Genetic mating patterns studied in pools with manipulated nest site availability in two populations of Pomatoschistus minutus

A. Singer, *C. Kvarnemo,* K. Lindström† & O. Svensson*

*Department of Zoology, Stockholm University, Stockholm, Sweden
†Department of Biological and Environmental Sciences, University of Helsinki, Finland

Keywords:
alternative mating tactics;
Baltic Sea;
extra pair paternity;
nest availability;
piracy;
sand goby;
sneaking;
sperm competition;
strategies.

Abstract

Pomatoschistus minutus show paternal care in a resource defence mating system. We investigated the effect of nest-site availability on parasitic spawning. Each experimental pool contained four potentially nest-building males, four females and nests-sites in shortage (2) or excess (6). Both treatments were conducted in two populations; one with natural nest-sites in excess, one with a nest-site shortage. Microsatellite-DNA revealed that all nest-holders had fertilized most of the eggs they tended. Yet, 35% of the nests contained eggs fertilized by another male and 14.4% of the males had performed parasitic spawning. There was no site or treatment effect. Several females spawned in two nests, which coincided with parasitic spawnings, suggesting a cost to the nest-holder in terms of lost mating success. Nest-holders with and without eggs and non-nesting males all spawned parasitically, generating a significantly lower opportunity for sexual selection compared to if there had been no parasitic spawning.

Introduction

External fertilization provides an opportunity for several males to spawn simultaneously (Petersen & Warner, 1998); which may be a reason why sperm competition is so common in fish. It also seems to have opened up opportunities for alternative mating tactics to evolve (Taborsky, 1994; Gross, 1996; Stockley et al., 1997); for example, males may monopolize resources such as spawning sites and nests, cooperate with resource holders, or exploit the resource of others (reproductive parasitism). One form of reproductive parasitism is sneaking, where the sneaker male is stealing fertilization from the resource-holding male by inconspicuously entering the nest or interfering with the spawning pair and releasing sperm. Sneaking commonly occurs among fish species with male parental care. In such species the potential for resource holding (being able to successfully defend a nest site and protect, fan and clean the eggs) is crucial for successful breeding, and females should prefer males with a high resource holding potential. Individuals with a weak potential have to use another tactic to get access to mates. By doing so they also avoid the costs that come with the effort of paternal care (Taborsky, 1994).

Environmental factors, such as predation risk and spawning site availability, often affect mating tactics. This has been shown, for example through experimentally altered predation risk on Poecilia reticulata (guppy) (Godin, 1995; Kelly et al., 1999). In Rhodeus sericeus (European bitterling), mating tactics are affected by male density, since this influences the relative availability of living freshwater mussels, into which this species spawns (Mills & Reynolds, 2003; Reichard et al., 2004). Furthermore, Gonçalves et al. (1996) described female mimicry by males in a population of the blenniid Salaria pavo with extreme nest site scarcity, a mating tactic that has never been observed in areas with normal nest site availability. In the gobiids Pomatoschistus microps (common goby) (Borg et al., 2002), and Rhinogobius sp. or (N. Okuda, personal communication; Okuda et al., 2003), male mating tactic co-varies with nest site availability in the field. Similarly, in the cooperatively breeding cichlid...
Neolamprologus pulcher, the number of helpers, which may spawn parasitically (Dierkes et al., 1999), is positively correlated with the number of shelters (Balshine et al., 2001).

However, successful males with a territory, and therefore mating opportunities, may also steal fertilizations from other territory owners (van den Assem, 1967; Karino & Nakazono, 1993; Magnhagen, 1998; Avise et al., 2002). They thereby resemble bird systems in which paired males constantly seek extra pair copulations (reviewed in Birkhead & Möller, 1992). Taborsky (1994) lists 18 teleost species from six families in which males have been documented to leave their territories to spawn parasitically in a neighbouring territory, which suggests that such behaviour may be quite common in teleost fish. These males may even be dominant over the nest-holding males that remain in their nests (van den Berghe, 1988; Magnhagen, 1998; Mboko & Kohda, 1999). Therefore, the effect of changes in population density and, e.g. nest site availability on parasitic spawning behaviour may not be clear-cut. For example, low nest site densities may result in few parasitically spawning neighbours but many sneaker males.

A marine and a brackish population of Pomatoschistus minutus (sand goby) have been found to differ in the mode of sexual selection due to marked differences in availability of nest sites, a result that was corroborated experimentally (Forsgren et al., 1996b). Pomatoschistus minutus uses mussel shells as nest sites, and these are typically very abundant in the marine water at the Swedish west coast, but scarce in the brackish water of the Baltic Sea (Remane & Schlieper, 1971). In their experiment, Forsgren et al. (1996b) found intra-sexual selection to be more intense among males under nest site shortage, due to nest site competition, whereas inter-sexual selection was more intense under nest site excess, thanks to a greater potential for female choice under such conditions. However, Jones et al. (2001b) found no difference in rate of parasitic spawning in nests collected from the field at these two study sites. This was contrary to the expectation that there would be a higher rate of parasitic spawning at sites where there is a shortage of nests, as there are more non-nesting males present. Yet, that study suffered from both methodological and ecological differences between the sites. Therefore, in the present study we revisited the same sites using a rigorous experimental protocol and genetic paternity analyses. We compared manipulated nest site shortage to nest site excess in outdoor pools, for both populations.

Our main predictions regarding the nest site availability (excess and shortage) for this study were that (1) in the nest site shortage treatment, a larger number of males become sneakers as they are without nests; (2) if nest-holders spawn parasitically, they should do it more often in the nest excess treatment; (3) the mating success of nest-holding males was predicted to be higher in the nest site shortage treatment compared to the nest site excess treatment, since there should be fewer built nests available for females to choose among; (4) nest-holding males were predicted to be larger than non-nest-holding males and (5) particularly so in the nest site shortage treatment because of the high intra-sexual competition for nest sites. In addition, if there is any effect at all of the origin of the fish, our predictions would be that; (6) if nest-holding males spawn parasitically, males from the nest shortage site would do so to a lesser extent than males from the nest excess site. This is because in a population characterized by nest site shortage nest-holders should be less likely to leave their nests to cuckold others, whether due to selection or previous experience, since an intense competition for nest sites should create high costs of leaving a nest unattended. (7) Because of a severe egg predation pressure by marine invertebrates at the nest-excess site, the difference in parasitic spawning behaviour between males with and without eggs should be more pronounced in this population compared to the nest limited population, in which those invertebrates are lacking (all treatments were without invertebrates). Finally, opportunity for sexual selection is an estimate of potential sexual selection that is based on the variance in reproductive success among individuals of a sex. In a model, Jones et al. (2001a) showed that the opportunity for sexual selection in P. minutus should be substantially reduced if parasitic spawning was only performed by unsuccessful males, compared to if there was no parasitic spawning or if it was done only by egg tending males, whereas the decrease should be less if successful egg tending males also spawned parasitically. To validate the predictions made by this model, we calculated the opportunity for sexual selection within each of the experimental pools, based on the genetically determined reproductive success of all males, and compared our outcome to the prediction of the model.

Materials and methods

Study species

The sand goby, P. minutus (Pallas), is a small marine fish. It breeds on shallow sand bottom areas during spring and early summer. Males build nests under empty bivalve shells or stones by excavating underneath and covering them with sand. Males attract females with a courtship display. After approving of the male and his nest, the female attaches her eggs in a monolayer on the ceiling of the nest, where they are fertilized. The male cares for the eggs alone by cleaning, fanning and protecting them until hatching. In the study area, P. minutus usually has one breeding season only. Males often simultaneously guard eggs from more than one female and go through several brood cycles over this season, while females can mature several new clutches of eggs (Forsgren, 1999; Lindström, 2001). In the field, half of all nests contain
eggs fertilized by male other than the nest-holder and one tenth of all eggs are fertilized parasitically (Jones et al., 2001a, b). For each of the study areas, nest site availability has been documented based on the proportion of natural nest sites that were occupied, and on the time until artificial nest sites became colonised (Forsgren et al., 1996b).

Study sites

The experiment was done on two *P. minutus* populations, collected nearby Klubban Biological Station on the Swedish west coast (58°15′N, 11°28′E), which is marine and Tvärminne Zoological Station on the Finnish coast of the Baltic Sea (59°50′N, 23°15′E), which has brackish water (Fig. 1). Each of these two sites is highly representative for its region. They will hereafter be referred to as the marine site and the brackish site, respectively. The two sites differ in several environmental factors associated with the difference in salinity (2–3% at the marine site and 0.4% at the brackish site). Because of the brackish environment in the Baltic Sea the mussel shells, which *P. minutus* rely on for nesting, are fragile, small and few (nest site density 0.2 sites per m²) whereas at the marine site the mussel shells are bigger and occur in large numbers (nest site density 9.6 sites per m²) (Forsgren et al., 1996b). Since the *P. minutus* densities are roughly equal at the two sites, there is a nest site shortage at the brackish site and nest site excess at the marine site. Nest site availability was also found to differ in terms of proportion of natural nest sites that were occupied, measured along multiple transects, and time to colonisation of artificial nest sites. Even the *P. minutus* are probably affected by the low salinity at the brackish site, as they are smaller at this site than at the marine site (Forsgren et al., 1996b).

Experiment

The breeding season is earlier at the marine site and therefore the experiment was carried out there during May 2002 and at the brackish site during June 2002. Fish were caught by hand trawl and in artificial nests by hand net. Only potential nest-holders with fully developed breeding coloration were used. A true sneaker male morph (large gonads, small and pale) was recently discovered in marine waters of the Swedish west coast (C. Kvarnemo & O. Svensson, unpublished data), but such fish were not used in the present study. The experimental pools measured 1.5 m in diameter and water depth was maintained at 20 cm. They were provided with a continuous flow of natural seawater and a layer of 4 cm fine sand on the bottom. In half of the replicates, six halved clay flower pots (diameter of 6 cm) were provided as nest sites (marine site, \( n = 10 \), brackish site \( n = 8 \)), and in the other half, two nest sites were provided (marine site, \( n = 10 \), brackish site \( n = 9 \)). Four males were introduced into each pool and four mature females were placed in a transparent net-cage in the middle of the pool. The fish were left for 12 h over night to acclimatize and allow the males to build nests. Thereafter, nest building was noted and females were released to join the males. During the experiment, further nest-building activity among males in each pool was recorded every morning. Each replicate was ended after 3 days. Males that were observed to guard nests were considered nest-holders and nest-holders with eggs were moved to separate tanks to continue care for another three days to ensure a sufficient degree of embryo development for micro-satellite DNA analysis. All eggs in a nest were then collected and stored in buffer (20% DMSO, 250 mM EDTA, pH 7.5, saturated NaCl) or in 95% alcohol. Egg volume was used as a measure of total number of offspring. All fish were killed and stored at −20 °C after their total body lengths had been measured to the nearest mm. We analysed 75 males and 78 females from the marine site and 57 males and 57 females from the brackish site, and assigned parentage for 20 randomly chosen eggs from each of 51 successful nests using micro-satellite-DNA analysis.
In each of two pool replicates, we failed to identify two nest-holders that did not have eggs, because such males can be very hard to catch together with their nests. These pools were deleted in the analyses concerned with male size and nest building. In another three pools, one nest-holder without eggs was not identified. In these cases, the average size of the males caught outside nests was used to replace the missing value. In two pools the only egg-guarding male ate all eggs (filial cannibalism) after being moved, and in one pool, the PCR on the eggs did not work, despite repeated attempts. Therefore, ten nest excess replicates and eight nest shortage replicates at the marine site and seven nest excess replicates and nine nest shortage replicates at the brackish site were successful and used in the micro-satellite paternity part of this study. Furthermore, eggs from another six males that received eggs could not be analysed due to full clutch cannibalism. However, these replicates were not deleted since there were other males in these pools that received eggs that were successfully sampled.

Micro-satellites DNA analyses and paternity

DNA was extracted using the Gloor et al. (1993) protocol (the incubation step for fin clips was doubled to 60 min and samples were vortexed a few times during incubation). Samples were diluted 1:10 and stored at \(-18^\circ\text{C}\) awaiting polymerase chain reaction (PCR). Micro-satellite DNA locus \(P_{\text{min}05}\), which has been shown to be highly polymorphic (expected heterozygosity 0.97, Jones et al., 2001a, b) was used. Each 20 \(\mu\)L PCR contained 1 \(\times\) Fermenta PCR Buffer, 1.5 \(\text{mM}\) \(\text{MgCl}_2\), 0.2 \(\text{mM}\) each dNTP, 0.5 units Fermenta Taq DNA polymerase, 0.7 \(\mu\)L each primer and 2 \(\mu\)L DNA. The PCR reaction was preceded by an initial denaturation step of 2 min at 95 \(^\circ\text{C}\), followed by 29 cycles of 1 min at 95 \(^\circ\text{C}\), 30 s at 63 \(^\circ\text{C}\) and 30 s at 72 \(^\circ\text{C}\). Cycling was followed by 3 min at 72 \(^\circ\text{C}\). The forward primer was fluorescence-labelled at the 5’ end during the commercial synthesis. PCR samples were assayed using MegaBACE\textsuperscript{TM} 1000 Genotyping System. 1 \(\mu\)L of each PCR, 0.25 \(\mu\)L MegaBACE\textsuperscript{TM} ET400-R Size Standard and 7.75 \(\mu\)L \(\text{H}_2\text{O}\) was mixed, centrifuged and denatured for 4 min at 95 \(^\circ\text{C}\), and placed on ice before injection. The sizes of the fragment were measured by using MegaBACE\textsuperscript{TM} 1000 DNA Analyser software. In 11.5% of the cases, it was impossible to exclude one other candidate male. In these cases, the progeny were considered to belong to the nest-holding male as long as he shared an allele with the progeny.

Statistics

In most tests, treatment was entered as a factor. Although the two sites were not replicated, we also used site as a factor in the analyses, to allow us to see if the origin of the fish influenced the outcome. Parametric tests were used whenever the conditions for these were fulfilled. Because of our experimental design we used two factor ANOVA to analyse the effects of site and nest availability. When the assumptions of the parametric ANOVA were violated, we used the Scheirer-Ray–Hare factorial extension of the Kruskal–Wallis rank-based nonparametric ANOVA (pp. 440–447, Sokal & Rohlf, 1995), which is justified by nonsignificant interaction terms (Seaman et al., 1994). Ties were corrected for using the method described in Meddis (1984). Other nonparametric tests were used when appropriate. Some of our predictions required that we compared males of different status within the same pools. For example, we predicted that nest holding males should be bigger than males without nests. In this case, the two categories of males (averages within each pool) cannot be considered as independent samples and therefore we have used repeated measures ANOVA in these situations with male status as the repeated measure. When treatments or populations are pooled, this is due to low sample size, or significant differences in variances. In parametric tests, male length and the number of parasitically fertilized eggs were square root transformed and each male’s parasitic spawning success was arcsine square root transformed to achieve normality. However, untransformed means and standard deviations are given in the text.

Results

Nest-building and mating success

The number of built nests per pool ranged between one and four (median: two nests). However, in only one of these were four nests built. There was no difference between the two study sites regarding the number of built nests but more nests were built in the nest excess treatment than in the nest shortage treatment (nonparametric two-way ANOVA, site \(H = 0.76, P = 0.38\), treatment \(H = 15.40, P < 0.0001\), interaction \(H = 0.27\) and \(P = 0.60\)).

There was significant heterogeneity in variances of male length between the two populations, regarding both nest-holding and non-nest-holding males, with a higher variance at the marine site compared to the brackish. As homogeneity of variances is an assumption for ANOVA, we were not able to test differences between the sites. Instead, we had to do the tests within sites and thereafter on the whole data set (sites pooled). When testing only within populations, nest-holding males of both populations were larger than non-nest-holding males, although not significantly so (repeated measures ANOVA, within subjects test of nesting status, marine: \(F_{1,16} = 3.96, P = 0.064\), brackish: \(F_{1,13} = 4.30, P = 0.059\), Fig. 2). However, when testing on the whole data set, nest-holding males were significantly larger than males without nests (\(F_{1,31} = 7.17, P = 0.01\), Fig. 2).

There was an effect of nest availability on the size difference between nest-holders and non-nest-holders in the brackish population (repeated measures ANOVA, within subjects nesting status by treatment interaction:

\[ F_{J,31} = 7.17, P = 0.01 \]
Parasitic spawning and nest site availability

In a total of 51 nests that were analysed with microsatellites, 18 nests contained eggs fertilized by another male than the nest-holder and 19 males were found to have performed parasitic spawnings. Site and treatment had no effect on the mean number of nests (i.e. males) in each pool that suffered from parasitic spawning (non-parametric two-way ANOVA, site \( H = 2.35, P = 0.12 \), treatment \( H = 2.38, P = 0.12 \), interaction \( H = 0.009, P = 0.92 \), Fig. 3). The number of eggs fertilized para-

![Fig. 2](image_url) In each pool, nest-holding sand goby males were larger in size (body length, mean ± SE mm) than males that did not hold a nest site, regardless of study site or nest site availability treatment. Sample sizes are showing number of pools.

![Fig. 3](image_url) Proportion (mean ± SE) of sand goby nests suffering from parasitic spawning in each treatment at each site. \( n = 8, 9, 7 \) and 9 pools, respectively.
sitionally did not differ between treatments or study sites (nonparametric two-way ANOVA, site: $H = 0.25$, $P = 0.62$, treatment: $H = 1.87$, $P = 0.17$, interaction: $H = 0.37$, $P = 0.54$). The proportion of males spawning parasitically did not differ significantly between the brackish site (12 of 57) and the marine site (7 of 75). Treatment had no effect (logistic likelihood ratio test, site: $\chi^2 = 3.66$, $P = 0.056$, treatment: $\chi^2 = 0.29$, $P = 0.59$, Fig. 4). Similarly, the individual parasitically spawning male was not more successful at the marine site compared to the brackish site (the average number was used for one male, which had spawned parasitically in two different nests, marine site: $19 \pm 16\%$, brackish site: $12 \pm 10\%$, t-test, $t = 1.20$, d.f. = 18, $P = 0.25$).

The males were categorized into three groups, non-nest-holders, unsuccessful-nest-holders (without eggs in their nests) and successful-nest-holders (with eggs in their nests). Since we were unable to catch all nest-holders without eggs in their nests, seven of these (or possibly fewer, as males sometimes build more than one nest) are included in the non-nest-holder group. The number of parasitically spawning males in each group was compared between the two treatments at each study site. No difference was found neither in the pools at the marine site (logistic likelihood ratio test, nest status: $\chi^2 = 0.49$, $P = 0.78$, treatment: $\chi^2 = 0.12$, $P = 0.72$) nor in the pools at the brackish site (logistic likelihood ratio test, nest status: $\chi^2 = 1.12$, $P = 0.57$, treatment: $\chi^2 = 0.08$, $P = 0.78$). Thus, regardless of site and treatment, nest-holders with and without eggs were as likely to act as parasitic spawners as were non-nest-holders.

All but two nest-holders certainly had a nest when they spawned parasitically and at least some already had eggs. We know this because all six males that received eggs and spawned parasitically had nests when the females were released. In addition, in one pool two nest-holders spawned parasitically in each other’s nests. Of the three nest-holding males that did not receive eggs but spawned parasitically, two did not have a nest when the females were released, but as both had nests 24 hours later it is possible that they too had nests when spawning occurred. The numbers of females spawning in the nest of a parasitic spawner were similar to the numbers spawning in the parasitized nest in all six cases (parasitic: $2.7 \pm 1.0$ females, parasitized: $3.2 \pm 1.2$ females, Wilcoxon matched pairs test $Z = 1.21$, $n = 6$, $P = 0.22$). The high mean values are due to split clutches. Males that spawned parasitically did not differ in size from the ones they parasitized (parasitic: $52 \pm 4$ mm, parasitized: $53 \pm 7$ mm), regardless of whether they had a nest or not (the two males which spawned in each other’s nests are excluded, repeated measures ANOVA, within subjects nesting status by treatment interaction, $F_{1,15} = 0.21$, $P = 0.65$). Finally, between males with and without nests that did fertilize parasitically, there was no difference in parasitic fertilization success (one-way ANOVA, $F_{1,17} = 0.05$, $P = 0.83$).

**Opportunity for sexual selection**

Site and treatment had no effect on any of our measures of opportunity for sexual selection but it was larger for males than for females. This was true whether the variance in breeding success was measured as the amount of eggs each male fertilized in his own nest compared to the amount of eggs each female laid (repeated measures ANOVA, between subjects, site $F_{1,30} = 2.04$, $P = 0.16$, treatment $F_{1,30} = 0.49$, $P = 0.49$, within subjects, sex $F_{1,30} = 35.57$, $P < 0.001$); the total amount of eggs each male fertilized, including eggs fertilized parasitically, compared to the amount of eggs each female laid (between subjects, site $F_{1,30} = 1.70$, $P = 0.20$, treatment $F_{1,30} = 0.80$, $P = 0.38$, within subjects, sex $F_{1,30} = 28.69$, $P < 0.001$); the number of females spawning in each male’s nest and the number of nests each female spawned in (between subjects, site $F_{1,30} = 0.51$, $P = 0.48$, treatment $F_{1,30} = 0.27$, $P = 0.61$, within subjects, sex $F_{1,30} = 37.12$, $P < 0.001$); or as the number of females each male spawned with (including parasitic spawning) compared with the number of males each female spawned with (including parasitic spawners) (between subjects, site $F_{1,30} = 0.49$, $P = 0.36$, treatment $F_{1,30} = 0.66$, $P = 0.42$, within subjects, sex $F_{1,30} = 19.58$, $P < 0.001$). The opportunity for sexual selection based on the total number of eggs each male fertilized including parasitically spawned eggs was lower than the opportunity for sexual selection based on only the amount of eggs a male cared for (between subjects, site $F_{1,30} = 2.07$, $P = 0.16$, treatment $F_{1,30} = 0.22$, $P = 0.64$, within subjects, eggs in nest/fertilized eggs $F_{1,30} = 4.79$, $P = 0.037$).
Discussion

In the present study, we found no difference in the proportion of nests suffering from parasitic spawning or the number of parasitically fertilized eggs between the experimental pools with high and low nest site availability or between the populations at the marine site at the Swedish west coast and the brackish site in the Baltic Sea. This was against our first prediction, since we expected the treatments to influence male–male competition and therefore the frequency of individuals that would have to resort to sneak mating. The fact that even nest-holders with eggs performed parasitic spawning, and that there appears to be no difference in parasitic fertilization success between the three types of males (males without a nest, males with a nest but no eggs and males guarding eggs in his nest), is a very likely explanation for the lack of treatment effect. This result may also explain why Jones et al. (2001b) found no difference either in parasitic spawning in their previous field study of the same two *P. minutus* populations as in the current study. In addition, in the closely related *P. microps*, males that have a nest have been found to perform parasitic spawning (Magnhagen, 1998). Thus, we have no reason to believe that our result is an outcome of an artificial situation. However, as suggested by Jones et al. (2001b) other factors than nest site availability could be important in influencing alternative mating tactics. For example, in the Mediterranean wrasse, *Symphodus ocellatus*, both males and females are unwilling to spawn when sneaker males are present. This decreases the parasitic spawners’ mating success, as well as the nest-holding male’s immediate mating success, possibly in favour of his future reproduction success (Alonzo & Warner, 1999). Predation risk could also affect the cost of sneaking versus conventional mating behaviour. In *P. reticulata* (guppy), parasitic mating behaviour increased as a response to the presence of a predator (Godin, 1995), whereas it decreased in *P. microps* (Magnhagen, 1995).

It is probably risky for a *P. minutus* male to leave his nest (Jones et al., 2001a), even for a short period of time. If the nest contains eggs, egg predators, including other *P. minutus*, threaten the eggs. Furthermore, even when the nest is empty of eggs there is a danger in leaving it without protection, not only because other males could steal the nest (Lindström & Pampoulie, 2005), but also because parasitic entries even before spawning can result in successful fertilizations (Svensson, 2004). Despite these risks, some of the nest-holding males managed to further increase their reproductive success by parasitic spawning. Contrary to the second prediction, these males were no less inclined than non-nest-holders to spawn parasitically in the nest shortage treatment, in which the risk of leaving the nest was assumed to be the highest. Consistently, our sixth and seventh predictions about population differences were also proven wrong: Nest-holders from the nest shortage site were not less likely to spawn parasitically than males from the nest excess site, indicating our assumption about selection against such behaviour in this population to be inaccurate. Also, despite a high density of *Hinia reticulata* (netted dogwhelk) that are fierce predators of *P. minutus* eggs (personal observations) at the marine nest-excess site, we found no support for prediction seven of a population difference in parasitic spawning rate between males with and without eggs. It is possible that the two different population specific risks associated with parasitic mating by egg guarding males’ balance out resulting in no net difference between populations. However, it does not explain why non-nest-holders should not behave differently.

The mating behaviour of *P. minutus* males has earlier been considered a conditional strategy (Malavasi et al., 2001) in which the tactic depends on what is most profitable under current circumstances. The present study clearly shows that variation in sexual behaviour exists even within individual males at the same time, since they can act both as parasitic spawners and nest-holders during the same breeding cycle, most likely even when occupied with paternal care. These males that succeeded in both holding a nest of their own and at the same time fertilize eggs in other males’ nests seem to have exploited all possible opportunities to maximize their reproductive success. Based on the variance in reproductive success in these closed populations in which all males were measured, our result shows that parasitic spawnings significantly decrease the opportunity for sexual selection, compared to if no males spawn parasitically. This result is consistent with predictions made in the model by Jones et al. (2001a), and, quite importantly, it shows that parasitic (or extra-pair) fertilizations do not necessarily increase the opportunity for sexual selection, as is commonly proposed (Gibbs et al., 1990; Kempenaers et al., 1992; Möller & Birkhead, 1994; Yezerinac et al., 1995; Möller & Ninni, 1998; Sheldon & Ellgren, 1999). Still, since not only males without a nest but also nest-holding males with eggs spawned parasitically, the measured opportunity for sexual selection is likely to have been slightly higher compared to what it would have been if only non-nest-holders had acted as cuckolders (Jones et al., 2001a).

We predicted that the mating success of nest-holding males should be higher in the nest shortage treatment because of fewer nests for females to spawn in. This was, however, not the case as the number of females that spawned in each nest was equal between treatments, mainly because eggs were found in only two of the nests in most of the pools. This behaviour may be explained by a female preference for spawning in nests that already contain eggs, shown by many species of fish including the *P. minutus* (Forsgren et al., 1996a). By doing so, a female may experience a two-fold benefit. Other eggs in the nest will dilute the risk that her eggs are the ones that
get eaten if the egg-tending male or another egg predator eats some eggs. Full clutch cannibalism by the egg-tending male is most common by far on small broods (reviewed in Manica, 2002), and hence spawning in a nest already containing eggs may substantially reduce this risk. Nevertheless, twenty percent of the females split their clutches into two nests. One possibility is that the preferred nest did not provide enough room for the last female’s whole clutch. However, in several of the pools more than one female split their clutches between the nests, making this an unlikely scenario. In addition, even though there is a strong correlation between female body size and fecundity (Kvarnemo, 1997), no size difference was found between females that spawned in one and two nests. Thus, larger females did not spread their eggs more than small females did. Another possibility is that the females spread the risk of infertility or inbreeding or to get genetically variable offspring (reviewed in Griffith et al., 2002). Although we cannot rule out the latter, the other two are unlikely benefits for P. minutus females because of a high fertilization rate (personal observation), pelagic fry (low philopatry) and huge population sizes. A more likely explanation is that these females got disturbed by other males. Indeed, more than ninety percent of the females that split their clutches spawned with males suffering from parasitic spawning, whereas this was the case in only 27% of the females that did not split their clutches. This suggests that disturbance from parasitically spawning males can give rise to a substantial cost to the nest-holder in terms of lost mating success.

In accordance with our fourth prediction and a previous study, done with a similar set up and on fish from the same populations (Forsgren et al., 1996b), nest-holding males in the present study were generally larger than non-nest-holding males. Also, in line with prediction five, there was a treatment effect with larger size difference in the nest shortage treatment in the brackish population, i.e. the population with a high level of nest site competition (the nonsignificant difference in the marine population is in the same direction). This indicates that male size is more important in acquiring a nest when there is a shortage of nest sites. On the other hand, we found no difference in size between egg guarders and non-egg-guarders (non-egg-guarders were larger regardless of treatment, although not significantly so). This is a result partially in contrast with the previous study, in which egg guarders were shown to be larger than nest-holders without eggs in a corresponding nest excess treatment (Forsgren et al., 1996b). However, due to few males having nests but no eggs in our study, it is possible that we did not have the power to detect such a difference.

Three-spined stickleback sperm have been shown to have a very long longevity, which also varies with salinity and among males from different salinities (Elofsson et al., 2003). Furthermore, in fish, sperm morphology and ejaculate characteristics correlate with sperm competition (Stockley et al., 1997; Balshine et al., 2001). Similar differences between, e.g. salinities and populations are possible in P. minutus, in particular in relation to mucus trails: Like nest-holders of three other species of gobies (Marconato et al., 1996; Ota et al., 1996; Scagigante et al., 1999; Rasotto & Mazzoldi, 2002), P. minutus males have been show to put down sperm-containing mucus trails inside their nests (Svensson & Kvarnemo, 2005). These mucus trails are probably an adaptation to sperm competition since they slowly release sperm for hours (Ota et al., 1996), and since males increase their activity preparing the nest with mucus trails in the presence of sneaker males (Svensson & Kvarnemo, 2005). Therefore, it is feasible that the parasitic fertilization dynamics differ in the two environments due to genetic or phenotypic differences. However, in the present study the proportion of parasitically spawning males was lower but not significantly so in the pools at the marine site compared to the brackish site, whereas the frequency of nests suffering from parasitic fertilizations or the number of eggs fertilized parasitically did not differ between the two studied populations. However, the lack of significant differences and the co-occurrence of low salinity and nest site shortage prevent us from drawing conclusions about parasitic fertilization dynamics.

In conclusion, we found that virtually any male may spawn parasitically, independent of whether they have a nest or not, and whether they guard eggs or not. There was even no difference in successful parasitic fertilizations between these groups, which is a likely explanation for the lack of treatment effect in the present study. One way to look at the males in this study is that they all have the same tactic, which different males manage to utilize to different extent. Furthermore, several females spawned in two nests. This can probably be explained by disturbance from parasitically spawning males. In addition, we have recently discovered what seems to be a true sneaker male morph, namely small males (smaller than occurred in the present study) that lack breeding coloration but have testes more than three times as large as those of males in breeding coloration (C. Kvarnemo. & O. Svensson, unpublished data). Therefore, the P. minutus mating system is clearly much more complex than previously thought.

Acknowledgments

We thank Outi Ala-Honkola who helped in the Helsinki DNA laboratory, Daniel Simonsson, Jenni Puntila and numerous other people that helped in the field at Klubban and Tvärminne, and Sigal Balshine, Bertil Borg, Shaun Denney, Martin Plenderleith and two anonymous referees for valuable comments on the manuscript. The helpful staffs at Klubban and Tvärminne deserve a special gratitude. Financial support was given by Stockholm University, Nordplus (AS), the Swedish Research Council
(CK) the Finnish Academy (KL), and the Swedish Royal Academy of Sciences (OS).

References


Received 25 January 2006; accepted 7 February 2006