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Genetic divergence of the seminal signal–receptor system in houseflies: the footprints of sexually antagonistic coevolution?

José A. Andrés* **and Göran Arnqvist**

Department of Ecology and Environmental Science, Animal Ecology, University of Umeå, SE-901 87 Umeå, Sweden

To understand fully the significance of cryptic female choice, we need to focus on each of those postmating processes in females which create variance in fitness among males. Earlier studies have focused almost exclusively on the proportion of a female's eggs fertilized by different males (sperm precedence). Yet, variance in male postmating reproductive success may also arise from differences in ability to stimulate female oviposition and to delay female remating. Here, we present a series of reciprocal mating experiments among genetically differentiated wild-type strains of the housefly *Musca domestica*. We compared the effects of male and female genotype on oviposition and remating by females. The genotype of each sex affected both female oviposition and remating rates, demonstrating that the signal–receptor system involved has indeed diverged among these strains. Further, there was a significant interaction between the effects of male and female genotype on oviposition rate. We discuss ways in which the pattern of such interactions provides insights into the coevolutionary mechanism involved. Females in our experiments generally exhibited the weakest, rather than the strongest, response to males with which they are coevolved. These results support the hypothesis that coevolution of male seminal signals and female receptors is sexually antagonistic.

Keywords: antagonistic coevolution; *Musca domestica*; oviposition; postmating sexual selection; remating; sexual conflict

1. INTRODUCTION

The concept of sexual selection, traditionally occupied with studying variance in male mating success (Andersson 1994), has gradually been expanded, and it is now widely recognized that there is variance also in male ability to succeed in achieving fertilizations (Lewis & Austad 1990; Eberhard 1996; Birkhead & Møller 1998). Whenever females mate with more than one male, postmating sexual selection will result if males vary with regard to reaching any of several different goals. First, males need to ensure that their gametes are successfully transferred to the female and transported or allowed to migrate to the sites of sperm storage and/or fertilization (Eberhard 1996; Birkhead & Møller 1998; Arnqvist & Danielsson 1999*a*; Chapman *et al.* 2000; Pizzari & Birkhead 2000). Second, males will benefit from stimulating female reproductive rate, to maximize the number of female gametes produced while their sperm are at a competitive advantage over sperm from other males (Thornhill 1983; Pitnick 1991; Chapman *et al.* 1995; Eberhard 1996; Wedell 1996). Third, males will benefit from delaying female remating as long as possible, to avoid future sperm competition from subsequent males (Simmons & Gwynne 1991; Eady 1995; Eberhard 1996).

The first of these routes to postmating sexual selection can be measured as the short-term relative fertilization success of males (e.g. P_2), and the large amount of empirical attention that this has been given has generated an emerging understanding of the processes involved and their implications (see Birkhead & Møller 1998). Most importantly, it is clear that at least part of the variance in short-term fertilization success is due to male genotype (Lewis & Austad 1990; Clark *et al.* 1995; Hughes 1997; Chapman *et al.* 2000; Civetta & Clark 2000). Female genotype is also of importance (Wilson *et al.* 1997; Clark & Begun 1998), and is known to sometimes interact with male genotype in determining male short-term fertilization success (Clark *et al.* 1999). In contrast, our understanding of the second and third routes mentioned above is much more limited. Males of a wide range of taxa are known to achieve these ambitions by transferring various peptides and proteins to the female with their ejaculate, some of which stimulate egg production in females and/or cause female non-receptivity to further matings (refractoriness) (see Chen 1984; Eberhard 1996). The general occurrence of seminal signals, observations of selection on loci coding for accessory seminal substances and the rapid rate at which such proteins evolve (Thomas & Singh 1992; Civetta & Singh 1995; Aguadé 1998, 1999; Tsaur *et al.* 1998) collectively suggest that these signals are mediators of important evolutionary processes. Despite this fact, our understanding of variation in male ability to elicit such favourable responses in females is very limited (but see Gromko & Newport 1988; Fukui & Gromko 1991; Service & Vossbrink 1996).

The current study represents an assessment of the role of intraspecific genetic variation in affecting the efficiency with which males elicit postmating responses in females. By crossing three potentially differentiated wild-type strains of the housefly *Musca domestica* reciprocally, we are able to separately estimate the effects of male and female genotypes, as well as their interaction, on induction of female oviposition and refractoriness. Further, we use the emerging pattern of genetic cross-compatibility to provide insights into the mechanisms of coevolution

*Author for correspondence (jose.andres@eg.umu.se).

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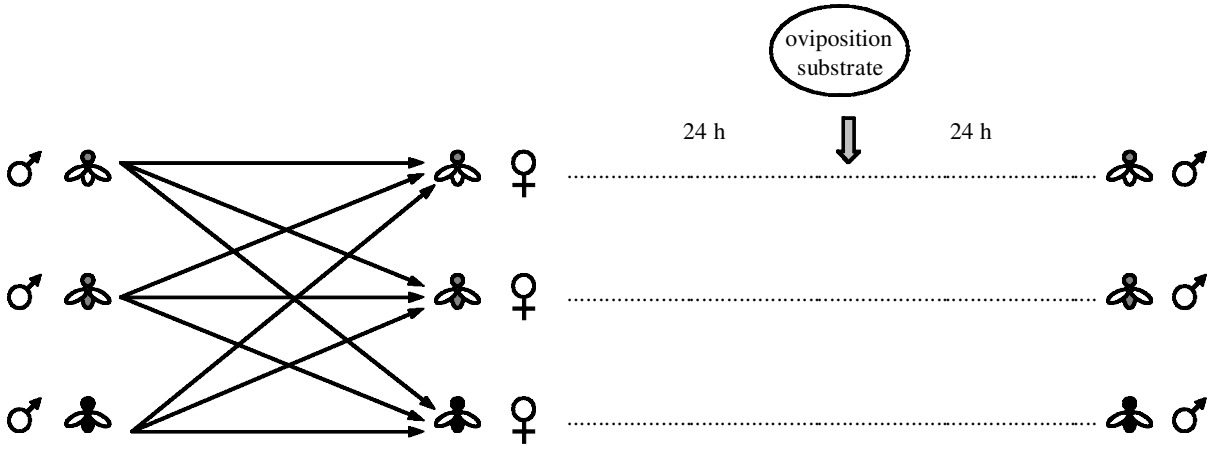

Figure 1. A flow chart describing the experimental design, involving three different wild-type housefly strains. Each female was mated once and then allowed to oviposit. First matings were reciprocal, so that females of all strains were mated to males of all strains. Females were then allowed an opportunity to remate with males of their own strain (see § 2).

between male seminal signals and female receptors to these. In particular, whenever an interaction is found, the relative female response to males of their own strain is key. Under a conventional female mate choice scenario we expect females to prefer males with which they are coevolved (e.g. Andersson 1994), whereas a process of antagonistic coevolution between the sexes would generate female resistance to such males (e.g. Rice 1996; Holland & Rice 1998; Parker & Partridge 1998) (see § 4).

2. MATERIAL AND METHODS

(a) *Study organism*

The female housefly (*Musca domestica*) has a very low remating rate. Laboratory experiments have shown that 2–14% of the females remate after an uninterrupted first mating (Riemann *et al.* 1967; Riemann & Thorson 1969; Leopold *et al.* 1971*a*). Previous experimental studies have shown that both induction of female refractoriness (i.e. loss of receptivity to further matings) and induction and stimulation of oviposition are caused by the male accessory seminal products (Riemann *et al.* 1967; Adams & Nelson 1968; Riemann & Thorson 1969; Leopold *et al.* 1971*a*,*b*). Copulation duration is typically over 1 h, and radioactive labelling experiments have shown that the process of ejaculate transfer is temporally structured. While complete sperm transfer is usually achieved in less than 10 min (Murvosh *et al.* 1964), the accessory seminal products are transferred separately and do not reach the vaginal pouches of the females until after this time. The transfer of seminal products seems to be completed in about 40 min (Leopold *et al.* 1971*b*). The effects of the ejaculate products on female physiology and behaviour are dose-dependent (Riemann & Thorson 1969). Thus, female oviposition rate is reduced (Riemann & Thorson 1969) and the receptivity to further matings increases (Riemann *et al.* 1967) when copulations are experimentally interrupted.

(b) *Stocks and rearing methods*

We used three wild-type housefly strains, derived from three sources. One strain (S) was founded by flies (80–100 individuals) collected by the authors from farms around Umeå in northern Sweden. The other two strains were laboratory wild-type stocks, derived from wild populations in Denmark (D) and Minnesota, USA (M). These were obtained from the Department of Entomology, Danish Pest Infestation Laboratory, Denmark and the Department of Entomology, University of Minnesota, USA, respectively.

The flies were reared at 25–27 °C and at a relative humidity of 60–70%, under a 12 L:12 D regime. These conditions were favourable for egg laying, larval development and hatching of pupae. All mating experiments, however, were performed at 22–23 °C. Larvae were reared in artificial medium containing water, wheat bran, alfalfa meal, baker's yeast and malt (for a full description of the rearing medium see Keiding & Arevad (1964)). To standardize conditions and prevent the formation of mould, the medium was stirred once every day until pupation. Under these rearing conditions, pupation was completed by day 7 to day 9 after seeding the eggs. At this time, pupae were separated from the medium by soaking the medium in water (25 °C), skimming and washing the floating pupae and air-drying these on filter paper. Each new generation was founded by 400 randomly chosen pupae. Flies hatched from day 2 to day 4 after isolation of pupae, and adults were maintained with sugar cubes, dry food (powdered milk–iced sugar–dried yeast, 100:100:2) and a continuous supply of water.

Seven to ten days after the peak of emergence, vials with filter paper soaked in milk or rearing medium were introduced into the insectaries as oviposition substrate. Egg laying was allowed for 4–5 h and each new jar of larval medium was seeded with 250 mg of eggs (about 3400 eggs). The total generation time was thus about three weeks.

(c) *Effects of male and female strain on induction of refractoriness and oviposition*

One way of assessing the importance of genetic variation for postmating processes is to compare the effects of mating with different male genotypes on female postmating reproductive behaviour. If male seminal products and female receptivity to these coevolve by postmating sexual selection, we should expect both male and female genotypes to affect the induction of oviposition and/or monandry in experiments involving discrete clusters of genotypes (e.g. genetically differentiated populations) (cf. Clark *et al.* 1999).

Here, we performed a series of reciprocal mating experiments involving the three wild-type housefly strains described above. They differ in their geographical origin, and are hence potentially genetically differentiated. Our experimental design aimed

at comparing the effects of seminal products on female postmating behaviour when mated to males of their own or of a different strain. Females of all strains were mated reciprocally with males of all strains (orthogonal design) and then tested for oviposition and remating following the protocol described in figure 1. In order to minimize any maternal effects, all strains were reared for at least six generations under the rearing conditions described above (§2(b)) prior to the start of the mating experiments.

For each of the strains, a random sample of pupae was introduced into a virgin chamber for hatching. The sexes were separated under brief CO_2 anaesthesia shortly after the emergence of virgin adults. Males were isolated individually, while females were kept together in small insectaries. All individuals used in the experiments were six to ten days post-eclosion. In the focal matings, each female was placed for 60 min with her first mate in a mating chamber consisting of a net cylinder (7.5 cm high, 9 cm diameter) provided with water and dry food. If mating was initiated within this time, the pair was allowed to copulate for 30 min, after which the copulation was interrupted by aspirating the mating pair out of the mating chamber and gently separating them by hand. If no mating occurred within 60 min, the male was removed and the female was used for a second trial with a different male. No female was exposed three times to putative first mates. The purpose of interrupting these focal matings was to elevate the overall level of remating in females, by reducing the amount of seminal products transferred to the female while ensuring full transfer of sperm (Murvosh *et al.* 1964). Remating rates are known to be very low in housefly females after uninterrupted copulations, but are dramatically increased if copulations are interrupted (Riemann & Thorson 1969). For example, Riemann *et al.* (1967) showed a dramatic increase in remating rates when copulations were interrupted after 30 min. Thus, interrupting matings should increase the power of our experimental design.

Following the first mating, each mated female was isolated in her mating chamber for 48 h, during the last 24 h of which a Petri dish (3.5 cm diameter) filled with oviposition substrate (as rearing medium but with 1.5% fat milk instead of water) was kept in the chamber. Remating rates were measured by offering each of these females a second male for 60 min, 48 h after the first mating (see figure 1). In order to be able to independently evaluate the effects of male genotype in inducing refractoriness in females within each female genotype, the second male was always of the female's own strain. In this way, the first males within a given female strain all competed against a constant background. All pairs were observed continuously and we recorded whether copulation (genitalia engaged) occurred or not. Subsequent to these remating assays, we recorded whether the female had oviposited or not during the intermating period by recording the presence or absence of eggs in the oviposition substrates.

Any differences in female response to males detected with the experimental protocol described above could in theory be due to (i) male copulatory courtship behaviour, and/or (ii) accessory seminal products transferred to the female (Eberhard 1996). However, while there is no visible male copulatory courtship behaviour in houseflies (Murvosh *et al.* 1964; J. A. Andrés, unpublished data), there is ample experimental evidence showing that seminal products induce both refractoriness and oviposition and that these effects are dose dependent (e.g. Riemann *et al.* 1967; Adams & Nelson 1968; Riemann & Thorson 1969; Leopold *et al.* 1971*a*,*b*). We shall therefore assume that any detected effects are primarily due to seminal products.

Table 1. *Results of generalized linear models of the effects of male and female genotype on the postmating reproductive behaviour of female houseflies*

	deviance	LLR[a]	d.f.	*p*
remating rate	216.52	—	162	—
female strain	—	8.90	2	0.012
male strain	—	7.12	2	0.028
male strain × female strain	—	2.47	4	0.650
oviposition rate	183.60	—	166	—
female strain	—	28.60	2	< 0.001
male strain	—	10.00	2	0.007
male strain × female strain	—	10.50	4	0.033

[a] LLR is the value of the log-likelihood ratio test.

(d) *Data analysis*

Data on remating and oviposition rates were dichotomous. We therefore modelled the variance in these response variables in generalized linear models, using binomial error distributions and logit link functions (McCullagh & Nelder 1989; Crawley 1993). The validity of all models was assessed by visual inspection of residuals, but no deviant cells were detected. All analyses were carried out using GLIM 3.77[1] . The effects of male and female genotype were tested in log-likelihood ratio tests comparing the deviance of a model including both factors with a model excluding the one being tested. The interaction between male and female genotype was tested by comparing the deviance of a full model with a model excluding the interaction term (McCullagh & Nelder 1989; Crawley 1993).

3. RESULTS

Female remating rate was strongly influenced by both female and male strain, but these factors did not significantly interact with one another (test of full model; $\chi^2 = 20.39$, 8 d.f., $p = 0.009$; table 1). Females showed the highest remating rates when previously mated to D males, and D females exhibited the highest overall remating rate (figure 2*a*).

Female oviposition rate was also affected by both female and male strain, but, in addition, these factors interacted significantly in their effect (test of full model; $\chi^2 = 55.97$, 8 d.f., $p < 0.0001$; table 1). While D and S females responded very differently to males of different strains ($\chi^2 = 11.90$, 2 d.f., $p = 0.003$ and $\chi^2 = 8.27$, 2 d.f., $p = 0.016$, respectively), this was not true for M females ($\chi^2 = 0.35$, 2 d.f., $p = 0.839$). In no case, however, did females respond significantly more strongly to their own males compared with males of other strains. On the contrary, in both of the strains where male genotype significantly affected female oviposition, males of the females' own strain were actually less able to induce oviposition than were males with which females were not coevolved (figure 2*b*). Planned post hoc contrasts showed that this pattern was significant among D females ($\chi^2 = 10.90$, 1 d.f., $p_{\alpha/2} < 0.001$) and marginally significant among S females ($\chi^2 = 2.20$, 1 d.f., $p_{\alpha/2} = 0.069$).

4. DISCUSSION

Several authors have stressed the need for increasing our understanding of intraspecific variation in postmating

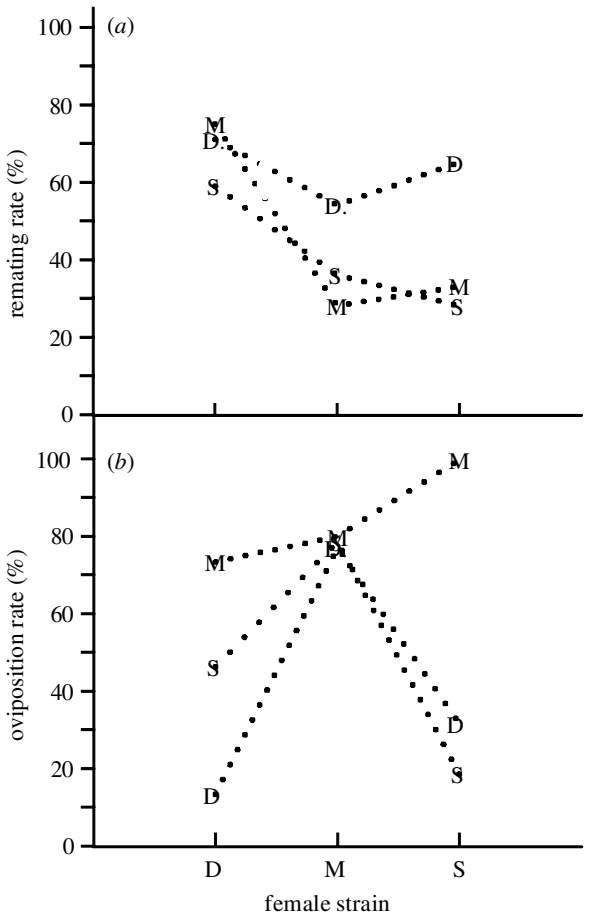

Figure 2. The effects of male and female strain on (*a*) the proportion of females remating with males of their own strain 48 h after the focal mating and (*b*) the proportion of females ovipositing 24–48 h after the focal mating. Letters inside the panels represent male strain. See table 1 for statistical evaluation.

processes (for reviews see Eberhard 1996; Birkhead & Møller 1998). Previous studies have dealt almost exclusively with the proportion of eggs fertilized by a certain male (e.g. P_2) (e.g. Lewis & Austad 1990; Eberhard 1996; Simmons *et al*. 1996; Cook *et al*. 1997; Arnqvist & Danielsson 1999*a*), despite the obvious fact that a simple proportion is a relatively poor measure of male reproductive success. For example, if P_2 is negatively related to female egg production rate, as may be expected if females experience shortage of sperm, low P_2-values may actually be associated with high numbers of offspring fathered. We therefore need to broaden the concept of postmating sexual selection, to include other components of postmating reproductive success. Our study exposed large intraspecific variation in male ability to trigger postmating responses in females, and offers several novel and important insights. First, the genotype of both sexes was of importance for the postmating reproductive behaviour of female houseflies, showing that the signal–receptor system involved has indeed diverged between the populations used in our experiment. Genetic variation in male ability to elicit favourable responses in females has previously been observed in *Drosophila* fruitflies (e.g. Van Vianen & Bijlsma 1993; Rice 1996; Service & Vossbrink 1996; Sgró *et al*. 1998). These studies have also shown that there is genetic variance for female responsiveness to male seminal stimuli. Such variation, however, could in theory simply be due to quantitative variation in the amount of seminal signals transferred by males and the sensitivity to these in females (i.e. the dose–response function) (Service & Vossbrink 1996; Arnqvist & Danielsson 1999*b*).

Our experiment also revealed an interaction between male and female genotype in their effect on female postmating reproductive behaviour (cf. Clark *et al*. 1999). We are unaware of any other study showing such an interaction. The existence of male × female interactions is very informative for at least three reasons. First, a significant interaction shows that females vary in their response to the seminal products (signals) provided by different males, and hence indicates a role for females in determining the effects of a mating on her postmating behaviour. Thus, one could argue that such an interaction is evidence of cryptic female choice (Arnqvist & Danielsson 1999*a*; Birkhead 2000; Pitnick & Brown 2000). Second, the pattern of the interaction offers potential insights into the ultimate mechanisms by which male signals and female receptors coevolve (see below). Third, the mere existence of an interaction strongly suggests that a relatively complex signal–receptor system determines male ability to induce female postmating behaviour. In a simple signal–receptor system, involving only quantitative variation in a single signal, populations could only diverge with regard to the strength and amount of the signal and the sensitivity of the receptor. In such situations, genetic variance for both the signal and the receptor may be observed, but males of different genotypes should rank similarly among the different female genotypes and we should see no interaction. An example of this is male ability to induce female refractoriness in our data, where male and female strain both affected remating rates but where no significant interaction was observed. In contrast, a more complex signal–receptor system would allow populations to diverge and coevolve with regard to the strength or amount of any of several signals and any multiple components of female sensitivity. Such complexity would thus tend to generate male × female interactions. There are reasons to believe that most of the signal–receptor systems involved in determining the effects of male seminal products on female postmating behaviour actually include several signals and receptors. It has been shown that the ejaculate of many taxa contains a wide range of substances with putative effects on females, mostly in the form of various proteins and peptides (Chen 1984; Eberhard & Cordero 1995; Eberhard 1996). For example, the ejaculate of the housefly is known to contain at least 12 different proteins, which have multiple target sites within the female (Terranova *et al*. 1972).

(a) *The pattern of male × female interactions: What does it tell us about male–female coevolution?*

In theory, male × female interactions could be due to any of three different associations. First, female response to males may differ, but the relative rankings across populations could be random. This would be inconsistent with earlier observations of selection acting on the loci involved (e.g. Aguadé 1998, 1999), since it would suggest that founder effects and/or genetic drift are responsible for the divergence between strains and populations.

Second, females could tend to respond strongest, or at least stronger than on average, to males of their own strain. Such positive functional matching would be expected if male signals and female receptivity coevolve by a cryptic female choice process analogous to conventional female mate choice. Under this form of sexual selection, females benefit from responding to males that provide the strongest stimuli, since this would increase the fitness of their offspring (e.g. Andersson 1994; Iwasa & Pomiankowski 1991; Pomiankowski *et al*. 1991). Given that male signals and female receptors coevolve, females would in essence tend to evolve a 'preference' for males of their own strain. Third, females could show the weakest response, or at least weaker than average, to males of their own strain. Such negative functional matching is predicted if male signals and female receptivity evolve by cryptic female choice driven by sexually antagonistic coevolution (Holland & Rice 1999; Gavrilets *et al*. 2001). This assumes that there are important and general differences in the postmating interests of the sexes. Males clearly stand to gain from any modification of the seminal signals that increases their sperm competition success, elevates female short-term egg production rate and/or decreases female remating rate (Eberhard 1996). This is true even if these benefits are achieved at the expense of female fitness (Chapman *et al*. 1995; Rice 1996). Whenever female interests are compromised by male seminal stimuli, females will evolve to depress these costs, by evolving 'resistance' to the signals. Given that male signals and female receptors coevolve, females could then be expected to exhibit the highest resistance to the seminal stimuli of males of their own strain (Parker & Partridge 1998; Gavrilets *et al*. 2001).

Our results showed that the ability of male houseflies to induce oviposition in females depended on the genotype of the female. Females of one of the strains used did not respond differently to males from different strains, while females of the two remaining strains showed the weakest response to males of their own strain. This result allows us to reject conventional female mate choice as the coevolutionary process responsible for the observed genetic divergence in seminal signals and receptors (cf. Eberhard 1993). Our experiment instead supports the hypothesis that male seminal signals and female receptors coevolve by sexually antagonistic coevolution and that females resist signals from males with which they are coevolved (see also Clark *et al*. 1999). Female fitness may be compromised in at least three different ways by male seminal signals, and in all of these cases we expect females to evolve resistance to male seminal substances. First, some substances in the ejaculate are known to be toxic to females, and to significantly reduce their life span (Chapman *et al*. 1995; Rice 1996). Although this deleterious effect is assumed to be a side-effect of substances with other functions (Chapman *et al*. 1995; Keller 1995; Holland & Rice 1999), it is worth noting that some of the peptides that males transfer are structurally similar to potent neurotoxins found in spider venom (Wolfner *et al*. 1997) and that others are very similar to peptides with known degenerative effects (Smid 1997). Second, female lifetime fitness will be optimized when egg production rate is at an intermediate level, reflecting the trade-off between the costs and benefits involved (Chapman *et al*. 1998). Male seminal signals are known to increase female short-term egg production rate (Chen 1984; Eberhard 1996), and may elevate female reproductive rate beyond that optimal for females (Arnqvist & Nilsson 2000). Third, female insects (especially dipterans) benefit from remating in terms of an increased rate of fertility (for a review, see Arnqvist & Nilsson 2000), presumably because this provides females with viable sperm. Seminal signals in males that depress female remating rate (Simmons & Gwynne 1991; Eberhard 1996) may therefore also reduce female fertility. It has also been suggested that males may cause direct harm to females as a strategy to delay remating (Johnstone & Keller 2000). In houseflies, the ejaculate contains substances which break down cells in the walls of the vaginal pouches (Leopold *et al*. 1971*b*), and such intrusive effects may obviously cause harm in females.

It is worth noting that postmating sexual antagonisms are apparently important in houseflies, despite their low remating rates. In theory, such conflicts are dissolved under strict monandry (Holland & Rice 1999; Arnqvist *et al*. 2000). However, theory also tells us that even a low degree of female remating could generate sexual selection among males to depress female remating rate and to elevate female oviposition. Female houseflies are not strictly monandrous. Evidently, the remating rates exhibited (2–14%) create sufficient variance in male reproductive success to found postmating sexual antagonisms.

There is a great need for studies assessing the patterns of intraspecific variation in the effects of seminal substances on female reproductive behaviour (cf. Howard 1999). If future studies confirm the pattern documented here, there are reasons to believe that sexually antagonistic coevolution is an important and general generator of divergent evolution of these signal–receptor systems. Therefore, one important implication of this scenario is that reproductive isolation (i.e. speciation) may be a common, but incidental, by-product of sexually antagonistic coevolution (Rice 1998). This is consistent with the relatively high rate of speciation documented in insect clades with an opportunity for postmating sexual antagonisms compared with those without such opportunity (Arnqvist *et al*. 2000).

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.