Male–male pheromone signalling in a lekking *Drosophila*

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Interest in sex pheromones has mainly been focused on mate finding, while relatively little attention has been given to the role of sex pheromones in mate choice and almost none to competition over mates. Here, we study male response to male pheromones in the lekking *Drosophila grimshawi*, where males deposit long-lasting pheromone streaks that attract males and females to the leks and influence mate assessment. We used two stocks of flies and both stocks adjusted their pheromone depositing behaviour in response to experimental manipulation, strongly indicating male ability to distinguish between competitors from qualitative differences in pheromone streaks alone. This is the first example of an insect distinguishing between individual odour signatures. Pheromone signalling influenced competition over mates, as males adjusted their investment in pheromone deposition in response to foreign pheromone streaks. Both sexes adapt their behaviour according to information from olfactory cues in *D. grimshawi*, but the relative benefits from male–female, as compared to male–male signalling, remain unknown. It seems likely that the pheromone signalling system originally evolved for attracting females to leks. The transition to a signalling system for conveying information about individuals may well, however, at least in part have been driven by benefits from male–male signalling.

Keywords: individual recognition; scent matching; male competition; mate choice; lekking

1. INTRODUCTION

Pheromonal signalling has been reported for a wide range of taxa including mammals, birds, reptiles, fish, crustaceans, fungi and bacteria (see references in Wyatt 2003). In particular, pheromones that influence sexual behaviours, sex pheromones, have received attention from students of animal communication. Most of this interest has, however, been directed at mate finding and recognizing conspecifics of the opposite sex. The rapid growth of evolutionary ecology over the past decades has resulted in a shift towards studies of individual variation and pheromones as signals of mate quality among members of the same species and sex. Such mate assessment pheromones require individual variation in order to convey information and the variation may be correlated directly or indirectly to traits such as condition, fertility, female reproductive status, age, parasite load, nutritional status, maturity or immunocompetence (Johansson & Jones submitted).

If the role of pheromones in mate choice has received little interest until recently, even less attention has been given to the role of sex pheromones in competition over mates. Individuals competing over matings (usually males) may benefit by adapting their behaviour according to information gathered by ‘eavesdropping’ when competitors broadcast honest signals of quality to choosing individuals (usually females). In this manner, a dual function of sex pheromones for conveying information about individual quality may evolve. Obviously, benefits associated with signalling identity or quality between

competitors may also lead to the evolution of novel signalling systems without pre-existing mate assessment signals. One of the best-studied cases where competitors adjust their reproductive behaviours in response to sex pheromones released by individuals of their own sex is the cockroach, *Nauphoeta cinerea*. Here, male pheromones signal social status to both males and females (Moore 1988), but females use olfactory cues to discriminate against the most aggressive and socially dominant males (Moore *et al.* 2001). Other examples come from voles, where males invest more in sperm competition in response to the smell of competitors (delBarco-Trillo & Ferkin 2004), and beetles where males may cease pheromone emission altogether if they detect the presence of other males (Bashir *et al.* 2003).

Drosophila grimshawi is a lekking Hawaiian fruitfly, where males deposit long-lasting pheromone streaks that attract males and females to the leks (Droney 1994) and that are involved in mate assessment (Droney & Hock 1998). We have previously shown that social environment influences male pheromone deposition in this species (Johansson *et al.* 2005). Here, we investigate the signalling system of *D. grimshawi* further by looking at whether exposure to sex pheromones alone may have an effect on pheromone deposition. Moreover, we test whether males can discriminate between self and non-self and if there is a stronger response to depositions from several competitors as compared to one. Our experiments shed further light on the function of the sex pheromone in *D. grimshawi* and how males allocate resources to pheromone signalling in this lekking system, where both sexes show preferences for the sex pheromone.

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2. MATERIAL AND METHODS

(a) *Study animal*

Male *D. grimshawi* aggregate to form leks on leaves, tree trunks, etc. and perform complex displays directed at conspecifics of both sexes. The leks hold no resources and are visited by females for the sole purpose of mating (Spieth 1984). Leks formed in the laboratory vary in size from 2 to 10 males and the males can also perform solitarily (Droney 1994), a situation that probably occurs also in natural populations (Spieth 1984). Lekking males utilize a wide array of tactile, visual and chemical signals (Ringo & Hodosh 1978; Spieth 1984). Among these, secretions from a pair of anal glands play a prominent part (Hodosh *et al.* 1979). The males walk over the lekking arena repeatedly dragging the tip of their abdomen against the surface (Spieth 1984), depositing secretions in the form of clearly visible streaks a couple of millimetres long (Droney & Hock 1998). The oily streaks remain for months and release volatile substances that attract both males and females from a distance (Droney 1994). Neither the composition of the actual pheromone nor the rate of emission from the streaks is known. Apart from acting as a calling signal, the pheromone might also function as an honest indicator of mate quality as males that deposit many streaks enjoy a greater mating success (Droney & Hock 1998) and have a reduced lifespan (Johansson *et al.* 2005). Males do not defend territories on the leks (Droney 1992) and may deposit secretions on top of streaks already deposited by themselves or other males.

We used two stocks of flies in this study. The 'wild-stock' was derived from five wild-caught females from the Makawao Forest reserve north of the Haleakala crater, Maui, Hawaii in 2000, and bred in the lab for nine generations prior to experiments. The 'lab stock' is an isofemale line, also known as the G1-stock, collected from Auwahi on the south slope of the Haleakala crater in 1965 (K. Kaneshiro 1999, personal communication). It has been maintained at high population numbers since and has been bred in our lab since 1999. For details on standard rearing techniques, see Johansson *et al.* (2005). Male *D. grimshawi* become sexually mature at about 10 days following eclosion (Carson *et al.* 1970). While collecting individuals for the experiment, all emerging adults were sexed and separated within 2 days of eclosion to ensure virginity and prevent prior experience from other males' displays. Males were placed individually in standard food vials lined with damp, double-thickness tissue paper (20 × 30 mm), containing 5 ml of standard food medium at the bottom. A male's food vial was changed twice weekly for the duration of his life. Only males that had started to deposit pheromones in their individual vials were later used in the experiment (age 17–24 days). All individuals were held at constant temperature (20 ± 5 °C) and relative humidity (60%) on a 14 : 10 h light : dark photoperiod (07.30–21.30).

(b) *Self versus non-self trial*

On the first day of the experiment, males were randomly assigned to one of three groups and placed singly in fresh food vials and left to deposit pheromones for 24 h. To collect pheromone depositions, we covered the interior surface of each food vial with a rolled up, rectangular sheath of transparent plastic (overhead) film (90 × 85 mm). During the second day of the experiment, the plastic films were removed and photographed, while the flies remained in the vials. During the third day of the experiment, remaining flies from the first two groups received fresh food vials, the

interiors of which were covered with either the same plastic film as they had deposited on the previous day (treatment A) or a film with depositions from another male (treatment B). The third group of males was used only as donors for treatment B. The males were left to deposit pheromones on these films for another 24 h, after which the films were once again photographed and the flies killed.

(c) *One or two competitors trial*

We used the films resulting from treatments A and B further, by exposing novel males to them. On the fifth experimental day, we used a new set of males that received fresh food vials with films containing 2 days of pheromone depositions from either one male (treatment C; films from treatment A) or two males (treatment D, films from treatment B). The males were left in the vials for 24 h, after which the films were photographed and the males killed.

(d) *Image processing*

Plastic films were photographed using a flatbed scanner (Agfa Snapscan 1212) and the area of pheromone depositions was estimated using the IMAGEJ (v. 1.30g) image processing software (for further details on the procedure, see Johansson *et al.* (2005)). To check the consistency of this method of measurement, we repeated the process at three different days with a sample of 12 images. The repeatability (Lessels & Boag 1987) for this sample was 0.98. The amount of pheromone deposited by male *D. grimshawi* has previously been estimated by manual counting of pheromone streaks (Droney & Hock 1998). We repeated this procedure for a small sub-sample ($n=20$) of our pheromone films and found a positive correlation between number of streaks and area measured as above ($r^2=0.35$, $t=3.12$, $p=0.006$).

(e) *Statistical analysis*

The effects of the experimental manipulations in the first part of the experiment were analysed using ANOVAs. For treatment A, the 'stimulus area' reflects propensity to deposit pheromones and previous investment, while stimulus area is independent of previous investment and male condition in treatment B, where males received films from other males. Thus, including 'stimulus size' as a covariate would control for different things in the experimental treatments and ANCOVAs could not be used. Unequal variances across stocks made it necessary to analyse the stocks independently for the first part of the experiment. Residual analysis revealed an extreme outlier among lab males in treatment A; this data point was included only in the non-parametric comparison of stimulus area between stocks. In the second part of the experiment, both treatments contained novel males and we controlled for stimulus size, by using it as a covariate in an ANCOVA. *Post hoc* tests (Tukey's unequal N) were used to contrast the behaviours of flies belonging to the different stocks and treatments in the full model of the second part of the experiment. Statistical analyses were performed using STATISTICA 7 (StatSoft, Inc., Tulsa, USA).

3. RESULTS

Males belonging to the wild-stock deposited more pheromones than lab males (Mann-Whitney; $n=51$, 42; $Z=3.97$, $p=0.00005$), but there were no differences in deposited areas between three groups of males within stocks during the first day (ANOVAs; $n=51$ or 42;

Figure 1. Total area of pheromone deposited (mm^2) over the stimulus day and experimental day in the first part of the experiment. Males in treatment A were exposed to their own pheromone deposits, while males in treatment B were exposed to streaks from a strange male. Boxes are s.e. and whiskers 95% confidence intervals. $n=13 A_{\text{wild}}$, $13 B_{\text{wild}}$, $17 A_{\text{lab}}$ and $14 B_{\text{lab}}$.

$F<1.36$; $p>0.26$). The 'stimulus areas' did not differ significantly in size between treatment A (stimulus=own deposits) and treatment B (stimulus=deposits from a third group of males) within stocks (wild: $F_{1,36}=1.31$, $p=0.26$; lab: $F_{1,26}=0.16$, $p=0.70$). Total area of pheromone streaks after 2 days of depositing was significantly larger for males presented with foreign streaks (treatment B) than for males presented with their own streaks (treatment A) in the lab stock ($F_{1,24}=4.87$, $p=0.04$) (figure 1). There was no significant difference between treatments A and B in pheromone area after 2 days of depositing for the wild-stock ($F_{1,30}=1.58$, $p=0.22$).

There were no significant main effects in the second part of the experiment, but there was a significant interaction between stock and treatment (table 1, figure 2). The interaction stems from wild-stock males depositing significantly less pheromone when encountering streaks from two males than from one male ($p=0.0005$) and from lab males behaving in the opposite manner, i.e. depositing significantly more pheromone when encountering streaks from two foreign males than from one ($p=0.0004$). Wild males deposited more pheromones than lab males in the presence of tracks from one male ($p=0.0002$), but there was no difference between the stocks in the presence of streaks from two males ($p=0.52$). The significant effect of stimulus size is trivial, as the groups and stocks differed to begin with (above).

4. DISCUSSION

Lab males deposited more pheromone in the presence of streaks from a strange male than when exposed to their own streaks and invested more when encountering streaks from two as compared to one strange male. This suggests a substantial increase in pheromone deposition in competitive situations, as compared to when depositing alone. An alternative explanation to the pattern in the first part of the experiment would be that males for some reason less often

Table 1. The effect of stimulus pheromones from one or two strange males on pheromone deposited. (Measured as total area corrected for stimulus area using ANCOVA. SS, sum of squares.)

variable	SS	d.f.	<i>F</i>	<i>p</i>
intercept	354 469	1	15.16	0.0003
stimulus area	2 423 964	1	103.67	0.0000
stock	52 492	1	2.8	0.14
treatment	7456	1	0.9	0.57
stock $\times$ treatment	180 885	1	8.8	0.008
error	1 215 776	52		

place their streaks on top of deposits from strange males, as compared to on top of their own streaks. The effect in the second part of the experiment suggests that the significant difference in the first part of the experiment is not due to males avoiding to mark on streaks from strange males.

Wild males showed no response to the manipulation in the first part of the experiment, but deposited much less in the presence of streaks from two as compared to one strange male in the second part. This may seem difficult to explain, but a possible proximate explanation is that wild males require larger amounts of pheromone from a single strange male in order to adjust their depositing behaviour. Data from this and previous experiments (B. G. Johansson 2003, unpublished results) suggest that wild males deposit more pheromone than lab males, and that wild flies require larger amounts of pheromone for being attracted over a short distance.

Clearly, both stocks of *D. grimshawi* adjusted their pheromone depositing behaviour in response to experimental manipulation, showing male ability to distinguish between competitors from qualitative differences in pheromone streaks alone. Individual variation in sex pheromones may be achieved through quantitative or

Figure 2. Amount of pheromone deposited (mm^2), corrected for previous depositions, on the experimental day in the second part of the experiment. Males were exposed to pheromones from a single male deposited over 2 days (treatment C) or streaks deposited by two males on 1 day each (treatment D). Squares signify the lab stock, circles the wild-stock. Whiskers are 95% confidence intervals. $n=17$ C_{wild} , 17 D_{wild} , 12 C_{lab} and 11 D_{lab} .

qualitative differences in the pheromone blend, or via differential release rates (Johansson & Jones submitted). In general, the quantity of pheromone released varies more than the ratios of its components. *Drosophila grimshawi* is a lekking species, but males do not defend display territories or pheromone streaks (Droney 1992). Males are tightly clustered on the leks and often deposit pheromones on top of streaks already present. Female *D. grimshawi* have been shown to preferentially mate with males that invest heavily in pheromone depositing behaviour, and pheromone depositing is correlated to other forms of courtship (Droney & Hock 1998). Female ability to sample the aggregate pheromone streaks on a lek and determine relative male investment will provide females with an effective way of assessing male investment in lekking over a much longer time period than a female visit. Time spent on the lek is a good predictor of male investment and success in *D. grimshawi* (Droney 1992) as in most other lekking species (Höglund & Alatalo 1995). Thus, males need not signal competitive ability directly using qualitative variation in the pheromone and females need not prefer specific qualitative pheromone traits; female ability to distinguish between deposits from different individuals in a lekking situation will suffice for assessing male quality. In this system, individual recognition of pheromone signatures might evolve without any benefits to females from mating with males displaying certain chemical qualities in their pheromone depositions.

Male ability to determine male identity from pheromonal deposits may be beneficial even if males were unable to determine the relative investment of the competitors. Large leks usually mean increased chances of encountering prospective mates (Höglund & Alatalo 1995) and may also result in better chances for lower ranking males to obtain matings (Widemo & Owens 1995, 1999). Thus, it will be beneficial for males sampling leks to be able to determine the number of males that have deposited pheromones. Being able to determine not only how many males that have been present but also how much they have invested will,

obviously, enable males to assess the investment in lekking activities from competitors. Matings in *D. grimshawi* often last more than a minute (F. Widemo 2003, unpublished results) and males often attempt to disrupt matings. An additional benefit to high quality males from investing in pheromone deposition could be that lower ranking males may be less likely to disrupt such males. Male interactions in lekking species often have significant impact on mating success, as males are tightly clustered in space. Here, signalling systems for conveying information between males are especially likely to evolve (e.g. Widemo 1997). It seems likely that the pheromone signalling system in *D. grimshawi* originally evolved for attracting females to leks. The transition to a signalling system for conveying information about individuals may well, however, at least in part have been driven by benefits from male–male signalling.

The benefits from distinguishing between the pheromone from different males for males and females that we have discussed are reminiscent of the scent-matching hypothesis (Gosling & Roberts 2001) being put forward to explain territorial marking in mammals. According to this hypothesis, intruders match the scent marks on a territory with the owner in order to assess his ownership and competitive abilities. This might be beneficial for males to avoid fights they are likely to lose (Gosling *et al.* 1996) and for females in mate choice (Rich & Hurst 1998). Lekking antelope have been shown to use olfactory cues from the soil on male territories to determine past success of the territory holder (Deutsch & Nefdt 1992), and the scent-matching hypothesis has been suggested to work also in non-territorial species (Wyatt 2003). Whether male or female *D. grimshawi* are able to match individual males to streaks deposited remains to be tested. Both males and females have the opportunity to sample old and freshly deposited streaks on the lek, however, as males do not defend territories.

To conclude, the pheromone signalling system in *D. grimshawi* is multifaceted, with both males and females being attracted to the pheromone and being able to distinguish between individuals from pheromone deposits alone. Pheromone signalling influences competition over mates, as males adjust their investment in pheromone deposition in response to pheromone streaks from strange males. Both sexes adapt their behaviour according to information from olfactory cues, but the relative benefits from male–female as compared to male–male signalling remain unknown. Further studies of the sex pheromone in *D. grimshawi* may help us understand how olfactory signals simultaneously used in mate choice and competition over mates may shape signalling systems.

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