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

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14

15 **Abstract**

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

32 processing related to stressor appraisal. These results suggest that stressor predictability
33 plays a key role in the activation of the stress response in a teleost fish, hence highlighting
34 the role of cognitive processes in fish stress.

35

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

38

39

40 **Introduction**

41 The literature on stress biology has long established the role of cognitive factors on
42 triggering the stress response, defined as a response of the organism to regain homeostasis
43 when exposed to a homeostasis threatening stimulus or event (aka stressor) (Koolhaas et al.
44 2011). Since the 1970's it became clear that the cognitive appraisal of stimuli is a key
45 mechanism in the activation of the stress response (Mason 1968; Weiss 1972). According
46 to this perspective, it is not the intrinsic physical characteristics of the stimulus that trigger
47 a response but rather the evaluation of what that stimulus or event means to that organism
48 at that moment in time, which depends on stored information in memory about relations
49 between stimuli (i.e. stimulus-stimulus learning or classic conditioning) and about relations
50 between responses and stimuli (i.e. stimulus-response learning or instrumental
51 conditioning) (Ursin and Eriksen 2004). Therefore, the same stimulus may elicit or not a
52 stress response depending on how it is appraised by the individual. An "alarm" response
53 would occur when expectancies, based on perceived contingencies between stimuli (i.e.
54 stimulus expectancies) and between stimulus and response (i.e. response expectancies), are
55 not met (i.e. when there is a discrepancy between expected situation and perceived
56 situation). Hence, stimulus predictability, which refers to high levels of perceived
57 probability of occurrence of the expected event, and stimulus controllability, which refers
58 to high levels of perceived probability for response outcomes, play a major role on the
59 appraisal of stimuli as aversive or not. Interestingly, the role of cognitive variables in the
60 activation of a stress response was first investigated in laboratory animals, in particular in
61 rodents (e.g. Weiss, 1970), and then extended to humans (e.g. Lazarus, 1999).

62 In the last decades the role of cognitive variables in the activation of stress
63 responses as well as in triggering responses to appetitive events has been framed under a
64 theory of cognitive appraisal. According to this theory individuals continuously monitor the
65 environment using a set of stimulus evaluation checks (e.g. intrinsic valence, novelty,
66 prediction error, capacity for control) in order to evaluate the valence (positive/ negative)
67 and salience (high/ low) of detected stimuli (primary appraisal), and also assess the
68 available organismal resources to deal with them (secondary appraisal) (Faustino et al.
69 2015; Mendl et al. 2010; Moors et al. 2013). While the appraisal concept has already been
70 applied to the study of stress and emotional behaviour in animals, mainly in mammals (see
71 Faustino et al. 2015 for a recent review), in fish the whole concept of psychological stress
72 has been rarely addressed (Galhardo 2009; Galhardo et al. 2011; Madaro et al. 2016, 2015;
73 Vindas et al. 2014a,b). However, empirical evidence for the occurrence of each of the
74 stimulus evaluation checks involved in primary appraisal has been described in fish. The
75 appraisal of the intrinsic valence of stimuli can be demonstrated by learned
76 approach/avoidance behaviours, and these have been described in different fish species
77 (Millot et al. 2014a,b). The use of the three cues that signal stimulus novelty have also been
78 documented in fish: the effects of predictability in modulating the behavioural and
79 physiological response to both aversive and appetitive stimuli have been described in the
80 Mozambique tilapia (*Oreochromis mossambicus*) (Galhardo et al. 2011); familiarity with
81 conspecifics has been shown to modulate both exploratory behavior and the response to a
82 territorial intrusion, also in Tilapia (Aires et al. 2015; Galhardo et al. 2012); and the effect
83 of controllability can be illustrated by rainbow trout (*Oncorhynchus mykiss*) that have the
84 chance to actively avoid being defeated by a larger conspecific in a conditioning paradigm
85 exhibiting a lower cortisol response to the conditioned stimulus, than those that cannot
86 escape social defeat (Carpenter and Summers 2009). Finally, prediction error has recently
87 been documented both in rainbow trout and in Atlantic salmon (*Salmo salar*) using a
88 reward omission paradigm (Vindas et al. 2012, 2014a,b). However, this evidence has so far
89 not been explicitly presented as supporting the occurrence of cognitive appraisal in fish and
90 the proximate (i.e. neural/physiological) bases of these cognitive appraisal processes have
91 not been investigated yet in fish. Given the expected universality of stimulus evaluation
92 checks across animals, it is now timely to characterize their occurrence across species and

93 to implement comparative studies on the underlying neural mechanisms. Teleost fish offer
94 an excellent opportunity for such comparative approach, given the divergent evolutionary
95 path between ray-finned fish and tetrapods (Venkatesh et al. 2001), and the homologies that
96 have already been established between teleost and mammalian brain regions, that include
97 some of the areas known to be involved in cognitive appraisal in mammals (i.e. amygdala
98 and hippocampus, Broglio et al. 2005; Ganz et al. 2012, 2015). Thus, the study of cognitive
99 appraisal in fish will allow testing if the same cognitive appraisal processes are present in
100 evolutionary divergent vertebrate taxa and if they share homologue neural mechanisms.

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

115

116 **Material and methods**

117

118 *Experimental fish and maintenance*

119 A batch of seabass with an initial body weight of 0.5 ± 0.3 g (mean \pm SD) hatched
120 at the experimental research station of IFREMER in Palavas-les-Flots (France) were
121 transported to CCMAR Research Station (Faro, Portugal). Fish were reared in 500 L tanks
122 in an open water circuit with constant aeration through air stones (temperature of 21 ± 5 °C,

123 salinity of 35 ± 1 ‰, dissolved oxygen above 75 %, and a 12L:12D photoperiod) during 10
124 months before the experiments. Fish were initially fed at 10 % of body weight with
125 commercial diets (Aquagold, Aquasoja, Sorgal SA, Portugal), and later food amount was
126 readjusted until 3 % of body weight in accordance with their growth. A total of 96 fish with
127 a body weight of 44.58 ± 6.36 g (mean \pm SD) at the start of the experiments were used.

128
129 *Experimental design and conditioning procedures*

130 Given the fact that seabass are a shoaling species the effects of predictability on the
131 stress response were tested both in isolated fish (Experiment 1) and in fish kept in social
132 groups (Experiment 2). The two experiments occurred between May and June of 2013.
133 Twelve experimental glass aquaria (70 x 40 x 30 cm) were used under the same housing
134 conditions as described above, except for the fact that no air flow was supplied, since the
135 water flow rate of 2.5 L min^{-1} was sufficient to guarantee oxygen saturation. A net, with the
136 same dimension as the lateral wall, was settled in one side of each aquarium at the
137 beginning of the experiment to be used as a confinement net. All aquaria walls were
138 covered with opaque plastic to avert visual contact between the animals and the
139 experimenters. The fish were fed at 3 \% Bw^{-1} daily, divided by two meals at 08:00 h and
140 18:00 h. Water quality was analysed for nitrites ($<0.1 \text{ mg.L}^{-1}$) and ammonia ($<0.1 \text{ mg.L}^{-1}$)
141 every three days. Temperature, oxygen saturation and pH were daily checked before the
142 cleaning routines performed 1 h after the second meal.

143 One month before the experiments, 96 fish were tagged under anaesthesia with a 1
144 cm long floy tag (Floy Tag Manufacturing Inc, Seattle, USA) and with a multicolour pearl
145 attached behind the dorsal fin. Two experimental conditions were tested in each
146 experiment: predictability (PRD) and unpredictability (UnPRD). In Experiment 1 (social
147 isolation), which lasted 3 days, 24 fish were tested in each experimental condition
148 (predictable asocial, PRDa; unpredictable asocial, UnPRDa). In Experiment 2 (social
149 groups), which lasted 14 days, six groups of 4 fish each were used in each experimental
150 condition (predictable social, PRDs; unpredictable social, UnPRDs; $n = 24$ fish/treatment).
151 The differences in the duration of the two experiments are due to the fact that preliminary
152 trials had shown that seabass kept in small shoals needs between 8 to 12 days to show

153 signals of adaptation to the experimental environment, whether seabass kept in social
154 isolation does not survive that long (e.g. individuals die before resuming feeding). Because
155 of this difference in duration between the two experiments their results cannot be directly
156 compared (see statistical analysis below). After this variable acclimation period, the
157 experimental period occurred in the last 2 days for both experiments and involved 4
158 training sessions in the first day (at 10:00 h, 12:00 h, 14:00 h and 16:00 h) and 3 training
159 sessions (at 10:00 h, 12:00 h, and 14:00 h) and one test session (at 16:00 h) on the second
160 day (see Fig. 1 for an overview of experimental procedures). To create the predictable and
161 unpredictable treatments two different training procedures were used. In the predictable
162 treatment a delay conditioning protocol was used for fish to learn to associate a visual cue
163 (CS), which consisted of a yellow and black striped card with the same size as the lateral
164 wall of the aquaria (40 x 30 cm), with a stressor (US: confinement). The CS was presented
165 on the opposite wall of the confinement net (US), remained in view for 1 min before the
166 occurrence of US, and overlapped 1 min with it. The confinement was obtained by moving
167 the net into the visual cue direction until it reached 15% of the aquarium volume. In the
168 unpredictable treatment fish were presented with the same visual sign but temporally
169 dissociated from the stressor (i.e. 30 min before or after in a random way). The number of
170 conditioning trials used was based on preliminary tests that indicated that 5-6 trials were
171 enough for aversive conditioning in this species. In the test session, fish from both
172 experimental treatments (PRD and UnPRD) were exposed to the visual cue together with
173 the stressor.

174

175 *Behavioural observations*

176 Fish behaviour was video recorded right before the first training session and during
177 the test session using video cameras (TVCCD-623-COL, Monacor®, Denmark and
178 webcams HD C310 Logitech) positioned 1 m above the tank. Videos were subsequently
179 analysed using multi-event recorder software (Observer XT® from Noldus, Netherlands).
180 The response to the visual cue was assessed using the following behavioural
181 measurements: (1) time spent in freezing behaviour (i.e. time fish spent immobile, with or
182 without fin movements, either on the bottom or in the water column); (2) escape behaviour
183 (i.e. fish swimming strongly, going close to the tank walls or moving the body in a way

184 that looked like escape attempts); (3) shoal cohesion, measured in an arbitrary scale (1 =
185 low, 2 = medium; 3 = high); and (4) exploratory behaviour following the formula:

$$186 \quad A / t_{\text{maximum}}$$

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

192

193 *Blood Sampling and plasma cortisol analysis*

194 In both experiments, 30 min after the test session, fish were rapidly caught and
195 euthanized with an overdose of 2-phenoxyethanol (1‰, Sigma-Aldrich) and blood was
196 immediately collected from the caudal vein and centrifuged at RT for 25 minutes at 2000
197 g. Plasma was stored at -80 °C until further processing. Plasma cortisol levels were
198 measured using a commercial ELISA kit (RE52061, IBL Hamburg, Germany), with a
199 sensitivity of 2.5 ng ml⁻¹ and intra- and inter-assay coefficients of variation (CV) were
200 2.9% and 3.5 %, respectively.

201

202 *Brain microdissection and gene expression analysis*

203 In both experiments 8 individuals from each experimental treatment were randomly
204 selected for the assessment of immediate early genes (IEGs) mRNA expression in brain
205 regions of interest (see below). Fish were sacrificed and the skull with the brain inside was
206 immediately imbedded in Tissue-Tek® and kept at -80°C until further processing. Brain
207 telencephalon was sliced through 150 µm thick cryostat (Leica, CM 3050S) coronal
208 sections, from which the medial part of the dorsal telencephalon (Dm), the Dorsal division
209 of the lateral telencephalon (Dld) and the Ventral division of the lateral telencephalon (Dlv)
210 (see supplementary material, Fig. S1) were microdissected with modified 25G steel needles
211 using a micropunching technique previously established in the lab (Teles et al. 2015).
212 These regions of interest in the brain were identified and classified following the available
213 brain atlas for seabass (Cerdeira-Reverter et al. 2001). Total tissue was collected directly into

214 lysis buffer from Qiagen Lipid Tissue Mini Kit (#74804; Valencia, CA) and total RNA
215 extracted from the samples, with some adjustments to the manufacturer's instructions (see
216 electronic supplementary material for detailed procedures). RNA from each sample was
217 then reverse transcribed to cDNA (BioRad iScript cDNA Synthesis Kit; Valencia, CA)
218 accordingly to manufacturer's instructions and used as a template for quantitative
219 polymerase chain reactions (qPCR) of *egr-1*, *c-fos*, *bdnf* and *npas4*, using the geometric
220 mean of the expression of two previously established housekeeping genes, *ee1a* and *18S*
221 (see electronic supplementary material Table S1 for primer sequences and for qPCR
222 conditions). The abundance of the internal control genes was stable across experimental
223 treatments. All reactions were run in duplicate and controls without DNA templates were
224 run to verify the absence of cDNA contamination. Fluorescence cycle thresholds (CT) were
225 automatically measured and relative expression of the target genes were calculated using
226 the $2^{-\Delta Ct}$ method (Livak and Schmittgen 2001). Primers efficiency was calculated for each
227 qRT-PCR reaction using Light Cycler 480 II inner software.

228

229 *Statistical analysis*

230 Given the differences in duration between experiments 1 and 2 the social effects on
231 the stress response were not formally investigated, and the results from the 2 experiments
232 were analysed separately. Parametric assumptions of normality and homoscedasticity of the
233 data were confirmed by analysis of the residuals. Homogeneity of variances was checked
234 by Levene's test. Log, log (X+1) or arcsine transformations were used to match parametric
235 assumptions when required [Experiment 1: time in freezing (arc-sin transformed), escape
236 behaviour and exploratory behaviour (log (X+1) transformed), plasma cortisol
237 concentration and IEGs mRNA expression (log transformed); Experiment 2: time in
238 freezing (arc-sin transformed), exploratory behaviour (log (X+1) transformed, IEGs mRNA
239 expression (log transformed)]. In experiment 1 (social isolation) a T-test was used to
240 compare the two experimental treatments (PRD vs. UnPRD). In experiment 2 (social
241 group), since multiple individuals came from the same social group, a two-way ANOVA
242 with experimental treatment (PRDs vs. UnPRDs) and social group (1-6) as independent
243 variables, was used to assess differences in the behavioural variables and in cortisol. Data
244 on the expression of immediate early genes was also analysed with T-tests in experiment 2

245 given the fact that only a subset of randomly chose fish was used and therefore most social
246 group only contributed with one fish to the sample. Pearson test was used to assess
247 correlations among variables. Descriptive statistics are expressed as mean \pm standard error
248 of the mean (SEM). All statistics were performed using IBM SPSS® statistics v19.0.
249 GraphPad and GraphPad Prism® v6.0 for windows was used for chart building and figures
250 layout.

251

252 **Results**

253 *Effects of stressor predictability on fish behaviour*

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

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

268 Moreover, in this experiment a positive correlation was found between exploratory
269 behaviour and escape attempts (R = 0.429, n = 48, p = 0.002). In Experiment 2 (social
270 group) fish in the predictable treatment spent less time in freezing ($F_{(1,36)} = 11.34$, p =
271 0.001), and showed less escape attempts ($F_{(1,36)} = 10.79$, p = 0.002) and more exploratory
272 behaviour ($F_{(1,36)} = 151.54$, p < 0.001) than fish in the unpredictable treatment (Fig. 2a-c).
273 In experiment 2, time in freezing and escape attempts were positively correlated (R =
274 0.656, n = 45, p < 0.001) and exploratory behaviour and time in freezing were negatively

275 correlated ($R = -0.325$, $n = 48$, $p = 0.024$). Finally, shoal cohesion (measured only for
276 experiment 2) was higher in the unpredictable treatment ($F_{(1,36)} = 27.98$, $p < 0.001$) (Fig.
277 2d). Moreover, there was a negative correlation between shoal cohesion and exploratory
278 behaviour ($R = -0.491$, $n = 48$, $p < 0.001$).

279

280

281 *Effects of stressor predictability on fish physiology*

282 In both experiments fish exposed to unpredictable stressors had higher cortisol
283 levels than fish exposed to predictable stressors (Fig. 3; PRDa vs. UnPRDa: t-test = -2.06,
284 $df = 46$; $p = 0.04$; PRDs vs. UnPRDs: $F_{(1,36)} = 24.79$, $p < 0.001$). In Experiment 2 cortisol
285 was positively correlated with both time in freezing and shoal cohesion ($R_p = 0.351$, $n =$
286 45 , $p = 0.018$; $R = 0.310$, $n = 45$, $p = 0.038$), whereas a negative correlation was found with
287 exploratory behaviour ($R = -0.491$, $n = 45$, $p = 0.001$).

288

289 *Effects of stressor predictability on brain activation*

290 In experiment 1 (social isolation) both Dm and Dlv exhibited changes driven by
291 predictability, with higher expression levels of IEGs in the unpredictable treatment (i.e. of
292 *c-fos* and *bdnf* at both brain nuclei and of *egr-1* and *npas4* at Dlv; Fig. 4). In this
293 experiment, positive correlations were found at the Dm between *egr-1* expression and that
294 of either *c-fos*, *bdnf* or *npas4* ($R_p = 0.881$, $n = 16$, $p < 0.001$; $R = 0.771$, $n = 16$, $p < 0.001$;
295 $R = 0.648$, $n = 15$, $p = 0.009$, respectively), and between *c-fos* and both *bdnf* and *npas4* (R
296 $= 0.804$, $n = 16$, $p < 0.001$; $R = 0.549$, $n = 15$, $p = 0.034$, respectively). In the Dlv, positive
297 correlations were also found between *egr-1* expression and that of either *c-fos*, *bdnf* or
298 *npas4* ($R = 0.963$, $n = 15$, $p < 0.001$; $R = 0.746$, $n = 15$, $p = 0.001$; $R = 0.594$, $n = 15$, $p =$
299 0.019 , respectively), and between *c-fos* and both *bdnf* and *npas4* ($R = 0.794$, $n = 16$, $p <$
300 0.001 ; $R = 0.597$, $n = 15$, $p = 0.015$, respectively).

301 In experiment 2 (social groups) only *egr-1* was up-regulated at the Dm under
302 unpredictable conditions (Fig. 4). Additionally, positive correlations were found between
303 this gene and *c-fos*, *bdnf* and *npas4* at Dm ($R = 0.893$, $n = 16$, $p < 0.001$; $R = 0.707$, $n = 16$,
304 $p = 0.002$; $R = 0.567$, $n = 16$, $p = 0.022$, respectively).

305

306 *Correlations between predictability-driven behavioural, physiological and brain activation*
307 *measures*

308 In experiment 1 (social isolation), negative correlations were found between cortisol
309 and escape behaviour ($R = -0.509$, $n = 16$, $p = 0.044$) and between escape behaviour and
310 *bdnf* expression in Dm ($R = -0.590$, $n = 16$, $p = 0.016$). Moreover, a positive correlation
311 between escape attempts and exploratory behaviour was found ($R = 0.702$, $n = 16$, $p =$
312 0.002). In experiment 2 (social groups), a positive correlation was found between time in
313 freezing and escape behaviour ($R = 0.850$, $n = 16$, $p < 0.001$), and a negative correlation
314 was found between exploratory behaviour and shoal cohesion ($R = -0.720$, $n = 16$, $p =$
315 0.002). Finally, a positive correlation between escape behaviour and the *c-fos* expression in
316 the Dlv was also found ($R = 0.584$, $n = 12$, $p = 0.046$).

317

318 **Discussion**

319 In this study we have shown that stressor predictability modulates the stress
320 response measured at the behavioural, physiological and neural levels, both in fish exposed
321 to the stressor in social isolation and in social groups. In social isolation fish exposed to an
322 unpredictable stressor exhibited less exploratory and escape behaviours, higher cortisol
323 levels and more neuronal activity in the Dm and the Dlv, as indicated by the expression of
324 IEGs (*egr1*, *c-fos* and *bdnf* for Dm; and *egr1*, *c-fos*, *bdnf* and *npas4* for Dlv), than those
325 exposed to a predictable stressor. When in social groups, fish exposed to the unpredictable
326 stressor showed higher freezing and more escape behaviours, higher shoal cohesion, less
327 exploratory behaviour, higher physiological reactivity and more activation of the Dm as
328 indicated by the expression of *egr1*. Therefore, in both social contexts (i.e. social isolation
329 and social group) the exposure to a unpredictable stressor seems to trigger a milder stress
330 response both in terms of the activation of the hypothalamic-pituitary-interrenal axis (HPI)
331 and of the activation of the brain regions putatively involved in the appraisal of the stressor,
332 such as the Dm (fish homologue of the tetrapod pallial amygdala) and the Dlv (fish
333 homologue of the hippocampus). In contrast, at the behavioural level the social context
334 seems to produce antagonistic responses, with stressor unpredictability increasing shoal
335 cohesion, freezing and escape responses. Thus, predictability seems to reduce the

336 behavioural response to stress in social groups but not in isolated fish. However, since it
337 was not possible to match the duration of the two experiments, time is a confounding
338 variable and the inference of social effects, albeit plausible, cannot be firmly established
339 here.

340 The effects of stressor predictability have been extensively studied both in humans
341 and in animals, and consistently the results have shown that prediction reduces the stress
342 effects of aversive experiences (Lovallo 2005; Sapolsky 2004). For example, in the rat,
343 which was the original model in which stressor predictability has been studied, it reduces
344 the behavioral responses to stress, as well as detrimental consequences of stress such as
345 pain reactivity, immunosuppression, gastric ulceration, and colonic motility (e.g. Weiss,
346 1970; Gliner, 1972; Hymowitz, 1979; Guile and McCutcheon, 1984; Mormede et al. 1988;
347 Tyler et al. 2007). Similar results have been found subsequently in other mammalian
348 species (e.g. sheep: Greiveldinger et al. 2007; dogs: Dess and et al. 1983; horses: Thomas
349 2010; pigs: Carlstead 1986). However, fewer studies have addressed such effects in non-
350 mammalian vertebrates (e.g. birds: Bauer et al. 2011), and among fish the few studies
351 available have produced contrasting results. While in this study, in conformity with
352 previous studies in Mozambique tilapia or in rainbow trout (e.g. Galhardo et al. 2011;
353 Vindas et al. 2014a), stressor predictability buffers the stress response, in Atlantic salmon
354 no effect has been found (e.g. Madaro et al. 2016). Given the fact that the two contrasting
355 results occur within the same family (Salmonidae), these differences do not seem reflect a
356 phylogenetic difference but rather a species-specific effect. Interestingly, classic studies in
357 this field have shown that when rats are given a choice between a signalled and an
358 unsignalled foot shock they prefer the former (Arabian and Desiderato 1975; Lockard
359 1963), despite the fact that signaled shocks are perceived as more intense than unsignalled
360 ones (Miller et al. 1983). Thus, it looks like during primary appraisal different stimulus
361 evaluation checks are not equally weighted, as in this case appraisal of stimulus
362 predictability seems to have overridden the perception of stimulus intensity. It is therefore
363 important to extend the study of stressor predictability, and of cognitive appraisal in
364 general, to other vertebrate species in order to assess how evolutionary conserved these
365 stimulus evaluation mechanisms are.

366 The higher activation found in this study of brain areas that are homologous to the
367 mammalian pallial amygdala (i.e. Dm) and to the hippocampus (i.e. DIv) (Ganz et al. 2015;
368 Maximino et al. 2013) in response to unpredictable stressors, suggests a conserved role of
369 these areas in the cognitive appraisal of stressors. Indeed these two brain regions, together
370 with the prefrontal cortex and the mesoaccumbens dopamine system have been implicated
371 in the cognitive modulation of the stress response in mammals (Belova et al. 2007; Cabib
372 and Puglisi-Allegra 2012; Maier and Watkins 2010; Pruessner et al. 2008). Given the lack
373 of a neocortex and the absence of midbrain dopaminergic neurons (Dahlström-Füxe's A10
374 nucleus, homologous to the mammalian mesolimbic ventral tegmental area) in fish (e.g.
375 Panula et al. 2010; Tay et al. 2011; Yamamoto and Vernier 2011), in this study we have
376 focused on the putative fish homologues of the mammalian amygdala and hippocampus. In
377 mammals the amygdala plays a central role in emotional processes since it receives multi-
378 modal sensory information, as well as inputs from the frontal cortex and the hippocampus,
379 hence allowing it to assess the valence/ salience of environmental stimuli in relation to
380 expectations and to information in memory; and projects to the hypothalamus, striatum,
381 hippocampus, and cortex, thus coordinating physiological, cognitive, and behavioral
382 responses (Davis 2000; LeDoux 2000). Similarly, in fish the Dm also receives multimodal
383 sensory inputs (e.g. olfactory, mechanosensory, auditory, electrosensory; Figueira et al.
384 2004a, b; Striedter 1991; Yamamoto and Ito 2005, 2008), and has reciprocal connections
385 with the hypothalamus (Braford 1995; Ehteler and Saidel 1981; Murakami et al. 1983;
386 Striedter 1991). Moreover, experimental lesions of Dm also impair emotional learning in
387 fish, thus suggesting also a functional similarity between the teleost Dm and the
388 mammalian amygdala (Portavella et al. 2002). Our results further support the role of the
389 Dm in emotional processes in fish, in particular the processing of aversive stimulus salience
390 in fish. In mammals the role of the hippocampus has been linked to the storage of repeated
391 experiences, in particular spatial memory (Eichenbaum et al. 1999). In teleost fish DI has
392 been established as a homologue of the mammalian hippocampus, with experimental
393 lesions in this area leading deficits in spatial learning, but not emotional or cue learning
394 (Portavella et al. 2002; Rodriguez et al. 2002). Our results suggest that a sub-region of the
395 DI in seabream (DIv) is also involved in stimulus appraisal, possibly due to its role in
396 memory storage of the predictable stimulus.

397 Finally, it should be mentioned that the loss of predictability (predictable followed
398 by unpredictable conditions) has also been reported to act as a stressor by itself, being even
399 more detrimental than unpredictable regimes (Bassett and Buchanan-Smith 2007; Gilbert-
400 Norton et al. 2009). In fish, a recent work has demonstrated that Atlantic salmon, increase
401 aggressive behavior after reward omission (Vindas et al. 2014a). Thus, predictability not
402 only of aversive but also of appetitive stimuli (e.g. feeding regimes) seem to play a major
403 role in stress management and should be taken into account in the handling of farmed fish
404 as a way to stress reduction (see Bassett and Buchanan-Smith, 2007 for a review on the
405 impact of predictability of animal welfare). In summary, in this study we have shown that
406 stressor predictability modulates the stress response at multiple levels (behavioural,
407 physiological, neuronal) in Sea Bass, which supports the occurrence of cognitive appraisal
408 of environmental stimuli in fish and highlights the need to consider psychological stress in
409 the handling of farmed-fish.

410

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418

419 **Compliance with ethical standards**

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

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

423

424 **Figure Captions**

425

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

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

437 **Fig. 3** Plasma cortisol responses (mean \pm SEM) of fish socially isolated and in social
438 groups towards predictable and unpredictable aversive stimuli (PRDa = predictable asocial;
439 UnPRDa = unpredictable asocial; PRDs = predictable social; UnPRDs = unpredictable
440 social). Significant differences between treatments are indicated by asterisks (* $p < 0.05$; **
441 $p < 0.01$; *** $p < 0.001$).

442

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

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