Plasticity in secondary sexual characteristics in the male of the river blenny *Salaria fluviatilis*

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Abstract

Alternative reproductive tactics (ARTs) driven by environmental factors are common among fish. However, the flexibility of fish to adopt distinct tactics in response to the characteristics of their environment has received little attention. The aim of this study was to study phenotypic plasticity in the adoption of dominant behaviour ("bourgeois tactic") by the male of the river blenny *Salaria fluviatilis* (Asso, 1801). For this purpose, two simultaneous experiments in aquaria were performed to examine the effect of social cues and nest abundance on the acquisition of secondary sexual characteristics (SSCs). Experiments were conducted with small (individuals without SSCs), medium-sized (one year old individuals), and large older dominant males (more than two years old), all collected in the wild. In Experiment 1 the three sizes of males were combined to compare their SSCs development depending on intrasexual context. In Experiment 2 the effect of nest abundance (two nests vs. six nests) was tested for each size of male. Medium-sized males showed phenotypic plasticity in response to the environmental conditions simulated in the two experiments. The absence of larger dominant males was found to be the main factor enhancing SSCs and the onset of parental behaviour. Nest shortage also influenced the degree of cephalic crest development among medium-sized males. This knowledge helps to understand how the population of *S.fluviatilis* still persists when it is reduced to young individuals during the summer droughts in Mediterranean streams.

Key words: Alternative reproductive tactics, freshwater blenny, nest abundance, social interactions, phenotypic plasticity.

Introduction

Alternative reproductive tactics (ARTs; Taborsky 1998) among individuals with the same genetic architecture are frequent and are directly influenced by environmental factors (Taborsky 2001). The last published count of ARTs in fish identified 170 species from 32 different families (Taborsky 2008). Usually, two alternative male morphs can be recognized in fish species with ARTs: (1) dominant individuals that compete for access to mates and show typical male behaviour, such as building elaborate nests, courting females or displaying ornaments (i.e. “bourgeois” males); and (2) parasitic males that take advantage of the success of dominant males in attracting females and attempt to steal fertilizations (i.e. sneakers and satellites). The size of a male is a strong determinant of reproductive tactics in fish (Taborsky 1998; Sato et al. 2004). Smaller and usually younger males behave as sneakers, whereas larger and older ones (more successful in male-male competition for nest sites) are dominant (Magnhagen and Kvarnemo 1989; Lindström 1992).

Alternative male morphs differ in morphology, physiology, and behaviour (Taborsky 2001). Morphological differences are based on the development (or not) of secondary sexual characteristics (SSCs). Changes in the environment can induce changes in the development of these traits. Several factors, such as the social context, competition for a suitable reproductive site, and female availability, can induce changes in male reproductive tactics in fish (De Fraipont et al. 1993; Taborsky 1994; Burmeister et al. 2005). For example, in the absence of male-male competition, sneakers of the black goby *Gobius niger* L., 1758 change their behaviour and develop SSCs as territorial males (Immler et al. 2004). Similar results were obtained for the grass goby *Zosterisessor ophiocephalus* (Pallas, 1814) (Scaggiante et al. 2004) and for the sand goby *Pomatoschistus minutus* (Pallas, 1770) (Takegaki et al. 2012).

In species with resource-defence mating systems (i.e., in which the male defends a nest), the primary factor behind male-male competition for mates is resource availability. Nest availability limits the number of parental males in marine blennies (Almada et al. 1994; Saraiva et al. 2009) and it is also associated with maturation in young male fish (Takahashi 2008). High nest abundance drives males into actively courting females and decreases the relative frequency of sneakers reproducing parasitically (Almada et al. 1995; Kvarnemo and Ahnesjö 1996; Saraiva et al. 2012). When nest sites are scarce, nests become a valuable resource and only the most competitive males succeed in occupying one (Almada et al. 1994; Saraiva et al. 2010).

The capacity to adopt reproductive tactics in response to different environmental conditions may be an adaptive trait in fish species that inhabit fluctuating environments. In fact, phenotypic plasticity is favoured in populations of fish living in heterogeneous habitats (Salonen and Peuhkuri 2007). Mediterranean intertidal areas and freshwater systems inhabited by blenniids are good examples of these environments. The co-occurrence of ARTs and male sexual polymorphism has been described for several species of this family (Neat et al. 2003; Oliveira et al. 2009). Although some works have approached how environmental factors can influence male sexual polymorphism (see for example Saraiva et al. 2012) it is necessary to better understand their role in freshwater ecosystems where fish populations are under strong stochastic processes (e.g. droughts and floods).

The river blenny *Salaria fluviatilis* (Asso, 1801) is a freshwater species of Blenniid which mainly inhabits Mediterranean-type streams. In these environments, parameters such as population age structure, fish density, and mate availability fluctuate, and reproductive populations can often be reduced to one-year old individuals (Vinyoles and Sostoa 2007). During the breeding season, which extends from May to the end of July (Vinyoles and Sostoa 2007), males excavate a nest cavity under a stone, and several females lay clutches of eggs in a monolayer on the underside of the stone. After egg fertilization, males provide sole care to the eggs by fanning and protecting them until they hatch. The SSCs of dominant parental males are comprised of a cephalic crest and two anal glands covering the first spinous rays of the anal fin. In this species, small ("obligatory sneakers", without SSCs) and medium-sized ("facultative sneakers", temporally without a nest) males show sneaking behaviour (Neat et al. 2003).

The aim of this work was to study the phenotypic plasticity of *S. fluviatilis* males to adopt the bourgeois tactic in response to controlled environmental conditions in the laboratory. For this purpose, two experiments were performed to study the effect of social cues (male-male size interaction experiment) and nest abundance (nest shortage experiment) in the adoption of the dominant tactic. Given that *S. fluviatilis* is an endangered species in Spain (Doadrio et al. 2011), the contribution of this study to future conservation plans will also be discussed.

Materials and methods

Two simultaneous experiments were performed in a total of 9 260-L aquaria in order to study how the size of neighbouring males (Experiment 1: "male-male size interaction") and the abundance of nests (Experiment 2: "nest shortage") affect the acquisition of the bourgeois tactic in male blennies. All aquaria had the same equipment and were maintained at 21 ± 0.5 ºC (mean ± SD) under 12L: 12D. These conditions are within those experienced by wild populations of this species during the breeding season (Vinyoles and Sostoa 2007). The substrate comprised a mix of sand, gravel and coral (proportion 2: 2: 1), the latter to prevent water acidification. The water consisted of 95% conventional depurated freshwater and 5% seawater (water final salinity was 2 ppm). Low levels of nitrogenous compounds and an adequate pH were ensured by periodical cleaning and partial water renovation.

The scientific procedure of this work was approved by the Animal Ethic Committee of the University of Barcelona (Registration nº 201/07) which follows European Directive 2010/63/UE on the protection of animals used for scientific purposes.

Field collection

Specimens were caught by electrofishing on 23 October 2007 in the River Segre (a tributary of the River Ebro in Catalunya, Spain), in a place where the species is abundant. A total of 162 individuals (81 males and 81 females), ranging from 40 to 110 mm in total length (TL), were collected. Some individuals (15 males and 15 females) were not used directly in the experiments (these fish were reserved to replace those that died, thus keeping the same conditions in the two experiments). None of the replacement fish were included in the analysis. Sex was determined in the field by examination of the external morphology of the anogenital area. The caught males were classified in three age categories from their TL (mm) at the time of capture following Vinyoles and Sostoa (2007). These categories were (in TL, mm): large: 80-110 (old males more than two years old, with SSCs); medium: 53-66 (young males one year old, with SSCs); and small: 40-49 (young males less than one year old, without SSCs). The older mature category of males in this study acted as dominant. The medium category of males resembled “facultative sneakers” in size, and the small one “obligatory sneakers”, as described by Neat et al. (2003). Gravid females (ranging from 66 to 70 mm in TL) were used in both experiments to stimulate male sexual activity. After finishing the two experiments (late July), the fish were returned to the stretch of river where they had been caught, with the permission of the Department of Environment of the Generalitat de Catalunya (Permit nº 3988).

Mesocosm experiments

Once in the laboratory, male and female blennies were placed simultaneously in the aquaria following the two experimental designs explained below. Artificial refuges (PVC tubes) were provided to monitor the health of fish and acclimation to captive conditions over two weeks. After this period, PVC refuges were replaced by flattened stones suitable as nests. Nests ranged from 143 to 374 cm^2 (mean area ± SD = 233.7 ± 56 cm^2) and were a similar size to those in the field (see Freeman et al. 1990). Since no relationship between male size and nest stone size has been found in field studies for this species (Côté et al. 1999) not different sizes of stones were provided for aquaria keeping males of different size categories. Three elongated pebbles (about 8 x 2 cm) were placed under three sides of each stone for support, thus preventing stone sinkage into the substrate and providing a suitable surface to lay the eggs. The side of the stones without a supporting pebble, facing an angle of 30° relative to the flow direction (0°) in the aquaria, was adapted as a nest entrance (see Vinyoles et al. 2002), and synthetic algae were placed between adjacent stones to provide visual barriers between nests. Fish readily accepted these features. Fish were fed twice a day at the same time in the morning and in the afternoon. The diet consisted of frozen red chironomid larvae and fresh food such as mussels, shrimp or squid.

Since this species nests in aggregations (Freeman et al. 1990; Côté et al. 1999) we considered important that the experiments simulate a “realistic” mesocosm that resembled the social interactions found in their natural habitat (i.e. many conspecifics interacting with each other). Small groups of individuals (males and females) were placed in the aquaria to better reproduce this condition in the laboratory. In Experiment 1 (“male-male size interaction”), three aquaria were set up with 12 nest stones each one distributed in two parallel rows of six nests each. A total of 60 fish (30 males and 30 females) were used in this experiment. Each aquarium had 10 males of two size categories (and 10 adult females of assorted sizes) distributed as follows: aquarium 1: five large and five medium males; aquarium 2: five large and five small males; and aquarium 3: five medium and five small males. The abundance of nest stones (12 nests for 10 males, at least one per male), sex ratio (1: 1), and fish density in this experiment were similar to those found during the breeding season in Mediterranean-type environments (Vinyoles and Sostoa 2007). The size of nest stones was not different between the three aquaria (Mann-Whitney *U* test; z= 0.24; *P*>0.05) of this experiment.

In Experiment 2 (“nest shortage”), two aquaria types (one with two nests and one with six nests) were assigned to each size category of males (large, medium and small) to test the effect of nest abundance on SSCs development. A total of 72 fish (36 males and 36 females) were used. As in Experiment 1, the aquaria held the same number of males and females. Thus, six males of each size category (along with six females) were distributed in three aquaria with two nests each (aquarium 4: six large males; aquarium 5: six medium males; and aquarium 6: six small males). This simulated the nest-shortage condition. Simultaneously, the same composition of fish was distributed in another three aquaria with six nests each (aquarium 7: six large males; aquarium 8: six medium males; and aquarium 9: six small males). One nest per male was considered as a non-limiting resource, as in a natural habitat *S. fluviatilis* males have not been observed simultaneously defending more than one nest (Freeman et al. 1990). The size of nest stones was not different between the three aquaria with six nests (Mann-Whitney *U* test; $z= 0.24$; $P>0.05$) of this experiment. Increases in length and SSCs development for the males in Experiment 2 (kept alone with females and nests) was also monitored to further elucidate male plasticity when no interaction with males belonging to other size categories is involved.

Males were measured once a month beginning in November (when both experiments started) and finishing two months after the last clutches were detected (in May for Experiment 1 and in July for Experiment 2). During measurement sessions, all fish were removed from their aquaria with small nets and temporarily placed in containers with oxygen supply. After anesthetizing the fish with MS-222 (tricaine methanesulfonate) and measuring TL, each male was placed in a small methacrylate box with graph paper at the back, and photographs were taken (both from the left and ventral side of the individuals). Using the Sigma Scan Pro 5 programme, SSCs measurements (height of cephalic crest and diameter of the anal glands) were determined on the basis of these photos (supplementary Fig. S1)[1]. The cephalic crest height (mm) was measured as the distance from the middle of the cranium to the top of the crest. The diameters of the first and second anal gland (mm) were calculated as an average of two measurements (width and length), thus avoiding bias caused by the irregular or non-circular shape of these glands. Individual recognition was also possible through the identification of traits like crest shape, mucous pore location and body pigmentation pattern. Before specimens were returned to the aquaria, nest stones were checked for clutches. No changes in fish behaviour were observed after manipulation.

The data considered in the analysis were the peaks reached in the development of SSCs (crest height and anal gland diameters) in the period of confinement of fish in the laboratory. Monitoring changes in SSCs development was supported by observations of male behaviour during the two experiments. These observations consisted of watching fish in periods of 10 min/aquarium once a week. In this study, observations on male behaviour were restricted to their classification in “dominant” (bourgeois) or “non-dominant”. Dominant males were those expressing courtship signals (nuptial coloration, body display, nodding, courtship-quiver and nest-showing) following the descriptions made by Neat et al. (2003), and defending a nest, while the non-dominant did not present these characteristics (Taborsky 1998, 2001). A male was definitively classified as dominant when it was observed to defend the same nest (chasing out other males and attracting females) for two consecutive observations (that means, a whole week). The observations also served to detect sneaky attempts.

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[1] Supplementary Fig. S1 is available with the article through the journal Web site

Our mesocosm approach provided a satisfactory simulation of interactions established between fish in nature. This design assumed there was no aquarium effect. In order to guarantee that this assumption was applicable to our study we performed a previous similar experiment with five replicate aquaria containing only one size of males (each one had eight medium males and eight females - in the range of length of fishes from experiment 1 and 2- and 10 nests) housed under the same environmental conditions.

No significant differences between aquaria were found for any of the three variables considered in this study (male length, crest height and anal gland diameters) in the peak of male's development (one-way-ANOVA random-effects model, $P>0.05$). The results of the previous experiment permitted us to assume that the use of one aquarium per condition was enough. Replication would have supposed to duplicate or triplicate the number of fish required and this could have compromised the reproduction of the species according to the fish density reported by Sostoa et al. (2010).

Statistical analyses

Given that the normality assumption was not guaranteed and because of the small sample size, data were tested by using a two-sample randomization test (hereafter, RT) with RUNDOM 3.14 LITE programme (Jadwiszczack, 2003). These statistical tests permitted us to control for pseudoreplication arising from data dependencies (Palagi 2009; Stiver et al. 2009). Significance was determined by comparing the observed mean difference with the distribution of 10,000 differences obtained by randomly allocating the observed data to two samples of size 5 (male-

male size interaction) or to two samples of size 6 (nest shortage). The significance level is the percentage of randomized statistical tests as large as or larger than the observed values indicated by the *P* values provided in the results.

Results

No significant differences in the initial values of the variables were found between male blennies of the same size category housed in separate aquaria neither in the Experiment 1 nor in the Experiment 2 (for each experiment and male size: RT, *P* > 0.05 for all variables). Initial values for each size category of males at the beginning of both experiments are summarized in Table 1. While for large and medium-sized males the initial values of the variables were those obtained in the first measurement in November, for the small males, as a result of the lack of developed SSCs in that moment, the initial values considered corresponded to December. Three fish in the Experiment 1 died (one medium male, one small male and one female in distinct aquaria) and five in the Experiment 2 (one large male, two small males, and two females). The SSCs development over time was different for each kind of male in both experiments (supplementary Fig. S2, Fig. S3, Fig. S4)[2].

Experiment 1: male-male size interaction

The large males reached the maximum development for all SSCs (crest height and anal gland diameters) in February (after being in the aquaria for four months).

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[2] Supplementary Fig. S1, S2 and S3 are available with the article through the journal Web site

These males reached a similar degree of SSCs development (RT, $P > 0.05$ for all the variables; Figs. 1b-1d) in both experimental conditions (that is, they were housed with medium-sized and with small males, respectively). Behavioural observations confirmed that all large males reached bourgeois status. Although these males defended a nest and showed characteristic courtship behaviour, not all of them were successful in reproducing. In February, four of the ten large males (two in aquarium 1 and two in aquarium 2) had clutches.

For the medium-sized males, SSCs development peaked in January (i.e., a month earlier than the large males). In contrast, these males had significantly more developed SSCs when housed with small males than when sharing the aquarium with large ones (RT, $P < 0.05$ for all variables; Figs. 1b-1d). All medium-sized males housed with small males adopted a dominant status and defended a nest. In January, three of these five medium-sized males had clutches. Conversely, medium-sized males kept with large males had underdeveloped SSCs and did not adopt parental behaviour. Two of these medium males showed a vigilant attitude towards occupied nests and tried sneaky attempts.

The maximum development in SSCs for the small-sized males of *S. fluviatilis* was recorded seven months into the experiment (in May). Small males that were kept with large males showed greater development of the two anal glands than those kept with medium males (RT, $P < 0.05$ for both anal glands; Figs. 1c-1d). However, no significant differences in cephalic crest height were found for the small males of the two experimental aquariums in May (RT, $P > 0.05$; Fig. 1b). None of these fish presented parental status or were observed performing sneaker behaviours.

At the end of the experiment (in May), large and small males within each size category reached the same size in the different conditions (RT, $P > 0.05$ in both cases; Fig. 1a). In contrast, medium-sized males kept with small ones grew significantly more than those housed with large males (RT, $P < 0.05$; Fig. 1a). No parental male was displaced from his nest by other males or defended more than one nest

Experiment 2: nest shortage

The abundance of nets had no effect on the development of SCCs, neither in large- nor in small-sized males (RT, $P > 0.05$ for all the variables and the two sizes of males; Figs. 2b-2d). For large males, SSCs development (crest height and anal gland diameters) peaked in May (seven months after fish entered the aquaria and about three months after males peaked in Experiment 1). Small males still took more time (nine months) to reach maximum development of these traits (which occurred in July, and also later than small males in Experiment 1). In contrast, medium-sized males had a more developed cephalic crest when they were under the nest shortage condition in May (when this variable peaked) than under the non-limiting nests condition, that peaked in July (RT, $P < 0.05$; Fig. 2b). The medium-sized males in the Experiment 2 reached maximum SSCs development between four and six months after those in Experiment 1. Crest development SD of medium males was higher in the nest-shortage (mean ± SD = 2.10 ± 1.01 mm) than in the non-limiting nest treatment (mean ± SD = 1.08 ± 0.46 mm). This was due to an exaggerated development of this trait in three of the males (3.12; 2.72; 2.88 mm) and non-development in one male (0.42 mm) in the nest-shortage aquarium. At the end of the Experiment 2 (in July), all male blennies reached a similar TL under the two experimental conditions (RT, $P > 0.05$ for each size category; Fig. 2a). Note, however, that small males grew more in TL in Experiment 2 than in Experiment 1 (RT, *P*<0.05, Fig. 1a and Fig. 2a).

The bourgeois tactic was adopted by large and medium-sized males in the same proportion as the number of nests in their aquaria (i.e., two males in aquaria with two nests and six males in aquaria with six nests). Of these bourgeois males, two large males had clutches in the nest shortage condition while only one had a clutch under non-limiting nest availability. Two medium males had clutches in the nest shortage condition and only one had a clutch in the aquarium with a non-limiting number of nests. All clutches appeared between April and May. Although females were mature and ready to spawn (presenting courtship coloration and abdominal distension) throughout the study period, small males did not adopt the bourgeois tactic nor did they have clutches. Nest stones with clutches found for medium males were not different in size than the ones found for large males, taking into account data from both experiments (Mann-Whitney *U* test; *U* = 9.0; *P*>0.05).

Discussion

Results from Experiment 1 revealed that *S. fluviatilis* males showed phenotypic plasticity in response to the simulated experimental social cues. Medium-sized males (about 60 mm in TL) became dominant (all of them had well-developed SSCs and defended a nest, while the majority of them protected their eggs) when they were the largest males in the aquarium. Therefore, the absence of larger dominant males promoted the adoption of the bourgeois tactic. The changes observed in SSCs development (accompanied by a change in behaviour) were associated with the acquisition of the dominant status (Oliveira and Almada 1998). Medium-sized male blennies also grew faster in length as established by Hofmann et al. (1999). According to these authors, fish ascending in social rank experience an increased growth rate. Male size is a critical trait for nest defence (Magnhagen and Kvarnemo 1989; Fujii et al. 2005), and the females of the species with paternal care strategies show a preference for larger mates (Kolm 2001).

Conversely, in the presence of large males, medium-sized males showed inhibited expression of SSCs. These males did not show parental behaviour and at least two of them were observed to adopt the sneaker tactic. According to the findings of Neat et al. (2003), the medium-sized males of *S. fluviatilis* might be facultative sneakers. Consistent with Immler et al. (2004), Scaggiante et al. (2004) and Takegaki et al. (2012), males of *S. fluviatilis* (medium-sized males) were able to adopt parental behaviour in the absence of larger males.

Larger and older males (longer than 80 mm) were consistently dominant and it should be noted that these fish were always housed with smaller males. Presumably, all these males had displayed dominant behaviour prior to being caught in the river and therefore showed less plasticity. In contrast, small male blennies showed moderate development of anal glands when housed with large males but not in the presence of medium-sized males. These observations suggest that different interactions might occur between fish depending on their respective sizes as described by Sneddon et al. (2006). This could be related to differences in hormonal responses affecting SSCs development (Oliveira and Almada 1998). Differences in sperm competition could also explain the differences in anal gland development between small males depending on male social conditions (Saraiva 2009). Although it was not clear whether small males act as sneakers, this study found that these males did not have a developed cephalic crest.

Nest abundance had little effect on the expression of SSCs in *S. fluviatilis*. In Experiment 2, only medium-sized males kept under the nest shortage condition, which could have increased male-male competition, had a more developed cephalic crest than those who were maintained under a non-limiting number of nests. An exaggerated development of SSCs when nests are scarce due to male-male competition has been already reported by Saraiva (2009). Our finding suggests that the cephalic crest is a key feature in male intra-sexual competition as it is also a signal of dominance over other males rather than serving merely to attract females. This possible function of the crest has been already proposed by Oliveira et al. (1999). SSCs can be simultaneously subjected to both intra- and inter-sexual pressure in many species, among these at least 14 fish species (Hunt et al. 2009). The fact that all medium males (except one) developed the cephalic crest in the nest-shortage treatment might indicate that in this competitive context (when males are the same size) the majority of males adopted the parental status. In nature these males might have opportunities to reproduce at the end of the reproductive period, by moving to other places or replacing parental males in case they disappear.

As in Experiment 1, all the males in Experiment 2 achieved some degree of SSCs development and grew in length. However, the time of maximum SSCs development was delayed several months with respect to fish in Experiment 1. This delay suggests that the social context is a greater stimulus of SSCs development than nest abundance. In *S. fluviatilis,* the effect of nest abundance on SSCs expression is presumably driven by social interactions, and in such interactions male size plays a major role (Forsgren et al. 1996; Natsumeda 2001). Small males had underdeveloped crests and they did not adopt the dominant status even when not housed with other male size categories. Were these males “obligate sneakers”, as suggested Neat et al. (2003)? In *Aidablennius sphinx* (Valenciennes, 1836), when nests are abundant even small males show parental behaviour and are successful mating at the end of the reproductive season when the nests belonging to large males are full of clutches (Neat and Locatello 2002). Similarly, in *S. fluviatilis*, when in a fluctuating stream the population was reduced to young individuals (males and females) one year old, males measuring from 46 to 59 mm were observed to act as parentals (Quirós and Vinyoles, unpublished data). In this study, however, other factors not considered may have influenced the non-adoption of dominant behaviour in small fish, for example a possible intersexual competition to occupy nests as shelters by large females. lso it is possible that small males need longer time to change tactic when nests are available due to the high energy cost it supposes to them (Takegaki et al. 2013). These males might prefer to invest in growth than in intrasexual competition, courtship and parental care. According to the gonadal cycle of *S. fluviatilis*, in nature males and females one year old spawn later in the reproductive season (during June and July) than the older ones (April-June) and they invest in growing fast during spring (Vinyoles 1993). These hypotheses might explain why these small males did not present a well-developed cephalic crest and did not act as parental over the present experiments. Future studies should be addressed to understand better the plasticity of small-sized males. The possibility that these males need longer time to adopt the dominant tactic should be taken into account.

The results have implications for conservation measures for *S. fluviatilis* and other fish species with similar reproduction systems. Firstly, breeding captivity programmes may enhance the male parental role by stimulating their potential to become dominant. Secondly, reintroduction programmes could be improved by considering that an appropriate mix of male sizes favours ARTs. Such enhancement may contribute to younger males adopting parental roles when the older males die (which, as suggested Vinyoles and Sostoa 2007, is a common occurrence in streams subjected to severe summer droughts). The detrimental effect of human activities (such as gravel extraction) on the reproductive habitat and the occurrence of nesting males of *S. fluviatilis* have been reported by Côté et al. (1999). The fact that two clutches were obtained in the nest-shortage condition compared to only one in the non-limiting nest condition is not possible to interpret. However, it could be related to a major social stimulation in nest-shortage experiment. The majority of medium-sized males in nest-shortage seemed to adopt the parental status even if there were not enough nests. This result suggests that the absence of larger males is a stronger stimulus for medium males' development than the availability of nests. However this must be validated in future works. The fact that in this study the bourgeois tactic was adopted by large and medium-sized males in the same proportion as the number of nests in their aquaria suggests that stone substrata preservation is a crucial element for the conservation of this species.

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Table 1. Values (means ± SD, mm) in total length (TL), crest height, and anal gland diameters for each size category of river blenny males (*Salaria fluviatilis*) at the beginning of both experiments are given. Measurements were made on November 20 for large and medium-sized males and on December 20 for small males (since the latter had no measurable SSCs in November).

	Male size	**TL**	**Crest height**	**1st anal gland**	**2nd anal gland**
Experiment 1	Large	90.8±7.9	0.9±0.2	1.2±0.3	1.4±0.3
(“male-male size interaction”)	Medium	61.2±3.8	0.5±0.3	0.9±0.1	1.2±0.2
	Small	46.5±2.7	0.2±0.1	0.6±0.2	0.5±0.1
Experiment 2	Large	88.6±8.9	0.8±0.2	1.3±0.3	1.6±0.4
(“nest shortage”)	Medium	59.4±3.4	0.5±0.2	0.8±0.1	1.1±0.2
	Small	49.3±3.6	0.3±0.1	0.7±0.2	0.6±0.2

Figure captions

Fig. 1. Maximum variable values reached for the three size categories of male river blennies (*Salaria fluviatilis*) in Experiment 1. Pairs of bars compare each size type of male (large, medium and small) when housed with (W/) the other two type sizes of males. Significant differences for $P < 0.05$ are marked by an asterisk.

Fig. 2. Maximum variable values reached for the three size categories of male river blennies (*Salaria fluviatilis*) in Experiment 2. Pairs of bars compare each size type of male (large, medium and small) under nest shortage (two nests) and under non-limiting nests (six nests). Significant differences for $P < 0.05$ are marked by an asterisk.