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Chemosensory cues allow male *Tenebrio molitor* beetles to assess the reproductive status of potential mates

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Males of many insect species, including beetles, choose their mates according to their reproductive status. However, the ways in which male beetles evaluate female reproductive status have received little attention. We tested the existence of male mate choice in the mealworm beetle, *Tenebrio molitor*, by observing mating and courtship behaviour of males given simultaneous access to pairs of females differing in their reproductive status: (1) mature versus immature; (2) virgin versus previously mated; (3) familiar (mated with the experimental male) versus unfamiliar (mated with a different male). Males courted and mated preferentially with mature and virgin females. To determine whether chemical cues played a role in these discriminations, we exposed males to filter paper squares bearing chemical cues from different types of females: (1) virgin versus mated; (2) mature versus immature. Males were significantly more attracted to those squares bearing chemical cues from virgin and mature females, suggesting that males can assess female reproductive status on the basis of chemical cues alone.

Sexual selection studies have generally emphasized mate choice in females (Andersson 1994; Ben-Ari 2000). In contrast, male mate choice has been relatively neglected (Bonduriansky 2001). This skewed approach to the study of mate choice probably reflects a biological fact: the costs of reproduction are typically higher for females, which tend to be more discriminating than males about which individuals they select as potential mates (Trivers 1972; Andersson 1994). However, male reproduction is not without costs. Sperm production, mate searching, courtship, mating and mate guarding can all increase the costs of male reproduction (Dewsbury 1982; Andersson 1994; Bonduriansky 2001). Thus, when the benefits outweigh the costs of being choosy (e.g. when female quality varies), male mate choice is expected to evolve (Verrell 1990; Andersson 1994; Bonduriansky 2001).

Sexual selection theory predicts the evolution of male preferences for females that maximize male fertilization success (Bonduriansky 2001). Hence, selection should favour males that choose female phenotypes associated with high fecundity or reduced sperm competition. In insect species in which females mate with multiple partners, the reproductive status of females can affect their fecundity or the potential for sperm competition. For example, immature females tend to be less fecund than sexually mature females (Ridley 1989), and mating with them could reduce reproductive benefits for males. Furthermore, for species in which second-male sperm precedence is not complete, unmated females are usually associated with reduced sperm competition (Simmons & Siva-Jothy 1998; Simmons 2001; Siva-Jothy & Stutt 2003). There is thus consistent theoretical and empirical background to suggest that female reproductive status influences male reproductive success. Therefore, males may be expected to evolve mechanisms that enable them to assess female reproductive status (Lewis & Iannini 1995; Arnaud & Haubruge 1999; Bonduriansky 2001; Wedell et al. 2002; Rypstra et al. 2003). Male mate choice based on female reproductive status is common in insects (Barrows & Gordh 1978; Johnson & Hubbell 1984; Wiklund & Forsberg 1985; Bonduriansky 2001; Sauter & Brown 2001; Wedell et al. 2002; Siva-Jothy & Stutt 2003), including beetles (Barrows & Gordh 1978; Johnson & Hubbell 1984; Brown & Stanford 1992; Lewis & Iannini 1995; Arnaud & Haubruge 1999). However, no studies have specifically addressed the stimuli that male beetles use to assess female reproductive status.

Chemical cues are important in mate selection in many kinds of organisms (Wyatt 2003). Typically, research on insect sex pheromones has emphasized their role as sex attractants, often assuming that they allow males to make simple discriminations, for example, to determine whether another individual is a male or a female, or either a conspecific or a heterospecific. However, growing

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evidence indicates that chemical cues may be used by one or both sexes to derive complex information about potential mates. Thus, insect chemical cues have been shown to convey information regarding social status (Moore et al. 1997), fluctuating asymmetry (Thornhill 1992), parasite load (Worden et al. 2000), immunocompetence (Rantala et al. 2002), familiarity (Bateman 1998; Arnaud & Haubruge 1999) and degree of relatedness (Simmons 1989; Smith & Breed 1995). Chemical assessment of female reproductive status by males has been reported in spiders (Rypstra et al. 2003) and several insect orders (Diptera: Tompkins & Hall 1981; Scott et al. 1988; Scott & Jackson 1990; Mair & Blackwell 1998; Lepidoptera: Gilbert 1976; Andersson et al. 2000; Hemiptera: Kukuk 1985). Given their critical role in the mating systems of many beetles, chemical cues similarly offer a potential source of information that males could use to discriminate between females differing in their reproductive status.

We examined the effect of female reproductive status on male mate choice in a polygynandrous beetle, *Tenebrio molitor*. We also tested the hypothesis that male *T. molitor* beetles can assess female reproductive status from chemical cues alone.

METHODS

Beetle Culture

We used beetles originating from stock cultures maintained in the Ethology Laboratory at the University of Valencia, Spain. These cultures have been running for more than 10 years with regular contributions from other cultures and from wild stock. All growth stages are kept together in single plastic containers with a rearing medium consisting of white flour and wheat bran to which chunks of fruit, bread and various vegetables are added periodically. The surface of the culture is covered with filter paper that is sprayed with water to provide moisture on a daily basis. All containers are stored in well-ventilated, dark places, at ambient humidity and under temperature-controlled conditions (22–25 °C).

We haphazardly collected beetle pupae from the stock cultures and sexed them by examining developing genitalia on the ventral side of the eighth abdominal segment (Bhattacharya et al. 1970). Individuals were examined under a dissecting scope both as pupae and after eclosion; we did not use beetles with obvious malformations. Sexed adults of the same age were kept separately in plastic containers (ca. 13 × 20 cm and 15 cm high) until used in the experiments. To ensure adequate motivation, before each trial males received a 24-h mating period with a nonexperimental female in a petri dish with 0.5 mm of wheat bran as substrate; at least one successful copulation was observed in all cases. Except in the case of females taking part in the mature versus immature test (see below), all male and female beetles involved in the same trial were approximately the same age. Trials were conducted at 22–25 °C, at ambient humidity and under dim red lighting.

Male Mate Choice Tests

To test for male mate choice, we investigated reproductive behaviour in adult nonvirgin males given simultaneous access to pairs of females of different reproductive status. We observed each male in the presence of one of the following pairs of females: (1) mature and immature virgin females; (2) mature virgin female and mature nonvirgin female; and (3) mature female mated to the experimental male (familiar) and mature female mated to another male (unfamiliar).

Immediately before each trial, females arbitrarily assigned to the nonvirgin group received a 24-h mating period with a nonexperimental male in a petri dish with 0.5 mm of wheat bran as substrate; at least one successful copulation was observed in all cases. Familiar females differed from unfamiliar females in that they were allowed a 24-h mating period with the experimental male. Mature females were at least 1 week posteclosion, whereas immature females were 24–48 h posteclosion (Tschinkel et al. 1967; Happ & Wheeler 1969). We marked pairs of females taking part in the same trial by applying one or two droplets of correction fluid (Liquid Paper) to different parts of their elytra.

All trials were run in a circular arena consisting of a glass dish 19 cm in diameter inverted over a piece of white filter paper (21 × 26 cm; experimental design follows Worden et al. 2000). Males were placed in the centre of the arena and restrained under a plastic dish 8.8 cm in diameter for 5 min. During this acclimation period, both females were left free in the remaining part of the arena. The plastic dish was removed at the beginning of each trial, which lasted 30 min. In *T. molitor*, courtship behaviour involves a sequence of highly stereotyped discrete action patterns, most of which are shown or initiated by males (Font & Desfilis 2003). The occurrence of any of these action patterns (Table 1) was considered as an indicator of male courtship. For each trial, we scored: (1) the reproductive status of the female with which the male copulated; (2) the number of complete or incomplete courtship sequences directed to each female; and (3) the total time the male devoted to courting each female. All behaviours were recorded on a laptop microcomputer equipped with event-recorder software (JWatcher 0.9, D. Blumstein, http://galliform.psy.mq.edu.au/jwatcher/).

Odour Preference Tests

To test male preferences for chemical cues from females with different reproductive status, we exposed each male to one of the following combinations of odour cues: (1) mature virgin female and mature nonvirgin female; and (2) mature and immature virgin females. We collected odours by placing one donor female in a plastic petri dish 5 cm in diameter with two 1-cm^2 pieces of white filter paper for a 24-h period immediately before each trial. All trials were conducted in an arena identical to that used in the male mate choice tests. Males were introduced into the arena with four 1-cm^2 pieces of filter paper (two from each type of odour donor) placed equidistant from each other and from the centre of the arena in an alternating

Table 1. Male *Tenebrio molitor* courtship and mating behaviour patterns recorded during mate choice tests

Behaviour patterns	Description
Courtship	
Tattoo	The male taps the body of the female with its antennae. The antennae move rapidly and in a rhythmic way
Mount	The male climbs on top of the female making rapid forward–backward scraping movements with its prothoracic legs against the female's sides; often combined with tattoo
Probing	The male moves its copulatory organ from side to side across the female's rear end until achieving intromission; sometimes a male will mount from the female's anterior end and will start probing the female's head until it is in the correct position
Mating	
Copulation	The male introduces its copulatory organ and the pair remains attached by the genitalia for a variable length of time, usually 1–2 min; copulation ends when the male withdraws its copulatory organ

See Font & Desfilis 2003 for a complete description of courtship and mating in this species.

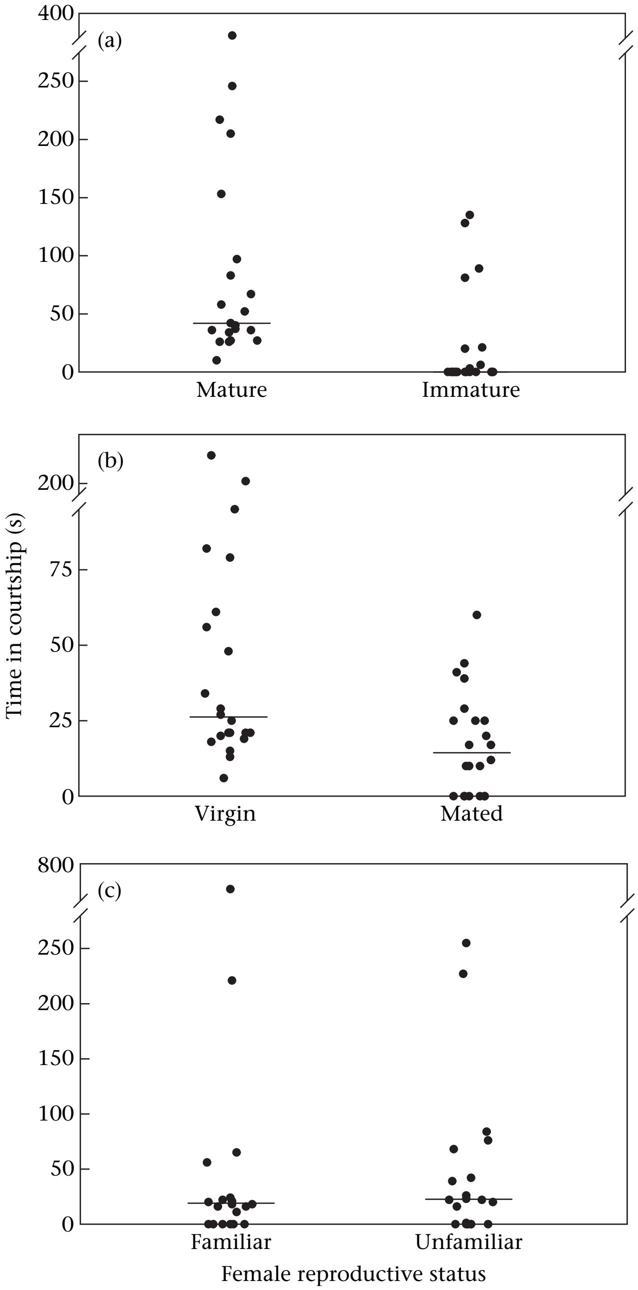

Figure 1. Total time that males spent courting each type of female: (a) mature versus immature; (b) virgin versus previously mated; and (c) familiar versus unfamiliar. Graphs show all the data points for each test. To display multiple data points with the same numerical value, we moved the locations of symbols by adding noise to the *X* coordinate of each data point. Noise was generated from a uniform distribution on a small interval centred on zero (Cleveland 1993). Horizontal lines represent median values.

pattern (Worden et al. 2000). After a 5-min acclimation period, we removed the plastic dish restraining the male and the trial began. Each trial lasted 10 min, during which we recorded both the number of contacts and the time the male spent in contact with each piece of filter paper.

Data Analysis

As we could not assume that data were normally distributed, we used two-tailed nonparametric statistical tests. To test for male mate choice, we used the sign test for paired measures (Siegel & Castellan 1989). Other comparisons were conducted with the Wilcoxon signed-ranks test for paired replicates (Siegel & Castellan 1989). All tests were corrected for experimentwise error rate using the sequential Bonferroni method described by Holm (1979). Although uncorrected values are given in the results, those tests reported as significant remained so after applying the Bonferroni correction.

RESULTS

Male Mate Choice Tests

Males presented simultaneously with mature and immature females mated only with mature females in all the tests (sign test: $k = 0$, $N = 19$, $P < 0.001$). Males also spent significantly more time courting mature than immature females (Wilcoxon test: $Z = 2.52$, $N = 21$, $P = 0.012$; Fig. 1a). A similar trend was observed for the number of courtship sequences directed to each female. However, because of the significant correlation between this variable and time spent courting (Spearman correlation: $r_S = 0.789$, $N = 42$, $P < 0.001$), we used only time spent courting in our analyses.

When given a choice between virgin and mated females, males mated significantly more often with virgin females (sign test: $k = 4$, $N = 20$, $P = 0.012$). Two males mated with both females and their data were not used in the analysis. The time that males allocated to courtship was also significantly higher with virgin females (Wilcoxon test: $Z = -3.864$, $N = 22$, $P < 0.001$; Fig. 1b). Finally, there were no differences in mate choice (sign test: $k = 8$, $N = 20$, $P = 0.252$) or time spent in courtship (Wilcoxon test: $Z = -0.710$, $N = 20$, $P = 0.478$) when males were given a simultaneous choice between familiar and unfamiliar females (Fig. 1c).

The higher proportion of males mating with virgins could be an artefact caused by virgin females being more receptive than mated females. Although we could not directly test for this possibility, we did verify that the proportion of unsuccessful copulation attempts (i.e. copulation sequences that were interrupted by the female) did not differ between mature virgin females (from the mature versus immature test) and mature mated females (pooled from the familiar versus unfamiliar test; *G* test: $G_{\text{adj}1} = 0.092$, $P > 0.7$).

Odour Preference Tests

Males spent significantly more time in contact with filter paper squares bearing chemical cues from virgin females than with those bearing chemical cues from mated females (Wilcoxon test: $Z = 2.94$, $N = 39$, $P = 0.003$; Fig. 2a). Although a similar trend was observed in the number of contacts with each type of square, only time in contact was used in the analysis of data from odour preference tests, since these two variables were correlated (Spearman correlation: $r_S = 0.758$, $N = 78$, $P < 0.001$). Males also spent significantly more time in contact with squares bearing chemical cues from mature than from immature females (Wilcoxon test: $Z = 3.16$, $N = 18$, $P = 0.002$; Fig. 2b).

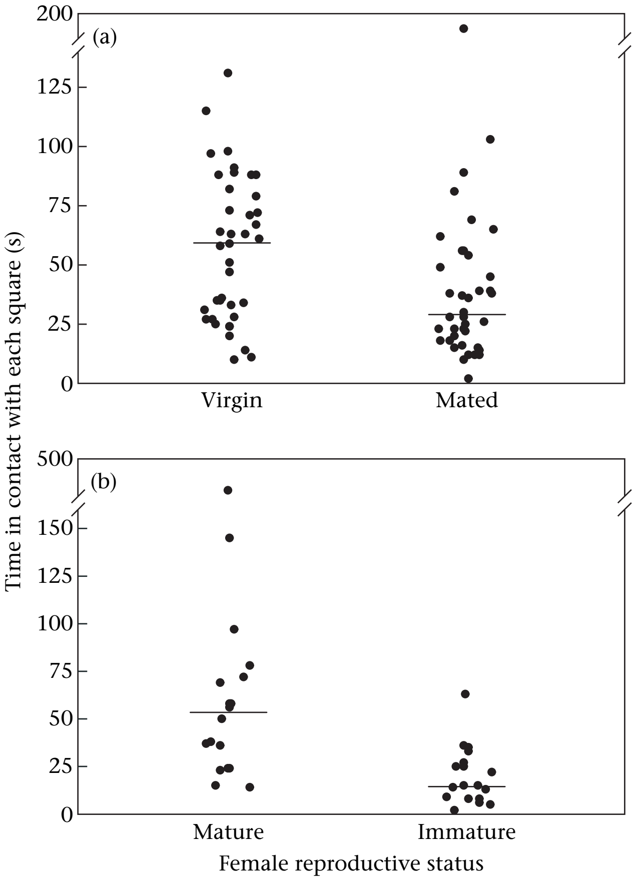

Figure 2. Time that males spent inspecting square filter papers bearing chemical cues from (a) virgin versus mated females, and (b) mature versus immature females. For details, see legend to Fig. 1.

DISCUSSION

Male Mate Choice

The results of the male mate choice tests suggest that male *T. molitor* beetles prefer mature to immature females. In polygynandrous species, mating typically occurs after a period of maturation during which the oocytes grow (Ridley 1989). Thus, immature females tend to have underdeveloped oocytes, which may curtail their fecundity. Newly emerged female mealworm beetles do not reach sexual maturity until approximately 5 days posteclosion (Valentine 1931; Happ & Wheeler 1969). Hence, by avoiding recently emerged females, those *T. molitor* males that discriminate in favour of mature females are likely to benefit by selecting the better (i.e. more fecund) mates.

Although immature females are capable of mating (Drnevich et al. 2001; Drnevich 2003), they seem to be less receptive than mature females (personal observation). Therefore, the finding that males mated exclusively with mature females may be explained by a combination of male mate choice and female receptivity effects. However, males also devoted disproportionate courtship to mature females. In *T. molitor* mating interactions, female resistance to mate (i.e. reduced receptivity) usually gives rise to prolonged courtship by the male (personal observation). Thus, the fact that males courted mature females more than immature females does not seem to be a consequence of reduced receptivity of immature females. On the contrary, this evidence suggests that *T. molitor* males are more attracted to mature females.

Our results further suggest that males prefer to mate with virgin females. *Tenebrio molitor* beetles have a polygynandrous mating system with a potential for intense sperm competition (Drnevich et al. 2000, 2001; Worden & Parker 2001). Because second-male sperm precedence in *T. molitor* is complete only if the remating intervals are very short (Drnevich et al. 2000; Drnevich 2003), males mating with nonvirgin females may have to share fertilizations with other males, and the ensuing sperm competition will tend to lower the fertilization success of nondiscriminating males (Simmons & Siva-Jothy 1998). Thus, female mating status can be crucial to male mealworm beetles, whose reproductive success will rest partly on their ability to choose virgin females, which minimize the risk of sperm competition (Simmons et al. 1994; Lewis & Iannini 1995).

Copulating with a previous mate may be ineffective, since males could be gaining precedence over their own ejaculate. In accordance with this hypothesis, Arnaud & Haubruge (1999) found that *Tribolium castaneum* males prefer unfamiliar (i.e. previously inseminated by other males) to familiar females (with which they have previously mated), a phenomenon known as the ‘Coolidge effect' (Dewsbury 1981; Wyatt 2003). Although we predicted a similar preference in *T. molitor*, we did not find evidence for a Coolidge effect, as males did not prefer unfamiliar to familiar females. Mating repeatedly with the same female has also been described as a way to ensure sperm precedence by facilitating dilution or displacement of rival sperm (Danielsson 1998; Simmons 2001). Recent evidence suggests that dilution of rival sperm may be an important mechanism of sperm competition in *T. molitor* (Gage & Baker 1992; Drnevich 2003). The benefits obtained by dilution of rival sperm could thus compensate for the costs associated with mating repeatedly with the same female, but we currently lack the data required to assess their relative contributions in *T. molitor*.

The Role of Chemical Cues

The results of the odour preference tests provided support for our prediction that male *T. molitor* are able to assess female reproductive status from chemical cues alone. In particular, males discriminated in favour of pieces of filter paper bearing chemical cues from virgin females over those bearing chemical cues from mated females. Previous studies have proposed the existence of a male-produced volatile antiaphrodisiac that would act by deterring other mealworm males from attempting copulation with a recently mated female (Happ 1969). The existence of such an antiaphrodisiac could explain male preferences for virgin chemical cues in *T. molitor* (see also Lewis & Iannini 1995). However, in our experiment odour donor females were allowed to label pieces of filter paper over a 24-h period preceding each odour preference trial, during which time they had no contact with males. It seems unlikely that, after such long delays, a volatile antiaphrodisiac could be responsible for male assessment of female reproductive status. Furthermore, the proposed male-produced antiaphrodisiac has not yet been isolated or identified, and recent evidence argues against its existence (Seybold & Vanderwel 2003).

An alternative hypothesis involving changing levels of female-produced chemical cues is more consistent with our results. In *T. molitor*, two sex pheromones act synergistically to trigger male mating behaviour: a nonvolatile pheromone present in both sexes, but more abundant in females, and a female-produced volatile pheromone identified as 4-methyl-1-nonanol by Tanaka et al. (1986, 1989). Growing evidence indicates that mating can affect pheromone production in insects (Ramaswamy et al. 1994), and a few studies have addressed this phenomenon in *T. molitor* (Happ 1969; Happ & Wheeler 1969; Seybold & Vanderwel 2003). Seybold & Vanderwel (2003) reported a transient decrease in the 'copulation release' (CR) activity of extracts (including volatile and nonvolatile compounds) from females that had just mated. Gas chromatographic analyses indicated that the decrease in CR activity was accompanied by a drop in 4-methyl-1-nonanol levels from 40 ng per individual in virgin females, to below levels of detection 12 h after mating (Seybold & Vanderwel 2003). Given that the presence of 4-methyl-1-nonanol is crucial to male mating behaviour, it seems likely that a drop in this sex pheromone is the cause of reduced CR activity in extracts from mated females. In many insect species, females stop producing sex pheromones (pheromonostasis) after mating (Ramaswamy et al. 1994; Ando et al. 1996). We propose that a similar mechanism may be at work in *T. molitor*, the reduced attractiveness of mated females being caused by a reduction in 4-methy-l-nonanol levels.

The results from the mature versus immature odour preference test also suggest the existence of a chemically based discrimination mechanism underlying male preferences for mature females. Pheromone emission in *T. molitor* virgin females is highly dependent on maturation: mature virgin females emit considerably more 4-methyl-1-nonanol than immature virgin females (Valentine 1931; Happ & Wheeler 1969). Those females with increased production of 4-methyl-1-nonanol are likely to be more attractive to males; if so, this would explain the observed male preferences for mature female chemical cues. Thus, the available evidence suggests that changes in the production of 4-methyl-1-nonanol, alone or as part of a pheromone blend (Johnston 2000), may explain patterns of male mate choice in the mealworm beetle.

To conclude, our results strongly suggest that male *T. molitor* beetles have evolved the ability to use female-produced chemical cues as an indicator of female reproductive status (i.e. maturity and virginity). That males find mature females more attractive than immature ones may benefit both mature females (e.g. via mate attraction) and immature females (e.g. via reduced sexual harassment). However, male preference for virgin females (or their chemical cues) is, at least from the female's point of view, intriguing. Given that both virgin and previously mated *T. molitor* females apparently benefit from mating with multiple males (Drnevich et al. 2001; Worden & Parker 2001), it is not obvious that signalling their reproductive status is in the females' best interests (Svensson 1996). Thus, our results provide evidence not of 'true communication' (i.e. where information benefits both sender and receiver), but of specialization restricted to the receiver, also termed 'spying' or 'eavesdropping' (Bradbury & Vehrencamp 1998; Sorensen & Stacey 1999; Wyatt 2003). Future studies should address these issues to gain a more complete understanding of the selective forces shaping chemical communication in *T. molitor* and in other beetles.

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