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Habituation and conditioning in gilthead sea bream (*Sparus aurata*): Effects of aversive stimuli, reward and social hierarchies

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Abstract

To tailor the farming environment to a fish species, we should understand the species-specific responses to stimuli, including the degree of adaption and learning. Groups of gilthead sea bream were given a delay Pavlovian conditioning regime using a conditioning stimulus (CS) of light flashes signalling arrival of food. Controls were exposed to light flashes unrelated to feeding. Fish in both treatments showed an initial fear response of moving away from the CS combined with reduced swimming speed. In subsequent trials, the Control fish largely habituated the fleeing response but sustained to respond by reducing the swimming speed. The Conditioning fish also stopped to escape from the CS, but opposed to the Control group they gradually increased their swimming speed in response to the CS. In addition, the number of fish in the feeding/CS area increased and became similar to basal level after around 16 trials. A small and variable proportion of the fish displayed black vertical bands on their body and territorial behaviour, and a social hierarchy could interfere with the processes of habituation and conditioning. The swimming speed of the fish increased with number of dark individuals, but this was not found during the CS and the light stimulus thus seemed to overrule the effect of territorial behaviour. The persistent negative response to light flashes in the Control suggests that fish seemingly adapted to repetitive stressors are still in a state of alertness. The change in the response to light shows the potential for rewarding aversive stimuli to reduce stress.

KEYWORDS
anticipatory behaviour, classical conditioning, feeding behaviour, habituation, learning, welfare

1 | INTRODUCTION

To tailor the farming environment to a given fish species, it is crucial to learn more about the initial response to various stimuli as well as the subsequent conditioning to biologically relevant stimuli and habituation to stimuli without any consequences. The capacity fish have for conditioning and habituation will determine how well fish cope with repetitive husbandry procedures and fluctuating environmental conditions. The learnt association between biologically relevant stimuli (unconditioned stimuli, US) and initially neutral cues (conditioned stimuli, CS), i.e. Pavlovian conditioning, induces a conditioned response (CR) at presentation of the cue alone (Lieberman, 2000). Pavlovian conditioning has been demonstrated in numerous fish species relevant for farming and research such as zebrafish (*Danio rerio*) (Manabe, Dooling & Takaku, 2013), rainbow trout (*Oncorhynchus mykiss*) (Colson et al., 2015; Nordgreen, Janczak,

Hovland, Ranheim & Horsberg, 2010), Atlantic salmon (*Salmo salar*) (Bratland et al., 2010), Atlantic cod (*Gadus morhua*) (Nilsson, Kristiansen, Fosseidengen, Fernö & van den Bos, 2008a,b) and Atlantic halibut (*Hippoglossus hippoglossus*) (Nilsson et al., 2010). Signalization of food arrival could be used in everyday farming to make feeding more predictable and to assess the appetite in fish groups (Bassett & Buchanan-Smith, 2007; Fernö, Huse, Jakobsen, Kristiansen & Nilsson, 2011). Anticipatory behaviour may also be used as an operative welfare indicator (Folkedal, Stien et al., 2012). However, findings from one species and one life stage cannot directly be transferred to another species (Martins et al., 2012). The cruising predator cod approaches the CS and the feeding area (Nilsson et al., 2008a,b), whereas the "sit-and-wait" predator halibut only responds by subtle positional changes (Nilsson et al., 2010), and the conditioned response of parr and post-smolt of Atlantic salmon are also markedly different (Folkedal, Stien, et al., 2012; Folkedal, Torgersen, et al., 2012).

Farmed fish are repeatedly exposed to stimuli that are initially perceived as aversive but are without any biological consequences which makes the fish to habituate to the stimulation (Folkedal, Torgersen, Nilsson & Oppedal, 2010; Madaro et al., 2016). In contrast, when fish are repeatedly exposed to a stimulus associated with reward or punishment, they will develop a conditioned response. Cues used in conditioning may initially release a fright reaction and may only after some time result in a positive response—"from fright to anticipation" (Bratland et al., 2010; Nilsson, Stien, Fosseidengen, Olsen & Kristiansen, 2012). How habituation modulates the response to the initial frightening stimulus when the stimulus is rewarded versus unrewarded is, however, not addressed in previous studies. This knowledge is required in understanding the scope for using reward to accelerate accommodation processes.

Farmed fish are reared in high densities and agonistic behaviour has been observed in several species (Barreto, Boscolo & Gonçalves-de-Freitas, 2015; Fernö & Holm, 1986; Jobling, 1983; Solstorm et al., 2016). Aggressive individuals should be expected to influence the baseline behaviour and stress level of subordinate fish and could thereby interfere with the processes of habituation and conditioning. The effect of social hierarchies has, however, been devoted little attention in earlier studies of learning in fish groups.

Gilthead sea bream (*Sparus aurata*) is widely cultured in the Mediterranean. Sea bream is a grazing species feeding mainly on shellfish, snails and mobile prey like fish (Andrade, Erzini & Palma, 1996; Pita, Gamito & Erzini, 2002). Farmed sea bream has been observed to increase swimming speed both during feeding (Andrew, Noble, Kadri, Jewell & Huntingford, 2002) and before scheduled feeding (Sánchez, López-Olmeda, Blanco-Vives & Sánchez-Vázques, 2009), suggesting anticipation for food. However, the appetitive behaviour has not been studied in detail, and to the best of our knowledge no detailed description of the anticipatory behaviour of sea bream during reward conditioning exists. A striking feature of gilthead sea bream and other sparidaes is transient changes in skin colouration in dominant individuals as displayed by vertical dark bands on the light hued bodies (Papadakis, Glaropoulos,

Alvanopoulou & Kentouri, 2016; Pavlidis & Mylonas, 2011). This overt marker may be used to assess the effect of the social environment on learning.

Here, we investigated habituation and Pavlovian anticipatory behaviour of gilthead sea bream exposed to an initially aversive flashing light that either announced a food reward (habituation and conditioning) or was unrewarded (control, habituation only). We hypothesized that, compared to unrewarded sea bream, rewarded sea bream would habituate more rapidly and eventually express food anticipation to the light flashes. We further predicted that social hierarchies would influence the anticipatory behaviour.

2 | MATERIALS AND METHODS

The experiment was carried out at the Matre Research Station (Institute of Marine Research, Norway) in April 2012, using commercially bred juvenile gilthead sea bream (Ferme Marine de Douhet, Ile d'Oléron, France) that were reared in Spain prior transportation to Norway in January 2012. The fish were kept in indoor tanks (500 L) in groups of ~28 fish per tank, under a 12D:12L photoperiod and fed a daily ration of 2% of biomass (ad libitum) (4.5 mm dry pellets, EFICO YM 54 N[0], BioMar, Spain). All tanks were supplied with seawater ($20.0 \pm 0.1°C$, 33 ‰ salinity) with the flow rate adjusted to keep a minimum O_2 saturation of 80% in the effluent water. The fish were given an acclimation period of 3 months after arrival, and 4 days before the experiment started the standard length and weight were 22.3 ± 1.0 (mean $\pm$ SD) cm and 246.8 ± 41.6 (mean $\pm$ SD) g respectively.

To secure a high feeding motivation, the daily feed ration was reduced to 1.4% of biomass 2 days before experimental start and kept at this level throughout the experiment. The conditioning experiment lasted 4 days. Automatic feeders (arvotech.fi), delivering the pellets in one corner of each tank, were set to distribute the daily ration over 10 equally sized meals (300 s), every hour from 09:00 until 18:00. The conditioning stimulus (CS) consisted of flashing (1 s on and 2 s off) with a light bulb (12 V, 21 W) positioned 5 cm above the surface in the feeding area, and food was given as the unconditioned stimulus (US). The conditioning regime consisted of the CS-light flashing from 30 s before feeding to 10 s after start of feeding (i.e. delay conditioning). A triplicate tank group was given the conditioning regime (Conditioning group), whereas a triplicate Control group was fed 30 min after the CS, that is, the CS had no predictive or incentive value. On Day 1 in trials 3, 4 and 5, all feeders in the Conditioning group failed to deliver food during the CS. We therefore stopped the conditioning procedure for Day 1 after trial 6, resulting in only 6 trials this day. On the last day of the experiment (Day 4), the conditioning procedure was stopped after the 5th meal, resulting in a total of 28 trials.

Video recordings were made of all trials by a submerged camera in each tank that allowed behaviour observations of about 75% of the tank volume from bottom to surface, including the feeding

area. Videos of every second trial were for all tanks (three tanks per treatment) analysed for fish spatial distribution 10 s before and 10 s after the CS flashes started. This was carried out by counting the number of fish in the quarter of the tank defined as the feeding area in a frozen video image. For the same time points and for all tanks, a swimming speed analysis (body lengths/s) was carried out on 10 randomly selected individuals by measuring the time from when the snout and tail passed a virtual vertical line. This line was set by the observer in the video image tank centre to provide a best possible side view of the fish, and allowed for recording of fish in any vertical position. Using the same start video frame for all 10 individuals per tank per observation time point, we avoided multiple recordings of the same fish. Individual fish with darker skin pigmentation (vertical bands, assumed to reflect territorial behaviour) were not included in the swimming speed analysis as their speed was markedly slower than non-territorial fish. To study how the presence of fish that appeared with darker skin pigmentation affected the average swimming speed of other fish in the tank, the number of dark fish in the tank was recorded 10 s before the CS flashes started.

Welch two-sample t-test was used to test differences between treatment groups in swimming speed and number of fish in the feeding area, before and during the CS. Kendall's tau rank correlation test was used to test whether swimming speed and number of fish in the feeding area before and during the CS were correlated with trial number, for each treatment group separately. Change in swimming speed from baseline to during the CS in each treatment group was tested with paired t-test. Effect of treatment, number of dark individuals and their interaction on the swimming speed before and during the CS was tested with linear model (lm). As number of dark fish is count data, effect of treatment, trial and their interaction on the number of dark fish in the tank was tested with generalized linear model (glm) for Poisson distribution. Also, the effect of number of dark individuals in the tank on the number of fish in the feeding area before and during the CS was tested with glm for Poisson distribution. All statistical analyses were done with R software system Version 3.2.1 (Copyright 2015, The R Foundation for Statistical Computing, Vienna, Austria), and the level of significance was set to 0.05.

3 | RESULTS

The number of fish in the feeding area before the CS did not differ between the Conditioning and the Control groups (Welch two-sample t-test, $t = 0.51$, $p > .1$) and did not change over trials in neither the Conditioning (Kendall's tau rank correlation, tau = .066, $p > .1$) nor the Control (tau = .022, $p > .1$) group (Figure 1a). Both treatment groups showed initially a negative response to the CS light flashes by fleeing from the feeding/CS area, but the strength of the response declined with trial number for both groups and the number of fish in the feeding area 10 s after the onset of the CS increased with trial number in both the Conditioning (Kendall's tau rank correlation, tau = .68, $p < .001$) and Control (tau = .76, $p < .001$) groups (Figure 1b). The number of fish in the feeding area during the CS was significantly higher in the Conditioning group than in the Control (Welch two-sample t-test, $t = 4.70$, $p < .001$) group, especially from around 10 trials onwards (Figure 1b). The Control group did not reach baseline levels of presence in the feeding area during the CS period (Figure 1). In contrast, although the Conditioning group never clearly aggregated in the feeding area during the CS flashes, the number of fish in the feeding area was slightly higher than baseline level after approximately 16 trials onwards (Figure 1).

The baseline swimming speed was highly variable over the trials, and did not differ between the treatment groups (Welch two-sample t-test, $t = 1.36$, $p > .1$, Figure 2a). Both groups initially responded to the CS flashes by decreased swimming speed to below baseline levels (paired t-test; Conditioning group: $t = 6.37$, $p < .001$; Control group: $t = 7.34$, $p < .001$, Figure 2). The Control group sustained the reduced swimming speed during the CS flashes throughout the study (Figure 2b), with no correlation between swimming speed during the CS and trial number (Kendall's tau rank correlation, tau = 0.11, $p > .1$). In contrast, the swimming speed during the CS in the Conditioning group increased with trial number (tau = .40, $p < .001$), and eventually the swimming speed during the CS was similar to baseline levels from around trial 16 (Figure 2). Comparing the treatment groups during the CS, the swimming speed of the Conditioning group was significantly higher than that of the Control group (Welch two-sample t-test, $t = 8.82$, $p < .001$, Figure 2b). Although difficult
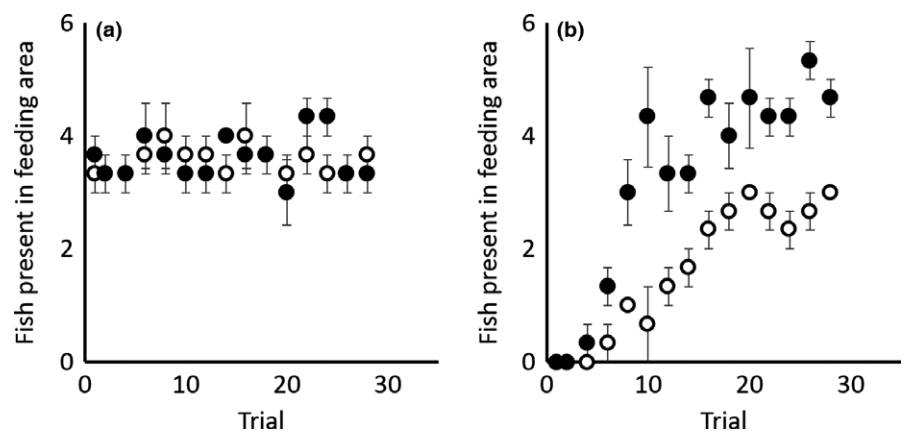

FIGURE 1 Mean ± S.E. number of fish present in CS/feeding area during the course of the experiments at: (a) baseline (10 s before the CS), and (b) 10 s into the CS period. Open circles indicate Control group, filled circles indicate Conditioning group
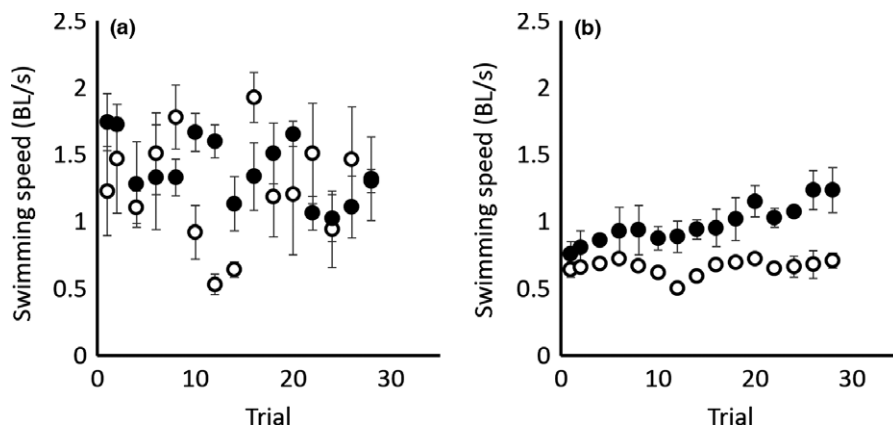
FIGURE 2 Mean ± S.E. swimming speed (body lengths/s) at: (a) baseline (10 s before the CS), and (b) 10 s into the CS period. Open circles indicate Control group, filled circles indicate Conditioning group
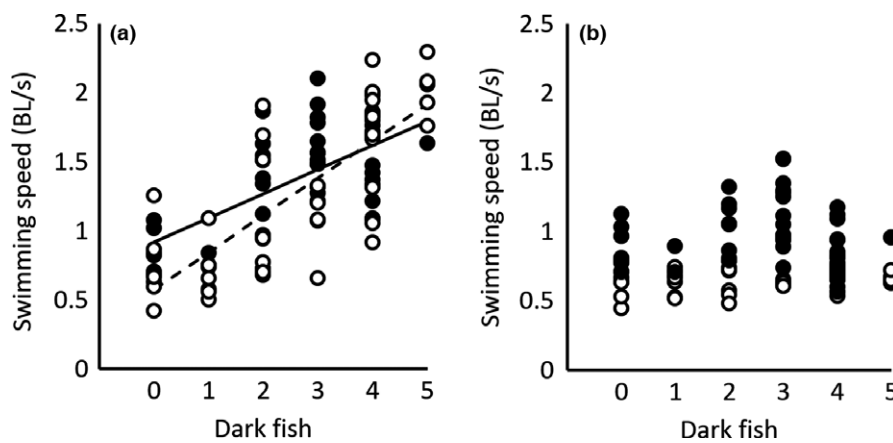

FIGURE 3 Correlation between the number of dark fish in the tank and average swimming speed. (a) Average baseline swimming speed (10 s before the CS). The solid line shows the regression for the Conditioning group: Swimming speed = 0.175 dark fish + 0.917, and the dashed line the regression for the Control group: Swimming speed = 0.269 dark fish + 0.576. (b) Average swimming speed during the CS (10 s into the CS). Open circles indicate Control group, filled circles indicate Conditioning group

to quantify from the video observations, the anticipatory swimming pattern of the Conditioning group became much more erratic during the CS flashes compared to the baseline behaviour.

The number of individuals displaying vertical dark bands on their body varied both between and within tanks, but dark fish were present in all tanks in most trials (Figure 3). These fish mainly stayed close to the tank bottom, maintained a slow swimming speed and frequently performed mock charges towards fish near-by. There was no effect (glm, Poisson distribution, $p > .1$) of trial number or procedure, and no interaction effect, on the number of dark individuals before the onset of the CS. There was, however, a positive effect of number of dark individuals in the tank before the CS on the baseline swimming speed (lm, $p < .001$, Figure 3a). No general effect of procedure was found ($p > .1$) although there was an interaction effect of number of dark individuals and procedure ($p < .05$). In contrast, during the CS no effect of number of dark individuals in the tank on swimming speed was found ($p > .1$), and there was no interaction effect, although there was an effect ($p < .001$) of procedure with fish in the Conditioning group swimming faster than fish in the Control group (Figure 3b). There was no effect of number of dark individuals in the tank before the onset of the CS on number of fish in the feeding area neither before (glm, Poisson distribution, $p > .1$) nor during ($p > .1$) the CS.

4 | DISCUSSION

In both the Conditioning and Control groups, the reaction to the light flashes changed markedly over time. Initially, sea bream in both groups showed a fright reaction to the light flashes. However, whereas the fish in the Control group habituated the initial response of fleeing from the CS light flashes, they still responded by lower swimming speed during the light flashes. Habituation is not necessarily total, that is, the response may decline, but not towards zero (Christoffersen, 1997), as earlier shown for salmon parr (Folkedal et al., 2010; Madaro et al., 2016), and the persistent response could reflect a prolonged alertness with a shift in attention in an otherwise monotonous environment (Mendl, 1999). In the Conditioning group, the interplay between aversive and reward stimuli gradually progressed to a quite different response, with no reduction in swimming speed during the light flashes and attraction of fish to the feeding area. The fish thus switched from fright to anticipation after reward conditioning. This has previously been demonstrated in several species, including Chinook salmon (*Oncorhynchus tshawytscha*) (Schreck, Johnson, Feist & Reno, 1995), Atlantic salmon (Bratland et al., 2010) and Atlantic cod (Nilsson et al., 2012), but the discrepancy between subtotal habituation of the behavioural response to a non-rewarded stimulus versus anticipation when the very same stimulus signalled

reward is to our knowledge a novel finding. In cod, the swimming speed response to a splashing dip net became totally habituated, whereas the oxygen hyperconsumption response partly persisted, suggesting that alertness towards the moving object indeed was sustained (Nilsson et al., 2012). The method used in Nilsson et al. (2012) did, however, not allow for a direct comparison of swimming speed during the CS period between rewarded and non-rewarded fish. The current study using gilthead sea bream does demonstrate a large scope for catalyzing behavioural habituation by reward conditioning, where the Control group did not habituate their swimming speed response versus full habituation of the response in the Conditioned group.

The weak positive CS response of fish aggregating in the feeding area observed for the Conditioning group after around 16 trials suggests associative learning. The fish were, however, relatively spread in the tank both before and during the CS light flashes, and sea bream did thus not show a strong or consistent response of moving into the feeding area in response to the CS. This contrasts previous observations in cod (Nilsson et al., 2008a,b) and salmon (Bratland et al., 2010; Folkedal, Stien, et al., 2012; Folkedal, Torgersen, et al., 2012). Furthermore, the lack of increased swimming speed above baseline in the Conditioning group was unexpected, as this has been observed during conventional and demand feeding (Andrew et al., 2002), and during the hours before a daily meal in tanks (Sánchez et al., 2009). We ascribe much of the lack in spatial responsiveness to the small size of the tanks used; at any distance from the defined feeding area, the fish could attack pellets shortly after arrival. With regard to swimming speed, the food arrived in one tank corner, and attacking the pellets was carried out in a "burst and stop" motion to prevent crashing into the tank wall. A high swimming speed during feeding may thus not be as efficient as within the vast volume of sea cages (Andrew et al., 2002). The currently observed shift to an erratic swimming pattern during the CS in the Conditioned group all the same indicates search behaviour and food anticipation.

The observed variations in number of fish with dark vertical bands between trials within the same tank suggest that this is a highly flexible trait, in line with the findings of Papadakis et al. (2016) describing territorial behaviour in gilthead sea bream in a similar tank and social environment. The positive relationship found between the number of dark territorial individuals and baseline swimming speed suggests that the social hierarchy influenced the whole group by increasing the general activity level. There was a weak but significant interaction effect with a somewhat stronger influence of dark individuals on swimming speed in the Control group. Still, the general pattern that the presence of dark individuals largely determines swimming speed was similar in both groups and cannot solely be an effect of conditioning procedure. The number and swimming speed of the fish in the feeding area during the CS flashes was not related to the presence of dark individuals before the onset of the CS, suggesting that the light stimulus overruled the effect of territorial behaviour. Similarly, Papadakis et al. (2016) reported minimal territorial behaviour during feeding compared to before and after. This may explain why social hierarchies did not

influence the anticipatory behaviour as we predicted. However, when social hierarchies have large effect on variation in baseline behaviour as in the present study, differences between baseline levels and CS levels may be more difficult to detect and anticipation more challenging to quantify even if it is not reduced *per se*.

We have shown that sea bream like other investigated aquaculture species can form an association between an initially aversive CS and a food-US, as displayed by anticipatory behaviour. Based on the relatively small changes in behaviour of fish in the small tanks in the present set-up, it is difficult to conclude if anticipatory behaviour could be used as an operative welfare indicator in sea bream. Studies in larger rearing units should resolve this. The persistence of the negative response to light flashes in sea bream not rewarded by food could mean that fish even seemingly adapted to repetitive stressors in aquaculture are still in a state of prolonged alertness. The difference between the Conditioning and the Control groups in habituation to the flashing light shows the potential for positive stress conditioning in aquaculture, boosting adaptation by pairing stressors with reward.

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