Spatial learning based on visual landmarks in the freshwater blenny *Salaria fluviatilis* (Asso, 1801).

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Abstract

The use and learning of visual landmarks seems to be strongly influenced by ecological demands and vary according to habitat, sex and, presumably, dominance status. In blennies, sexes differ in their home-range extension and this could predispose them to use different mechanisms to navigate. The main aim of this study was to investigate if \textit{Salaria fluviatilis} (a benthonic and polyginic freshwater blenny) was able to solve a learning maze using direct visual landmarks. Performances between sexes were compared and the possible relationship between the males’ secondary sexual characteristics (SSCs) development and their spatial ability was considered. In this species the SSCs consist of a cephalic crest and a pair of anal glands. Fish were subjected to 10 sessions (each one consisting of 11 consecutive trials) in a specially designed apparatus where they had to find the correct exit using direct visual landmarks. The proportion of males that learned the task (80\%) was higher than that of females (30\%). Cephalic crest development of the male was associated with a higher readiness in solving the spatial task. These results support previous works stating the importance of ecological demands on shaping the species spatial abilities. They also provide a comprehensive perspective that would link dominance (through cephalic crest development), learning and sexual selection.

\textbf{Keywords:} dominance, fish navigation, river blenny, secondary sexual characteristics, spatial ability
1. Introduction

Fish rely on their capacity of navigation to perform a series of vital activities such as foraging (Hughes & Blight, 1999), locating spawning sites (Mazeroll & Montgomery, 1998), or eluding predators (Aronson, 1971; Markel, 1994). Their navigation skills are based on different external stimulus such as water flow direction (Braithwaite & Girvan, 2003), electromagnetism (Walker et al., 1997), polarized light (Hawryshyn, Arnold, Bowering, & Cole, 1990) and odours (Stabell, 1984). While the most complex navigation (true navigation) consists in a map-and-compass mechanism, other navigation phenomena such as pilotage are simpler. The latter allows animals to steer home on the basis of a topographic map without resorting to a compass (Papi, 2006).

In this context, pilotage depends mostly on spatial learning and memory (Salas et al., 2006). The ability to include previous experiences within spatial behaviour is essential for certain fish to survive and to adapt to environmental changes (Dodson, 1988). This permits them to perform behaviours such as homing behaviour (Thyssen, 2010; Jorge, Almada, Gonçalves, Duarte-Coelho, & Almada, 2012) and to remember and use visual landmarks (Burt & Macias García, 2003).

An extensive bibliography on spatial learning based on visual landmarks in fish is available. It has been described that they can use visual cues as direct and indirect reference points (Warburton, 1990; López, Broglio, Rodríguez, Thinus-Blanc, & Salas, 1999) and as sequences (Mazeroll & Montgomery, 1998). They are able to gather information from different directions (Rodríguez, Duran, Vargas, Torres, & Salas, 1994) and, finally, they can establish complex geometric relationships between them (e.g. Braithwaite & De Perera, 2006; Sovrano, Bisazza, & Vallortigara, 2007; Lee,
Vallortigara, Ruga, & Sovrano, 2012)). The understanding of such mechanisms is growing steadily and some studies reveal that certain spatial abilities of fishes are comparable to those of adult humans and higher than those found in children and rats (Sovrano, Bisazza, & Vallortigara, 2002). This suggests that spatial information use could be more influenced by the species ecological demands than by its phylogeny.

The habitat where a fish population occurs shapes the importance of visual landmarks in their navigation. In experiments with sticklebacks *Gasterosteus aculeatus* it was observed that visual landmarks were used more by individuals from 1) a lake population as compared to a river population (Odling-Smee & Braithwaite, 2003), 2) a population dwelling in clear and static waters as compared to a population living in eutrophic waters of poor visibility (Girvan & Braithwaite, 1998) and 3) a population living under a high-predation pressure as compared to a low-predation risk population (Huntingford & Wright, 1989). Moreover, it has been suggested that the benthonic species of *Gasterosteus* would be more prone to use visual landmarks than the limnetic ones (Odling-Smee, Boughman, & Braithwaite, 2008).

In certain species, males and females differ in home-range size (Wittenberger, 1981) and this entails differences in ecological demands and spatial ability (Gaulin & FitzGerald, 1986, 1989). In promiscuous or polygynous mating systems, males are frequently the most mobile sex (Brown, 1966; Lockie, 1966; Ewer, 1968; Trivers, 1972) and they present more complex spatial abilities than females (Gray & Buffery, 1971; Vallortigara, 1996). However, in blennies, this circumstance is reversed; while the females have a broad home-range (Costa et al., 2011), males are restricted to their nest (Neat & Lengkeek, 2009) and they only make short excursions away from it in
order to feed and to defend their territory (Almada, Gonçalves, De Oliveira, & Barata, 1992; Gonçalves & Almada, 1998). Distinct preference predispositions between the sexes concerning the use of landmarks for orientation could have evolved as a consequence of such home range differences. Previous works support the hypothesis that males might rely upon local landmarks more than females as has been already suggested for chicks (Tommasi & Vallortigara, 2004). In at least two species of blennies, females have a bigger telencephalon than males and, thus, they can rely more in geometric features than in local beacons (Costa et al., 2011).

Animal personality may also influence spatial learning. Boldness (defined as the propensity to take risks) has been associated to a higher learning ability (Dugatkin & Alfieri, 2003) as well as to a dominant status acquisition (Dahlbom, Lagman, Lundstedt-Enkel, Sundström, & Winberg, 2011). These associations may have a physiological basis; fish that grow faster and have a better response to stress are more prone to explore their surroundings (Biro & Stamps, 2010; Archard, Earley, Hanninen, & Braithwaite, 2012) and to become dominant (Hofmann, Benson, & Fernald, 1999; Pottinger & Garrick, 2001). Putting these facts together, it seems reasonable to expect that dominance and spatial learning might be related and, furthermore, that dominant males (in territorial species) might be more motivated to use visual landmarks than non-dominant males (without a territory). Among fish, visual landmarks are frequently used to define territorial limits (Heap, Byrne, & Stuart-Fox, 2012). In species with alternative reproductive tactics (ARTs) the dominant status is associated to a higher development of the secondary sexual characteristics (SSCs) (Fleming & Gross, 1994; Oliveira & Almada, 1998; Oliveira, Carneiro, Gonçalves, Canario, & Grober, 2001).
Salaria fluviatilis is one of the few blenny species that inhabits freshwaters and occurs in rivers and lakes of the Mediterranean basin. It is a benthic and speleophil-nest spawner fish (Balon, 1975) that has been described as endangered in many Mediterranean countries where it occurs (see Vinyoles & Sostoa, 2007). During the reproductive period, which extends from May to the end of July (Vinyoles & Sostoa, 2007), males excavate a cavity under a stone and several females lay monolayer clutches on the underside of the stone. A good topographic knowledge of the familiar area, a good memory and a particular curiosity to explore has been attributed to this species by Wickler (1957), but the role of visual landmarks was not studied. Repeated incursions to the nest probably enable visual recognition of neighboring objects by males. In a species with similar habits, the shanny (Liphophyris pholis), the use of visual landmarks has already been described (Burt de Perera & Guilford, 2008). S. fluviatilis represents also an appropriate model to study the relationship between spatial ability and SSCs development. During the reproduction period, males develop a cephalic crest and a pair of anal glands. Recently, boldness has been associated with the cephalic crest in this species (Fabre, García-Galea, & Vinyoles, 2014) but it is still unknown if SSCs are related to spatial ability.

The main objective of this study was to define whether S. fluviatilis is able to use solely direct visual landmarks for navigation and if there are differences between the sexes. Then, if visual landmark use is found for males, the relationship between SSCs development and spatial ability will be explored.

2. Material and methods

2.1. Fish field collection and maintenance
Fish were caught in the river Segre, a tributary of the Ebro River, close to the village of Camarasa (Spain) in November 2010. 80 specimens were collected overall, consisting of 40 males and 25 females of similar sizes (ranging from 60 to 70 mm in total length, TL), and 15 large females (ranging from 80 to 90 mm in TL).

Animals were placed in five 260 L aquaria provided with a biological filter, air diffuser and substrata (consisting of a mixture of sand, gravel and coral in proportion 2:2:1). Each aquarium contained 10 artificial nests (distributed in two five-nest parallel rows) that function both as refuges and nests for the fish. Nests consisted of transparent plastic boxes (13.5 cm width x 7 cm high x 12 cm depth) with inner sides covered with black acetate sheets to make them opaque, an opening in the front (nest entry) and a glass surface substituting the ceiling. Previous experiments with these artificial nests confirmed that the species readily accepted them as nesting sites (unpublished data).

The water in the aquaria consisted of 95% depurated freshwater and 5% seawater (with a final salinity of 2 ppm) and was partially renewed every two weeks in order to maintain adequate pH, NO$_2$ and NH$_4$ levels. Temperature (mean ± SD = 22.96 ± 1.15 ºC) and light regime 12L: 12D were maintained constant throughout the experiment.

The proportion of fish in each aquarium was the following: eight males, five median females and three large females. Only median-sized fish were used in the experiment (all males and the five median females). Large females just acted as sexual stimulus in the aquaria (in order to simulate the female size variability found in nature, Vinyoles and Sostoa (2007)). Animals were fed “ad libitum” with red quironomidae larvae (the principal prey of the species following Vinyoles (1993)) once a day. Fresh mussels were added to the diet once a week.
The experiment was performed from August to September 2011 (coinciding with the end of the reproductive period). The long period of time that fish were maintained in the aquaria before the experiment allowed them to habituate to captivity and manipulation (manipulation consisted in capturing them once a month with small hand-nets). Fish individual recognition was possible through the identification of certain traits such as the cephalic crest shape, mucous pore location and body pigmentation pattern. After the experiments fish were released into the river.

2.2. Apparatus

The experimental apparatus used to perform the learning task was similar to the design of the set described in Ingle and Saharian (1973) and used later on by other authors (Salas et al., 1996; López, Broglio, Rodríguez, Thinus-Blanc, & Salas, 1999, 2000). This structure consisted of a rhomboidal box (hereafter referred to as decision-box) with an area of 196 cm\(^2\) and 20 cm in height, constructed with PVC pieces (0.5 cm thick) and whose vertices were opened with 5 cm openings (Fig. 1). Two of these openings (opposite to each other) gave access to a cylindrical compartment that consisted of a vertical PVC tube (11 cm in diameter x 20 cm in height) uncovered at the top. A grey plastic sliding door (intended for opening and closing the compartment) was placed between each cylindrical compartment and the decision-box. During the trials, one of the cylindrical compartments remained closed (the non-used one) while the other received the individual that had to start the trial. The other two openings from the decision-box acted as exits of the apparatus and the fish had to decide throughout the trial which one to use. These exits had sliding doors as well, but in this occasion they were made with transparent plastic (to avoid fish visual detection of the
closed door). During the trials one of the exits was opened (correct exit) while the other was blocked by the transparent sliding door (wrong exit). The visual landmarks used to indicate the correct exit were two similar flattered stones (6.6 x 4.5 cm and 5.4 x 4.5 cm respectively). They were placed on the floor of the decision-box, one at each side of the correct exit (at a distance of 4.5 cm from the exit and at 2.5 cm from the closest apparatus wall). When the fish that was performing the trial succeed to exit the apparatus, it found two small PVC tubes outside, one at each side (4 cm diameter x 13 cm length) that acted as refuges. Although these tubes were invisible to the fish until it exit the apparatus, they represented a positive stimulus.

The experimental apparatus plus the refuges were placed in a rectangular opaque plastic tank (47 x 56 cm base x 40.5 cm height, hereafter referred to as experimental tank) covered with the same substrata as the 260 L aquaria. Illumination was provided by two neon lights (98 x 12 cm, 60 W) placed 25 cm above each cylindrical compartment. Direct illumination of the cylindrical compartments prevented the fish from adopting the compartment as a refuge and stimulated them to seek safer places. The experimental tank was surrounded by curtains (to avoid interactions with the researcher). In order to record fish movements, a video camera (Sony Handycam HDR-SR1E) was placed above the apparatus (at a height of 140 cm). During the trials the experimental tank was filled with water (same composition and temperature as that in the aquaria) to a level of 15 cm and the lab lights were switched off.

2.3. Learning task

All fish (other than the large females) underwent the experiment. They were separated randomly from the rest in groups of two males and two females during the
period they were subjected to the experimental sessions (in a separate zone of 30 x 40 cm surface created by placing a divider in the aquarium). This procedure was done in order to facilitate their daily capture and to prevent unnecessary manipulation of individuals that were not going to be tested. After two habituation days in the separate zone, the learning task started. Each fish performed a maximum of 10 sessions (consisting of 11 consecutive trials each) during three days. Every trial consisted of placing the fish into one of the cylindrical compartments (see the assignation sequence below) and waiting until the fish chose one of the two exits. When the first decision of the fish was to exit through the “correct exit” the choice was considered “correct” while in the opposite circumstance (when it hit the closed transparent door) the choice was considered “wrong”. Following López, Broglio, Rodríguez, Thinus-Blanc, & Salas (2000) recommendations, if a fish did not emerge from the cylindrical compartment within 5 minutes it was lightly prodded with a glass rod to motivate an exit. Fish that did not emerge their heads out of the cylindrical compartment and that were immobile during the first 15 minutes of the first session were discarded from the experiment. This decision was based on previous observations that indicate that fish that presented these behaviors did not leave the cylindrical compartment during the following hour. Fish that were not discarded had no time limitation to perform the trials. Each trial finished when the fish had left the apparatus through the correct exit and hid itself in one of the PVC refuges. After 30 seconds, the fish was captured and a new trial started until the session (11 trials) was completed.

The apparatus configuration changed between trials; the cylindrical compartments (A or B) and the correct exits (1 or 2) were switched in a pseudorandom sequence. Thus,
correct direction and correct turn were in a random order but with the imperative that
50% of turns were to the right and the other half to the left to avoid the possibility of
fish conditioning.

The two stones were moved to always indicate the correct exit and were the only
reliable landmark to learn the task. During the 30 seconds between trials, the
correspondent configuration was settled and the substratum in front of the apparatus
exits was also stirred to avoid the use of any of its elements to orientate. At the end of
the session, the fish was left in a 10 L bucket provided with an air-diffuser and a PVC
refuge to rest. Each fish was submitted to 4 sessions during the first and second days
and to two sessions during the third day, with an interval of 2-3 hours between
sessions during the same day. After the sessions of the day, fish were released into
their respective aquaria and fed.

2.4. Measured variables

Fish were considered to have learnt (i.e. they reached the acquisition criterion) when
they attained 9 correct choices in a session. In previous essays, it was seen that fish
that reached this punctuation, maintained or increased it in successive sessions;
therefore, when a fish attained the acquisition criterion it was not subjected to more
sessions (This entailed that not all fish required to perform the 10 sessions). Each fish
was assigned a learning category (yes, no) depending on whether they had reached the
acquisition criterion within a maximum of the 10 sessions or not. For the fish in the
learning category “yes” learning rapidity was evaluated through the number of
sessions performed to attain the acquisition criterion. The time each fish needed to
complete a session was recorded in order to calculate the average time per session (s)
for each sex and learning category. The average time per session refers to the sum of times for all the sessions (each one covering from the moment we introduced a fish inside the cylinder for the first time until it finished the 11th trial), divided by the total number of sessions it required to learn. Except for discarded fish, individuals emerged almost immediately from the cylinder (once the fish emerged, even if still inside the cylinder, they started to explore the decision-box from this position). Time was kept also constant between trials and between fish. Therefore, the observed time differences are a good estimator of fish exploration and they are inversely associated to impulsivity.

At the end of the experiment, individuals were measured in a small transparent container with a graph paper at the back. The variables considered were: TL for both sexes and SSC development for males (cephalic crest height and gland mean diameter). Cephalic crest height (Cr) was measured as the distance from the middle of the cranium to the top of the crest (mm) and the gland mean diameter (Gld) was obtained as the average of the first and second anal gland diameter. Both SSCs development variables were divided by TL, thus obtaining ratioCr (Cr/TL) and ratioGld (Gld/TL). All variables were log (x +1) transformed before statistical analyses.

2.5. Statistical analyses

In order to analyze whether the development of SSCs was related to the number of sessions required attaining the acquisition criterion, a generalized linear model (GLM) with a Poisson distribution of errors (with the link function log) was used. In this model, “number of sessions until acquisition” was the dependent variable (ranging from 1 to 10, where the fish that did not learn were assigned a 10). Each model
included initially as covariates: log(TL), log(ratioCr) and log(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 & Anderson, 2002).
Error adjustment was verified by visual inspection of the normal probability plots.
Statistical analyses were performed with the glm function in the package stats from
the free software R (R Core Team, 2012). The best model was found by using
sequentially the drop1 function.

3. Results

There were no significant differences in TL (mean ± SD, mm) between males (91.6 ±
7.9) and females (90.5 ± 3.2) when the experiment was conducted
(t=0.41, d.f.=18, P=0.69). Only 10 individuals of each sex out of the 40 males and 25
females subjected to the learning task were able to achieve it. The remaining
individuals were discarded because they did not emerge from the cylindrical
compartment (Fig. 1) during the first session. There were no significant differences in
the proportion of discarded males versus females (G-test=2.34, d.f.=1, P=0.13). Before
the experiment, six fish (3 males and 3 females) were submitted to 10 sessions without
landmarks. None of them attained the learning criterion and they maintained a
random error tax. In addition, all the fish used in the experiment (including those that
did learn), when committing an error, they pumped against the transparent door as in
the first session. Therefore, these facts altogether confirm that fish could not detect
the door.
The number of correct choices in the first session followed a random pattern for all individuals (binomial distribution P>0.20). Approximately more than half of the fish that were able to perform the experiment attained the acquisition criterion (55%). Among the fish that solved the task within 10 sessions there were 8 males (80% of males) and 3 females (30% of females), implying that males showed a major capacity than females in finding the exit of the apparatus by learning the position of the visual landmarks (G-test= 5.3, d.f.=1, P=0.021). The three females that reached the acquisition criterion required 7, 7 and 9 sessions respectively. The number of sessions that each of the eight males needed to learn the task can be found in table 1. There were no significant differences in the average time per session (s) between sexes (Fig 2a; Mann–Whitney U-test, n = 10, P >0.05) and between fish depending on their category of learning (yes, no) (Fig 2b; Mann–Whitney U-test, n = 10, P =0.07), although in this case the result is marginally significant. There were no differences in length between fish that learn and fish that did not (t=-0.49,d.f.=18,P=0.63).

The best GLM model for the number of sessions depending on TL and SSC development included only the variable log(ratioCr), which had a negative effect on the number of sessions (table 2). The males that had a major cephalic crest development during the session were those who learned faster (i.e. they needed fewer sessions to attain the acquisition criterion). It should be noted, in addition, that the three males that learned faster (they only needed the 4 first sessions) tended to have, apart from a large cephalic crest, larger glands than the rest (Table 1).

4. Discussion
The present study is the first experimental approximation to the use and learning of direct visual landmarks in *S. fluviatilis*. The first finding was that the species used visual landmarks to navigate and that males and females appear to differ in the learning task resolution. These differences might be attributed to distinct spatial abilities in the sexes adjusted to their distinct ecological demands (Odling Smee & Braithwaite, 2003).

This result supports the initial hypothesis of *S. fluviatilis* males being more prone than females to use visual landmarks, because males need to return to their nests after the short excursions they make in search for food or to defend their territories. However it is not clear whether there are other mechanisms at work, their relative importance or the interaction between them. Females seemed to use visual landmarks less than males. In some blennies, it has been found that females have a bigger telencephalon than males (Carneiro, Andrade, Oliveira, & Kotrschal, 2001; Costa et al., 2011).

Telencephalon is related to memory and spatial learning in fish (Overmier & Hollis, 1990; Broglio, Rodríguez, & Salas, 2003; Rodríguez et al., 2002) and it is important in the construction of the spatial map (Salas et al. 1996; López, Broglio, Rodríguez, Thinus-Blanc, & Salas, 1999, 2000). Spatial maps could be constituted by a series of submaps obtained through different sensory systems-(Sovrano, Bisazza, & Vallortigara, 2003; Quinn, 2005). The task presented in this experiment involved the use of only a single direct visual landmark (position of two stones indicating the correct exit of the maze). Females could have had a more global perception of the surroundings and they could have relied more in other stimulus that were not fixed throughout the experiment (owing to the apparatus change of configuration). Female blennies have larger home ranges than males (Costa et al., 2011) and they need to travel long distances in order to visit and reproduce with several males (Kraak, 1996; Fagundes,
Gonçalves, & Oliveira, 2007). In this context, it is likely that they prefer a wider signal repertory in order to navigate in large areas. Future studies are needed in order to ascertain if these hypotheses are correct by more strictly adjusting the learning task to the females’ ecological demands.

An association between learning and dominance on the one hand (Zhuikov, 1993) and between learning and boldness on the other (Dugatkin & Alfieri, 2003) has been established. Cephalic crest in blennies has been related both to dominance (Oliveira, Carneiro, Gonçalves, Canario, & Grober, 2001) and boldness (Fabre, García-Galea, & Vinyoles, 2014). Based on this premise, the positive relationship between cephalic crest development and learning ability found in this experiment seems coherent. The relationship we found between cephalic crest size and speed of learning cannot be attributed to age differences. All males had the same length and age at the beginning of the experiment. Age determination (through the observation of opercular bones and back-calculations in length following Vinyoles & Sostoa (2007)) permits us to ascertain that these fish were one year old. Differences in crest development could otherwise be influenced by other variables such as androgen concentration (Oliveira, Carneiro, Gonçalves, Canario, & Grober, 2001). Androgen concentration has already been related to spatial memory in rats (Benice & Raber, 2009).

In a study performed with guppies (Poecilia reticulata) it was found that females preferred to reproduce with males that solved a spatial task easier (Shohet & Watt, 2009). Some studies with blennies found that the cephalic crest is a sexual characteristic preferred by females (Gonçalves & Oliveira 2003; Fagundes, Gonçalves, & Oliveira, 2007) and this could give support to the crest as a cue used by females to
select bold and fast-learning males. However, other studies suggest that the cephalic crest plays an important role in intrasexual competition (Oliveira, Almada, Forsgren, & Gonçalves, 1999; Fabre, García-Galea, & Vinyoles, 2014).

Previous works studying visual landmark learning in fish through resolution of mazes show that some species such as the goldfish (Carassius auratus) adapt suitably to methodologies similar to that used in the present study (Salas et al., 1996; López, Broglio, Rodríguez, Thinus-Blanc, & Salas 1999, 2000). Contrarily, S. fluviatilis presented difficulties in adapting to the learning task. Despite the fact that the fish used in this experiment were specifically habituated to captivity and manipulation, only a 30.8% of them were able to perform the task. This produced a small sample size, so that obtained results must be interpreted with caution. It must be highlighted that in different animal species the adaptability to learning tasks and experimental conditions presents a high variability (Shettleworth, 1993). An exhaustive training, such as the one described here, has never been performed before to a speleophil-nest spawner fish and it is likely that fishes with this reproductive behaviour would be more sensitive to manipulation. A difference in emotional responsiveness between sexes as an explanation of the observed differences in this experiment is unlikely, as the proportion of discarded fish was not different between sexes. According to this, preliminary data from a previous experiment (Fabre et al., unpublished data) suggest that there are not differences in boldness (propensity to take risks) between males and females. The present results apply to those males and females that accepted the experimental design (probably, bold individuals) but future studies are required to investigate if these results still hold with shyer individuals. They also provide relevant information for the conservation of this endangered species (e.g. habitat...
requirements) and make a good start point for future research on spatial learning in fish. Increasing the habituation period to the apparatus or using other similar fish models (with a conservation status allowing the use of a higher number of individuals) should be taken into account.

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