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Intrasexual competition between males and intersexual mate choice by females may have complementary or antagonistic effects on the evolution of sexually-selected traits. We used open-aquarium experiments to test for the effects of male body size and male secondary sexual characters on male–male competition and on female mate choice in the fathead minnow *Pimephales promelas*. Larger males were more successful than smaller males in competing for nesting substrates. Larger males were also preferentially chosen by females for spawning. Secondary sexual characters (tubercle number, banding pattern, and dorsal pad development) were not associated with success in male–male contests and were not preferred by females. In a separate experiment, we found that female choice was not influenced by past male reproductive success. We confirmed genetic paternity of fertilized

1871; Andersson, 1994). An underlying assumption in sexual selection research has been that intra- and intersexual processes of sexual selection are complementary: the trait or suite of traits that determines the outcome of intrasexual competition accurately conveys the quality of potential suitors during intersexual mate choice (Candolin, 1999; Berglund & Rosenqvist, 2001). Indeed, success during intrasexual competition can be indicative of a suitor's quality (Bisazza et al., 1989; Montgomerie & Thornhill, 1989; Alatalo et al., 1991; Kodric-Brown, 1996), and there is empirical evidence that

sex characters, and behaviour on male–male competition for nesting substrates and female mate choice in the fathead minnow (*Pimephales promelas*). Male–male competition and female preference both favoured large male body size, suggesting that these mechanisms of sexual selection are complimentary with respect to male body size.

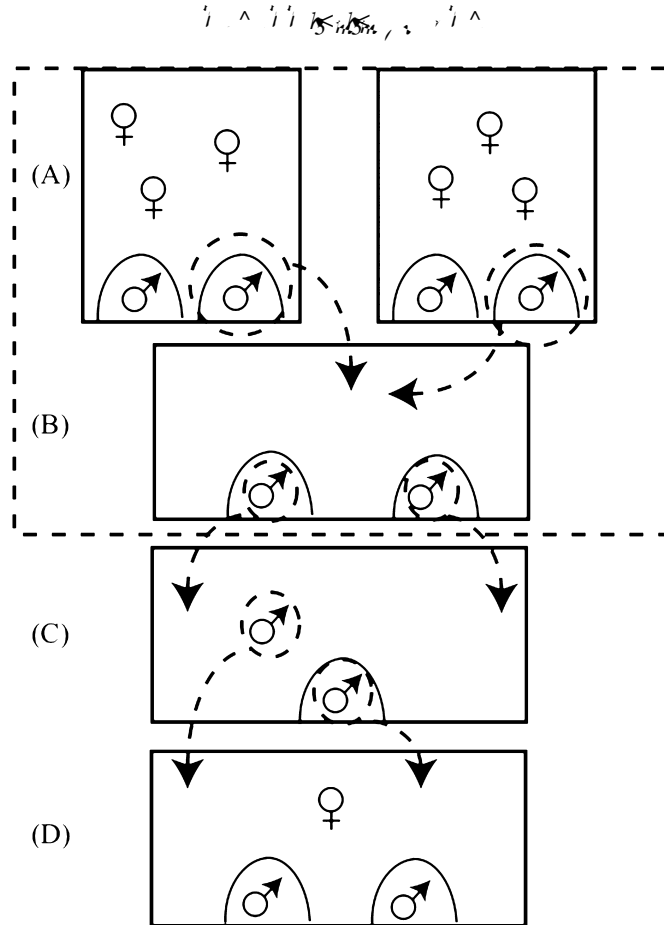
The fathead minnow is a freshwater cyprinid that inhabits lakes, ponds, and slow-moving streams (Andrews, 1970; Lee et al., 1980). Its geographic range is from southern Canada through the entire continental United States and into northern Mexico (Andrews, 1970; Lee et al., 1980). The sexes are indistinguishable as juveniles, but considerable sexual dimorphism is evident in adults approximately 30 days before spawning begins (Flickinger, 1969). Males develop breeding tubercles (horny projections on the snout and lower jaw), a pad of thickened, mucous-secreting epidermal cells between the head and the dorsal fin, and a contrasting pattern of alternating dark and light bands (Isaak, 1961; McMillan & Smith, 1974; Unger, 1983). Sexually mature males move into shallow water and compete to defend nesting territories established on the undersides of rocks or in stable vegetation (Markus, 1934; Isaak, 1961; Andrews, 1970; McMillan & Smith, 1974). Nesting territories are vigorously defended from other males, as well as from non-gravid females, juveniles, and other potential egg predators (Markus, 1934; Unger, 1983; Pyron & Beitinger, 1989; Sargent, 1989). Males confront nest intruders (egg predators and other males) either with a behavioural display or by butting them with their tubercles. Mature females move singly or in small groups through areas where males have established territories to make spawning decisions. Territorial male fathead minnows court females with a behavioural repertoire that includes approaches, lateral displays, and leading behaviours (Cole & Smith, 1987). Females enter the nests of territorial males and deposit their eggs on the ceiling of the nest cavity (Markus, 1934; Isaak, 1961; Andrews, 1970). Males fertilize and subsequently care for the eggs until they hatch.

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Fathead minnows used in this experiment were obtained from Aquatic Research Organisms (Hampton, NH, USA). The fish were housed in 114-l stock tanks until they could be sexed based on external morphology (Flickinger, 1969). Males and females were then separated and housed in 76-l aquaria. Aquaria were maintained at an ambient water temperature of  $23 \pm 2$  C and a 16 h/8 h (light/dark) photoperiod to maintain adults in reproductive condition (Denny, 1987). All fish were fed twice daily using flake food or frozen brine shrimp. This project was carried out in accordance with the methods described in protocols 01-105 and 04-126 approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Vermont.

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We used a paired contest, open-aquarium design (Houde, 1997) to determine how body size (standard length, SL; wet mass, WM), secondary sex characters (banding pattern, BP; tubercle number, TN; dorsal pad development, DP), and courtship behaviour related to male success during both intrasex-



Experimental design and tank arrangement used for Experiment 1. Males were transferred from stock aquaria to 38-l preparation tanks and allowed to interact with three sexually mature females until one member of the pair spawned successfully. When two males were simultaneously guarding eggs in separate preparation tanks (A), they were transferred to an experimental tank and allowed to acclimate (B). Using dyadic contests, we monitored success during intrasexual interactions (C). Using open choice spawning trials we monitored spawning success (D).

and one male was found guarding eggs. To establish dyads for our experiment, we randomly paired two reproductively successful males from separate preparation tanks. Each male was anesthetized using MS-222 (0.3 g/l), measured for standard length and wet mass, and marked for individual identification by clipping a small portion of either the top or bottom lobe of the caudal fin. Both males were placed in a 1-litre chamber until they fully recov-

ered from anaesthesia (approx. 30 min). Once both males had recovered, we released them into a 76-l aquarium containing two identical nest substrates and allowed them to acclimate for approx. 12 h (Figure 1B).

#### Male–male competition ( $n = 50$ dyads)

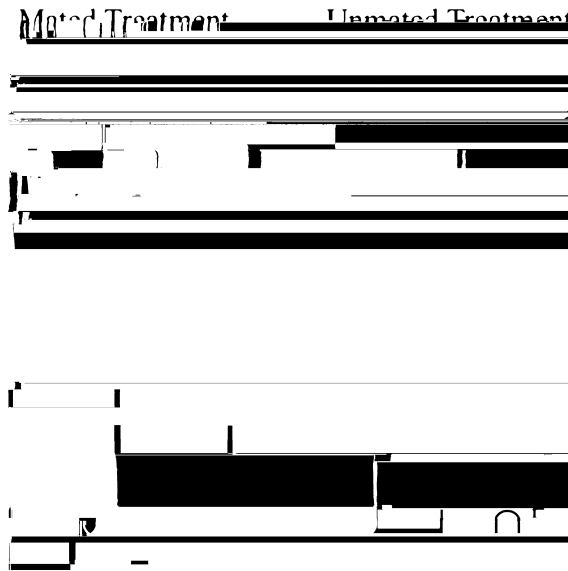
Following the acclimation period, we scored the banding pattern of each male on a scale of 1 (=lowest intensity) to 4 (highest intensity; Unger, 1983; see also Danylchuk & Tonn, 2001), removed the two nest substrates, and replaced them with a single fresh substrate in the centre of the tank (Figure 1C). After the introduction of the new nest substrate, we quantified the number of seconds each male spent guarding the nest over a 15-min. observation period (=resource holding power, RHP). All replicates resulted in a clear winner.

#### Female choice ( $n = 34$ dyads)

During the female choice stage, we removed the central nest from the tank containing the two males and replaced it with two new nests (Figure 1D). We then introduced a gravid virgin female (unfamiliar to both males) in the tank and allowed her to swim freely and acclimate for approx. 8 h. We quantified male–female behavioural interactions by videotaping 17 randomly selected replicates for 10 min during the morning (0800–1000) and during the evening (1600–1800) each day until spawning occurred. We later scored the videotaped interactions for the number of courtship approaches the male made toward the female (Cole & Smith, 1987; Vives, 1988) and the amount of time the male spent guarding his nest. Males and females included in this stage of the experiment were 4(e)14.9(xp399.4(acclim(54(.)]T4eI)0.74a34(acce)-204.21.9(ob)-0.f(acest)-228.)-327

Preparation ( $n = 20$  dyads)

During the preparation stage, we selected two similarly-sized virgin males from a 114-l single-sex stock tank. Each male was anaesthetized and measured as in Experiment 1. We alternately assigned the larger of the two males to mated and unmated treatments ( $n = 9$  larger males and  $n = 11$  smaller males mated; sample sizes were initially equal, but one or both males died in two replicates in which the larger male was assigned to the mated treatment). This design ensured that the average size difference between males in the mated and unmated treatments did not differ significantly from 0 (matched pairs analysis: mean difference in standard length =  $-0.804$  mm,  $t = -1.28$ ,  $df = 19$ ,  $P = 0.22$ ; mean difference in wet mass =  $-0.002$  g,  $t = -0.02$ ,  $df = 19$ ,  $P = 0.98$ ), thus allowing us to detect subtle effects of size on male mating success. After measuring the males, we released them into a 1-l chamber to recover from anaesthesia for 30 minutes and then transferred them into their respective 38-l aquaria for the duration of the preparation stage. Mated males were then released into an aquarium containing a



Experimental design for examining the relationship between male reproductive history and female preference. Large and small males were alternately assigned to the mated or unmated treatments. Mated males were housed in a 38-l aquarium containing a nest substrate and three adult females until at least one female had spawned (A) whereas unmated males were housed alone with a nest substrate (B). Upon successful spawning, both males



contests over a single nest site. We compared the frequency of winning males that were chosen by females to a null expectation of 0.50.

We used matched-pairs analysis to determine whether body size, or secondary sex characters were associated with the outcome of female choice trials. For each predictor variable, we tested the null hypothesis that the mean difference between chosen and rejected males would be 0. We used the Dunn-Sidak method of stepwise Bonferonni correction (Sokal & Rohlf, 1995) to set the experimentwise  $\alpha = 0.05$ . Uncorrected  $P$ -values are reported in the text with results that were significant after Dunn-Sidak correction indicated by an asterisk (\*).

#### Experiment 2. Male reproductive history and female choice

We tested the hypothesis that male reproductive success in a current mating event was independent of reproductive history by counting the number of previously successful males that were chosen and rejected by females in our experiment. We compared the observed frequencies to our null expectation that females would mate randomly with respect to male reproductive history using a likelihood ratio test.

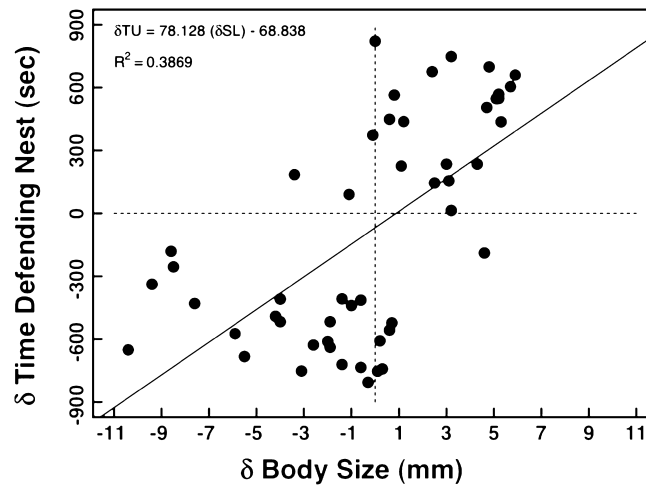
#### Paternity analysis

Because male fathead minnows that are not guarding eggs may usurp the nests of males that are guarding (Unger, 1983; Sargent, 1988; Unger & Sargent, 1988), we used microsatellites to confirm the genetic paternity of the eggs spawned during the female choice trials in Experiment 1 and, therefore, eliminate the possibility that the putative father had stolen the clutch from the genetic father.

We used the extraction protocol for animal tissues from a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) to isolate DNA from tissue collected from both putative fathers and 10 embryos for each of the 35 replicates that resulted in spawning during Experiment 1. We used primers for two previously developed markers (PPRO 118 and PPRO 171; Bessert & Orti, 2003) to amplify microsatellites in single-primer-pair polymerase chain reactions (PCR) in an Eppendorf Mastercycler gradient thermalcycler under the optimized conditions suggested by Bessert & Orti (2003). Forward primers were labelled with fluorescent dye (HEX, 6FAM), and PCR products from each locus were combined within an individual or clutch for multiplex fragment

analysis. We successfully isolated DNA from both the tissue and embryos for 26 of 35 replicates. Paternity for each replicate was determined by visual inspection of the electropherograms using GeneMapper software (v 3.75; Applied Biosystems). We used DNA fingerprinting analysis (sensu Burke et al., 1989; see also Avise, 2004) because paternity had to be determined for only two individuals and maternity was known for all replicates. Briefly, because we did not genotype the females from each replicate, those bands (=alleles) in the progeny that could not have been inherited from either putative father were identified as maternally derived and excluded from our analysis. We then scored the potential contribution of each male to the observed clutch genotype by counting the number of shared bands at each locus and calculating the probability of paternity using the formula

$$\text{Pr}[\text{Male}_i] = \frac{c_{m1} \times c_{m2}}{c_{m1} + c_{m2}}$$



Relationship between the difference in body size between males and the difference in the time each male spent guarding the nest during the male–male competition experiment.

$df = 4$ ,  $p = 0.001$ ). The best morphological predictor of male success in intrasexual contests was body size ( $F = 19.116$ ,  $df = 1$ ,  $p = 0.0001$ ; Figure 3), with no influence of banding pattern ( $F = 1.204$ ,  $df = 1$ ,  $p = 0.28$ ,  $\eta^2 = 0.000$ ), dorsal pad development ( $F = 0.303$ ,  $df = 1$ ,  $p = 0.59$ ,  $\eta^2 = 0.007$ ), or tubercle number ( $F = 0.289$ ,  $df = 1$ ,  $p = 0.60$ ,  $\eta^2 = 0.044$ ).

#### Female choice

Female fathead minnows spawned with males that won their intrasexual contest during 57% (20/35) of our trials, which did not differ significantly from the expected frequency of 50% (two-tailed binomial test,  $p = 0.69$ ).

Although female fathead minnows did not preferentially spawn with males that won intrasexual contests, male morphology was associated with reproductive status. Specifically, large male body size (mean difference = 2.21,  $F = 2.93$ ,  $df = 34$ ,  $p = 0.0061$ ; Figure 4A) and a dark banding pattern (mean difference = 0.579;  $df = 34$ ;  $p = 0.0016$ ; Figure 4B) were associated with male reproductive success, whereas dorsal pad development (mean difference = 0.014,  $df = 34$ ,  $p = 0.82$ ) and tubercle number (mean difference = 1.03,  $df = 34$ ,  $p = 0.34$ ) were not.

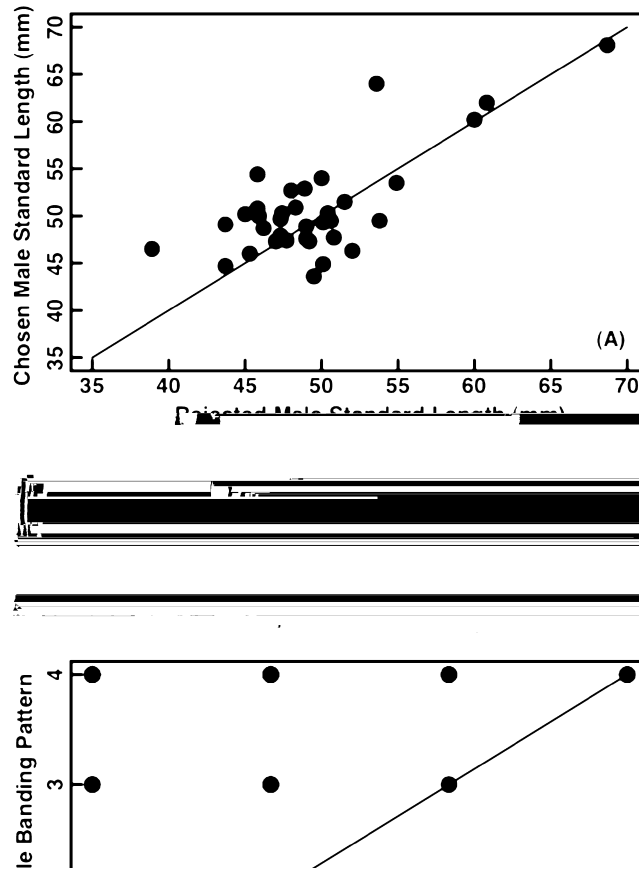


Fig. 1. Results of multiple matched-pairs analyses of morphological characters and their association with male mating success. Body size (A,  $P = 0.0061$ ) and banding pattern (B,  $P = 0.0016$ ) were significantly associated with male reproductive success.

#### Male behaviour and female choice

Males that were chosen by females spent 86% of their time ( $\bar{x} = 492 \pm 144$  s) guarding their nests and made an average of  $3.9 \pm 4.4$  approaches toward the female during the female choice stage of our experiment. Rejected males spent 71% of their time ( $\bar{x} = 426 \pm 204$  s) guarding their nests and made an average of  $1.9 \pm 3.1$  approaches toward the female. There were no significant differences between chosen and rejected males in nest attentiveness and courtship (multiple logistic regression,  $\chi^2 = 3.65$ ,  $df = 2$ ,  $P = 0.16$ ).

### Paternity analysis

We successfully amplified two microsatellite loci (PPRO118 and PPRO171; Bessert & Orti, 2003) for 74.2% (26/35) of the replicates that yielded a clutch of eggs during Experiment 1. Of these, paternity was confirmed for 100% (26/26) of the males observed to be guarding the eggs (Table 1).

### Female preference for a previously mated male

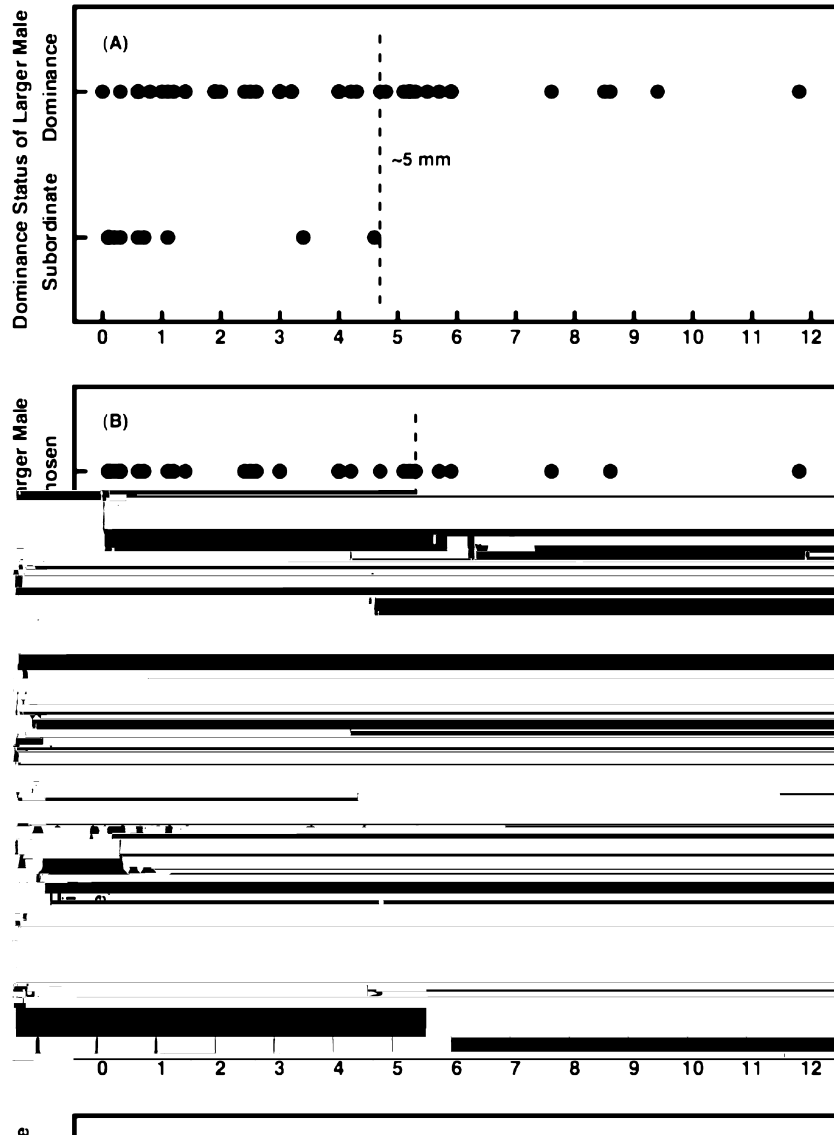
Female fathead minnows chose to spawn with the previously mated male in 45% (9/20) of the choice trials. Hence, female preference for a given male fathead minnow was not predicted by his reproductive history (log likelihood  $\chi^2 = 0.20$ ,  $df = 1$ ,  $P = 0.655$ ). As in Experiment 1, however, female fathead minnows chose to spawn with the larger of the two males in 85% (17/20) of the choice trials. Thus, the result that female fathead minnows prefer relatively large males was independently corroborated with our second experiment (log likelihood  $\chi^2 = 10.25$ ,  $df = 1$ ,  $P = 0.0015$ ).

Results for paternity analysis by DNA fingerprinting for 26 replicates for which there were female choice data and we were able to successfully amplify microsatellites from both putative fathers and a sample of the eggs. For the eggs, the numbers indicate the number of paternally derived alleles at each of two microsatellite loci (Loc 1 and Loc 2). For the chosen and rejected male of each female preference replicate, the numbers indicate the number of alleles shared with the eggs at each microsatellite locus, and the probability of paternity ( $P$ ) is indicated for each male.

Rep	Eggs		Rejected male			Chosen male		
	Loc 1	Loc 2	Loc 1	Loc 2	$P$	Loc 1	Loc 2	$P$
1	1	2	0	1		1	2	1.00
2	1	1	0	0		1	1	1.00
3	1	1	0	1		1	2	1.00
4	1	2	0	0		1	2	1.00
5	1	1	0	1		1	1	1.00
6	2	3	1	1	0.17	2	3	1.00
7	2	2	1	0		2	2	1.00
8	1	2	0	0		1	2	1.00
9	3	2	2	0		1	2	0.33
10	1	1	0	0		1	1	1.00
11	1	3	1	1	0.33	1	3	1.00
12	1	1	0	0		1	1	1.00
13	1	2	0	1		1	2	1.00
14	1	2	1	1	0.50	1	2	1.00
15	1	2	1	1	0.50	1	2	1.00
16	1	2	1	0		1	2	1.00
17	2	2	0	0		2	2	1.00
18	1	3	0	2		1	2	0.67
19	1	2	1	0		1	2	1.00
20	2	2	1	0		2	2	1.00
21	1	1	1	0		1	1	1.00
22	1	2	0	0		1	2	1.00
23	1	3	0	1		1	2	0.67
24	1	2	1	1		1	2	1.00
25	1	3	0	1		1	2	0.67
26	1	2	0	0		1	2	1.00

If dominance relations between males remained unchanged over the duration of our experiment, and dominance relations interfered with female preference, then our result should have been complete agreement between dominance status and mating success. This, however, was not the case. More-

over, our behavioural data suggested that, although male–male interactions did occur during the female preference stage of our experiment, they were of relatively low intensity in comparison to the interactions during the intrasexual contest stage, and that dominance relations remained consistent through-



Graphical comparisons of male success on body size difference during both intra and intersexual stages of our experiments. Overall, large male body size was favoured during intrasexual contests (A), and intersexual mate choice (B), but was absolutely predictive only over a threshold size of approximately 5 mm. Below 2 mm females apparently preferred to mate with subordinate males (C) suggesting that the interaction between processes of sexual selection shift with respect to the size structure of the mating population.





tubercles are apparently broadly distributed in the Cyprinidae, it is possible that this trait is phylogenetically constrained and differentially expressed depending on the breeding biology of the species, rather than a secondary sex character that has evolved by sexual selection.

Figure 1. Male behaviour toward females and reproductive success.

We found no association between male behaviour toward females and reproductive success. Cole & Smith (1987) suggested that male fathead minnows

and a growing number of studies in an array of taxa have led to a better understanding of the interactions between and relative contributions of intra- and intersexual mechanisms of sexual selection (e.g., Moore & Moore, 1999; Moore et al., 2001; Lopez et al., 2002; Candolin, 2004; Wong, 2004; Shackleton et al., 2005; Borg et al., 2006). Our results from fathead minnow show that large male body size is favoured during male–male competition for nests and is preferred by females choosing mates when size differences between males were greater than 5 mm. When males differed by 2 mm, however, females preferred subordinate males. Female fathead minnows, therefore, may adjust their priorities for choosing mates based on the size structure of the males defending territories, thus maintaining genetic variation for choice criteria.

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