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

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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. Predictability is a key 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*). Groups of four fish were exposed to a predictable (signalled) or unpredictable (unsignalled) stressor. 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, we monitored the activity of three sea bass brain regions known to be implicated in stressor appraisal: the dorsomedian telencephalon, Dm (putative homologue of the pallial amygdala); and the dorsal (Dld) and ventral (Dlv) subareas of the dorsolateral telencephalon (putative homologue of the hippocampus). The activity of both the Dm and Dlv significantly responded to stressor predictability, suggesting an evolutionarily conserved role of these two brain regions in information processing related to stressor appraisal. These results indicate 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.

1. 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) [1]. Since the 1970s, it became clear that the cognitive appraisal of stimuli is a key mechanism in the activation of the stress response [2,3]. 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 classical conditioning) and about relations between responses and stimuli (i.e. stimulus-response learning or instrumental conditioning) [4]. 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 [5]. 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. [6]), and then extended to humans (e.g. [7]).

In recent 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 and 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) [8–10]. While the appraisal concept has already been applied to the study of stress and emotional behaviour in animals, mainly in mammals (see [8] for a recent review), in fish, the whole concept of psychological stress has been rarely addressed [5,11–16]. 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 [17,18]. 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*) [12]; familiarity with conspecifics has been shown to modulate both exploratory behaviour and the response to a territorial intrusion, also in tilapia [19,20]; 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 [21]. Finally, prediction error has recently been documented both in rainbow trout and in Atlantic salmon (*Salmo salar*) using a reward omission paradigm [15,16,22]. 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 [23], 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 [24–26]). Thus, the study of cognitive appraisal in fish will allow testing if the same cognitive appraisal processes are present in evolutionarily 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*). Sea bass was used as a model in this study given its wide use in European aquaculture, which makes the results presented 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 (IEGs) 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 (Dm, putative teleost homologue of the mammalian amygdala) and the dorsolateral telencephalon (Dl, putative teleost homologue of the mammalian hippocampus), in order to test if brain regions involved in cognitive appraisal are evolutionarily 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.

2. Material and methods

(a) Experimental fish and maintenance

A batch of sea bass with an initial body weight of 0.5 ± 0.3 g (mean ± s.d.) hatched at the experimental research station of IFREMER in Palavas-les-Flots (France) were transported to Ramalhete Research Station (CCMAR, 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 12 L : 12D photoperiod) during ten 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 72 fish with a body weight of 44.58 ± 6.36 g (mean ± s.d.) at the start of the experiments were used.

(b) Experimental design and conditioning procedures

The effects of predictability on the fish stress response were tested in groups of four individuals randomly chosen from the reared tank. The experiment occurred between May and June of 2013 ($T(°C) = 21.89 \pm 1.77$, $DO(\%) = 86 \pm 6$ and $pH = 8.13 \pm 0.15$). Eighteen experimental glass aquaria ($70 \times 40 \times 30$ cm) were used under the same housing conditions as described above, except for the fact that no airflow 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 (less than 0.1 mg l^{-1}) and ammonia (less than 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, 72 fish were tagged under anaesthesia with a 1 cm floy tag (Floy Tag Manufacturing, Seattle, USA) and with a multicolour pearl attached behind the

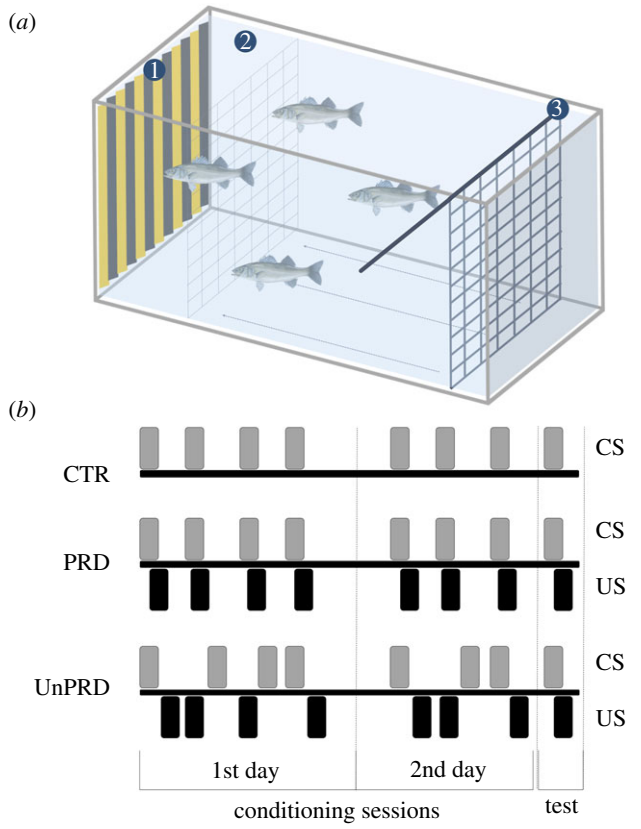

Figure 1. Overview of the protocol and experimental conditions used to test predictability as an appraisal modulator of aversive events in sea bass, *Dicentrarchus labrax*. (*a*) Schematic of the experimental tank. 1. Visual cue CS, settled at the time of conditioning. 2. Confinement area, correspondingly to 15% of the aquarium volume. 3. Confinement net US, settled at the beginning of the trial. (*b*) Description of the procedures used to test predictability: CTR, control; PRD, predictability; UnPRD, unpredictability. (Online version in colour.)

dorsal fin. Three experimental conditions were tested: control (CTR), predictability (PRD) and unpredictability (UnPRD). The experiment lasted 14 days, in which six groups of 4 fish each were used in each experimental condition (control conditions, CTR; predictable conditions, PRD; unpredictable conditions, UnPRD; $n = 24$ fish per treatment). After the acclimation period of 12 days, the experimental period occurred in the following 2 days and involved four training sessions in the first day (at 10.00 h, 12.00 h, 14.00 h and 16.00 h), and three 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 figure 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 × 30 cm), with a stressor (US: confinement promoted by a rigid net of the same size). The net was settled on the opposite side from the CS, and the US obtained by moving the net towards the CS until the fish were restrained in 15% of the aquarium volume. During the procedure, the CS remained in view for 1 min before the occurrence of US, and overlapped 1 min with it (see figure 1 for the schematic of the experimental tank and conditioning procedures)'. 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). In the control groups, fish were only subjected to the CS without US presentation. 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, and fish from control were subjected only to the CS to discard the effect of the CS on fish responses during the test session.

(c) 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, that is increase of fish swimming speed and movements towards the bottom of the tanks or towards the tank walls, or moving the body against the tank walls); (3) shoal cohesion, quantified through a proximity metric, defined as the distance variation between individuals within the shoal structure, and measured in an arbitrary scale (1 = low, mean distance greater than 15 cm apart; 2 = medium, 5 cm < mean distance > 15 cm apart; 3 = high, mean distance < 5 cm apart); and (4) exploratory behaviour, measured according to Galhardo *et al*. [12], following the formula:

$$\frac{A}{t_{\text{maximum}}},$$

where A is the arithmetic mean of the time fish spent in each one of three previously delimited areas of the tank (confinement net area; centre of the tank; and visual cue area), and t_{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.

(d) Blood sampling and plasma cortisol analysis

For each treatment, 30 min after the test session, fish were rapidly caught at the same time through a soft net with the same width as the experimental tank to reduce net chasing bias; they were 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 min 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.

(e) Brain microdissection and gene expression analysis

Eight individuals from each experimental treatment were randomly selected for the assessment of 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 electronic supplementary material, figure S1) were microdissected with modified 25 G steel needles using a micropunching technique previously established in the

laboratory [27]. These regions of interest in the brain were identified and classified following the available brain atlas for sea bass [28]. Total tissue was collected directly into lysis buffer from Qiagen Lipid Tissue Mini Kit (no. 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 (CTs) were automatically measured and relative expression of the target genes were calculated using the $2^{-\Delta Ct}$ method [29]. Primers efficiency was calculated for each qRT-PCR reaction using Light Cycler 480 II inner software.

(f) Statistical analysis

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 (time in freezing (arc-sin transformed), escape behaviour and exploratory behaviour (log$(X+1)$ transformed), Shoal cohesion, plasma cortisol concentration and IEGs mRNA expression (log-transformed)). LMM analyses were used to assess the effect of predictability (i.e. PRD versus CTR; PRD versus UnPRD; CTR versus UnPRD) on the behavioural variables before any stimulation. The same analysis was performed for a test session on the behavioural variables, on cortisol levels and on IEGs mRNA expression (*egr-1*, *c-fos*, *bdnf* and *npas4*) in each brain region (Dm, Dld and Dlv). Given that we have used more than one individual from the same experimental tank in each treatment, pseudo replication concerns could be raised. We accounted for sampling dependence by adding a random effect for the 'tank' factor in each LMM. In general, we did not find an effect of the 'tank' variable on the measured responses. All LMM were estimated using the restricted maximum-likelihood method. *A priori* planned comparisons with p-values adjusted following the Benjamini and Hochberg's method were used to test for specific differences between experimental conditions, namely: PRD versus CTR; PRD versus UnPRD; CTR versus UnPRD. Pearson test was used to assess correlations among variables. Descriptive statistics are expressed as mean ± s.e.m. The LMM and planned comparisons were performed using R (R Development Core Team) and GraphPad Prism v. 6.0 for windows was used for chart building and figures layout.

3. Results

(a) 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 the experimental treatments PRD, UnPRD and CTR (time freezing: $F_{(2,54)} = 0.36$, $p = 0.69$; escape events: $F_{(2,54)} = 0.44$, $p = 0.64$; exploratory behaviour: $F_{(2,54)} = 0.31$, $p = 0.73$). In the same way, shoal cohesion before training also did not show differences between experimental conditions ($F_{(2,54)} = 0.01$, $p = 0.98$).

The behaviour displayed by fish during exposure to the visual cue in the test session was markedly different between experimental treatments (figure 2; electronic supplementary material, table S2). Fish in the predictable treatment spent less time in freezing, and showed less escape attempts and more exploratory behaviour than fish in the unpredictable treatment (figure 2*a–c*; electronic supplementary material, table S2). Moreover, in this experiment, time in freezing and escape attempts were positively correlated ($R_p = 0.721$, $n = 72$, $p < 0.001$) and exploratory behaviour and time in freezing were negatively correlated ($R_p = -0.299$, $n = 48$, $p = 0.011$). Finally, shoal cohesion was higher in the unpredictable treatment over predictable and control conditions (figure 2*d*; electronic supplementary material, table S2). Moreover, there was a negative correlation between shoal cohesion and exploratory behaviour ($R_p = -0.427$, $n = 72$, $p < 0.001$).

(b) Effects of stressor predictability on fish physiology

Fish exposed to unpredictable stressors had higher cortisol levels than fish exposed to predictable stressors and to the levels found for the control group (electronic supplementary material, table S2; figure 3). Cortisol was positively correlated with time in freezing, shoal cohesion and frequency of escape events ($R_p = 0.355$, $n = 68$, $p = 0.003$; $R_p = 0.371$, $n = 68$, $p = 0.002$ and $R_p = 0.327$, $n = 68$, $p = 0.006$, respectively), whereas a negative correlation was found with exploratory behaviour ($R_p = -0.656$, $n = 68$, $p = 0.001$).

(c) Effects of stressor predictability on brain activation

Stressor predictability induced significant changes in the expression levels of IEGs, with an upregulation of *egr-1* at Dm associated with stressor unpredictability and a decrease of *npas4* at Dlv associated with stressor predictability (figure 4). Moreover, both predictable and unpredictable stress-induced an upregulation of *egr-1* in Dld and a downregulation of *c-fos* in Dlv (figure 4). *Bdnf* expression did not respond to any of the stress treatments (electronic supplementary material, table S2).

(d) Correlations between predictability-driven behavioural, physiological and brain activation measures

A positive correlation was found between time in freezing and escape behaviour ($R_p = 0.853$, $n = 24$, $p < 0.001$), and a negative correlation was found between exploratory behaviour and both shoal cohesion ($R_p = -0.593$, $n = 24$, $p = 0.002$) and cortisol ($R_p = -0.581$, $n = 24$, $p = 0.003$). Regarding neuronal plasticity, a negative correlation between cortisol and both *c-fos* and *bdnf* in the Dlv was also found ($R_p = -0.487$, $n = 20$, $p = 0.029$; $R_p = -0.473$, $n = 22$, $p = 0.026$, respectively).

4. Discussion

In this study, we have shown that stressor predictability modulates the stress response measured at the behavioural, physiological and neural levels. Fish exposed to the unpredictable stressor showed higher freezing and more escape behaviours, higher shoal cohesion, less exploratory

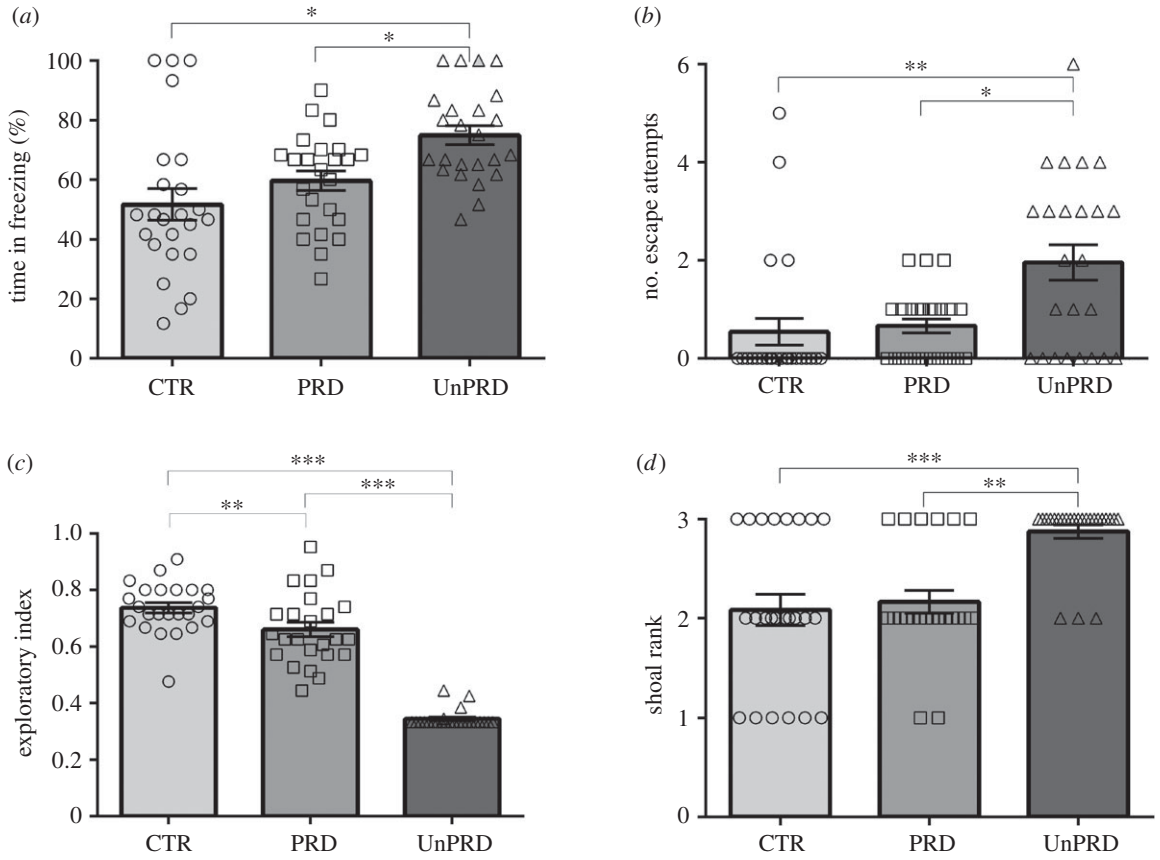

Figure 2. Behavioural responses (mean ± s.e.m.) of fish in social groups towards predictable and unpredictable aversive stimuli (CTR, control; PRD, predictable; UnPRD, unpredictable): (*a*) time in freezing; (*b*) escape attempts; (*c*) exploratory behaviour (measured by the ratio between the arithmetic mean of the time spent in each area of the experimental tank, by the higher time spent measured of such areas); 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 ± s.e.m.

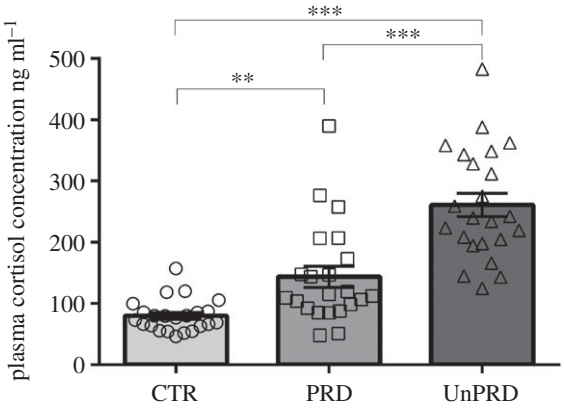

Figure 3. Plasma cortisol responses (mean ± s.e.m.) of fish in social groups towards predictable and unpredictable aversive stimuli (CTR, control; PRD, predictable; UnPRD, unpredictable). Significant differences between treatments are indicated by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

behaviour, higher physiological reactivity and more activation of the Dm as indicated by the expression of *egr1*. Therefore, an unpredictable stressor seems to trigger a higher stress response both in terms of the activation of the hypothalamic–pituitary–interrenal axis (HPI) and of the activation of a brain region putatively involved in the appraisal of the stressor, such as the Dm (fish homologue of the tetrapod pallial amygdala). Thus, predictability seems to reduce the behavioural response to stress.

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 [30,31]. For example, in the rat, which was the original model in which stressor predictability has been studied, it reduces the behavioural responses to stress, as well as detrimental consequences of stress such as pain reactivity, immunosuppression, gastric ulceration and colonic motility (e.g. [6,32–36]). Similar results have been found subsequently in other mammalian species (e.g. sheep [37]; dogs [38]; horses [39]; pigs [40]). However, fewer studies have addressed such effects in non-mammalian vertebrates (e.g. birds [41]), and among fish, the few studies available have produced contrasting results. While in this study, in conformity with previous studies in Mozambique tilapia, Gilthead sea bream or in rainbow trout (e.g. [5,12,16]), stressor predictability buffers the stress response, in Atlantic salmon no effect has been found (e.g. [13]). 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 [42,43], despite the fact that signalled shocks are perceived as more intense than unsignalled ones [44]. 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

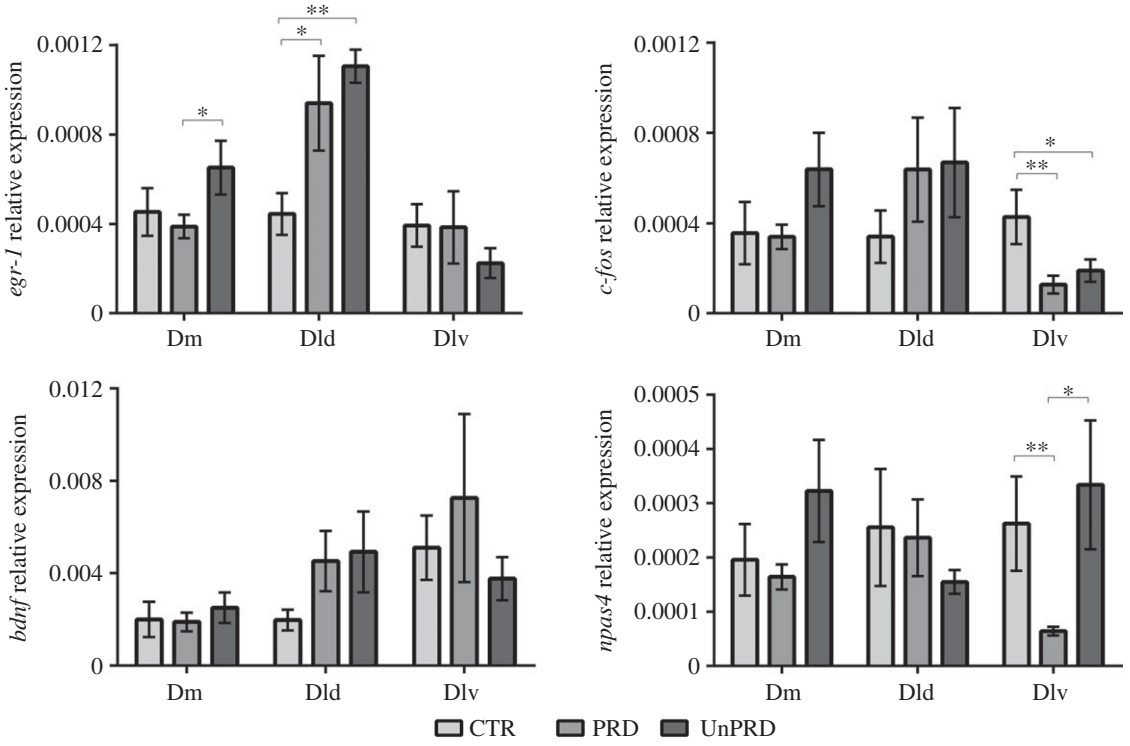

Figure 4. Expression (mean ± s.e.m.) of the immediate early genes *egr-1*, *c-fos*, *bdnf* and *npas4* in different brain nuclei (Dm, medial part of the dorsal telencephalon; Dld, dorsal division of the lateral telencephalon; Dlv, ventral division of the lateral telencephalon) of fish in social groups towards predictable and unpredictable aversive stimuli (CTR, control; PRD, predictable; UnPRD, unpredictable). Significant differences in expression levels between experimental conditions (i.e. PRD versus UnPRD; PRD versus CTR; UnPRD versus CTR) are indicated by asterisks: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

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 evolutionarily conserved these stimulus evaluation mechanisms are.

The amygdala together with the prefrontal cortex and the mesoaccumbens dopamine system have been implicated in the cognitive modulation of the stress response in mammals [45–48]. 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. [49–51]), in this study, we have focused on the putative fish homologues of the mammalian amygdala (Dm) and hippocampus (Dl).

In mammals, the amygdala plays a central role in emotional processes since it receives multi-modal 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 behavioural responses [52,53]. Similarly, in fish, the Dm also receives multimodal sensory inputs (e.g. olfactory, mechanosensory, auditory, electrosensory [54–58]), and has reciprocal connections with the hypothalamus [56,59–61]. 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 [62]. Hence, the teleost Dm has been considered a putative homologue of the mammalian pallial amygdala [26,63]. The higher activation of Dm found in this study suggests a conserved role of this area in the cognitive appraisal of stressors. Our results further support the role of the Dm in emotional processes in fish, in particular, the processing of aversive stimulus salience.

In mammals, the role of the hippocampus has been linked to the storage of repeated experiences, in particular, spatial memory [64]. 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 [62,65]. However, more precise analysis of the available evidence, based on extensive connections with septal nuclei and the preoptic area, the distribution patterns of histochemical and molecular markers and the patterns of neurogenesis and interneuron migration, suggests that this homology should be restricted to its ventral subdivision (Dlv) [66–68]. On the other hand, the dorsal subdivision of Dl (Dld) seems to be specialized in the processing of visual information via a tectal loop and in the multimodal integration of visual information with other sensory modalities, given its afferents to other sensory organs [66,68]. Our results suggest that Dlv is also involved in stimulus appraisal, possibly due to its role in (reduced) memory storage of the predictable stimulus. Interestingly, the expression of *npas4*, an IEG involved in contextual learning [69], is significantly decreased in the predictable stressor treatment, suggesting a role for contextual learning of predictable stressors. Recently, it has been shown that *npas4* plays a critical role in experience-dependent regulation of structural and functional plasticity at mossy fibres–CA3 synapses in the mammalian hippocampus, during contextual memory formation [70].

It is interesting to note that both stressors (i.e. either predictable or unpredictable) elicited in parallel an increase in the expression of *egr-1* in the Dld and a decrease in expression of *c-fos* in Dlv. Although both *c-fos* and *egr-1* are transiently

expressed in response to neuronal activity, hence being widely used in the field as markers of neuronal activity, *c-fos* has usually a more ubiquitous expression with egr-1 being regionally more restricted (e.g. [71]), and each of these genes plays different roles in neural plasticity. *C-fos* is involved in the regulation of transcription, and may mediate long-term effects of growth factors and membrane-depolarizing signals on neural activity [72]. *Egr-1* belongs to a family of transcriptional regulators (i.e. *egr-1*, *egr-2* and *egr-3*) involved in memory and learning [73]. Evidence from mutant mice suggests that *egr-1* is specifically required for long-term memory consolidation (e.g. [74–76]). The increase of activity in Dld (as reported by the upregulation of *egr-1*) in response to both stressor treatments can be associated with sustained higher arousal when repeatedly exposed to stressors, given the role of this area in the processing and integration of sensory stimuli. The decrease in activity in the Dlv (as reported by the downregulation of *c-fos*) suggests a reduced hippocampal-like memory storage during stress exposure, which is apparently further reduced in the predictable stressor treatment, as indicated by the reduction in *npas4* expression in the Dlv in the predictable stressor treatment discussed above.

Given the established role of *bdnf* in stress-induced neural plasticity [77], the lack of effects of stressor exposure on *bdnf* expression may be seen as surprising. However, in rodents, there are conflicting results regarding the effect of acute restraint as a stressor on *bdnf* expression, with some studies reporting increases and others decreases in expression, with variation also regarding brain regions [77]. Moreover, in rodents, the increase in *bdnf* expression has been detected in the hippocampus 1 h after stressor exposure [78]. Thus, our sampling time point may have failed to capture a putative *bdnf* response to our stressor treatments.

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 [79,80]. In fish, a recent work has demonstrated that Atlantic salmon, increase aggressive behaviour after reward omission [15]. 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 considered in the handling of farmed fish as a way to stress reduction (see [79] for a review on the impact of predictability of animal welfare). In line with this, controllability, another key component of stimuli appraisal, is recognized to increase coping ability by combining the individual's affective state and the environmental conditions for the appraisal process [81,82]. In fish, it was demonstrated that an aversive event is less stressful when the animal exerts control over it, likely reducing negative emotional responses and permitting adjusting their coping responses to the environmental conditions [83].

In summary, in this study, we have shown that stressor predictability modulates the stress response at multiple levels (behavioural, physiological and 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.

Ethics. All applicable international, national and/or institutional guidelines, with the permit no. 0420/00/000-n.9909/11/2009, for the care and use of animals were followed.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Authors' contributions. R.O., S.M. and M.C. designed research; M.C. and S.M. performed research; M.C. and A.F. carried out the molecular laboratory work; M.C., T.S. and G.A.O. carried out the statistical analyses; C.C.V.O., S.R. and S.M. critically revised the manuscript; R.O. and M.C. wrote the paper.

Competing interests. We declare we have no competing interests.

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