhythmic characteristics (i.e. inter-pulse interval (IPI), and sinusoidal IPI cycle) in the song (see also RITCHIE *et al.* 1999). The IPI cycle is encoded by the *period* (*per*) gene in the male (KYRIACOU and HALL 1980; WHEELER *et al.* 1991). I also found that female mating preference for song is affected by this gene using a null mutant strain and a transgenic strain with *per* expression abolished in the peripheral nervous system (PNS). As discussed in Chapter 2, *per* does not have a strict genetically coupled effect, but provides a plausible example of pleiotropy. In the follow-up experiment I tested further the idea that cells in the PNS could be important for song preference, by specifically examining the effect of *per* expression in the Johnston Organ (JO) hearing neurons using RNA interference (RNAi). Unfortunately, the results from this experiment remain inconclusive. Although, according to the predictions, the two knockdown strains tested did not show significant preference for conspecific song, neither did the parental control strains, making it impossible to distinguish the effect of RNAi from that of the genetic background. Therefore other RNAi lines should be tested in the future to discover a suitable parental strain that demonstrates wild-type behaviour. With such strains it could be possible to test conclusively the effect of *per* expression in the JO neurons. Identifying the cells important for song preference can help to discover its regulatory mechanism and pin down the genetic architecture underlying this trait.

The genes for female response to courtship signals are largely unknown in *Drosophila* (but see e.g. LAWNICZAK and BEGUN 2004), and thus the *per* gene

represents an important candidate. In chapter 3 I used microarrays to detect expression changes in the female head, with the attempt to identify novel genes that respond to attractive courtship song. An interesting finding in this chapter was the detection of expression changes in several genes previously identified to respond to mating, including several members from the family of *Turandot* (*Tot*) immunity genes. This suggests these genes can be transcribed independent of the act of mating or seminal transfer. As I chose to test the gene expression in females after 15 min song stimulation, it is unlikely that the genes identified are involved in song preference *per se* (which may or may not require transcriptional changes). Moreover, detailed examination (using qPCR) of two of the *Tot* genes detected on the arrays showed no induced expression after 5 min, but only after 15min. This suggests that these genes respond only after the females become stimulated by the song, perhaps in 'preparation' to mating-related changes in females. As I tested the female gene expression using only acoustic signal stimulation, in isolation from other male traits, another interesting result was to find expression changes in genes involved in functions related to pheromone detection. To my knowledge this is the first indication that a single courtship signal could simultaneously trigger a response in several sensory modalities. It would be interesting to also test the gene expression response to other courtship signals in isolation, such as pheromones.

Chapter 4 explored further how the two *Tot* genes identified in Chapter 3 may be involved in post-mating fecundity in females. Using RNAi, I found indications that reduced expression of either *TotM* or *–C* increases immediate egg-laying rate. Future studies should focus on understanding better how these signalling genes that are also related to immune response (EKENGREN and HULTMARK 2001; EKENGREN *et al.* 2001) may have a pleiotropic influence on egg laying. For example, it would be important to

know which tissues may be involved. According to FlyAtlas, *TotM* and *TotC* both show expression enrichment in the fat body in the head and in female spermatheca. A starting point could therefore be to test how knocking down *Tot* gene expression in these tissues affects the immediate egg laying rate, by using tissue-specific *GAL4* drivers for RNAi (rather than a generic one used in the present study). These results also generate new questions of how *Tot* genes could be involved in interactions with male seminal fluid molecules to control female egg laying, which could be adressed in the future.

Overall, my findings highlight how it would be valuable to study further the functions of genes identified using transcriptomic approaches. During the past decade, microarray studies of female post-mating responses in *D. melanogaster* have produced long lists of such genes candidates (e.g. INNOCENTI and MORROW 2009; LAWNICZAK and BEGUN 2004; MCGRAW *et al.* 2008; MCGRAW *et al.* 2004). However, little effort has been made to understand further their function in female mating responses and how they might interact with male molecules. More work should therefore be directed in the future for studying such genes in females. This may also help with interpreting whether such genes are involved in antagonistic interactions that are often associated with male seminal fluid molecules (e.g. CHAPMAN and DAVIES 2004).

The role of sexual selection in speciation is a timely topic (BUTLIN *et al.* 2012), and although sexual selection (particularly in the form of sperm competition) has been implicated in several studies of molecular evolution, its effect on generating divergence has not been directly demonstrated in an experimental context. In chapter 5 I used experimentally evolved females of *D. pseudoobscura* to test how variation in the level of polyandry, and thus in the intensity of sexual selection, has caused gene

expression divergence. I demonstrated that up to 43% of the analysed transcriptome show gene expression differences between polyandrous and monandrous females after 100 generations of experimental evolution. This represents a substantial proportion of the genome, but is in line with other studies that have found gene expression patterns to differ when populations have been subjected to strong selection. For example, divergent artificial selection on mating speed in *D. melanogaster* resulted in differential gene expression in 21% of the probes analysed after just 29 generations (MACKAY *et al.* 2005). It is unlikely that all the genes identified in my study (or in Mackay *et al* 2005) would be subject to divergent selection. The genomic era has demonstrated that gene-gene interaction networks are a defining feature of genomes (DE VISSER *et al.* 2011; TYLER *et al.* 2009). Such epistatic interactions could explain the large number of genes that show expression differences in both studies: selection may have targeted some genes upstream in such networks (e.g. transcriptions factors), which will consequently have a knock-on effect on many others that are not under direct selection. It is important to note that epistatic gene regulatory networks are likely to underlie complex behaviours, and therefore selection can indirectly affect large numbers of genes when targeting a focal behaviour. But because not all the participating genes will require sequence changes, it would be useful to focus on identifying the (regulatory) genes that do. Such work could, for example, use expression QTLs, which aim to associate expression profiles with SNP markers (i.e. sequence variation). This could be applied to the whole transcriptome but also to interesting functional networks, such as oogenesis related genes identified in my study, and can help to find the polymorphic DNA regions associated with the expression differences between the experimental treatment groups. Such regions can subsequently be sequenced to study the types of mutations that underlie the eQTLs.

Researchers generally agree on the importance of gene regulation in creating species differences (e.g. STERN and ORGOGOZO 2008). However, there is a major debate over the relative importance of coding versus *cis*-regulatory sequence divergence (which both affect gene expression) in organismal evolution (HOEKSTRA and COYNE 2007; STERN and ORGOGOZO 2008; STERN and ORGOGOZO 2009). Therefore identifying the relative quantities of *cis*-regulatory and coding region mutations affecting gene expression divergence in E and M lines would be valuable for this debate, but also for the recently raised discussion on the relevance of experimental evolution studies for understanding molecular changes associated with long-term evolution and speciation (STERN and ORGOGOZO 2008; STERN and ORGOGOZO 2009).

The most exciting finding in Chapter 5 was that selection due to polyandry has mainly targeted the expression of female-biased genes, increasing their expression and concomitantly reducing the expression of male-biased genes in polyandrous females relative to monandrous. These results are in line with predictions of sexual selection theory and to my knowledge provide the first experimental evidence that sexual selection increases sexual dimorphism at the gene expression level. Sex-biased genes are thought to contribute to the development of sex-specific traits, which may be subject to sexual selection. This is the interpretation that many microarray studies have adopted when observing sex-dependent differences in gene expression (ELLEGREN and PARSCH 2007). However, such studies have recently been criticised for directly comparing males and females, because some of the patterns may arise as an artefact of dimorphism in body/organ type, size and shape (STEWART *et al.* 2010). My findings provide confirmation that expression level difference between the sexes is a reliable indication of gender-specific roles for sex-biased genes related to sexual interactions.

In chapter 5 the functional enrichment analysis of differentially expressed genes suggested that polyandrous females show higher expression of fecundity related genes. This suggests a positive fitness effect of increased polyandry on females. Theory provides many suggestions of how females can benefit from mate choice, and observations in *D. pseudoobscura* (CRUDGINGTON *et al.* 2005; GOWATY *et al.* 2010) and other species (ARNQVIST and NILSSON 2000; SLATYER *et al.* 2011) offer empirical evidence for small but positive effect of multiple mating on female fitness. Mating can also be costly to females (e.g. CRUDGINGTON *et al.* 2010 for E/M system), not least because of male seminal fluid molecules (CHAPMAN 2001; CHAPMAN 2006; CHAPMAN and DAVIES 2004). Although several studies have looked at the effect of polyandry on female fitness (using currencies like fecundity, female survival, number of adult offspring and offspring survival), the way polyandry affects females via pre- and postcopulatory sexual selection remains poorly understood. For example, how much does selection from pre- versus post-copulatory interactions affect females? How much of the selection arises from antagonistic effects and can co-evolution with males that cause antagonistic effects (on non-coevolved females) also benefit females. Moreover, we know relatively little about the impact of sexual selection and potential conflict on female-specific traits other than those used for measuring fitness. What are the traits that mediate such effects (e.g. physiological and morphological related to egg-laying, or perhaps life-history trade-offs related to survival)? Answers to such questions will not only increase our understanding of the roles of females in sexual selection, but they will also help us understand why such a large proportion of the genome appears involved, as identified in my study. The experimentally evolved M and E lines provide an ideal system to further study the fitness consequences of polyandry on females, with a possibility to associate the phenotypic differences

between the selection lines with underlying genetic variation. For this, detailed observations of behaviour (e.g. mating behaviour), morphology (e.g. reproductive tract) and life-history (e.g. egg-laying, development time) could be collected and associated with the sequence polymorphism (e.g. with GWAS technique using a SNP Chip) and gene expression. This could not only reveal the underlying genetic basis for the trait variation in females, but may also tell us more about potential pleiotropic influences of genes on multiple traits. Studies that take advantage of controlled experimental evolution and molecular techniques have the potential to greatly advance our understanding of how mating system variation can change patterns of sexual and natural selection on females.

Future studies on the M and E system could also focus further on the sexually dimorphic gene expression, for example by exploring the expression patterns in both sexes in different tissues and at different stages of sexual interactions. Targeted expression analyses of specific tissues could provide information on how sexual selection acts on genes expressed in different tissues and help identify changes in less abundant (tissue-wise) transcripts, that otherwise may not be detected in whole-body samples. Also, an unexpected finding in Chapter 5 was the relatively small effect of male sexual selection regime on female gene expression responses to mating, and it would be interesting to test whether this is more pronounced at other time points after mating than that used in the present study. Moreover, it would be interesting to know if the patterns of sex-bias (i.e. loss, gain and reverse of sex-bias) show differences between the experimental groups. Some switches in sex-bias between the monogamy and polyandry treatments were indicated in my comparison of expression profiles with wild-type flies (see for example the heatmaps). Examining such genes more closely with expression information also from E and M males would be valuable for

finding candidate genes experiencing particularly rapid selection, with a possible implication for intra-locus conflict. Genes that normally are female-biased and rapidly evolve into male-biased could potentially have detrimental effects on females, and *vice versa* on males for genes with similar switches that are favourable only to females.

Microarrays as a tool for studying sexual interactions: the future?

Microarray technology provides a powerful way to simultaneously obtain information from thousands of genes to test responses to a controlled environmental/experimental stimulus, or differences between populations and species. During the past decade this technique has been applied to understand the genomic complexities underpinning behaviours such as sexual interactions (BAILEY *et al.* 2011; CARNEY 2007; DALTON *et al.* 2010; ELLIS and CARNEY 2009; ELLIS and CARNEY 2010; ELLIS and CARNEY 2011; IMMONEN and RITCHIE 2012; INNOCENTI and MORROW 2009; LAWNICZAK and BEGUN 2004; MACK *et al.* 2006; MCGRAW *et al.* 2008; MCGRAW *et al.* 2004; MCGRAW *et al.* 2009), circadian rhythms (ALLADA and CHUNG 2010; CERIANI *et al.* 2002; CLARIDGE-CHANG *et al.* 2001; DOHERTY and KAY 2010; FRENKEL and CERIANI 2011; KEEGAN *et al.* 2007) and memory formation (GUAN *et al.* 2011; JIANG *et al.* 2011). How useful is this approach for generating new knowledge of traits involved in sexual interactions important for evolutionary questions, and what are its limitations? Behaviours result from the integration of internal information (DNA, RNA, proteins, non-neuronal tissues, neuronal circuitry) with the external environment. Behaviours are often considered to be polygenic and therefore very challenging to study, and the prospect of capturing genome-wide information associated with them appears attractive. When analysing changes at the RNA level we hope to obtain information

about the genes that are important for a particular phenotype. Cross talk between genes and environment via changes in gene expression forms the basis for why transcriptomic techniques can be invaluable for studies of sexual interactions. Each sex forms the social environment for the other, and the impact of such 'environmental effects' on gene expression have already been studied in both males and females, in both pre- and post-copulatory contexts (BAILEY *et al.* 2011; CARNEY 2007; DALTON *et al.* 2010; ELLIS and CARNEY 2009; ELLIS and CARNEY 2010; ELLIS and CARNEY 2011; IMMONEN and RITCHIE 2012; INNOCENTI and MORROW 2009; LAWNICZAK and BEGUN 2004; MACK *et al.* 2006; MCGRAW *et al.* 2008; MCGRAW *et al.* 2004; MCGRAW *et al.* 2009 and chapter 5 of this thesis). However the sensitivity to environment also poses a challenge for experimental design to control for unwanted noise, and can also limit comparisons between experiments. Multiple experiments on the same phenomenon should be encouraged to be able to see which gene expression changes are robust and due to the focal treatment *per se*, and which are due to plasticity of the transcriptome (which can be of interest on its own right). Experimental evolution studies that use replication of the selection regime represent a powerful way of testing robust evolutionary effects (chapter 5), but also comparisons between experiments made in different laboratories are very useful.

A second issue related to studying genetic basis for sexual behaviours is that we do not really know whether their manifestation requires changes in gene expression, or do they become hard-wired during development. Related to this, it is also unclear how fast relevant RNA changes occur and how long they last. These issues will most likely depend on the behaviours in question, and can be particularly challenging for traits such as female mating decision. Female preferences are hard enough to measure on the phenotypic level (CHENOWETH and BLOWS 2006; WAGNER

1998), and unless females express their preference with a detectable and repeatable behaviour it is impossible to know when is the best moment to sample the gene expression profiles. This could potentially be overcome by focusing on study species with a clear preference signal, and also obtaining expression profiles across multiple time points could be a highly useful approach.

A key feature of transcriptomic approaches is that the phenotypic effects are interpreted from the genetic information and not *vice versa*. This relies on the ability to extract relevant information from tens of thousands of probes by finding a balance between a true biological signal and the risk of false negatives, and a meaningful interpretation of the data with functional annotation tools. A wealth of statistical tools help with the issues related to deciding which genes show differential expression, however, another crucial part is the annotation information available. Model organisms such as *D. melanogaster* and house mouse have relatively well annotated genomes, however, it is not always clear how meaningful the functional information of focal genes is for the particular trait of interest (especially behaviour) and not the least because of the pleiotropic nature of many genes. Moreover, using functional information from related species can also be misleading if gene functions are not conserved. However, the annotation information available for data interpretation is rapidly improving, with new annotations being updated to the gene ontology databases also from behaviour-oriented experiments. One such database has been generated solely for information of sex-bias in gene expression (GNAD and PARSCH 2006), which adds a novel type of annotation available to use in conjunction with functional annotation terms (chapter 5). Another way forward is to focus on specific tissues instead of whole bodies (chapter 3). This should narrow down the number of expressed genes discovered and allow better detection of rare transcripts, thereby

improving the accuracy of functional interpretation of the data (e.g. DALTON *et al.* 2010; MACK *et al.* 2006). The choice of tissue will of course depend on the question at hand, and whole-body samples can also be a good starting point, for example for understanding differences between populations and species (chapter 5).

Microarrays are exploratory and their key strength is to point the direction for future experiments by providing novel hypotheses. The development of deep sequencing techniques for RNA (RNAseq) adds to the tool kit available for transcriptome analysis. RNAseq will be particularly useful for identifying novel splice-variants for genes (MALONE and OLIVER 2011), which could provide new insights, for example, for the way genes are used in different environments or in different sexes. These techniques show reassuringly good agreement over a broad range of expression levels (apart from extremes), however, microarrays still outcompete RNAseq techniques in price and the availability of well-developed tools for robust statistical analyses (MALONE and OLIVER 2011).

Transcriptomic techniques go a long way as a tool for generating new discoveries of the genetic basis of fascinating traits. However, I believe care should be taken when generalising findings based on functional annotations to interpret the roles of genes in a behavioural context, and where possible, other techniques such as RNAi need to be employed to further test the hypothesis generated with gene expression data. In the future, more focus should also be given for protein level changes (proteomics). However, for evolutionary studies of sexual interactions, perhaps the biggest promise is in elegant experimental designs that can associate expression profiles with phenotypic fitness measurements (e.g. INNOCENTI and MORROW 2010), selection pressures (e.g. MACKAY *et al.* 2005; MOGHADAM *et al.* 2012) or tease apart genotypic and environmental effects associated with sexual selection (Chapter 5).

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