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Research paper

The brain-gut axis of fish: Rainbow trout with low and high cortisol response show innate differences in intestinal integrity and brain gene expression

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ABSTRACT

In fish, the stress hormone cortisol is released through the action of the hypothalamic pituitary interrenal axis (HPI-axis). The reactivity of this axis differs between individuals and previous studies have linked this to different behavioural characteristics and stress coping styles. In the current study, low and high responding (LR and HR) rainbow trout in terms of cortisol release during stress were identified, using a repeated confinements stress test. The expression of stress related genes in the forebrain and the integrity of the stress sensitive primary barrier of the intestine was examined. The HR trout displayed higher expression levels of mineralocorticoid and serotonergic receptors and serotonergic re-uptake pumps in the telencephalon during both basal and stressed conditions. This confirms that HPI-axis reactivity is linked also to other neuronal behavioural modulators, as both the serotonergic and the corticoid system in the telencephalon are involved in behavioural reactivity and cognitive processes. Involvement of the HPI-axis in the brain-gut-axis was also found. LR trout displayed a lower integrity in the primary barrier of the intestine during basal conditions compared to the HR trout. However, following stress exposure, LR trout showed an unexpected increase in intestinal integrity whereas the HR trout instead suffered a reduction. This could make the LR individuals more susceptible to pathogens during basal conditions where instead HR individuals would be more vulnerable during stressed conditions. We hypothesize that these barrier differences are caused by regulation/effects on tight junction proteins possibly controlled by secondary effects of cortisol on the intestinal immune barrier or differences in parasympathetic reactivity.

1. Introduction

In vertebrates, the primary physiological stress response involves the release of stress hormones, such as catecholamines and corticosteroids (Axelrod and Reisine, 1984). In fish, the amount of corticosteroids released during stress depends on the reactivity of the hypothalamic pituitary interrenal axis (HPI-axis). Low and high responding (LR and HR) individuals, regarding plasma corticosteroid release during stress have been identified in a wide range of vertebrates (Koolhaas et al., 1999). In rainbow trout (*Oncorhynchus mykiss*), this has been shown to be a stable and heritable trait and it has been coupled to the concept of stress coping styles (Schjolden et al., 2005; Koolhaas et al., 1999; Pottinger and Carrick, 1999). HR individuals are often described to have a reactive stress coping style, showing low aggression and subordination and to respond to stress with a behavioural "conservational/withdrawal response", whereas LR individuals often are considered more proactive, showing high aggression and dominance and to respond to stress with a "fight or flight" behaviour (Schjolden et al., 2005; Koolhaas et al., 2010, 1999; Øverli et al., 2005).

Cortisol is the main corticosteroid in teleosts and it acts through two types of corticoid receptors, the glucocorticoid receptors (GR)

Abbreviations: P_{app}, Apparent permeability; CNS, Central nervous system; CV, Coefficients of variation; GR, Glucocortocoidreceptor; HR, High responding; HPI-axis, Hypothalamic-pituitary-interrenal axis; LR, Low responding; MR, Mineralcorticoid receptors; Oprd, Opioid receptor Delta; PITtags, Passive integrated transponders; Sert, Serotonin transporter; SCC, Short circuit current; TEP, Transepithelial potential; TER, Transepithelial resistance; Tph, Tryptophan hydroxylase; 5HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine.

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and the mineralocorticoid receptors (MR), which are abundantly expressed in most tissues, including all major parts of the salmonid brain (Barton 2002; Barton and Iwama, 1991; Bury and Sturm, 2007; Johansen et al., 2011). Within the central nervous system (CNS), cortisol is known to act via both MR and GR and to be involved in processes, such as memory retention, behavioural reactivity and further modulation of the stress response (Oitz et al., 1997; Schjolden et al., 2009; Sørensen et al., 2013). Another component within the CNS suggested to be interlinked with the HPI-axis is the serotonergic system (Chaouloff 2000; Schjolden and Winberg, 2007; Winberg et al., 1997; Winberg and Nilsson 1993; Øverli et al., 2001). Stress commonly leads to an increased activity in the serotonin (5-HT)-system, which has been shown to play a role in stress coping in both mammals and fish, modulating locomotion, temperament and aggression (Koolhaas et al., 2010; Øverli et al., 1999, 2004b, 2005). Previous studies on HPI-axis reactivity and stress coping have mainly focused on the concentrations and ratios of 5-HT and its metabolite 5HIAA. However, the investigation of other serotonergic components such as the level of tryptophan hydroxylase enzymes (Tph) involved in 5-HT synthesis, or the amount of 5-HT-receptors and 5-HT re-uptake pumps are of importance to gain a more comprehensive picture of the involvement of the serotonergic system (Andersson and Höglund, 2012; Lillesaar 2011; Schjolden et al., 2006b; Øverli et al., 2005).

In mammals, the term brain-gut axis is a concept used to describe the bi-directional communication between the central nervous system and intestinal function (Aziz and Thompson, 1998). In both mammals and fish it has been shown that acute and long term stress can affect the integrity of the intestinal epithelium, leading to increased pathogen entry and infection rate (Fridell et al., 2007; Olsen et al., 2005; Sundh et al., 2010; Velin et al., 2004). Cortisol has several both central and systemic effects that are adaptive and mainly positive, however if the stressor is severe or chronic, cortisol can cause deleterious secondary and tertiary effects and it has been suggested as one possible mediator of the stress-induced increases in intestinal permeability (Fridell et al., 2007; Pickering and Pottinger, 1989; Spitz et al., 1996; Sundh et al., 2010; Söderholm and Perdue, 2001). This raises the question whether the intestinal barrier function and its sensitivity to stress differs between LR and HR individuals.

The aim of the current study is to investigate inherent traits in brain signaling pathways and intestinal barrier function, linked to diverging stress reactivity and how/if these traits are affected by stress. We hypothesize that the LR and HR individuals differ in their neuronal arrangement in the telencephalon area of the CNS. Furthermore, we hypothesize that the higher HPI-axis reactivity among HR fish make them more susceptible to stress-induced decreases in intestinal barrier function. Information on the neurobiology and intestinal barrier function of individuals with contrasting stress reactivity can help us understand individual variation in stress and disease resistance.

2. Materials & methods

2.1. Experimental animals

169 under-yearling rainbow trout (*Oncorhynchus mykiss*) of mixed sex (Weight; 106.7 ± 1.6 g Length; 21.3 ± 0.9 cm) was obtained from a commercial fish farm (Vänneåns fiskodling, Sweden) and transported to the experimental animal facility at The Department of Biological and Environmental Sciences at The University of Gothenburg, Sweden. The fish were randomly divided between three identical, well aerated, circular tanks (500 L, 56 fish/tank) with re-circulating fresh water held at 10–11 °C. The fish were acclimated to lab conditions for 18 days before the experiments were initiated (2014-09-15). The light were set to a 12:12 h photoperiod with an automated 30 min dawn and dusk at 07:00 and 19:00, respectively. The fish were fed commercial pellets (EWOS ST40), three times a week. Animals were cared for in accordance with the "Guide for the Care and Use of Laboratory Animals" (1996), and the experiments were approved by the regional ethical committees on animal experiments under license no. 85-2012 and 343-2012.

2.2. Identification of high and low responding individuals

Two experimental groups with contrasting HPI-axis reactivity (low and high responding) were created by sorting the fish on the basis of plasma cortisol excretion following a standardized confinement stress tests performed twice with three weeks interval (Pottinger et al., 1992; Øverli et al., 2004a). The fish were randomly netted from the holding tank and placed in individual opaque confinement boxes (Height; 6 cm, Width 8 cm, Length ~25 cm; the length of the box was individually fitted to correspond to the fish length plus ~2 cm. The set-up contained 6 boxes, all supplied with flow-through, well-aerated water from the main system. After 30 min confinement, the fish were placed in anesthesia, (metomidate, 6 mg L^{-1}) and body weight and fork length was noted. Blood samples (0.2 mL) were taken from the caudal vein using a heparinized syringe, and centrifuged at 7000 rpm for 5 min), the plasma was stored in −80 C° until analyses. Following the first confinement trial, all fish were individually marked using passive integrated transponders, PIT-tags (Biomark, Inc., Meridian, Idaho, USA) that were manually inserted into the body cavity through a small (<3 mm) incision in the body wall, made using a scalpel.

The confinement trials were performed on all 169 fish (confinement 1) and then repeated three weeks later (confinement 2) and completed over three days on both occasions. Fish were ranked in order of plasma cortisol levels, using the individual mean value from the two confinement trials, using a maximum standard deviation of 30 ng mL^{-1} between the two trials to maintain their ranking. The 31 highest ranked fish (stdev <30 ng mL^{-1}) and the 32 lowest ranked fish (stdev <20 ng mL^{-1}) were assigned to the high responding (HR) and the low responding (LR) group, respectively. The intermediate group (106 fish) was discarded. The HR and LR fish were placed in mixed groups in two identical tanks (~31 fish/-tank), tank design is described in Section 2.1.

2.3. Stress exposure

The main objective of the current study is to examine different inherent traits and how these respond to stress in LR and HR individuals. In order to comprise with animal welfare recommendations according to 3R (refine, reduce, replace), two main objectives were examined on the same fish using a combined experimental protocol. The first objective involved an elaborate handling protocol comprised of the combination of respirometry measurements, chasing stress and recordings of behavioural data (open field trials), followed by a final sampling of blood, brain tissue and intestinal preparations.

The effects of the handling protocol on primary stress responses, mRNA brain gene expression levels and intestinal barrier functions are presented in this study, whereas data collected during the handling protocol are prepared for publication in a separate manuscript.

The handling protocol consisted in detail of the following steps. The fish were netted from the holding tanks and individually placed into 4 cylindrical respirometry chambers (3 L), isolated from outer disturbance. After 23 h they were individually transferred to a flow-through well aerated 75 L circular tank (diameter 65 cm) and subjected to a 5 min chasing stress protocol, performed

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by hand (wearing a rubber glove), during which the fish were not allowed to recover or rest (Norin and Clark, 2016). The fish were then immediately placed back into the respirometers for measurements of maximum metabolic rate. After 30 minutes, the fish were transferred from the respirometers into 4 individual open field arenas (45 × 68 cm, water level: 15 cm), supplied with aerated flow-through water from the main system. The fish were left undisturbed and monitored by overhead video recording for 3.5 h and thereafter sampled as described in Sections 2.4–2.7. These trials were performed between 2014-10-29 and 2014-11-27.

2.4. End sampling for the stress-exposed and basal group

The fish forming the basal group (n_{HR} = 15, n_{LR} = 15) were sampled straight from the holding tanks, while the fish forming the stress exposed group (n_{HR} = 15, n_{LR} = 16) were netted straight from the open field arenas. The fish were immediately euthanized by an overdose of anesthesia, metomidate (12 mg L^{-1}). Extra care was taken not to disturb the fish before sampling and blood samples were taken within 1 minute and thus before any expected rise in cortisol due to handling (Gamperl et al., 1994).

Fork length, body weight and PIT tag identification number were noted and blood samples treated as described in Section 2.2. Whole brains was dissected out and placed in RNAlater. The brains were fixated at 4 °C for 24 h and then stored at −20 °C, until further processing. The intestine was dissected out from the body using blunt dissection, cleared from mesenteric fat, cut opened longitudinally and separated into its proximal and distal parts and placed in ice-cold ringer solution (140 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM $MgSO_4 \cdot 7\ H_2O$, 15 mM $NaHCO_3$, 1.0 mM KH_2PO_4, 0.5 mM L-proline, 0.5 mM L-lysine, 20 mM glutamine, 10 mM glucose, 5.0 mM Hepes). One HR individual (basal) and one LR individual (stress) were excluded from all further analysis since they were anemic, showed low condition factor and high level of fin damage.

2.5. Plasma cortisol measurements

Plasma cortisol concentration was measured using radioimmunoassay (Young, 1986) modified by Sundh et al. (2011) using sheep anti-cortisol antibodies (Code: S020; Lot: 1014–180182; Guildhay Ltd., Guildford, Surrey, UK). Tritiated hydrocortisone-[1,2,6,7-^{3}H(N)] (NET 396, NEN Life Sciences Products, Inc., Boston, Massachusetts, USA) was used as tracer and radioactivity was measured in a β–counter (Wallac 1409 Liquid Scintillation Counter, Turku, Finland). Non-radiolabeled cortisol standards were prepared from hydrocortisone (Sigma-Aldrich, St. Louis, Missouri, USA). The detection limit of the assay was 0.7 ng mL^{-1} and intra- and inter-assay coefficients of variation (CV) for cortisol assays in our lab have previously been assessed to be 3.9% and 5.4%, respectively (Sundh et al., 2011).

2.6. Intestinal barrier function

The intestinal barrier function was examined *in vitro* using an Ussing chamber method (Sundell et al., 2003; Sundell and Sundh 2012). This methodology assesses the integrity of the intestinal epithelia using electrophysiological parameters as well as diffusion and transport rates of specific marker molecules. The transepithelial electrical resistance (TER) estimates the paracellular permeability of charged molecules, while the apparent permeability (P_{app}) of the hydrophilic marker molecule mannitol estimates the paracellular permeability of un-charged, water soluble and inert substances. Nutrient transport rate was assessed in the anterior intestine using an exemplifying amino acid, L-lysine.

In short, the intestine was dissected out, cut open longitudinally and separated into its anterior and posterior parts. The serosa was peeled off using fine forceps and thereafter each segment (0.75 cm^2) was mounted between two half chambers, representing the mucosal (luminal) and the serosal (blood) side. In a few cases the intestinal preparations was damaged during mounting and these were excluded from final analyses. After mounting, 4 ml of oxygenated ringer solution (11 °C) was added to each half- chamber. The preparations were allowed a stabilization period of 60 min, after which the Ringer solution was renewed on both sides and radioactive labelled mannitol (^{14}C-mannitol, 56.5 Ci $mmol^{-1}$, 3.7 MBq mL^{-1}) and L-lysine (^{3}H-Lysine, 91.6 Ci $mmol^{-1}$, 37 MBq mL^{-1}), (NEN/Amersham, St. Luis, USA) was added to the mucosal side. Sequential samples were taken from the serosal side (50 μL) at t = 0, 20, 25, 30, 55, 80, 85 and 90 min (replacing removed volume with fresh Ringer solution). The samples were put into vials, adding 4.5 mL of liquid scintillation fluid (ULTIMA GOLD™, PerkinElmer, Inc, USA), and radioactivity was measured in a β–counter (Wallac 1409 Liquid Scintillation Counter, Turku, Finland).

The diffusion rate of ^{14}C-mannitol, P_{app} (cm s^{-1}) and the transfer rate of L-Lysine (nmol min^{-1} cm^{-2}) was calculated according to Vidakovic et al (2016). Individual TER values were calculated from the mean value over the last 30 min of the total measuring period (60–90 min).

The transepithelial potential (TEP), and short circuit current (SCC) were measured every 5 min throughout the whole experiment, 60 min of stabilization followed by 90 min experimental period, to validate the viability of the intestinal preparations.

2.7. Analysis of mRNA brain gene expression using quantitative PCR (qPCR)

Eleven target genes were selected for the mRNA expression studies, 4 corticoid receptor genes (MRA, MRB, GR1, GR2), six serotonergic genes (two 5-HT producing enzymes: Tph1, Tph2; two 5-HT receptor isoforms: 5HT1Aα, 5HT1Aβ and two 5-HT re-uptake pumps (SertA, SertB) and finally one opioid receptor (Oprd1b) was quantified as preliminary studies has shown a possible connection between this receptor and behavioural stress responses in zebrafish (Sarah McCarrick, Ove Thörnqvist and Svante Winberg unpublished).

In addition, based on previously published studies, four reference genes was chosen, Histone H3 (HH3), elongation factor EF1 alpha (Ef1a), hypoxanthine phosphoribosyl transferase 1 (Hprt1) and ribosomal protein (S20) and they were tested for expression stability using geNorm (Dang and Sun, 2011; Olsvik et al., 2005, Thörnqvist et al., 2015; Vandesompele et al., 2002b; Øvergård et al., 2010). Table 1 displays the target and reference genes together with their primer design. The primers were designed using NCBI primer blast with a melting point around 60 °C and ordered from Invitrogen (Thermo Scientific, USA).

The telencephalon was separated from whole brain by dissection and individually sonicated in lysis buffer for 5–10 s (Sonifier cell disruptor B-30). Three samples were lost during handling, two HR (basal) and one LR (stress). mRNA was extracted from individual samples using GenElute mammalian total RNA mini prep kit (Sigma, RTN70-1KT, St. Louis, MO, USA) and removal of genomic DNA was executed using, Ambion RNA, TURBO DNAfree, (Life Technologies, CA, USA). The quality and quantity of the RNA was assessed using spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). cDNA was prepared from 0.8 μg of total RNA (Maxima First Strand cDNA Synthesis kit for RT-qPCR, Thermo Scientific, USA), using oligo (dT) and random hexamer primers provided by the kit.

For the qPCR reaction, the following components were added to each well plate; 4 μl (4 ng) of diluted cDNA (1:40), 5 μL Maxima SYBR Green/Rox Master Mix (Thermo Scientific), 0.5 μL of each pri-

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Table 1
Genes, accession numbers, primer sequences and amplicon sizes for qPCR analysis.

Gene	Accession no.	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)
5HT1Aα	KP334154.2	TCCGCCCGTAGAGGACCAG	TCAGCCAGGACCCGGGCTAC	87
5HT1Aβ	CCAF0100015582	GAGGACCAACGGGGACCCGA	AATCGCCGTGCTTGACCGCA	92
GR1	NM_001124730.1	ACGACGATGGAGCCGAAC	ATGGCTTTGAGCAGGGATAG	107
GR2	NM_001124482.1	TGGTGGGCTGCTGGATTTCTGC	CTCCCTGTCTCCCTCTGTCA	240
MRA	NM_001124483	AGACTCGACCCCCACCAAG	CGTTAGTGGGACTGGTGCTC	102
MRB	NM_001124740.1	TACGGGGTTGTTACATGCGG	AGGTAGTTGTGCTGGCCTTC	80
Oprd1a	CCAF010045521	GCTCTCGTGTGGAGTTGCG	TCAACAGCACGAACCCCTAT	199
SERTA	CCAF010142484	TCTGCCGTTCTCTTGCGATG	TGACGCAGTCAGTGGTGATG	83
SERTB	CCAF010024691	ATGGCGATTTTTGGAGGGGT	TGCCTTTGAAGATGGGGCAT	112
EF1Aa	NM_001124339.1	GCAGGAAAAGAACCCAACG	AGTTACCAGCAGCTTTCTTCC	133
HH3	X01064.1 GI:64324	TCCGTCGTTACCAGAAGTCC	AGGTTGGTGTCCTCGAACAG	176
hprt	BT043501.1	CGTGGCTCTCTGCGTGCTA	TGGAGCGGTCGCTGTTACGG	92
20S	BT073292.1	GCGGACCTTATCCGTGGAG	TGGTGATGCGCAGAGTCTTG	262

5HT1Aα, serotonin 1a-like receptor; *5HT1Aβ*, WGS project data, contig 15582, containing serotonin 1b-like receptor; *GR1*, glucocorticoid receptor (nr3c1); *GR2*, glucocorticoid receptor 2; *MRA*, mineralocorticoid receptor form A; *MRB*, mineralocorticoid receptor form B; *Oprd1a*, Opioid receptor 1 Delta b; *SERTA*, neurotransmitter transporter, member 4 (slc6a4); *SERTB*, neurotransmitter transporter, member 4 (slc6a4); *EF1Aa*, elongation factor EF1 alpha; *HH3*, Histone H3; *hprt*, hypoxanthine phosphoribosyltransferase 1; *20S*, ribosomal protein S20.

mer (5 μM), ending with a total reaction volume of 10 μL. Amplification efficiencies were calculated from the slope of a cDNA standard curve, using triplicate 4-fold cDNA dilution series (range 4.0 ng μl^{-1} to 3.9 pg μl^{-1}), and the efficiencies were between 97 and 99%. To control for primer and DNA contamination, "no template controls" were used on each plate and for one of the reference gene, exclusion of reverse transcriptase confirmed high efficiency of the DNAase treatment. The samples were prepared in triplicates on 384-well plates using an initial denaturation at 95° C for 10 min, followed by a three step cycling protocol (40 cycles): 95 °C for 30 s + 60 °C for 30 s + 72 °C for 30 s (ABI 7900HT fast system, Applied Biosystem, Sweden). After the thermal cycling, a melting curve analysis was performed at 95 °C for identity and specificity of the PCR product. An agarose-gel electrophoresis was run to validate the PCR product-size and to confirm that no primer-dimers were formed. The use of four reference genes for the normalization of the qPCR data gave the lowest variation across treatments (>0.15) and they were therefore all included in the calculation of the target gene expression levels using geNorm (Vandesompele et al., 2002a).

2.8. Data treatment and Statistics

2.8.1. General

All continuous data sets were checked for normality distribution and homogeneity of variances, using visuals inspection of histograms and boxplots as well as, Shapiro-Wilk's test of normality and Levene's test for equality of variances (Zuur et al., 2009). If these assumptions were not met this is stated in the text. When needed, data were either sqrt- or Log10-transformed (depending on the skewness of the data) to achieve normal distribution and homogeneity of variances before further analyses. Significant p-value was set to below 0.05 and in some data sets, FDR correction for multiple comparisons are also presented (Benjamini and Hochberg, 1995). The data is presented as the mean ± standard error.

All statistical analyses were performed in IBM SPSS Statistics 22 (SPSS, Inc., an IBM Company, Armonk, New York).

2.8.2. Correlation analyses and t-test

Pearson's product-moment correlations were used to assess the relationship between plasma cortisol during confinement trial 1 and 2. A 2-tailed independent samples *t*-test was conducted on the individual cortisol average from the two confinement trials using *type* (LR and HR) as the grouping variable. Equal variances were not assumed, since the HR group displayed slightly higher variation.

Pearson's product-moment correlations were also used to assess the relationship between plasma cortisol levels and intestinal parameters, as well as between the mRNA expression of different genes and between mRNA gene expression and plasma cortisol. The latter analysis included 264 comparisons and the correlations that are presented as significant in the result section are based on family wise False Discovery Rate (FDR) error correction for multiple comparisons (Benjamini and Hochberg, 1995).

2.8.3. General Linear models

Two-way Anovas was conducted on plasma cortisol, intestinal parameters, growth and mRNA gene expression data. They were all designed using *Treatment* (basal and stress) and *Type* (HR and LR) as fixed factors together with the interaction (*Treatment*×*Type*). Non-significant interaction terms were removed from the final analyses. When a significant interaction was found, pairwise comparisons (Bonferroni) were used for each simple main effect. This was also performed on significant main treatment or type effect, when further investigation of the data was warranted.

Concerning mRNA gene expression data, original and adjusted p-values using FDR error correction for multiple comparisons (clustering 5-HT related genes, and corticoid receptors in two separate groups) were calculated (Benjamini and Hochberg, 1995).

The four combinations of MR/GR mRNA ratios were calculated by dividing MR (A and B) values by the GR (1 and 2) values.

In two sets of TER data (from anterior intestine of the LR/stress group, and the posterior intestine of the HR/stress group) transformation reduced the skewness of the data but, failed to obtain normality distribution. Also concerning Tph1 and Tph2, transformations reduced the skewness of the data but failed to obtain normality distribution for all type and treatment combinations, as well as homogeneity of variances for Tph1. However, the two-way Anova analyses were performed on the transformed data, trusting the robustness of the Anova concerning at least violations of normality (Schmider et al., 2010).

3. Results

3.1. Identifying high and low responding individuals

There was a positive correlation between plasma cortisol levels from confinement trial 1 and 2, ($r_{167} = 0.50$, $p = 6.68 \cdot 10^{-12;}$ Fig. 1A). The group average of cortisol for the two confinement trials was

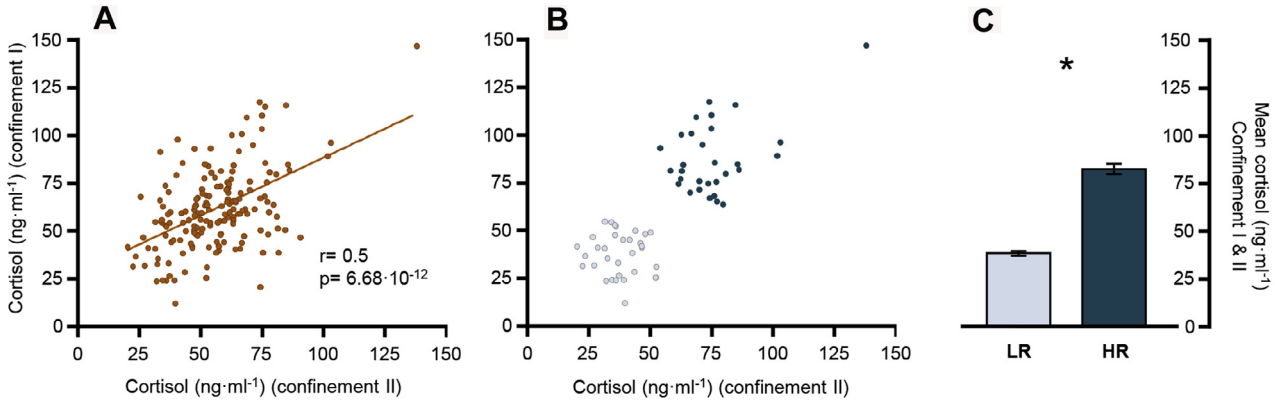

Fig. 1. Plasma cortisol response during confinement stress I and II. (A) Cortisol dispersal between confinement stress test 1 (y-axis) and test 2 (x-axis), r = Pearson's product-moment correlation, N = 167. (B) Cortisol dispersal in individuals characterized as LR and HR during confinement test 1 and 2. (C) The overall mean plasma cortisol response of the LR and HR individuals from the two confinement tests, $n_{HR} = 30$, $n_{LR} = 28$. Bars show averages ± SE, statistical significance is either shown by exact p-values or denoted by asterisk (*=$p < 0.05$).

twice as high in the HR type compared to the LR type ($t_{(61)} = 14.68$, $p = 1.02 \cdot 10^{-7}$; Fig. 1B and C).

3.2. Body size and growth

No difference was found in final body size between the groups, $HR_{weight} = 117.8 \pm 3.46$ g, $LR_{weight} = 117.4 \pm 3.82$ g, $HR_{length} = 225.9 \pm 1.95$ mm and $LR_{length} = 224.1 \pm 2.45$ mm. Furthermore, no differences were found in initial size of the fish or in length or mass specific growth rates (Table S1).

3.3. End sampling: Plasma cortisol

The stress treated group showed significantly higher plasma cortisol levels compared to the basal group ($F_{1,58} = 44.64$, $p = 1.01 \cdot 10^{-8}$), while there was no effect of HR/LR type ($F_{1,58} = 0.3$, $p = 0.59$); Fig. 2). Two LR individuals from the basal treatment group were excluded from further analyses since they had cortisol levels well above resting levels that could be traced back to human error during sampling (Iwama 1998).

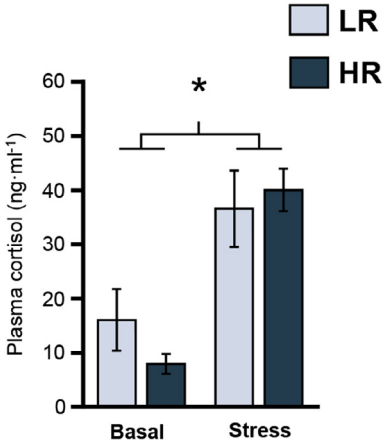

Fig. 2. Plasma cortisol of the LR and HR individuals during basal and stressed conditions (following 24 h handling and stress exposure). Basal; $n_{HR} = 15$ $n_{LR} = 15$, stress; $n_{HR} = 15$, $n_{LR} = 16$. Bars show averages ± SE, statistical significance is denoted by asterisk (*=$p < 0.05$).

3.4. Brain gene expression

3.4.1. General

For the interpretation of the mRNA gene expression data it was decided to use the original p-values due to the clear pattern and distribution of the data and to avoid increasing the risk of making type II errors (Armstrong 2014; Perneger 1998). Original and adjusted p-values using family wise FDR error correction for multiple comparisons are presented in Table S2.

3.4.2. Corticoid receptors

HR fish showed significantly higher expression levels of MRA ($F_{1,53} = 6.03$, $p = 0.017$; Fig. 3A) compared to LR fish. There was also a trend towards higher expression levels of GR2 in the HR group ($F_{1,53} = 3.32$, $p = 0.074$; Fig. 3D). There was no statistical difference in MRB (Fig. 4B) or GR1 levels (Fig. 3C) between the groups (Table S2) but the data follows the same pattern as MRA and GR2, with higher mean expression levels in the HR group, independent of treatment. There were no trends or significant effects due to stress exposure for any corticoid receptor in either group (Table S2). There were no differences in the MR/GR ratios between LR and HR group, either during basal or stressed conditions (Table S3).

3.4.3. The serotonin system and Oprd1b

HR fish showed significantly higher expression levels of the 5HT1 Aβ receptor ($F_{1,53} = 8.72$, $p = 0.005$; Fig. 4A) and the re-uptake pump SertB ($F_{1,53} = 4.95$, $p = 0.03$; 4F) compared to the LR fish, but no significant effects were seen due to stress exposure in either group (Table S2). The 5HT1Aα receptor on the other hand showed a trend towards lowered expression after stress exposure ($F_{1,53} = 3.35$, $p = 0.073$; Fig. 4C) and even though there was no significant interaction effect, visual examination of the data reveals that this trend is probably mainly driven by low expression levels in the LR group following stress exposure. There were no significant differences in re-uptake pump SertA levels (Table S2; Fig. 4E) or in the 5-HT producing enzymes Tph1 and Tph2 (Table S2; Fig. 4A and B). There were no statistical differences in Oprd1b levels (Table S2; Fig. S1).

3.4.4. Gene expression correlations

During basal conditions strong significant positive correlations were found both within and between glucocorticoid (GR1, GR2) and the mineral corticoid receptors (MRA, MRB) in both groups (HR and LR). Following stress exposure, significant correlations

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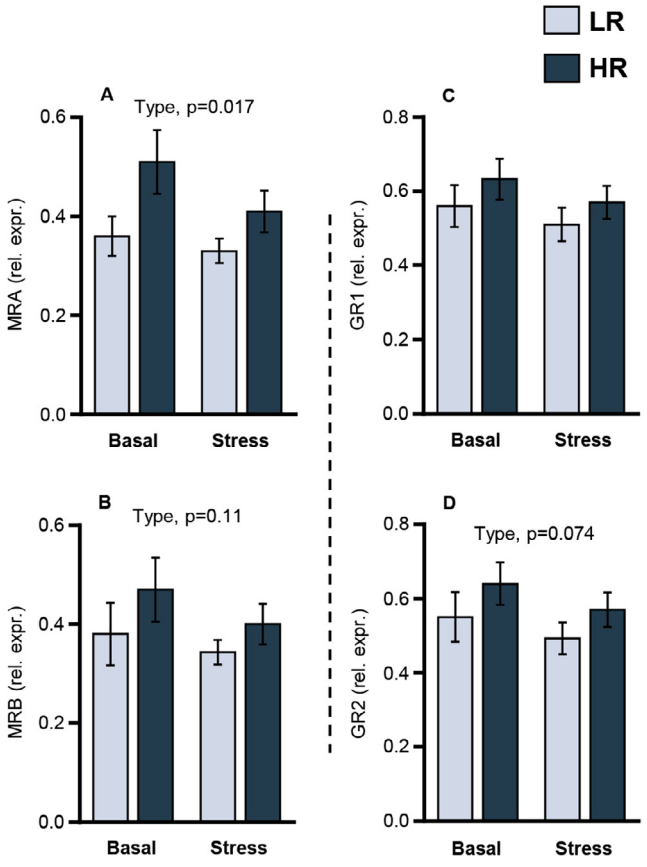

Fig. 3. Brain gene expression of corticoid receptors. mRNA expression levels in the telencephalon of LR and HR fish sampled under basal and stress conditions, (A, B) mineralocorticoid receptor A & B (MRA, MRB), (C, D) glucocorticoid receptor 1 & 2 (GR1, GR2). The y-axis shows the relative expression normalized to 4 reference genes. Bars show averages ± SE, statistical significance is shown by exact p-values (Type = LR /HR group). Basal; $n_{HR} = 13$, $n_{LR} = 13$, stress; $n_{HR} = 15$, $n_{LR} = 15$.

are less numerous and for the LR group, the only correlation that is still apparent is between the two GR receptors, suggesting a possible uncoupling between the mRNA expressions of the different MR type receptors (Table S4; Fig S2A&B&C).

In the LR and the HR group, trends towards and significant positive correlations between corticoid and 5-HT receptors were found (Table S4, Fig. S3A).

For the HR group during stress and the LR group during basal conditions, there was also a positive relationship between 5HT1Aβ and SertB (Table S4, Fig. S3B).

In the HR group there was a negative correlation between the opioid receptor Oprd1br and 5HT1Aα, both during basal and stressed conditions (Table S4; Fig. S3C).

In the LR group, during basal conditions, there was a strong positive correlation between corticoid receptors (MRB and GR2) and SertB (Table S4, Fig. S4A&B).

3.5. Intestinal barrier function

The transepithelial resistance (TER) of the posterior intestine showed a significant interaction effect ($F_{1,53} = 4.44$, $p = 0.040$), but no main effect of treatment or type (Table S5; Fig. 5B). The interaction effect seems to be driven by a lower TER in the LR group compared to the HR group during basal levels, followed by the opposite relationship post-stress. Pairwise comparisons also revealed a close to significant difference between the HR and LR type following stress exposure ($F_{1,53} = 3.56$, $p = 0.065$).

Regarding the paracellular permeability of mannitol (P_{app}), in the posterior intestine, there was, likewise, a significant interaction effect ($F_{1,52} = 9.37$, $p = 0.003$) but no factorial treatment or type effect (Table S5; Fig. 5D). Pairwise comparisons revealed a higher P_{app} for the LR type compared to the HR type during basal conditions ($F_{1,52} = 4.40$, $p = 0.041$) and the opposite relationship following stress exposure ($F_{1,52} = 4.98$, $p = 0.030$). For the LR group, P_{app} increased after stress exposure ($F_{1,52} = 6.40$, $p = 0.014$) and for the HR group there was a trend towards a decrease in P_{app} following stress exposure ($F_{1,52} = 3.19$, $p = 0.080$). This data supports the TER data indicating a weaker intestinal barrier in the LR group compared to the HR group during basal conditions but with a stronger barrier following stress exposure.

For TER and P_{app} in the anterior intestine there were no significant interaction, treatment or type effects (Table S5; Fig. 5A and C).

The up-take rate of the amino acid L-Lysine in the anterior intestine showed no significant interaction, treatment or type effects (Fig. S5).

In the posterior intestine of the LR group there were also significant negative correlations between TER and P_{app} during both basal and stressed conditions and for the HR group during stressed conditions but not during basal conditions. In the anterior intestine there was also a significant negative correlation between TER and P_{app} in the LR group during stressed conditions (For statistical details see, Table S6).

3.6. Intestinal barrier function and plasma cortisol correlations

In the posterior intestine of the LR group there was a clear positive correlation between cortisol and P_{app}, $r_{13} = 0.73$, $p = 0.005$ (Fig. 6C) which was matched by a strong negative relationship between cortisol and TER, ($r_{13} = 0.69$, $p = 0.009$ (Fig. 6A). In the anterior intestine of the same group (LR) there was also a trend towards a negative correlation between cortisol and TER, $r_{13} = 0.51$, $p = 0.078$. No correlations between cortisol and intestinal parameters were found in the HR group (Table S7; Fig. 6B and C). During stressed conditions no significant correlations between cortisol and intestinal parameters was found in either group or intestinal segment (Table S7).

4. Discussion

The current study presents novel data, linking HPI-axis reactivity to inherent physiological traits such as brain signaling pathways and intestinal barrier function. The HR fish showed higher mRNA expression of corticoid and 5-HT receptors in combination with higher expression of 5-HT re-uptake transporters in the telencephalon compared to LR fish, presenting novel data on how the serotonergic synapse arrangement could be linked to HPI-axis reactivity. Furthermore, the HR group showed a higher intestinal integrity during basal conditions compared to the LR group. After stress, HR fish instead showed impaired integrity, whereas the LR group showed increased integrity.

4.1. Mineral and glucocorticoid receptors and cortisol

4.1.1. Mineral and glucocorticoid receptors

The HR group showed higher mRNA expression levels of the MRA and a trend for higher levels of GR2, compared to the LR group. Furthermore, despite lack of statistical significance, the MRB and GR2 data follow the same pattern, with a tendency towards higher mRNA levels in the HR group. These results confirm that the LR and HR group are two distinct groups with contrasting cortisol signaling profiles. Furthermore, it suggests that the higher

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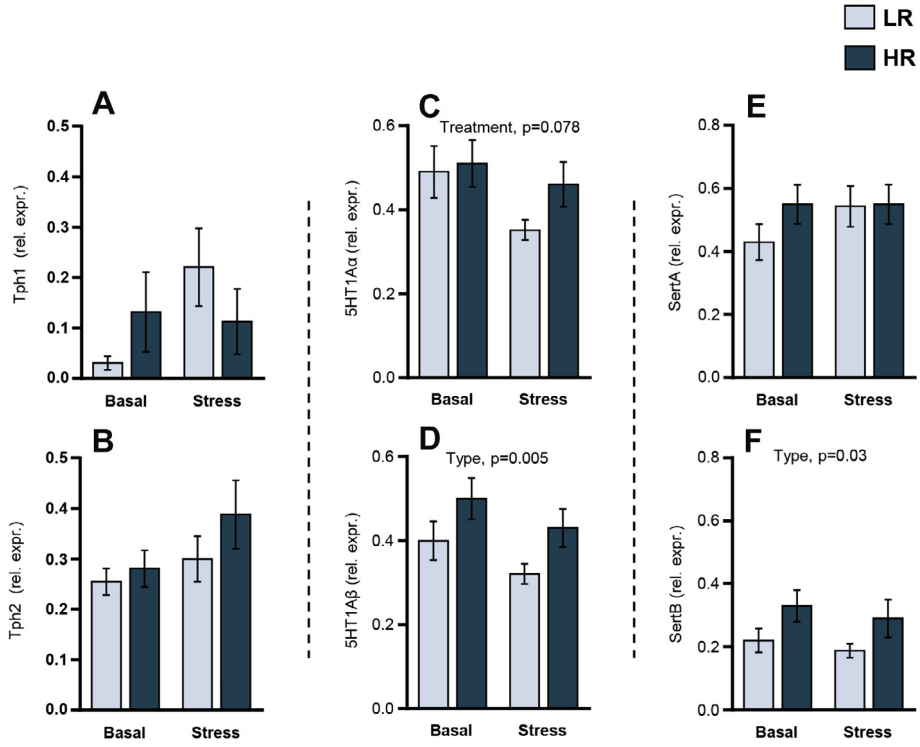

Fig. 4. Brain gene expression of the serotonergic system. mRNA expression levels in the telencephalon of LR and HR fish sampled under basal and stress conditions, (A, B) tryptofanhydroxylase 1 & 2 (Tph1, Tph 2), (C, D) serotonin receptors (5HT1Aα, 5HT1Aβ), (E, F) serotonin re-uptake pumps (SertA, SertB). The y-axis shows the relative expression normalized to 4 reference genes. Bars show averages ± SE, statistical significance is shown by exact p-values (Type = LR/HR group, Treatment = Basal/Stress). Basal; $n_{HR} = 13$, $n_{LR} = 13$, stress; $n_{HR} = 15$, $n_{LR} = 15$.

HPI-axis reactivity in HR individuals may also be coupled to higher corticoid sensitivity in the telencephalon. This fits well with the common notion that LR and HR individuals differ in their "personalities" and stress coping styles, as corticoid signals and receptors located in the CNS and the telencephalon are believed to be involved in processes such as behavioural reactivity, memory storage, neural plasticity and stress response modulation (Johansen et al., 2011; Oitz et al., 1997; Schjolden et al., 2009; Sørensen et al., 2013). The higher MRA expression levels in the HR group are however in contrast to previous results from rainbow trout strains that are selectively bred on HPI-axis reactivity (Pottinger and Carrick; 1999). Using these LR/HR strains, LR trout instead showed higher levels of MR mRNA expression in several brain regions, including the telencephalon (Johansen et al., 2011). It is possible that the discrepancy between the studies is due to inbreeding or "genetic hitchhiking" caused by the selective breeding for the LR and HR strains (Koolhaas et al., 2010; Smith and Haigh, 2009). In order to restore corticoid signaling functionality within the CNS, MR expression might have increased in the LR bred strain and/or decreased in the HR bred strain, through regulation of negative feedback mechanisms (Wendelaar Bonga, 1997).

Generally, strong correlations between GR and MR mRNA levels were found in the brain, with one exception in the LR group. Following stress exposure in this group, no correlations between the two MRs or between the MRs and GRs were found, suggesting a possible stress related uncoupling between MR mRNA expressions levels in LR individuals. Interestingly it has been shown in mammals that MR and GR activation have opposing effects on neural excitability, where MR activation leads to maintained excitation and GR activation leads to decreased neuronal excitation. Differences in the MR:GR ratio has been suggested to control memory formation, behavioral reactivity and the development of routines, however no differences were found regarding this ratio in the current study (Sørensen et al., 2013).

4.1.2. Plasma cortisol

Unlike expected, there was no difference in plasma cortisol between the LR and HR fish after stress, i.e. after the completion of the handling protocol. While this result may contradict the establishment of a low and high responding group, it should be kept in mind that these cortisol levels only shows the endpoint of the cortisol profile over a handling and stress exposure period that lasted for more than 24 h. In previous studies on the selected LR and HR strains it has been shown that during sustained stress, initial differences in plasma cortisol levels between the selected LR and HR strains disappeared after 24 h (Pottinger et al., 1994; Trenzado et al., 2003). Therefore, the assumption in the present study is that the LR and HR groups initially showed diverging cortisol responses that probably leveled out during the sustained stress exposure. The HR and LR groups defined in the present study are identified to be of truly diverging stress responsiveness based on the convincing correlation between confinement stress trial one and two as well as on the differences found in the expression levels of stress related genes.

4.2. Serotonin

The HR group showed higher expression levels of the 5HT1Aβ-receptor and the SertB pump compared to the LR group during both basal conditions and following stress exposure. The higher receptor levels show a potential for a stronger postsynaptic 5-HT response in the HR group. While the higher SertB levels can lead to a more rapid transient effect of released 5-HT, through faster clearance, it also enables its reuse, suggesting that the HR group have a higher serotonergic turn-over rate in the synaptic cleft compared to the LR group (Winberg and Nilsson, 1993). These results fits well with previously published studies on fish where high serotonergic activity was linked to e.g. subordination, low spontaneous locomotion and suppression of aggressive behaviour, characteris-

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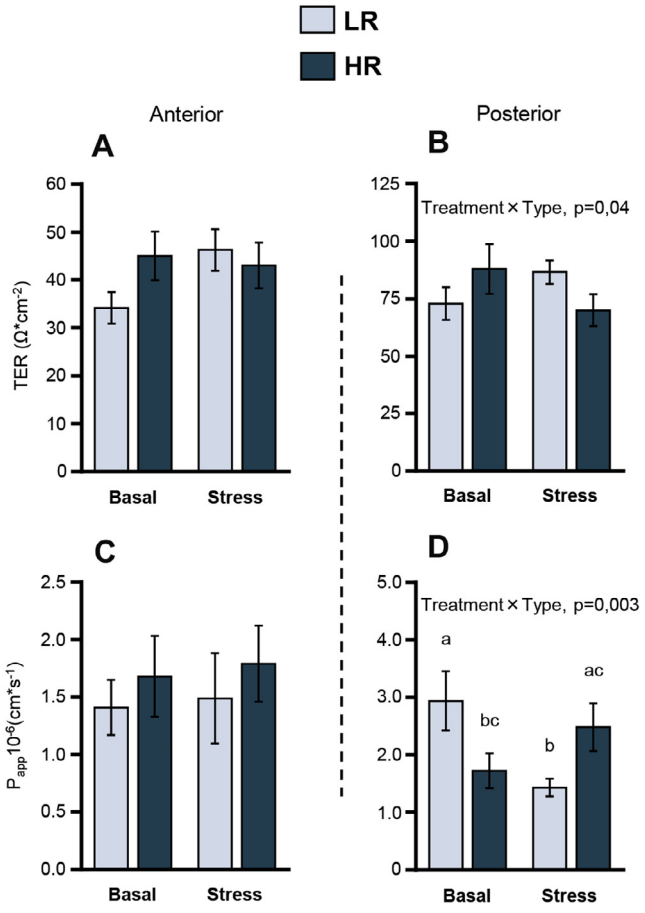

Fig. 5. Intestinal barrier function. Intestinal integrity of LR and HR fish under basal conditions and after stress exposure measured through TER (A, B) and P_{app} (C, D), in the anterior and posterior part of the intestine. TER = transepitelial resistance, P_{app} = permeability of the paracellular marker molecule, ^{14}C-mannitol. Bars show averages ± SE, statistical significance of main effects are shown by exact p-values and for pairwise comparisons through different letters (Type = LR /HR group, Treatment = Basal/Stress). Anterior intestine: basal; $n_{HR} = 14$, $n_{LR} = 13$, stress; $n_{HR} = 14$, $n_{LR} = 14$, posterior intestine: basal; $n_{HR} = 15$, $n_{LR} = 13$, stress; $n_{HR} = 15$, $n_{LR} = 14$.

tics often attributed to HR individuals (Andersson and Höglund, 2012; Popova 2006; Schjolden et al., 2006a; Winberg and Nilsson 1993; Øverli et al., 1999, 2004b). In contrast to this, the selectively bred LR trout strain has been suggested to have a higher serotonergic activity in the telencephalon when assessed through concentrations and ratios of 5-HT and its metabolite, 5-HIAA (Schjolden et al., 2006b; Øverli et al., 2005). However, assessing neural activity solemnly through 5-HT and 5-HIAA has limitations, as it is difficult to assign the effect to differences in production and/or clearance rates of the neurotransmitter (Chaouloff 2000, 2005). As the present study do not provide data on 5-HT and 5-HIAA, direct comparison to the studies on the selectively bred LR/HR strains are difficult. However, the similar expression levels of Tph1 and Tph2 (rate limiting enzymes in 5HT synthesis) suggests that there are no differences in production rates of 5-HT between the LR and HR fish, see Popova (2006).

There was a general trend for a positive correlation between the expression levels of 5HT1Aβ receptors and SertB pumps (significant in the LR basal and the HR stress groups), suggesting that high expression levels of 5-HT receptors is generally coupled to high levels of 5-HT re-uptake. During basal conditions, there were positive correlations between expression levels of the 5HT1Aα receptor and MRB, in the HR group, and between SertB and both GR2 and MRA, in the LR group. It is possible that the corticoid and the serotonergic systems interconnect at levels not measured here, or that the differences seen between the groups have been shaped by processes acting at a longer time-scale. None of the measured serotonergic components changed to any major extent due to the stress exposure and while previous data show that serotonergic activity can stimulate cortisol release and vice versa, there were no correlations between cortisol release and serotonergic activity in the present study (Chaouloff, 2000; Winberg et al., 1997; Øverli et al., 1999, 2005).

4.3. Opioid receptor

There were no differences in opioid receptor mRNA expression between the LR and HR fish or between treatments. There was, however, a significant negative correlation between the mRNA expression of 5HT1Aα and the Oprd1b receptor in the HR group during both basal and stressed conditions, which could be of interest as stimulation of the opioid delta receptors have been suggested to decrease anxiety-like behavior in zebrafish (Stewart et al., 2011).

4.4. Brain gut axis

The current study shows that rainbow trout with diverging HPI-axis reactivity, express differences in the integrity of the posterior intestinal primary barrier. In this region, the LR group exhibited a lower epithelial integrity during basal conditions, as shown by the significantly higher permeability for mannitol (Papp) and by the general pattern and significant interaction effect concerning the transepithelial resistance (TER) data. During basal levels a negative correlation between cortisol and TER, and a positive correlation, between cortisol and P_{app} was found in the LR group. These results indicate a possible higher sensitivity towards cortisol for the LR group during basal conditions. No such a relationship was found in the HR group and due to the vast number of target tissues for cortisol, individuals with a higher HPI-reactivity (HR individuals) could gain from desensitization of certain sensitive tissues, such as the intestine. However, following stress exposure, where both groups showed an increase in plasma cortisol, the HR group showed a reduction in intestinal integrity compared to the LR group, while the LR group at the same time showed strengthened intestinal integrity compared to basal conditions (as shown by a fifty percent decrease in P_{app}). In the anterior intestine there were no differences between the groups either during basal or stressed conditions. The results from the anterior intestine fits well with previous data showing that this region is less sensitive to stress (Rosengren et al., 2016; Sundh et al., 2010).

The strengthened integrity of the posterior intestine displayed by the LR group after stress is unexpected as the only previously found barrier enhancement effects following stress, are transient increases in mucus release, which usually does not affect P_{app} (Castagliuolo et al., 1998; Meaney and O'Driscoll, 1999; Olsen et al., 2005; Schipper et al., 1999). The clear negative correlation found between TER and P_{app} also indicates that it is the functional permeability of tight junction proteins (and not mucus production) that mainly influences the paracellular permeability (Schneeberger and Lynch 2004).

The reduction in integrity of the posterior intestine in the HR group after stress are in line with previous studies, as negative effects due to stress on the intestinal barrier function are well established (Meddings and Swain, 2000; Olsen et al., 2005; Segner et al., 2012; Sundh et al., 2010; Velin et al., 2004). The intestinal primary barrier is comprised of a single layer of innervated epithelial cells, connected to each other by tight junction

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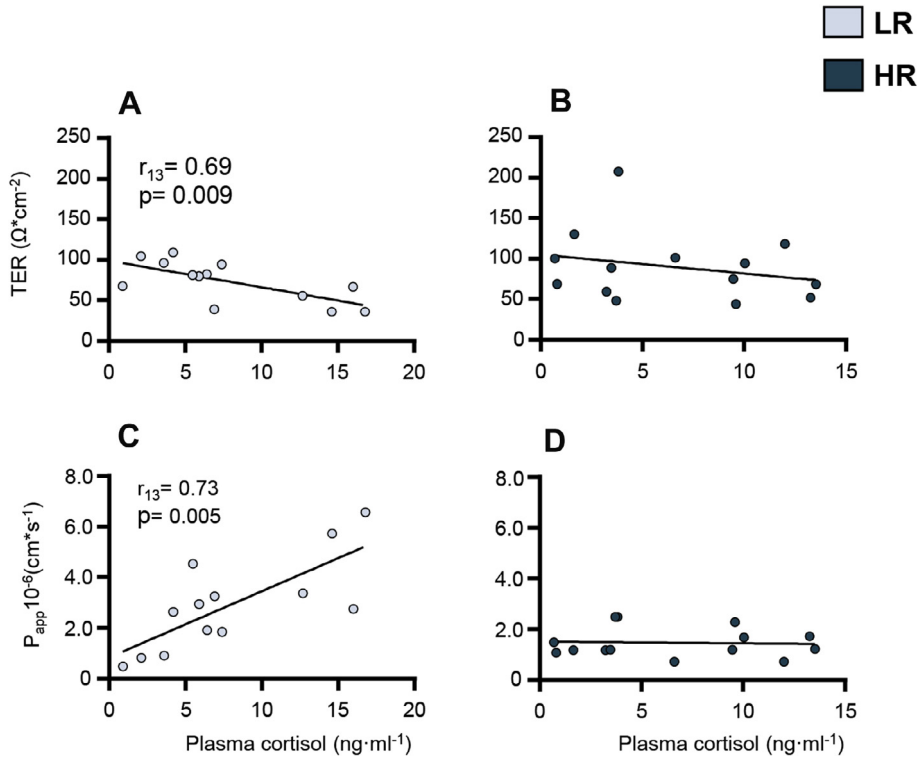

Fig. 6. Correlations between plasma cortisol and TER (A, B) and P_{app} (C, D) for LR (A, C) and HR fish (B, D) in the posterior intestine during basal conditions. TER = transepitelial resistance, P_{app} = permeability of the paracellular marker molecule, ^{14}C-mannitol (n_{HR} = 14, n_{LR} = 13).

proteins, the epithelium is in turn protected by associated immune cells and an extrinsic mucus layer. Effects on all these components can result in modulations of the intestinal integrity (Olsen et al., 2005; Saunders et al., 1994; Segner et al., 2012). Stress, cortisol administration and pathogen infections are all factors known to negatively affect the intestinal integrity in fish (Segner et al., 2012; Sundh 2009; Sundh et al., 2009). Therefore, one possibility is that the differences between the groups stem from secondary immune effects driven by cortisol. Cortisol administration has been shown to trigger degranulation of teleost mast cells and in mammals, degranulation and release of e.g. histamine from mast cells has been linked to loss of intestinal barrier function (Perdue et al., 1991; Reite 1997; Santos et al., 1998, 2001). It has also been shown that in mammals, corticoids can suppress intestinal secretory antibodies, leading to increased interaction between luminal bacteria and the epithelium, in turn resulting in loss of intestinal integrity (Spitz et al., 1996). Interestingly, links between high corticoid reactivity and intestinal dysfunction has also previously been found in mammals. The Wistar-Kyoto rat, a strain with attenuated corticoid reactivity, displayed an increase in intestinal permeability and ulcers following stress exposure compared to a strain with normal corticoid reactivity (Paré and Redei, 1993; Redei et al., 1994; Saunders et al., 1997). This was found to be linked to enteric nerves receiving input from the parasympathetic nervous system (Kiliaan et al., 1998; Saunders et al., 1994, 1997). In both mammals and fish it has been shown that acetylcholine and CRH, released by enteric neurons, can decrease intestinal integrity by contraction of the epithelial cytoskeleton and/or degranulation of mast cells (Bakker and Groot, 1989; Perdue et al., 1991; Santos et al., 1999). The permeability increase during stress in the HR fish could therefore also be an effect of parasympathetic activity as HR and reactive individuals are often suggested to have a stronger parasympathetic reactivity compared to LR and proactive individuals (Koolhaas et al., 1999).

5. Conclusion

The current study contributes new information on secondary traits and stress effects linked to HPI-axis reactivity in fish. The HR group displayed both higher expression levels of mineralcorticoid and serotonergic receptors as well as serotonergic re-uptake pumps, providing novel data on how telencephalon neuronal organization is linked to HPI-axis reactivity and stress coping. This also show that the teleost telencephalon is a major site for classical 5-HT neurotransmission, showing expression of 5-HT receptor and re-uptake pumps (Serts) and Tph. As both the serotonergic and the corticoid systems in the CNS are involved in cognitive processes, the higher MR expression levels and the organization of the serotonergic system (suggesting a potentially stronger postsynaptic 5-HT response coupled to a faster clearance rate) fits well with the notion of HR/reactive individuals as behaviorally flexible and more responsive to environmental stimuli compared to LR/proactive individuals (Koolhaas et al., 1999, 2010; Moreira et al., 2004; Ruiz-Gomez et al., 2011; Sørensen et al., 2013).

Furthermore, this is the first study reporting differences in intestinal sensitivity to stress in fish with diverging corticoid reactivity, indicating that the HPI-axis is involved in the brain-gut-axis in fish. We hypothesize that these intestinal differences are caused by regulation/effects on tight junction proteins possibly linked to secondary effect of cortisol on intestinal immune activity or differences in parasympathetic reactivity. Since even small changes and decreases in paracellular permeability is known to affect e.g. antigen and pathogen translocation rates this could make the LR group more susceptible to inflammation and infections compared to the HR group during basal conditions, whereas the HR group would be more vulnerable during stressed conditions (Fridell et al., 2007; Sundh et al., 2009; Velin et al., 2004). This highlights the LR/HR model as an interesting model to study mechanisms controlling and affecting intestinal barrier function in fish. The pre-

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sented difference in performance between situations and contexts also fits well with the evolutionary basis for co-existence of parallel stress coping styles (Careau et al., 2008).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2017.09.020.

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