Applied Animal Behaviour Science 154 (2014) 104–111

Contents lists available at ScienceDirect

Applied Animal Behaviour Science

journal homepage: www.elsevier.com/locate/applanim

Use of conditioned place preference/avoidance tests to assess affective states in fish

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ARTICLE INFO

Article history:
Available online 20 February 2014

Keywords:
Sparus aurata
Conditioning
Swimming activity
Cortisol
Place learning
Welfare

ABSTRACT

Animal welfare has been defined as the balance between positive and negative experiences or affective states. Despite the growing evidence of complex cognitive abilities and the expression of affective states such as pain and fear, very little is known about ability to experience memory based affective states in non-mammalian animal models. The goal of this study was to validate conditioned place preference/avoidance (CPP/CPA) tests as a method to assess the affective valence of environmental stimuli in teleost fishes. Physiological and behavioural indicators of affective state were used to characterise the response to *a priori* appetitive and aversive stimuli in CPP/CPA tests in gilthead sea bream (*Sparus aurata*). Fish were tested individually in a CPP/CPA tank divided into two halves, with one half uniformly white and one half marked by dotted wall patterns. During an initial habituation phase fish were placed in a central alley for 10 min and afterwards allowed to swim freely throughout the whole tank during 20 min in order to determine their initial preferred and non-preferred side (IPS/INPS). During the training phase, fish were presented either with a single aversive stimulus in the IPS (chasing with a dip net) or with a repeated appetitive stimulus (release of pellets) in the INPS. The test phase consisted of the same procedure as the habituation phase. The behaviour of each individual was video-recorded and analysed with video-tracking software. Fish submitted to appetitive stimulus increased significantly the time spent and the distance moved in the stimulation side, while fish exposed to aversive stimulus decreased significantly the time spent in the stimulation side, increased the distance moved in the non-stimulation side and showed an increase in cortisol level. Therefore, the use of behavioural (individual swimming activity) and physiological (plasma cortisol concentration) indicators of affective state during the CPP/CPA test allowed to validate the use of this test as a way to assess the affective valence attributed by fish to different environmental stimuli. Finally, this study also shows that fish are able to retain memories of events with positive/negative valence which are retrieved by environmental cues.

1. Introduction

We have in recent years witnessed a considerable increase in the public and scientific debate regarding

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http://dx.doi.org/10.1016/j.applanim.2014.02.004
0168-1591/

the welfare of animals in human custody. Current concepts of animal welfare acknowledge the fact that welfare incorporates not only physical well-being but also mental well-being and the possibility of animals to live according to their nature and to express their full behavioural repertoire (Brambell, 1965; Korte et al., 2007; Ohl and van der Staay, 2012). This view implies that animals may experience feelings (*i.e.* affective states *sensu* Mendl et al., 2010), such that they will attempt to minimise negative affective states (*e.g.* fear) while seeking positive ones (*e.g.* pleasure; Dawkins, 1983; Duncan, 1996; Boissy et al., 2007). Affective states are a set of neural responses that occur more or less unconsciously when the brain detects certain challenging or rewarding situations. These automatic responses occur within the brain, involving changes in arousal levels and in cognitive functions such as attention, memory processing and decision strategy, and in the body in the form of endocrine, autonomic and musculoskeletal responses (Kandel et al., 2012). These reactions are not equivalent to or indicative of intention, but may affect and promote action tendencies, subjective feelings, thoughts and behaviour when such abilities are present (Frijda, 1986).

However, affective states are not directly observable, and behavioural and physiological proxies have to be used in order to probe animal affective state. Preference/avoidance and motivation tests have been used for this purpose, based on the assumption that affective states are linked to motivation/preference and ultimately drive behaviour (Kirkden and Pajor, 2006). In these tests the animal is given some control over its environment, so we can observe their choices in preference tests, or how much they are willing to work to access or avoid given resources or threats in operant motivation tests (Yue et al., 2008; Endo et al., 2002; Herrero et al., 2005; Kirkden and Pajor, 2006).

Teleost fishes are currently central in the animal welfare debate, considering the vast numbers of individuals reared in aquaculture (Ashley, 2007; Huntingford and Kadri, 2009). Fish are also gradually introduced as an alternative to small mammals in biomedical and behavioural research (Thorgaard et al., 2002; Epstein and Epstein, 2005; Steenbergen et al., 2011). In this context, it is discussed to what degree fishes possess conscious awareness and mental capacities to experience pain and discomfort (Rose, 2002; Chandroo et al., 2004; Huntingford et al., 2006; Bekoff, 2007; Galhardo and Oliveira, 2009; Browman and Skiftesvik, 2011). Despite the increasing interest in fish welfare and the recent evidence that fish have the capability to experience affective states (Chandroo et al., 2004; Galhardo and Oliveira, 2009), the quantification of fish motivation as an indicator of its needs has been only measured using a push-door paradigm test (Galhardo et al., 2011). Generalisation of the use of motivation tests across different fish species has been hampered by the fact that the operant task needs to fit the natural behaviour of each species, which varies to a great extent among fish. For example, the successful use of the push-door operant task in tilapia mentioned above took advantage of the robustness of its snout that is used by males in mouth-digging of nests and in mouth-fighting, and would probably not be appropriate for other species. Therefore, it would be of great value to develop a common motivation test to be used across different species.

The conditioned place preference (CPP) paradigm is a commonly used technique in behavioural neuroscience to evaluate rewarding and aversive effects of addictive drugs (Tzschentke, 2007; Prus et al., 2009). In general, this task involves the establishment of an association between a specific environmental stimulus and a positive or a negative reward. Typically, a positive or negative reward is repeatedly paired with a location marked with a cue so that the animal associates the marked location with the reward, and eventually develops a preference or avoidance (conditioned place avoidance, CPA) even in the absence of the stimulus (Mathur et al., 2011a). Therefore, CPP and CPA paradigms offer the possibility to assess the reward or aversive value that animals attribute to different environmental stimuli in general and can easily be used across different species. In fish, CPP has been used to determine the reinforcing effects of natural rewards, such as food, or social context (Lau et al., 2006; Zala and Määttänen, 2013), but much more frequently of addictive drugs (*e.g.* Mattioli et al., 1998; Coelho et al., 2001; Darland and Dowling, 2001; Ninkovic and Bally-Cuif, 2006; Mathur et al., 2011a,b; Klee et al., 2011). This test has rarely been used in the field of animal welfare as a tool to evaluate the value that fish attribute to husbandry stimuli, hence allowing an indirect assessment of their affective state.

In this study we investigated how Gilthead sea bream (*Sparus aurata*), one of the most important commercial species in Europe, evaluated an *a priori* appetitive (food) or aversive (net chasing) stimuli in a CPP/CPA paradigm. In order to validate this paradigm as a measure of valence attributed by the fish to the appetitive/aversive stimuli, we used physiological (cortisol, glucose and lactate levels) and behavioural (distance travelled and swimming activity) variables independent of the CPP/CPA. The broader goal of this work was to validate the use of CPP/CPA test as a method to assess the value that fish attribute to stimuli with different valences by using several physiological and behavioural indicators of internal state.

2. Materials and methods

Experimental procedures were conducted in accordance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals, and under a "Group-1" licence from the Portuguese competent authority for the protection of experimental animals (Direção Geral da Alimentação e Veterinária, Portugal; permit number 0420/000/000-n.99-09/11/2009).

2.1. Experimental fish, housing and feeding

Fish (10 ± 3 g; mean $\pm$ SE) were obtained from a commercial fish farming (MARESA Mariscos de Esteros SA, Huelva, Spain) and transported to Ramalhete Research Station (Faro, Portugal). They were housed in stock tanks (500 L) under standard rearing conditions during 3 months before the start of the experimental procedures (rearing density from 2 kg m^{-3} to 9 kg m^{-3}). Fish were fed a

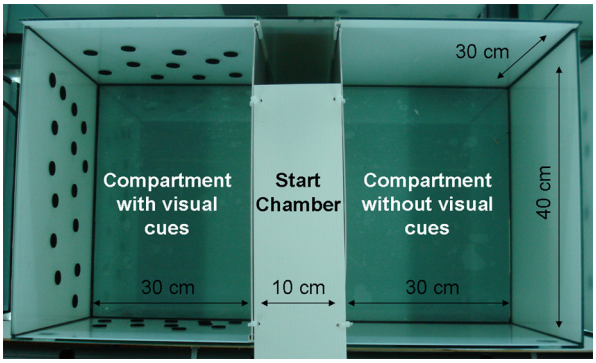

Fig. 1. Experimental tank. CPP/CPA test glass aquarium (80 L) divided in three compartments: one start chamber with grey walls and sheltered and two lateral compartments with white walls and with or without visual cues (black spots).

commercial diet (Aquagold 3 mm, Aquasoja, Sorgal SA, Portugal; 44% crude protein, 14% crude fat, 8% ash, 2.5% crude fibres, 1.0% phosphorus) using automatic feeders (1.5% BW day^{-1}). They were reared in open water circuit tanks, with a temperature of 20 ± 3 °C, salinity of 34 ± 2‰ and dissolved oxygen above 95%, and a 12L:12D photoperiod was employed with light on at 08:00. One month before the start of experimental procedures, 120 fish were randomly selected, anaesthetised with 2-phenoxyethanol (0.3‰, Sigma–Aldrich) and individually identified with a visible implant elastomer tag (VIE; Northwest Marine Technology, USA) in the caudal fin. At the start of the experiment the body mass of the fish was 45 ± 2 g (mean ± SE).

2.2. Set up and experimental procedures

Two days before the start of the conditioned place preference/avoidance test (CPP/CPA), 4 groups of 6 fish each (with distinct VIE) were placed in 4 different 100 L home tanks located in the experimental room. This was done to acclimatise fish to their new environment. The photoperiod and water temperature, salinity and oxygenation were the same as in rearing tanks. This procedure was repeated 5 consecutive times in order to test 30 fish per treatment: appetitive (APP), control appetitive (APPctr), aversive (AVER) and control aversive (AVERctr).

The CPP/CPA test was performed in 6 glass aquaria of 80 L (70 cm length × 40 cm width and 30 cm depth). Each aquarium was divided into two halves by a 10 cm wide grey and sheltered central alley (start chamber): one half marked by white and the other half marked by dotted wall patterns (Fig. 1). One infrared LED projector (IR-294S/60, Monacor®) was placed beneath each aquarium.

The CPP/CPA test consisted of three experimental phases conducted over a 3–5 days period. An initial habituation phase was performed on the first day. During this phase, each fish was placed individually in the sheltered start chamber for 10 min. Afterwards, partitions between the start chamber and the lateral compartments were gently removed. Fish were allowed to swim freely throughout the whole tank for 10 min. For each individual the initial preference for white or dotted side (IPS > 50% of the total time) was then assessed through a 20 min recording, after which the fish was put back in the home tank. Fish that showed a very strong initial preference (>90% time spent) in either side or strong freezing behaviour (<500 cm distance moved) were excluded from the study. Therefore, individuals that showed an initial preference between 50.1% and 89.9% for either side and which had moved more than 500 cm were used for data analyses. Similarly to Lau et al. (2006), Darland and Dowling (2001) and Kily et al. (2008) we used a single exposure to the CPP/CPA test to establish the fish baseline preference. Mathur et al. (2011a) have shown in zebrafish that repeated exposure to the tank did not significantly alter the baseline preference in a CPP testing.

The habituation phase was followed by a conditioning phase, during which treatments differed between the appetitive and aversive stimulus groups. For the aversive stimulus (AVER), fish (not food deprived to not add a negative event) was placed in the same aquarium as during the habituation phase but retained in the IPS for 10 min, hereafter termed the stimulation side (SS). Afterwards, the fish was chased with a net during 10 s each 2 min for a period of 10 min. For the appetitive stimulus (APP), fish (food deprived for 24 h to increase the reward effect) was placed in the non-IPS (new SS) for 10 min and then received one food pellet each 30 s during 10 min. Based on preliminary results, the aversive treatment was performed only one day in order to avoid fish habituation to the stimulus (decrease of flight response towards the net and avoidance of the stimulation zone since the second stimulus exposition) contrary to the appetitive treatment which was repeated during three consecutive days for an improved conditioned response. The control fish (AVERctr; APPctr) were handled exactly the same way as the tested animals except that the stimulus was omitted during the training phase. After each treatment, fish was put back in the home tank.

The test phase was performed on the last day of the experiment (the third day for the aversive stimulus or the fifth day for the appetitive one) and consisted exactly of the same procedure as the habituation phase in order to record any behavioural changes.

After this last phase, fish were immediately caught and euthanized with an overdose of 2-phenoxyethanol (1‰, Sigma–Aldrich) and vertebral column cut just behind the head after blood sampling. Blood was thus sampled 30 min after fish were transferred to the experimental aquarium (based on Arends et al., 1999). Blood was withdrawn within 3 min from the caudal vein using heparinised syringes and centrifuges at 2000 × *g* for 20 min at room temperature. After centrifugation plasma was frozen in dry ice and stored at −80 °C for glucose (QCA, Spain) and lactate (Spinreact, Spain) kits analysis. Plasma cortisol levels were measured by means of a commercial ELISA kit RE52061 (IBL, Hamburg, Germany), with a sensitivity of 2.5 ng ml^{-1}, and intra and inter-assay coefficients of variation (CV) of 2.9 and 3.5%, respectively. After blood sampling, fish were identified and measured for standard length (cm).

During each phase, individual behaviour was recorded by infrared sensitive video camera (TVCCD-623-COL, Monacor®, Denmark) equipped with infrared filter (dark red, Schneider Optics, USA) and positioned 1 m above the

tank. The videos were stored in AVI files on a hard drive and analysed afterwards with the Lolitrack 2.0 software (Loligo® Systems, Denmark). Before each video analyses, the background image of each tank was divided into three arenas (Arena 1 = white side, Arena 2 = grey middle alley, Arena 3 = dotted side). For each tank the background was calibrated by marking the length of the Arena 2 in the image and entering its actual value (10 cm). The Lolitrack 2.0 software tracks the fish as a dark object on a light background. By using infrared light (which is not visible for fish) beneath the tank we avoid light reflexion on the water surface and optimise the fish tracking by the software. The following parameters were quantified by the software: time spent in each arena (min), distance travelled in each arena (m) and the swimming speed in each arena ($cm\,s^{-1}$). In order to remove the influence of fish size in swimming speed data, these values were transformed in body length per second ($BL\,s^{-1}$).

2.3. Statistics

Statistical analyses were performed using Statistica 7 software (Statsoft, USA). The results were expressed as mean ± standard error (SE). Data were analysed for normality with a Shapiro–Wilk test and for homoscedasticity with a Bartlett's test.

For each treatment (*i.e.* CPP test for appetitive and CPA test for aversive) repeated-measures ANOVA's were used to analyse: (1) the differences in time spent by the experimental and control fish in the stimulation side (SS) before and after the conditioning phase (2-levels repeated factor: before *vs.* after; categorical variable: experimental *vs.* control fish); and (2) the differences in distance travelled (m) and swimming speed ($BL\,s^{-1}$) between experimental and control fish, experimental phases (*i.e.* before and after conditioning phase) and tank sides (4-levels repeated factor: before SS *vs.* before non-stimulation side (nSS) *vs.* after SS *vs.* after nSS; categorical variables: experimental *vs.* control fish). Planned comparisons were subsequently used to test differences between the habituation and the test phase of each treatment and between the control and the experimental groups both at the habituation and at the test phase of each treatment.

One way ANOVA and planned comparisons were used to analyse the differences in plasma concentrations of cortisol ($ng\,ml^{-1}$), glucose ($mmol\,l^{-1}$) and lactate ($mmol\,l^{-1}$) between experimental and control fish for each treatment (*i.e.* CPP test for appetitive and CPA test for aversive).

3. Results

From the 120 fish tested in this study, 20 fish did not comply with our acceptance criteria and were thus rejected from the analysis. This resulted in 25 fish being analysed per treatment.

A null model of side preference was tested by comparing the observed fish distribution to the theoretical homogeneous distribution in stimulation side and non-stimulation side (50% in each zone) by a Kolmogorov–Smirnov test. No systematic side preference was observed during the habituation phase (stimulus conditioning was performed 44 times in the white side *vs.* 56 times in the dotted side; $d = 0.26$; $p > 0.05$).

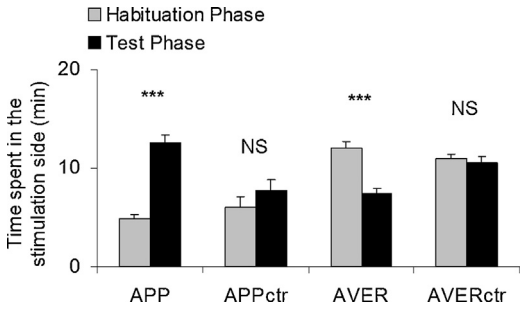

Fig. 2. Time spent in the stimulation side. Time spent (mean ± SE; in min) by the fish in the stimulation side during the habituation and test phases for each treatment. Repeated ANOVA, NS, non-significant; *** <0.001.

3.1. Time spent in the stimulation side

On average fish subjected to the appetitive stimulus showed a high increase of the time spent in the SS during the test phase (Fig. 2), whereas fish exposed to the aversive stimulus showed a decrease of the time spent in this side (Fig. 2). Both for the appetitive and for the aversive test the repeated measures ANOVA's revealed no main effect of treatment (overall control *vs.* experimental group) and significant effects of the repeated factor (before *vs.* after exposure to stimulus) and of the interaction between treatment and the repeated factor (Table 1). Planned comparison analyses showed that in the appetitive test there was a significant increase in the time spent in the SS for experimental group but not for the control group (Table 1). Conversely, in the aversive test there was a significant decrease in the time spent in the SS for the experimental group but not for the control group (Table 1).

3.2. Distance travelled

The main effect of treatment (*i.e.* overall control *vs.* experimental group) on the distance travelled was not significant, but there was a significant main effect of the repeated measure (*i.e.* before SS *vs.* before nSS *vs.* after SS *vs.* after nSS) and a significant interaction between treatment and the repeated measure, both for the appetitive and for the aversive experiments (Table 2).

In the appetitive experiment fish increased the distance travelled in the SS in the test phase both in the control and in the experimental group (*i.e.* exposed to the appetitive stimulus), whereas the distance travelled in the nSS increased only in the control treatment (Table 2 and Fig. 3). There was a significant difference in the distance travelled between the experimental and the control treatment only in the habituation phase for the nSS (Table 2 and Fig. 3).

In the aversive experiment the distance travelled increased in the test phase for the experimental treatment in the nSS and for the control treatment in SS (Table 2 and Fig. 3). Furthermore, there were no significant differences in the distance travelled between the experimental and the control treatments either in the habituation or in the test phase for either the SS or the nSS (Table 2 and Fig. 3).

Table 1
Results of repeated-measures ANOVA and planned comparisons used to analyse the differences in time spent by the experimental and control fish in the stimulation side (SS) before and after the conditioning phase (2-levels repeated factor: before *vs.* after; categorical variable: experimental (E) *vs.* control (C) fish).

	Appetitive treatment		Aversive treatment	
	$F_{1,48}$	*P*-value	$F_{1,48}$	*P*-value
Treatment (T) main effect	2.36	0.13	3.17	0.08
Repeated factor (R) main effect	65.55	<0.001*	18.69	<0.001*
T × R interaction	27.93	<0.001*	14.23	<0.001*
Planned comparisons				
C_{before} *vs.* C_{after}	3.95	0.053	0.15	0.698
E_{before} *vs.* E_{after}	89.53	<0.001*	32.77	<0.001*
C_{before} *vs.* E_{before}	1.05	0.31	2.09	0.16
C_{after} *vs.* E_{after}	11.96	<0.001*	11.42	<0.01*

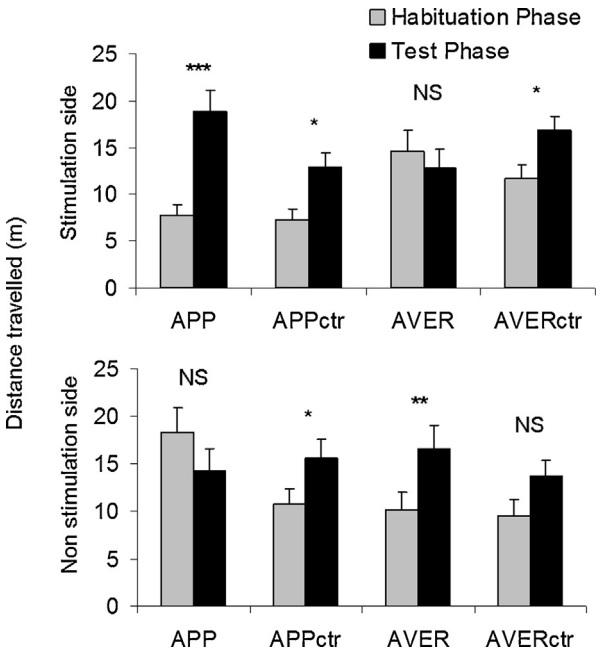

Fig. 3. Distance travelled. Distance travelled (mean ± SE; in m) by the fish in the stimulation side and the non-stimulation side during the habituation and test phases for each treatment. Repeated ANOVA, NS, non-significant; * <0.5; ** <0.01; *** <0.001.

3.3. Swimming speed

In the appetitive experiment neither the main effect of treatment (*i.e.* overall control *vs.* experimental group), nor the main effect of the repeated measure (*i.e.* before SS *vs.* before nSS *vs.* after SS *vs.* after nSS), nor interaction between treatment and the repeated measure, were significant on the swimming speed (Table 3). However, swimming speed significantly increased from the habituation to the test phase in SS for the control treatment and in the nSS for the experimental treatment (*i.e.* exposed to the appetitive stimulus; Table 3 and Fig. 4). There were no significant differences between the control and the experimental treatments either in the habituation or the test phase, either for the SS or for the nSS (Table 3 and Fig. 4).

In the aversive experiment there was a main effect of the repeated factor on swimming speed (*i.e.* before SS *vs.* before nSS *vs.* after SS *vs.* after nSS), but neither the main effect of the treatment (*i.e.* overall control *vs.* experimental group) nor the interaction between the treatment and the repeated factor were significant (Table 3). Swimming speed only increased from the habituation to the test phase in the control treatment in the SS (Table 3 and Fig. 4). There were no significant differences between the control and the experimental (*i.e.* exposed to the aversive stimulus) treatments either in the habituation or the test phase, either for the SS or for the nSS (Table 3 and Fig. 4).

Table 2
Results of repeated-measures ANOVA and planned comparisons used to analyse the differences in distance travelled (m) between experimental (E) and control (C) fish, experimental phases (before and after conditioning phase) and tank sides (stimulation side (SS) and non-stimulation side (nSS) (4-levels repeated factor: before SS *vs.* before nSS *vs.* after SS *vs.* after nSS; categorical variables: E *vs.* C fish).

	Appetitive treatment		Aversive treatment	
	F statistic	*P*-value	*F* statistic	*P*-value
Treatment (T) main effect	$F_{1,48}$ = 2.50	0.12	$F_{1,48}$ = 0.09	0.77
Repeated factor (R) main effect	$F_{3,144}$ = 9.65	<0.001*	$F_{3,144}$ = 7.06	<0.001*
T × R interaction	$F_{3,144}$ = 4.98	<0.01*	$F_{3,144}$ = 3.13	<0.05*
Planned comparisons ($F_{1,48}$)				
C_{before} *vs.* C_{after} in SS	5.55	<0.05*	6.67	<0.05*
E_{before} *vs.* E_{after} in SS	25.02	<0.001*	0.79	0.38
C_{before} *vs.* C_{after} in nSS	6.02	<0.05*	4.02	0.051
E_{before} *vs.* E_{after} in nSS	3.72	0.06	9.38	<0.01*
C_{before} *vs.* E_{before} in SS	0.54	0.46	1.21	0.28
C_{after} *vs.* E_{after} in SS	2.19	0.15	2.68	0.11
C_{before} *vs.* E_{before} in nSS	10.54	<0.01*	0.07	0.79
C_{after} *vs.* E_{after} in nSS	0.005	0.95	0.92	0.34

Table 3
Results of repeated-measures ANOVA and planned comparisons used to analyse the differences in swimming speed (BL s^{-1}) between experimental (E) and control (C) fish, experimental phases (before and after conditioning phase) and tank sides (stimulation side (SS) and non-stimulation side (nSS) (4-levels repeated factor: before SS *vs.* before nSS *vs.* after SS *vs.* after nSS; categorical variables: E *vs.* C fish).

	Appetitive treatment		Aversive treatment	
	F statistic	*P*-value	*F* statistic	*P*-value
Treatment (T) main effect	$F_{1,48}$ = 0.83	0.37	$F_{1,48}$ = 0.73	0.40
Repeated factor (R) main effect	$F_{3,144}$ = 2.44	0.07	$F_{3,144}$ = 4.80	<0.01*
T × R interaction	$F_{3,144}$ = 2.52	0.06	$F_{3,144}$ = 1.58	0.20
Planned comparisons ($F_{1,48}$)				
C_{before} *vs.* C_{after} in SS	12.79	<0.001*	7.90	<0.01*
E_{before} *vs.* E_{after} in SS	0.05	0.83	2.09	0.16
C_{before} *vs.* C_{after} in nSS	0.03	0.88	4.03	0.05
E_{before} *vs.* E_{after} in nSS	4.21	<0.05*	0.15	0.70
C_{before} *vs.* E_{before} in SS	3.95	0.053	1.22	0.28
C_{after} *vs.* E_{after} in SS	0.67	0.42	0.003	0.96
C_{before} *vs.* E_{before} in nSS	0.005	0.95	2.82	0.10
C_{after} *vs.* E_{after} in nSS	3.98	0.052	0.0009	0.98

3.4. Blood parameters

The plasma cortisol was not significantly different between the control and the experimental fish in the appetitive experiment ($F_{1,91}$ = 0.13, p = 0.72; Fig. 5). In the aversive experiment fish from the experimental group (*i.e.* exposed to the aversive stimulus) showed significantly higher plasma cortisol levels than fish from the control group ($F_{1,91}$ = 5.83, p < 0.05). However, cortisol levels of the experimental groups did not differ between the appetitive and aversive experiments ($F_{1,91}$ = 0.28, p = 0.60), but cortisol levels of the control groups were higher in the appetitive than in the aversive experiment ($F_{1,91}$ = 5.12, p < 0.05; Fig. 5).

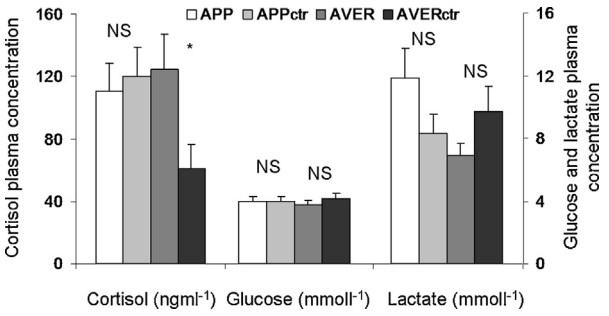

Fig. 5. Physiological analysis. Plasma concentration (mean ± SE) of cortisol (mg ml^{-1}), glucose (mmol l^{-1}) and lactate (mmol l^{-1}) for each treatment. One way ANOVA, NS, non-significant; * <0.05.

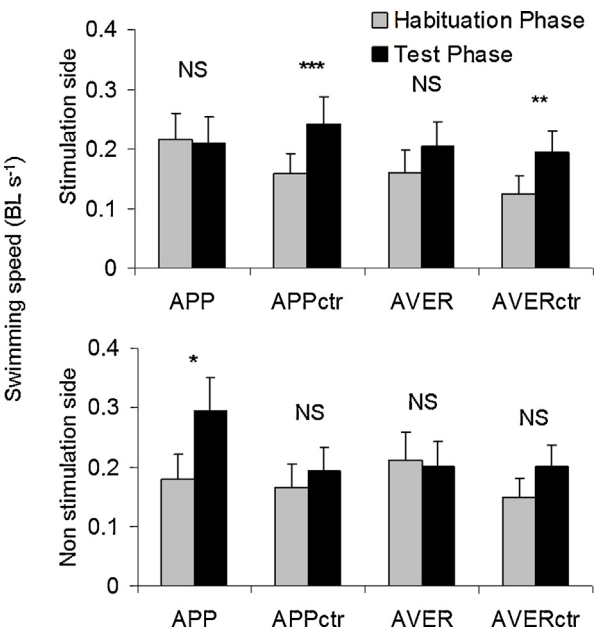

Fig. 4. Swimming speed. Swimming speed (mean ± SE; in BL s^{-1}) by the fish in the stimulation side and the non-stimulation side during the habituation and test phases for each treatment. Repeated ANOVA, NS, non-significant; * <0.5; ** <0.01; *** <0.001.

There were no significant differences in plasma concentrations of glucose between control and experimental fish, neither for the appetitive ($F_{1,86}$ = 0.0006, p = 0.98) nor for the aversive experiment ($F_{1,86}$ = 1.08, p = 0.30). Furthermore, glucose levels did not differ between the appetitive and the aversive experiments neither for the experimental ($F_{1,86}$ = 0.31, p = 0.58) nor for the control groups ($F_{1,86}$ = 0.23, p = 0.63).

There were no significant differences in plasma concentrations of lactate between control and experimental fish, neither for the appetitive ($F_{1,86}$ = 3.19, p = 0.08) nor for the aversive experiment ($F_{1,86}$ = 2.12, p = 0.15). However, lactate levels were significantly higher in the appetitive than in the aversive experimental group ($F_{1,86}$ = 6.08, p < 0.05), but not between controls ($F_{1,86}$ = 0.55, p = 0.46).

4. Discussion

The mechanisms that regulate food reward-associated behaviours have been traditionally studied in rodent models and very seldom in fish. To the best of our knowledge, there are two studies in zebrafish using food as reward in CPP test (Lau et al., 2006; Zala and Määttänen, 2013). Lau et al. (2006) established a CPP paradigm and used it to demonstrate that fish exhibit a robust preference for morphine as well as for food. Zala and Määttänen (2013) used the CPP paradigm to study social learning. However, these

authors focused on the change in time spent in the stimulation side, and did not analyse the overall behaviour change and the fish physiological response to the stimulus.

The present study showed that during the test phase fish previously exposed to an appetitive stimulus increased significantly the time spent in the stimulation side. Such place preference suggests that fish attribute a positive valence to the presence of food in this area. In addition, these fish highly increased the distance travelled in the stimulation side. Visual observations clearly revealed that fish explored the bottom of the stimulation side in the test phase as if they were in search of pellets, suggesting a food anticipatory activity (Galhardo et al., 2011; Bassett and Buchanan-Smith, 2007). These fish were also characterised by an increase of swimming speed in the non-stimulation side due to their quick passages in this zone, which could be interpreted as a loss of interest for this area. These results suggest that the changes of behaviour observed for these fish during the test phase are due to their associative learning of the visual cues with the reward, and when placed again in the experimental tank these fish assess the stimulation side as an area of potential food delivery.

No differences in plasma cortisol, glucose and lactate concentrations were observed between fish exposed to the appetitive conditioned treatment and the control ones. The relatively high concentration of plasma cortisol for both experimental and control fish in the appetitive experiment can be explained by the handling stress involved in the experimental procedure (each fish was transferred daily from their home tank to the CPP tank for 5 days in a row). Indeed, the values measured in this study are comparable to those reported by Barton et al. (2005) for sea bream exposed to handling stress during 30 s. Therefore, the plasma cortisol concentrations measured for the appetitive treatment seem to be more representative of the handling procedure, rather than elicited by the intrinsic valence of the stimulus.

Studies based on stress-associated behaviour or avoidance learning are relatively abundant in fish and traditionally used the association between different types of conditioned stimulus (CS; *e.g.* light on, water flow stop, air bubbles) and unconditioned stimulus (US, *e.g.* social aggression, confinement, electric shock). Yue et al. (2004) for instance, revealed that trout are able to learn the association between turning on a light and a net plunging into the water. Trout shuttled from one side of the tank to the other depending on where the light went on. Dunlop et al. (2006) showed that trout and goldfish have the capacity to learn to associate a particular area with a noxious stimulus and retain that learned information. More recently, Martins et al. (2011) showed that *Nile tilapia* exhibits avoidance learning when exposed to confinement stress. However, fish behavioural and physiological responses towards an aversive stimulus have never been accessed before with a CPA test.

This study demonstrated that a single exposure to a chasing net significantly decreased the time spent by sea bream in the stimulation side and increased highly the distance travelled in the non-stimulation side. These behavioural changes clearly suggest a strong avoidance of the stimulation side and a search for an escape in the non-stimulation side. These results propose that the changes of behaviour observed in the aversive treatment during the test phase are due to associative learning of the visual cues with the stressor, and when placed again in the experimental tank these fish still assess the stimulation side as an area of potential aversive event.

These results are confirmed by the physiological data. Indeed, even if plasma concentrations of glucose and lactate were not different between experimental and control fish, the plasma cortisol concentration was two times higher for the experimental fish. This result highlights that fish which were previously exposed to the stressor, when placed in the same environment, show a high physiological stress response due to the expectation of an aversive stimulus.

In this study, the control fish provided also interesting findings. As expected control fish in both experiments (appetitive and aversive) did not show any differences in the time spent in both sides during the habituation or test phase. However, fish slightly increased the distance travelled and the swimming speed in both sides during the test phase. The fact that these changes of behaviour happened on both sides of the tank revealed that the control fish did not have any preference for one or the other compartment, and that the increase in swimming activity can be interpreted as a reduction in anxiety during the test phase due to habituation to the experimental set-up. Indeed, during the test phase the control fish of the appetitive experiment had already been exposed 4 times to the set up and the control fish of the aversive experiment 2 times. Consequently it is expectable that the fish would be less stressed during the test phase than during the habituation phase. This highlights the need to minimise as much as possible the fish exposition to the CPP/CPA set-up in order to avoid habituation which may impact fish behaviour and hamper data interpretation.

In conclusion, this study showed that fish exposed to appetitive stimulus in a certain side of the tank induced a preference and higher exploratory behaviour for that side even in the absence of the stimulus (conditioned place preference). In contrary, fish exposed to aversive stimulus (even only one time) in a certain side of the tank exhibit avoidance behaviour for that side and a stress physiological response even in absence of the stimulus (conditioned place avoidance). Thus, this study showed that the CPP/CPA paradigm can be used in fish to assess the valence (positive *vs.* negative) that they attribute to different stimuli.

Determining carefully the fish behaviour and physiological changes after experience is essential in the evaluation of positive and negative stimuli. In this study, food induced a more marked affect than net chasing stimulus. This could be explained by the repeated presentation to food compare to the single experience of chasing. Thus, the effect could be presumably determined both by the intrinsic valence of the stimulus and by the number of exposures, which together contribute to its affective value. However, as already mentioned in this study, the behaviour of the fish may change when experiencing the tank environment repeatedly (decrease of flight response towards the net and avoidance of the stimulation zone) and it is important to restrict the use of multiple exposures. It could be possible

anyway that the test becomes more sensitive if a larger area is available for the fish to express slighter behavioural differences. Different intensity of the same stimulus could be also tested in order to determine a sensitivity threshold. Therefore, optimising the technique may allow the assessment of affective value of even weaker stimuli, making it possible to map stimuli in an affective space of the fish.

Acknowledgements

This research project has been support by the European Commission under the 7th Framework Programme FP7-KBBE-2010-4 Contract n°: 265957 Copewell. The work of Sandie Millot is supported by the Portuguese Fundação para a Ciência e Tecnologia (FCT) Grant n°: FRH/BPD/72952/2010. The work of Marco Cerqueira is supported by the FCT grant n°: SFRH/BD/80029/2011. The authors are grateful to João Reis and Miguel Viegas for technical assistance and two anonymous referees for their valuable comments.

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