

Article

Do Sand Smelt (*Atherina presbyter* Cuvier, 1829) Larvae Discriminate among Conspecifics Using Different Sensory Cues?

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Abstract: The ability of shoaling fish to recognise and differentiate between potential groupmates may affect their fitness and survival. Fish are capable of social recognition and multiple sensory cues mediate the recognition mechanisms. This has been comprehensively studied for juvenile and adult freshwater species. However, the recognition ability and mechanisms intervening during the larval phase of marine species are yet poorly understood. Fish larvae are capable of discriminating conspecifics from heterospecifics based on chemical and/or visual cues, but whether this recognition occurs at finer scales, such as discerning among conspecifics of different reefs, is yet understudied. Here, we tested the hypothesis that larvae of a marine fish species, the sand smelt (*Atherina presbyter* Cuvier, 1829), are able to recognise and associate with conspecifics of their natal reef versus conspecifics of a non-natal reef based on three sensory modalities—chemical, visual, and chemical and visual simultaneously. Results do not support our hypothesis, but still provide evidence of group cohesion and indicate large differences in the relative importance of the different senses when associating with conspecifics, with visual cues playing a more important role than chemical cues alone.

Keywords: chemical cues; visual cues; shoals; conspecifics; social recognition



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1. Introduction

Social species rely on recognition mechanisms to discriminate and associate with individuals similar to themselves, a phenomenon common to multiple taxa across the animal kingdom [1]. This has also been confirmed for multiple fish species, for which social recognition and attraction can be mediated by sex [2], body size [3], shoal size [4], body coloration [5], health status (e.g., external indicators of parasitism [6]), behaviour [7], relatedness [8], and familiarity [9]. Associating with either kin (genetically related) or familiar (with sufficient amount of time of interaction and sharing of activities between the individuals) fishes has several benefits for individuals' fitness and survival, as it promotes growth [10], enhances cooperative behaviour [11], improves group antipredator behaviour [12], enhances foraging activity [13], reduces aggression between individuals within the group [14], and even promotes learning abilities [14–16].

While kin recognition may mostly rely on the use of chemical cues that make up the individuals' chemical signature, such as cues mediated by the major histocompatibility complex (MHC) [17], recognition of familiar individuals may be based on a more general, group-specific pattern [17,18], or may rely specifically on individual recognition [1,19]. These expressions of social preferences can be mediated using a range of sensory cues, of which chemical and visual cues seem to be the ones that play the most significant role. While visual cues are only dependent on the physical appearance (phenotype) or behaviour of individuals [20], chemical cues provide additional information, either intrinsic (e.g., genotype (MHC related compounds)) [17] or extrinsic (e.g., habitat or diet) [21,22].

Several studies have reported preferences to associate with kin, and/or familiar conspecifics across a wide range of fish species (e.g., guppies [23], sticklebacks [24], rainbowfish [25], cichlids [8]), and addressed the sensory modalities involved in this association. For instance, adult three-spined sticklebacks associate with familiar shoals when relying only on chemical cues [26] and also when relying on chemical and visual cues simultaneously [22], but they show no familiar preference when only using visual cues [22]. On the other hand, adult banded killifish prefer to associate with shoals relying only on visual cues [27]. For several species, such as European minnows and guppies, a stronger social recognition of juvenile and adult conspecifics is expressed when fish are provided with both chemical and visual cues [17,18]. Despite the growing body of literature on fish social recognition mechanisms, most studies are on freshwater species, and focus on juvenile and adult fish. In contrast with freshwater species, many marine fish species have a complex, bipartite life cycle, with a pelagic, dispersive larval stage, followed by a benthic juvenile and adult stage. Historically, larvae were assumed to drift away from the reef to the open ocean and its distribution and settlement would only be driven by currents and other oceanographic conditions. Nevertheless, evidence of natal homing and self-recruitment in several reef species (e.g., [27–30]), as well as genetic-based evidence of high sibling association at settlement stage [31,32], indicates that fish might travel together during the pelagic larval phase, and therefore may have social recognition mechanisms to maintain social cohesion already during such a critical life period.

There is wide evidence that fish larvae are capable of discriminating conspecifics from heterospecifics based on chemical and visual cues (e.g., [32–35]). However, whether this recognition occurs at finer scales, such as discerning among conspecifics of different reefs, remains poorly understood. Here, we investigated whether larvae of sand smelt, a coastal marine species, can discriminate among conspecifics of natal and non-natal reefs. Sand smelt spawns large benthic eggs laid in close proximity [36], and larvae hatch well developed (6.5–7.5 mm SL, [36]), with good swimming abilities [37], and are able to use chemical cues to discriminate among habitats and conspecifics [38]. Therefore, it all seems to play in favor for a strong social cohesion. Indeed, dense shoals of different size classes are frequently observed close to the nearshore (A.M.F., pers. obs.). Here, we investigated if sand smelt larvae are able to use a combination of chemical and visual cues to maximize social recognition and group cohesion. To test these hypotheses, we ran multiple choice tests in which the focal larva is allowed to interact with two stimulus shoals (natal reef vs. non-natal reef) using different cues (chemical, visual, or both) and assessing the time and number of visits to each shoal.

2. Materials and Methods

2.1. Collection and Handling of Sand Smelt Larvae

Larvae were collected on July 2019 in the west coast of Portugal at two rocky reefs, one located at Avencas Marine Protected Area (Avencas, 38°40′58.74″ N, 9°20′19.01″ W) off the Tagus estuary, and the other at Arrábida Marine Park (Arrábida, 38°28′57.13″ N, 8°58′35.68″ W) off the Sado estuary. These sites are 35 km apart.

Larvae were collected using 1 mm mesh dip nets, and immediately transported to MARE-ISPA fish facilities in 30 L buckets with gentle aeration and water from the rocky reef. On arrival, larvae were randomly distributed through eight 30 L tanks, each with approximately 80 individuals. Tanks were separated with opaque white sheets, to prevent visual contact between larvae. Tanks were filled with artificial seawater (Marine Salt—Sal para Acuarios Marinos, ICA), equipped with an artificial filter, and maintained at a constant water temperature and salinity of 20 °C and 35, respectively, to match the conditions in the field. The photoperiod was of 14 h L:10 h D to simulate the natural photoperiod in Portugal in July.

Experimental tests were completed within 48 h after fish collection. Larvae were kept unfed until the completion of trials to avoid diet-based chemical cues from shaping conspecific recognition and preference [38]. Tests previously done with sand smelt showed

that larval condition and behaviour are not affected by this period of food deprivation (A.M.F., pers. obs.).

2.2. Experimental Tanks

To investigate the sensory cues involved in the expression of a preference for individuals of the natal reef vs. non-natal reef, three sensory modalities were assessed: chemical cues, visual cues, and chemical and visual cues simultaneously. For this purpose, three test tanks were used (Figure 1). Each test tank (400 mm × 250 mm × 300 mm—length, width, depth) was divided in three compartments using Plexiglas sheets, placed 70 mm from either end along the longest axis, leaving a 260 mm wide central compartment.

To investigate chemical cues, two opaque and perforated Plexiglas barriers (holes' diameter approximately 1 mm, 5 ± 1 holes/cm²) were used, whereas to exclude chemical communication and allow visual communication, barriers were replaced by two transparent and nonperforated Plexiglas sheets. To test chemical and visual cues simultaneously, two transparent and perforated Plexiglas sheets were used. Black lines were drawn outside of each test tank, to mark two 40 mm preference zones at either end of the central compartment. This 40 mm distance is the average distance kept between individuals in free shoals [39], which represents three body-lengths of a 13 mm larvae (total length). To prevent any external influence, the tank lateral walls were covered with opaque Plexiglas sheets, and during the trials, tanks were surrounded by a covered structure (black curtains).

Before the experiments, dyes were used to ensure that chemical cues passed through the perforated Plexiglas sheets in the tanks for chemical, and chemical and visual cues tests, and that they did not pass through the transparent and non-perforated Plexiglas sheet for visual cues tests.

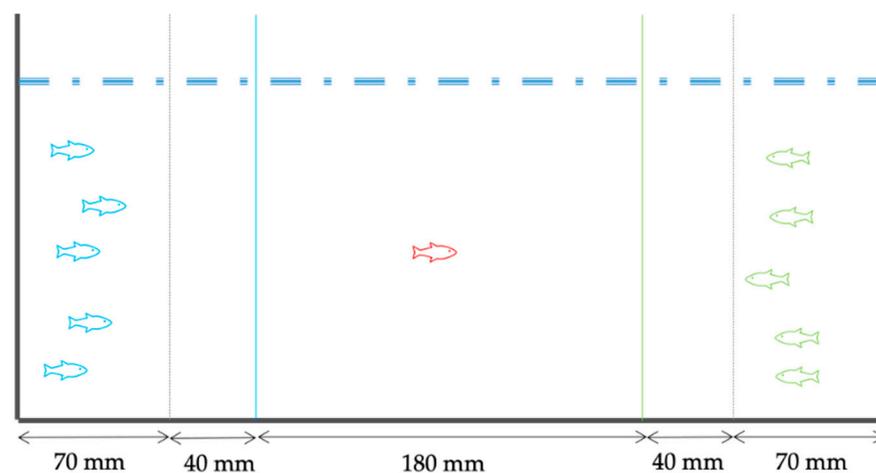


Figure 1. Scheme of the test tank (not drawn to scale). The focal fish is allowed to explore the central compartment and interact with the stimulus shoals placed at the two end compartments (70 mm). Focal and stimulus fish are separated by Plexiglas barriers (perforated and opaque, non-perforated and transparent and perforated and transparent for chemical, visual, and chemical and visual simultaneous tests, respectively). The two preference zones for each of the shoals are also marked (40 mm).

2.3. Experimental Protocol

Only larvae from Arrábida were used as focal fish, whereas larvae from Arrábida (natal reef) and Avencas (non-natal reef) were used as stimulus shoals. The single focal fish was always taken from the same tank as the Arrábida stimulus shoals. Two shoals composed of five larvae each were added to the two outer compartments of the test tank, one from natal reef and one from non-natal reef. Stimulus shoals were left for a 10 min habituation period, after which a focal fish was introduced in the central compartment of the test tank, inside a transparent and perforated cylinder with diameter of 40 mm. The focal fish was left inside the cylinder for a 5 min habituation period, after which the

cylinder was gently removed, and fish behaviour was recorded during 10 min with a video camera (SONY handycam DCR-SR58E).

After each trial, the focal fish and shoals were removed, and the test tank was washed with tap water before another trial. Each focal fish and stimulus shoal were used only once. For control purposes, and to account for possible side preference, a further experiment was performed without any stimulus shoals at either end of the experimental tank. Furthermore, the side of the tank occupied by the natal reef shoal was randomised between replicates, and the order of the tests (chemical, visual, chemical and visual simultaneously) was also kept randomised.

Experiments were run twice in time (two batches, two weeks apart), in a total of 18 replicate trials to assess chemical cues (9 replicates in each batch), 18 replicate trials to assess visual cues (9 replicates in each batch), and 17 replicate trials to assess chemical and visual cues simultaneously (8 replicates in batch 1 and 9 replicates in batch 2). Additionally, we carried out a total of 11 replicates for the control experiment.

For each experimental trial, the focal fish and the conspecifics forming the stimulus shoals matched as closely as possible for standard length (SL) (Supplementary Table S1). However, in batch 2, larvae from the non-natal reef were slightly larger. In batch 1, the size of larvae from the natal reef (focal fish included, as it was removed from the same group) and from the non-natal reef did not differ (one-way ANOVA: $F_{(1,40)} = 2.732$, $p = 0.106$), and averaged (\pm standard error) 11.1 ± 0.2 mm and 11.6 ± 0.2 mm, respectively; in batch 2, however, the size of shoals of the natal and the non-natal reefs differed significantly (one-way ANOVA: $F_{(1,29,555)} = 39.701$, $p = 0.000$), and averaged (\pm standard error) 11.9 ± 0.2 mm and 14.8 ± 0.4 mm, respectively.

2.4. Video Analysis

Videos were analyzed using the video tracking software The Observer XT 7.0. A total of 64 videos of 10 min each were analyzed. For each video, the time spent by the focal fish in the central compartment and within the preference zone of each stimulus shoal was registered, as well as the number of visits to each zone.

2.5. Data Analysis

Normality and homogeneity of variances were visually assessed with Q-Q plots, Shapiro–Wilks and Levene tests, but were not assumed for the majority of data. A Mann–Whitney U test tested for differences in larval preferences for each sensory modality between the two batches (batch 1, batch 2). There were no differences in larval preferences, and therefore data for each sensory modality (chemical, visual, and chemical and visual simultaneously) were pooled for the final analysis (Supplementary Tables S2 and S3). The preference of the focal larva for a stimulus shoal (or empty compartments, in the case of the control test) was tested using the non-parametric Wilcoxon matched-pairs signed-ranks test.

A generalized linear mixed model (GLMM) was constructed to analyze the contribution of each sensorial cue (visual, chemical, visual and chemical) on the percentage of time spent close to each stimulus shoal, as well as percentage of visits to each shoal. Cue was inserted as fixed factor, and Batch was assigned as a random factor. Tukey post hoc tests were used for further pairwise comparisons.

p -values below 0.05 were considered significant, although our analysis places a greater emphasis on graphical representation of the data due to the imprecise nature of p -values ([40]) and low sample sizes in our study. Values are reported as means \pm Standard Error (S.E.).

All data analysis was conducted in R statistical software (version R 3.5.2) and R Studio (version 1.1.463), using the ‘glmer’ function of the ‘lme4’ R package for running GLMM models.

3. Results

3.1. Overall Preferences

In the control experiment, when both end compartments of the experimental tank were empty, sand smelt larvae spent approximately the same time on each preference zone ($10.811 \pm 2.077\%$ vs. $14.811 \pm 3.132\%$, $Z_{(11)} = 1.067$, $p = 0.286$) and performed the same number of visits to each preference zone ($22.035 \pm 3.402\%$ vs. $26.097 \pm 3.289\%$, $Z_{(11)} = 0.711$, $p = 0.477$).

When considering the three sensory modalities together (chemical, visual, and chemical and visual simultaneously), sand smelt larvae spent $22.130 \pm 4.301\%$ of the time close to the natal reef shoal and $12.830 \pm 2.732\%$ of time close to the non-natal reef shoal ($Z_{(44)} = 1.37$, $p = 0.168$, Tables 1 and 2, Figure 2A). Considering the number of visits to each shoal, sand smelt larvae visited the natal reef shoals $23.130 \pm 2.644\%$ and $19.340 \pm 2.516\%$ the non-natal reef shoals ($Z_{(49)} = 0.91$, $p = 0.360$, Tables 1 and 2, Figure 2B).

Table 1. Summary of the mean and the standard error (SE) for each of the tests performed.

Shoal Preference	Shoal	Mean	SE
<i>Control</i> % time	Natal	10.811	2.077
	Non-natal	14.811	3.131
% visits	Natal	21.910	3.399
	Non-natal	27.000	3.267
<i>All cues together</i> % time	Natal	22.151	4.301
	Non-natal	12.604	2.739
% visits	Natal	22.755	2.616
	Non-natal	18.769	2.526
<i>Chemical cues</i> % time	Natal	4.390	2.535
	Non-natal	8.222	3.859
% visits	Natal	13.060	4.127
	Non-natal	21.278	4.375
<i>Visual cues</i> % time	Natal	34.278	8.830
	Non-natal	14.611	4.362
% visits	Natal	26.889	4.300
	Non-natal	20.222	4.370
<i>Chemical and visual cues simultaneously</i> % time	Natal	28.118	7.715
	Non-natal	15.117	5.996
% visits	Natal	28.647	4.457
	Non-natal	14.294	4.450

Table 2. Summary statistics for differences in percentage of time and visits to each stimulus shoal, according to the sensory cues under test (visual, chemical, chemical and visual simultaneously), using Wilcoxon matched-pairs signed-ranks; test value, sample size (N), and the p -value (p).

Shoal Preference	Test Value	N	p
<i>Control</i> % time	1.067	11	0.286
	0.711	11	0.477
<i>All cues together</i> % time	1.377	44	0.168
	0.915	49	0.360
<i>Chemical cues</i> % time	0.489	11	0.625
	1.164	15	0.244
<i>Visual cues</i> % time	1.219	18	0.222
	0.980	18	0.327
<i>Chemical and visual cues simultaneously</i> % time	1.079	15	0.280
	1.474	16	0.140

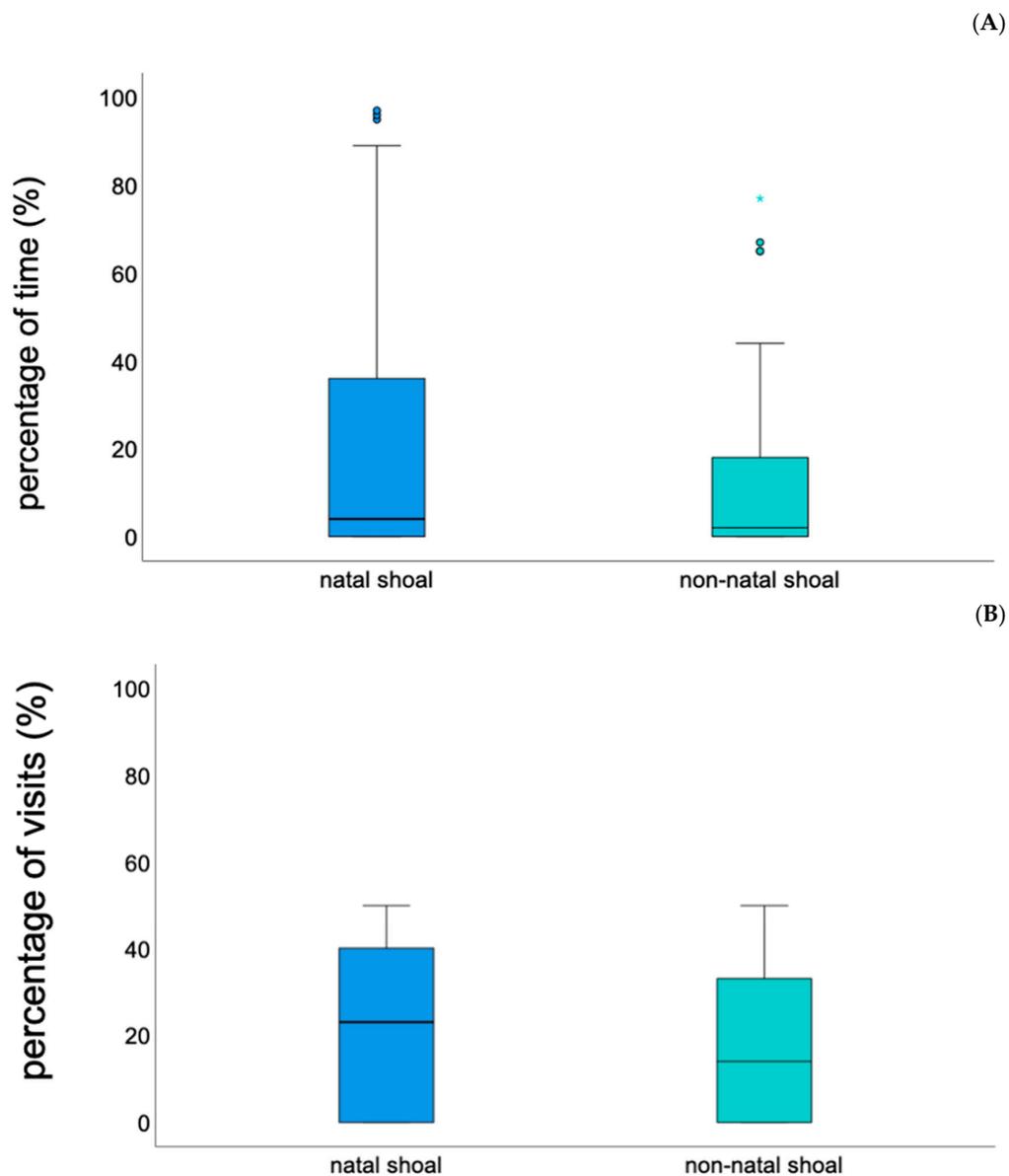


Figure 2. (A) Percentage of time (%) spent by the focal fish near the natal and non-natal shoal; (B) Percentage of visits (%) performed by the focal fish to the natal and non-natal shoal. The boundaries of the box closest to and farthest from zero indicate the 1st and 3rd quartile, respectively; the black line within the box marks the median; whiskers below and above indicate the minimum and maximum value of the data set, respectively; circles indicate outliers; asterisks indicate extremes.

3.2. Sensory Modalities

When comparing the relative importance of each sensory modality in the choice for natal reef shoals, sand smelt used the three cues differently (Figure 3). Larvae spend considerably more time close to the natal reef individuals when visual cues alone were present ($34.28 \pm 8.83\%$) compared with chemical and visual cues simultaneously ($28.11 \pm 7.71\%$) (GLMM, $Z_{(53)} = -3.251$, $p = 0.001$), and compared with chemical cues alone ($4.39 \pm 2.53\%$) (GLMM, $Z_{(53)} = -17.201$, $p < 0.0001$) (Figure 3A). Sand smelt larvae also spend more time close to the natal reef individuals when both chemical and visual cues were present, compared with chemical cues alone (GLMM, $Z_{(53)} = 15.293$, $p < 0.001$) (Figure 3A). A similar pattern was observed regarding the frequency of the visits by the focal fish to the natal reef shoal, making more visits to familiar individuals when visual cues alone were present ($26.89 \pm 4.30\%$) compared with chemical cues alone ($13.06 \pm 4.13\%$) (GLMM, $Z_{(53)} = -9.087$,

$p < 0.0001$), although the frequency of visits did not differ when both chemical and visual cues were present ($28.65 \pm 4.46\%$) (GLMM, $Z_{(53)} = 0.987$, $p = 0.324$) (Figure 3B).

When associating with non-natal reef shoals, results follow a similar pattern (Figure 4). Time spent close to the stimulus shoals, as well as the number of visits, differs depending on the sensory modality involved, with larvae spending more time close to non-natal reef shoals when visual cues alone are present ($14.61 \pm 4.36\%$), compared with chemical cues alone ($8.22 \pm 3.86\%$) (GLMM, $Z_{(53)} = -5.60$, $p < 0.0001$), but not differing when both chemical and visual cues are present ($15.12 \pm 5.99\%$) (GLMM, $Z_{(53)} = 0.987$, $p = 0.324$) (Figure 4A). Sand smelt visited the non-natal reef shoals more frequently when visual cues are present ($20.22 \pm 4.37\%$), compared to when both chemical and visual cues were available ($14.29 \pm 4.45\%$) (GLMM, $Z_{(53)} = -4.19$, $p < 0.0001$), but frequency of visits did not differ when chemical cues alone were present ($21.28 \pm 4.37\%$) (GLMM, $Z_{(53)} = 0.695$, $p = 0.487$) (Figure 4B).

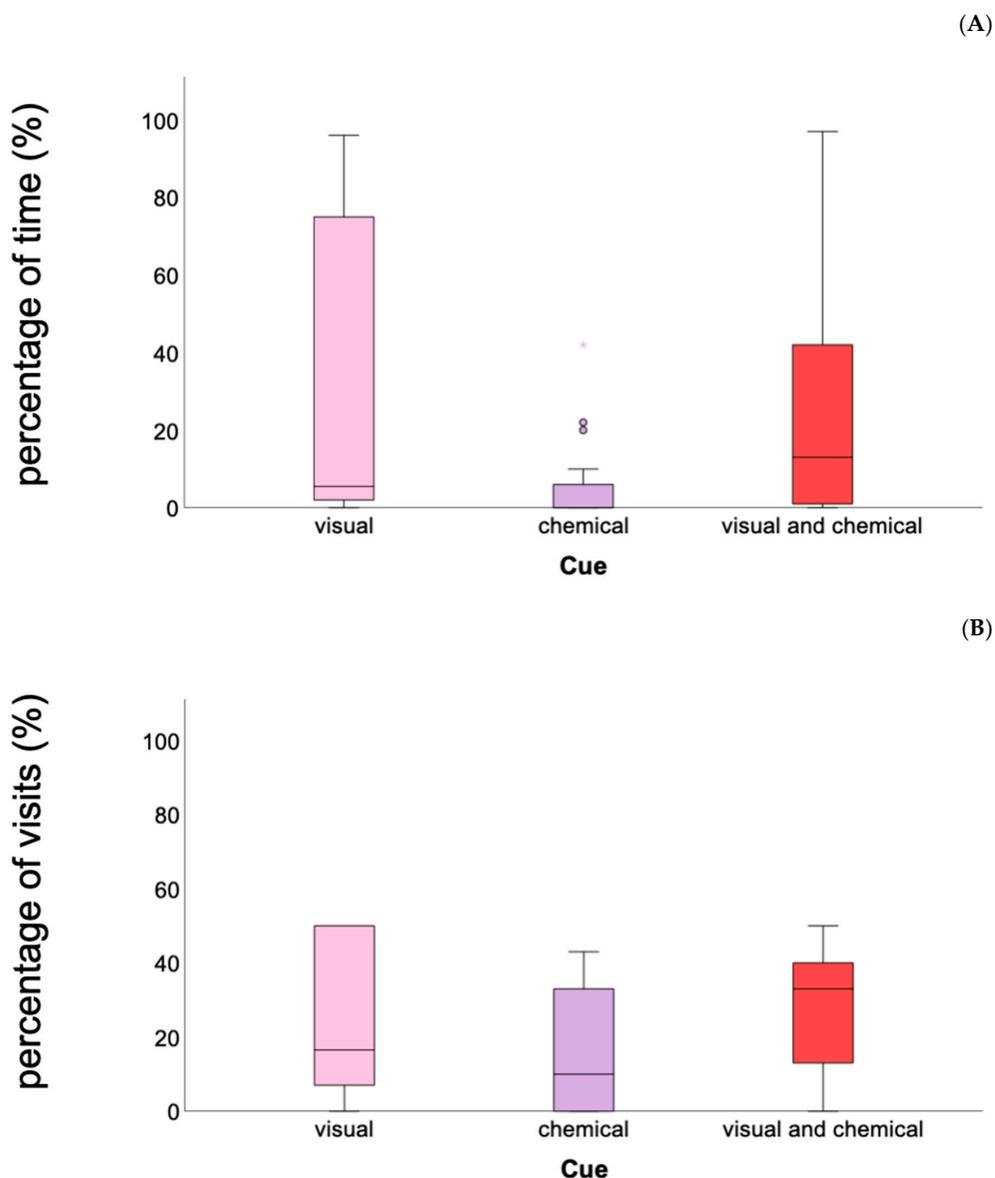


Figure 3. (A) Percentage of time (%) spent by the focal fish near the natal shoal; (B) Percentage of visits (%) performed by the focal fish to the natal shoal. The boundaries of the box closest to and farthest from zero indicate the 1st and 3rd quartile, respectively; the black line within the box marks the median; whiskers below and above indicate the minimum and maximum value of the data set, respectively; circles indicate outliers; asterisks indicate extremes.

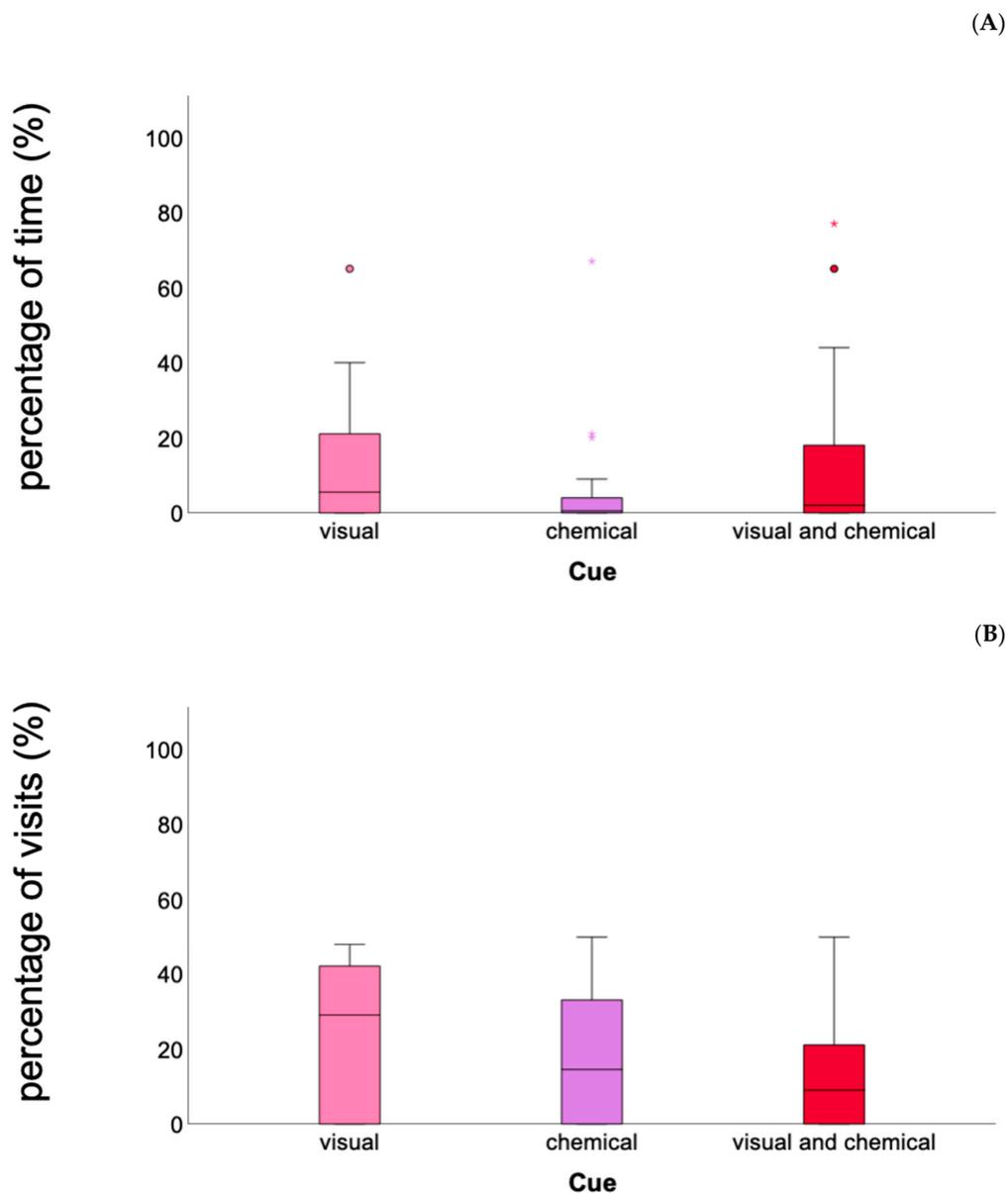


Figure 4. (A) Percentage of time (%) spent by the focal fish near the non-natal shoal; (B) Percentage of visits (%) performed by the focal fish to the non-natal shoal. The boundaries of the box closest to and farthest from zero indicate the 1st and 3rd quartile, respectively; the black line within the box marks the median; whiskers below and above indicate the minimum and maximum value of the data set, respectively; circles indicate outliers; asterisks indicate extremes.

3.2.1. Visual Cues

When only visual cues were present, sand smelt spent twice the time close to natal reef shoals, compared with non-natal shoals ($34.22 \pm 8.84\%$ vs. $15.39 \pm 4.28\%$, $Z_{(18)} = 1.219$, $p = 0.222$; see Tables 1 and 2, Figures 3A and 4A). However, the frequency of visits to natal and non-natal reef individuals was very similar ($26.89 \pm 4.30\%$ vs. $22.11 \pm 4.26\%$, respectively; $Z_{(18)} = 0.980$, $p = 0.327$; see Tables 1 and 2, Figures 3B and 4B).

3.2.2. Chemical Cues

When only chemical cues were available, sand smelt larvae spent little time close to natal reef fish ($4.390 \pm 2.535\%$) and non-natal reef fish ($8.110 \pm 3.870\%$) ($Z_{(11)} = 0.489$, $p = 0.625$; see Tables 1 and 2, Figures 3A and 4A). The frequency of visits to natal and non-natal stimulus shoals averaged $13.060 \pm 4.127\%$ vs. $21.330 \pm 4.374\%$, respectively ($Z_{(15)} = 1.164$, $p = 0.244$; see Tables 1 and 2, Figures 3B and 4B).

3.2.3. Chemical and Visual Cues Simultaneously

When both chemical and visual cues were present, sand smelt spent almost twice the time close to the natal reef fish comparatively to the non-natal shoal ($28.12 \pm 7.71\%$ vs. $15.12 \pm 5.99\%$, respectively, $Z_{(15)} = 1.079$, $p = 0.280$; see Tables 1 and 2, Figures 3A and 4A), and performed twice the number of visits to natal reef fish, compared with non-natal reef fish ($29.89 \pm 4.52\%$ vs. $14.29 \pm 4.45\%$, respectively; $Z_{(16)} = 1.474$, $p = 0.141$; see Tables 1 and 2, Figures 3B and 4B).

4. Discussion

Contrary to our expectations, results from the present study do not support the hypothesis that sand smelt is capable of fine-scale discrimination to the point of being able to discriminate between conspecifics of different reefs. Our initial hypothesis was based on both field and behavioural observations that suggest strong school cohesion soon after hatching (A.M.F. pers. obs.), considerable swimming abilities [37], and ability to discriminate among habitats and conspecifics based on chemical cues [38]. Moreover, the fact that sand smelt spawn benthic eggs attached to vegetation implies that individuals are in close proximity since the embryonic stage, and they can be either kin (genetically related) or familiars (with a sufficient amount of time interacting and sharing activities) [41]. Together, this evidence suggested that sand smelt larvae could show strong shoal cohesion and be able to discriminate between conspecifics of different reefs. This was not the case, although we did see that sand smelt spent twice the average time associating with natal reef individuals compared with non-natal reef fish when visual cues were present. However, there was also high interindividual variability associated with the observed responses, particularly when visual cues were available (alone and simultaneous with chemical cues), suggesting that some fish are more sociable than others. Ref. [42] examined the within-species variation in grouping tendency in the western mosquitofish, and scored individuals based on their social personality types (sociability), describing both highly social types, and asocial individuals, who shoaled less and swam more between shoals.

One possible explanation for the observed lack of preference for either shoal (natal or non-natal) might have to do with time that the focal fish spent with the natal shoal individuals before being tested in the choice arena. Familiarity (preference to associate with conspecifics based on prior social experience) develops gradually, over a period of time [14,19]. In our experiment we chose to test larvae within 48 h after they were collected in the field, because we wanted to avoid feeding the individuals, as a previous study showed that diet-based chemical cues shaped conspecific recognition and preference in this species [38]. We might, therefore, prevented larvae from gaining familiarity with fish from their natal site. Future studies should address this hypothesis.

We cannot rule out the possibility that the experimental tank also influenced the association preferences. The test involves placing the focal fish in a novel environment, an open arena where there is no refuge, and this environment must be perceived as a dangerous environment. In this case, associating with a shoal of conspecifics, independently of being familiar or not, might be more advantageous than being alone. We did not observe any particular stressful behaviours, though, such as erratic swimming, or clinging to the walls of the tank, suggesting that this might not be the case.

Despite the lack of preference to associate with natal or non-natal reef individuals, our results indicate that sand smelt larvae use visual and chemical cues differently, to associate with conspecifics. We observed that visual cues were more important to associate with conspecifics (regardless of being natal or non-natal reef shoals), followed by chemical and visual cues simultaneously, and finally by chemical cues (Figures 3 and 4).

4.1. Visual Cues

Aquatic environments are quite variable in terms of visual and olfactory conditions, due to varying turbidity (which reduces the efficiency of visual cues) and currents (which disrupts chemical cues). There is evidence that larval fish can use both chemical and

visual information to find a habitat to settle [33,43], and discriminate between conspecifics and heterospecifics [34], suggesting that there might be a wide variability in the relative importance of chemical and visual cues. However, very few studies have addressed the relative importance of the different sensory modalities in fish preferences (e.g., [44]).

For sand smelt larvae, similarly for most marine fish larvae, vision is likely to play a critical role in locating food and detecting predators [33], and it is reasonable to assume that vision also plays a key role in social affiliation [45]. Visual cues provide to fish information about phenotype (specific traits, such as color and shape [41]) and body motion [46] of the individuals, and this information can be used by fish to decide whether or not to interact.

4.2. Chemical Cues

Opposite to visual cues, chemical cues alone played a minor role in sand smelt association to conspecifics, when compared with visual cues and visual and chemical cues simultaneously. This was surprising, not only because a large number of studies in the literature points to the use of chemical cues in kin and conspecific recognition [47,48], but also because previous research provided evidence that chemical cues can trigger a preference response in sand smelt larvae [38]. This inconsistency is likely due to differences in the experimental approaches. The previous study tested chemical cues in a two-choice flume chamber, where cues were presented in higher concentrations, and the shoal providing the cues was six times larger in number (30 individuals) [38]. In the current study, although we confirmed, using dye tests, that water flew from the shoal compartments to the central arena, it might be that the chemical cues released by five individuals is insufficient to elicit a stronger association response. Nevertheless, there are also examples of fish requiring visual cues or a combination of visual and chemical cues for conspecific recognition [49]. A possible explanation for relying more on visual than chemical cues might be related to ecological characteristics of the habitat where sand smelt larvae inhabit. Sand smelt larvae are usually found in the transparent, surface waters of the very nearshore (A.M.F. pers. obs.), thus depending more on vision.

4.3. Chemical and Visual Cues Simultaneously

When both chemical and visual cues were present, it was expected that the response to associate with conspecifics would be stronger or, at least, equal comparatively to when in the presence of only one of the modalities of cues, chemical or visual. This was the case when associating with non-natal reef shoals (the two cues simultaneously were as important as visual cues), however, when associating with natal conspecifics, visual cues alone still played a more important role when deciding to associate with conspecifics. It might be the case that when larvae were presented with both cues, they spent more time exploring the experimental arena (supported by the observed higher percentage of visits to each shoal, which did not differ between visual cues alone and chemical and visual cues simultaneously).

An experimental caveat of our study, which could potentially influence the relative importance of each sensory modality when associating with conspecifics, relates to the different body sizes of the stimulus shoals, that differed between the two batches of experiments. In batch 2, fish from the non-natal reef were significantly larger than the focal fish and fish from the natal reef. Body size is known influence the decision to associate with a particular group of fish, and many species prefer to associate with conspecifics of similar size [3,26]. Additionally, it is likely that larger fish will provide a higher concentration of chemical cues. However, we consider that this body size difference is unlikely to have affected overall preferences. Not only we did not see a batch effect on our model, indicating that between batches, larvae processed the sensory information in similar ways, but our field observations ([36], A.M.F. pers. obs.) indicate that sand smelt larvae shoals with conspecifics of different sizes and ages ([36], A.M.F. pers. obs.), thus discrediting the possibility that sand smelt larvae express a preference to associate with similar size fish.

4.4. Overall Insights into Marine Fish Larval Ecology

Preferential shoaling with individuals from the natal reef since early life may contribute to the maintenance of social structure within natural populations. The benefits of such social organization include reduced competition and aggression between individuals [14,50], and allow larvae to spend energy on more important activities, such foraging [51], avoiding predators [13], and even enabling social learning [16]. Our current data does not support the hypothesis that sand smelt larvae is capable of fine-scale discrimination between conspecifics from different reefs. However, a few experimental limitations might have shaped these overall results, in particular, the fact that we did not allow larvae to gain familiarity while in the laboratory. A future study should address this experimental limitation. Moreover, we only tested the response of one population (natal reef, Arrábida), therefore we cannot rule out any idiosyncrasy associated with that population. Running experiments with wild individuals has several logistical constraints, which we cannot control, namely the number of individuals we can collect, as well as size range. Replicating the exact same experiment with focal fish from another reef (Avencas) would be very challenging as it would require a large number of individuals, that are difficult to obtain from that foreign reef. However, the fact that we conducted two batches of experiments, separated in time, and obtained similar results, gives us confidence on our data.

Despite not finding support for our hypotheses, results still provide evidence of strong school cohesion, based on different sensory modalities. Future research directions should then include identifying fitness and survival benefits of such social organization, and further understand genetic relatedness of cohorts arriving to coastal habitats for settlement. Fish larval siblings have been found in close association after the planktonic dispersal phase, suggesting that they have travelled together throughout this period of time [30,31]. Moreover, whether kin/familiar association remains throughout juvenile and adult stages should be investigated, as this social recognition will be essential to avoid inbreeding. This overall knowledge will provide critical information on species dynamics and populations structure, which has fundamental implications for species conservation and management of marine protected areas.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/oceans2040038/s1>, Table S1. Summary of the mean and the standard error (S.E.) of the larvae (standard length in mm) of both shoals and batches used in the study; Table S2. Summary of the mean and standard error (S.E.) of the preference of the focal larva (% time and % visits) for each stimulus shoal (natal and non-natal), sensory cues (visual, chemical, chemical and visual simultaneously), and batch of experiments (1 and 2); Table S3. Summary statistics for differences in percentage of time and visits to each stimulus shoal, according to the sensory cues under test (visual, chemical, chemical and visual simultaneously) and between batches (1 and 2), using Mann–Whitney U test; test value (U), Z-score, sample size (n in each batch), and the *p*-value (*p*).

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