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Physiological responses during acute stress recovery depend on stress coping style in European sea bass, *Dicentrarchus labrax*.

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Abstract

Individual stress coping style (reactive, intermediate and proactive) was determined in 3 groups of 120 pit tagged European seabass using the hypoxia avoidance test. The same three groups (no change in social composition) were then reared according to the standards recommended for this species. Then, 127 days later, individuals initially characterized as reactive, intermediate or proactive were submitted to an acute confinement stress for 30 min. Blood samples were taken to measure plasma cortisol levels 30 min (Stress30) or 150 min (Stress150) after the end of the confinement stress. Individuals were then sacrificed to sample the telencephalon in order to measure the main monoamines and their catabolites (at Stress30 only). Individuals from Stress150 were sampled for whole brain for a transcriptomic analysis. The main results showed that reactive individuals had a lower body mass than intermediate individuals which did not differ from proactive individuals. The physiological cortisol response did not differ between coping style at Stress30 but at Stress150 when intermediate and proactive individuals had recovered pre stress levels, reactive individuals showed a significant higher level illustrating a modulation of stress recovery by coping style. Serotonin turnover ratio was higher in proactive and reactive individuals compared to intermediate individuals and a significant positive correlation was observed with cortisol levels whatever the coping style. Further, the confinement stress led to a general increase in the serotonin turnover comparable between coping styles. Stress150 had a significant effect on target mRNA copy number (*Gapdh* mRNA copy number decreased while *ifrd1* mRNA copy number increased) and such changes tended to depend upon coping style.

Key words: *fish, behaviour, physiology, personality, brain, transcriptomic*

***Highlights**:*

- Reactive fish showed a slower stress recovery after acute confinement stress
- Serotonin turnover ratio was affected by acute confinement stress
- A positive correlation was shown between plasma cortisol and serotonin turnover ratio

1. Introduction

Recently, the scientific community has seen a surging interest in the evolution and development of links between different variable traits and consistent variation between individuals across time and contexts. Adaptive phenotypic variation is principally expressed as correlated behavioural and physiological profiles, in turn conferring a variable vulnerability to competition, stress and disease. Physiological-behavioural trait associations are commonly referred to as stress coping styles (Koolhaas et al, 1999). These individual coping styles occur within a population along an axis (also called a continuum) between pro- and reactive extremes. For simplification, most studies only consider the extreme phenotypes but it should be understood within this continuum and relative to the population composition and situation. Proactive animals tend to engage in active avoidance or cope with stressful stimuli through a “fight or flight” response contrary to reactive ones which display a passive behaviour through a “freeze and hide” response (Koolhaas et al, 1999; Koolhaas, 2008).

Physiologically, a proactive fish is characterized with a lower hypothalamus-pituitary-inter-renal (HPI) activity (Øverli et al, 2005, 2007; Silva et al, 2010) and higher sympathetic reactivity compared to a reactive fish (reviewed by Øverli et al, 2007; Castanheira et al, 2013; Sørensen et al, 2013). Therefore, proactive fish typically have lower basal concentrations of glucocorticoids (the principal hormones involved in the stress response and the ultimate product of HPI axis activation) and lower stress-induced glucocorticoid concentrations (Øverli et al, 2007) than reactive individuals. In further details, the main endocrine components of stress are an immediate increase in the release of catecholamines (epinephrine and norepinephrine [NE]) into the circulation, accompanied by increased sympathetic tone, and a slightly delayed (within minutes) increase in the release of glucocorticoid hormones (mainly cortisol in teleost fish). The monoamines serotonin (5-hydroxytryptamin, 5-HT), dopamine (DA) and norepinephrine (NE), have been studied as neurotransmitters or neuromodulators potentially involved in the mediation of physiological as well as behavioural stress responses (Winberg et al, 1991; Winberg and Nilsson, 1992, 1993; Lillesaar, 2011; Vindas et al, 2014; Thornqvist et al,. 2018). The effects of monoaminergic neurotransmitters are terminated by uptake into presynaptic nerve and possibly glial cells. Following uptake, monoamines are deaminated to their main catabolites by monoamine oxydase (MAO). 5-HT is transformed to 5-hydroxyindoleacetic acid (5-HIAA) and DA to 3,4-dihydroxyphenylacetic acid (DOPAC). Both for 5-HT and the catecholamines, the ratio of the tissue concentration of their metabolites to that of the parent monoamine is frequently used as an index of neural activity, increased concentration of the metabolite being taken to indicate increased release and turnover of the neurotransmitter (reviewed in Shannon et al, 1986; Fillenz, 1993).

Behavioural inhibition in reactive animal has been suggested to be mediated partly by a stress induced elevation of brain serotoninergic activity (Winberg et al, 1993a, b; Øverli et al, 1998) but the opposite, a reduced serotoninergic activity, has also been observed (Alfonso et al 2019). 5-HT has also been suggested to stimulate HPI axis in teleosts (reviewed in Höglund et al, 2000). The release of catecholamines is also integral part of the physiological response to stressors in all vertebrates (Hart et al, 1989). The function of catecholamines includes modulation of respiratory and cardiovascular systems, blood oxygen transport capacity, blood glucose and free fatty acid levels. Moreover, in fish, brain dopamine seems to have a generally suppressive effect on HPI axis activity and 5-HT signalling (Höglund et al, 2001).

Molecular regulation at the level of the transcriptome underpins the behavioural and physiological characteristics of the two main coping styles described above (MacKenzie et al, 2009). This regulation insures correct adaptive response to changing environmental conditions and include a scaled suite of multi-directional regulatory processes, from transcriptome to behaviour, that interact to optimize individual fitness (Rey et al, 2013). Thus, a transcriptome or gene expression profile is a collection of mRNAs within a cell, tissue or organism that represents the available transcripts at a specific point in time (i.e. phenotype shaped by the organism’s genotype, Rey et al, 2013). In previous studies, prior screening for coping style before experimentation increased the value of gene expression data and their interpretation (MacKenzie et al, 2009; Rey et al 2016). Such methodology allows for targeted candidate gene expression analysis in follow-up studies (e.g. the current report).

Here we present a multidisciplinary study, assessing coping styles at the level of behavioural and physiological responses with the aim to constitute an integrative design to better understand underlying mechanisms related to adaptive phenotypic variation in sea bass (*Dicentrarchus labrax*). Indeed, in this particular species, genetic correlations between weight and risk taking traits showed negative values i.e. reactive individual mass was heavier (Ferrari et al, 2016) contrary to a recent study performed in Atlantic salmon (Damsgård et al, 2019).

The objectives of this study were to assess physiological, neurochemical and mRNA expression of selected brain target genes of fish from divergent coping style after an acute confinement stress and during recovery. To achieve this, sea bass were screened by using a hypoxia avoidance test (Laursen et al 2011, translated to sea bass by Ferrari et al, 2015) and were assigned to reactive, intermediate and proactive categories. Although this test does not allow distinguishing between exploration and hypoxia tolerance, it is relevant for coping style assessment in its physiological dimension since hypoxia tolerance is a determinant of individual fitness in sea bass (Joyce et al., 2016) and correlations between risk taking and hypoxia tolerance were shown in sea bass (Ferrari et al. 2016, Alfonso et al. 2019).

Following this, fish were reared under standard conditions and were exposed to a confinement stress. Brain and blood were sampled, plasma were analysed for cortisol, telencephalon for monoaminergic neurochemistry and gene expression of target genes were analysed in whole brains. Data obtained for each individual were cross-correlated in order to explore causal links of phenotypic traits.

2. Material and methods

In this study, PIT tagged sea bass were screened using the hypoxia tolerance test (see below). They were then reared for 4 months without changing group composition. At the end of the rearing period, a confinement stress protocol was applied resulting in three treatments (Control, Stress30 and Stress150, **Figure 1**). Blood samples were taken to assess cortisol levels, and fish were dissected to sample telencephalon to quantify monoamines whereas target mRNA expression was measured in whole brain.

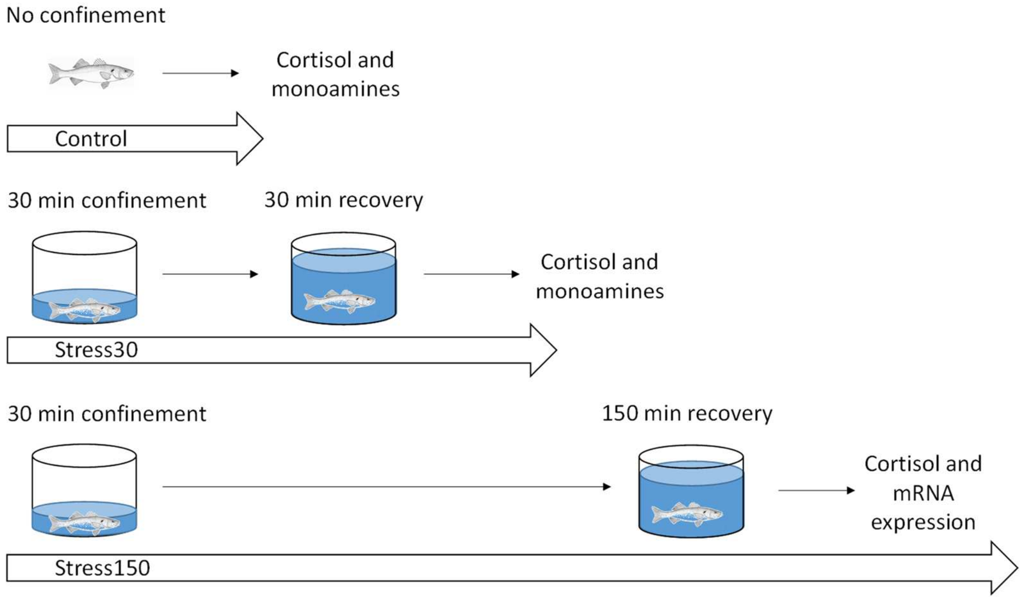


Figure 1: Scheme of the three treatment conditions (Control, Stress30 and Stress150). “Control” corresponds to cortisol and monoamine sampling just after netting in the home tank. “Stress30” corresponds to cortisol and monoamine sampling after 30 minutes confinement and 30 minutes recovery. “Stress150” corresponds to Cortisol and gene expression sampling after 30 minutes confinement and 150 minutes recovery.

2.1. Fish and experimental conditions

Fish were hatched and reared at the experimental research station of Ifremer (Palavas-les-Flots, France) according to sea bass rearing standard (Chatain, 1994). Three groups of each 120 fish were used in this experiment (N total=360). Each triplicate was placed in a 1.5 m^3 tank under sand filtered open flow system.

2.2. Growth follow-up

All fish were weighed five times under anaesthesia ((benzocaine, 200 ppm, after tranquilization in the rearing tank with 70 ppm Benzocaine), at 215 days post hatching (dph), 251 dph, 285 dph, 314 dph and 342 dph (hereafter termed BW_215dph, BW_285dph etc…). Specific Growth Rate was calculated as follows (SGR=100*ln(BW_f)-ln(BW_i)/t, in %) with BW_f corresponding to final body mass and BW_i to initial body mass and t corresponded to time in days between two successive mass measurements. Four SGRs were calculated: SGR_1 (215-251 dph), SGR_2 (251-285 dph), SGR_3 (285-314 dph) and SGR_4 (314-342 dph). At the end of the experiment, fish were euthanatized with an overdose of anaesthetic for further sampling (see following sections) and phenotypic sex was determined according to the method described by Ferrari et al (2014). Four fish with undetermined sex were removed from statistical analyses.

2.3. Behavioural screening

Three groups of 120 individuals were screened at 215 dph (mass 25.91 ± 0.51 g) for hypoxia tolerance (adapted from Laursen et al, 2011; translated to sea bass by Ferrari et al, 2015) in order to assess their coping styles and then placed back in their home tank without modifying the group composition. Briefly, in the hypoxia test, oxygen concentration was decreased in one out of two adjacent chambers of a test tank, and the escape from the hypoxic to the normoxic compartment was recorded. The test apparatus consisted of two identical circular tanks (70 l, h: 48 cm, diameter: 49.5 cm,) attached to each other via a transparent acrylic pipe (diameter: 11 cm, length: 30 cm, height from bottom: 23 cm, see Castanheira et al, (2013) for a detailed diagram of the apparatus). Each tank was considered a separate environment individually equipped with oxygen and air supply that were switched off during the trials in the hypoxia tank (see below). Sixty fish were placed in one chamber of the tank (which subsequently became the hypoxia tank) and were allowed to acclimate to the conditions for 30 minutes before the start of the experiment. The hypoxia tank was supplied with nitrogen, to induce hypoxic conditions during the experiment (nitrogen bubbling decreased oxygen saturation from 90 % to 8 % in 1 hour). The second chamber of the tank, which was supplied with oxygen, is referred to as the normoxia tank. Once an individual escaped from the hypoxic tank into the initially empty normoxia tank, it was immediately netted, then placed in a separate tank before being anesthetized (benzocaine, 200 ppm), tagged with 12 mm ISO PIT tags, measured for mass and replaced into the home rearing tank. Within the coping style continuum, assignment to categories was performed based on Ferrari el al. (2015) previous behavioural and physiological characterisation which demonstrated that hypoxia avoider fish had lower cortisol concentration, higher levels of activity and took more risks, three characteristics of a proactive coping style. Hence it was done as follows: The 20 first fish escaping hypoxic conditions were referred to as proactive (P), the ~20 followers were referred to as intermediate (I) and the last fish that did not escape hypoxic conditions were referred to as reactive (R). The hypoxia test ended when two third of the fish had escaped from the hypoxia tank or when 8% oxygen saturation was reached (water temperature 20°C, salinity 26.9). This operation was repeated until the three triplicates of 120 fish were screened. In total the characterization from each replicate rearing tank yielded: Tank 1: 40 P, 39 I and 40 R (one fish died), Tank 2: 40 P, 61 I and 19 R and finally Tank 3: 40 P, 46 I and 34 R.

2.4. Stress treatment

In total, 276 fish were subsampled at 342 dph (mean mass 89.2 ± 31.8 g) after applying a confinement stress protocol resulting in three treatments: Control, Stress30 and Stress150 (**Figure 1**). Fish were fasted 24 hrs prior to sampling. After decreasing the water level in their home tank, fish were lightly anaesthetised (70 ppm of Benzocaine), netted and transferred in a smaller well aerated holding tank still under light anaesthesia. PIT tags were read, then mass and length were measured and depending on their previously known coping styles and assigned treatment, fish were dispatched in 9 identical tanks (70 l each: Proactive, Intermediate, Reactive x Control, Stress30 and Stress150). Fish thereafter called Control were immediately deeply anaesthetized using 200 ppm of benzocaine and blood samples were obtained from the caudal vein with heparinised syringes within 3 minutes. Thereafter, fish were killed using an overdose of anaesthetic and body kept on ice for further brain dissection. Fish from Stress30 group were placed under confinement (water level at 1/4 in the 70l tanks) during 30 min then allowed to recover (water level back to maximum) during 30 min and were then directly anaesthetized for the same sampling procedure as above. Fish from Stress150 group were submitted to the same stress procedure, but allowed 150 min for recovery and then underwent the same sampling procedure.

2.5. Plasma cortisol

Fish were sampled at 342 dph for circulating cortisol (40 individuals per coping style for the control treatment, 20 individuals per coping styles for Stress30 and Stress150 treatments). The blood was centrifuged (5 min at 1500 g) to obtain plasma samples, which were stored at -22°C for further analyses. Plasma cortisol concentration was determined with an ELISA kit (RE52061, IBL, Germany) following manufacturing instructions.

2.6. Telencephalon monoamine neurochemistry

A subset of 10 individuals per coping styles from Control and Stress30 treatment were selected for analysis of monoamine neurochemistry. Brains were dissected out from each fish in order to extract the telencephalon which was frozen individually in liquid nitrogen and kept at -80°C for later analysis. Before analysis, each frozen telencephalon was individually weighed. After weighing, the brain part was homogenised in a homogenising reagent (4% perchloric acid, 0.2% Ethylene diamine tetraacetic acid, 40 ng ml^{-1} dihydroxi benzylamine hydroxide solution). The solvent was then centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was assayed by High Performance Liquid Chromatography (HPLC) with electrochemical detection to quantify the concentration of 5-HT and its catabolite 5-HIAA, DA and its catabolite DOPAC and norepinephrine NE. The HPLC system consisted of a mobile phase (buffer solution; 10.35 g l^{-1} sodium phosphate, 0.3252 g l^{-1} sodium octyl sulphate, 0.0037 g l^{-1} EDTA, 7% acetonitril in deionised water), a solvent delivery system (Shimadzu, LC-10AD), an auto injector (Famos, Spark), a reverse phase column (4.6 mm 100 mm, Hichrom, C18, 3.5 mm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at -40 mV and +320 mV. A conditioning electrode with a potential of +40 mV is used to oxidize possible contaminants before analysis. Brain 5-HT, 5-HIAA, DA, DOPAC and NE were quantified by comparing them with standard solutions of known concentrations and corrected for recovery of the internal standard using HPLC software (CSW, DataApex Ltd, Czech Republic).

2.7 Brain gene expression patterns

A subset of 20 individuals per coping styles from Control and Stress150 treatment were prepared for whole brain gene expression analysis. Brains were extracted and immediately frozen with liquid nitrogen. Total RNA was extracted from the brain using TriReagent (Molecular Research Center) following the manufacturer's instructions and verified for quantity using a NanoDrop ND-1000 (Thermo Scientific) and quality visualized under UV light in a 1% agarose gel containing 1 µg.ml^{-1} ethidium bromide. 1 µg of total RNA was taken from each individual to synthesize cDNA with SuperScript III RNase Transcriptase (Invitrogen) and oligo-dT primer (Promega). Selected target transcripts were cloned and sequenced. Conventional PCR products were visualized under UV light in a 1.2 % agarose gel containing 1.5 µg/ml ethidium bromide, purified using PCR clean-up Gel extraction MN (Cultek), cloned into pGEM-T Easy Vector (Promega) by T/A cloning and transfected into competent Escherichia coli DH5α strain TM Competent Cells, Invitrogen (Promega). Plasmid DNA was isolated by Nucleospin Quickpure (Marcherey Nagel). All constructs were verified by DNA sequencing (GATC Biotech).

Absolute quantification was performed and the copy number of each transcript, derived from the standard dilution curve obtained from target plasmids was analysed using a Thermocycler Stratagene Mx3005P (Agilent, USA). Each sample was tested in triplicate in a 96-well plate. The reaction mix (20 µl final volume) consisted of 10 µl of SYBR Green mix (Aligent, USA), 0.5 µl of each primer (20 µM), 7 µl of H_2O and 2 µl of a 1/10 dilution of the cDNA sample. The thermocycling program consisted of one hold at 95 °C for 3 min, followed by three-step 35 cycles of 15 s at 95 °C, 10 s at 58 °C and 10 s at 72 °C. No template controls (NTCs) were used to assure no false positive signals were calculated. Thresholds were normalized for all genes with the gene project software.

Identification of Target mRNAs

Taking a comparative evolutionary approach, common mRNA transcripts differentially expressed in zebrafish screened for coping styles (Rey et al 2013) were used to identify target genes in *D. labrax*. The zebrafish specific target transcripts that display coping style specific variance were cloned ’in silico’. Cloning was carried out using genomic resources from all available public libraries. To identify specific European Sea bass targets parallel scale BLAST (Basic Local Alignment Search Tool) was executed using the filtered target set from the zebrafish resource against the species of interest. Curated lists of mRNA transcripts were used for primer design and validated using pools of fish whole brain cDNAs with the objective of individual absolute quantification of gene expression. From this collection, 4 mRNAs for sea bass were chosen for the gene expression study. The genes selected for the sea bass study were sodium/potassium-transporting *atp*ase subunit alpha-3: *atp*aseα3, glyceraldehyde-3-phosphate dehydrogenase: *gapdh*, Interferon-related developmental regulator 1: *ifrd1*, and *nedd8* precursor factor: *nedd8* (see Table 1).

Table1. mRNA target sequence names, primers, amplicon size and database accession number used for European sea bass.

	Primer Sequence	**Amplicon (bp)**	**NCBI seq ID**
ATPase alpha3_For	AGAACATGGTGCCTCAGCAA	146	AY532637.1
ATPase alpha3_Rev	GCCATGAGCAGAAACAACCC		
Dlabrax_IFRD1_For	GTGACACCACCAGTGTAGCA	237	NM_001076555.1|
Dlabrax_IFRD1_Rev	TGCCTTTCTTGAGGCATCGT		
Dlabrax_GAPDH_for	CTGTCCGTCTGGAGAAACCC	210	AY863148.1
Dlabrax_GAPDH_rev	TGTCGTACCATGTGACCAGC		
Dlabrax_NEDD8_for	TTGAGCCCACAGACAAGGTG	148	XM_003457410.2
Dlabrax_NEDD8_rev	ACTGAGCCTCCCTGGATCTT		

2.8. Data analyses

Analyses of growth were carried out using a two factor ANOVA without replication with Sex and Coping style (Proactive, Reactive and Intermediate) as fixed factors after checking for normality and homogeneity of variances. Significant ANOVA outcomes were then followed by post hoc Newman-Keuls test. Plasma cortisol levels and brain monoamine neurochemistry in telencephalon were analyzed using a two factor ANOVA without replication with Treatments (Control, Stress30 (for monoamines), and Stress30 and Stress150 (for cortisol)) and Coping style (Proactive, Intermediate, Reactive) as fixed factors. A Pearson correlation was used to assess links between plasmatic cortisol concentration and ratio of [5-HIAA]/[5-HT]. In order to fulfil the assumption of normal distribution, data on plasma [cortisol], brain [NE], [DA], [DOPAC], [5-HT], [5-HIAA] and ratios of [5-HIAA]/[5-HT] and [DOPAC]/[DA] were log-transformed. For data analysis of gene expression, brain mass and gene copy number were checked with an ANOVA test and a post-hoc Scheffé test was performed for specific significances. A blind analysis on individual gene expression data for the whole subpopulation with a K-means cluster was performed. These analyses were performed with SPSS v19 (IBM®) and Statistica. For correlations between coping styles selected by behavioural screenings and coping styles identified by clustering of individual gene expression, analyses were carried out using a two factor ANOVA without replication with Sex and Coping style as fixed factors after checking for normality and homogeneity of variances. In case of non-normality or non-homogeneity of variances, data were log transformed. Significant ANOVA were then analyzed using post hoc Newman-Keuls test. Other statistical analyses were performed with Statistica for windows (Statsoft, USA).

3. Results

3.1. Growth performances

Coping styles were equally distributed between sexes (Chi^2=1.25, Df=6, p=0.97) and females sea bass were bigger than males all along the experiment duration (Table 2).

Already at the beginning of the experiment (just after the behavioural screening), fish body mass (BW) were different between coping styles (at 215 dph; Table 2, Figure 2). Post hoc NK test showed that reactive fish had lower BW than intermediate and proactive fish ($p<0.05$, Figure 2). At the second measuring point, differences in body mass were still observed between coping styles (at 251 dph, Table 2) and post hoc test showed that reactive and proactive fish were similar and reactive fish had lower BW than intermediate fish ($p<0.05$, Figure 2). At the third measuring point, five weeks later, differences in body mass had developed (at 285dph, Table 2, Figure2) and post hoc test showed the same situation ($p<0.01$, Figure 2). At the fourth measuring point, body mass were still different between coping styles (at 314dph, Table 2, Figure 2) and post hoc test showed that reactive fish had lower BW than intermediate and proactive fish (p<0.05 or 0.01, Figure 2). At the final sampling point, the situation was the same (at 342dph, Table 2, Figure 2), post hoc test showed that reactive fish had lower BW than intermediate and proactive fish (p<0.01 and p<0.05 respectively, Figure 2).

Concerning specific growth rate, no Sex or Coping styles effects were observed on SGR.

Table 2. Results of a two factor ANOVA without replication of Body mass (BW) and Specific Growth Rate (SGR) in relation to Sex and Coping style. NS: non significant.

	Sex		*Coping style*	
Fish age (dph)	*f*	*p*	*f*	*p*
BW_215	48.45	**<0.001**	3.57	**<0.05**
BW_251	44.90	**<0.001**	3.71	**<0.05**
BW_285	36.30	**<0.001**	5.42	**<0.05**
BW_314	39.26	**<0.001**	5.47	**<0.05**
BW_342	33.24	**<0.001**	5.21	**<0.05**
SGR_215_251	NS		NS	
SGR_251_285	NS		NS	
SGR_285_314	NS		NS	
SGR_314_342	NS		NS	

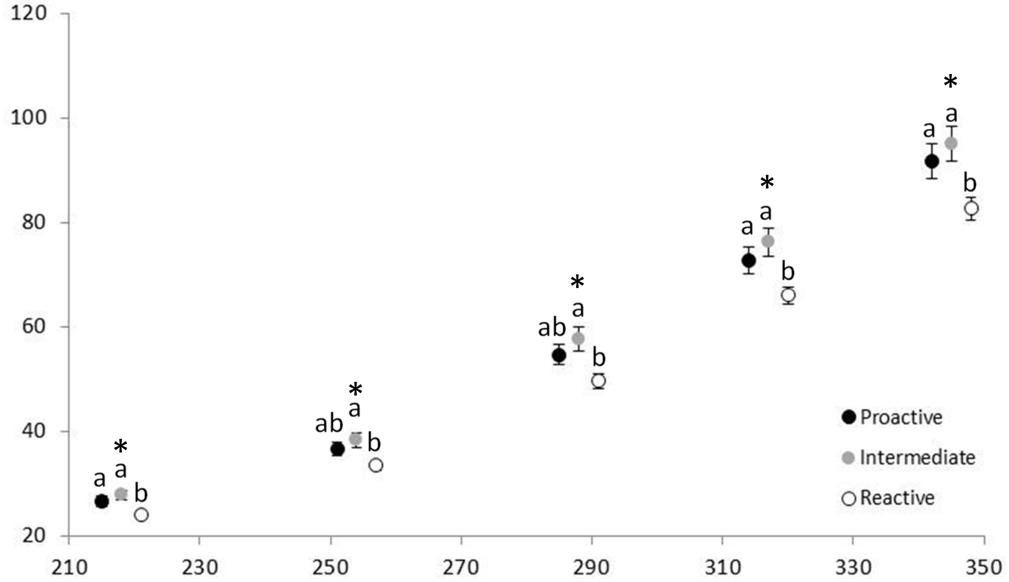


Figure 2: Fish body mass according to age (dph) and coping style (mean ± SEM). * indicate significant differences between coping styles and letters indicate Newman-Keuls post hoc test results.

3.2. Physiological status

3.2.1. Plasma cortisol

No significant sex effect was observed on plasma cortisol concentration for Control, Stress30 and Stress150 groups tested separately ($F_{(1,113)}=2.59$, $p=0.11$; $F_{(1,55)}=0.16$, $p=0.69$ and $F_{(1,55)}= 0.41$, $p=0.52$ respectively). No correlations were observed between cortisol values and fish BW.

Cortisol data were then analysed using Treatment (Control, Stress30 and Stress150, Figure 3) and Coping styles (Proactive, Reactive and Intermediate) as fixed factors. No effect of treatment or coping style alone was seen, but a significant interaction between treatment and coping style was present ($F_{(4,229)}=2.55$, $p<0.05$). Post hoc test showed that Stress150-Proactive and Stress150-Intermediate fish had significantly lower plasmatic cortisol concentrations than Stress150-Reactive fish (Figure 3). However, plasma cortisol concentrations were not different for any coping style at baseline and Stress30 sampling points. The lowest values were observed for Stress150-Proactive fish (561.93 ± 42.80 ng ml^{-1}) whereas the highest values were observed for Stress30-Reactive fish (668.16 ± 33.95 ng ml^{-1}).

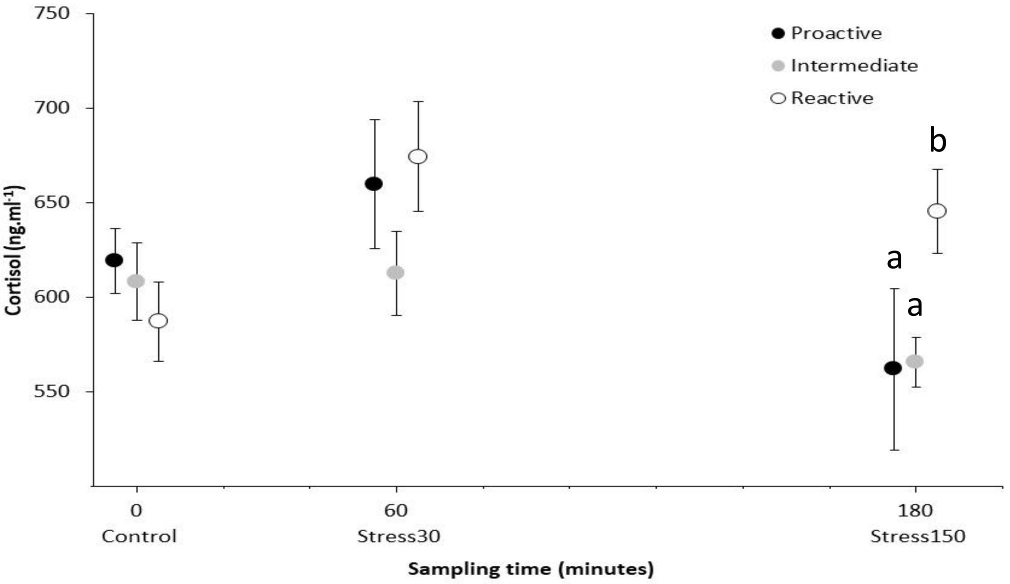


Figure 3: Plasma cortisol concentration according to treatment (sampling time post confinement stress) and coping style (mean ± SEM). Letters indicate significant differences within treatment.

3.2.2. Monoamine neurochemistry

Data on monoamine neurochemistry in the telencephalon are shown in table 3. A significant interaction was observed between coping style and treatment on the ratio [5-HIAA/5HT] ($F_{(2,49)}$=4.83, p=0.01, Figure 4), specifically in that intermediate fish showed lower serotonergic turnover than both proactive and reactive categories under control (unstressed) conditions. 5-HIAA/5-HT ratios increased in response to acute stress (sampling time: “Stress30”) in all groups, and at this time point there were no longer any significant effect of coping style on this indicator. The stress-induced increase in 5-HIAA/5-HT ratios could be ascribed to a rise in catabolite (5-HIAA) concentrations in all groups, with a near significant effect of coping style (i.e. reactive fish showed a trend [p=0.06] towards generally higher 5-HIAA concentrations, see table 3 for full summary of statistics and effects).

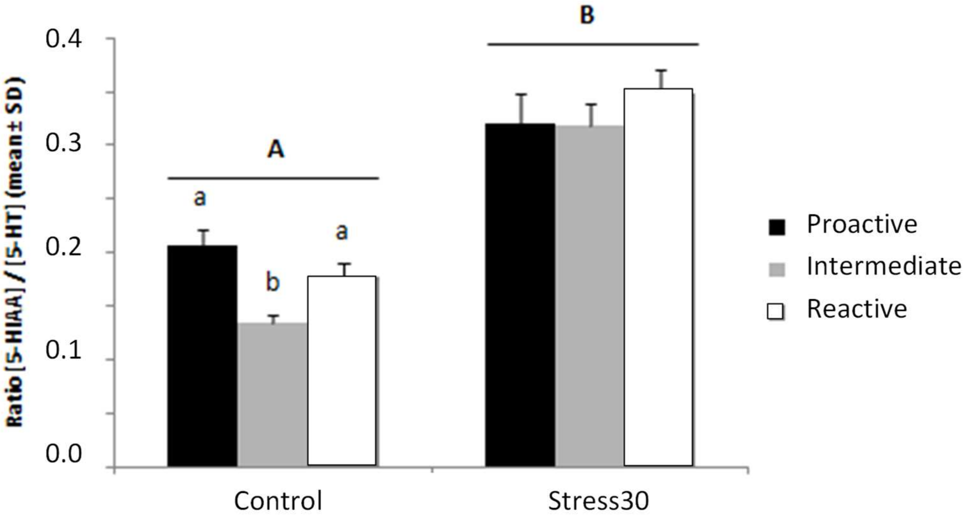
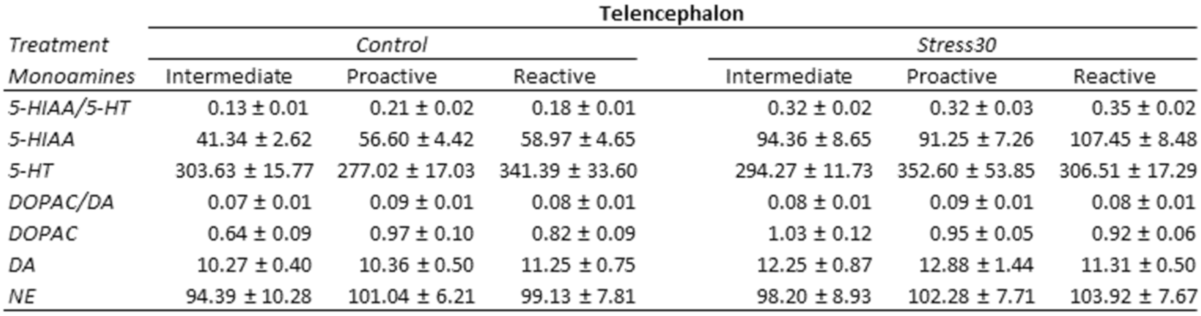


Figure 4: Mean value of the ratio between the concentrations of [5-HIAA/5-HT] in telencephalon according to treatment and coping style (mean ± SEM). Lower case letters indicate significant differences within treatment and upper case letter indicate significant differences between treatments.

For the DA system, a stress-induced increase in the DA catabolite DOPAC was accompanied by a corresponding increase in concentrations of the parent monoamine, leaving DOPAC/DA ratios unaffected by stress. Norepinephrine [NE] concentrations remained unaffected by stress, and NE metabolites were undetectable in the current analytical set-up. None of the catecholamine indicators were affected by coping style or an interaction between coping style and stress. (Table 3).

Table 3. Neurochemistry in the telencephalon according to treatment and coping style (mean ±SEM).



	Telencephalon					
Treatment	*Control*			*Stress30*		
Monoamines	Intermediate	Proactive	Reactive	Intermediate	Proactive	Reactive
5-HIAA/5-HT	0.13 ± 0.01	0.21 ± 0.02	0.18 ± 0.01	0.32 ± 0.02	0.32 ± 0.03	0.35 ± 0.02
5-HIAA	41.34 ± 2.62	56.60 ± 4.42	58.97 ± 4.65	94.36 ± 8.65	91.25 ± 7.26	107.45 ± 8.48
5-HT	303.63 ± 15.77	277.02 ± 17.03	341.39 ± 33.60	294.27 ± 11.73	352.60 ± 53.85	306.51 ± 17.29
DOPAC/DA	0.07 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
DOPAC	0.64 ± 0.09	0.97 ± 0.10	0.82 ± 0.09	1.03 ± 0.12	0.95 ± 0.05	0.92 ± 0.06
DA	10.27 ± 0.40	10.36 ± 0.50	11.25 ± 0.75	12.25 ± 0.87	12.88 ± 1.44	11.31 ± 0.50
NE	94.39 ± 10.28	101.04 ± 6.21	99.13 ± 7.81	98.20 ± 8.93	102.28 ± 7.71	103.92 ± 7.67

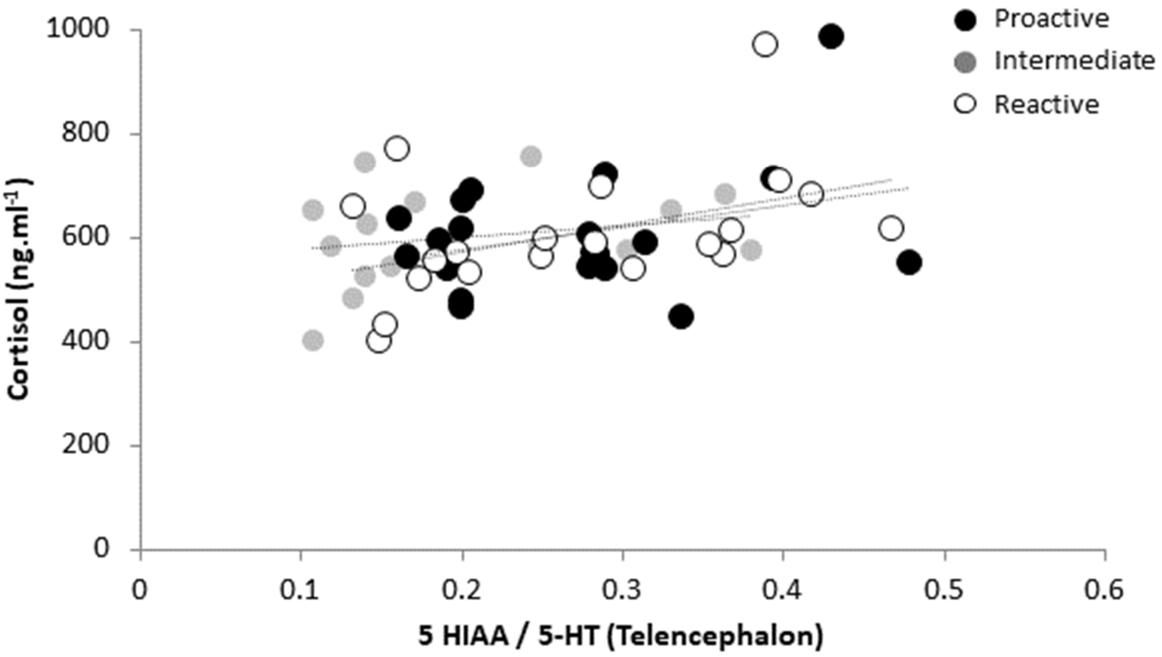


Figure 5: Pearson correlation between plasmatic cortisol concentration and ratio of [5-HIAA / 5-HT] (N=54, r_p=0.34, p<0.01), different colours indicate different coping style.

A significant positive correlation (N=54, r_p=0.34, p<0.01) was observed between plasmatic cortisol concentration and ratio of [5-HIAA / 5-HT], (Figure 5), the correlation did not differ between coping style.

3.2.3. Gene expression

Stress150, 150 min post confinement, had a significant effect on target mRNA copy number that was in several cases dependent upon coping style (ANCOVA, $F_{4,96}=30.574$; $p<0.001$). *Gapdh* mRNA copy number decreased after the stress treatment (Post-hoc, $p<0.001$) whereas *ifrd1* transcripts increased (Post-hoc, $p<0.01$). No statistical differences were observed for the other two mRNAs studied however there were observable tendencies for measured mRNA abundances dependent upon coping styles with the intermediate fish being the most different in gene expression (see Figure 6a, b, c and d). No significant interactions between coping style and treatment were observed.

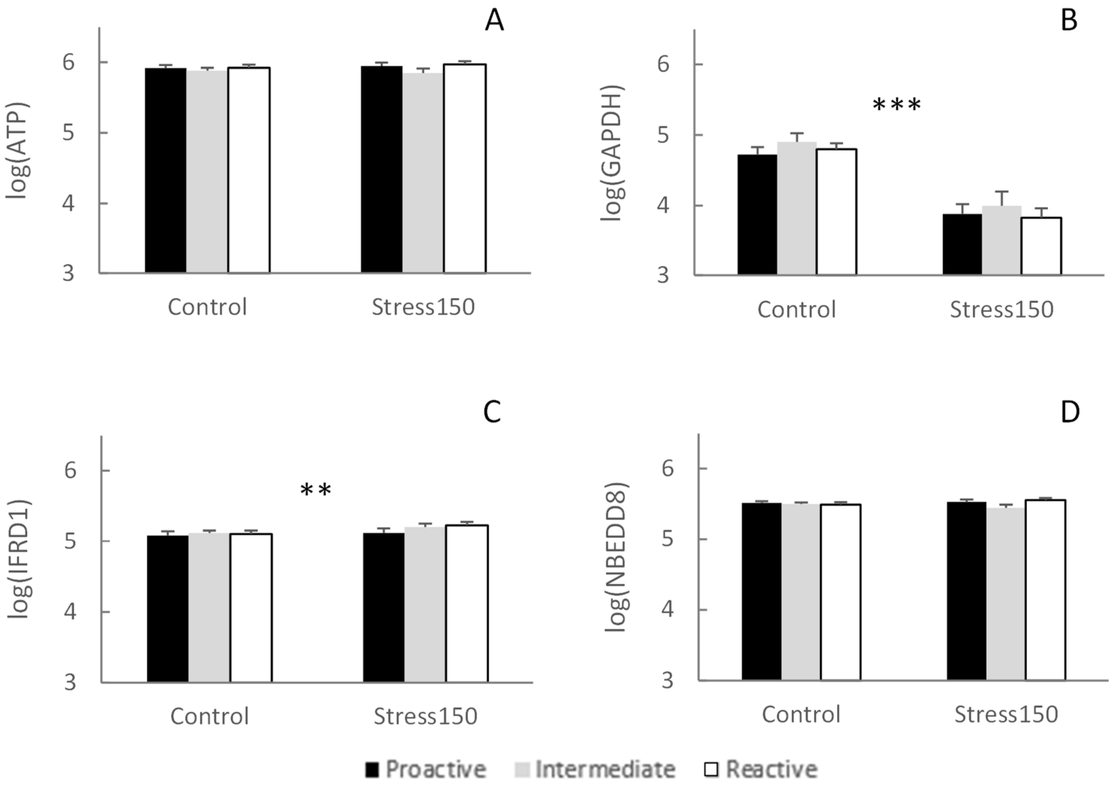


Figure 6: Differential mRNA expression transcripts for fish with different coping style under control conditions and after a confinement stress treatment (Stress150). **A.** Log copy number for *atpaseα3*, **B.** Log copy number for *gapdh*, **C.** Log copy number for ifrd1 and **D**. Log copy number for *nedd8* gene.

4. Discussion

Sea bass are one of the highest commercial value species for European aquaculture, with a current mean European production of about 125,000 metric tons year^{-1} (Tveteras and Nystoyl, 2011).

In addition, sea bass domestication is still in its infancy and studying physiological and behavioural responses of fish from divergent coping style should allow improving domestication process and selecting fish with higher adaptation abilities to rearing conditions. Indeed European sea bass stress coping styles have been characterized and resemble most fish species studied (Ferrari et al 2015, 2016; Samaras et al 2016a; Alfonso et al 2019) and when held in groups and fed to satiation, the species does not display aggressive social hierarchy but is rather showing a producers-scroungers social organization instead of a hierarchical one (Di Poï et al 2007, Ferrari et al 2014). Further, the high stress responsiveness of this species (Samaras et al 2016b) makes it a good marine teleost model to study the dynamics of cortisol signalling. Here a medium term experiment (140 days) was performed and specifically targeted neural and transcriptional activity, behaviour and cortisol levels to evaluate stress recovery, a rarely investigated interaction (Wong et al 2019).

Differentially expressed traits were observed regarding growth with intermediate fish growing larger and showing a lower baseline serotonergic activity. Confinement stress induced an immediate and higher serotonergic activity whatever the coping style and a slower cortisol recovery rate in reactive fish. Finally mRNA copy numbers for some genes associated to metabolism were also differentially affected.

In further details, all along the experiment duration, reactive fish had lower body mass than intermediate fish which were the larger fish but most often not significantly different from proactive ones. This is an interesting result which echoes the findings of Millot (2008) who observed, when comparing selected *versus* wild strain that proactive sea bass from wild population had lower body mass than reactive ones. On the opposite, proactive sea bass issued from selected for growth strain had higher body mass than reactive ones. In addition, Ferrari et al (2016) observed that reactive fish from an unselected sea bass population (close to wild fish) had higher body mass than proactive ones. Overall, this divergent growth potential leads to think that hatchery selection and/or domestication process and/or husbandry practices promote growth of proactive coping style, as already observed in salmonids (Sundström et al, 2004; Huntingford and Adams, 2005, Damsgård et al 2019). Further, the high growth observed in intermediate fish has already been observed in emerging rainbow trout fry where early and late emerging individuals grew less than intermediate emerging individuals (Andersson et al 2013). The authors hypothesized that intermediate emerging individuals had a 'stress coping style lying between the proactive and reactive extremes in the pro-reactive continuum', our results corroborate their findings. Further, intermediate fish showed both enhanced growth and reduced resting 5-HT activation under basal conditions. In a previous study, Cubitt et al (2008) showed that slower growing salmon in aquaculture were characterised by enhanced 5-HT neurotransmission, attributing this observation to the presence of a size hierarchy even in relatively large groups of fish. Our contrasting results highlight the fact that interactions between coping style, body size, and social status still little explored outside the salmonids family should be investigated further. The present results, in summary, suggest that intermediate fish with less pronounced coping styles were best adapted to current rearing conditions.

No difference in circulating cortisol levels were observed between the different coping styles fish in the Control and Stress30 treatments. This, on one hand, confirms the high susceptibility to stress of this species (Samaras et al 2016b), and demonstrates that a different protocol such as water cortisol (Fanouraki et al 2008) should be favoured to analyse basal cortisol since any manipulation of the fish leads to an immediate increase of plasmatic cortisol concentration. On the other hand, a similar result has recently been observed in rainbow trout where coping style divergent fish had similar cortisol levels both at basal levels and after an acute confinement stress when a confinement stress-challenge test was performed several weeks after the coping style characterization (Gesto 2019). As mentioned by Gesto (2019), the known high phenotypical plasticity of fish could also have affected the individual differences during that time. Nonetheless, what pleads for a correct coping style characterization is that after a recovery period (Stress150 treatment, 150 minutes recovery), proactive and intermediate fish had a lower cortisol level than reactive ones. This shows that these individuals were able to recover faster from the stress than reactive ones, highlighting that proactive and reactive sea bass display differential hypothalamus-pitutary-interrenal (HPI) axis reactivity as already observed in rainbow trout (Øverli et al 2005). In other word, proactive and intermediate as opposed to reactive fish have higher capacity to downregulate HPI activity, in line with data from other species such as zebrafish (Tudorache et al, 2013, 2015, Wong et al 2019). Such involved mechanisms, leading to a faster HPI axis down regulation of proactive individuals, may be an evolutionary adaptive process for the proactive individuals to be faster prepared to unpredictable stress since proactive behavioural responses are known to be maladaptive under repeated, uncontrollable or unpredictable stress (Øverli et al 2007). From an operational perspective, the hypoxia tolerance test is thus relevant to assess coping style in sea bass and predict cortisol response after an acute stress (herein a 30 min confinement stress). In our study, fish were screened for coping style 127 days before stress protocol was applied and blood sampling performed, and physiological differences between coping styles were still observed at least for plasmatic cortisol. This underlines that the cortisol response is relatively stable over time (Samaras et al, 2016b), contrary to some personality traits which could be shaped by environmental factors, age or experience (Ferrari et al, 2015, 2016; Alfonso et al 2019).

In the present experiment, confinement stress lead to an increase in telencephalic 5-HIAA concentrations and 5-HIAA/5-HT ratios, indicating a general activation of 5-HT neurotransmission in this brain part similar for all behavioural coping styles. Elevated brain serotonergic activity is a general indicator of aversive experiences in all vertebrates and has in fishes been shown to occur after for instance social stress (Øverli et al 1999), predator exposure (Winberg et al 1993b), confinement stress (Øverli et al 2001), salmon louse infestation (Øverli et al 2014) and suboptimal rearing conditions (Laursen et al 2013). Therefore, in all likelihood the telencephalon response reflects a general increase in 5-HT activity throughout the brain at Stress30 following the onset of stress. Here, we however focus on the telencephalon which also in fish contains limbic systems assumed to mediate hippocampal and amygdala like functions (Portavella et al 1998, 2002; Demski 2013; Maximino et al 2013; Silva et al 2015). Altered brain 5-HT dynamics in these areas may influence animal welfare through its role in mood control and emotion (Cools et al. 2008; Dayan and Huys 2009; Carhart-Harris and Nutt 2017), neurogenesis, and neural plasticity (Gould 1999; Mahar et al 2014, Castrén et al 2017). In particular, the ability to respond to further acute stressors is an essential indicator of compromised animal welfare (Vindas et al 2016). Therefore, measurements of immediate responsiveness of the 5-HT system are indicative to reveal any effect of contrasting coping ability on animal welfare. These differences did however not translate into an altered ability to respond to further acute stress, i.e. indicative of allostatic overload (Vindas et al 2016). All groups showed significant cortisol, serotonergic and dopaminergic responses 30 min post-stress. No significant effect of coping style was observed after stress, although there was a trend towards reactive fish showing a slightly enhanced response in terms of elevated 5-HIAA concentrations. This differs from the responses observed in shy sea bass after an open field test (Alfonso et al. 2019) and might reveals some context specificities. Relevance of the sampling protocol is illustrated by significant correlation in all groups between telencephalon 5-HIAA/5-HT and cortisol, suggesting co-ordinated activation of these neuroendocrine systems under stress.

The dopaminergic system was not differentially activated across coping style as also observed in European sea bass in another context by Alfonso et al (2019). Interestingly, regarding the DA system, it should be noted that a stress induced increase in DOPAC did not result in elevated DOPAC/DA ratios, due to a compensatory simultaneous increase in concentrations of the parent monoamine DA. This again suggests robust coping ability at least under acute stress in our tested fish, and also illustrates the importance of observing both relative and absolute amounts of analytes in neurochemical studies.

The genes selected for this study were functionally related to different physiological processes: *atp*ase α3 is related to osmotic regulation as major mediator of cellular transmembrane ionic gradients. It also plays an important role in signal transduction in the nervous system. It has been found necessary for brain ventricle formation and development in early brain morphogenesis (brain lumen inflation). *Ifrd1* is related to development and alternate splicing results in multiple transcript variants. *Gapdh* is related to general metabolic processes. *Need8* is a neural precursor for Ubiquitin-like protein, which plays an important role in cell cycle control and embryogenesis.

The stress treatment had a significant effect on target mRNA copy number with different reaction norms for each mRNA and that were in several cases dependent on coping style. This was mainly due to the differential gene expression of *gapdh* and *ifrd1*; *gapdh* mRNA copy number decreased for all three coping styles in the same way and similar magnitude after the stress treatment showing an effect of stress over the expression of this metabolic gene whereas *ifrd1* transcripts increased. *Ifrd1* is an immediate early gene that encodes a protein related to interferon-gamma. This protein may function as a transcriptional co-activator/repressor that controls the growth and differentiation of specific cell types during embryonic development and tissue regeneration. Mutations in this gene are associated with sensory/motor neuropathy with ataxia. The general increase of *Ifrd1* transcripts for all coping styles could be related to their role in the immune system (Langevin et al 2013) with an immediate response. *Ifrd1* has also been identified in Cyprinids as being able to discern between proactive and reactive stress coping styles (Mackenzie et al 2009) under stress situations. In sea bass there were differences between coping styles but not significantly different in any case. The tendency was for the proactive fish to have higher numbers of mRNA transcripts than the reactive both under control and stress conditions. For the other genes studied, both *atp*aseα3 and *nedd8* are quite ubiquitous genes and may play specific roles in cell control but maybe in this case they had not enough time (sampling after 150 min post stress) to be fully differentially expressed or they just are not so affected by this specific confinement stress applied. However, for *atp*aseα3 a tendency was observed to increase the number of transcripts after the stress situation for Proactive and Reactive fish, and to decrease for intermediate fish. The same response was observed between intermediate and reactive fish for *nedd8.*

Conclusions

In the present study as well as in previous ones, we have shown that different coping styles are associated with different growth potential closely linked with the domestication level, husbandry conditions and likely the social context; here intermediate and proactive coping styles appeared favoured. In previous work it was shown that European sea bass extreme coping style (reactive vs. proactive) displayed associated differences in the HPI axis, the serotonergic and noradrenergic system reactivity, and in neurogenesis at one time point immediately post challenge (Alfonso et al 2019). The present work highlight the importance of investigating not only the immediate neuroendocrine components responses of coping styles, especially in such a highly stress responsive species, but the post stress recovery phase which is even more discriminant as for the stress axis reactivity and the metabolic transcriptional activity. This implies allowing sufficient time to test the ability to both respond and down regulate and hence tackle the allostatic reaction norm of the species in any particular and well defined context.

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