

Boldness is related to the development of the cephalic crest in the male of the river blenny *Salaria fluviatilis* (Asso, 1801)

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Abstract Boldness is defined as the tendency of an individual to take risks when exposed to novel objects or situations. The main aim of this work was to elucidate if boldness was related to the development of the secondary sexual traits (SSTs) in the male of the river blenny *Salaria fluviatilis*, a freshwater fish which takes care of eggs. As a second objective SSTs effect on parental status adoption was also explored. Wild young fish were caught in the Segre River (Ebro basin) in November 2010. Fish were kept in five aquaria in groups of eight males and eight females with artificial nests. Boldness (boldness score and hesitancy) of males (initially with little SSTs development) was tested as the time required to exit a refuge when exposed to a new environment. Fish were maintained in the same aquaria for several months, and males' SSTs development and reproductive behaviour were monitored. Boldness score was negatively associated to cephalic crest development. Hesitancy was negatively related to male length and was also influenced by the interaction between male length and cephalic crest. Parental status acquisition was positively related to the male's length, marginally related to anal gland development, and non-related to cephalic crest. These results suggest an intrasexual role of the crest as an informative trait of boldness (honest signal) which might not necessarily be chosen by the females. It is also discussed that correlational selection could be responsible for linking boldness and growth rate in this species. Sexual selection, in addition to having directed the evolution of secondary sexual traits, might have favored certain personality traits (e.g. boldness) associated with the achievement of a larger body length [*Current Zoology* 60 (3): 373–380, 2014].

Keywords Animal personality, Behavioural syndromes, Freshwater blenny, Male reproductive success, Shyness

Animal personality is defined as a set of behavioural responses (behavioural syndrome) that differs between individuals of the same species and exhibits consistency through time and in different contexts (Sih et al., 2004; Réale et al., 2007). Animal personality is generally described in five general categories: activity, shyness-boldness, exploration-avoidance, aggressiveness and sociability (Réale et al., 2007). One of the most thoroughly studied is the shyness-boldness continuum, which is described as the propensity (boldness) or aversion (shyness) to take risks in front of novel objects or situations (Sloan Wilson et al., 1994). Boldness can be evaluated through different behavioural responses such as the willingness to approach predators (Godin and Dugatkin, 1996), the tendency to exploit new sources of food or to interact with unfamiliar objects (Wilson, 1998), exploration of a new environment (Dzieweczynski and Crovo, 2011) or foraging activity under risk (Bell, 2005). In fish, positive relations regularly exist between boldness and other behaviours: learning capacity (Dugatkin and

Alfieri, 2003), dispersal (Rehage and Sih, 2004), innovation (Harcourt et al., 2009), feeding rate and growth (Biro and Stamps, 2008), survival (Smith and Blumstein, 2008), physical condition (Pellegrini et al., 2010), aggressiveness (Tulley and Huntingford, 1988) and dominance (Sundström et al., 2004).

Coloration also has been related to boldness. The eumelanin-based coloration from the males has been associated to boldness and aggressivity in many vertebrates (Ducrest et al., 2008; Mafli et al., 2011; Mateos-González and Senar, 2012). In the guppy fish *Poecilia reticulata*, the boldest males were found to have a brighter coloration than shy males (Godin and Dugatkin, 1996). Among fish (and other vertebrates) it is not known, however, if boldness is related to the development of secondary sexual traits (SSTs) of the male based on the development of special morphological structures (e.g. cephalic crests, breeding tubercles or anal glands). Are bold males more likely to develop their SSTs than shy males? Taking into account that

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hormones (androgens) implicated in dominance and aggressiveness are associated both to SSTs development (Oliveira et al., 2001) and to boldness (Daisley et al., 2005), it is quite probable that a relation between SSTs and boldness could exist.

In some species, females choose males' boldness directly (Godin and Dugatkin, 1996) while in others they select indirect characteristics related to it, for instance the courtship intensity (Reaney and Backwell, 2007). Choosing bold males might be advantageous to females because these males would be more likely to have fitness advantages related to this personality trait and transmit them to their offspring (Drent et al., 2003; Brown et al., 2007). Female choice often is based on the SSTs in order to assess male's quality (Candolin, 2000; Ottova et al., 2005). It remains to be answered whether the degree of development of the SSTs could also be influenced by a personality trait such as boldness.

The river blenny *Salaria fluviatilis* is a freshwater fish of the Blenniidae family with parental care of eggs. During the breeding season, which in nature 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 in a monolayer on the underside of the stone. This species adapts easily to captivity conditions and is thus suitable to study the relation between boldness and SSTs development. Among blennies the presence of at least two alternative reproductive tactics (Oliveira et al., 2001) is common: dominant males (territorial, with developed SSTs) and sneaker males (often with female appearance and with non-developed SSTs). In *S. fluviatilis* these two reproductive tactics (dominant and sneaker) were described by Neat et al. (2003). The male SSTs consist of the development of a cephalic crest and two anal glands covering the first spines of the anal fin. Generally the degree of development of these SSTs has been described as a male characteristic preferred by the females (Gonçalves and Oliveira, 2003; Serrano et al., 2008).

The main objective of our study was to investigate (in wild fish maintained under experimental conditions) if boldness in the males of *S. fluviatilis* was related to the degree of their SSTs development. We will consider whether or not acquisition of parental status among males (defined here as the obtention of clutches inside the nest) was related to their SSTs development. *S. fluviatilis* is a species classified as vulnerable or threatened in many countries where it occurs (see Vinyoles and Sostoa, 2007). Therefore the results from our work could bring information for the future management and

conservation of this species.

1 Materials and Methods

1.1 Field collection and fish maintenance

We caught the fish by electrofishing on November 2010 in the River Segre (a tributary of the River Ebro), near Camarasa (Catalonia). Males chosen during fishing ($n=48$) were one year old ranging from 60 to 70 mm in total length (TL). These characteristics ensured that, at the time of reproduction, the fish were sexually mature according to Vinyoles and Sostoa (2007) and, subsequently, could develop SSTs during the experiment. Females ($n=53$) had a larger range of lengths (from 60 to 90 mm TL) and took part in the study as a sexual stimulus for the males. At their arrival to the animal facility for aquatic animals (in the University of Barcelona) we measured the fish (TL) and distributed them into five experimental aquaria as follows: eight males, five small females (from 60 to 70 mm TL) and three large females (from 80 to 90 mm TL). An extra aquarium served to maintain the remaining fish which were used to replace experimental individuals that died during the experiment. None of these replacement fish were included in the analyses.

We tagged experimental males with Visible Implant Elastomer (VIE) following the guidelines of Northwest Marine Technology Inc. (Shaw Island, Washington). Just one colour was used (fluorescent red), only visible under ultraviolet light. We made a small mark on each male in the base of one of the impair fins (first dorsal, second dorsal, anal or caudal) and in one of the two sides (right or left). This permitted us to differentiate the eight males from each aquarium. Marks apparently did not affect fish behaviour nor health.

All experimental aquaria (260L) were in a climatized room and had the same equipment: one biological filter, air diffusor and substrata (consisting of a mix of sand, gravel and coral in proportion 2:2:1). They were maintained under constant Mean (\pm SD) temperature (22.96 ± 1.15 °C) and water physicochemical conditions (pH = 7.4 ± 0.21 , $\text{NO}_2^- = 0.05 \pm 0.00$ mg/L, $\text{NO}_3^- = 3.4 \pm 0.84$ mg/L, $\text{NH}_4^+ = 0.00 \pm 0.00$ mg/L; hardness: 19). Light regime was 12:12 (light: dark). Each aquarium was isolated by opaque curtains to prevent any interaction with external features. We placed 10 nests in each aquarium (distributed in two parallel rows of five nests each). Nests were artificial structures that had been already accepted as nesting sites by males of the same species in previous experiments (unpublished data). They consisted of transparent plastic boxes (13.5 cm

width \times 7 cm high \times 12 cm depth) open in the front (nest entry) and with the ceiling substituted by a glass surface. Their inner walls were covered by black acetate sheets fixed to the structure with plastic clips. Nest entries faced the aquaria walls in order to facilitate clutch detection and observations. We fed fish “ad-libitum” with frozen red chironomid every morning (10:00 a.m.). Once a week fresh mussels were added to the diet.

1.2 Experimental protocol

The aquarium where fish boldness was tested (boldness aquarium) measured $45 \times 45 \times 45$ cm and had similar conditions to the ones of the experimental aquaria (temperature, water characteristics and substrata). This aquarium was also isolated with opaque curtains to prevent possible interferences produced by our presence on fish behaviour during the tests. We designed the boldness aquarium (Fig. 1) following Brown et al. (2007). We installed a video camera (Sony Handycam HDR-SR1E) 120 cm above the aquarium to record the tests and posteriorly visualize them with the computer.

Boldness tests were performed from 19 to 27 December (from 8:00 a.m to 3:00 p.m), approximately one month after fish capture. At this time males’ SSTs were little developed. During this experimental phase we fed the fish daily at 7:00 p.m. (to prevent possible interactions between feeding and behaviour) and we temporarily divided the aquaria in two compartments (one with the fish to test and the other one with the ones that had it already done). We tested each fish only once, follow-

ing Brown and Braithwaite (2004), and its turn to perform the test was randomly established. We caught each male with a hand net and placed it inside the “boldness box” (Fig. 1) which top was immediately closed with a plastic cover. After a 5 minutes settling time, we opened the boldness box window by raising the transparent trapdoor (Fig. 1) and fish were free to emerge. Test duration was 55 minutes and all of them were video-recorded. After the test (and before being returned to their aquarium), we anesthetized (with MS-222, tricaine methanesulfonate), measured (TL), and photographed (lateral and ventral view) the male inside a small methacrylate recipient with a graph paper at the back. These pictures permitted us to measure the SSTs development (cephalic crest and anal glands) by using the Sigma Scan Pro 5 programme. When we returned the male to the aquarium, we placed it in the compartment for fish which had already been tested. Before we introduced a new individual into the boldness aquarium, we renewed the water (always maintaining the same level of 27 cm depth). Once all the fish had performed the test, we removed the divisions from the experimental aquaria. Throughout the experiment we measured fish TL (both sexes) and SSTs development (only males) every 45 days from November 2010 to July 2011. Measures were done following the same steps as those indicated for the males after the boldness test, but females were just measured in TL and not photographed.

We monitored clutch laying every morning (9:00 a.m) from January to the end of the experiment in July. When we found a clutch we classified the correspondent male as “parental”. We considered the males that did not obtain clutches during the experiment to be “non-parental”.

1.3 Measured variables

In this study we considered the same behavioural variables as the ones used in Brown et al. (2005): 1) Boldness score (b1) defined as the time taken for the fish’s snout to emerge from the box, and 2) Hesitancy (b2) defined as the time the fish took to cross the black arc minus the time it took for the fish to emerge from the box (Fig. 1). We assigned the maximum time for the test (3300 seconds) to the fish where no time for b1 was registered (because they did not emerge from the boldness box during the test). We did not assign a hesitancy score to the fish that did not cross the black arc following Brown et al. (2005) recommendations. We obtained variables b1 and b2 through the posterior visualization of the video recordings and log-transformed them for the analyses.

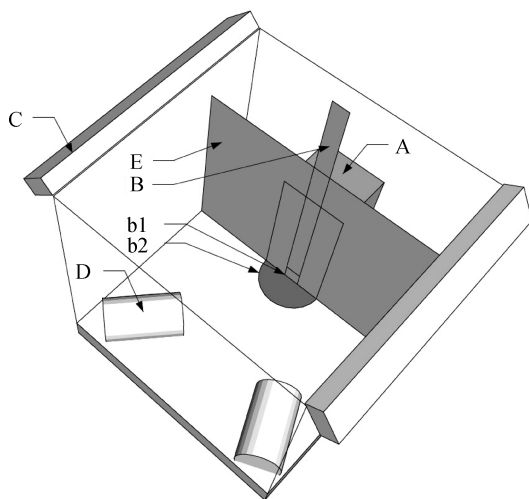


Fig. 1 Diagrammatic representation of the boldness aquarium

A: Boldness box (30 \times 11 \times 11 cm) with a top opening to introduce fish and an inferior small window (3 cm) for to exit. B: transparent trapdoor (39 \times 4.5 cm). b1: line that defined boldness score (emergence time). b2: arc that define hesitancy time. C: neon lamp. D: PVC tube (4 cm diameter \times 13 cm length). E: methacrylate wall.

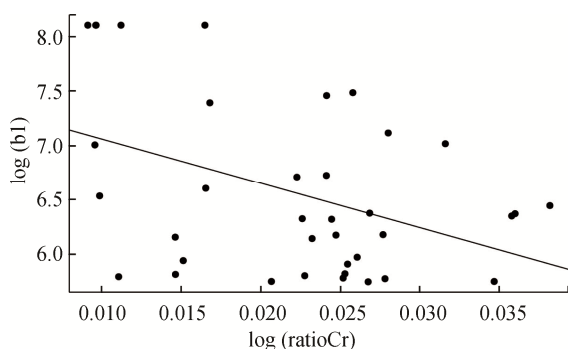


Fig. 2 Relation between the development of the cephalic crest, $\log(\text{ratioCr})$, and boldness score, $\log(\text{b1})$

The line was adjusted with the estimations obtained in the linear mixed model.

The SSTs measures we took for the males were: the cephalic crest height (Cr, mm, measured as the distance from the middle of the cranium to the top of the crest) and the gland mean diameter (Gl, calculated as the average of the first and second anal gland diameters). We expressed the SSTs variables as the log-transformation of ratioCr (Cr/TL) and ratioGl (Gl/TL) in the analyses.

The study protocol of this work was approved by the Research Ethics Committee of the University of Barcelona (Registration n° 221/11). In October 2011 we returned the fish to the stretch of river where they had been caught.

1.4 Statistical analyses

We analyzed whether boldness (b1) and hesitancy (b2) were related to SSTs development by means of two linear mixed models (LMMs, one for b1 and one for b2) with aquarium ID as a random variable to control for data dependency. Each model included initially as covariates: $\log(\text{TL})$, $\log(\text{ratioCr})$ and $\log(\text{ratioGl})$, with all the paired interactions between them. The best model was found by extracting parameters using the AIC criterion corrected for small samples sizes (AICc) (Burnham and Anderson, 2002).

To analyze whether or not the development of SSTs was related to obtaining a clutch we used a general linear mixed model (GLMM) with a binomial distribution of errors and the link function logit. In this model “Pa-

rental status” was the dependent binary variable (0 = non-parental, 1 = parental) and aquarium the random component.

Error adjustment was verified by visual inspection of the normal probability plots. Statistical analyses were performed with the free software R (R Core Team, 2012). For the linear mixed models estimations lmer and glmer functions from the package lme4 (Bates et al., 2012) were used. The best model was found by using sequentially the drop1 function.

2 Results

At the beginning of the experiment (in November) we found no significant differences between aquaria for the TL neither for females ($F_{4,36} = 0.06$; $P > 0.05$; Mean \pm $SD = 73.00 \pm 9.57$ mm) nor for males ($F_{4,36} = 0.31$; $P > 0.05$). In November males had little SSTs development (Table 1) and this development was similar in all the aquaria (all $P > 0.05$). In December, when boldness tests were performed, for fish that emerged, we observed a high variability for b1, Mean (\pm SD) = 659.72 ± 423.60 seconds (min = 313, max = 1781 seconds) and for b2, Mean (\pm SD) = 145.00 ± 278.31 seconds (min = 3, max = 1218 seconds). Five males did not emerge from the boldness box and from the ones who emerged only two did not cross the black arc.

We observed that the month of maximum SSTs development for all the males ($n = 37$) was in February (Table 1). At that time males' TL, Cr and Gl were similar between aquaria (all $P > 0.05$). Similar results were obtained for females' TL ($F_{4,34} = 0.13$; $P > 0.05$; Mean \pm $SD = 74.95 \pm 10.04$ mm). February also coincided with the beginning of the reproductive period that lasted until April. 40.5% of males ($n = 15$) acted as parents (i.e. they had clutches in their nests). Distribution of parental males in the five aquaria was: three in three aquariums, two in one aquarium and four in another aquarium (at most half of males acted as parentals in each aquarium). No parental male was displaced from his nest by other males or defended more than one nest during the experiment. There was one parental male (7% of parentals)

Table 1 Total length (TL), cephalic crest height (Cr) and mean glandular diameter (Gl) for the males of *S. fluviatilis* in November (with underdeveloped SSTs) and February (parental and non-parental males)

Month	Male status	<i>n</i>	TL (mm)	Cr (mm)	Gl (mm)
November	Underdeveloped	40	65.98 ± 3.39 (60–70)	0.71 ± 0.21 (0.29–1.38)	1.14 ± 0.23 (0.54–1.60)
February	Parental	15	78.33 ± 2.89 (72–82)	2 ± 0.75 (0.75–3.19)	2.09 ± 0.26 (1.58–2.53)
	Non-parental	22	71.95 ± 5.37 (61–81)	1.46 ± 0.57 (0.61–2.68)	1.74 ± 0.27 (1.24–2.21)

Means (\pm SD , and minimum-maximum values in parentheses) are shown.

and four non-parental males (18% of non-parentals) that did not emerge from the boldness box. Throughout the experiment three males and one female died (in different aquaria) and we substituted them with fish of similar characteristics (not considered in the analyses).

The best LMM for log(b1) only included the covariate log(ratioCr) which has a significant negative effect on log(b1) (Table 2). The interpretation of this result is that a higher crest development is associated to less time required to emerge from the boldness box (boldness score, Fig. 2). The best LMM for log(b2) included log(TL) (significant negative effect) and log(TL):log(ratioCr) interaction with a positive effect (Table 2).

The best GLMM model for parental status included log(TL) and log(ratioGI), log(TL) had a significant positive effect and log(ratioGI) a marginal positive effect (Table 2). Parental males were longer and had a tendency to have more glandular development than non-parentals males. Crest height did not have an effect on the condition of parental status.

3 Discussion

In this work we found that bolder males of *S. fluviatilis* showed a higher degree of cephalic crest development. Who would benefit from this? The males or the females? The cephalic crest function remains still unclear among blennies. In *S. pavo* the degree of development of this trait has been related to the male reproductive success (Gonçalves and Oliveira, 2003). Conversely, in the latter species, this relationship was not found by Oliveira et al. (1999). These authors suggested that the cephalic crest in blennies could play an important role in male-male competition rather than merely attracting females. Though, this suggestion would be

supported by our results obtained with *S. fluviatilis* (the cephalic crest size was not related to parental status of males). Boldness has been related to growth (Biro and Stamps, 2010), aggressiveness (Huntingford, 1976), dominance (Sundström et al., 2004; Colléter and Brown, 2011), resistance to infections (Locatello et al., 2012) and to a major ability to win fights (Fuxjager et al., 2010). Larger size (body length) and aggressiveness are known to offer advantages in the intrasexual competition (Fujii et al., 2005; Larson et al., 2006). Thus, if the size of the cephalic crest is associated to boldness, males could use this information to assess aggressiveness and dominance of competitors before a potential confrontation. In this case the cephalic crest could function as an honest signal (see Maynard-Smith and Harper, 1995). Using this kind of signal allows animals to avoid potentially costly fights that they are unlikely to win. It is probable that *S. fluviatilis* males use these signals since in blennies fights lead to higher energy expenditure and increase the risk of injury (in the fins and body) that might compromise their survival and reproduction (Santos and Nash, 1996).

Females, otherwise, could prefer characteristics that make a male a good father rather than a dominant individual (Forsgren, 1997; Wong, 2004). We observed here that males which succeeded in obtaining clutches in their nests were those with bigger sizes. Male size has been often related to female choice in fish (Kolm, 2001). In fish species with a resource-based mating system, male size has been associated with benefits in nest defence and greater ability in taking care of clutches (Fujii et al., 2005). In our study, males with higher hesitancy times were larger at the time of reproduction, and this suggests they could have grown faster. In this sense,

Table 2 Results for the best Linear Mixed Models (for b1 and b2) and for the best General Linear Mixed Model (Parental)

Dep	Fixed effects	Estimates \pm SE	F	df	P
log(b1)	Intercept	7.47 \pm 0.35	–	–	–
	Log (ratioCr)	-40.8 \pm 14.72	8.21	1	0.00*
log(b2)	Intercept	-108.14 \pm 67.55	–	–	–
	Log (TL)	-24.00 \pm 15.55	5.39	1	0.02*
	Log (ratioCr)	-5699.61 \pm 2683.17	0.04	1	0.83
	Log(TL):log(ratioCr)	1310.71 \pm 616.08	4.53	1	0.03*
Parental	Intercept	-40.78 \pm 14.01	–	–	–
	Log (ratioGI)	345.57 \pm 185.71	3.46	1	0.06
	Log (TL)	0.42 \pm 0.15	7.77	1	0.01*

“Dep” indicates the dependent variables for each model: log(b1), boldness score; log(b2), hesitancy; and Parental, males’ parental status. Considered covariates were: log(ratioCr), cephalic crest height; log(ratioGI), mean glandular diameter; and log(TL), total length. Significant fixed effects are indicated (*).

personality seemed to have played an important role on length which positively influenced the adoption of parental status. According to Biro and Stamps (2010), individuals with a more active metabolism and faster growth have a higher energy expenditure and therefore should take more risks when they are foraging. The interaction found between male length and cephalic crest size explaining hesitancy is more difficult to interpret. This interaction would indicate that the effect of male length on the hesitancy would be mitigated by the cephalic crest size. We also found a marginally significant effect of anal glands size on parental status acquisition. It has been described that these glands are responsible for pheromones that are released to attract females (Barata et al., 2008) and for antimicrobial secretions to prevent egg infections (Pizzolon et al., 2010). Our study, however, was not addressed to investigate female choice. Further studies are required to better understand female choice on male morphological traits (SSTs) and male boldness behaviour.

The variability of personality traits between individuals suggests that the optimal behaviour for a species is not unique (Dall et al., 2004; Stamps, 2007; Wolf and Weissing, 2010). The individual position in the shyness-boldness axis affects its fitness differently depending on the environmental context (Dingemanse et al., 2004; Dingemanse and Réale, 2005; Sinn et al., 2006). In the male of *S. fluviatilis*, where Neat et al. (2003) have described the presence of two reproductive tactics (parental and sneakers), boldness could favour dominant behaviours against other males in the nest selection process. Animal personality traits might influence the adoption of a specific tactic, because tactics are related to the presence or absence of developed SSTs. Alternative reproductive tactics in fish are, in fact, influenced by environmental factors such as the social context, competition for a suitable reproductive site, and female availability (Burmeister et al., 2005).

The pace-of-life syndrome (POLS) states that individual behavioural differences should be linked to life-history differences (Réale et al., 2010). Our results indicate that in this species correlational selection might be favoring the linkage between boldness and life-history traits such as fast growth and probably early reproduction (due to the advantage of larger males to become parental). Our results also suggest that intrasexual and intersexual selection might not only drive the evolution of morphological traits, but thereby also favor behavioral types that allow males to reach larger body sizes. Increasingly, knowledge of animal persona-

lity is taken into account to improve the success of reintroductions and conservation programs in the field (McDougall et al., 2006). Our study allows us to understand better how boldness is related to the development of SSTs and the latter with the acquisition of parental status. In other fish species latency to exit the refuge was found to be repeatable (e.g. Wilson and Godin, 2009; Harris et al., 2010). However, future studies need to test whether boldness is repeatable in *S. fluviatilis* and to determine whether these processes also occur in nature.

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