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Mating patterns of female Leon Springs pupfish *Cyprinodon bovinus*

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In a field study of Leon Springs pupfish *Cyprinodon bovinus*, two questions about female promiscuity were investigated. First, were females selective in the males with whom they spawned or were they unselective, spawning randomly among males? Second, how promiscuous were the females, *i.e.* with how many males did they spawn? If simply spawning with many males maximized a female's reproductive success, then females might be expected to spawn randomly with as many males as possible. Alternatively, if females were selective but engaged in multiple mating, they would limit their spawning to preferred males. In the only wild population of this endangered fish, breeding males defend closely associated territories in the shallow margins of a single desert pool. No territories were observed elsewhere in the pool. Therefore, all territorial males were present simultaneously and females could survey all of them, depositing any number of eggs with one, a few or many males. Rather than spawning randomly, females surveyed many males first, visited relatively few males and ultimately spawned with a small fraction of those available males. With increasing numbers of spawns, however, females increased the number of different mates with whom they spawned. Thus, females showed a bet-hedging tactic of having a narrow mate preference while also laying eggs in the territories of other males, possibly to reduce egg predation and to avoid inbreeding.

Key words: female mate choice; promiscuity; territoriality.

INTRODUCTION

Unlike females in polygynous breeding systems that mate with only one male, promiscuous females mate with multiple males, often in rapid succession. The potential benefits to females of mating with multiple partners include assurance of fertilization (Birkhead & Fletcher, 1995; Uller & Olsson, 2005), promotion of sperm competition (Keller & Reeve, 1995) or increase in offspring quality (Sheldon, 2000; Head *et al.*, 2006). For example, Evans & Magurran (2000) found that promiscuous female guppies *Poecilia reticulate* Peters 1859 produced more and higher-quality offspring than those females that mated with single males (Trexler *et al.*, 1997). Likewise, Draud &

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Itzkowitz (2004) argued that the act of mating with multiple males enhanced the reproductive success of female variegated pupfish *Cyprinodon variegatus* Lacépède 1803. Each of these studies suggests that the advantages of multiple mating are not dependent on a female's ability to compare quality among prospective mates before breeding with multiple males.

The ability to mate with multiple males, however, does not necessarily imply that females should not be or are not choosy in their mating decisions. Females that mate with multiple males can be highly selective, and choosy promiscuity may be a mechanism by which females improve mate quality (Gabor & Halliday, 1997; Jennions & Petrie, 2000). For instance, in captive populations of *P. reticulate*, females were likely to mate with a second male if this male was more attractive than the females' first mate (Pitcher *et al.*, 2003) and females copulated longer with and had more offspring sired by more attractive males (Pilastro *et al.*, 2007). Because *P. reticulate* encountered potential mates sequentially, it is unclear whether females would be promiscuous if they were able to make a simultaneous, direct comparison between males, choosing only the best one with which to breed.

Here, the breeding patterns of females in a wild population of the Leon Springs pupfish *Cyprinodon bovinus* Baird & Girard 1853 are examined. This species is well suited to address questions of multiple mating, because unlike most studies of promiscuity in fishes (Evans *et al.*, 2003), pupfishes have external fertilization and females lay one egg per spawning event (Leiser *et al.*, 2006). Females can spawn several times daily for a period of several weeks (M. Itzkowitz, pers. obs.) or many (up to *c.*100) times during a single spawning session (Leiser, 2003). Large adult males maintain territories but provide no parental care other than defending the territory from egg predators (Leiser, 2003; Gumm *et al.*, 2008). In *C. bovinus*, large males defend small territories that are tightly packed together onto a small shelf of shallow water in a single desert pool; this cluster of contiguous territories closely resembles a lek (Leiser & Itzkowitz, 2003). Small adult males present in the population are cryptically coloured and swim among the territories of the large males in attempts to sneak spawns with females (Leiser *et al.*, 2006). Thus, females are capable of swimming through a number of territories, quickly comparing a number of territorial and sneaker males, and spawning once or multiple times with any number of them. This flexibility in female spawning also means that a female can spawn with any particular (territorial or sneaker) male, leave the territory to spawn (or not) with other males and then return to the first male.

Females were observed as they entered the shallow shelf of breeding territories and their behaviours were followed with two questions in mind. First, it was asked whether females were selective with whom they spawned, spawning more times with some, higher-quality males over others, or whether the females spawned randomly? If females spawn randomly, it was predicted that territorial males would all receive similar numbers of spawns and that spawning with sneaker males would be relatively common. Random spawning would indicate that the benefit to females of mating with multiple males was independent of male quality. For example, laying eggs in multiple territories may mitigate the risk of egg predation (Gumm *et al.*, 2008, 2011) or decrease the likelihood of inbreeding (Hosken & Blanckenhorn, 1999).

Most studies of pupfishes, however, suggest that females do not spawn randomly but deposit their eggs based on the characteristics of the males (Kodric-Brown, 1981, 1983) or their territories (Itzkowitz, 1978). In laboratory studies, female *C. variegatus* preferred the larger of two males in the absence of male–male competition (Draud &

Itzkowitz, 2004) and territorial males received eggs in proportion to the sizes of the area they defended (Itzkowitz, 1978). For this reason, the relationship between the males' body sizes and territory areas relative to the number of females that spawned with them was examined. If females mated selectively, they should have been expected to swim through a number of territories without spawning and spawn only with a subset of those males visited. In this case, spawning with the smaller sneaker males should have been rare.

Second, it was asked to what degree did females engage in multiple mating? That is, the number of different males with whom females spawned was counted. If females attempted to acquire as many mates as possible (Evans & Magurran, 2000), it was predicted that those females that spawned the most would spawn with the greatest number of different males and that the number of eggs a female deposited in each territory would correlate with the total number of males with whom she spawned. In addressing these two questions, the presence of a mating preference (question 1) was not considered to be exclusive of the degree of promiscuity (question 2). If a female preferred a particular male (Gabor & Halliday, 1997), then the number of times she spawned with him would be high and unrelated to her total number of mates. In this instance, females could possibly be both choosy and promiscuous, preferentially mating with one male or a few males (visiting these territories more and depositing more eggs therein), while still breeding (although less frequently) with other, less preferred territorial or sneaker males.

MATERIALS AND METHODS

Cyprinodon bovinus is one of the many highly endangered pupfish species that occur in scattered springs in the arid south-western U.S.A. and northern Mexico (Kodric-Brown, 1981). *Cyprinodon bovinus* was studied in an outflow pool of the Diamond Y Spring, located *c.* 20 km north of Fort Stockton, Texas (30·9° N; 102·9° W). The pool is *c.* 20 m in diameter and has a shallow open shelf (5–15 cm deep) along its northern shoreline where males defend territories during the warmer months of the year (March to October). With the exception of 1 year (2001; out of 10 years, 2000–2009) when males defended territories in another shallow weedy location that had been flooded by a particularly wet spring, no males have ever been seen defending territories or spawning in any areas of the pool other than the territorial shelf (Leiser & Itzkowitz, 2003; Leiser *et al.*, 2006). The shelf is oblong, following the shoreline for *c.* 4 m and extending outward *c.* 1 m and normally supports 15–25 territories during the breeding season (Leiser & Itzkowitz, 2003; Leiser *et al.*, 2006). At any moment, there are as many different females (>25) visible in the shallow area as there are males and females enter and exit the shallows frequently. This allows clear observation of the spawning activity in the population with minimal disturbance to the fish's behaviour.

Females that are ready to spawn can be easily seen at the periphery of the shelf before they swim into the territorial area. After entering the shelf, females swim near the surface of the water and pass over a number of territories. When the female is ready to spawn, she enters an individual male's territory and rapidly descends to the substratum. If the male is not nearby, the female waits briefly for his approach. When the male approaches, he positions himself parallel to the female with his flank in contact with hers. At this time, the female may leave the territory or may stay with the male to spawn. If the female chooses to spawn with the male, the pair glides forward, typically one-to-several cm, where the female stops, nips the substratum and places her anal fin near the bitten area. The male then wraps his anal fin around the female's anal fin, both fish forming an s-shaped posture and performing a single jerking movement to complete the spawn. After the initial spawn, the female may glide forward and spawn again with the same male, or she may leave the male's territory to spawn with other males or to exit the shelf entirely.

Such spawning events are brief, occurring singly or in sequence, with the female ovipositing one egg per spawn (Itzkowitz, 1974; Kodric-Brown, 1988).

Breeding activity on the territorial shelf was observed in late May and early June 2002. Before beginning behavioural observations, a survey of the territories on the shelf was taken and drawn in small scale onto a sheet of paper. The territories were numbered on the map and a corresponding small numbered tag (2·5 cm × 5·0 cm) was placed in the centre of each territory. The tags did not appear to interfere with the fish's behaviour, allowed for rapid and accurate identification of the territories during behavioural observations and were used as size references to estimate male size.

A total of 233 behavioural observations were conducted on females within the pool. Remaining onshore using a hand-held camcorder, a researcher located an individual female as she approached the periphery of the territorial shelf. Her movements were then recorded as she entered the shelf and traversed the territories, with the researcher narrating her behaviour aloud into the camera to accompany the visual record. The narrative emphasized the female's location within particular territories and whether or not she spawned with the territorial and sneaker males. The term visit was used to describe any time a female was observed entering a male's territory and descending to the substratum, whether or not the pair spawned. A visit was not recorded if a female simply swam over a territory near the water's surface. Spawns and egg laying were recorded interchangeably.

Each female was observed for as long as she was visible on the territorial shelf. As females were lost from sight after they left the shelf for deeper water, it is uncertain whether a particular female returned to the shelf another time; thus, each time a female was watched while on the shelf was treated as a separate mate searching bout. No study has examined the fecundity of pupfish females under natural conditions, but observations of *C. variegates* in aquaria indicate that females spawn many times during the breeding season (Raney *et al.*, 1953; Itzkowitz, 1974, 1978; Leiser & Itzkowitz, 2004*a*, *b*).

In addition to observations of females, the territory of each male was video-recorded for a period of 15 min. During these recording sessions, the video camera was positioned on a tripod and situated as vertically as possible over the male's territory; video cameras do not appear to disturb the males' behaviours (Leiser, 2003; Leiser *et al.*, 2006). In this way, all 19 territorial males that were present on the shelf during 2002 were recorded. From the videos, estimates of the males' body and territory sizes were confirmed and the location of each male's territory was recorded with respect to others on the shelf: along the shoreline, in the middle of the shallow shelf or along the drop-off edge to deeper water. In addition, the numbers of times territorial males displayed against or chased conspecific neighbours as well as the numbers of times they chased conspecific and heterospecific intruders in their territories were recorded.

Pearson product–moment correlations were used to compare aspects of visitation and spawning patterns of females and to compare female behavioural patterns with attributes of the territorial males (*e.g.* body length, territory area and aggression). A one-way repeated-measures ANOVA was used to examine the egg-laying pattern of females that spawned with multiple males during a single mate searching bout. Comparisons of females that spawned *v.* those that did not were evaluated with *t*-tests. Likewise, *t*-tests were used to evaluate comparisons of male body size and territory area as well as differences in aggression among different males. The authors felt justified in the use of parametric analyses given the robustness of these tests (Zar, 1999) and the relative homogeneity of group variances within the data; nonetheless, a significance level of $\alpha = 0{\cdot}025$ was adopted to account for any potential increase in type I error caused by unequal sample sizes (Keppel, 1991). Statistical analyses were performed by Statistica (StatSoft; www.statsoft.com).

RESULTS

A total of 233 mate searching bouts by females visiting the territorial shelf were recorded. During nearly 72% (166) of these bouts, females spawned with at least one male. In 17 of these 166 bouts (*c.* 10%), females spawned only with sneaker males; in 104 bouts (*c.* 63%), females spawned only with territorial males and in 45 bouts

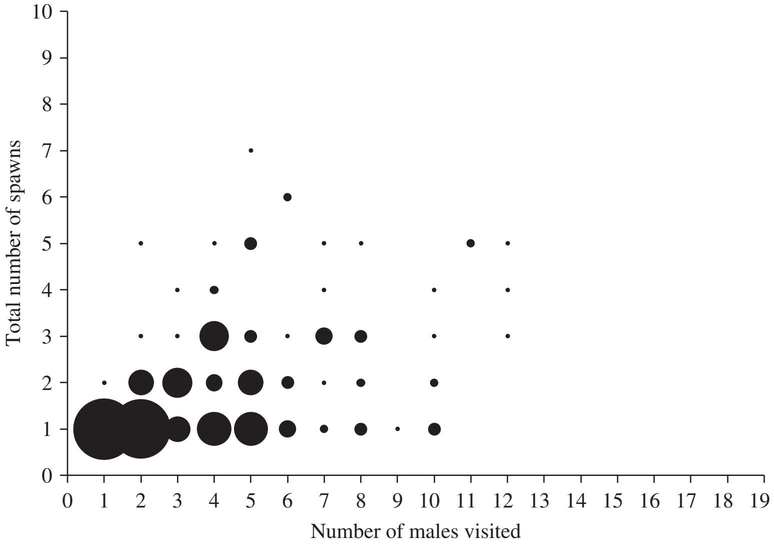

FIG. 1. The number of territorial male *Cyprinodon bovinus* (out of 19 possible territories) visited by females and the total number of times that each female spawned; the number of males visited was significantly correlated to a female's total number of spawns ($P < 0{\cdot}001$). Point markers have been weighted relative to the number of times each point was recorded.

(*c.* 27%), females spawned with both territorial and sneaker males. A total of 620 different spawning events were observed; the majority of spawns (498; *c.* 80%) were with territorial males.

While all females swam above the territories of a number of different males, those females that did spawn during a given mate searching bout visited significantly more males (mean ± S.E. = 4·61 ± 0·24) than those females that did not spawn (3·07 ± 0·25; *t*-test, $t_{231} = 5{\cdot}31$, $P < 0{\cdot}001$). Not all spawning females visited multiple males. Of the 166 females that spawned, 35 spawned with the first (and only) male they visited. On 131 occasions, females visited multiple territories before spawning with one or more of those males visited.

For spawning females, there was a positive correlation between the number of males visited during a mate searching bout and the total number of times the female spawned ($n = 166$, $r = 0{\cdot}38$, $P < 0{\cdot}001$; Fig. 1) and there was a positive correlation between the number of times a female spawned and the number of different males with whom she spawned ($n = 166$, $r = 0{\cdot}60$, $P < 0{\cdot}001$; Fig. 2). Females that spawned and visited many males tended to spawn with many different males; however, not all females that visited multiple males spawned with multiple males. Of the 131 bouts during which females visited multiple males before spawning, the females spawned with only one male on 73 occasions. During 58 mate searching bouts, females (*c.* 25% of all observed) spawned with two or more males.

The females that did spawn with more than one male did not spawn equally with those males. For example, females that spawned with two males during a single mate searching bout ($n = 37$) laid twice the number of eggs with the one compared to the other male [one-way repeated-measures ANOVA, $F_{1,48} = 14{\cdot}38$, $P < 0{\cdot}001$; Fig. 3(a)]. Females that spawned with three different males ($n = 12$) showed a linear decline in

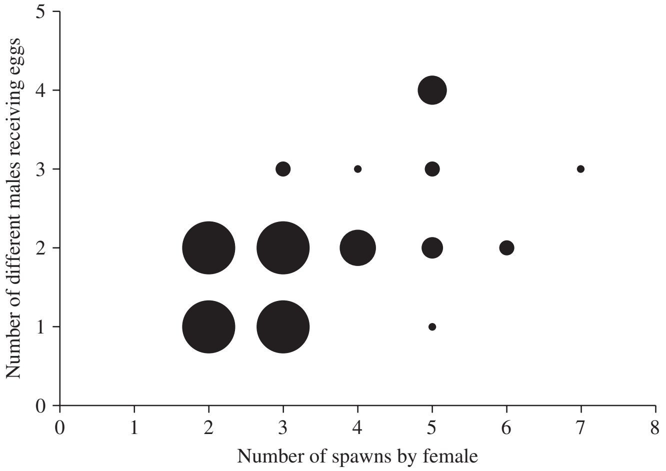

FIG. 2. The total number of times a female *Cyprinodon bovinus* spawned and the number of different males with whom she spawned. The number of spawns was related to the number of males receiving eggs ($P < 0{\cdot}001$). Point markers have been weighted relative to the number of times each point was recorded.

the number of eggs they laid with one of the males compared to the other two males with whom they spawned [linear trend comparison within the ANOVA, $F_{1,11} = 13{\cdot}15$, $P < 0{\cdot}01$; overall ANOVA, $F_{2,22} = 9{\cdot}93$, $P < 0{\cdot}01$; Fig. 3(b)]. In addition, females that spawned with four different males ($n = 9$) laid eggs unequally among those males (overall ANOVA, $F_{3,15} = 12{\cdot}14$, $P < 0{\cdot}001$). Ranking the males with respect to the number of times females spawned with each, these females laid significantly more eggs with one of the males compared with the other males [comparison of second *v.* third and fourth ranked male: $F_{1,5} = 1{\cdot}00$, $P > 0{\cdot}05$; comparison of first *v.* second, third and fourth ranked males: $F_{1,5} = 10{\cdot}00$, $P < 0{\cdot}05$; Fig. 3(c)]. Among females who spawned with both territorial and sneaker males, none spawned more times with any single sneaker than with a territorial male.

Females that visited multiple males during a single mate searching bout but spawned with only one and females that spawned with multiple males (but unequally so) during a mate searching bout appeared to show a preference for certain males over others. To determine whether or not all females showed a preference for particular males, territorial males were ranked based on the number of times they were visited by females that spawned and by females that did not spawn. Some territorial males were clearly visited more often than others, with the three highest-ranked males accounting for 36% of all visits. Non-spawning and spawning females appeared to show similar preferences for visiting particular males over others ($n = 19$, $r = 0{\cdot}84$, $P < 0{\cdot}001$; Fig. 4). Similarly, when territorial males were ranked according to the number of spawns in which they engaged, it was clear that females preferred to mate with some males over others. The three highest-ranked males received 65% of all spawns. The males that were visited the most frequently also received the greatest number of spawns ($n = 19$, $r = 0{\cdot}85$, $P < 0{\cdot}001$; Fig. 5), with the top three most frequently visited males (male numbers 12, 15 and 17) receiving the most spawns (15, 12 and 17).

Although a number of factors may have influenced the females' preference for particular males, it did not appear that their preference was based on the sizes of the

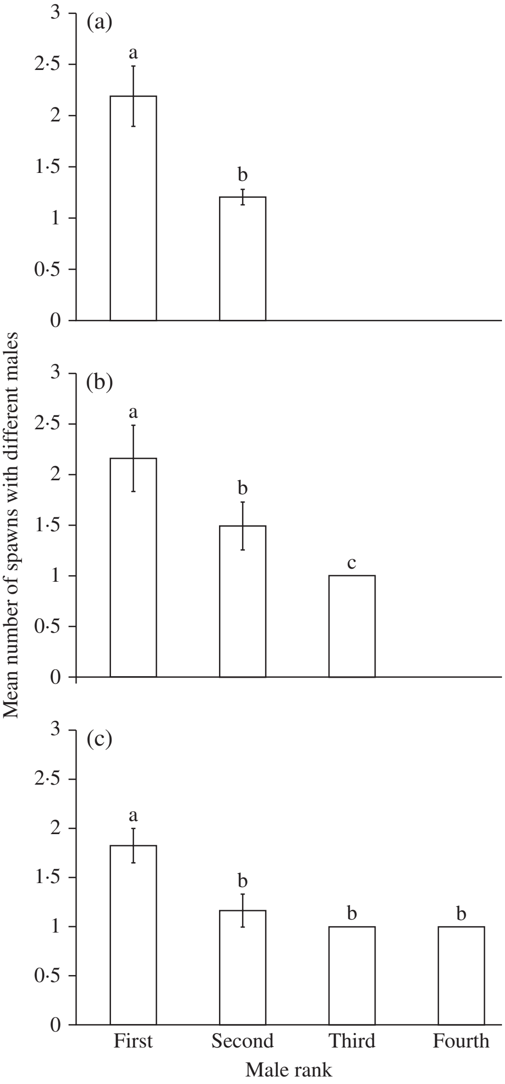

Fig. 3. Mean ± s.e. number of spawns that *Cyprinodon bovinus* females allocated to different males for females spawning with (a) two, (b) three and (c) four different males. Females spawned more often with the highest-ranked male than with others. Different lower-case letters indicate significant ($P < 0.05$) difference between means.

males' territories. Neither the total number of visits ($r = -0.11$, $P > 0.05$) nor the total number of spawns ($r = -0.02$, $P > 0.05$) a male received was correlated with the size of his territory and the territories of the three most-preferred males were of similar size (mean ± s.e. = 4000 ± 200 cm^2) compared with the territories of the other males (3900 ± 900 cm^2; *t*-test, $t_{17} = 0.06$, $P > 0.05$). The location of the males' territories on the shelf did not appear to matter as the three most-preferred males defended territories in different locations; male 17 defended a shoreline territory, male 15 defended a central territory and male 12 defended a drop-off edge territory. In addition, all territorial

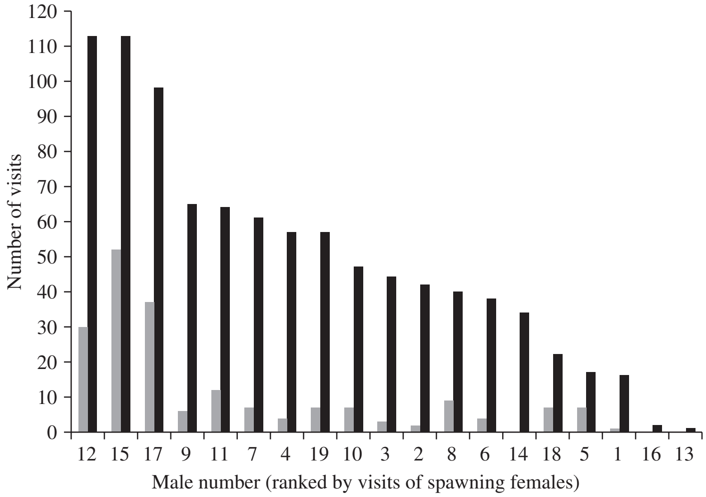

FIG. 4. Territorial *Cyprinodon bovinus* males ranked for the total number of females that visited their territories. Males are presented as their tag number and ranked based on visits by females that did spawn (■), although the visitation pattern was similar for females that did not spawn (▨).

males were within 1·5 cm (4·5–6·0 cm) total length (L_T) of one another and the three most-preferred males were not larger than the other males on the shelf (t-test, $t_{17} = 1{\cdot}32$, $P > 0{\cdot}05$). The number of times that the three most-preferred males attacked conspecific opponents (mean ± S.E. = 34·67 ± 12·72) was similar to the number of times the other males attacked conspecific opponents (27·67 ± 5·69; t-test, $t_{17} = 0{\cdot}58$, $P > 0{\cdot}05$). Likewise, the amount of aggression against heterospecific fish was similar for the three

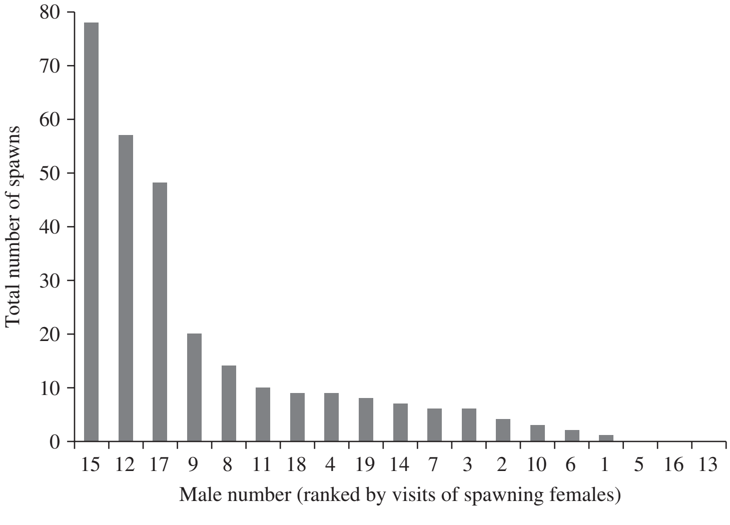

FIG. 5. Territorial *Cyprinodon bovinus* males ranked for the total number of spawns received from different females. The three highest-ranked males received 65% of all spawns.

most-preferred males (mean ± S.E. = 15·67 ± 4·41) and the other males on the shelf (20·67 ± 3·84; t-test, $t_{17} = 0·69$, $P > 0·05$).

DISCUSSION

In summary, during mate searching bouts made to the shelf of shallow water, females swam over the territories of a number of different males but visited only a small sub-set of the territories. Females that did not spawn visited fewer males than those that did, but whether or not they spawned, all females showed a preference for visiting certain territories. Females spawned more with territorial males than with sneakers and females that did spawn visited more than twice the number of males than those with whom they spawned. Most often (79% of the time), these females visited multiple males before spawning with one or more of those visited. Once a female had spawned with a particular male, she either: spawned with this male again, left the male's territory for another male to spawn (or not) or left the shelf for deeper water. As spawn frequency increased, so too did the number of different males with whom females spawned. Multiple spawning females preferentially spawned with one male, laying more eggs in his territory than in the territories of the other males.

Female *C. bovinus* discriminated among potential mates by surveying many males during their mate searching bouts but spawning with only a small sub-set of those males available to them. The precise criteria for this mate preference are uncertain; however, many females preferred the same individual males. These males did not defend larger territories; nor were the males themselves larger compared with other territorial males. In this sense, this species deviates from the more widely distributed *C. variegatus* (Leiser & Itzkowitz, 2004*b*) and other congeners (Kodric-Brown, 1981, 1983; Leiser & Itzkowitz, 2002) where male size is correlated with territory size and the largest males (and territories) receive the most spawns. It is possible that certain aspects of the microhabitat, such as fine features of the substratum, were preferred by females, but such differences among the territories could not be identified; the three most-preferred males occupied territories in different areas of the shelf. In addition, as the males were closely matched with respect to territory area, body size and level of aggression, the females may have selected males based on other cues such as the presence (or amount) of eggs in a male's territory (Jamieson, 1995), male colouration (Kodric-Brown, 1983; Ludlow *et al.*, 2001) or other aspects of territorial defence (Kodric-Brown, 1988; Leiser & Itzkowitz, 2004*a*, Leiser *et al.*, 2006).

Regardless of the precise male characteristics they preferred, female *C. bovinus* showed a combination of selectivity and promiscuity in their mating patterns. During any given mate searching bout, females had opportunity to assess all of the males prior to mating and could have deposited all of their eggs with any one male. In this way, their multiple mating behaviour was not consistent with the trade-up hypothesis in systems where females encounter mates sequentially and mate with subsequent males only when each new male's quality is higher than the quality of the previously encountered mate (Gabor & Halliday, 1997; Pitcher *et al.*, 2003). It is also unclear how spawning in less preferred territories or with smaller sneaker males would directly improve the quality of a female's offspring (as in *P. reticulate*; Evans & Magurran, 2000). There may be, however, two (or more) potentially interrelated benefits to females for having a narrow mating preference while exhibiting some, more-limited, spawning with other males.

First, laying eggs in multiple territories may mitigate the risks of egg predation. The Pecos gambusia *Gambusia nobilis* (Baird & Girard 1853) is an opportunistic egg predator and individual fish converge in large numbers on spawning pairs of *C. bovinus* (Leiser & Itzkowitz, 2003; Gumm *et al.*, 2011). Similarly, individuals of other gambusia species hover around the territories of the Comanche Springs pupfish *Cyprinodon elegans* Baird & Girard 1853 (Leiser & Itzkowitz, 2002, 2003) and *C. variegatus* (Leiser, 2003), closely following spawning pairs and preying on recently deposited eggs. These egg predators represent a threat to the majority of the relatively few eggs that a female might lay in a given male's territory and higher densities of the egg predators have been observed in those territories where more spawning occurs (Gumm *et al.*, 2008, 2011). As a result, females probably face a fitness trade-off between the superior genes of preferred males and the decreased probability of offspring survival when they spawn in a territory with many egg predators. Consequently, females may compromise by laying most of their eggs with carefully selected males and a few eggs with less preferred territorial or sneaker males in locations where the risks of egg predation are lower.

Second, mating with multiple males may reduce the potential costs of inbreeding in this species (as in others, Stockley *et al.*, 1993; Hosken & Blanckenhorn, 1999; Varian-Ramos & Webster, 2012). *Cyprinodon bovinus* is highly endangered and lives in an extremely restricted range (*i.e.* the outflow pool of the Diamond Y Spring). As a result, its population size is very low and individuals have no ability to disperse. It is likely that females cannot avoid spawning with relatives and spawning with multiple males increases the probability that at least some offspring will be outbred (Stockley *et al.*, 1993; Richardson *et al.*, 2004). The 'inbreeding avoidance hypothesis' (Hosken & Blanckenhorn, 1999), however, does not consider that females might be both selective and promiscuous. The model assumes that inbreeding is associated with low fitness (Wildt *et al.*, 1987; Mullard *et al.*, 2009) and that random multiple mating by females increases the genetic diversity and therefore fitness, of her offspring relative to monogamous mating.

In *C. bovinus*, spawning with multiple males despite having a narrow mate preference resembles a pattern of differential allocation of reproductive effort (Head *et al.*, 2006). In this form of post-copulatory mate choice, females tend to lay more eggs with or invest more energy in the offspring of more attractive males (de Lope & Moller, 1993; Eberhard, 2000). Such differential allocation may increase offspring quality (Sheldon, 2000) if a positive genetic correlation arises between male attractiveness and female preference. In this manner, the selective promiscuity of *C. bovinus* females appears to balance the fitness consequences of spawning with preferred, but possibly related, males with the necessity to ensure outbreeding. This form of bet-hedging mate choice may also explain the persistence of the conditional male mating tactics within this species.

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