Cognitive appraisal in fish: stressor predictability modulates the physiological and neurobehavioural stress response in seabass

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Abstract

The role of cognitive factors in triggering the stress response is well established in humans and mammals (aka cognitive appraisal theory) but very seldom studied in other vertebrate taxa. According to cognitive appraisal theory it is not the intrinsic characteristics of the stimulus that trigger a response but rather the evaluation of what that stimulus means to that organism at that moment in time in terms of ecological threat or opportunity. Predictability is often used as a factor of the cognitive evaluation of stimuli. In this study we tested the effects of stressor predictability on behavioral, physiological and neuromolecular responses in the European Sea Bass (*Dicentrarchus labrax*). Fish were exposed to a predictable (signalled) or unpredictable (unsignalled) stressor, either in social groups or in social isolation. Stressor predictability elicited a lower behavioural response and reduced cortisol levels. Using the expression of immediate early genes (*c-fos*, *egr-1*, *bdnf* and *npas4*) as markers of neuronal activity, the activity of two Sea Bass brain regions [Dm (putative homologue of the pallial amygdala) and Dl (putative homologue of the hippocampus)], known to be implicated in stressor appraisal, were monitored. The activity of both the Dm and the ventral area of Dl significantly responded to stressor predictability, suggesting an evolutionary conserved role of these two brain regions in information...
processing related to stressor appraisal. These results suggest that stressor predictability plays a key role in the activation of the stress response in a teleost fish, hence highlighting the role of cognitive processes in fish stress.

**Keywords:** Behavioural contagion, Environmental perception, Psychological components; Stimulus regularity; Social contagion.

**Introduction**

The literature on stress biology has long established the role of cognitive factors on triggering the stress response, defined as a response of the organism to regain homeostasis when exposed to a homeostasis threatening stimulus or event (aka stressor) (Koolhaas et al. 2011). Since the 1970’s it became clear that the cognitive appraisal of stimuli is a key mechanism in the activation of the stress response (Mason 1968; Weiss 1972). According to this perspective, it is not the intrinsic physical characteristics of the stimulus that trigger a response but rather the evaluation of what that stimulus or event means to that organism at that moment in time, which depends on stored information in memory about relations between stimuli (i.e. stimulus-stimulus learning or classic conditioning) and about relations between responses and stimuli (i.e. stimulus-response learning or instrumental conditioning) (Ursin and Eriksen 2004). Therefore, the same stimulus may elicit or not a stress response depending on how it is appraised by the individual. An “alarm” response would occur when expectancies, based on perceived contingencies between stimuli (i.e. stimulus expectancies) and between stimulus and response (i.e. response expectancies), are not met (i.e. when there is a discrepancy between expected situation and perceived situation). Hence, stimulus predictability, which refers to high levels of perceived probability of occurrence of the expected event, and stimulus controllability, which refers to high levels of perceived probability for response outcomes, play a major role on the appraisal of stimuli as aversive or not. Interestingly, the role of cognitive variables in the activation of a stress response was first investigated in laboratory animals, in particular in rodents (e.g. Weiss, 1970), and then extended to humans (e.g. Lazarus, 1999).
In the last decades the role of cognitive variables in the activation of stress responses as well as in triggering responses to appetitive events has been framed under a theory of cognitive appraisal. According to this theory individuals continuously monitor the environment using a set of stimulus evaluation checks (e.g. intrinsic valence, novelty, prediction error, capacity for control) in order to evaluate the valence (positive/ negative) and salience (high/ low) of detected stimuli (primary appraisal), and also assess the available organismal resources to deal with them (secondary appraisal) (Faustino et al. 2015; Mendl et al. 2010; Moors et al. 2013). While the appraisal concept has already been applied to the study of stress and emotional behaviour in animals, mainly in mammals (see Faustino et al. 2015 for a recent review), in fish the whole concept of psychological stress has been rarely addressed (Galhardo 2009; Galhardo et al. 2011; Madaro et al. 2016, 2015; Vindas et al. 2014a,b). However, empirical evidence for the occurrence of each of the stimulus evaluation checks involved in primary appraisal has been described in fish. The appraisal of the intrinsic valence of stimuli can be demonstrated by learned approach/avoidance behaviours, and these have been described in different fish species (Millot et al. 2014a,b). The use of the three cues that signal stimulus novelty have also been documented in fish: the effects of predictability in modulating the behavioural and physiological response to both aversive and appetitive stimuli have been described in the Mozambique tilapia (*Oreochromis mossambicus*) (Galhardo et al. 2011); familiarity with conspecifics has been shown to modulate both exploratory behavior and the response to a territorial intrusion, also in Tilapia (Aires et al. 2015; Galhardo et al. 2012); and the effect of controllability can be illustrated by rainbow trout (*Oncorhynchus mykiss*) that have the chance to actively avoid being defeated by a larger conspecific in a conditioning paradigm exhibiting a lower cortisol response to the conditioned stimulus, than those that cannot escape social defeat (Carpenter and Summers 2009). Finally, prediction error has recently been documented both in rainbow trout and in Atlantic salmon (*Salmo salar*) using a reward omission paradigm (Vindas et al. 2012, 2014a,b). However, this evidence has so far not been explicitly presented as supporting the occurrence of cognitive appraisal in fish and the proximate (i.e. neural/physiological) bases of these cognitive appraisal processes have not been investigated yet in fish. Given the expected universality of stimulus evaluation checks across animals, it is now timely to characterize their occurrence across species and
to implement comparative studies on the underlying neural mechanisms. Teleost fish offer an excellent opportunity for such comparative approach, given the divergent evolutionary path between ray-finned fish and tetrapods (Venkatesh et al. 2001), and the homologies that have already been established between teleost and mammalian brain regions, that include some of the areas known to be involved in cognitive appraisal in mammals (i.e. amygdala and hippocampus, Broglio et al. 2005; Ganz et al. 2012, 2015). Thus, the study of cognitive appraisal in fish will allow testing if the same cognitive appraisal processes are present in evolutionary divergent vertebrate taxa and if they share homologue neural mechanisms.

In this study we tested the effect of predictability of a stressor on the behavioural and physiological stress response of European sea bass (*Dicentrarchus labrax*). Seabass was used as a model in this study given its wide use in European aquaculture, which makes the results present here not only of importance for the basic biology of fish stress but also to have translational value for the improvement of welfare of farmed fish. We have also characterized the pattern of neuronal activation (using the expression of immediate early genes as markers of neuronal activation) of two brain regions that are homologous to mammalian brain regions known to be involved in cognitive appraisal in mammals, namely the dorsomedial telencephalon (putative teleost homologue of the mammalian amygdala) and the dorsolateral telencephalon (putative teleost homologue of the mammalian hippocampus), in order to test if brain regions involved in cognitive appraisal are evolutionary conserved. Given that predictability is a key stimulus evaluation check in cognitive appraisal theory, its occurrence in fish will also be proof for the occurrence of cognitive appraisal in fish.

### Material and methods

#### Experimental fish and maintenance

A batch of seabass with an initial body weight of 0.5 ± 0.3 g (mean ± SD) hatched at the experimental research station of IFREMER in Palavas-les-Flots (France) were transported to CCMAR Research Station (Faro, Portugal). Fish were reared in 500 L tanks in an open water circuit with constant aeration through air stones (temperature of 21 ± 5 °C,
salinity of 35 ± 1 ‰, dissolved oxygen above 75 ‰, and a 12L:12D photoperiod) during 10
months before the experiments. Fish were initially fed at 10 % of body weight with
commercial diets (Aquagold, Aquasoja, Sorgal SA, Portugal), and later food amount was
readjusted until 3 % of body weight in accordance with their growth. A total of 96 fish with
a body weight of 44.58 ± 6.36 g (mean ± SD) at the start of the experiments were used.

Experimental design and conditioning procedures

Given the fact that seabass are a shoaling species the effects of predictability on the
stress response were tested both in isolated fish (Experiment 1) and in fish kept in social
groups (Experiment 2). The two experiments occurred between May and June of 2013.
Twelve experimental glass aquaria (70 x 40 x 30 cm) were used under the same housing
conditions as described above, except for the fact that no air flow was supplied, since the
water flow rate of 2.5 L min⁻¹ was sufficient to guarantee oxygen saturation. A net, with the
same dimension as the lateral wall, was settled in one side of each aquarium at the
beginning of the experiment to be used as a confinement net. All aquaria walls were
covered with opaque plastic to avert visual contact between the animals and the
experimenters. The fish were fed at 3 % Bw⁻¹ daily, divided by two meals at 08:00 h and
18:00 h. Water quality was analysed for nitrites (<0.1 mg.L⁻¹) and ammonia (<0.1 mg.L⁻¹)
every three days. Temperature, oxygen saturation and pH were daily checked before the
cleaning routines performed 1 h after the second meal.

One month before the experiments, 96 fish were tagged under anaesthesia with a 1
cm long floy tag (Floy Tag Manufacturing Inc, Seatle, USA) and with a multicolour pearl
attached behind the dorsal fin. Two experimental conditions were tested in each
experiment: predictability (PRD) and unpredictability (UnPRD). In Experiment 1 (social
isolation), which lasted 3 days, 24 fish were tested in each experimental condition
(predictable asocial, PRDa; unpredictable asocial, UnPRDa). In Experiment 2 (social
groups), which lasted 14 days, six groups of 4 fish each were used in each experimental
condition (predictable social, PRDs; unpredictable social, UnPRDs; n = 24 fish/treatment).
The differences in the duration of the two experiments are due to the fact that preliminary
trials had shown that seabass kept in small shoals needs between 8 to 12 days to show
signals of adaptation to the experimental environment, whether seabass kept in social isolation does not survive that long (e.g. individuals die before resuming feeding). Because of this difference in duration between the two experiments their results cannot be directly compared (see statistical analysis below). After this variable acclimation period, the experimental period occurred in the last 2 days for both experiments and involved 4 training sessions in the first day (at 10:00 h, 12:00 h, 14:00 h and 16:00 h) and 3 training sessions (at 10:00 h, 12:00 h, and 14:00 h) and one test session (at 16:00 h) on the second day (see Fig. 1 for an overview of experimental procedures). To create the predictable and unpredictable treatments two different training procedures were used. In the predictable treatment a delay conditioning protocol was used for fish to learn to associate a visual cue (CS), which consisted of a yellow and black stripped card with the same size as the lateral wall of the aquaria (40 x 30 cm), with a stressor (US: confinement). The CS was presented on the opposite wall of the confinement net (US), remained in view for 1 min before the occurrence of US, and overlapped 1 min with it. The confinement was obtained by moving the net into the visual cue direction until it reached 15% of the aquarium volume. In the unpredictable treatment fish were presented with the same visual sign but temporally dissociated from the stressor (i.e. 30 min before or after in a random way). The number of conditioning trials used was based on preliminary tests that indicated that 5-6 trials were enough for aversive conditioning in this species. In the test session, fish from both experimental treatments (PRD and UnPRD) were exposed to the visual cue together with the stressor.

**Behavioural observations**

Fish behaviour was video recorded right before the first training session and during the test session using video cameras (TVCCD-623-COL, Monacor®, Denmark and webcams HD C310 Logitech) positioned 1 m above the tank. Videos were subsequently analysed using multi-event recorder software (Observer XT® from Noldus, Netherlands). The response to the visual cue was assessed using the following behavioural measurements: (1) time spent in freezing behaviour (i.e. time fish spent immobile, with or without fin movements, either on the bottom or in the water column); (2) escape behaviour (i.e. fish swimming strongly, going close to the tank walls or moving the body in a way
that looked like escape attempts); (3) shoal cohesion, measured in an arbitrary scale (1 = low, 2 = medium; 3 = high); and (4) exploratory behaviour following the formula:

\[ \frac{A}{t_{\text{maximum}}} \]

where \( A \) is the arithmetic mean of the time fish spent in each one of 3 previously delimited areas of the tank (confinement net area; centre of the tank; and visual cue area), and \( t_{\text{maximum}} \) is the maximum time found for any of the areas tested. When this ratio is close to 1 it indicates high exploratory behaviour, and when it is close to 0 it indicates low exploratory behaviour.

**Blood Sampling and plasma cortisol analysis**

In both experiments, 30 min after the test session, fish were rapidly caught and euthanized with an overdose of 2-phenoxyethanol (1‰, Sigma-Aldrich) and blood was immediately collected from the caudal vein and centrifuged at RT for 25 minutes at 2000 g. Plasma was stored at -80 °C until further processing. Plasma cortisol levels were measured using 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) were 2.9% and 3.5%, respectively.

**Brain microdissection and gene expression analysis**

In both experiments 8 individuals from each experimental treatment were randomly selected for the assessment of immediate early genes (IEGs) mRNA expression in brain regions of interest (see below). Fish were sacrificed and the skull with the brain inside was immediately imbedded in Tissue-Tek® and kept at -80°C until further processing. Brain telencephalon was sliced through 150 µm thick cryostat (Leica, CM 3050S) coronal sections, from which the medial part of the dorsal telencephalon (Dm), the Dorsal division of the lateral telencephalon (Dld) and the Ventral division of the lateral telencephalon (Dlv) (see supplementary material, Fig. S1) were microdissected with modified 25G steel needles using a micropunching technique previously established in the lab (Teles et al. 2015). These regions of interest in the brain were identified and classified following the available brain atlas for seabass (Cerda-Reverter et al. 2001). Total tissue was collected directly into
alysis buffer from Qiagen Lipid Tissue Mini Kit (#74804; Valencia, CA) and total RNA extracted from the samples, with some adjustments to the manufacturer’s instructions (see electronic supplementary material for detailed procedures). RNA from each sample was then reverse transcribed to cDNA (BioRad iScript cDNA Synthesis Kit; Valencia, CA) accordingly to manufacturer’s instructions and used as a template for quantitative polymerase chain reactions (qPCR) of egr-1, c-fos, bdnf and npas4, using the geometric mean of the expression of two previously established housekeeping genes, eef1a and 18S (see electronic supplementary material Table S1 for primer sequences and for qPCR conditions). The abundance of the internal control genes was stable across experimental treatments. All reactions were run in duplicate and controls without DNA templates were run to verify the absence of cDNA contamination. Fluorescence cycle thresholds (CT) were automatically measured and relative expression of the target genes were calculated using the 2\(^{-\Delta\Delta CT}\) method (Livak and Schmittgen 2001). Primers efficiency was calculated for each qRT-PCR reaction using Light Cycler 480 II inner software.

Statistical analysis

Given the differences in duration between experiments 1 and 2 the social effects on the stress response were not formally investigated, and the results from the 2 experiments were analysed separately. Parametric assumptions of normality and homoscedasticity of the data were confirmed by analysis of the residuals. Homogeneity of variances was checked by Levene’s test. Log, log (X+1) or arcsine transformations were used to match parametric assumptions when required [Experiment 1: time in freezing (arc-sin transformed), escape behaviour and exploratory behaviour (log (X+1) transformed), plasma cortisol concentration and IEGs mRNA expression (log transformed); Experiment 2: time in freezing (arc-sin transformed), exploratory behaviour (log (X+1) transformed, IEGs mRNA expression (log transformed)). In experiment 1 (social isolation) a T-test was used to compare the two experimental treatments (PRD vs. UnPRD). In experiment 2 (social group), since multiple individuals came from the same social group, a two-way ANOVA with experimental treatment (PRDs vs. UnPRDs) and social group (1-6) as independent variables, was used to assess differences in the behavioural variables and in cortisol. Data on the expression of immediate early genes was also analysed with T-tests in experiment 2.
given the fact that only a subset of randomly chosen fish was used and therefore most social
group only contributed with one fish to the sample. Pearson test was used to assess
correlations among variables. Descriptive statistics are expressed as mean ± standard error
of the mean (SEM). All statistics were performed using IBM SPSS® statistics v19.0.
GraphPad and GraphPad Prism® v6.0 for windows was used for chart building and figures
layout.

Results

Effects of stressor predictability on fish behaviour

Analyses of fish behaviour during the 2 min preceding the first training session (i.e.
before any stimulation or manipulation of the fish) showed no significant differences
between PRD and UnPRD in any of the two experiments [Experiment 1 (time freezing: t-
test = 0.28 (df = 46); p = 0.77; escape events: no events occurred; exploratory behaviour: t-
test = -0.09 (df = 46); p = 0.92); Experiment 2 (time freezing: F_{(1,36)} = 0.43, p = 0.57;
escape events: F_{(1,36)} = 0.36, p = 0.55; exploratory behaviour: F_{(1,36)} = 0.04, p = 0.88)]. In
experiment 2 shoal cohesion before training also did not show differences between PRD
and UnPRD (F_{(1,36)} = 0.11, p = 0.74).

The behaviour displayed by fish during exposure to the visual cue in the test session
was markedly different between experimental treatments in both experiments (Fig. 2). In
Experiment 1 (social isolation) there was no significant difference in time spent freezing
between the two experimental treatments (t-test = -1.02 (df = 46); p = 0.31), but fish in the
unpredictable treatment showed less escape attempts (t-test = 3.91 (df = 46); p < 0.001) and
less exploratory behaviour (t-test = 5.78 (df = 46); p < 0.001) (Fig. 2a-c).

Moreover, in this experiment a positive correlation was found between exploratory
behaviour and escape attempts (R = 0.429, n = 48, p = 0.002). In Experiment 2 (social
group) fish in the predictable treatment spent less time in freezing (F_{(1,36)} = 11.34, p =
0.001), and showed less escape attempts (F_{(1,36)} = 10.79, p = 0.002) and more exploratory
behaviour (F_{(1,36)} = 151.54, p < 0.001) than fish in the unpredictable treatment (Fig. 2a-c).
In experiment 2, time in freezing and escape attempts were positively correlated (R =
0.656, n = 45, p < 0.001) and exploratory behaviour and time in freezing were negatively
correlated ($R = -0.325$, $n = 48$, $p = 0.024$). Finally, shoal cohesion (measured only for experiment 2) was higher in the unpredictable treatment ($F_{(1,36)} = 27.98$, $p < 0.001$) (Fig. 2d). Moreover, there was a negative correlation between shoal cohesion and exploratory behaviour ($R = -0.491$, $n = 48$, $p < 0.001$).

**Effects of stressor predictability on fish physiology**

In both experiments fish exposed to unpredictable stressors had higher cortisol levels than fish exposed to predictable stressors (Fig. 3; PRDa vs. UnPRDa: t-test = -2.06, df = 46; $p = 0.04$; PRDs vs. UnPRDs: $F_{(1,36)} = 24.79$, $p < 0.001$). In Experiment 2 cortisol was positively correlated with both time in freezing and shoal cohesion ($R_p = 0.351$, $n = 45$, $p = 0.018$; $R = 0.310$, $n = 45$, $p = 0.038$), whereas a negative correlation was found with exploratory behaviour ($R = -0.491$, $n = 45$, $p = 0.001$).

**Effects of stressor predictability on brain activation**

In experiment 1 (social isolation) both Dm and Dlv exhibited changes driven by predictability, with higher expression levels of IEGs in the unpredictable treatment (i.e. of *c-fos* and *bdnf* at both brain nuclei and of *egr-1* and *npas4* at Dlv; Fig. 4). In this experiment, positive correlations were found at the Dm between *egr-1* expression and that of either *c-fos*, *bdnf* or *npas4* ($R_p = 0.881$, $n = 16$, $p < 0.001$; $R = 0.771$, $n = 16$, $p < 0.001$; $R = 0.648$, $n = 15$, $p = 0.009$, respectively), and between *c-fos* and both *bdnf* and *npas4* ($R = 0.804$, $n = 16$, $p < 0.001$; $R = 0.549$, $n = 15$, $p = 0.034$, respectively). In the Dlv, positive correlations were also found between *egr-1* expression and that of either *c-fos*, *bdnf* or *npas4* ($R = 0.963$, $n = 15$, $p < 0.001$; $R = 0.746$, $n = 15$, $p = 0.001$; $R = 0.594$, $n = 15$, $p = 0.019$, respectively), and between *c-fos* and both *bdnf* and *npas4* ($R = 0.794$, $n = 16$, $p < 0.001$; $R = 0.597$, $n = 15$, $p = 0.015$, respectively).

In experiment 2 (social groups) only *egr-1* was up-regulated at the Dm under unpredictable conditions (Fig. 4). Additionally, positive correlations were found between this gene and *c-fos*, *bdnf* and *npas4* at Dm ($R = 0.893$, $n = 16$, $p < 0.001$; $R = 0.707$, $n = 16$, $p = 0.002$; $R = 0.567$, $n = 16$, $p = 0.022$, respectively).
Correlations between predictability-driven behavioural, physiological and brain activation measures

In experiment 1 (social isolation), negative correlations were found between cortisol and escape behaviour ($R = -0.509$, $n = 16$, $p = 0.044$) and between escape behaviour and $bdnf$ expression in Dm ($R = -0.590$, $n = 16$, $p = 0.016$). Moreover, a positive correlation between escape attempts and exploratory behaviour was found ($R = 0.702$, $n = 16$, $p = 0.002$). In experiment 2 (social groups), a positive correlation was found between time in freezing and escape behaviour ($R = 0.850$, $n = 16$, $p < 0.001$), and a negative correlation was found between exploratory behaviour and shoal cohesion ($R = -0.720$, $n = 16$, $p = 0.002$). Finally, a positive correlation between escape behaviour and the $c-fos$ expression in the Dlv was also found ($R = 0.584$, $n = 12$, $p = 0.046$).

Discussion

In this study we have shown that stressor predictability modulates the stress response measured at the behavioural, physiological and neural levels, both in fish exposed to the stressor in social isolation and in social groups. In social isolation fish exposed to an unpredictable stressor exhibited less exploratory and escape behaviours, higher cortisol levels and more neuronal activity in the Dm and the Dlv, as indicated by the expression of IEGs ($egr1$, $c-fos$ and $bdnf$ for Dm; and $egr1$, $c-fos$, $bdnf$ and $npas4$ for Dlv), than those exposed to a predictable stressor. When in social groups, fish exposed to the unpredictable stressor showed higher freezing and more escape behaviours, higher shoal cohesion, less exploratory behaviour, higher physiological reactivity and more activation of the Dm as indicated by the expression of $egr1$. Therefore, in both social contexts (i.e. social isolation and social group) the exposure to a unpredictable stressor seems to trigger a milder stress response both in terms of the activation of the hypothalamic-pituitary-interrenal axis (HPI) and of the activation of the brain regions putatively involved in the appraisal of the stressor, such as the Dm (fish homologue of the tetrapod pallial amygdala) and the Dlv (fish homologue of the hippocampus). In contrast, at the behavioural level the social context seems to produce antagonic responses, with stressor unpredictability increasing shoal cohesion, freezing and escape responses. Thus, predictability seems to reduce the
behavioural response to stress in social groups but not in isolated fish. However, since it was not possible to match the duration of the two experiments, time is a confounding variable and the inference of social effects, albeit plausible, cannot be firmly established here.

The effects of stressor predictability have been extensively studied both in humans and in animals, and consistently the results have shown that prediction reduces the stress effects of aversive experiences (Lovallo 2005; Sapolsky 2004). For example, in the rat, which was the original model in which stressor predictability has been studied, it reduces the behavioral responses to stress, as well as detrimental consequences of stress such as pain reactivity, immunosuppression, gastric ulceration, and colonic motility (e.g. Weiss, 1970; Gliner, 1972; Hymowitz, 1979; Guile and McCutcheon, 1984; Mormede et al. 1988; Tyler et al. 2007). Similar results have been found subsequently in other mammalian species (e.g. sheep: Greiveldinger et al. 2007; dogs: Dess and et al. 1983; horses: Thomas 2010; pigs: Carlstead 1986). However, fewer studies have addressed such effects in non-mammalian vertebrates (e.g. birds: Bauer et al. 2011), and among fish the few studies available have produced contrasting results. While in this study, in conformity with previous studies in Mozambique tilapia or in rainbow trout (e.g. Galhardo et al. 2011; Vindas et al. 2014a), stressor predictability buffers the stress response, in Atlantic salmon no effect has been found (e.g. Madaro et al. 2016). Given the fact that the two contrasting results occur within the same family (Salmonidae), these differences do not seem reflect a phylogenetic difference but rather a species-specific effect. Interestingly, classic studies in this field have shown that when rats are given a choice between a signalled and an unsignalled foot shock they prefer the former (Arabian and Desiderato 1975; Lockard 1963), despite the fact that signaled shocks are perceived as more intense than unsignalled ones (Miller et al. 1983). Thus, it looks like during primary appraisal different stimulus evaluation checks are not equally weighted, as in this case appraisal of stimulus predictability seems to have overridden the perception of stimulus intensity. It is therefore important to extend the study of stressor predictability, and of cognitive appraisal in general, to other vertebrate species in order to assess how evolutionary conserved these stimulus evaluation mechanisms are.
The higher activation found in this study of brain areas that are homologous to the mammalian pallial amygdala (i.e. Dm) and to the hippocampus (i.e. Dlv) (Ganz et al. 2015; Maximino et al. 2013) in response to unpredictable stressors, suggests a conserved role of these areas in the cognitive appraisal of stressors. Indeed these two brain regions, together with the prefrontal cortex and the mesoaccumbens dopamine system have been implicated in the cognitive modulation of the stress response in mammals (Belova et al. 2007; Cabib and Puglisi-Allegra 2012; Maier and Watkins 2010; Pruessner et al. 2008). Given the lack of a neocortex and the absence of midbrain dopaminergic neurons (Dahlström-Füxe’s A10 nucleus, homologous to the mammalian mesolimbic ventral tegmental area) in fish (e.g. Panula et al. 2010; Tay et al. 2011; Yamamoto and Vernier 2011), in this study we have focused on the putative fish homologues of the mammalian amygdala and hippocampus. In mammals the amygdala plays a central role in emotional processes since it receives multimodal sensory information, as well as inputs from the frontal cortex and the hippocampus, hence allowing it to assess the valence/ salience of environmental stimuli in relation to expectations and to information in memory; and projects to the hypothalamus, striatum, hippocampus, and cortex, thus coordinating physiological, cognitive, and behavioral responses (Davis 2000; LeDoux 2000). Similarly, in fish the Dm also receives multimodal sensory inputs (e.g. olfactory, mechanosensory, auditory, electrosensory; Folgueira et al. 2004a, b; Striedter 1991; Yamamoto and Ito 2005, 2008), and has reciprocal connections with the hypothalamus (Braford 1995; Echteler and Saidel 1981; Murakami et al. 1983; Striedter 1991). Moreover, experimental lesions of Dm also impair emotional learning in fish, thus suggesting also a functional similarity between the teleost Dm and the mammalian amygdala (Portavella et al. 2002). Our results further support the role of the Dm in emotional processes in fish, in particular the processing of aversive stimulus salience in fish. In mammals the role of the hippocampus has been linked to the storage of repeated experiences, in particular spatial memory (Eichenbaum et al. 1999). In teleost fish Dl has been established as a homologue of the mammalian hippocampus, with experimental lesions in this area leading deficits in spatial learning, but not emotional or cue learning (Portavella et al. 2002; Rodriguez et al. 2002). Our results suggest that a sub-region of the Dl in seabream (Dlv) is also involved in stimulus appraisal, possibly due to its role in memory storage of the predictable stimulus.
Finally, it should be mentioned that the loss of predictability (predictable followed by unpredictable conditions) has also been reported to act as a stressor by itself, being even more detrimental than unpredictable regimes (Bassett and Buchanan-Smith 2007; Gilbert-Norton et al. 2009). In fish, a recent work has demonstrated that Atlantic salmon, increase aggressive behavior after reward omission (Vindas et al. 2014a). Thus, predictability not only of aversive but also of appetitive stimuli (e.g. feeding regimes) seem to play a major role in stress management and should be taken into account in the handling of farmed fish as a way to stress reduction (see Basset and Buchanan-Smith, 2007 for a review on the impact of predictability of animal welfare). In summary, in this study we have shown that stressor predictability modulates the stress response at multiple levels (behavioural, physiological, neuronal) in Sea Bass, which supports the occurrence of cognitive appraisal of environmental stimuli in fish and highlights the need to consider psychological stress in the handling of farmed-fish.

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Compliance with ethical standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.
Figure Captions

Fig. 1 Overview of the protocol and experimental conditions used to test predictability as an appraisal modulator of aversive events in seabass *Dicentrarchus labrax*.

Fig. 2 Behavioural responses (mean ± SEM) of fish socially isolated and in social groups towards predictable and unpredictable aversive stimuli (PRDa = predictable asocial; UnPRDa = unpredictable asocial; PRDs = predictable social; UnPRDs = unpredictable social): (a) time in freezing; (b) escape attempts; (c) Exploratory behaviour (measured by the arithmetic mean of the time spent in each area of the experimental tank / higher time) and (d) Shoal cohesion rank for fish tested under social conditions (1 – low cohesion; 2 – medium cohesion; 3 – high cohesion). Significant differences between treatments are indicated by asterisks (* p < 0.05; ** p < 0.01; *** p < 0.001). All descriptive statistics are mean ± SEM.

Fig. 3 Plasma cortisol responses (mean ± SEM) of fish socially isolated and in social groups towards predictable and unpredictable aversive stimuli (PRDa = predictable asocial; UnPRDa = unpredictable asocial; PRDs = predictable social; UnPRDs = unpredictable social). Significant differences between treatments are indicated by asterisks (* p < 0.05; ** p < 0.01; *** p < 0.001).

Fig. 4 Expression (mean ± SEM) of the immediate early genes *egr-1*, *c-fos*, *bdnf* and *npas4* in different brain nuclei (Dm = medial part of the dorsal telencephalon; Dld = dorsal division of the lateral telencephalon; Dlv = ventral division of the lateral telencephalon) of fish socially isolated and in social groups towards predictable and unpredictable aversive stimuli (PRDa = predictable asocial; UnPRDa = unpredictable asocial; PRDs = predictable social; UnPRDs = unpredictable social). Significant differences in expression levels between experimental conditions (i.e. PRDs vs. UnPRDs; PRDa vs. UnPRDa) are indicated by asterisks: * p < 0.05; ** p < 0.01; *** p < 0.001.
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