Do male guppies distinguish virgin females from recently mated ones?

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In some mating systems males should benefit from mating with virgin females because of their higher reproductive value. We determined experimentally whether and how males distinguish between virgin and recently mated females in the guppy, *Poecilia reticulata*, a promiscuous livebearer. In a free-swimming experiment, males showed flexible mating behaviour by adjusting their tactics according to the mating status of the female they encountered, virgin or mated. Males followed, nipped and copulated with virgins more than with mated females, but they performed more sneaky copulations with mated females, possibly because the latter were more reluctant to mate than virgin females. When, in another set of experiments, males received only the visual cues of both virgins and mated females they showed no preference for either, but when they were exposed only to the female olfactory cues, they associated considerably more with the smell of virgin females. These results suggest that male guppies assess female behavioural and olfactory cues to determine female virginity and then use different mating tactics depending on the female's status. It is possible that the changes in male mating behaviour increase male reproductive success.

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The traditional view of the sex roles (Darwin 1871), in which females are the choosier sex and males mate indiscriminately, has changed markedly during the last decade (Kokko & Jennions 2008) and research has also started to focus on the choosiness of males (Bonduriansky 2001). Male mate preference for specific female phenotypes should evolve when there is variation in female reproductive value and the costs of mate discrimination are low (Andersson 1994). Empirical and theoretical studies have confirmed this and demonstrated male mate choice, even when the sex roles are not reversed (Dewsbury 1982; Bonduriansky 2001; Servedio & Lande 2006). In an effort to maximize their reproductive success, males often prefer more fecund females (Sargent et al. 1986; Erlandsson & Johannesson 1994; Cuadrado 1998; Bonduriansky 2001; Jones et al. 2001). However, another important determinant of female reproductive value is their receptivity and mating status: males that spend time courting and pursuing pregnant or nonreceptive females may incur energetic or reproductive costs (Kelso & Verrell 2002). Additionally, polyandry can affect male reproductive success by reducing the female fecundity accessible to each male (Wedell et al. 2002). Consequently, males are expected to discriminate between females based on their mating status.

Across species, males use a variety of cues to assess female quality, usually in the form of female fecundity and mating status (Andersson 1994; Bonduriansky 2001). Fecundity in females is frequently correlated with phenotypic traits such as size (Roff 1982; Honek 1993). In addition, mating status and receptivity are revealed by specific cues such as body coloration (Galan 2000), olfactory cues (Miranda et al. 2005; Friberg 2006), auditory cues (Semple & McComb 2000), behavioural signals (such as an increase in female receptivity; Engelhardt et al. 2005), and even social indicators (Dosen & Montgomerie 2004b).

Male preference for virgin or mated females has been observed in several different mating systems. Virgin females are preferred when they carry mature eggs ready to be fertilized (Bonduriansky 2001), when females are highly receptive (King et al. 2005), when there is a first-male advantage in sperm competition (Wedell et al. 2002; Engqvist & Reinhold 2006), and when there is a decreased chance of contracting sexually transmitted diseases (Knell & Webberley 2004). In contrast, mated females are preferred when these females are more fecund because they are older and/or larger (DeClercq & Degheele 1997), when there is last-male sperm precedence (Birkhead & Hunter 1990), and when the number of previous partners can serve as an accurate cue of female quality (Schlupp & Ryan 1997).

The best evidence of male mate choice for virgin females comes from work on a variety of invertebrates, such as spiders (Riechert & Singer 1995; Herberstein et al. 2002; Gaskett 2007) and insects (crickets: Wedell 1998; Bateman & Ferguson 2004; Thomas &

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Simmons 2007; beetles: Carazo et al. 2004; damselflies: Uhia & Rivera 2005; butterflies: Wiklund & Forsberg 1986; Wedell & Cook 1999), but has been less often described in vertebrates. Even though male vertebrates are able to evaluate certain characteristics of female mating status (Schwagmeyer & Parker 1990; Saether et al. 2001), little is known about the ability of male vertebrates to distinguish between virgin and mated females (but see Whitfield 1990) and in particular what cues this may involve.

We investigated whether male guppies, *Poecilia reticulata*, can discriminate between females on the basis of their mating status, specifically, whether males can differentiate between virgin and recently mated females and, if so, what cues they use. Male guppies attempt to inseminate females via two alternative tactics: they either court females by performing a sigmoid display followed by an attempt to copulate (i.e. courtship or solicited copulation), or they perform forced copulations by thrusting the gonopodium towards the gonopore area of the female without previously performing courtship display (sneaky copulation; Houde 1997). The probability of sperm transfer is higher in solicited copulations, but males can still gain some success via forced copulations (Pilastro & Bisazza 1999; Matthews & Magurran 2000; Evans et al. 2003). Females solicit copulations when they are receptive, that is, virgin females, virgins that have recently mated (up to 5 days) or postpartum females (i.e. during a few days after giving birth; Liley 1966; Liley & Wishlow 1974). Males are known to prefer unfamiliar (Kelley et al. 1999), larger (Dosen & Montgomerie 2004a) and nonpregnant females (i.e. females with immature, early yolked and mature eggs (stages 1–3); Ojanguren & Magurran 2004). Additionally, male guppies prefer the sexual pheromone produced by postpartum females over the odour of mid-cycle pregnant females (Crow & Liley 1979), but it is not known whether virgin females also secrete this sexual pheromone. Furthermore, it is not known whether males can detect whether a female has previously mated or not. Many studies of invertebrates have shown that males are able to identify virgin females, but there is less evidence for vertebrates (Bonduriansky 2001). Given that female guppies have a distinct receptivity cycle (Houde 1997) and because virgins are always receptive to males (Liley & Wishlow 1974), males could benefit by identifying virgin females and attempting courtship copulations with them, rather than courting choosier mated females. Guppies therefore provide an excellent opportunity to increase the studies of vertebrates that test whether males are able to recognize and respond differentially to females based on their mating status.

We tested the hypothesis that males adjust their mating behaviour depending on whether the female they encounter is virgin or has recently mated (henceforth 'mated females'). First, we allowed males access to virgin and mated females in a free-swimming experiment where they received all the cues from the females, and we recorded male and female behaviour. Here we predicted that males should court and copulate with virgin females more than mated females, and, if mated females are less receptive, males should attempt forced copulations with them more than with virgin females. In a second set of experiments, we measured male preference for visual or olfactory cues from virgin and mated females independently in dichotomous choice trials. Since female guppies secrete a sexual pheromone when they are receptive (Crow & Liley 1979), we predicted that males would prefer the olfactory cues from virgin females and that visual cues would be less important in this discrimination.

METHODS

Population and Fish Maintenance

We used wild-type descendants of a population from Trinidad kept at the University of Leicester Botanical Gardens since the late 1970s. It is not known which population the fish came from in Trinidad. Fish were raised under laboratory conditions in a recirculation system at the Department of Animal and Plant Sciences, University of Sheffield, where they were maintained at 27 °C under a 12:12 h light:dark photoperiod and fed twice a day ad libitum with live brine shrimp naupilii (*Artemia salina*) and commercial flakes. Water used in the experiments was dechlorinated, aerated and kept at the same temperature as the stock aquaria. An 18 W natural daylight fluorescent tube (Arcadia, Croydon, U.K.) with a spectral composition of 320–730 nm provided even lighting for all trials. Experimental fish were randomly chosen from the population, did not have experience in previous experiments and were used only once. After the trials, the fish were used as breeding stock. All procedures were conducted under Home Office licence.

Virgins and Mated Females

Females were visually isolated from males at the first sign of maturity (i.e. presence of dark coloration in gonopore area; 4–6 weeks old); however, females received olfactory cues from both females and males because of the recycling system where they were housed. Virgin females were housed in groups of 15 ± 3 females and they had visual access to virgin females in adjacent tanks. Once mature (ca. 5–7 months old), isolated females were randomly assigned to one of two treatment groups: virgin or mated. Female guppies show a well-defined reproductive cycle, which is linked to female receptivity: pregnant nonreceptive females become receptive to males after giving birth (Liley 1966); 3 days after parturition, receptivity drops sharply and females avoid males again (Liley 1966). Virgin females are always receptive in their initial encounters with males, but after a few days of continuous exposure to males (even without insemination), receptivity drops and females show the cycle of pregnant females (Liley & Wishlow 1974).

Females in both treatment groups were stimulated by males over a 2-day period. For the virgin group, a batch of five females was placed next to 10 males isolated by a glass partition, allowing females to watch males courting but preventing copulation. We used water that had previously contained males, so females also had access to male pheromones. For the mated group, five females were placed in a tank with 10 males where they could interact and mate. As a result, both virgin and mated females were receptive on the third day (Liley & Wishlow 1974), when the experiments, or the collection of cues, took place (see below). Virgin and mated females were the same age, similar in size (we used pairs of females that did not differ in length by more than 0.3 mm), and were raised under the same conditions. Following their use in experiments, we isolated virgin and mated females for 2 months to record parturition. Of 64 mated females used in this study, 58 produced offspring, and none of the virgin females produced offspring.

Free-swimming Experiment

To test whether males distinguish between virgin and recently mated females and adjust their mating tactics accordingly, we used a free-swimming experimental design in which we allowed a single male to court two females, one virgin and one mated. Virgin and mated females were paired according to size (standard length SL; the pair of females did not differ by more than 1 mm) and marked by clipping their caudal fin: in one female the fin was clipped dorsally, whereas in the other it was clipped ventrally. The clipping style was alternated, so that over the course of the experiment the same number of virgin and mated females were clipped in both ways. Once females were clipped, they were monitored and allowed to recover for 2 days before being used in trials. No females showed any signs of distress as a result of this procedure.

Trials were conducted in a tank (33 × 18 cm and 18 cm high) filled to a depth of 15 cm with water and covered on three sides with green opaque cardboard. We placed a video camera (Sony DCR-TRV245E) in front of the exposed side of the tank to record the trials. In each trial, we placed one male and a pair of females (i.e. one virgin and one mated) into the tank. We recorded male mating behaviour towards each female during 1 h from the time when the male first courted one of the females. For males we recorded: (1) time following: the time that the male spent at one body length from each female; (2) time courting: the duration of all the sigmoid displays directed to each female; (3) nipping behaviour: the number of times the male directed his mouth to each female's gonopore area, which may be an indication of the male smelling the female (Herdman et al. 2004); (4) the number of copulation attempts: male approaching a female immediately after courting; (5) the number of successful copulations (i.e. with transmission of sperm): considered as male contact with a female followed by characteristic male body jerks; and (6) the number of forced mating attempts: gonopodial thrusts to the gonopore area of a female without courtship. Female behaviour was also recorded, in terms of receptivity and fleeing behaviour. Receptivity in female guppies is expressed in terms of the 'glide' behaviour, where the female swims using only her pectoral fins to move in a smooth motion (Liley 1966). We used gliding response (number of glides/total number of times she was courted) to quantify female receptivity. Female fleeing behaviour was quantified as the proportion of times a female rejected a male attempting to copulate, specifically when females fled in response to an approaching male immediately after courting. A more detailed description of the reproductive behaviour of the guppy can be found in Liley (1966). We tested 19 males (mean SL ± SE = 20.04 ± 0.33 mm), with 19 virgins (SL = 27.14 ± 0.19 mm) and 19 mated females (SL = 27.03 ± 0.18 mm). Virgins and mated females did not differ in size (paired t test: $t_{35} = 0.41$, $P = 0.68$).

Visual Experiment

To examine whether males use visual cues to identify virgins, we paired females of similar size; half of them were assigned to the virgin treatment and half to the mated treatment. Trials were conducted in a tank (48 × 19 cm and 22 cm high) with two sealed glass compartments (10 × 19 cm and 20 cm high) at both extremes. The rest of the tank was divided into two preference zones, marked by a line drawn at 5 cm from each compartment, and one middle neutral zone (18 cm). The tank was covered on three sides by green opaque cardboard and data were recorded by a single observer hidden behind a screen that was placed in front of the open side of the tank.

A virgin and a mated female were each placed in one of the two glass compartments, and the side the virgin was on was alternated between trials. The male was placed within a cylinder (20 cm high, 7 cm in diameter) in the centre of the neutral zone and after a 10 min acclimation period for both male and females, the cylinder was removed and recording began. We recorded the time the male spent in each preference zone over a 10 min period. The position of the females was then reversed and recording continued for another 10 min, so that each male was recorded for a total of 20 min. Females did not show receptive behaviour during the trials and they did not differ in size (virgin females: SL = 19.98 ± 0.32 mm; mated females: SL = 20.17 ± 0.31 mm; paired t test: $t_{47} = -0.42$, $P = 0.67$). A total of 25 males were tested (SL = 17.27 ± 0.25 mm).

Olfactory Experiment

To examine whether males use olfactory cues to discriminate between virgin and mated females, we collected female olfactory cues from 5-litre tanks holding five females (virgins or mated). We kept the females in the tank for 24 h, where they had visual contact with five males in an adjacent tank. After that period, we took the females out of the tank and the holding water was used as the olfactory cue in the trials; trials were conducted the day females were removed from the holding water. Fish were not fed during the period of olfactory cue production, but were fed before and after. We produced four replicates of olfactory cue per treatment, using four different batches of five virgin females and four different batches of five mated females (i.e. 20 females per treatment). Virgin (SL = 20.01 ± 0.30 mm) and mated females (SL = 20.17 ± 0.32 mm) did not differ in size (paired t test: $t_{37} = -0.36$, $P = 0.71$).

Trials were carried out in a tank (36 × 22 cm and 21 cm high) that was divided by external lines into three 12 cm zones: two preference zones at the extreme ends of the tank and one middle neutral zone (Fig. 1). All the tank sides were covered by green opaque cardboard and a video camera (DCR-TRV245E), placed 60 cm over the experimental tank, was connected to a monitor away from the tank where the data were recorded. The olfactory cues were kept in plastic containers set 40 cm above and next to both ends of the experimental tank (Fig. 1). Each container had a tube connected to its base, and at the end of this tube was a tap that could be adjusted to allow the olfactory cues to flow into the tank at a rate of 2 ml/min. The rate at which the olfactory cues flowed was checked at the beginning of each trial. In each trial one male was placed into the neutral zone and allowed a 5 min acclimation period; following this we introduced the olfactory cues into the tank and recorded male behaviour. We recorded the total time that the male spent in each preference zone over a 10 min period. Tanks were cleaned thoroughly with 98% alcohol and dechlorinated water and filled again and the trial was repeated with the same fish but with the position of the olfactory cues reversed. We found no evidence that the cues remained after we changed their position. A total of 37 males (SL = 17.11 ± 1.20 mm) were tested.

Statistical Methods

In the olfactory and visual experiments we pooled the data from before and after reversing the position of the stimuli. We used Student's t tests and Wilcoxon signed-ranks tests according to whether the data were normally or non-normally distributed, respectively, to analyse the differences in male behaviour in
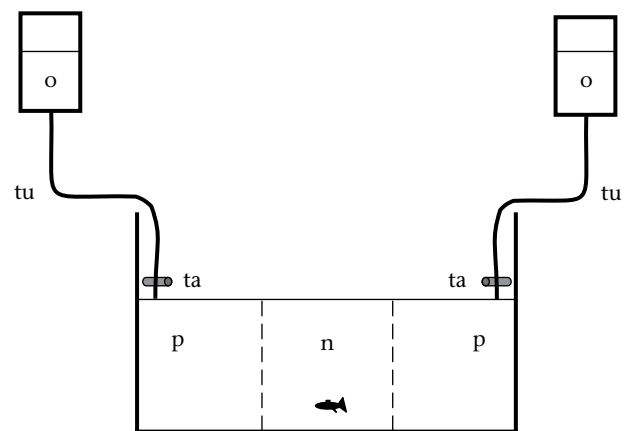

Figure 1. Experimental set-up used in the olfactory experiment. The experimental tank was divided into two preference zone (p) and one middle neutral zone (n). The olfactory cues were kept in individual containers (o), each with a tube (tu) connected to its base, and at the end of this tube was a tap (ta) that could be adjusted to allow the olfactory cues to flow into the tank. The focal male (here in black) was placed in the middle neutral zone at the beginning of the trial.

response to virgin and mated females. All tests were two tailed. In the free-swimming experiment, the number of copulations received by virgin and mated females was compared using a binomial test, and a logistic regression was used to analyse the effect of female reproductive status (virgin or mated) on female fleeing behaviour. We used the program R (R Foundation for Statistical Computing, Vienna, Austria) to analyse our data.

RESULTS

Free-swimming Experiment

Although males courted virgin and mated females at a similar rate (Student's t test: $t_{18} = 1.46$, $P = 0.16$; Fig. 2a), certain behaviours were used at different frequencies towards the two types of females (Fig. 2b–f). Males followed (Wilcoxon signed-ranks test: $T = 155.0$, $N = 19$, $P = 0.008$; Fig. 2b), nipped (Student's t test: $t_{18} = 2.62$, $P = 0.02$; Fig. 2c), attempted copulations (Student's t test: $t_{18} = 3.46$, $P = 0.003$; Fig. 2d) and copulated with (binomial test: $P = 0.006$; Fig. 2e) virgin females more than mated females. However, males performed more forced copulation attempts with mated females than with virgin females (Wilcoxon signed-ranks test: $T = 10.5$, $N = 18$, $P = 0.01$; Fig. 2f). Virgin and mated female receptivity, in terms of gliding response, did not differ during the experiment (Student's t test: $t_{18} = -1.30$, $P = 0.21$; Fig. 3a). However, females varied in their fleeing behaviour: mated females rejected males' attempts to copulate more often than virgin females (logistic regression: $F_{1,33} = 13.66$, $P < 0.001$; Fig. 3b).

Visual Experiment

There was no significant difference in the amount of time that males spent in the preference zone next to virgin females compared to mated females (Student's t test: $t_{24} = 0.78$, $P = 0.44$; Fig. 4a).

Olfactory Experiment

Males spent significantly more time associating with the olfactory cues of virgin females than mated females (Student's t test: $t_{36} = 3.13$, $P = 0.003$; Fig. 4b).

DISCUSSION

Males are likely to reduce reproductive costs by discriminating between females based on their mating status (Andersson 1994). In this study we tested whether male guppies could discriminate between virgin and recently mated females and whether they modified their behaviour according to female mating status. Male sexual behaviour differed towards these two groups of females: males not only followed virgin females more, but they also nipped, attempted copulations and copulated more often with them. Mated females, on the other hand, were the main target of forced mating attempts, possibly because these females were more resistant to male copulation attempts, and therefore less likely to respond to male courtship, than virgins. The plastic mating behaviour of males suggests that they can discriminate between females with differing mating status and differing reproductive value to the males
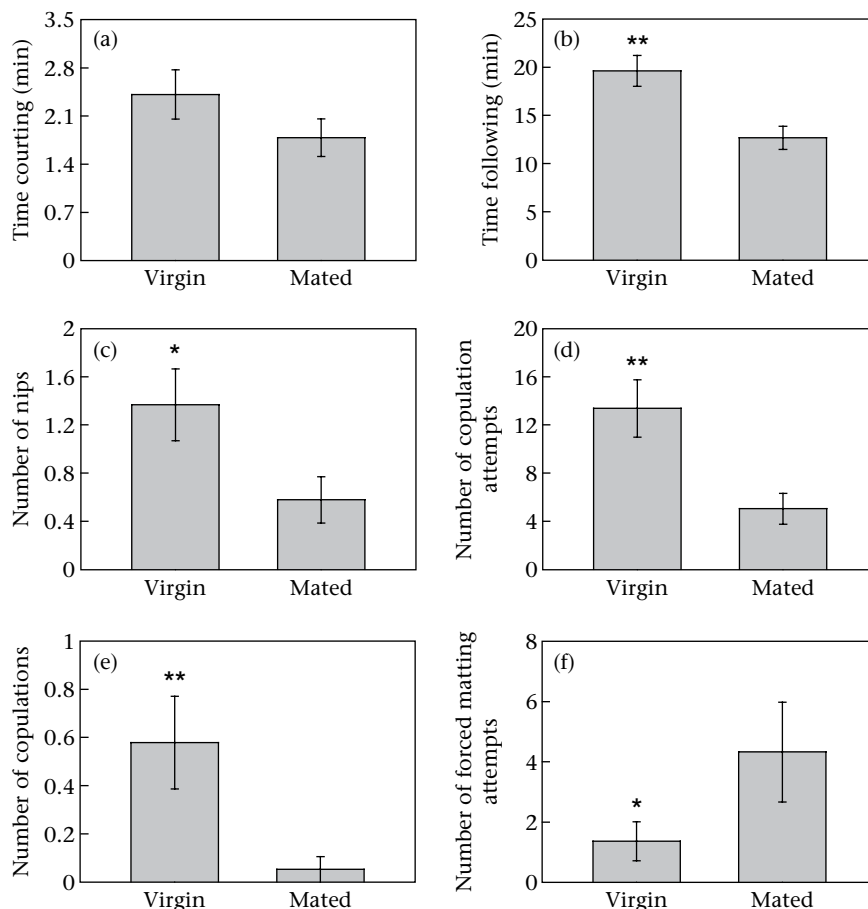

Figure 2. Mean ± SE male mating behaviours towards virgin and mated females during the free-swimming experiment ($N = 19$). (a) Duration of courtship (sigmoid display), (b) time spent following females, (c) number of times the male nipped the females, (d) number of copulation attempts, (e) number of copulations, (f) number of forced mating attempts (gonopodial thrusts). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.
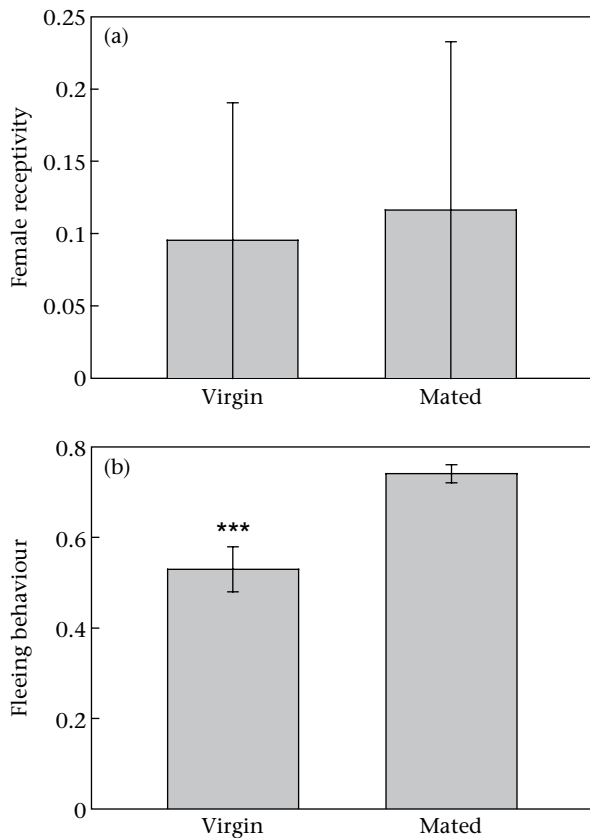
Figure 3. Mean ± SE female mating behaviours recorded in the free-swimming experiment (*N* = 19 per treatment). (a) Female receptivity measured as proportion of times a female accepted the male's courtship and (b) female choosiness measured as the number of times the female fled as the male approached. **P < 0.01.

Figure 4. Mean ± SE time that males spent associating with the (a) visual (*N* = 25) or (b) olfactory (*N* = 37) cues from virgin or mated females in a choice test. **P < 0.01.

(i.e. virgin and mated females). Similarly, Hibler & Houde (2006) also found that male guppies behave differently towards recently mated, receptive females and pregnant, nonreceptive females presented in succession to groups of males. In their study, they found that males displayed significantly less often to recently mated females than to pregnant females. Hibler & Houde (2006) suggested that this decrease in courtship rate was caused by males increasing the time spent following the receptive females and displaying to them more selectively. Our experimental design differs considerably from that of Hibler & Houde (2006), but it is interesting to note the difference in male response when males encounter females sequentially rather than simultaneously.

Males in our study preferentially associated with the odours of virgin females, but when odour cues were not available males did not preferentially associate with virgins. This suggests that males use olfactory cues to discriminate between females of differing mating status and visual cues may be less informative (which might be different in other species, e.g. Peden 1973). Nipping behaviour is thought to reflect male assessment of female reproductive status via olfactory cues (Herdman et al. 2004), and in the free-swimming experiment we recorded a higher rate of nipping behaviour towards virgins, providing additional support for the importance of olfactory cues during male assessment of females. The importance of female odour in male mate choice has been shown in other poeciliids, in which a male's preference is not expressed when males use solely visual cues (Brett & Grosse 1982; Sumner et al. 1994; Park & Propper 2002). Taken together, these results indicate that males alter their mating behaviour according to the mating status of the females and that olfactory, not visual, cues play an integral role in discrimination. The use of odour to recognize virgin
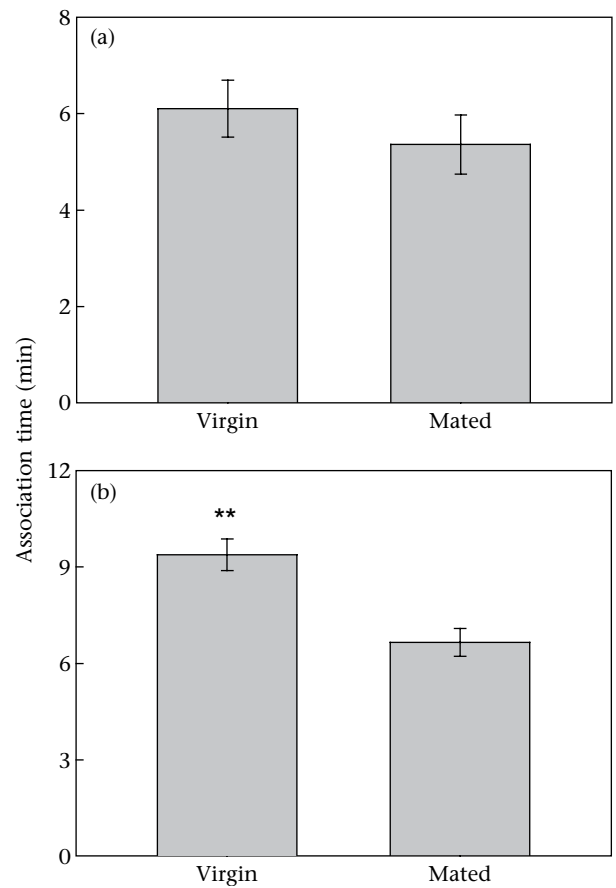
females has not, to our knowledge, been shown previously in male vertebrates.

Pheromones are known to contribute to male recognition of female mating status (Wyatt 2003). It is plausible that virgin and mated female guppies differ in the olfactory cues they produce. The secretion of a sexual pheromone in female guppies is correlated with their receptive period (Crow & Liley 1979), and this period decreases faster in mated females than in virgins that have been stimulated by male courtship, but not inseminated (Liley 1968). Therefore, it is possible that the male preference for virgin odour we found is attributable to a rapid decline in the production of a sexual pheromone after mating, as is the case in some insects (Tompkins & Hall 1981; Ayasse et al. 1999, 2001; Wedell 2005; Stoltz et al. 2007). Alternatively, females may change their pheromone composition after copulation, such as in the ground-nesting bee, *Andrena nigroaenea*, where females' odour attractiveness decreases because they produce chemicals that inhibit male interest (Schiestl & Ayasse 2000).

One alternative to the idea that males use female odours to assess their mating status is that mated females may carry chemicals from the previous male, in the form of sperm or ejaculate products, and that males are actually responding to these. For example, female butterflies of some *Pieris* species have a reduced rate of remating because of an antiaphrodisiac synthesized by males and transferred to females at mating; as a result, recently mated females are unattractive to other males (Andersson et al. 2000, 2003). Female guppies sometimes eject sperm after mating (Liley 1966) and this may provide a cue of female mating status. The importance of female odours in male mate choice in the guppy makes it a model system for future

studies wishing to identify female chemicals and/or odour profiles used in male mate choice.

The ultimate finding of this study is the plasticity of male mating tactics in response to female mating status. This strategic change in mating tactics may confer important benefits to males in terms of mating success. One benefit they might get is a reduction in energy and time expenditure by matching their mating behaviour to the female's mating status: if mated females are less likely to engage in copulation, males should detect such females and adjust their courtship strategies accordingly. However, if females are receptive and mate indiscriminately, as is the case for virgin females (Liley & Wishlow 1974), males would benefit by preferentially courting and copulating with them. Although males can forcibly copulate with females, insemination is more likely when females solicit copulations (Liley 1966; Luyten & Liley 1991) and, by mating through courtship copulations rather than forced copulations, males may appear more attractive to other females (Witte & Ryan 2002; Godin et al. 2005). Based on all these arguments, we suggest that males that detect receptive, virgin females could increase their reproductive success by directing courtship efforts towards them.

In conclusion, our results suggest that male guppies display plasticity in their mating behaviour in accordance with the mating status of the females they encounter. They use predominantly olfactory cues to discriminate between mated and virgin females and modify their mating tactics accordingly. This change in mating tactics is likely to increase male mating success and reduce the potential costs of attempting solicited copulations with non-receptive females.

Acknowledgments

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