Genetic evidence reveals density-dependent mediated success of alternative mating behaviours in the European bitterling (*Rhodeus sericeus*)

M. REICHARD,†* C. SMITH† and W. C. JORDAN‡
*Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Květná 8, 603 65 Brno, Czech Republic, †Department of Biology, University of Leicester, University Road, Leicester LE1 7RH, UK, ‡Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY, UK

Abstract

The reproductive success of alternative mating behaviours may vary within and among populations in relation to environmental factors and demographic parameters. We used behavioural and genetic data to investigate how male density affects reproductive success of territoriality and sneaking in the European bitterling (*Rhodeus sericeus*, Cyprinidae), a freshwater fish that spawns on the gills of living freshwater mussels. Keeping the number of spawning sites constant, we manipulated male densities in laboratory and mesocosm experiments. We showed that sneaked fertilizations were common in *R. sericeus*, and that they increased significantly with male density. Territorial mating was almost 17 times more successful than sneaking at the lowest male density treatment, and still 2–3 times more successful at intermediate densities. However, both behaviours conferred the same fitness pay-off at the highest male density. While the success of territorial males declined with male density, the success of individual sneaking males remained constant across densities. Notably, the capacity of territorial males to outcompete sneakers by preoviposition sperm loading was the best predictor of male reproductive success, rather than aggression, body size or postoviposition ejaculation.

Keywords: fish, mating tactics, paternity, sneaking, sperm competition, territoriality

Received 12 October 2003; revision received 16 January 2004; accepted 16 January 2004

Introduction

Alternative mating behaviours are common in nature, evolving through frequency- or condition-dependent sexual selection. The mating behaviour of an individual may be fixed, an expression of successive ontogenetic stages, or an opportunistic response to the probability of a fitness benefit in a particular situation (Gross 1996; Taborsky 1998). The diversity of reproductive behaviour in fishes is particularly great, and fishes show the widest range of sperm competition intensity of any animal group (Taborsky 1994, 1998). A typical mating system with alternative behaviours in fishes is the sneak-guard model

(Gross & Charnov 1980; Parker 1990, 1998) and its various derivations (reviewed by Taborsky 1994, 2001; DeWoody & Avise 2001). This system involves territorial (bourgeois) males that invest in mate attraction and courtship, defence of resources and sometimes parental care, along with nonterritorial (parasitic) males that try to steal the reproductive investment of territorial males by 'sneaking' fertilizations (Taborsky 1998).

In the typical sneak-guard model (Parker 1990), males adopt either a territorial or a sneaking behaviour, but not both. A fixed dichotomy in male mating behaviour appears to be met in some circumstances (for example, in bluegill sunfish *Lepomis macrochirus*; Gross & Charnov 1980). However, the adoption of alternative mating behaviour may be largely opportunistic, with a male switching between territoriality and sneaking in relation to a particular situation (Taborsky 1994, 1998). This opportunism in male reproductive behaviour has received little attention,

Correspondence: M. Reichard, Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Květná 8, 603 65 Brno, Czech Republic. Fax: + 420 543211 346; E-mail: reichard@brno.cas.cz

although it occurs commonly in fishes [e.g. three-spined stickleback *Gasterosteus aculeatus*, Rico *et al*. (1992); blenny *Aidablennius sphinx*, Neat & Locatello (2002); bitterling *Rhodeus* spp., Kanoh (2000); Smith *et al*. (2002)] and may be related to the relative success of alternative mating behaviour under different conditions.

Demographic parameters and resource availability are predicted to affect the relative success of alternative mating behaviour (Taborsky 2001; Neat & Locatello 2002). Increasing male density and male to resource ratio increase energetic demands on territory defence because territorial males are exposed to more frequent intrusions by competing males (Warner & Hoffman 1980; Reichard *et al*. 2004). However, the question of whether higher intrusion rates necessarily translate into a decrease in reproductive success remains unanswered.

Here, we investigate how male density affects the absolute and relative success of territoriality and sneaking in the European bitterling *Rhodeus sericeus*, a small freshwater fish with a resource-based breeding system. Male bitterling defend territories around living freshwater mussels and attract females to spawn on the mussel's gills (Smith *et al*. 2004). Males typically guard territories of 4–10 m² (Schaumburg 1989) around a mussel, although territories may be considerably smaller at high male densities, and may contain more than a single mussel at high mussel densities. Territory take-overs occur and males can hold a given territory from hours to weeks (M. Reichard & C. Smith pers. obs.). Mating is promiscuous; both males and females spawn repeatedly, with multiple partners. Females use an extended ovipositor to lay their eggs on the mussels' gills through the exhalant siphon of the mussel. Males release sperm over the inhalant siphon of the mussel so that water filtered by the mussel carries the sperm through the gills and fertilization occurs inside the gill chamber. Embryos hatch after 2 days but remain inside the mussel for about a month (Aldridge 1999). A typical breeding season fecundity of female bitterling is 80–250 eggs; females spawn in several bouts lasting 1–3 days and consisting of about five independent spawnings (separated by a resting interval of more than 15 min) each day (Smith *et al*. 2000a, 2004). One to six (usually two or three) eggs are laid during a single spawning (Smith *et al*. 2000a; Mills & Reynolds 2002). Bitterling spawn from late April to June, although most spawnings occur within a 3-week period (Douglas 2003). The number of spawnings in which a male may participate during a breeding season is not known, but is presumably higher than that of females, as more than one male usually participates in a typical spawning (Smith *et al*. 2002, 2003, 2004) and the population sex ratio is equal (Smith *et al*. 2000a). Females are choosy about where they spawn, because mussels used for oviposition and territory holders vary in their quality (Smith *et al*. 2000a,b). The extent of carotenoid-based red nuptial colouration (Candolin & Reynolds 2001), which correlates with male body size in our study population (Smith *et al*. 2002), signals male quality.

Sneaking behaviour, when one male releases sperm into a mussel guarded by another male, is common in bitterling (Smith *et al*. 2003, 2004). In addition to nonterritorial males, territory holders also commonly invade adjacent territories to sneak (Kanoh 1996, 2000; Candolin & Reynolds 2002a,b; Smith *et al*. 2002, 2003, 2004). There is no evidence of a genetic or morphological distinction between territorial and sneaking males (Smith *et al*. 2004). Both territorial and sneaking *R. sericeus* often perform preoviposition ejaculations, releasing sperm before a female spawns (Kanoh 1996; Candolin & Reynolds 2002a; Smith *et al*. 2002, 2003), presumably to obtain precedence for their sperm within the gill chamber of the mussel.

The bitterling is an ideal model for investigating the reproductive success of alternative mating behaviours in relation to spawning site availability, spawner density and operational sex ratio. Bitterling behaviour can be observed readily in the field and laboratory, and bitterling ejaculation is characteristic; the male sweeps forward and down quickly over the inhalant siphon of the mussel and a greyish cloud is sometimes visible upon sperm release. Also, bitterling spawning sites (mussels) can be manipulated readily.

To examine how male density affects the fitness pay-offs of alternative mating behaviours, we conducted two experiments. First, we studied the reproductive success of individual territorial and sneaker males when competing with a different number of rivals in a laboratory experiment. Second, we used a relatively large-scale mesocosm study to manipulate male density under seminatural conditions, estimating reproductive success of alternative mating behaviours across male densities at two spatial scales.

Materials and methods

Experiments

All experiments were conducted at the Institute of Vertebrate Biology (IVB), Brno, Czech Republic in May 2002. Fish used in the experiments were electrofished in the River Kyjovka in the southeast of the Czech Republic (the centre of geographical distribution of *R. sericeus* in Europe) and transported to the aquarium facilities at IVB. They were separated by sex and housed in seven aquaria (75 (length) × 40 (width) × 40 (depth) (cm) with an external filter and continuous aeration. Fish were held under a natural light cycle and fed *ad libitum* twice a day with a mixture of live, frozen and flake food. Water temperature matched natural conditions, varying between 18 and 21 °C. Two species of mussels, *Unio pictorum* and *U. tumidus*, were collected from an oxbow lake adjacent to the River Kyjovka before the start of the bitterling spawning season

and stored in sand-filled containers in a shaded garden pond, as described previously by Smith *et al*. (2003).

In the laboratory experiment, a randomly predetermined number of males [1–7; a natural range of males that participate in a typical spawning (Smith *et al*. 2003)] were stocked in experimental aquaria (75 × 40 × 40 cm), each with a 2 cm layer of sand substrate and two artificial plants as refuges. Experimental aquaria were isolated using opaque barriers so that fish in adjacent aquaria could not interact. One *U. tumidus* in a sand-filled flowerpot was placed in the centre of each aquarium. The second day after stocking (when territorial dominance by one male had been established), a female with an extended ovipositor was introduced into the aquarium and remained there until she spawned. Territorial males were recognized by their overt aggression and dominance, and were identified individually by obvious unique features (e.g. colour pattern, lost scales, external parasites). After a spawning occurred, the fish were not disturbed for at least a further 8 min; in previous studies it was shown that postoviposition ejaculation ceased after this interval (Smith *et al*. 2003). In some cases, spawnings were not monitored continuously and more than a single spawning may have occurred during a test. After spawning was completed, all fish were captured, measured to the nearest 1 mm for Standard Length (SL, from the tip of the snout to the end of caudal peduncle) and a fin clip taken and stored in 96% ethanol. Mussels were isolated for 7 days, after which developing bitterling embryos were removed by dissection and fixed in 96% ethanol. In a subset of replicates, male behaviour (aggression of territorial male, courting and leading of females and ejaculation of territorial and sneaking males) was recorded throughout the time the female was in the aquarium, with a distinction drawn between pre- and postoviposition behaviour.

The pool experiment was conducted in a large outdoor concrete pool at IVB. The pool measured 12.4 × 6.0 m, with a water depth of 0.6 m. Water temperature varied naturally between 17 and 24 °C and water visibility was always more than 3 m. Ten *U. pictorum* were placed separately in sand-filled flowerpots and distributed in pairs among five arenas around the pool perimeter. Arenas were at least 6 m apart and separated by artificial vegetation, such that territory holders from different arenas did not interact. Within 1 day of stocking, males had established territories around mussels and females formed large shoals and had begun visiting male territories to spawn. Fish grazed on a carpet of algae that established on the walls and floor of the pool and were additionally fed *ad libitum* daily on frozen bloodworm.

We examined the effects of male density at two spatial scales. We termed the total male density in the pool as 'global male density', while 'local male density' was defined as the proximity between territories in an arena. Two levels of each density treatment were tested. At the low global density treatment, 32 males were stocked in the pool and at the high global density 64 males were stocked. Based on intrusion rates between adjacent territory holders, we defined high local male density when mussels were positioned 75 cm apart (high intrusion rate) and low local density for a distance of 150 cm (low intrusion rate) (Reichard *et al*. 2004). Two *U. pictorum* were always placed 150 cm apart in each arena when observations were not taking place to ensure that territorial males were always present. Female density was constant throughout the experiment, with 170 females stocked.

For each experimental observation, the resident *U. pictorum* in a randomly chosen arena was replaced by a *U. tumidus* that contained no bitterling embryos. A different mussel species was used to prevent any mix-up between resident and experimental mussels during the experiment. Each observation was chosen in a random, predetermined order to be either a high (75 cm) or low (150 cm) local density. Due to logistic constraints, replicates within global density treatments were investigated sequentially; all replicates within the low global male density between 16 and 19 May and within the high global density between 21 and 25 May. However, each replicate was a single, independent spawning event with different territorial males, females and mussels. Consequently, in our analyses we treated all replicates (spawnings) as independent observations.

During each test, a diver recorded fish behaviour from a distance of approximately 1.5 m. Exact timing of ejaculations by territorial male and individually distinguished rivals was recorded. Male behaviour (aggression of territorial male, courting and leading females) and spawning interruptions were also recorded, following an established protocol (Reichard *et al*. 2004). Individual males were recognized according to unique features of their appearance (Smith *et al*. 2000b, 2002, 2003), because individual marking could affect fish behaviour and mate choice. Once a spawning occurred, the precise time was recorded and a cylindrical (diameter 80 cm) net set around each spawning arena was gently raised. After raising the net, males continued to ejaculate into mussels and observations lasted until this behaviour ceased (2–8 min after oviposition). All the fish retained in the net were captured using a hand net, measured for SL to the nearest 1 mm and fin-clipped. The mussel in which spawning had occurred was isolated and after 7 days embryos were removed and stored in 96% ethanol. Nonterritorial males were immediately returned to the pool, while territorial males were placed in an aquarium pending release and were not used again in the study. Territorial males were replaced with another male of equivalent size from a stock of males held in a 2 m length keep net in the pool. Thus, every male was observed only once (one spawning) in a territorial role. Females that had been caught and fin-clipped after spawning were similarly removed from the pool and were not used again. Newly

released males were seen to participate in spawnings and defend territories within 1 h following their release. Two divers observed spawning in the five arenas, with a maximum of one observation in 5 h at an arena and a maximum of eight observations each day in total.

Paternity analysis

Putative parents and embryos from 14 groups from the aquarium experiment and 17 spawnings from the pool experiment were genotyped. The 17 spawnings used in paternity estimates were a representative subsample from the 52 spawnings observed in total. No significant difference was found for the number of males that participated in spawning and male behaviour (the rates of territorial male aggression, courting and leading, territorial and sneaker male ejaculations, spawning interruptions and time to a successful spawning) between the spawnings for which paternity was analysed and the spawnings without paternity analysis (GLM ANOVA, d.f. = 1,50, all $P > 0.34$). No territory take-overs occurred during the course of the aquarium experiment. Samples were excluded from paternity analysis if the territorial male was not captured (pool experiment) or no embryo was recovered from the mussel (both experiments), which yielded 14 (of 42) and 17 (of 52) replicates for aquarium and pool experiments, respectively. Embryo mortality in the mussel gill cavity is common (Smith *et al.* 2000a; Mills & Reynolds 2002), and may have increased under the experimental protocol as a result of moving the mussels (pers. obs.). We assumed that embryo mortality was not biased towards offspring fertilized by particular males (Evans *et al.* 2003).

DNA from fin samples or embryos with yolk sac removed was isolated using QIAmp DNA Minikit extraction kit (Qiagen). DNA was amplified in a 9 µL reaction volume containing 0.6 U *Taq* polymerase (Invitrogen), 2.5 pmol of dye-labelled forward primer, 2.5 pmol of reverse primer, 0.5 mM $MgCl_2$ and 0.15 mM of each dNTP in the manufacturer's buffer. Polymerase chain reaction (PCR) cycling consisted of an initial 3 min denaturation at 94 °C, 30 cycles of 40 s at 94 °C, 30 s at 65 °C and 40 s at 74 °C, and a final extension at 72 °C for 7 min. PCR product, 0.4–0.8 µL, was mixed with 1.4 µL of deionized formamide, 0.25 µL Tamra size standard and 0.36 µL of loading buffer and run on 6% acrylamid gel on a 373A Applied Biosystem automated sequencer. Fragment lengths were determined using GENOTYPER 2.0 software. All individuals from one spawning were assayed on the same gels. Eight microsatellite loci were used: *Rser*01–06, *Rser*08 and *Rser*10 (Dawson *et al.* 2003) with a mean of 8 (range 4–24) alleles per one locus. Observed heterozygosities ranged from 0.29 to 0.81. Paternity was assigned using CERVUS 2.0 software (Marshall *et al.* 1998). Combined exclusionary power was 82% for samples without known maternal genotype and 96% when the mother was known (we possessed the maternal genotype for 91.2% of analysed embryos). Paternity was assigned with 95% confidence in 92 of 113 offspring and with a relaxed 80% confidence in an additional 12 cases. LOD scores (log-likelihood that putative parent is a true parent relative to other candidates) of the second most probable fathers in most of these 12 cases were negative, discounting their assignment as sires (Marshall *et al.* 1998). However, in the aquarium experiment, two sneaker males in three cases (one embryo from each replicate) and a sneaker and territorial male in two cases (two embryos from the same replicate) obtained similar LOD scores and did not mismatch the embryo genotype at any locus. In these cases, paternity was assigned to the male with the highest LOD score. Altering paternity assignment between candidate males with similar LOD scores did not affect the results of our subsequent analyses. In the pool experiment, four embryos from three spawnings did not match the genotype of any male captured (but all matched the maternal genotype) and these were designated as having been sired by a noncaptured sneaker male. The noncaptured males ejaculated within 1, 2 and 14 min before oviposition for the three spawnings, respectively. Exclusion of embryos sired by noncaptured males from the analyses did not affect the overall results, although it had the effect of decreasing the significance of statistical tests. Parent–embryo mismatches that occurred in three replicates were consistent with the occurrence of a null allele at locus *Rser*04.

Data analysis

The reproductive success of individual males was calculated as a percentage of embryos they sired in a given spawning. Each spawning (pool experiment) or group (aquarium experiment) were treated as independent replicates. The occurrence of cuckoldry refers to a situation when at least one embryo was not sired by the territory holder. To compare body size between territorial and sneaker males in the aquarium experiment, the mean SL of sneakers in a group was used. Similarly, average sneaker male success was used in comparisons of reproductive success between alternative behaviours across male densities to avoid pseudoreplication. Data were log-, square-root or arcsine-transformed to meet assumptions of parametric tests where necessary and nonparametric equivalents were used where transformations were not successful. All tests are two-tailed and all means and estimates are presented with 1 SEM or 1 SEE, respectively.

The number of replicates in some of our analyses was lower than planned because of embryo mortalities during incubation in mussels, although comparable to other fish parentage studies (DeWoody & Avise 2001). Consequently, we present the *post hoc* calculated power of our main statistical tests as recommended by Jennions & Møller (2003).

For nonparametric tests, we calculated the power of the respective parametric test (Zar 1999; Jennions & Møller 2003). Power values < 0.30 (Jennions & Møller 2003) for non-significant results were treated with caution (but see Hoenig & Heisey (2001) for a critique of *post hoc* power testing).

The number of effective sneakers is the number of sneakers that ejaculated successfully into the test mussel within the period 15 min before and 8 min after oviposition. The time of 15 min is a conservative measure. The sperm of *R. ocellatus* were shown to fertilize eggs *in vitro* up to 4 min after stripping from males (Kanoh 1996). However, we observed a successful fertilization by a male that ejaculated 14 min before oviposition (see above). It is possible that bitterling sperm have greater longevity in the environment of a mussel gill cavity.

To quantify sperm loading by bitterling males, we calculated the number of ejaculations per spawning. Figures refer to the whole period of the observation and sums pre- and postoviposition ejaculations. The proportion of ejaculations that occurred within 1 min before and after oviposition was calculated from data combined for all spawnings. The number of total effective ejaculations was calculated as the sum of effective preoviposition (15 min before oviposition) and effective postoviposition (8 min after oviposition) ejaculations. Ejaculation Index (*EI*) was calculated as the relative proportion of territorial and rival male's ejaculations, and took the form $EI = \Sigma e_{\text{T}}/(\Sigma e_{\text{T}} + \Sigma e_{\text{S}})$ where Σe_{T} is the sum of territorial male effective ejaculations and Σe_{S} is the sum of the effective ejaculations of all rival males. Thus, a value of 0.5 indicates equal effective ejaculation between territorial male and sneakers. A value < 0.5 shows that sneakers ejaculated relatively more frequently than the territorial male and a value > 0.5 vice versa.

The stepwise forward procedure of General Linear Multiple Regression was used to identify correlates of the reproductive success of an individual territorial male. At each step of the model construction, a variable that explained most of the variability was confronted with criteria for addition to the model, and included when it significantly improved the existing model. Before analysis, the number of sneakers and ejaculations for several time periods before and after oviposition were correlated with the reproductive success of the territorial male, and those that explained most variability was entered in the analysis (data not shown).

Results

Aquarium experiment

A total of 73 embryos from 14 groups were obtained from spawnings in aquaria, with 1–21 (median 3.5) embryos, which indicates that multiple spawnings occurred in some groups. The number of embryos recovered from mussels

Table 1 Treatment (number of sneakers in aquarium), number of embryos recovered and reproductive success (measured as a percentage of embryos sired) of territorial and sneaker males in aquarium experiment. Medians with interquartile range in parentheses are shown. Sneaker male success was calculated on an individual basis. Egg mortality during incubation reduced the number of successful replications. Consequently, treatments with 3–6 sneaker males were grouped to increase the power of *post hoc* tests. Further, behavioural observations of a subset of replicates revealed that not all males always participated in spawnings when sneaker density was > 3

Treatment	n	Embryos	Territorial male	Sneaker male
0	1	2	100	—
1	6	3 (1–4)	100 (67–100)	0 (0–33)
2	3	5 (3–6)	16.7 (0–60)	40 (0–83)
3–6	4	7 (3–16)	27.1 (7–58)	10 (0–19)
Total	14	3.5 (3–6)	63.3 (17–100)	10 (0–29)

was positively correlated with the number of males in aquaria (Spearman's correlation, $r_{\text{s}} = 0.632$, $n = 14$, $P = 0.015$) but our experimental design did not permit further investigation of this pattern. Multiple paternity occurred frequently (Table 1) but could not be linked directly to reproductive behaviour due to the lack of behavioural observations in a subset of replicates. The reproductive success of territorial males declined with an increasing number of sneakers. Territorial males that competed with more than one sneaker sired a significantly lower proportion of offspring than territorial males that competed with a single sneaker (ANOVA, $F_{2,10} = 5.44$, $P = 0.025$, power = 0.72; Tukey's HSD *post hoc* comparisons, $P = 0.006$). The reproductive success of individual sneaker males did not differ among male density treatments (ANOVA, $F_{2,10} = 1.02$, $P = 0.40$; Table 1), but the power of this test was low (power = 0.18) due to the high variation among individual sneakers. Territorial males sired a significantly higher proportion of embryos than individual sneaker males when competing with a single sneaker (paired *t*-test, $t_5 = 2.93$, $P = 0.033$, power = 0.65) but not when competing with two or more (paired *t*-test, $t_5 = 0.29$, $P = 0.782$, power = 0.06) (Table 1). Territorial males (SL, mean 48.1 ± 0.63 mm, range 41–55 mm, $n = 13$) were significantly larger than sneakers (SL, mean 40.6 ± 0.47 mm, range 36–47 mm, $n = 30$) (paired *t*-test, $t_{12} = 10.8$, $P < 0.001$). For the subset of groups for which behaviour was recorded during spawning, male behaviour was similar to that for males in the pool experiment (data not shown).

Pool experiment

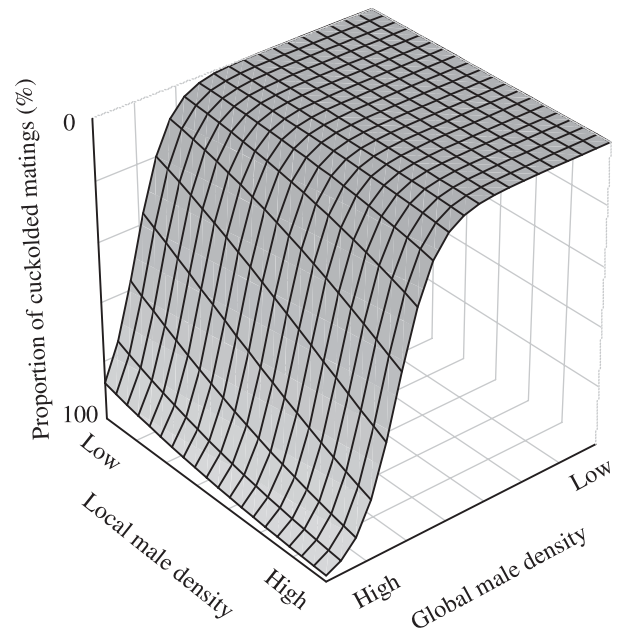The number of effective sneakers depended on male density treatment and increased with global (factorial
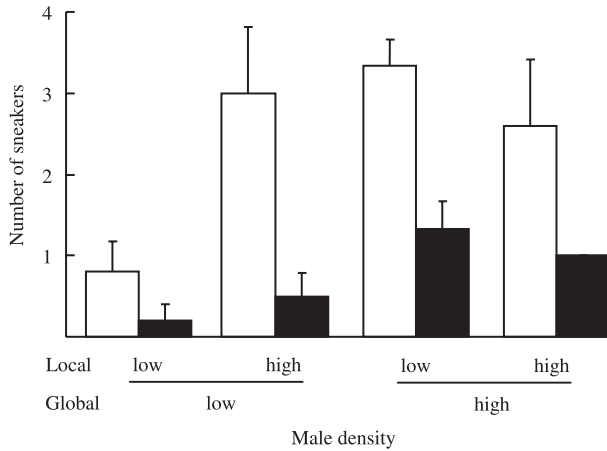
Fig. 1 Number of sneaker males that participated in analysed spawnings (white bars) and the number of successful sneakers (that sired at least one embryo; black bars) among four male density treatments in the pool experiment. Means (+ 1 SE) are shown. Note that there is no variance in the number of successful sneakers at high-high density.
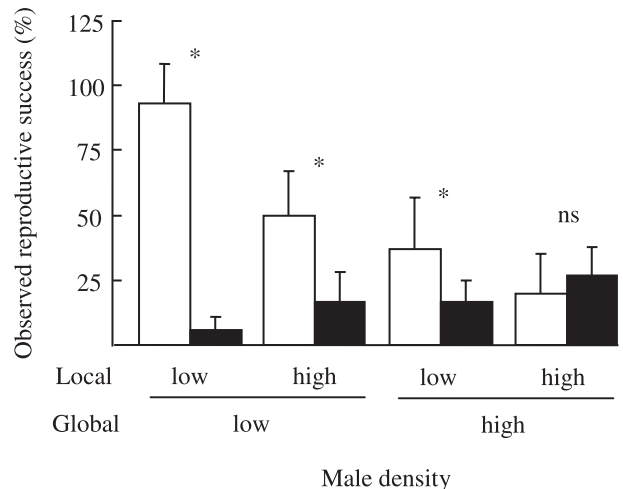

Fig. 2 The results of multiple logistic regression showing effects of global and local male densities on the occurrence of cuckoldry in the pool experiment.


Fig. 3 Mean (+ 1 SE) observed relative reproductive success of territorial (white) and sneaker (black) males among four male density treatments in the pool experiment. Asterisk indicates statistical differences between alternative behaviours at $\alpha = 0.05$. For differences in reproductive success within each reproductive behaviour, see Results.

GLM ANOVA, $F_{1,48} = 7.02$, $P = 0.011$, power = 0.74) but not local ($F_{1,48} = 3,76$, $P = 0.058$, power = 0.48) density. A significant interaction effect ($F_{1,48} = 4.97$, $P = 0.030$, power = 0.59) showed that the number of sneaker males increased with the local male density at low but not at high global density (Fig. 1). Territorial males (SL, mean 48.3 ± 0.75 mm, range 37–58 mm, $n = 39$) were larger than their rivals (SL, mean 43.1 ± 0.86 mm, range 35–54 mm, $n = 41$) (unpaired t-test, $t_{78} = 4.60$, $P < 0.001$). The mean number of ejaculations per spawning was 15 ± 1.3 for territorial males and 16 ± 2.6 for sneakers (all individuals pooled).

A total of 40 (mean 2.4 ± 0.31) embryos were obtained from 17 spawnings. Eleven (65%) spawnings were cuckolded. Male density significantly increased the occurrence of cuckoldry (multiple logistic regression, $\chi^2 = 11.35$, d.f. = 2, $P = 0.003$; Fig. 2) with a significant effect of global (logistic regression, $\chi^2 = 10.62$, d.f. = 1, $P = 0.001$), but not local density (logistic regression, $\chi^2 = 1.45$, d.f. = 1, $P = 0.229$). Indeed, only three of nine spawnings were cuckolded at low global density, compared with all eight spawnings cuckolded at high global density. Multiple paternity occurred in seven of 11 cuckolded spawnings. At least eight eggs from five spawnings could have been fertilized only by preoviposition ejaculation; in these cases the assigned sire did not ejaculate after oviposition (the last ejaculation of the sire in each case was 1, 2, 5, 13 and 14 min before oviposition). No fertilization could be assigned exclusively to postoviposition ejaculation.

The proportion of offspring sired by territorial males (Fig. 3) decreased with an increase in global male density (unpaired t-test, $t_{15} = 2.36$, $P = 0.032$, power = 0.60). An average of $74 \pm 14.5\%$ offspring were sired by territorial

males at low global density, but only $33 \pm 8.9\%$ at high global density. The effect of local male density was not significant (unpaired t-test, $t_{15} = 1.80$, $P = 0.092$, power = 0.39). Territorial males sired $72 \pm 12.0\%$ of offspring at the low local density and $39 \pm 13.9\%$ at high local density. The proportion of embryos sired by an individual sneaker male

Table 2 Correlation between reproductive success of territorial male and pre- and postoviposition ejaculation events, number of sneaker males, territorial male aggression and territorial male body size

	Preoviposition		Postoviposition		Total	
	r	P	r	P	r	P
Territorial male success						
Territorial male ejaculation*	−0.057	0.829	0.312	0.223	0.001	0.996
Rival males ejaculation*	−0.558	0.020	−0.221	0.392	0.528	0.029
Number of rival males	−0.508	0.038	−0.123	0.638	−0.470	0.057
Ejaculation index	0.592	0.012	0.404	0.108	0.530	0.029
Terr. male aggression rate*	0.014	0.958	0.408	0.109	0.286	0.266
Territorial male body length	—	—	—	—	−0.262	0.327
Number of rival males						
Territorial male ejaculation*	0.424	0.090	0.452	0.069	0.127	0.626
Rival males ejaculation*	0.923	< 0.001	0.896	< 0.001	0.911	< 0.001
Ejaculation index	−0.811	< 0.001	−0.773	< 0.001	−0.725	< 0.001

*Data were $\log_{10}$ transformed prior to analysis.
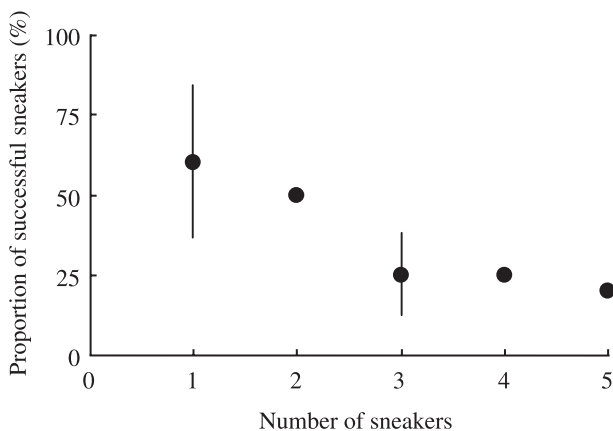
Fig. 4 Individual success of sneaking males (which fertilized at least one embryo) with varying number of rival sneakers in the pool experiment. Note that a territorial male also participated in spawnings at all sneaker abundances.

was significantly higher at high global (Mann–Whitney test, $n_{low} = 8$, $N_{high} = 9$, $Z = 2.27$, $P = 0.024$, power = 0.20) but not high local male density, although the result approached significance (Mann–Whitney test, $n_{low} = 8$, $n_{high} = 9$, $Zz = 1.77$, $P = 0.076$, power = 0.44; Fig. 3). Territoriality conferred a higher reproductive success than sneaking when global (paired t-test, $t_{15} = 2.45$, $P = 0.040$) or local (paired t-test, $t_{17} = 4.04$, $P = 0.005$) male density was low. No significant difference in reproductive success between alternative behaviours was detected at the high global (paired t-test, $t_{17} = 0.01$, $P = 0.989$) and high local (paired t-test, $t_{15} = 0.10$, $P = 0.921$) male densities. The number of successful sneakers increased with the number of sneakers involved in a spawning (Fig. 1), although the percentage of successful sneakers decreased (Fig. 4).

There was no relationship between the number of effective ejaculations, aggression or body size and territorial male reproductive success (Pearson's correlation; Table 2). However, both the number of sneakers engaging in effective ejaculations and the sum of sneakers' effective ejaculations decreased the proportion of offspring sired by territorial males (Pearson's correlation; Table 2). The reproductive success of territorial males increased with their participation in sperm loading (Pearson's correlation for *EI*; Table 2), although even with a 10-fold higher frequency of ejaculations compared with sneakers, territorial males did not always ensure their paternity in a spawning (Fig. 5). Preoviposition events explained the pattern of territorial male reproductive success better than postoviposition events (Table 2).

A model that best explained the proportion of embryos sired by territorial males (GLM stepwise forward regression, $R^2 = 0.350$, $F_{1,15} = 8.08$, $P = 0.012$; Table 3) contained preoviposition *EI* as a single explanatory variable (estimate: 96.2 ± 33.83, $t_{16} = 2.84$, $P = 0.012$). Other variables that correlated with territorial male success (Table 2) were also correlated with preoviposition *EI* and had no additional explanatory power when preoviposition *EI* was included in the model (Table 3). The intercept of the model was not significantly different from zero (estimate: $−10.5 \pm 10.35$, $t_{17} = 0.43$, $P = 0.627$) and was excluded from the final model, which took the form $RS_T = 96.2 * EI$.

Discussion

We used an experimental manipulation of male density in laboratory and mesocosm studies to show that the reproductive success of alternative mating behaviours in *R. sericeus* was density-dependent. We demonstrated
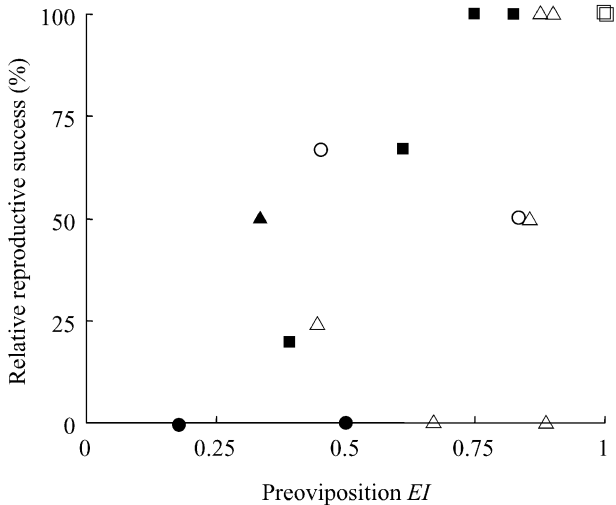
Fig. 5 The relationship between preoviposition ejaculation index (calculated as the relative proportion of territorial and rival male ejaculations) and proportion of embryos sired by territorial males. The value of 0.5 indicates equal ejaculation effort between territorial and sneaking males. Values < 0.5 show that sneakers outcompeted the territorial male and a value > 0.5 *vice versa*. Different points denote number of sneaking males present at a spawning (□: 0 rivals; △: 1 rival; ○: 2 rivals; ■: 3 rivals; ▲: 4 rivals; ●: 5 rivals.

Table 3 Results of general linear multiple regression model using a forward stepwise procedure for inclusion of explanatory variables

Variable	F	P	Outcome
Preoviposition EI	8.08	0.012	Included
Courting rate of territorial male	2.10	0.169	Not included
Body size of territorial male	0.70	0.416	Not included
Aggression rate of territorial male	0.43	0.521	Not included
Postoviposition EI	0.06	0.810	Not included
Ejaculations of sneaker males	0.05	0.827	Not included
Number of effective sneakers	0.05	0.829	Not included
Ejaculations of territorial male	0.02	0.895	Not included

that sneaked fertilizations were common, and increased with male density. In total, 65% of all spawnings in the seminatural pool experiment were cuckolded. At our lowest male density treatment territoriality was, on average, almost 17 times more successful than sneaking. The average success of territorial males at intermediate male densities was 2–3 times higher than that of sneaking males. At the highest male density, territoriality and sneaking conferred the same fitness pay-offs (Fig. 3). In a companion paper, we have shown that both global and local male densities affected bitterling spawning behaviour and spawning rate and that they interacted in their effects (Reichard *et al.* 2004). Territorial males became more aggressive at higher male densities when they encountered more rivals. This effect decreased the total spawning rate of the population, as territorial males often stopped courting a female when engaged in aggression directed at rivals, resulting in interrupted spawning. However, territorial aggression decreased at the highest male density, with a stabilizing effect on the spawning rate (Reichard *et al.* 2004). The present study shows that this response may be adaptive; males appeared to abandon energetically expensive territorial defence at the highest density, because the reproductive success of territorial males was no greater than that of a sneaker. Alonzo & Warner (1999) observed a comparable response by territorial males in the Mediterranean wrasse, *Symphodus ocellatus*, a species with paternal care. Territorial males failed to spawn with receptive females when the density of sneakers in the proximity to a nest was high, but they spawned immediately after sneakers were experimentally removed.

We found that in the pool experiment, the reproductive success of individual sneaking males was higher at the high global and high local male densities. In the aquarium experiment, we detected no difference in reproductive success with density treatment among males adopting the sneaking behaviour, although the power of the test in this case was low. Territorial males usually outcompeted a single sneaker, but the individual reproductive success of sneakers decreased as a function of the number of males that participated in a spawning (Fig. 4). The mean number of sneakers that participated in spawnings in the experimental pool varied from 0.9 to 3.1 (total range 0–7) across male density treatments (Fig. 1). These densities are comparable with natural situations where we have recorded 1.6 ± 0.31 (range 0–10) sneaker males per spawning in a field study (Smith *et al.* 2003) and a mean of 1.25 (range 0–7) males per spawning reported by Kanoh (2000) for *R. ocellatus*.

As male bitterling do not provide parental care to offspring, the cost of cuckolded spawning is lower than that of fish species with paternal care. However, the fact that the mortality of embryos inside the mussel is density-dependent (Smith *et al.* 2000a) means that there is a cost to offspring from future spawnings and also a decrease in mussel attractiveness to females (Smith *et al.* 2000b). Approximately one-third of nests have been shown to be cuckolded to differing degrees in fishes with extended male parental care, territorial males typically siring between 70 and 95% of offspring in their nests (reviewed by DeWoody & Avise 2001). These results are comparable with our lowest male density treatment. In species that do not provide care to their offspring, such as Atlantic salmon *Salmo salar*, the average reproductive success of dominant males may vary greatly, but is typically > 50% (Thomaz *et al.* 1997). Our results indicate that territoriality in bitterling may be adaptive even when the territorial male obtains

paternity of less than 50%, depending on male density (Fig. 3) and spawning site availability (Reichard *et al.* 2004).

Preoviposition ejaculation events were the best predictor of the outcome of sperm competition, with eggs from at least five (and potentially all 17) spawnings fertilized by sperm released before oviposition. Deposition of sperm at the spawning site before the eggs are laid is characteristic of bitterling (Taborsky 2001), and may be important in this species because the sperm must pass through mussel gills to reach the egg. Our results demonstrate that sperm may fertilize the eggs at least 14 min after ejaculation, showing that bitterling ejaculates may be adapted to the microenvironment of the mussel gill chamber. Further research is required to investigate sperm and ejaculate adaptation to mussel gills, and the mechanism of sperm precedence in fertilization.

Territorial males increased their probability of reproductive success by outcompeting sneaking males by sperm loading (Table 2, Fig. 5). However, they did not always engage in sperm loading, especially when more than two rivals competed for fertilization (Fig. 5; Parker *et al.* 1996; Candolin & Reynolds 2002a; Smith *et al.* 2003). In addition, the outcome of preoviposition sperm competition, although significantly affecting fertilization success, was not its ultimate predictor (Fig. 5). Males that engage in sneaking are generally predicted to be superior in sperm competition as they are expected to devote their reproductive investment to sperm production rather than mate acquisition (Parker 1990, 1998). In bluegill sunfish *Lepomis macrochirus*, sneaking males were shown to sire more offspring than territorials in spawnings in which they participated, although territorial males took part in more spawnings (Fu *et al.* 2001). At present, we have few data on sperm allocation differences among male bitterling. However, reproductive behaviour of male bitterling is opportunistic and territorial males often behave as sneakers in neighbouring territories (Kanoh 2000; Smith *et al.* 2004). Further, no variation in the number of sperms in ejaculates of individual *R. sericeus* was found by Candolin & Reynolds (2002a), suggesting that bitterling may, contrary to some other fishes (Warner *et al.* 1995; Pilastro *et al.* 2002; Zbinden *et al.* 2003), lack the capability to adjust ejaculate size. Relative and absolute sperm allocation and expenditure may vary greatly among individuals (Warner *et al.* 1995) but, contrary to the assumption of the sneak-guard model, larger males may invest relatively and absolutely more in sperm if they also engage in sneaking (Zbinden *et al.* 2001; Neat & Locatello 2002).

Sperm competition in *R. sericeus* is intense compared with other fishes. In our experiment, territorial males typically ejaculated 15 times per single spawning, a value comparable to field observations (Smith *et al.* 2003). As male *R. sericeus* mate repeatedly during each day throughout the breeding season, the high number of ejaculations for each spawning raises the possibility of sperm depletion (Warner *et al.* 1995; Weddel *et al.* 2002). The finding that the number of embryos that were recovered from mussels correlated with the number of males that participated at spawning suggests that there may be a direct benefit for females to mate with multiple males. Indeed, in a separate study we found that female bitterling actively solicited sneaking (C. Smith & M. Reichard unpubl. data). The question of whether the benefit stems from an insurance of fertilization (a particular problem for bitterling, given that sperm must pass through the mussel gill) or a decrease in probability of genetic incompatibility, calls for further investigation. Our future studies on bitterling will be directed at quantifying sperm allocation, expenditure and quality, as well as comparative studies on sperm adaptation to the mussel gill and male reproductive behaviour in species from Europe and Asia.

Acknowledgements

References

Aldridge DC (1999) Development of European bitterling in the gills of freshwater mussels. *Journal of Fish Biology*, **54**, 138–151.

Alonzo SH, Warner RR (1999) A trade-off generated by sexual conflict: Mediterranean wrasse males refuse present mates to increase future success. *Behavioral Ecology*, **10**, 105–111.

Candolin U, Reynolds JD (2002a) Adjustments of ejaculation rates in response to risk of sperm competition in a fish, the bitterling (*Rhodeus sericeus*). *Proceedings of the Royal Society London, Series B*, **269**, 1549–1553.

Candolin U, Reynolds JD (2001) Sexual signaling in the European bitterling: females learn the truth by direct inspection of the resource. *Behavioral Ecology*, **12**, 407–411.

Candolin U, Reynolds JD (2002b) Why do males tolerate sneakers? Tests with the European bitterling, *Rhodeus sericeus*. *Behavioural Ecology and Sociobiology*, **51**, 146–152.

Dawson DA, Burland TM, Douglas A, Le Comber SC, Bradshaw M (2003) Isolation of microsatellite loci in the freshwater fish, the bitterling *Rhodeus sericeus* (Teleostei: Cyprinidae). *Molecular Ecology Notes*, **3**, 199–202.

DeWoody JA, Avise JC (2001) Genetic perspectives on the natural history of fish mating systems. *Journal of Heredity*, **92**, 167–172.

Douglas A (2003) *Population ecology and behaviour of juvenile bitterling* (Rhodeus sericeus, *Pallas*). PhD Thesis, Queen Mary College, University of London.

Evans JP, Zane L, Francescato S, Pilastro A (2003) Directional post-copulatory sexual selection revealed by artificial insemination. *Nature*, **421**, 360–363.

Fu P, Neff BD, Gross MR (2001) Tactic-specific success in sperm competition. *Proceedings of the Royal Society London, Series B*, **268**, 1105–1112.

Gross MR (1996) Alternative mating strategies and tactics: diversity within sexes. *Trends in Ecology and Evolution*, **11**, 92–98.

Gross MR, Charnov EL (1980) Alternative male life histories in the bluegill sunfish. *Proceedings of the National Academy of Sciences USA*, **77**, 6937–6940.

Hoenig JM, Heisey DM (2001) The abuse of power: the pervasive fallacy of power calculations for power analysis. *American Statistician*, **55**, 19–24.

Jennions MD, Møller AP (2003) A survey of the statistical power of research in behavioral ecology and animal behavior. *Behavioral Ecology*, **14**, 438–445.

Kanoh Y (1996) Pre-oviposition ejaculation in externally fertilizing fish: how sneaker male rose bitterlings contrive to mate. *Ethology*, **102**, 883–899.

Kanoh Y (2000) Reproductive success associated with territoriality, sneaking, and grouping in male rose bitterling, *Rhodeus ocellatus* (Pisces: Cyprinidae). *Environmental Biology of Fishes*, **57**, 143–154.

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**, 639–655.

Mills SC, Reynolds JD (2002) Host species preferences by bitterling, *Rhodeus sericeus*, spawning in freshwater mussels and consequences for offspring survival. *Animal Behaviour*, **63**, 1029–1036.

Neat FC, Locatello L (2002) No reason to sneak: why males of all sizes can breed in the hole-nesting blenny, *Aidablennius sphinx*. *Behavioral Ecology and Sociobiology*, **52**, 99–73.

Parker GA (1990) Sperm competition games: sneaks and extrapair copulations. *Proceedings of the Royal Society London, Series B*, **242**, 127–133.

Parker GA (1998) Sperm competition and the evolution of ejaculates: towards a theory base. In: *Sperm Competition and Sexual Selection* (eds Birkhead TR, Møller AP), pp. 3–54. Academic Press, San Diego.

Parker GA, Ball MA, Stockley P, Gage MJG (1996) Sperm competition games: individual assessment of sperm competition intensity by group spawners. *Proceedings of the Royal Society London, Series B*, **263**, 1291–1297.

Pilastro A, Scaggiante M, Rasotto MB (2002) Individual adjustment of sperm expenditure accords with sperm competition theory. *Proceedings of the National Academy of Sciences USA*, **99**, 9913–9915.

Reichard M, Jurajda P, Smith C (2004) Male–male interference competition decreases spawning rate in the European bitterling (*Rhodeus sericeus*). *Behavioral Ecology and Sociobiology*, DOI 10.1007/s00265–004–00760–2.

Rico C, Kuhnlein U, FitzGerald GJ (1992) Male reproductive tactics in the threespine stickleback: an evaluation by DNA fingerprinting. *Molecular Ecology*, **1**, 79–87.

Schaumburg J (1989) Zur Ökolgie von Stichling *Gasterosteus aculeatus* L., Bitterling *Rhodeus sericeus amarus* Bloch, 1782 und Moderlischen *Leucaspius delineatus* (Heckel, 1843) — drei bestandsbedrohten, einheimischen Kleinfischarten. *Berichte der Akademie für Naturschutz und Landschaftspflege*, **13**, 145–194.

Smith C, Reynolds JD, Sutherland WJ (2000a) The population consequences of reproductive decisions. *Proceedings of the Royal Society London, Series B*, **267**, 1327–1334.

Smith C, Douglas A, Jurajda P (2002) Sexual conflict, sexual selection and sperm competition in the spawning decisions of bitterling (*Rhodeus sericeus*). *Behavioral Ecology and Sociobiology*, **51**, 433–439.

Smith C, Reichard M, Jurajda P (2003) Assessment of sperm competition by European bitterling, *Rhodeus sericeus*. *Behavioral Ecology and Sociobiology*, **53**, 206–213.

Smith C, Reichard M, Jurajda P, Przybylski M (2004) The reproductive ecology of the European bitterling (*Rhodeus sericeus*). *Journal of Zoology*, **262**, 107–124

Smith C, Reynolds JD, Sutherland WJ, Jurajda P (2000b) Adaptive host choice and avoidance of superparasitism in the spawning decisions of bitterling (*Rhodeus sericeus*). *Behavioral Ecology and Sociobiology*, **48**, 29–35.

Taborsky M (1994) Sneakers, satellites, and helpers: parasitic and cooperative behaviour in fish reproduction. *Advances in the Study of Behaviour*, **23**, 1–100.

Taborsky M (1998) Sperm competition in fish: bourgeois males and parasitic spawning. *Trends in Ecology and Evolution*, **13**, 222–227.

Taborsky M (2001) The evolution of bourgeois, parasitic and cooperative reproductive behaviors in fishes. *Journal of Heredity*, **92**, 100–110.

Thomaz D, Beall E, Burke T (1997) Alternative reproductive tactics in Atlantic salmon: factors affecting mature parr success. *Proceedings of the Royal Society London, Series B*, **264**, 219–226.

Warner RR, Shapiro DY, Marcanato A, Petersen CW (1995) Sexual conflict: males with the highest mating success convey the lowest fertilization benefits to females. *Proceedings of the Royal Society London, Series B*, **262**, 135–139.

Warner RR, Hoffman SG (1980) Population density and the economics of territorial defence in a coral reef fish. *Ecology*, **61**, 772–780.

Weddel N, Gage MJG, Parker GA (2002) Sperm competition, male prudence and sperm limited females. *Trends in Ecology and Evolution*, **17**, 313–320.

Zar JH (1999) *Biostatistical Analysis*, 4th edn. Prentice Hall, NJ.

Zbinden M, Largadièr CR, Bakker TCM (2001) Sperm allocation in the three-spined stickleback. *Journal of Fish Biology*, **59**, 1287–1297.

Zbinden M, Mazzi D, Künzler R, Largadièr CR, Bakker TCM (2003) Courting virtual rivals increase ejaculate size in sticklebacks (*Gasterosteus aculeatus*). *Behavioral Ecology and Sociobiology*, **54**, 205–209.