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Evolution of reduced post-copulatory molecular interactions in *Drosophila* populations lacking sperm competition

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Abstract

In many species with internal fertilization, molecules transferred in the male ejaculate trigger and interact with physiological changes in females. It is controversial to what extent these interactions between the sexes act synergistically to mediate the female switch to a reproductive state or instead reflect sexual antagonism evolved as a by product of sexual selection on males. To address this question, we eliminated sexual selection by enforcing monogamy in populations of *Drosophila melanogaster* for 65 generations and then measured the expression of male seminal fluid protein genes and genes involved in the female response to mating. In the absence of sperm competition, male and female reproductive interests are perfectly aligned and any antagonism should be reduced by natural selection. Consistent with this idea, males from monogamous populations showed reduced expression of seminal fluid protein genes, 16% less on average than in polygamous males. Further, we identified 428 genes that responded to mating in females. After mating, females with an evolutionary history of monogamy exhibited lower relative expression of genes that were up regulated in response to mating and higher expression of genes that were down-regulated – in other words, their post-mating transcriptome appeared more virgin-like. Surprisingly, these genes showed a similar pattern even before mating, suggesting that monogamous females evolved to be less poised for mating and the accompanying receipt of male seminal fluid proteins. This reduced investment by both monogamous males and females in molecules involved in post-copulatory interactions points to a pervasive role of sexual conflict in shaping these interactions.

Introduction

Females respond to mating in diverse ways that include physiological, anatomical and behavioural changes. In *Drosophila melanogaster*, the well-characterized female post-mating response involves changes in expression levels of thousands of genes (McGraw *et al.*, 2004) in both the reproductive tract (Mack *et al.*, 2006) and elsewhere in the soma (Dalton *et al.*, 2010), with a strong temporal pattern (McGraw *et al.*, 2008). This is accompanied by a suite of phenotypic changes, including an immune response (Lawniczak & Begun, 2004; McGraw *et al.*, 2004; Peng *et al.*, 2005; Mack *et al.*, 2006; Domanitskaya *et al.*, 2007; Kapelnikov *et al.*, 2008; Innocenti & Morrow, 2009), altered feeding behaviour (Carvalho *et al.*, 2006) and sleep and activity patterns (Isaac *et al.*, 2010), and reduced sexual receptivity (Manning, 1962; Chen *et al.*, 1988; Chapman *et al.*, 2003). Many of these changes are mediated by male seminal fluid proteins (SFPs) (McGraw *et al.*, 2004, 2008; Chapman, 2008; Wolfner, 2009; Avila *et al.*, 2011). It remains an open question to what extent these changes reflect synergy between male and female

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molecules generating a female's post-mating transition to a reproductive state versus antagonistic interactions fuelled by sexual conflict.

On the surface, sexual molecular interactions in *Drosophila* often appear to function cooperatively. For example, the switch to a reproductive state involves female processing of male-derived molecules (Park & Wolfner, 1995; Heifetz *et al.*, 2005; Ram *et al.*, 2006; Mueller *et al.*, 2008) that trigger ovulation (Monsma & Wolfner, 1988; Heifetz *et al.*, 2000). SFPs, but not sperm, are also necessary for conformational changes in female anatomy that allow sperm to enter storage (Heifetz & Wolfner, 2004; Adams & Wolfner, 2007; Avila & Wolfner, 2009). Some of this transition to storing sperm is regulated by interactions between SFPs and innervated stretches of the reproductive tract (Heifetz & Wolfner, 2004). Later, in order for fertilization to occur, both female secretions (Prokupek *et al.*, 2008) and male SFPs (Ram & Wolfner, 2007) are required. Perhaps the most compelling evidence of synergy comes from recent studies demonstrating a stepwise seminal fluid proteolytic cascade with diverse effects on reproductive processes (LaFlamme *et al.*, 2012) that requires both male and female contributions (LaFlamme *et al.*, 2014).

However, the female reproductive tract also acts as an arena for sexual conflict – the optimal outcome of sexual interactions is likely to differ between males and females. First, sperm and SFPs have presumably been selected to increase the male's success in direct sperm competition with the sperm of other males within the female reproductive tract (Parker, 1970). This function of SFPs is supported by the fact that males transfer more SFPs during mating if another male is present, implying a greater risk of sperm competition (Wigby *et al.*, 2009). Some of the female response to mating may exist to influence the outcome of this competition via cryptic female choice (Eberhard, 1996), but males would be selected to overcome this. Consistent with this notion, paternity is known to be affected by both male (Clark *et al.*, 1995; Hughes, 1997) and female (Clark & Begun, 1998; Giardina *et al.*, 2011; Lupold *et al.*, 2013) genotype, often in a nonadditive way (Clark *et al.*, 1999; Chow *et al.*, 2010). Second, SFPs influence female behaviour or physiology in a way that makes her less likely to mate again (Manning, 1962; Chen *et al.*, 1988; Chapman *et al.*, 2003). This is advantageous to the male, but may be disadvantageous to the female – females in insects usually experience a net benefit from multiple mating (Arnqvist & Nilsson, 2000). Third, SFPs inflict direct costs on females in terms of reduced lifetime reproduction or lifespan (Chapman *et al.*, 1995; Lung *et al.*, 2002; Wigby & Chapman, 2005; Mueller *et al.*, 2007). Even if some of those costs are compensated by improved offspring quality (Priest *et al.*, 2008), the majority of evidence suggests strong net costs for females upon receipt of SFPs; thus, selection should favour a female response that counteracts this effect. Molecular evolutionary patterns support this role for sexual conflict, as the family of SFP genes (comprising at least 140 members (Findlay *et al.*, 2008)) are rapidly evolving at the sequence level (Swanson & Vacquier, 2002). The female side of this putative arms race, involving primarily proteases expressed in the reproductive tract, also shows evidence of rapid evolution (Lawniczak & Begun, 2007) and a signature of strong selection (Panhuis & Swanson, 2006).

One way to test the extent to which male and female contributions are operating cooperatively or antagonistically is to study how they evolve if sexual selection is experimentally eliminated. Removing female choice and competition for mates eliminates sexual conflict – any amount of harm to females would become detrimental to the reproductive success of both sexes. Therefore, if antagonism dominates these post-copulatory molecular interactions, the levels of expression of male SFP genes and the magnitude of the female post-mating transcriptional response should both be reduced by the action of natural and sexual selection. In contrast, male SFP gene expression along with any elements of the female post-mating transcriptional response that control the switch to and maintenance of a reproductive state would be sexually synergistic and not expected to diminish in the absence of sperm competition. Consistent with the former prediction, Innocenti *et al.* (2014) reported that genes known from previous work (Innocenti & Morrow, 2009) to be up regulated after mating show lower expression in mated females originating from populations evolving without sperm competition; the opposite was observed for genes found to be down-regulated after mating. However, because female gene expression before mating was not measured, it is unclear to what extent these differences represent a weaker transcriptional response to mating rather than a baseline difference already present in virgin females. The latter possibility is predicted by the hypothesis that females are molecularly 'poised' for receipt of SFPs (Heifetz & Wolfner, 2004; McGraw *et al.*, 2004). We tested these alternative predictions about the relative roles of synergy and antagonism by allowing *D. melanogaster* populations to evolve either with or without sperm competition for 65 generations and then measuring the expression of genes involved in molecular interactions between the sexes, in both males and virgin and mated females.

Materials and methods

Experimental evolution

The evolving fly populations used in the experiments have been described previously (Hollis & Houle, 2011; Hollis & Kawecki, 2014). Three polygamous populations and three monogamous populations were established

from a long-term laboratory population and maintained with a census size of 200 adults per generation. In the monogamous populations, virgin females were randomly paired with virgin males. In contrast, groups of five virgin females were combined with groups of five virgin males in the polygamous populations. After 2 days of interaction, males from all populations were discarded and females placed into two bottles per population, each with 50 females. Females were then allowed to spend 3 days laying eggs in these bottles, which were the source of the next generation's flies. Under this design, polygamous populations experience competition for mates, both directly (e.g. scramble competition or aggressive interactions) and indirectly (e.g. sperm competition), as well as mate choice; monogamous populations experience no competition for mates. The six populations had undergone 65 generations of experimental evolution at the time of the experiments.

Gene expression profiling

All populations were first reared in the monogamous mating system for one generation to control for any nongenetic differences (e.g. maternal effects) that might be caused by the evolutionary regime. Virgin males and females were then collected and held in same sex groups of 10 individuals. After 3 days, 20 females from each population were individually paired with 20 males from the same population and observed until mating occurred. After mating, females and males were separated, and 24 h later, RNA was extracted from 10 mated females from each population. The remaining females and males were kept as virgins during these 24 h, and RNA was extracted from 10 virgin males and 10 virgin females at the same time as the mated females, when all flies were 4 days old. In all samples, total RNA was extracted from whole flies using RNAzol (Molecular Research Centre, Cincinnati, OH, USA). Double-stranded cDNA was then synthesized using Invitrogen Superscript II kit, fluorescently labelled, and hybridized to Roche Nimblegen 12x135k arrays. From each of the 6 experimentally evolved populations, virgin female, mated female and virgin male cDNA were each hybridized on separate arrays, for a total of 18 arrays used in the experiment. Data for virgins were previously used as part of another study (Hollis *et al.*, 2014).

Analysis

Raw signal intensity values for all probes were preprocessed using the RMA (Robust Multichip Average) algorithm (Bolstad *et al.*, 2003; Irizarry *et al.*, 2003). For genes with multiple probe sets, only the one with the highest average signal intensity was retained for analysis, leaving 13,995 genes total for downstream analysis. We filtered the bottom one-third of signal intensities from both virgin and mated female data, leaving 9,139 genes, to limit the number of statistical tests performed. All analyses were performed using PROC MIXED in SAS (SAS Institute, 2011).

For males, we examined 138 SFP genes from Findlay *et al.* (2008) that are present on the Nimblegen arrays. We modelled gene expression with a fixed effect for selection regime to test for differential expression of each SFP gene. We then used a single paired t-test to investigate whether there was a significant difference in the average expression level of this entire class of SFP genes between the two selection regimes.

For females, we fit a mixed model for each gene where female gene expression was predicted by the fixed effects of selection regime (monogamy versus polygamy) and female mating status (virgin versus mated) and their interaction, including replicate population nested within selection regime as a random effect. Power to detect individual genes with significantly different responses to mating in the two regimes (a selection regime x mating status interaction) after false discovery rate (FDR) correction is limited with only three replicate populations in each selection regime. We therefore focused our analysis on a broad set of genes that respond to mating (a significant mating status effect) at an FDR of 20%. We used paired t-tests to compare average gene expression in the two regimes for this class of mating-responsive genes, analysing genes up regulated after mating and genes down-regulated after mating separately, for both virgin and mated females. We used the same approach to compare the change in average gene expression after mating for these up and down-regulated genes. We tested this list of mating-responsive genes for enrichment of Gene Ontology terms using FlyMine (Lyne *et al.*, 2007) with default settings and used the FlyAtlas data set and classifications (Chintapalli *et al.*, 2007) to determine patterns of gene expression in the reproductive tract (ovaries and spermatheca).

Results

Male seminal fluid protein genes

Males evolved under monogamy expressed SFP genes 16% less on average than males from polygamous populations ($t_{137} = -8.39$, $P < 0.0001$, Fig. 1). In total, the estimates of expression levels of 80% of these SFP genes (111/138) were lower in monogamous than polygamous males, although no individual genes were significantly different between regimes at an FDR of 20% (Table S1).

Female post-mating response

We detected 428 genes that changed in expression after mating in our populations (based on the main effect of

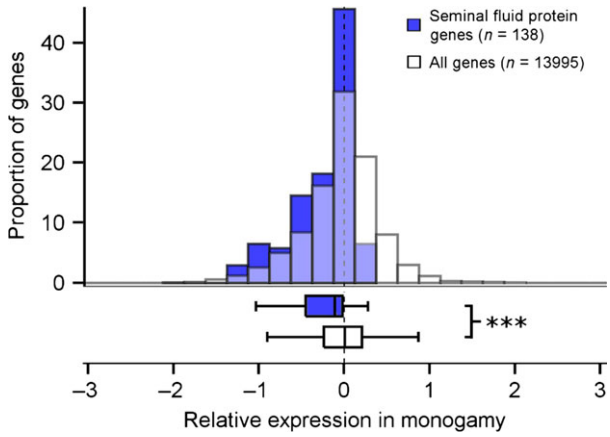

Fig. 1 Seminal fluid protein genes (n = 138) showed reduced expression in monogamous populations relative to polygamous populations. *** $P < 0.001$.

mating status across both regimes), 283 of which were up regulated and 145 down-regulated (Table S2). Of these detected genes, over half (220) show at least moderate expression in the reproductive tract based on FlyAtlas data (Chintapalli *et al.*, 2007). As expected, the 428 mating-responsive genes were significantly enriched in gene ontology categories associated with reproduction (Table S3).

On average, the relative expression of genes which are up regulated in response to mating was lower in females from monogamous than polygamous populations. This held for both premating (Fig. 2a, $t_{282} = 7.87$, $P < 0.0001$) and post-mating (Fig. 2b, $t_{282} = 13.43$, $P < 0.0001$) levels of expression. The opposite held for genes down-regulated in response to mating: monogamous females showed a trend towards higher relative expression before mating (Fig. 2d, $t_{144} = -1.84$, $P = 0.067$) and had significantly higher expression after mating (Fig. 2d, $t_{144} = -6.81$, $P < 0.0001$). Monoga-

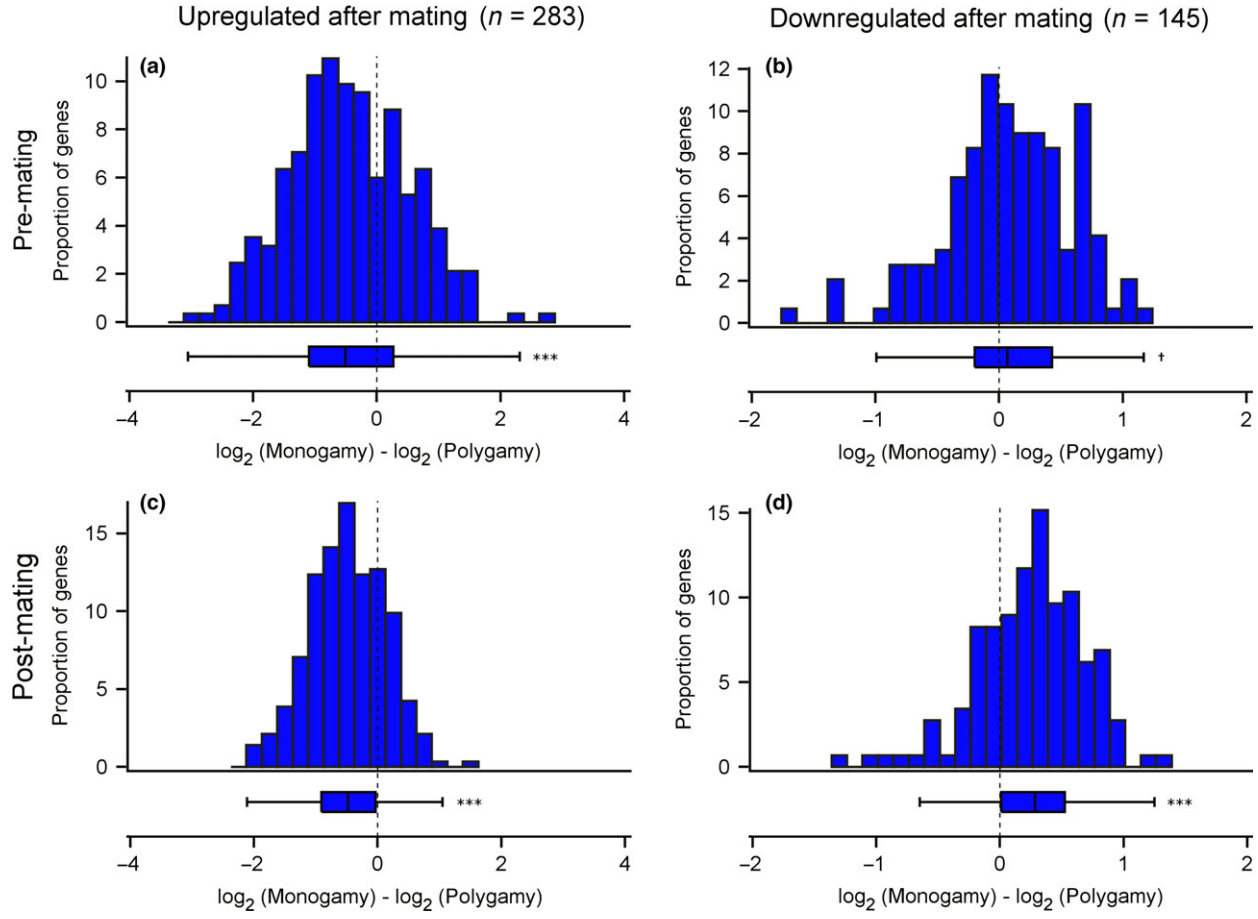

Fig. 2 Relative expression in monogamy ($\log_2$ Monogamy – $\log_2$ Polygamy) for those genes upregulated after mating (a, c) and down-regulated after mating (b, d). Monogamous females showed virgin-like expression profiles—lower premating (a) and post-mating (c) expression of genes that are upregulated in response to mating, as well as higher premating (b) and post-mating (d) expression of genes that are down-regulated in response to mating. ***$P < 0.001$.

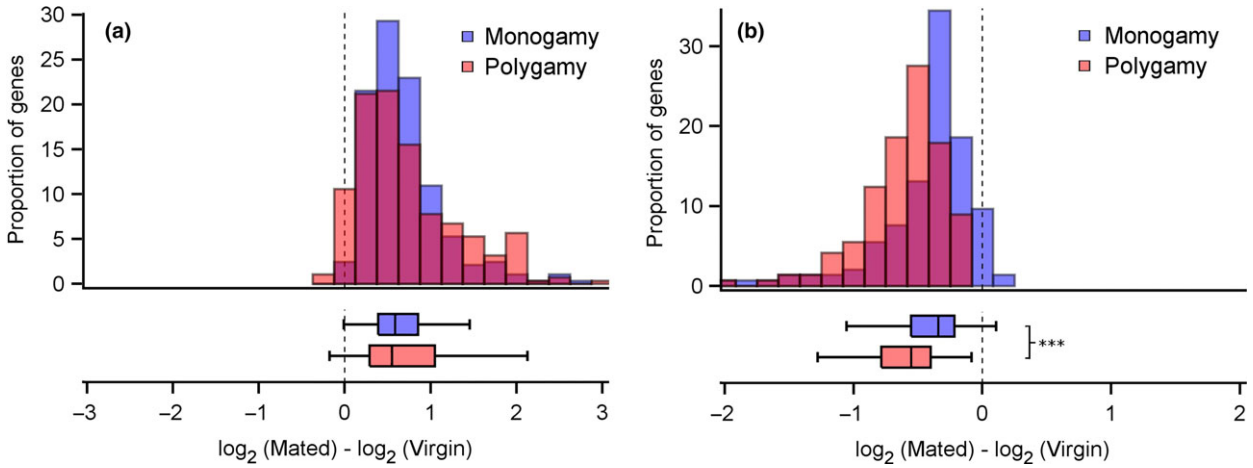

Fig. 3 The change in gene expression after mating for monogamous and polygamous selection regimes for those genes upregulated after mating (a) and down-regulated after mating (b). There was no difference between selection regimes in the strength of the response to mating for those genes upregulated after mating (a), but for those genes down-regulated after mating the monogamous regime showed a significantly weaker response (b). ***$P < 0.001$.

mous females were therefore more virgin-like in gene expression profile both before and after mating than were polygamous females.

The magnitude of change in expression of the class of genes up regulated in response to mating was not significantly different between monogamous and polygamous regimes (Fig. 3a, $t_{282} = -1.28$, $P = 0.201$). For genes down-regulated in response to mating, though, monogamous flies showed on average an 8% weaker transcriptional response to mating (Fig. 3b, $t_{144} = 6.36$, $P < 0.0001$). In fact, of the mating-responsive genes with the strongest evidence of evolutionary change in postmating response (selection regime x mating status interaction $P < 0.05$, Table S1), nearly all (30 of 33) showed a greater response to mating in polygamous than monogamous populations. The effect of mating on these 33 genes was on average 22% greater in polygamous populations.

Discussion

We studied how the expression of male seminal fluid protein genes and genes involved in female physiological response to mating evolved in the absence of sexual selection and sexual conflict. We tested alternative predictions based on the hypotheses that the male–female interactions these genes mediate are mostly synergistic, regulating the switch of female physiology to reproduction, or mostly antagonistic, driven by sexual conflict (Parker, 1979). The broad pattern of evolutionary change in the expression of genes involved in this interaction, evident in both males and females from populations maintained under enforced monogamy, supports a prevailing role for sexual conflict.

First, we found that males from monogamous populations showed an overall pattern of reduced expression of seminal fluid protein (SFP) genes. Levels of gene expression normally explain a large part of the variance in protein levels (de Sousa Abreu *et al.*, 2009), particularly for secreted products. This reduced investment in SFPs would not be expected if SFPs had a positive effect on female reproductive output, because under monogamy, male fitness is completely dependent on the individual female with whom he is paired. Rather, this result indicates that high expression of SFPs is favoured and maintained by sexual selection because it increases the male's paternity share, for example by allowing the male to mate with several females in quick succession (Sirot *et al.*, 2009), by affecting the outcome of sperm competition, or by inducing female unwillingness to mate with another male. Once the opportunity for sexual selection is removed by enforced monogamy, high SFP expression is disfavoured, indicating that SFP production is costly to male fitness under the monogamous regime. This could, in principle, be a cost that does not affect female fitness, but under our monogamy regime, the opportunity for such a male-only cost is mostly limited to effects on survival to adulthood. There is arguably more scope for a cost of high SFP levels in terms of reduced reproductive output of the pair, mediated by negative effects on male fertility (sperm quality or sperm number) or by direct negative effects of the female, especially given the evidence for the latter (Chapman *et al.*, 1995; Lung *et al.*, 2002; Wigby & Chapman, 2005; Mueller *et al.*, 2007). The reduced SFP expression observed in our experimental populations after evolution under monogamy is also in agreement with previous work showing reduced accessory gland size (the location of most SFP production) in

populations evolving in mating systems with reduced sexual selection (Crudgington *et al.*, 2009) and increased male competitive success in populations with relatively larger accessory glands (Wigby *et al.*, 2009). These results support the notion that the receipt of SFPs comes at a net cost to female fitness and, under monogamy, males have evolved to reduce these costs to their single reproductive partner.

Second, both premating and post-mating gene expression profiles of monogamous females were more virgin-like than those of females from polygamous populations. Specifically, genes that are up regulated after mating showed on average lower expression in both virgin and mated monogamous females. The opposite was the case for genes down-regulated after mating (although for virgin females this difference was only marginally significant). The post-mating expression profile was measured in females mated within-population, and the transcriptional response to mating in females is known to be regulated in part by male SFPs (McGraw *et al.*, 2004, 2008). Thus, the differences between monogamous and polygamous populations in post-mating expression profiles could, in principle, be mediated by lower SFP expression in monogamous males. This opens the possibility of future work to disentangle these effects by testing males and females from different evolved populations with one another. The differences we observed in premating expression cannot be influenced by males, however, and therefore must reflect evolutionary change directly affecting female gene expression. Such a systematic change would not be expected if these changes in expression mediated high fecundity or offspring investment – both selection regimes select for high reproductive output and larval competitive ability. It is possible that some of the changes in female premating gene expression evolved under monogamy as a result of relaxed sexual selection on female choice or female–female competition for males. However, given that the strength of sexual selection on females decreases after mating, premating expression of such genes should have evolved to be more similar to that of mated females, in contrast to the prevailing pattern. Thus, the most parsimonious explanation for the patterns in our data is that much of the female post-mating response functions as a costly defence mechanism against male antagonism and monogamy selects for reduced investment in this defence.

Our results are based on the relative expression of genes in the whole fly. Thus, we will detect changes in both the expression level on a per-cell basis, and in the relative size of structures such as the testis or female reproductive tract. Both of these types of changes are relevant to our predictions of changes in the total investment by males in SFPs and by females in the response to mating. Our power to detect changes relevant to evolution in monogamy will vary with the expression pattern of the gene involved. For example, if only expression in reproductive structures is relevant, then power increases with the level of gene expression in that reproductive tissue, and decreases with the level of expression in nonreproductive tissue that may be unaffected by experimental evolution. For female expression, our initial screen for changes in gene expression after mating will tend to filter out those genes for which power to see evolutionary changes is lowest. The set of 428 genes tested for changes in the female will be enriched for genes in which our power to detect evolutionary changes is relatively high.

Overall, our results provide strong evidence that much of the molecular interplay between the sexes that occurs after mating has been shaped by conflict between the sexes and is generally not cooperative in nature. The idea that antagonism dominates male–female interactions has received support from many previous experimental evolution studies (Rice, 1996; Holland & Rice, 1999; Crudgington *et al.*, 2010; Hollis & Houle, 2011). Our results add to this body of work by demonstrating the breadth of this antagonism in the expression of genes involved in post-copulatory interactions. In principle, the expression of only one or a few of these genes could be involved in antagonistic interactions, with the rest facilitating the onset of reproduction. Instead, even though many individual genes in our analysis do show different expression trends, the overall pattern is strong and consistent with sexual conflict being the main driver behind the evolution of much of the transcriptomic response to mating.

In the only other work to examine evolutionary change in mated female transcriptional profiles (Innocenti *et al.*, 2014), genes that had been identified as responsive to mating (Innocenti & Morrow, 2009) were overrepresented among those that subsequently evolved differential expression between alternative mating systems. The expression levels of these genes also appeared more virgin-like in mated monogamous females (expression prior to mating was not measured). In contrast to our populations (Hollis & Houle, 2011) and those from past similar manipulations (Holland & Rice, 1999), fecundity is reduced in the monogamous populations of Innocenti *et al.* (2014). This difference may be partially explained by differences in the experimental manipulation between studies. One key difference is that the monogamy treatment of Innocenti *et al.* (2014) allows only a single mating, elevating the importance of sperm storage because females spend several days without males before laying eggs for the next generation. However, despite differences in the manipulations, it is clear that some element of the evolution of mating-responsive gene expression in females (e.g. the post-copulatory virgin-like expression profile found in both studies) is robust against details of the selection regime.

Much of the research on the molecular basis of female defence against male antagonism, including this study, has focused on the magnitude of changes in expression after mating. However, it has been hypothesized that even before mating females are poised to receive male seminal fluid through anticipatory expression of genes whose products interact with male molecules (Heifetz & Wolfner, 2004; McGraw *et al*., 2004). This hypothesis gains direct experimental support from our results. We found that evolutionary change in these genes under monogamy not only occurred via reduction of the magnitude of response to mating, but also changes in baseline expression prior to mating. This indicates that virgin females are indeed poised for interaction with SFPs, and the degree of this anticipatory effect is reduced in females that co-evolve with males that invest less in SFPs. This opens the possibility that some aspects of female defence are fully on even before mating, and thus cannot be detected by looking at the plastic response to mating.

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Competing interests

We have no competing interests.

Author contributions

BH conceived of and performed the experiments, collected and analysed the data with TJK and wrote the manuscript with DH and TJK.

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Supporting information

Additional Supporting Information may be found in the online version of this article:
Table S1 SFP gene expression.
Table S2 Female post-mating response in the transcriptome.
Table S3 Female post-mating response Gene Ontology (GO) enrichment.

Data deposited at Dryad: doi: 105061/dryad.2m6s2

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