**General and Comparative Endocrinology**
October 2018, Volume 267, Pages 76-81
http://dx.doi.org/10.1016/j.ygcen.2018.06.001
http://archimer.ifremer.fr/doc/00444/55560/




# Enhanced brain expression of genes related to cell proliferation and neural differentiation is associated with cortisol receptor expression in fishes

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**Abstract :**

Stress enhances or inhibits neurogenesis in mammals and some fish species. The link between the two processes is still unclear. Most studies have been performed in very specific stressful or altered environments. Despite the known inter-individual divergence in coping abilities within populations, the relationship between the stress axis and neurogenesis has never been addressed in unstressed individuals. Here we correlate brain expression of the *pcna* (proliferating cell nuclear antigen) and *neurod1* (neurogenic differentiation factor 1) genes, two markers of neurogenesis, with transcripts of cortisol receptors in three fish species living in very distinct environments. Within the three species, individuals with the highest expression of neurogenesis genes were also those that expressed the high levels of cortisol receptors. Based on these correlations and the hypothesis that mRNA levels are proxies of protein levels, we hypothesize that within unstressed animals, individuals sensitive to cortisol perceive a similar environment to be more stimulating, leading to increased neurogenesis. Although it is difficult to determine whether it is sensitivity to cortisol that affects neurogenesis capacities or the opposite, the proposed pathway is a potentially fruitful avenue that warrants further mechanistic experiments.

**Highlights**

► Neurogenesis and cortisol receptors genes positively correlate in brain of fishes. ► Three fish species from different environments show similar patterns. ► Unstressed animals were considered.

**Keywords** : Neurogenesis, Stress, Glucocorticoid receptor, Mineralocorticoid receptor, Hypothalamo-pituitary-interrenal axis

## 1. Introduction

Coping with the environment is a major component of fitness traits, especially in a world with increased environmental variability and frequent extreme events (Bonier et al., 2009; Németh et al., 2013). The hypothalamo-pituitary-interrenal axis plays a central role in coping abilities of fishes, with the final product,

cortisol, acting as the endocrine activator of most physiological responses to perturbations (Schreck and Tort, 2016). Most importantly, it allocates energy initially directed towards the production of structures in the liver and muscles to fuel coping behavior, osmoregulatory mechanisms, and immune responses (Sadoul and Vijayan, 2016). Cortisol is also known to operate on the brain, mostly by regulating its own production (Fryer and Peter, 1977) and its transformation into the inactive metabolite, cortisone (Alderman and Vijayan, 2012), through a negative feedback loop. These effects occur primarily by the binding of cortisol to its receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). These two receptors are found in many fish tissues, including the brain (Faught et al., 2016).

Recent studies in fish have demonstrated that cortisol has a role in brain physiology other than simply its own regulation. Cortisol treatment during development was found to increase neurogenesis, affecting brain development, in treated zebrafish, *Danio rerio* (Best et al., 2017). In contrast, a study in adult rainbow trout, *Oncorhynchus mykiss,* demonstrated that elevated cortisol levels lead to reduced cell proliferation in the brain (Sørensen et al., 2011). In addition, inhibition of GR, reducing the actions of cortisol, was found to increase the expression of genes related to neurogenesis during development (Nesan and Vijayan, 2013). These studies highlight a link between the stress axis and neurogenesis in fishes. However, the results are still inconsistent and the mechanisms involved mostly unknown. It is still unclear whether such opposing results are the consequence of differences between species or experimental set up.

Here, we investigated the relationship between the expression of genes related to neurogenesis and those involved in cortisol signaling by exploring the natural variability between individuals of three fish species from very distinct environments: European sea bass, *Dicentrarchus labrax*, from temperate marine waters, tetra fish, *Odontostilbe pequira,* from neotropical freshwater, and rainbow trout, *O. mykiss*, from cold freshwater. Rainbow trout and European sea bass are two major aquacultured species, well-described in the literature, whereas *O. pequira* has only recently been used as a sentinel for the effect of ecotourism in Brazil (Balduino et al., 2017; Geffroy et al., 2018). The expression of PCNA and NeuroD1, generally considered to be the principal markers of cell proliferation and neural differentiation, respectively (Sørensen et al., 2013), were used in this study.

## 2 Materials and Methods

### 2.1 Study sites and sampling procedures

A) European sea bass (born the 17$^{th}$ of October 2016) were reared at the experimental station of Ifremer (Palavas-Les-Flots) for 10 months before being sampled (10$^{th}$ of August 2017). All fish (n = 27) were euthanized using a lethal dose of Ethyl-p-aminobenzoate (Benzocaine, E1501, Sigma, St Louis, MO, USA), before being weighed (47 g ± 15 g). Then, the brain of each fish was immediately extracted and placed in liquid nitrogen and stored at -80ºC prior to analysis.

B) Rainbow trout (n = 48) were sibs of fish used as part of another experiment aiming to assess the effects of early stress on fish behavior at the juvenile stage (Poisson et al. 2017). Early maternal, artificial or pheromone stress was applied

to juveniles (0-57 dpf). Fish were then reared at INRA (LPGP, Rennes) for seven months until sampling (11th of July 2016). All fish were euthanized using a lethal dose of 2-phenoxyethanol (1,000 ppm) and weighed (60 g ± 16 g). The brain of each fish was then immediately extracted and placed in liquid nitrogen and stored at -80ºC prior to analysis. The early stress treatment did not affect gene expression at the group level at seven months (*i.e.* no significant differences between groups for any investigated genes).

C) Tetra fish were collected as part of a previous study aiming to assess the impact of ecotourism on fish stress (Bessa et al., 2017). Fish (standard body length: 2.5 ± 0.5 cm; weight: 0.22 ± 0.1g), sampled from June to August 2014 in both a touristic (total n = 15) and control zone (without tourism, total n = 15) in the Cuiabazinho River Basin (Mato Grosso, Brazil), were flash frozen in liquid nitrogen, after euthanasia, and stored at -80° C in the laboratory of the Federal University of Mato Grosso, Brazil, before being shipped to Rennes, France, for gene expression measurements in the brain. The brain was placed in RNAlater® solution for one day and stored at -80º C prior to analysis. Fish from the touristic zone displayed higher MR and neurod1 gene expression than fish from the non-touristic zone, but we observed no interaction (*i.e.* fish with high levels of MR were those with high levels of neurod1 in both populations).

## 2.2 Gene expression analysis

### *2.2.1 Extraction and reverse transcription of total RNA from the brain*

For both European sea bass and rainbow trout, total RNA was extracted using Tri Reagent (Molecular Research Centre, Cincinnati, OH), according to the manufacturer's instructions. For tetra fish, total RNA was obtained following the

instructions of the SV Total RNA isolation System kit (Promega®). RNA was reverse transcribed using 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega), 2 µg random hexamers (Promega), and 25 U RNase inhibitor (RNasin; Promega) in a final volume of 25 µl. The products were then diluted 1:10 for quantitative real-time PCR (qPCR).

*2.2.2 Quantitative real-time PCR*

Primer sequences were retrieved from the literature (Backström et al., 2011; Crespo et al., 2013; Pavlidis et al., 2011) or the primers were specially-designed when the sequences were not available (Table 1). It was unknown whether *O. pequira* possessed two isoforms of GR, similar to rainbow trout and sea bass, or only one, such as for zebrafish. Based a phylogenetic analysis, the isoform amplified for Tetra fish was within the GR2 cluster and therefore designated GR2. QPCR was performed using the GoTaq® qPCR Master Mix (Promega) for tetra fish and the SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies) for the other two species. The Step One Plus system (Applied Biosystems, Foster City, CA) was used for all species. The hot start enzyme was activated 2 min at 95°C and amplification carried out using the following program: 95°C for 3 s and 60°C (for rainbow trout and the European sea bass) or 62°C (for tetra fish) for 30 s for 40 cycles. After amplification, a melting curve was obtained using the following protocol: 10 s holding at 55°C followed by sequential 0.05°C increases, repeated 80 times.

*2.2.3 Housekeeping Genes*

Various housekeeping genes were used, depending on their intra-species variation (assessed through the coefficient of variation: CV) and availability for

the target species. Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), ribosomal 18S (*18S*), ribosomal protein L13 (*L13*), eukaryotic translation elongation factor 1 alpha (*eef1-alpha*), and β-Actin (*β-Actin*) are all recommended reference genes for qPCR (Bland et al., 2012; Goidin et al., 2001; Radonić et al., 2004). For European sea bass, *L13* and *eef1-alpha* were used, with CVs of 2.6 and 3.3%, respectively. For rainbow trout, *eef1-alpha* and *β-Actin* were used, with a common CV of 1.1%. For tetra fish, *gapdh* and *18S* were used, with CVs of 5.9% and 3.4%, respectively. For all three species, the mean of the two selected genes was used to calculate the relative quantity of each target gene.

### 2.3 Ethical statement

Experiments were authorized by ethics committee agreements C35-238-6 of INRA-LPGP for rainbow trout, EEA# 34-192-6 and APAFIS #7098 for European sea bass, and 2071343 of ICMBio (Brazilian biodiversity protection organ) for tetra fish. All procedures involving animals were in accordance with the ethical standards of the institution and followed European Directive 2010/63 UE.

### 2.4 Statistical analyses

Statistical analyses were performed using R 3.1.0 software (R core team, 2014). All statistical analyses were carried out at a 95% level of significance. Two samples of European sea bass and three of tetra fish were discarded due to RNA extraction failure, resulting in the analysis of a total of 26 European sea bass, 48 rainbow trout, and 27 tetra fish.

We first performed linear regression analysis between the expression of neurogenesis genes (*i.e.*, *pcna* and *neurod1*) and that of cortisol receptors (*i.e.*, *mr*, *gr1*, and *gr2*) for each species. We also measured other genes to confirm that

false correlations were not generated. We thus performed a linear regression analysis between gene expression linked to neurogenesis (*i.e.*, *pcna* and *neurod1*) and that of genes not related to cortisol receptors (*i.e.*, *avt* for European sea bass and rainbow trout and *hsp70* for tetra fish).

## 3 Results

### 3.1 PCNA and cortisol receptors

The correlation by linear regression was significant between the expression of *pcna* and *mr* for European sea bass, ($p = 0.02$, $F = 6.15$, $R^2 = 0.17$), rainbow trout ($p < 0.001$, $F = 44.95$, $R^2 = 0.48$), and tetra fish ($p = 0.005$, $F = 9.38$, $R^2 = 0.24$, **Figure 1.A**). Similarly, the correlation between the expression of *pcna* and *gr2* was also significant for European sea bass ($p = 0.003$, $F = 10.64$, $R^2 = 0.28$), rainbow trout ($p < 0.001$, $F = 24.25$, $R^2 = 0.33$), and tetra fish ($p < 0.001$, $F = 34.39$, $R^2 = 0.56$, **Figure 1.B**) and for *pcna* and *gr1* for rainbow trout ($p < 0.001$, $F = 30.76$, $R^2 = 0.39$; **Figure 1.C**), but not European sea bass ($p = 0.76$, $F = 0.01$). For *avt*, the correlation with *pcna* was significant for rainbow trout ($p = 0.04$, $F = 4.38$, $R^2 = 0.07$), but not European sea bass ($p = 0.21$, $F = 1.63$). *hsp*70 expression did not correlate with that of *pcna* in tetra fish ($p = 0.69$, $F = 0.16$).

### 3.2 NeuroD1 and cortisol receptors

The correlation between the expression of *neurod1* and *mr* for European sea bass was not significant ($p = 0.10$, $F = 2.87$), but it was significant for rainbow trout ($p < 0.001$, $F = 38.60$, $R^2 = 0.44$) and tetra fish ($p < 0.001$, $F = 77.43$, $R^2 = 0.75$, **Figure 2.A**). The correlation between the expression of *neurod1* and *gr2* was not significant for European sea bass ($p = 0.25$, $F = 1.41$), but reached

194 significance for rainbow trout ($p < 0.001$, $F = 46.13$, $R^2 = 0.49$) and tetra fish ($p <$
195 $0.001$, $F = 30.39$, $R^2 = 0.53$; **Figure 2.B**) and for *neurod1* and *gr1* for rainbow
196 trout ($p < 0.001$, $F = 19.28$, $R^2 = 0.28$; **Figure 2.C**), but not European sea bass ($p =$
197 $0.93$, $F = 0.01$). Neither the expression of *avt* nor *hsp70* significantly correlated
198 with that of *neurod1* (European sea bass: p=0.12, F=2.58; rainbow trout: p=0.22,
199 F=1.23; tetra fish: p=0.61, F=0.27).
200

201 **4 Discussion**

202 This study shows, for the first time, that inter-individual variability in the
203 expression of brain cell proliferation and neural differentiation genes is
204 associated with the expression of cortisol receptors in three fish species. This
205 was observed in unstressed fishes randomly picked within a population.
206 Although we report only correlations, the strong effect highlighted here suggests
207 that individuals with higher expression of cortisol receptor transcripts display
208 stronger neurogenesis in a non-stressful environment.

209 Although molecular mechanisms of MR on neurogenesis are still poorly
210 understood, it is well-known GR directly influence the proliferation of neural
211 stem cells through genomic and non-genomic mechanisms, as recently reviewed
212 in Odaka et al. (2017). Additionally, it was previously suggested that moderate
213 stress may stimulate neurogenesis in fish (Sørensen et al., 2013). This was
214 illustrated in one study on rainbow trout in which short confinement stress led
215 to increased expression of brain-derived neurotrophic factor (*bdnf)* and *pcna* in
216 the brain (Johansen et al., 2012). Similarly, enriched environments, associated
217 with small increases in cortisol levels (von Krogh et al., 2010), were described as

stimulators of neurogenesis, shown by increased brain expression of *neurod* and *pcna* (Salvanes et al., 2013; von Krogh et al., 2010). At the group level, the strain of zebrafish with the highest baseline cortisol values also showed the strongest levels of *mr, gr-beta, pcna*, and *neurod* transcripts, implying a similar relationship (van den Bos et al., 2017). Social interactions were also found to increase neurogenesis in the electric fish, *Brachyhypopomus gauderio,* which was suggested to be mediated by GR (Dunlap et al., 2013). Comparable results were obtained in mice placed in enriched environments or subjected to mild physical activity, which both increased glucocorticoids levels, favoring neurogenesis (Saaltink and Vreugdenhil, 2014). Thus, overall, low activation of glucocorticoid receptors in the brain, through non-threatening stimuli or acute and rare stressors, was shown to enhance neurogenesis in both mammals and fish. Here, we speculate on another mode of action, independent of the environment, linking the expression of GR and MR mRNA with neurogenesis. Although previous results have demonstrated discrepancies between GR and MR transcript levels, protein levels, and binding capacities, they were obtained under artificial conditions that strongly affected plasma glucocorticoid levels, such as exogenous cortisol administration (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003), or chemical adrenalectomy (Medina et al., 2013). In contrast, mRNA and protein level of GR have been found to be highly consistent in unstressed mammalian species throughout the brain (Herman and Spencer, 1998) or during development (Owen and Matthews, 2003). We thus reasoned that GR and MR mRNA levels are reasonable proxies for respective protein expression in unstressed individuals, although this needs to be formally demonstrated. Consequently, we propose that a same stressor can lead to various levels of

neurogenesis due to variable levels of cortisol receptors in fishes. Whether this is due to differences between individuals in their perception of the environment or variability in their life history needs to be investigated further.

The strength of our data relies on the general pattern observed over three teleost species living in very different environments and therefore sampled in three highly separated regions. The observed common phenomenon suggests a general teleost mechanism of interconnection between neurogenesis and cortisol receptors. Such consistency among teleost species is not surprising, given the strong evolutionary conservation of brain structures across fishes (Ganz and Brand, 2016). By considering the whole brain, multiple regions with specific functions and patterns of gene expression (Ganz and Brand, 2016) were combined in this study. However, consistent effects of stress on cell proliferation have been observed in different brain regions (Maruska et al., 2012), suggesting that our results would be expected to be comparable for different brain regions.

Overall, our results further aid our understanding of how the environment can dynamically drive cellular and molecular changes in the brain, with associated ultimate consequences on experience-dependent behavior and fitness. We therefore propose that inter-individual variability in cortisol receptors partially explains differences in neurogenesis within a similar non-stressful environment.

## 5 Acknowledgments

We would like to thank Dr Patrick Prunet for providing the rainbow trout and for his help during the sampling. Additionally, we thank Sébastien Szlabowicz for performing the qPCR of the sea bass.

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**Table 1.** Primer sequences for gene expression analysis

| Species | Gene | GenBank accession numbers | Primers | Primer sequence |
|---|---|---|---|---|
| *Dicentrarchus labrax* | *gr1* | a* | gr1-F | 5'GAGATTTGGCAAGACCTTGACC3' |
| | | | gr1-R | 5'ACCACACCAGGCGTACTGA3' |
| | *gr2* | a* | gr2-F | 5'GACGCAGACCTCCACTACATTC3' |
| | | | gr2-R | 5'GCCGTTCATACTCTCAACCAC3' |
| | *mr* | JF824641.1 | mr-F | 5'GTTCCACAAAGAGCCCCAAG3' |
| | | | mr-R | 5'AGGAGGACTGGTGGTTGATG3' |
| | *pcna* | b* | pcna-F | 5'CAGAGCGGCTGGTTGCA3' |
| | | | pcna-R | 5'CACCAAAGTGGAGCGAACAA3' |
| | *neurod1* | c* | neurod1-F | 5'TTCTCCTTCAGCGTGCACTA3' |
| | | | neurod1-R | 5'GGTGCGAGTGTCCATCAAAG3' |
| | *eef1-alpha* | AJ866727.1 | ef1-F | 5'AGATGGGCTTGTTCAAGGGA' |
| | | | ef1-R | 5'TACAGTTCCAATACCGCCGA3' |
| | *L13* | DT044910.1 | L13-F | 5'TCTGGAGGACTGTCAGGGGCATGC3' |
| | | | L13-R | 5'AGACGCACAATCTTGAGAGCAG3' |
| | *avt* | c* | avt-F | 5'CAATAGTTTTCTCCTCCAGTGCA3' |
| | | | avt-R | 5'GGGGTGAGCAGGTAGTTCTC3' |
| *Oncorhynchus mykiss* | *gr1* | Z54210 | gr1-F | 5'CCATCGTCAAGCGGGAAGAG3' |
| | | | gr1-R | 5'GGAACTCCACGCTAAGGGATTTATTC3' |
| | *gr2* | AY495372 | gr2-F | 5'CTCCGCTTTCTCCAGCAGCTA3' |
| | | | gr2-R | 5'GTGAGCCACCCCGTAGTGACAG3' |
| | *mr* | AF209873 | mr-F | 5'GAAACAGATGATCCGCGTGGT3' |
| | | | mr-R | 5'TGGATCAGGGTGATTTGGTCCT3' |
| | *pcna* | d* | pcna-F | 5'ATGTGGACAAGGAGGA3' |
| | | | pcna-R | 5'CTATCTTGTACTCCACCA3' |
| | *neurod1* | e* | neurod1-F | 5'TTAGGAGAAGTGCGGATA3' |
| | | | neurod1-R | 5'GGCCCAAGTATTCGTTT3' |
| | *eef1-alpha* | AF498320.1 | ef1-F | 5'AGGCATTGACAAGAGAACCATT3' |
| | | | ef1-R | 5'TGATACCACGCTCCCTCTC3' |
| | *ß-Actin* | AF157514.1 | Actine-F | 5'AGAGCTACGAGCTGCCTGAC3' |
| | | | Actine-R | 5'GCAAGACTCCATACCGAGGA3' |
| | *avt* | f* | avt-F | 5'TGAACACACCCAGAATAGAGC3' |
| | | | avt-R | 5'TCTACTTCTGCTGTGTGTCTG3' |
| *Odontostilbe pequira* | *gr* | KU820856 | gr-F | 5'GGAACACGCAGCACTATGTC3' |
| | | | gr-R | 5'CCTCCCGACTGTTTTCATGT3' |
| | *mr* | KU820857 | mr-F | 5'TGAGTCCATGGGCATCTACA3' |
| | | | mr-R | 5'ATGGTGTTGGTGGAGCTTTC3' |
| | *pcna* | KU820860 | pcna-F | 5'GACCTGATCACCGAGGCYTGCTGGG3' |
| | | | pcna-R | 5'CTGTCGCAGCGGTAGGAGTCG3' |
| | *neurod1* | KU820859 | neurod1-F | 5'AGATGCGGCGCATGAAGGCGAACGC3' |
| | | | neurod1-R | 5'CGGAGSGTCTCGATCTTGGAGAGCT3' |
| | *gapdh* | KU820855 | gapdh-F | 5'CAATGACCCCTTCATTGACC3' |
| | | | gapdh-R | 5'TAGTCAGCACCAGCATCACC3' |
| | *18S* | KU820862 | 18S-F | 5'TCGCTAGTTGGCATCGTTTAT3' |
| | | | 18S-R | 5'CGGAGGTTCGAAGACGATCA3' |
| | *hsp70* | g* | hsp70-F | 5'GACAACCAGCCCGGTGTCCTG3' |
| | | | hsp70-R | 5'CTGCCCTTGTCGTTGGTGATGGTGA3' |

a* from Pavlidis et al. 2011
b* from Crespo et al. 2013
c* designed from the available European seebass genome from UCSC
d* PREDICTED: Oncorhynchus mykiss proliferating cell nuclear antigen (LOC110525832), mRNA Sequence ID: XM_021606282.1
e* PREDICTED: from Oncorhynchus mykiss neurogenic differentiation factor 1-like (LOC110520317), mRNA Sequence ID: XM_021597549.1
f* from Backström et al (2011)
g* Based on the zebrafish gene: AF210640.1 - Efficiency:1.9; R-squared: 0.93

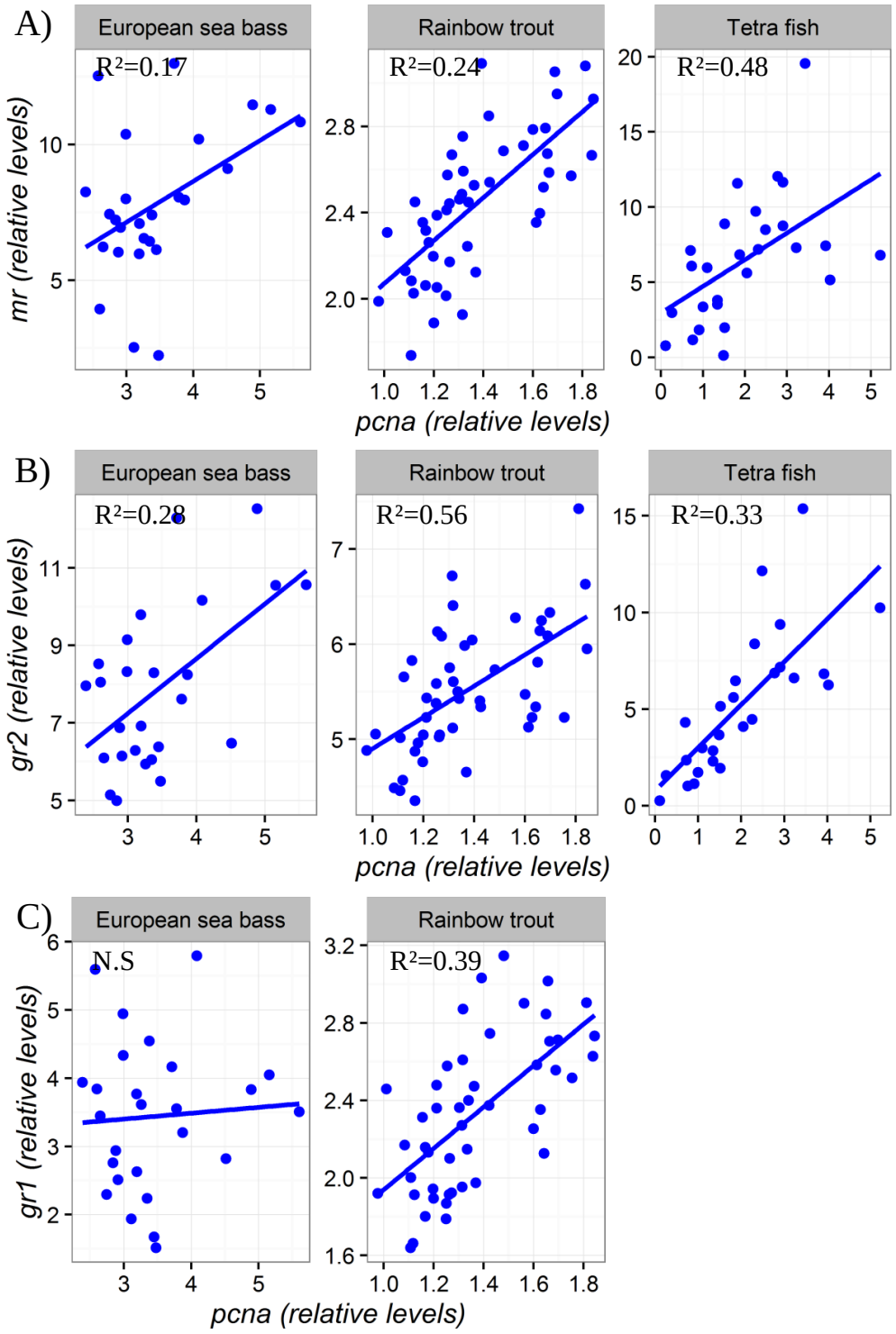


**Figure 1**. Gene expression of *pcna* in European sea bass *Dicentrarchus labrax* (n = 26), rainbow trout *Oncorhynchus mykiss* (n = 48), and tetra fish *Odonstostilbe pequira* (n = 27) as a function of (A) *mr*; (B) *gr2*, and (C) *gr1* gene expression. Gene expression was normalized against that of *eef1-alpha* and *l13* for European sea bass, *β-Actin* and *eef1-alpha* for rainbow trout, and *gapdh* and *18s* for tetra fish. $R^2$ is indicated for each significant linear regression. Otherwise N.S (not significant) is indicated.

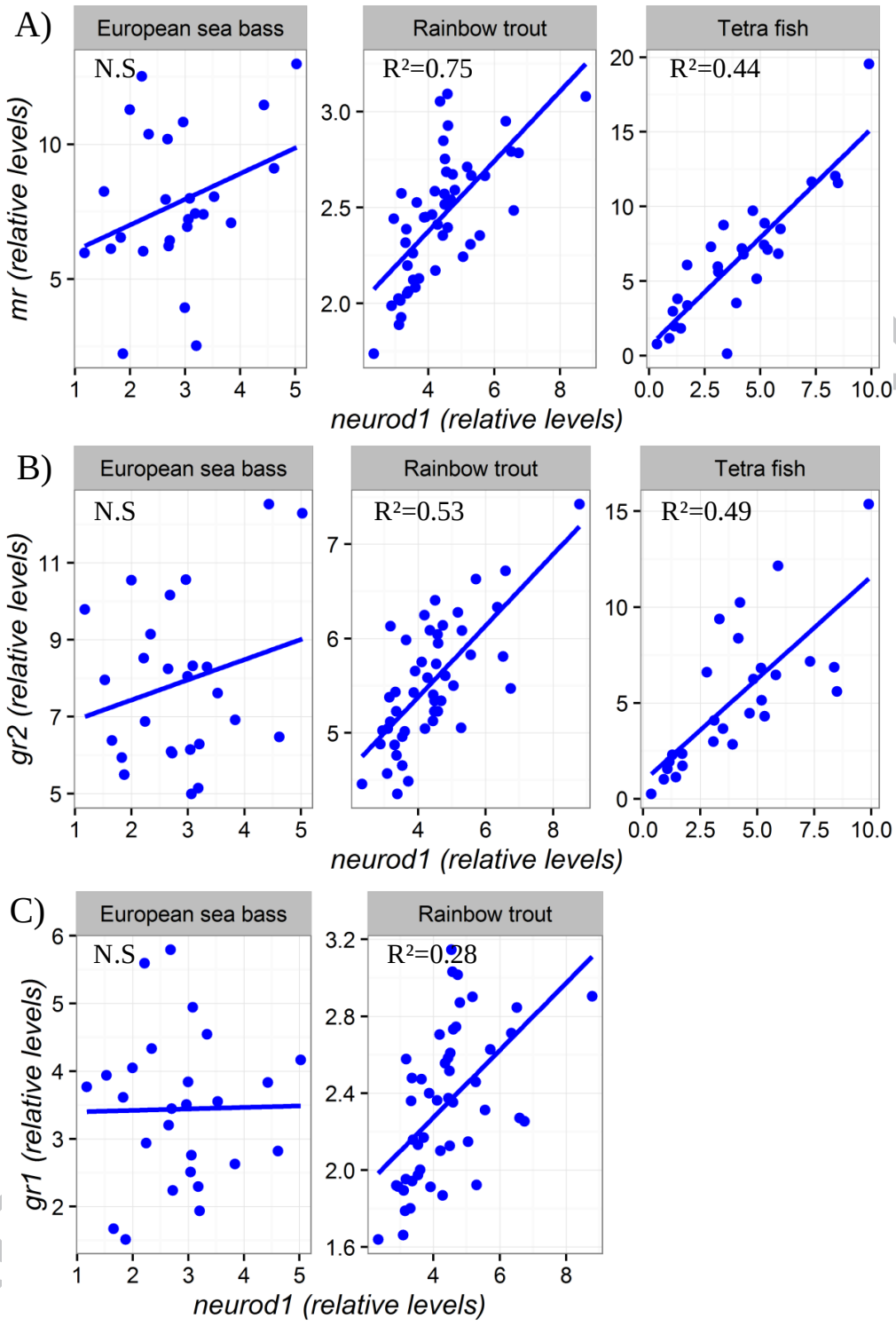


**Figure 2**. Gene expression of *neurod1* in European sea bass *Dicentrarchus labrax* (n = 26), rainbow trout *Oncorhynchus mykiss* (n = 48), and tetra fish *Odonstostilbe pequira* (n = 27) as a function of (A) *mr*, (B) *gr2*, and (C) *gr1* gene expression. Gene expression was normalized against that of *eef1-alpha* and *l13* for European sea bass, *β-Actin* and *eef1-alpha* for rainbow trout, and *gapdh* and *18s* for tetra fish. $R^2$ is indicated for each significant linear regression. Otherwise N.S (not significant) is indicated.

Neurogenesis and cortisol receptors genes positively correlate in brain of fishes.
Three fish species from different environments show similar patterns.
Unstressed animals were considered.
PCNA and neuroD are for the first time studied in *O. Pecquira*.