Male mate-searching strategies and female cues: how do male guppies find receptive females?

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Locating potential mates is critical to mating. We studied males' association with females and mate-searching patterns in the guppy, *Poecilia reticulata*, a promiscuous live-bearer. In the field, we examined whether male guppies respond differently to a shoal of conspecific fish based on the members of the shoal. We found that more males were attracted to shoals that contained receptive females than to shoals of nonreceptive females or males. We also conducted laboratory experiments to investigate how males use olfactory cues of nonreceptive and receptive females to search for and associate with females. We gave males the option to associate with nonreceptive females when olfactory cues of receptive or nonreceptive females were present and absent, and when olfactory cues were presented alone. Males associated with females most strongly when both cues were presented simultaneously, but when cues were presented separately males' association with females differed with respect to the olfactory cues that were added. Males associated with females equally with visual and olfactory cues presented separately when the odour cues were from receptive females. However, when the odour cues were from non-receptive females, males associated with females less with olfactory than visual cues. Searching activity increased when males had access only to olfactory cues. Taken together these results suggest that olfactory cues influence males' association with females and searching behaviour, and these changes in behaviour are likely to maximize a male's opportunity to encounter receptive females.

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Mate searching has broadly been observed in males rather than females (Andersson 1994). This disparity in searching effort between the sexes seems to be related to high multiple mating rates, and consequently high sperm competition, and a biased operational sex ratio rather than a difference in reproductive investment between the sexes (Kokko & Wong 2007). Finding potential mates is an essential component of a male's reproductive success. Males allocate a considerable amount of time searching for mates (Bondurianksy 2001) and by increasing mate-searching effort, males augment the probabilities of finding females and increase their mating opportunities (Real 1990). However, mate searching is costly not only in terms of time but also in terms of an increase in energy expenditure (Proctor 1992; Byers et al. 2005)

and it can increase male mortality as well (Gwynne 1987; Andrade 2003; Kasumovic et al. 2007; Kraus et al. 2008). For example, higher mate-searching activity by male wolf spiders, *Hygrolycosa rubrofasciata*, which increases their probability of finding females, also increases their risk of predation (Kotiaho et al. 1998). Variation in the costs of mate searching has been found to be an important selective agent in many systems. For instance, male searching effort varies with predation risk and males experiencing a higher risk of predation spend less time searching for females (DeRivera et al. 2003). Population density and sex ratio can also influence male mate-searching behaviour. This is the case for males of the fish ectoparasite *Argulus coregoni*, which stay in their host when there are females available, but when no females are present they tend to switch hosts (Bandilla et al. 2008). Indeed, at lower densities or when sex ratios are male biased, males are less likely to encounter females and alter their behaviour accordingly (Lawrence 1987; Carroll 1993; Mathews 2002; DeRivera et al. 2003; Bertin & Cezilly 2005; Kokko & Wong 2007).

In the face of the considerable costs of mate searching, males should prioritize their effort to find receptive females and avoid wasted effort on nonreceptive females (Real 1990). Discrimination between females then represents an important component of male

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fitness, and as a result many different discrimination mechanisms have evolved in males (Bonduriansky 2001). Most commonly, males use visual and/or odour cues to identify and find receptive females (Dunham 1978; Vane-Wright & Boppre 1993; Ayasse et al. 2001; Shine & Mason 2001; Diaz & Thiel 2004; Nahrung & Allen 2004; Aldridge et al. 2005; Head et al. 2005; Paxton 2005; Spiewok et al. 2006; Gaskett 2007; Toshova et al. 2007), but in some cases male searching involves integration of sensory mechanisms with highly developed memory (Wcislo 1992; Schwagmeyer 1995). Although animals use several cues during mate selection (Candolin 2003), some cues might play specific roles depending on the context in which they are used. For example, male garter snakes, *Thamnophis sirtalis parietalis*, differentiate females from males by means of dimorphic traits such as size, body temperature, muddiness and aggregation with conspecifics (Shine & Mason 2001). However, particular sensory modalities are used differently depending on the conditions in which females are found: when females are part of a mating ball males use principally odour cues to recognize them, but in a solitary context males rely mainly on visual cues (Shine & Mason 2001).

Despite the potential importance of accurate assessment of female receptivity to a male's reproductive success, there is little empirical evidence of male discrimination between receptive and nonreceptive females in natural conditions and how female cues influence male movement between groups of females. In the present study we examined male recognition of receptive females and the role of female olfactory cues in mate-searching strategies in the guppy, *Poecilia reticulata*, a small live-bearing fish with strong male-biased operational sex ratio (OSR) caused by asynchrony in female receptivity. Only virgin and postpartum females (i.e. females that have just given birth) are receptive to males (Liley 1966); pregnant females, in contrast, are nonreceptive and ignore males' continuous attempts to copulate (Liley 1966). Although considered a priori indiscriminate, males do exert mate choice: they prefer larger (Dosen & Montgomerie 2004; Herdman et al. 2004), unfamiliar (Kelley et al. 1999) and nonpregnant females (Ojanguren & Magurran 2004). As in other poeciliids (Brett & Grosse 1982; Sumner et al. 1994; Park & Propper 2002), male guppies are attracted to a female pheromone produced only by receptive females (Crow & Liley 1979; Guevara-Fiore et al. 2009). Males can achieve fertilization by solicited copulations after courting females or by forcing copulations (Liley 1966), and they allocate a significant proportion of their time to these mating activities (Magurran & Seghers 1994), but they also are known to search for females constantly (Houde 1997; Griffiths & Magurran 1998; Croft et al. 2003a, b). Whereas females are likely to school with familiar individuals and show site fidelity (Griffiths & Magurran 1998), male guppies tend to switch between shoals which results in high rates of shoal encounters (Croft et al. 2003b).

In this study we used both field and laboratory trials to test whether males discriminate between shoals of conspecifics based on sex and receptivity of the members within that shoal and how olfactory cues were used during the discrimination process. In the first experiment we tested whether males in the field were differentially attracted to shoals of fish that were composed of either males, nonreceptive or receptive females and we predicted that shoals of receptive females would attract the most males. In the second set of experiments, in the laboratory, we tested how olfactory cues from receptive and nonreceptive females influenced male movement and association behaviour in the presence of a shoal of size-matched nonreceptive females that was presented between three shoals of males (see below). We allowed males to associate with the female stimulus when olfactory cues and visual cues were presented alone or in combination. First, we predicted that males would spend longer periods associating with the female stimulus

when both odour and visual cues were presented together than when either was presented alone. However, we predicted that males would associate more with a female stimulus with receptive female odour present than with only visual cues, but that there would be no difference in association behaviour when the stimuli were a female with nonreceptive odour and a female without odour (i.e. visual cues only). Our second prediction was that males would be able to localize and move towards the shoal of females faster when males have complete information, that is, when both cues are present. Our third prediction was that male search activity should increase if males can smell females but no visual cues are available to them.

METHODS

Experimental Fish

Guppies in this study were from the Upper Aripo River (Grid Reference PS 931 817) in the Northern Range mountains of Trinidad. This is a low-predation locality where guppies coexist with the gape-limited cyprinodontid fish *Rivulus hartii* (Magurran 2005). Collections were made using a one-person seine net in May 2007. Fish were captured, and transported in groups of about 50 fish in covered 20-litre buckets to the laboratory at the University of the West Indies, St Augustine, Trinidad. Care was taken during handling to ensure that fish were not stressed. In the laboratory, fish were maintained on a 12:12 h light:dark regime at an ambient temperature (ca. 25 °C). Male and female guppies were housed together in large aerated aquaria (45 × 45 cm and 120 cm deep; ca. 150 individuals per tank) furnished with natural river gravel and were fed twice daily with commercial flake food. No fish died or showed no signs of stress throughout the study. After the experiment the fish remained in the laboratory as breeding stock. The study was approved by the University of the West Indies, Trinidad and Tobago.

During the study we individually isolated 60 females to record parturition; females were placed next to each other so that they had visual contact with other fish. Females were classified as nonreceptive when they were pregnant (15 ± 1 days after giving birth) or as receptive when females were postpartum, that is, selected during the first 3 days after giving birth. Female guppies store sperm and can use it several times to fertilize a new clutch of eggs (Liley 1966). Both receptive and nonreceptive females were isolated to control for possible differences between them caused by this procedure.

Field Experiment

To determine whether male guppies prefer to associate with shoals of receptive females we conducted an experiment in the Upper Aripo River in which we recorded the number of males in proximity to an enclosure containing different groups of fish. We grouped fish in four treatments: (1) no fish (control), (2) three males, (3) three nonreceptive females, and (4) three receptive females. Four different groups of fish were used as stimulus fish in the treatments (i.e. 12 males; mean ± SE standard length, SL = 18.05 ± 0.34 mm), 12 nonreceptive females (24.36 ± 0.26 mm) and 12 receptive females (24.28 ± 0.19 mm); nonreceptive females and receptive females presented in the same pool were matched by size (±2 mm). Fish were placed in a transparent and perforated plastic bottle (9 cm diameter, 11 cm high; perforations were ca. 2 mm diameter and were separated from each other ca. 1 cm evenly distributed around the bottle), which permitted the transmission of both visual and olfactory cues and this bottle was then placed into a pool within the river. A total of 14 pools were used and pools were chosen if they contained a shoal of guppies with at least 15 individuals and the water was no deeper than 13 cm.

All four treatments were repeated in each pool and the order of treatments was randomized. To begin the experiment we placed the bottle with fish or no fish into the water and allowed a 10 min acclimation period. After this time two people, one observing and the other recording, counted males in proximity (two body lengths) to the different treatments every 10 s for 10 min. Experiments took place between 1000 and 1700 hours.

Enclosure Experiments

To identify how male guppies decide when to join and leave a shoal of females using olfactory cues we conducted two independent experiments with the same methodology. In the first experiment (the 'receptive experiment') we used female odour from postpartum receptive females, and in the second experiment (the 'nonreceptive experiment') we used the odour from the same females but when they were pregnant and nonreceptive. We conducted experiments outdoors, between 0800 and 1700 hours in large plastic pools (118 cm diameter, 20 cm high) that had a 10×10 cm grid painted on the floor. Inside each pool we placed four water-tight, glass compartments (15×10 cm and 11.5 cm deep) at equidistant positions around the edge of the pool, and an active area was delimited 10 cm around each of the compartments (Fig. 1). Three of the compartments contained cues of males and the remaining compartment contained cues of females. Each experiment consisted of a repeated measures design, in which a male received a randomized sequence of three different treatments: (1) visual cues, (2) odour cues, and (3) a combination of visual and odour cues of fish. For the 'visual' treatment, we placed three fish in each of the four water-tight compartments. In the female stimulus compartment we used nonreceptive females in both the receptive and nonreceptive experiment (receptive experiment: $SL = 18.25 \pm 0.18$ mm; nonreceptive experiment: $SL = 18.27 \pm 0.14$ mm). This was to control for variation between receptive females in their behaviour during the trials and because we were interested in testing the effect of the female pheromone rather than female behaviour. For the 'odour' treatment we collected water from a 3-litre tank that had contained three fish for 24 h and dropped this water into the experimental pool at a rate of 2 ml/s in front of the empty compartments (females:
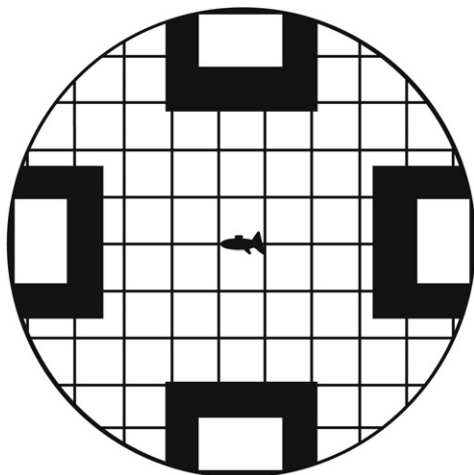

Figure 1. Experimental set-up. The circle represents the pool where the experiments took place. For simplicity, the diagram does not show the numbers in each of the individual squares of the grid. White rectangles represent the glass compartments used to isolate stimulus fish in the visual treatment. The black area around the rectangles represents the active area in which the focal male (here in the middle of the grid) was considered to be in association with the respective cues.

$SL = 24.31 \pm 0.16$ mm; males: $SL = 18.49 \pm 0.22$ mm). This method has been successfully used in smaller tanks (Crow & Liley 1979; Guevara-Fiore et al. 2009) and pilot experiments within these larger pools using dyed water showed that this was a good rate to keep a high concentration of the stimulus around the compartments. Finally, in the 'combined' treatment we used both the visual and odour stimulus at the same time as described above.

Trials began when the focal male was placed in the middle of the pool and allowed 10 min for acclimation before the cues were added (i.e. visual, olfactory or visual and olfactory cues). Then we recorded the position of the male in the grid every 10 s. Using these data, we were able to measure: (1) male association with the female stimulus, measured as the number of times a male was recorded in the active area near the female stimulus as a proportion of the total number of visits to all active areas; (2) maximum number of consecutive visits to the active area near the female stimulus as a proportion of the total time spent in the female active area; (3) whether the male visited the female active area first; and (4) male activity, measured as the number of times a male changed grid positions. We tested 20 males per experiment (40 males in total; receptive experiment: $SL = 18.25 \pm 0.18$; nonreceptive experiment: $SL = 18.27 \pm 0.14$), and each male was tested with all three treatments.

Statistical Analysis

For the field experiment, we tested the effect of treatment (bottle containing no fish, males, receptive females, nonreceptive females) on the number of males associating with the bottle using a linear mixed model. The response variable (number of males) was transformed using a Box–Cox power transformation to meet the assumptions of normality. Two random factors were included in the model: 'pool' was included to control for variation between pools in the number/composition of fish and the random factor of 'fish group inside the bottle' was used to control for any variation from different individual fish within the bottle.

For the enclosure experiments, we analysed the receptive and the nonreceptive experiments independently from each other because our objective was to compare how males' searching and association behaviour changed when visual and olfactory cues were presented in isolation and together. Additionally, because both experiments were conducted at two different periods using fish that were collected at different times, we cannot compare them directly. We used generalized linear models to analyse the effect of treatment, and we used the focal male identity as a random factor. Proportion of time spent in the active area near the female stimulus and the maximum number of consecutive visits to the female active area were tested with binomial models. The first visit to the active area near the female stimulus, and male activity (number of changes in grid position) were analysed using Poisson models.

In all models we compared treatment means using the mean difference and the 95% confidence interval, CI. If the 95% CI around the mean differences did not overlap zero we considered this evidence that there was a significant difference between the two treatment means being compared ($P < 0.05$). This approach was adopted to avoid conducting multiple post hoc t tests to compare treatment means. Also, confidence intervals are more informative than P values and hypothesis testing because they provide evidence of uncertainty around estimates and mean differences are easily translated into effect sizes which can be useful for future meta-analysis studies (Nakagawa & Cuthill 2007). All data analysis was carried out with R statistical package version 2.7.1 (R Core Development Team 2006).

Table 1
Differences between the mean number of males (and their 95% confidence intervals, CI) recorded near the enclosure that was either empty (E) or contained males (M), nonreceptive females (NR) or receptive females (R)

Comparison	Mean difference	Lower 95% CI	Upper 95% CI
E versus M	**1.1396**	**0.5809216**	**1.6982784**
E versus NR	**1.6752**	**1.1165216**	**2.2338784**
E versus R	**2.266**	**1.7073216**	**2.8246784**
NR versus M	−0.5356	−1.0942784	0.0230784
NR versus R	**− 0.5908**	**− 1.1494784**	**−0.0321216**

Bold text highlights differences between means, i.e. the mean difference and 95% CI do not overlap zero.

RESULTS

Field Experiment

The bottle containing the receptive females attracted the most males and this was more than the bottle containing nonreceptive females (Table 1, Fig. 2). The bottle containing nonreceptive females attracted similar numbers of males as the bottle containing males and the empty bottle attracted the fewest males, less than all the other treatments (Table 1, Fig. 2).

Enclosure Experiments

In the receptive experiment, males visited the female cues equally when visual and odour cues were presented separately, but visual and odour cues in combination obtained the highest number of visits from males (Table 2, Fig. 3a). However, in the nonreceptive experiment, males visited the female compartment less when odour cues were present than the other two treatments (Table 2, Fig. 3b). The different treatments (visual, odour, combined) had a similar effect on the maximum number of consecutive visits by the male to the female active area, independent of whether the odour was from a receptive or nonreceptive female (Table 2, Fig. 3c, d). Males stayed in the female area for a longer block of time when both visual and odour stimuli were present, more than visual only and least of all when odour cues were presented on their own. Males did not find the female active area faster using single cues or a combination of them (Table 2, Fig. 3e, f), and this was true for both the receptive and the nonreceptive experiment. Finally, male activity was much higher when males had access to odour cues
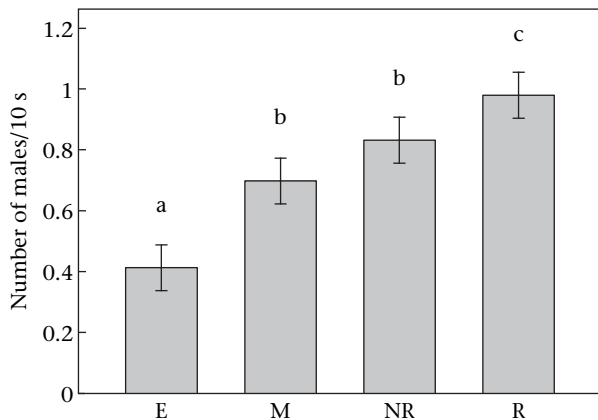
Table 2
Differences in mean male behaviour (and their 95% confidence intervals, CI) for each treatment in the receptive and nonreceptive experiments

Experiment	Comparison	Mean difference	Lower 95% CI	Upper 95% CI
Proportion of visits to female area				
Receptive	O versus V	0.02	−0.01528	0.05528
	O versus OV	**−0.13**	**−0.16528**	**−0.09472**
	V versus OV	**−0.15**	**−0.18528**	**−0.11472**
Nonreceptive	O versus V	**−0.15**	**−0.18724**	**−0.11276**
	O versus OV	**−0.17**	**−0.20724**	**−0.13276**
	V versus OV	−0.02	−0.05724	0.01724
Maximum no. of visits to female stimulus				
Receptive	O versus V	**−0.30**	**−0.36076**	**−0.23924**
	O versus OV	**−0.38**	**−0.9876**	**−0.31924**
	V versus OV	**−0.08**	**−0.6876**	**−0.01924**
Nonreceptive	O versus V	**−0.34**	**−0.4184**	**−0.2616**
	O versus OV	**−0.44**	**−0.5184**	**−0.3616**
	V versus OV	**−0.10**	**−0.1784**	**−0.0216**
First visit to female stimulus				
Receptive	O versus V	0.06	−0.2144	0.3344
	O versus OV	−0.04	−0.3144	0.2344
	V versus OV	−0.10	−0.3744	0.1744
Nonreceptive	O versus V	−0.17	−0.4934	0.1534
	O versus OV	−0.17	−0.4934	0.1534
	V versus OV	0.00	−0.3234	0.3234
Male activity (no. of changes in grid)				
Receptive	O versus V	**30.74**	**28.11262**	**33.36738**
	O versus OV	**33.04**	**30.41262**	**35.66738**
	V versus OV	2.30	−0.32738	4.92738
Nonreceptive	O versus V	**35.41**	**32.3230**	**38.4970**
	O versus OV	**44.98**	**41.8930**	**48.0670**
	V versus OV	**9.57**	**6.48300**	**12.6570**

Treatments consisted of odour only (O); visual only (V) or a combination of both (OV). Bold text highlights differences in means, i.e. the mean difference and 95% CI do not overlap zero.

only in both the receptive and nonreceptive experiments (Table 2, Fig. 3g, h). The presence of odour cues in the absence of visual confirmation of a female seemed to elicit increased search effort by males. In the receptive experiment, males' activity was the same in the presence of visual cues and a combination of both cues (Table 2, Fig. 3g), whereas in the nonreceptive experiment males reduced their activity in the presence of both visual and odour cues combined (Table 2, Fig. 3h).

DISCUSSION

In this study we investigated the association preferences and mate-searching behaviour of male guppies in the presence of receptive and nonreceptive female stimuli. The study demonstrated that males discriminate and prefer to associate with shoals of receptive females compared to nonreceptive females or males in the field. The study's findings also identified how olfactory cues from receptive and nonreceptive females influenced male association and mate-searching behaviour. Males associated with female odour cues as much as female visual cues but only when the odour was from receptive females. Males showed stronger association with females when both visual and olfactory cues were present, and this was independent of whether the olfactory cues were from receptive or nonreceptive females. Males did not localize a shoal of females faster using one specific cue (visual or olfactory cues) or the combination of both cues (visual and olfactory cues), and male searching effort increased when they had access to female odour (both of receptive and nonreceptive females) but no visual cues were available.
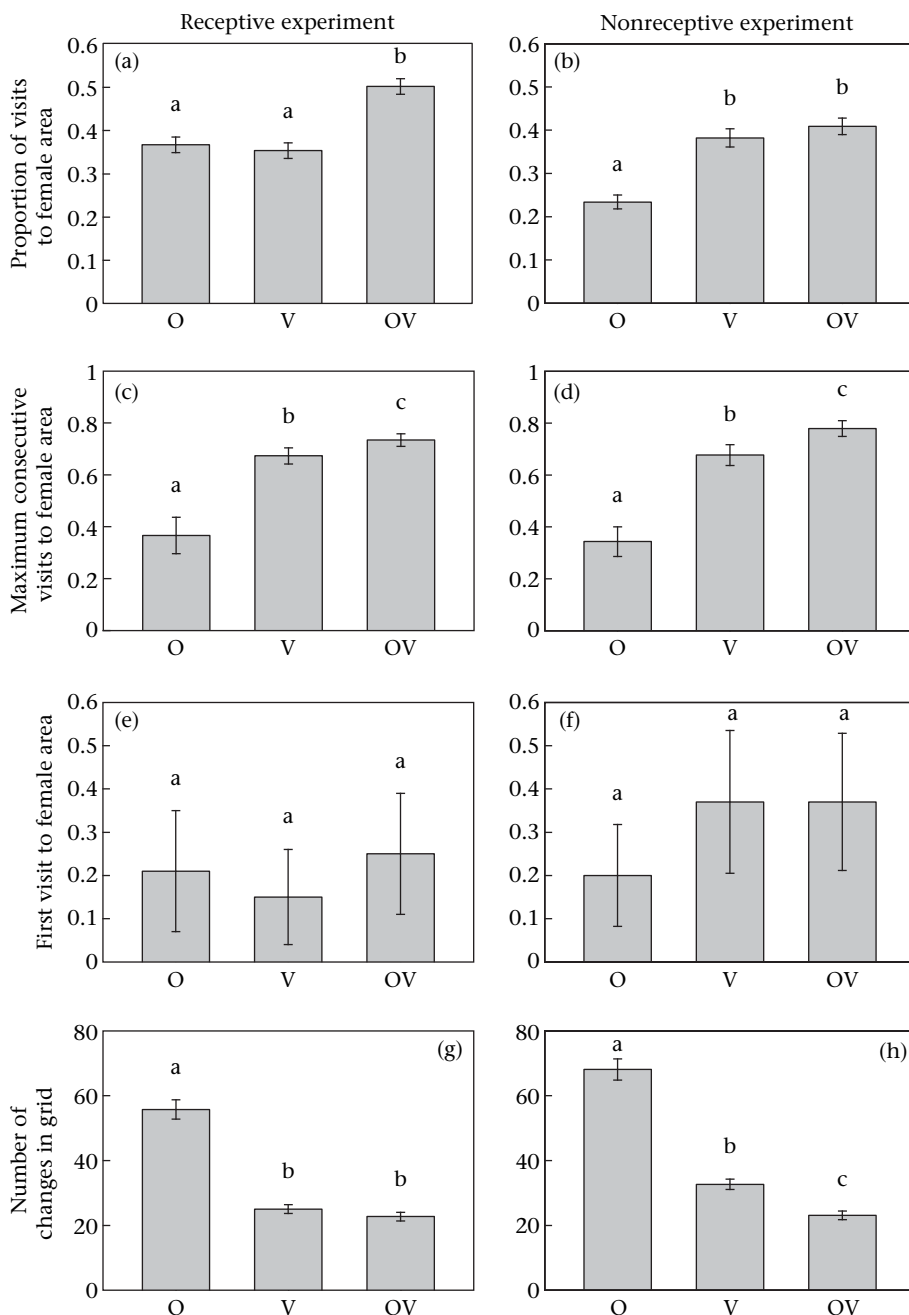

Figure 2. Field experiment. Mean ± SE proportion of males in proximity to (E) no fish, (M) three males, (NR) three nonreceptive females and (R) three receptive females. Different lowercase letters indicate statistical differences between means, based on mean differences and 95% confidence intervals presented in Table 1.

Figure 3. Laboratory experiments. (a, b) Mean ± SE proportion of times a male visited the female cues active area, (c, d) maximum number of consecutive visits to the female cues active area, (e, f) order of first visit to female cues active area and (g, h) male activity (number of changes in the grid). (a, c, e, g) Receptive odour experiment; (b, d, f, h) nonreceptive odour experiment. O: olfactory cues; V: visual cues: OV: olfactory and visual cues. Different lowercase letters indicate statistical differences between means, based on mean differences and 95% confidence intervals presented in Table 2.

Given the strong male-biased OSR in guppies, and an increase in predation risk when males court, selection should favour males that identify receptive females. Croft et al. (2003a) proposed that the continuous movement between shoals distinctive of male guppies may be advantageous for two reasons, namely finding novel females and emigrating to areas with a female-biased OSR. The results of our field experiment in the river pools demonstrated that groups of receptive females were more attractive than non-receptive females; to our knowledge, this is the first study to show this preference in the field. This finding suggests that male guppy movement between shoals is influenced by the receptivity of the

females and not only the presence of females. Similar results were found in a study on the milkweed beetle, *Tetraopes tetraophthalmus*, which showed that male emigration from a patch was dictated by the number of receptive females, and that males might use olfactory rather than visual cues to decide whether to abandon or remain in the patch (Lawrence 1987). Results from our laboratory experiments also indicate that female odour is an important cue in the dispersal decisions of male guppies. The fact that males stayed in areas in which they perceived the smell of receptive females as much as when they saw the females (but did not do the same when the odour was from nonreceptive females) suggests that the female

pheromone might motivate males to stay or to resample a specific area. An analogous example has been found in the case of male mate-guarding decisions (Carroll 1993; Mathews 2002). For instance, some male spiders assess the quality of the female (i.e. fecundity and receptivity) before deciding whether to stay and attempt to mate or to leave in search of a better opportunity to mate (Prenter et al. 1994).

We found that males did not distinguish females from males immediately using olfactory or visual cues separately or in combination (i.e. their first visit was random whether the container had males or females, Table 2), and this was irrespective of whether the olfactory cues were of receptive or nonreceptive females. This suggests that males do not discriminate females from males visually from a long distance (80 cm), and since they have to approach a shoal of fish to assess their olfactory cues, it seems that males might need to search actively and constantly change shoals to find females (Croft et al. 2003a). In our experiment, we used olfactory cues of receptive females associated with visual cues of nonreceptive females. In natural conditions, receptive females are rare (Houde 1997), so it is likely that a receptive female will be found in a shoal of nonreceptive females. In this case, receptive female olfactory cues would be present around a shoal containing mostly nonreceptive females. Obviously, nonreceptive female odour will also be present; however, it seems plausible that males would be more attuned to detecting the receptive female odours, because these would confer a greater benefit in terms of reproductive success. There are no obvious differences in the appearance of receptive and nonreceptive females; however, these two groups of females may differ in their behaviour (Guevara-Fiore 2009). An interesting extension to this study would be to use nonreceptive and receptive female visual cues, to see how males respond to a mismatch between odour and visual cues. In this experiment, however, we wanted to control for female behaviour by using the same visual stimuli across receptive and nonreceptive treatments. Although guppies do not live in habitats with totally still, isolated water, female pheromones could accumulate in small pools present in the river, and it is possible that this environmental condition may help males to detect whether a receptive female is in a pool and influence their decision to stay in a pool or continue to the next. Otherwise, in stream habitats, males might need to come in direct contact with each of the females in a shoal to recognize the presence of a receptive female. Assessment of olfactory cues might be particularly important when males search for females that are downstream.

Crow & Liley (1979), using dichotomous choice experiments, demonstrated that males are attracted to the odour of receptive females but do not respond to the odour of nonreceptive females. Contrary to Crow & Liley's findings, we found that males do respond to the odour of nonreceptive females: males spent longer periods in association with females (i.e. maximum number of consecutive visits to the female stimulus) when they had access to both visual and olfactory cues simultaneously, even when the odour was from nonreceptive females. We also found that males increased their searching activity (measured as mobility) when only odour cues were available, and again this was irrespective of the source of the odour cues. However, we identified a reduction in activity when nonreceptive odour and visual cues were combined compared to just visual cues (Fig. 3h). This pattern was not present in the receptive experiment. It has been suggested that sexual pheromones increase activity in males (Wyatt 2003), and this seems to be the case for male guppies (Crow & Liley 1979; Guevara-Fiore 2009), which explains the difference in patterns between the experiments.

In conclusion, our results confirm that even in promiscuous systems males do not allocate mating efforts randomly. This study suggests that male guppies do not associate with females indiscriminately, but prefer to approach receptive females and are more attracted to shoals containing receptive females. Although assessing potential mates might involve costs, identifying receptive females could increase male reproductive success. Our results also identified that olfactory cues of receptive and nonreceptive females influence male behaviour differently. Olfactory cues of receptive females increased male association with a shoal of females, even if that shoal contained visual cues of nonreceptive females. While the combined visual and olfactory cues were the strongest attractants, odours appeared to increase male search activity more than the other types of stimuli. Indeed, increased mobility has been proposed as a mating advantage for males to augment their mating success (Schwagmeyer 1988).

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