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Sexual conflict and female immune suppression in the cricket, *Allonemobious socius*

KENNETH M. FEDORKA & MARLENE ZUK

Department of Biology, University of California Riverside, Riverside, CA, USA

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Abstract

In many animal systems, females exhibit a localized immune response to insemination that helps defend against sexually transmitted disease. However, this response may also kill sperm, reducing a male's reproductive potential. If males could suppress this response, they may be able to increase their sperm's representation in the female's reproductive tract, thereby increasing their fitness. Here we address the hypothesis that, under conditions of sperm competition, males interfere with female immunity. To test our hypothesis, we manipulated levels of female mating frequency (single vs. multiply mated) and seminal diversity (monandrous vs. polyandrous) in the cricket, *Allonemobius socius* and measured female immune response. As mating frequency increased, female hemocyte load decreased, indicating a general reproductive cost. As seminal diversity increased, phenoloxidase (PO) activity (*in vitro* measure of 'potential' macroparasitic defense) increased and encapsulation ability (*in vivo* measure of 'realized' macroparasitic defense) decreased in polyandrous females. These results suggest that males may manipulate female immunity by interrupting the pro-PO cascade, which begins with the activation of PO and ends in the encapsulation of invading foreign bodies. In other words, female immune function may serve as a battleground over which a sexual conflict is fought.

Variance in both reproduction and immune defense are major determinants of fitness. Yet biologists have only recently begun to focus on how these systems interact in an evolutionary context (Sheldon & Verhulst, 1996; Schmid-Hempel, 2003; Rolff & Siva-Jothy, 2003). For instance, mounting evidence suggests that reproductive effort and immunity indirectly trade-off due to the competitive allocation of limited resources (McKean & Nunney, 2001; Rolf & Siva-Jothy, 2003; Fedorka *et al.*, 2004). As such, an increased investment in reproduction (or its components) may in part inhibit immune function. However, the fact that these systems also interact directly during mating has received relatively little attention from evolutionary biologists.

The direct interplay between reproduction and immunity is well documented in mammals, where females commonly marshal a number of immune cells into their reproductive tract in response to insemination (Barratt & Pockley, 1998). This immune build-up is characterized by a massive increase in the leukocyte count of the cervical mucus (Pandya & Cohen, 1985; Thompson *et al.*, 1991,1992), which has been suggested to serve as a primary defense against infectious agents passed during mating (Nunn *et al.*, 2000; Nunn, 2002). The additional observation that these leukocytes phagocytize spermatozoa (Yanagimachi & Chang, 1963; Mattner, 1969) has led to an extension of this hypothesis, which suggests that a female's immune response weeds out suboptimal sperm and serves to maximize both male and female fitness (Barratt & Pockley, 1998). It appears that invertebrates also undergo a similar immune reaction to insemination. A recent microarray study in *Drosophila melanogaster* indicated that both male sperm and accessory gland proteins (Acps) independently elicit the up-regulation of genes associated with antimicrobial defense in females (McGraw *et al.*, 2004). As one might expect, a delicate balance must be struck between the intensity of the female's immune response and the volume and

Correspondence: Kenneth M. Fedorka, Department of Genetics, University of Georgia, Athens, GA 30602, USA.
Tel.: +1-706-5428133, fax: +1-706-5423010,
e-mail: fedorka@uga.edu

composition of the male's ejaculate to ensure successful fertilization.

In polyandrous mating systems, however, where the likelihood of sperm competition is high, phagocytosis of spermatozoa could decrease a male's fertilization potential. This leads to a conflict of interest between the sexes because a male's reproductive success may be constrained by his mate's immune response. Although male mammalian seminal fluid has been noted to contain components that suppress female leukocyte proliferation (Dostal *et al.*, 1995; Kelly, 1995), these components are generally considered to be mutually adaptive, serving to usher sperm through an otherwise hostile reproductive environment (Barratt & Pockley, 1998). The alternative hypothesis, that males interfere with female immunity to the detriment of female fitness, has not been widely considered.

Selfish chemical coercion of females by males is not uncommon. For instance, male butterflies pass anti-aphrodisiacs (Andersson *et al.*, 2003) or nonfertilizing 'filler' sperm (Wedell, 2001) to the female in an effort to reduce remating propensity. Likewise, *Drosophila* males pass Acps during copulation that decrease a female's sexual appetite, incapacitate previously donated sperm, prevent future sperm displacement (see Chapman *et al.*, 1995 and references therein) and increase the rate of oviposition (Heifetz *et al.*, 2000). Moreover, Acps tend to decrease a female's overall life expectancy in a dose dependant manner (Chapman *et al.*, 1995; Lung *et al.*, 2002). This negative relationship between longevity and mating is a common cost of reproduction in many taxa (e.g. Hautekeete *et al.*, 2001; Wheelwright *et al.*, 2003; Fedorka *et al.*, 2004) and this pattern may be due in part to immunosuppressive components in the male's ejaculate. In other words, the local transfer of immune suppressants aimed at the female's reproductive tract may cause a systemic decrease in immune function leading to a decrease in female fitness.

In the striped ground cricket, *Allonemobius socius*, previous work suggests that the increased mortality associated with increased mating effort may be mediated through a reduction in female immunity (Fedorka *et al.*, 2004). In addition, females who mate polyandrously incur a higher mortality rate and lower fecundity than monoandrously mated females (Fedorka & Mousseau, 2002a). This is a curious pattern to explain, especially since the only obvious difference between these two strategies was in the composition of the ejaculate donations (females of both strategies were mated the same number of times and received an approximately equal amount of sperm). Here we address the hypothesis that, while holding mating rate constant, increased seminal diversity will lead to a reduction in female immunity in *A. socius*. To help disentangle the immune effects of increased mating frequency from increased seminal diversity, we mated females to multiple males in either a monoandrous or polyandrous fashion. We predicted that monandrously mated females would exhibit a decreased immunity when compared to singly mated females due to life-history trade-offs between reproduction and immune function. We predicted further that polyandrous females due to increased seminal diversity would incur an additional immune cost, suggesting that males may interfere with the female immune response.

Methods

Immune traits

To obtain a robust description of individual immunity, we assayed three immune responses including (1) lytic activity–estimates the amount of the hemolymph-bound enzyme lysozyme, which provides a measure of bacterial defense, (2) phenoloxidase activity (PO)–estimates the amount of the inactive hemolymph-bound enzyme pro-PO, which is a key component of invertebrate innate immunity and a precursor in the encapsulation response pathway and (3) encapsulation ability–estimates the degree to which a macroscopic invading body is melanized, providing a measure of macroparasitic defense. In addition, we measured hemocyte load, which estimates the concentration of circulating hemocytes in the hemolymph and is often associated with general parasitic resistance. These measures are commonly employed as 'quality' indicators of the invertebrate immune system (Eslin & Prevost, 1998; Siva-Jothy *et al.*, 2001; Rantala & Kortet, 2003).

Mating and immune assay

Allonemobius males are unique among crickets in that they posses a specialized spur on their hind tibia that the females chew during copulation, providing a hemolymph-based nuptial gift. Previous work has shown that the duration of spur chewing is positively related to the size of the gift, with larger males providing larger gifts (for a detailed description of mating behaviour, see Fedorka & Mousseau, 2002a). Experimental crickets were third generation lab-reared individuals derived from gravid, wild-caught females from central South Carolina. All crickets were maintained in $10 \times 10 \times 8$ cm plastic cages containing ground cat food, a carrot slice, dampened cheesecloth (water source and oviposition material) and strips of brown paper towel for cover. Every 2 days the food, carrot and paper towel were replaced. At this time, newly enclosed adults were separated and caged as sex-specific cohorts. All cages were kept in a constant environment at 28.5°C and a 12: 12 [L: D] photoperiod provided by a Precision incubator (Model 818; Precision Scientific, Chicago, IL).

To address the hypothesis that increased seminal diversity leads to a higher female immune cost, we created three treatments that varied in mating frequency and mate composition. The control treatment consisted

of 55 virgin females mated once to a single male. The monandrous treatment consisted of 44 virgin females mated to the same male on average four times. The polyandrous treatment consisted of 47 virgin females mated to on average four different males, one time each. These treatments allowed us to disentangle the effects due to increased reproduction (single vs. multiply mated treatments) from those due to increased sperm competition (monandrous vs. polyandrous). For all treatments individuals were randomly chosen from the stock population. The control females were mated 12 ± 1 days post-eclosion. Mating for the polyandrous and monandrous treatments began 8 ± 1 days after adult eclosion and females were provided with either a new or the same partner once per day for 5 days. Males were rotated within the polyandrous treatment so that mating partners had the same level of mating experience (i.e. 0–3 prior matings).

All matings were conducted in a mating arena constructed of a 75 mm Petri dish lined with filter paper. The duration (in minutes) that the female chewed on the tibial spur was recorded for each mating trial to obtain an estimate of nuptial gift size. In addition, the duration that the spermatophore (externally passed package containing sperm) was attached was recorded to obtain an estimate of sperm transfer. Body size estimates were obtained by measuring the length of the pronotum to the closest 0.01 mm using a camera-mounted dissecting microscope and NIH Image software. All individuals were 12 ± 1 days old upon completion of their respective treatments, whereupon they were placed in a 50 mm Petri dish along with a carrot slice and dampened cheesecloth (water source and oviposition material) and maintained as above. Individuals were then examined every 2 days until their death, at which time the cheesecloth was collected and the number of eggs counted to estimate female fecundity. Longevity was measured as the time from adult eclosion until death.

At 13 ± 1 days of age (24 h after the last mating), we assayed immune function in all individuals. Using a microsyringe (Hamilton Co., Reno, NV) we removed 2.5 μL of hemolymph from between the second and third abdominal sternites of cold anesthetized crickets (5 min on ice). Immediately following, we placed a 2 mm piece of microfilament (0.2 mm diameter fishing line roughed on sandpaper) directly into the hole created by the needle to simulate the invasion of a novel parasite. The microfilament was knotted at one end, allowing us to nondestructively remove the monofilament after 6 h to assess the degree of encapsulation (see below). Although the above procedure increases mortality rates by providing a costly immune challenge (K. M. Fedorka, unpublished data), all individuals in all treatments experienced this standardized procedure.

The hemolymph was separated into two samples by dispensing 1.5 μL into 20 μL of phosphate buffered saline (PBS) and dispensing 1.0 μL into 9 μL of anticoagulant (39 mg NaOH + 85 mg NaCl + 37 mg EDTA + 79 mg citric acid + 1 L distilled water). The PBS-bound hemolymph was frozen to induce cell lysis and maintained for several weeks at approximately –17°C, while the anticoagulant-bound hemolymph was immediately expelled onto a hemocytometer and the number of hemocytes per millilitre was estimated. To estimate PO activity, 90 μL of a 15 mM L-Dopa buffered solution were added to 10 μL of the frozen hemolymph sample. Likewise, 90 μL of a *Micrococcus leutus* buffered solution (3 mg of freeze-dried *M. leutus* per litre) were added to the remaining 10 μL of hemolymph to estimate lytic activity. The total change in optical density (OD) in both samples over 30 min served as our estimates, which were recorded at 490 nm using a microplate reader [Model 550, Bio-Rad, Hercules CA; OD ranges from 0.000 (transparent) to 3.500 (opaque)]. The above methods for estimating both PO and lytic activity were adopted from Rantala & Kortet (2003,2004).

Encapsulation ability was measured as the degree to which the monofilament was melanized after 6 h, which was quantified using a camera-mounted dissecting microscope and NIH Image software (available from the National Institutes of Health at: http://rsb.inof.nih.gov/nih-image). The degree of melanization was measured as the mean grey scale darkness of the pixels, with 0 being completely white and 255 being completely dark. This defense mechanism is commonly quantified by calculating the amount of melanin covering inert implants such as nylon monofilaments or Latex beads (König & Schmid-Hempel, 1995; Rantala *et al.*, 2000; Doums *et al.*, 2002; Rantala & Kortet, 2003).

Of the immune components measured, our estimates of lytic activity and PO activity represent an individual's 'potential' to mount an immune response. This is because our estimates were made *in vitro* by lysing all of the hemocytes in a given hemolymph sample. Hemocytes, however, perform a variety of physiological functions and the proportion of cells that would be allocated toward a future immune challenge is unknown. In contrast, encapsulation ability represents a 'realized' immune response because it was measured *in vivo* using a novel immune challenge. All statistical analyses were performed using SAS V. 8.12 (SAS Institute, 1999).

Results

The immune, behavioural and life-history variables were not normally distributed (Shapiro–Wilk test, all $P > 0.05$; with the exception of lytic activity and encapsulation ability) and were transformed to improve normality. Planned orthogonal comparisons were made between the single and multiply mated treatments to examine the effect of increased mating frequency. Planned orthogonal comparisons were also made between the monandrous and polyandrous treatments to examine the effect of increased seminal diversity. Although the monandrous and polyandrous groups differed in the genetic diversity

Table 1 Comparison of the multiply mated treatments. Mean ± SE was back-transformed from square root estimates.

	Number of matings	Chewing duration (min)	Spermatophore attachment time (min)
Monandrous	4.06 ± 0.23	71.1 ± 10.6	97.1 ± 11.9
Polyandrous	4.16 ± 0.24	79.5 ± 8.6	98.3 ± 10.6
d.f.	88.99	81.67	91
T	−0.58	−1.19	−0.15
P	NS	NS	NS

of their mates, there appeared to be no statistical difference between them in the number of matings or the total amount of gift and sperm transferred (Table 1). Thus any differences found between these groups were likely due to differences in male ejaculate composition or in the female response to insemination.

Considering that larger females are generally more fecund (Fedorka & Mousseau, 2002b) and longer-lived (K. M. Fedorka, unpublished data), we used body size as a covariate in the following analyses. Overall, female fecundity (square root transformed) significantly covaried with mating treatment (Fig. 1; ANCOVA: $F_{2,86} = 5.59$, $P < 0.01$; no significant interaction between treatment and body size). A planned orthogonal comparison showed a reproductive advantage for multiply mated females (monandrous and polyandrous) compared to singly mated females (ANCOVA: $F_{2,86} = 10.82$, $P < 0.01$; back-transformed least-squared mean ± SE: 78.11 ± 7.28 eggs vs. 39.96 ± 9.13 eggs, respectively). However, no relationship between seminal diversity and reproductive output was detected (planned comparison between monandrous and polyandrous treatments, ANCOVA: $F_{1,63} = 1.07$, $P =$ n.s.).

Female longevity (square root transformed) also significantly varied among mating treatments (Fig. 1; ANCOVA: $F_{2,121} = 7.87$, $P < 0.001$; no significant interaction between treatment and body size). As mating frequency increased, multiply mated females exhibited a significantly shorter life-span than singly mated females (ANCOVA: $F_{2,121} = 12.35$, $P < 0.001$; 22.4 ± 1.49 days vs. 31.1 ± 4.14 days, respectively), suggesting a survival cost to mating multiply. In addition, females who received a more diverse sperm donation exhibited a nonsignificant trend towards a lower life-span (ANCOVA: $F_{1,71} = 3.44$, $P = 0.075$), suggesting that polyandrous females might incur a higher survival cost than their monandrous counterparts (19.9 ± 2.03 days vs. 25.2 ± 2.28 days, respectively).

Female body size was not associated with any of the immune parameters (all $P > 0.22$), and was therefore not included in the following analyses. Previous work suggests that females with a higher hemocyte load generally exhibit a better lytic activity and encapsulation ability (Fedorka *et al.*, 2004). Therefore, these

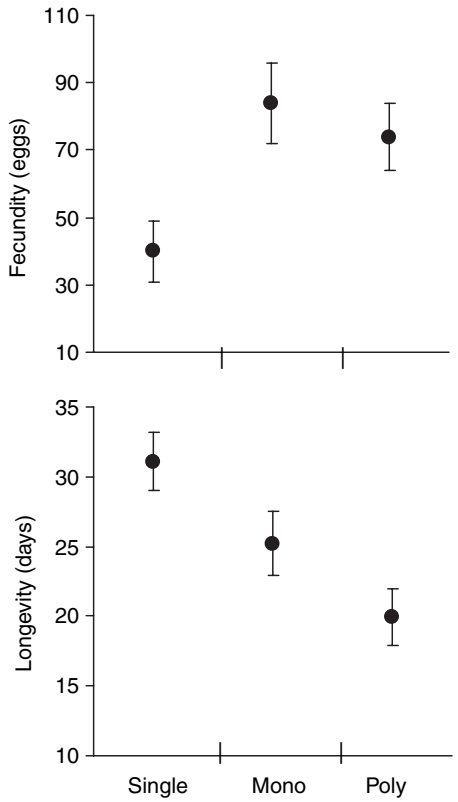

Fig. 1 The effect of reproductive strategy on female life histories. Increased mating frequency had a positive affect on female reproductive output, but a negative affect on female longevity. In addition, increased seminal diversity had a marginally negative effect on mean female life-span (ANCOVA: $F_{1,71} = 3.44$, $P = 0.0749$). These estimates represent back-transformed square root least-square means ± SE, controlled for female body size.

correlated response variables were included into the following MANOVA models where appropriate. We found that hemocyte load and lytic activity did not vary among the treatments (Fig. 2; MANOVA: $F_{2,71} = 1.03$, $P =$ n.s. and $F_{2,120} = 0.43$, $P =$ n.s., respectively). However, by using the above multivariate analysis, our ability to detect a difference between treatments in hemocyte load was severely limited by the small number of encapsulation estimates. Therefore, we removed encapsulation from the model to investigate further the difference between multiply mated and singly mated females. We found that increased mating frequency had a negative effect on hemocyte load ($F_{2,121} = 8.23$, $P < 0.001$; 52.2 ± 5.37 cells vs. 79.07 ± 10.7 cells for multiply and singly mated, respectively), indicating a general cost to reproduction. Seminal diversity, however, had no affect on hemocyte load ($F_{1,78} = 0.14$, $P =$ n.s.).

Both PO activity (: $F_{2,138} = 3.68$, $P < 0.05$) and encapsulation ability (MANOVA: $F_{2,80} = 4.88$, $P < 0.01$)

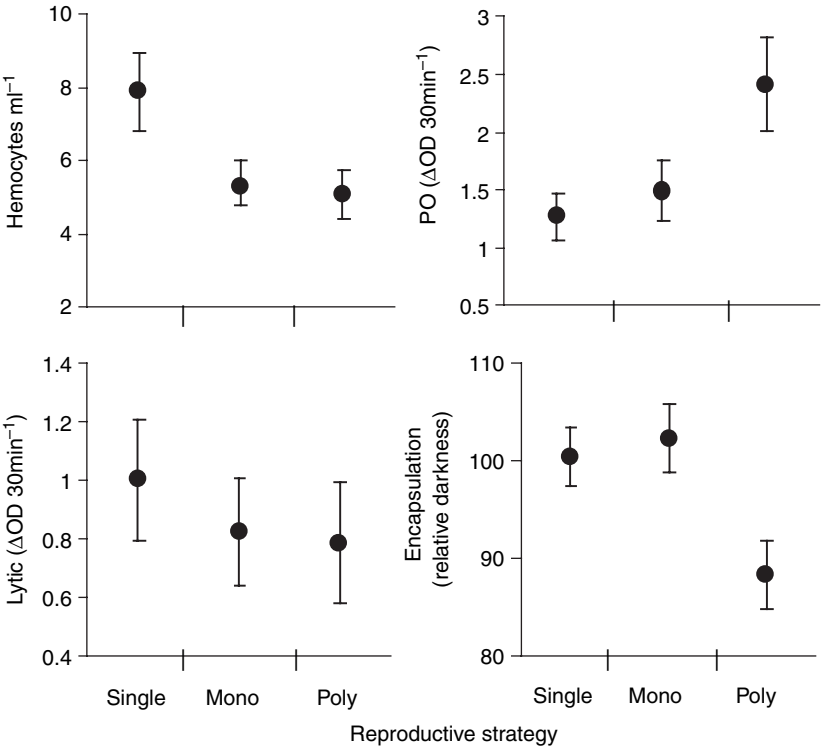

Fig. 2 Reproductive strategy and the female immune response. As mating frequency increased, female hemocyte load decreased, indicating a general cost to reproduction. Increased seminal diversity was associated with increased female phenoloxidase (PO) activity and decreased encapsulation ability, suggesting that the male ejaculate significantly influences female immunity. Lytic and PO activity were measured as the change in ocular density of the hemolymph sample over 30 min (Δ OD per 30 min), and were transformed by 10^{-2} to simplify presentation. The PO activity and hemocyte load were back-transformed from log and square root estimates, respectively.

varied significantly with regard to mating treatment (Fig. 2). There was no effect of increased mating frequency on either PO or encapsulation (all $P > 0.15$). Perhaps most striking, as seminal diversity increased PO increased (planned comparison ANOVA: $F_{1,89} = 5.42$, $P = 0.022$), however, female encapsulation ability decreased (MANOVA: $F_{1,50} = 8.63$, $P < 0.01$). Considering that encapsulation ability represents a 'realized' immune response, these data strongly suggest that a diverse seminal donation negatively affects a female's ability to defend against macroparasitic immune challenges.

Because the polyandrous treatment most closely represents the natural mating system, we analysed the associations between the life-history variables and encapsulation within this treatment. We found that longer-lived polyandrous females tended to lay more eggs, although this relationship was not significant ($r = 0.29$, $P = 0.056$). Even though polyandrous females exhibited the lowest encapsulation ability and mean longevity, we found no relationship between these traits within this treatment ($r = 0.26$, $P =$ n.s.). In contrast, females with a better encapsulation response did exhibit a higher fecundity (Pearson correlation: $r = 0.45$, $P < 0.05$; significant after a Bonferroni correction, $k = 3$). To visualize this relationship, we used a nonparametric cubic spline function that needs no a priori knowledge of the data's distribution (Fig. 3). These data suggest that decreased encapsulation ability significantly decreases reproductive output.

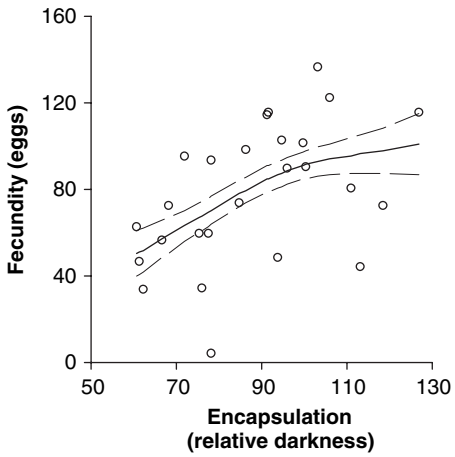

Fig. 3 Female reproductive fitness as a function of macroparasitic defense. As encapsulation ability increased, so did female fecundity. This relationship implies that females who are immune suppressed may exhibit a significantly reduced reproductive output. The function was bootstrapped 100 times to obtain a measure of variance (±1 SE) around the predicted response.

Discussion

Our data indicate that the increased seminal diversity accrued by females from a polyandrous mating strategy affects their immune ability. As seminal diversity

increased, PO activity increased and encapsulation ability decreased. Furthermore, encapsulation ability was positively associated with fecundity. In short, it appears that females who acquire additional mating from genetically diverse mates accrue a reproductive load on their immune system, reducing the ability to defend against macroparasitic invasion as well as reducing their reproductive potential. This pattern may be further exaggerated under field conditions, where individuals are most likely to encounter a greater number of immune challenges.

It should be noted that males may still maximize their fitness by suppressing female immunity, even though female fecundity (or longevity) was reduced. In a polyandrous mating system, where sperm competition is high, males are most concerned with the female's short-term reproductive output. This is true because *A. socius* females might mate with several males per day throughout adulthood. Thus, by getting his mate to store more sperm, he may have a better probability of fertilizing a female's eggs over the short-term before his sperm is significantly diluted by rival males.

Our results also imply that in vitro measures of 'potential' immunity (e.g. PO activity) may not accurately represent an individual's ability to clear an immune challenge (see Adamo, 2004a for review). This pattern is consistent with recent work in the field cricket, *Gryllus texensis*, where pre-infection estimates of PO and lysozyme activity were not correlated with resistance to an immune challenge (Adamo, 2004b). In contrast, our in vivo estimate of encapsulation ability represents a direct assessment of an individual's immunocompetence, implying that polyandrous females were significantly immune suppressed.

Why then, did we see a marked increased in PO ability with increased seminal diversity? Pro-PO is a major enzyme involved in the encapsulation pathway (Nayar & Knight, 1995) and is converted into its active form, PO, through proteolytic cleavage by a serine proteinase (Fig. 4; Chosa *et al.*, 1997; Söderhäll & Cerenius, 1998). The PO catalyzes the oxidation of phenols to quinones, which then convert to melanin during encapsulation (Söderhäll & Cerenius, 1998). This biochemical cascade produces highly toxic intermediates, causing PO activation to be regulated through proteinase inhibitors (Söderhäll & Cerenius, 1998). If male sperm are recognized as parasitic invading bodies and encapsulated, we might expect male interference with this response. In other words, male seminal products may interrupt the formation of PO, causing an increase in pro-PO due to the up-regulation of immune genes with insemination, while creating a marked decrease in the encapsulation response (Fig. 4). The fact that both male sperm and seminal proteins in *Drosophila* have been found to independently up-regulate several immune-related genes in females (McGraw *et al.*, 2004) and that *Drosophila* seminal compounds have been identified that resemble proteinase inhibitors (e.g. Acp62F; Lung *et al.*, 2002), suggest that this may be the case.

With regard to mean fecundity and longevity, our results show little difference between the polyandrous and monandrous females. This is a curious pattern, considering that we have previously found a monandrous female advantage for these life-history variables when compared to polyandrous females (Fedorka & Mousseau, 2002a). However, the fact that polyandrous females exhibited a reduction in encapsulation ability and that encapsulation was positively correlated to fecundity, which was in turn correlated to longevity, suggests that polyandrous females may still suffer a reduction in these life-history variables under natural conditions.

The question remains, however, whether female immune suppression is the result of a direct male adaptation or a negative pleiotropic effect (Morrow *et al.*, 2003). Pleiotropic harm could have evolved due to the indirect toxic consequences of other male adaptations that provide a selective advantage during sperm competition. The fact that the monandrous females' immunity was not compromised suggests that these immune effects may only occur in the presence of other 'non-self' ejaculates. This effect could be due to genetic differences in ejaculate composition, or to phenotypic differences in the ejaculate based on environmental cues. For instance, a male may reduce the number of sperm and seminal proteins that he invests in a particular copulation depending on the amount of sperm he has already allocated to the current female, a phenomenon known as the 'Coolidge Effect' (Pizzari, 2002). This behaviour optimizes the allocation of sperm across female partners in polyandrous systems.

There is evidence that male crickets can modify their ejaculate composition based on the perceived risk of sperm competition. For instance, in the crickets *Acheta domesticus* and *Gryllodes supplicans*, Gage & Barnard (1996) showed that as the risk of sperm competition increased, males modified the number of sperm (and possibly other seminal proteins) transferred in their spermatophore. Given that approximately 20 min pass between initial courtship and spermatophore production in *A. socius* (Fedorka & Mousseau, 2002a), there is ample opportunity for males to modify their ejaculate composition. Whether immune suppression is due to an increase in immunosuppressants or to an increase in other adaptive compounds that indirectly suppress immunity is unclear, leaving the distinction between the adaptive and pleiotropic harm hypotheses beyond the scope of this study.

As stated earlier, it is generally assumed that the increase in female immune parameters during reproduction serves to protect against sexually transmitted diseases (STDs; Barratt & Pockley, 1998). As such, one might expect that more promiscuous species have evolved a greater investment in immune function due

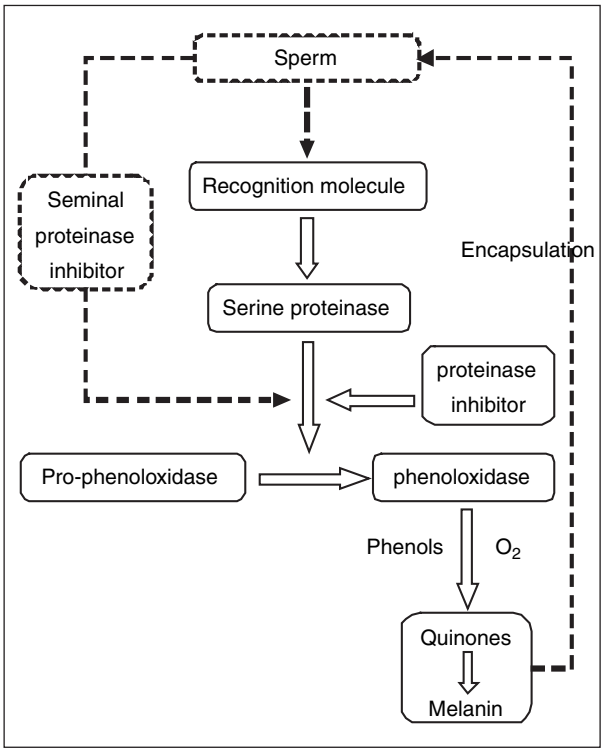

Fig. 4 Proposed interaction between the male ejaculate and the female's phenoloxidase (PO) cascade. After the recognition of foreign material, a serine proteinase converts pro-PO into PO. The PO then catalyzes the oxidation of phenols to quinones, which nonenzymatically polymerize into melanin. We suggest that the male ejaculate (dotted boxes) may elicit the activation of PO in the female, leading to the encapsulation of sperm. To circumvent this reaction, males may transfer proteinase inhibitors along with sperm to block the conversion of pro-PO. Dotted arrows represent the proposed interaction between the male ejaculate and female PO cascade.

to elevated STD transmission levels (Lockhart *et al.*, 1996; Thrall *et al.*, 2000). This prediction was supported by Nunn *et al.* (2000) who found that a female's total leukocyte count and, the degree of promiscuity are, positively correlated across a phylogenetically related group of primates. This result, however, is also consistent with our hypothesis. In other words, the positive association found between immune function and promiscuity across species might reflect an antagonistic coevolutionary arms race between the male ejaculate and female immunity due to sperm competition.

In summary, our results suggest that, as seminal diversity increased, females incurred a suppressed immunity. In addition, immune suppression was correlated with a reduction in reproductive fitness. Thus, there appears to be a sexual conflict between the male ejaculate and female immunity in this system. The extent to which males gain by impeding the female immune response, however, has yet to be addressed.

Acknowledgments

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