PATTERNS OF BRAIN ACTIVATION IN EUROPEAN SEABASS (DICENTRARCHUS LABRAX) WITH DIFFERENT COPING STYLES

André Filipe Martins de Almeida Tacão Monteiro

DISSERTAÇÃO

MESTRADO EM BIOLOGIA EVOLUTIVA E DO DESENVOLVIMENTO

2015
PATTERNS OF BRAIN ACTIVATION IN EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*) WITH DIFFERENT COPING STYLES

André Filipe Martins de Almeida Tacão Monteiro

DISSERTAÇÃO

MESTRADO EM BIOLOGIA EVOLUTIVA E DO DESENVOLVIMENTO

Dissertação orientada por:

Prof. Doutor José Élio da Silva Sucena (DBA/FCUL)

Prof. Doutor Rui Filipe Nunes Pais de Oliveira (ISPA/IGC/CNP)

2015
Acknowledgments

First, I would like to thank Professors Rui Oliveira and Élio Sucena for accepting to be my master thesis advisors. Especially to Professor Rui Oliveira for giving me the opportunity to integrate his research group.

I would also to thank Prof. Marie-Laure Bégout for providing the heads of the fish in which the coping styles were assessed.

To all members of IBBG lab for their support. A very special thanks to Ana Sofia Félix, whose guidance, knowledge, patience and friendship were essential during this project. To Magda Teles, for the great lab tips, particularly regarding micropunch dissections, for all the teaching about social network analyses, for all the useful discussions. To Sara Cardoso, who taught me everything I know about primer design and a lot about social network analyses. To Ana Faustino, for all the availability, support during the entire process, great discussions, for the thesis review, and for the friendship. To Rodrigo Abreu, with whom I had delightful discussions, thank you for all the support, friendship, and for the sincerity in all the moments, specially in the most difficult ones. To Gonçalo Oliveira, who destroyed my allure for Freud, but we still managed to become friends, thank you for all the answers to random questions and for all the support, especially during the writing of this thesis. To António Roleira, for all the support given during the long days spent in the lab. To the rest of the IBBG group Ana Cruz, João Sollari Lopes, José Miguel Simões, Júlia Pinho, Natália Madeira and Raquel Martins for all the help and support.

To Ana Catarina Morais, Marisa Rodrigues e Telma Laurentino for their friendship and support during our master degree.

To my dearest friends Alexandre Ginja, Ana Teresa Gabriel, Débora Braga, Helena Lages, João Costa, João Monteiro, Marta Costa, and Roberto Almeida for the friendship and the support throughout all the years we met.

Finally, I would like to thank my family, specially my parents, that always believed in me, and loved me unconditionally.

I would like to acknowledge the project: “COPEWELL: A new integrative framework for the study of fish welfare based on the concepts of allostasis, appraisal and coping styles” (EU-FP7 project # 265957) that funded this research.
“Alice thought to herself "I don't see how he can ever finish, if he doesn't begin.”

— Lewis Carroll, Alice's Adventures in Wonderland
Resumo

Numa população existem diferenças comportamentais entre os indivíduos que a constituem. No entanto essa variação não é aleatória, mas sim consistente tanto a um nível intra- como inter-individual e acompanhada por diferenças a um nível fisiológico. Existe uma consistência intra-individual, pois cada indivíduo possui um conjunto de comportamentos constante ao longo do tempo e em diferentes situações. A consistência inter-individual prende-se com o facto de existirem pelo menos dois grupos, cada grupo constituído por indivíduos que possuam o mesmo conjunto de comportamentos associados. A título de exemplo, animais mais agressivos são normalmente mais ousados (do inglês bold) na aproximação a um predador (associação entre comportamentos) enquanto que indivíduos menos agressivos também são menos ousados. Esse nível de agressividade é dirigida tanto a conspecíficos como indivíduos de outras espécies (consistência em diferentes situações) e ao longo do tempo.

Estas diferenças foram também observadas em resposta ao stress, e neste fala-se de coping style. Deste modo um coping style é um conjunto de comportamentos e alterações fisiológicas que ocorrem em resposta a um stress, consistente ao longo do tempo e em diferentes situações e é característico de um determinado grupo de indivíduos de uma população. Tendo em conta que os coping styles são um contínuo de estratégias, existem duas estratégias que se encontram nos dois extremos do contínuo: proactivos e reactivos. Estas estratégias encontram-se em diferentes taxa do reino animal. A um nível comportamental os indivíduos proactivos apresentam maiores níveis de agressividade, são mais ousados e têm baixa latência para atacar. Contrariamente, os indivíduos reactivos apresentam baixos níveis de agressividade, são menos ousados e têm uma alta latência para atacar. A um nível fisiológico, os proactivos em relação aos reactivos possuem uma menor reatividade do eixo hipotálamo-hipófise-adrenal (HPA, do inglês Hypothalamic–Pituitary–Adrenal), uma maior atividade do sistema nervoso simpático, uma menor produção de cortisol após uma situação de stress, uma menor densidade de projeções de neurónios libertadores de vasopressina (AVP) para o septo lateral, e consequentemente uma menor libertação de AVP para o septo lateral em condições de stress e níveis de mRNA de receptores de serotonina mais elevados no hipocampo.
Tendo em conta estas diferenças, nomeadamente as diferenças a nível neuronal, fomos averiguar se existiam diferentes padrões de atividade neural em robalo (*Dicentrarchus labrax*) com diferentes *coping styles*. Para isso seis áreas foram selecionadas: parte lateral do telencéfalo dorsal (Dl) [que foi dividida em divisão dorsal da Dl (Dld), e divisão ventral da Dl (Dlv)], a parte medial do telencéfalo dorsal (Dm), o núcleo ventral do telencéfalo ventral (Vv), o núcleo supracomissural do telencéfalo ventral (Vv) e a área pré-optica (POA).

As áreas candidatas foram escolhidas devido à sua importância não só para o sistema mesolímico de recompensa (do inglês *mesolymbic reward system*), parte mas também pela sua relevância na regulação do eixo HPI (homólogo ao eixo HPA dos mamíferos), importante para a regulação na resposta ao stress.

Estudos anteriores demonstraram que diferentes *coping styles* faziam diferentes avaliações de um mesmo estímulo, e o sistema mesolímico de recompensa é apontado como a rede neuronal onde a avaliação de um estímulo é processada. Fazem parte deste sistema a Dl, a Dm, a Vv e a Vs. Além disso, o septo lateral (homólogo em mamífero da Vv) e a *extendend amygdala* (homólogo em mamífero da Vs) fazem parte do circuito central de stress, um circuito do sistema nervoso central que regula a resposta ao stress. Desse circuito faz também parte a POA, que apesar de não pertencer ao sistema mesolímico de recompensa, foi escolhida como área candidata devido ao seu importante papel na regulação do eixo HPI.

Neste estudo os genes de ativação imediata (IEG; do inglês *immediate early gene*) foi utilizada como repórteres de atividade sináptica, devido às suas características. Os IEG são expressos de uma forma rápida e transitiva em resposta a estímulos extracelulares, sem a necessidade de síntese *de novo* de proteínas, pois utilizam factores de transcrição expressos constitutivamente. Nos neurónios, existe alteração de expressão de IEGs após ocorrer uma sinapse e por essa razão estes genes são bons repórteres de atividade sináptica. Os genes escolhidos foram o *c-fos* e o *egr-1*, IEGs que codificam factores de transcrição (existem também outros IEGs que codificam para proteínas executoras), pois ambos têm o pico máximo de expressão aos 30min após o final do estímulo. Vários estudos demonstraram que o stress altera a expressão de IEGs no cérebro, pelo que é possível que diferentes *coping styles* possuam diferentes padrões de ativação cerebral.

Este trabalho teve como objectivo averiguar se diferentes *coping styles* possuem diferentes padrões de ativação neuronal em zonas específicas do cérebro importantes para a regulação do stress e avaliação de estímulos. Para tal,
recorreremos à expressão de IEGs como repórteres de atividade sináptica. A espécie utilizada foi o robalo (*Dicentrarchus labrax*), uma espécie que tem tido cada vez mais importância a nível comercial, com o consumo de robalo a aumentar imenso nas últimas décadas, tanto o robalo selvagem como o criado em cativéiro, ou seja em aquacultura. A importância dos *coping styles* de animais criados em aquacultura prende-se com o facto do bem estar animal. Vários estudos têm apontado que os indivíduos proactivos têm uma maior facilidade em ser criados em cativéiro do que indivíduos reactivos que, devido às suas características, têm maior dificuldade em se alimentar, passando por longos períodos de jejum. Uma triagem preliminar de indivíduos proactivos e reactivos assim como estabelecer um rácio de indivíduos de cada um dos *coping styles* seria óptimo tanto para os animais como para os comerciantes, pois teriam menos perdas de animais.

A experiência comportamental deste trabalho foi realizada por outro laboratório e é consistido por duas partes: (i) triagem de *coping styles*, onde os indivíduos foram caracterizados como proactivos, intermédios e reactivos e (ii) uma experiência de stress onde indivíduos de cada *coping style* ficaram em confinamento durante trinta minutos e depois esperaram outros 30 minutos de forma a atingirem o pico de IEGs antes de serem sacrificados. Indivíduos controlo de cada *coping style* eram sacrificados sem passar pela experiência de stress.

Os resultados da expressão génica mostraram que o *c-fos* é um melhor repórter que o *egr-1* para esta situação de stress e para esta população de robalo. Para além disso, quando analisando cada área individualmente, existe efeito do stress, com aumento de expressão génica na Dld e diminuição na Vv, Vs e POA nos indivíduos stressados em relação aos controlos, o que sugere um aumento de atividade neural na Dld e um decréscimo de atividade na Vv, Vs e POA. No entanto, não existe efeito do *coping style*. A Vv, Vs e POA são áreas com um efeito inibitório do núcleo préoptico (NPO), onde se encontram os neurónios que libertam CRF (do inglês *Corticotropin-Releasing Factor*) e desencadeia a ativação do eixo hipotálamo-pituitária-interrenal (HPI; homólogo ao eixo HPA em mamíferos). Propomos então que, numa situação de stress, em conjunto com a ativação de áreas que promovem a atividade do NPO, há uma diminuição de atividade destas áreas inibitórias de forma a reforçar a ativação do NPO.

Quando analisamos cada fenótipo composto (i.e. combinação do tratamento e *coping style*) observamos uma diferença entre controlo e stress apenas para a POA dos indivíduos intermédios. Propomos então que os *coping styles* mais extremos (i.e. proactivos e reactivos) têm uma expressão de IEGs mais rápida e
transiente do que os indivíduos intermédios e que a POA poderá ser a última área a voltar ao estado basal numa resposta ao stress.

Finalmente, analisámos o estado neurogenómico, i.e. o número e padrão de co-activação das áreas cerebrais estudadas. Com esta análise voltámos a verificar que o c-fos é melhor repórter que o egr-1 nesta situação específica, pois apenas para o c-fos o estado neurogenómico era preditor tanto do coping style como do estado de stress do indivíduo. Tanto quanto sabemos este resultado é a primeira demonstração da teoria da rede de tomada de decisão social (do inglês social decision making network), onde o sistema mesolímico de recompensa é parte integrante, de que um fenótipo é melhor explicado pela interação da rede neuronal, do que observando cada área isoladamente.

**Palavras chave:** coping style; genes de ativação imediata; stress; padrões neurogenómicos; *Dicentrarchus labrax*
Abstract

Animals from the same populations react differently to a stressor. This reaction is both behavioural and physiological, with each group that react differently presenting a set of traits that is different from the other group and consistent over time and across situation, and is termed coping style. A distinction is usually made between two coping styles: proactive and reactive. Besides these behavioural and physiological differences, the patterns of brain activation of each coping style were never studied. In the present study the immediate early genes (IEGs) c-fos and egr-1 were used as a proxy of neural activity in brain areas from the mesolimbic reward system, the neural circuit responsible to code the stimulus’ salience – different coping styles show different salience to the same stimulus –, and the central stress circuitry, the circuitry from the central nervous system that control the stress response. c-fos mRNA expression was higher in Dld and lower in the Vv, Vs and POA of the stressed individuals when compared to the control ones, suggesting an increase of Dld activity and a decrease of Vv, Vs and POA activities. The mammalian homologues of the last three areas are known to have an inhibitory effect in the hypothalamic–pituitary–adrenal (HPA) axis and it is proposed that the decrease of activity in these areas during a stress may contribute to increase hypothalamic–pituitary–interrenal (HPI) – fish HPA homologue – reactivity. Furthermore, the neurogenomic state for each coping style was assessed for both stress and control individuals. These results showed that the neurogenomic state was predictive of both coping style and the stress state of the individual. To our knowledge, this is the first time that the Social Decision Making network theory that the analysis of the network of brain areas is a better explainer of the phenotype than the analysis of each area alone.

Key words: coping style; immediate early gene; stress; neurogenomic state; Dicentrarchus labrax
# Table of Contents

## Introduction
- 1.1. Coping Styles ........................................................................................................ 1
- 1.2. Regions of interest in the brain ........................................................................... 5
- 1.3. Immediate Early Genes .................................................................................. 8
- 1.4. *Dicentrarchus labrax* ..................................................................................... 11
- 1.5. Main Hypothesis .......................................................................................... 12

## Materials and Methods
- 2.1. Animals ........................................................................................................... 13
- 2.2. Micropunch dissections ................................................................................. 14
- 2.3. mRNA extraction and cDNA synthesis ....................................................... 14
- 2.4. Primers Design ............................................................................................ 15
- 2.5. Real-time qPCR .......................................................................................... 16
- 2.6. Statistical Analyses ...................................................................................... 17

## Results
- 3.1. Body Size and Weight .................................................................................... 20
- 3.2. Sex and Strain ............................................................................................... 22
- 3.3. Treatment and Coping Style ......................................................................... 23
- 3.4. Neurogenomic States .................................................................................... 26

## Discussion
- 4.1. Neurogenomic States .................................................................................... 37
- 4.2. Final Remarks ............................................................................................... 38

## References
- .......................................................................................................................... 41

## Supplementary Information
- .......................................................................................................................... 51
List of Figures

Fig. 1 – Influence of cellular gene expression by extracellular stimuli ........................................ 9
Fig. 2 – Consumption of seabass throughout XX and XXI centuries ........................................ 11
Fig. 3 – Fork length and weight distribution in the seabass population ...................................... 21
Fig. 4 – Differences of IEG mRNA expression between sexes and strains ................................. 23
Fig. 5 – IEG mRNA expression in control and stressed individuals for each brain area studied ............................................................................................................................................. 24
Fig. 6 – IEG mRNA expression in individuals with different coping styles for each brain area studied ............................................................................................................................................. 24
Fig. 7 – IEG mRNA expression in each composed phenotype for each brain area studied ............................................................................................................................................. 25
Fig. 8 – Heatmap of correlations between c-fos expressions of brain areas in each composed phenotype ............................................................................................................................................. 28
Fig. 9 – Heatmap of correlations between egr-1 expressions of brain areas in each composed phenotype ............................................................................................................................................. 30
Fig. 10 – Co-activation patterns of c-fos mRNA expression in a subset nuclei of the SDMN for each composed phenotype ............................................................................................................................................. 39
Fig. 11 – Co-activation patterns of egr-1 mRNA expression in a subset nuclei of the SDMN for each composed phenotype ............................................................................................................................................. 40
Fig S1 – Telecenphalic regions in the brain of seabass (Dicentrarchus labrax) and the location of micropunches dissections ............................................................................................................................................. 51
List of Tables

Table 1 – Main differences between proactive and reactive individuals .......... 2
Table 2 – Washes performed for mRNA extraction in RNeasy® column ............. 15
Table 3 – List of primers used for quantitative real-time PCR .................................. 16
Table 4 – Univariate main effects of one-way MANOVA for IEGs expression between sexes on each brain area ......................................................................................... 22
Table 5 – Univariate main effects of one-way MANOVA for IEGs expression between strains on each brain area ......................................................................................... 22
Table 6 – Results from planned comparisons between control and stress individuals’ c-fos mRNA expression of each coping style ............................................................................. 27
Table 7 – Results from planned comparisons between control and stress individuals’ egr-1 mRNA expression of each coping style ............................................................................. 27
Table 8 – Comparison between two composed phenotype through QAP Correlations for c-fos and egr-1 .................................................................................................................. 31
Table 9 – Eigenvector centrality for c-fos expression in each brain area for each composed phenotype .................................................................................................................. 32
Table 10 – Eigenvector centrality for egr-1 expression in each brain area for each composed phenotype .................................................................................................................. 32
Table 11 – Density of connections for each composed phenotype for c-fos mRNA expression ......................................................................................................................... 33
Table 12 – Density of connections for each composed phenotype for c-fos mRNA expression ......................................................................................................................... 33
Table 13 – Comparison of densities of connections between groups of two composed phenotypes ..................................................................................................................... 34
Table S1 – Univariate main effects of one-way MANOVA for fork length and weight between sexes .................................................................................................................. 52
Table S2 – Univariate main effects of one-way MANOVA for fork length and weight between strains .................................................................................................................. 52
Table S3 – Univariate main effects of factorial MANOVA for fork length and weight between treatments and coping styles .................................................................................. 52
Table S4 – Planned comparisons results between control and stress individuals’ fork length and weight of each coping style
Introduction

1.1. Coping Styles

Individual differences in behaviour are a widespread phenomena in the animal kingdom (Carere et al., 2010). These differences do not derive from a stochastic combination of different behaviours, but rather result from a consistent set of behaviours associated with each other. This consistency occurs both between- and within-individuals. Between-individual consistency occurs when individuals differ in the set of associated behaviours, which is associated with different physiological traits. Within-individual consistency occurs when individuals show a consistent set of behaviours over time and across situations (Sih and Bell, 2008). When these behavioural and physiological differences that are consistent throughout time and in different situations occur in response to stress, it is called coping style (Koolhaas et al., 1999). In literature, this phenomenon is also referred to as temperament, animal personality, behaviour syndromes, tendencies, axes, strategies, or constructs (reviewed by Réale et al., 2007).

Huntingford (1976) conducted one of the first studies documenting individual variation in behaviour, in which she studied the differences in aggressiveness towards territorial intruders in the three-spined stickleback (*Gasterosteus aculeatus*). She observed that individuals that were more aggressive to conspecific intruders during the breeding season were also more aggressive towards heterospecifics. Additionally, the most aggressive individuals in the breeding season were also the boldest when approaching a predator outside the breeding season. Although the term “coping style” was never applied throughout the article, the study fulfils the definition: the correlation between a consistency over time was observed – the relative levels of aggressiveness and boldness were maintained throughout the breeding season – as well as consistency across situations – the more aggressive individuals towards conspecifics were also more aggressive towards heterospecifics. Furthermore, a correlation between behaviours was found – the more aggressive individuals towards a conspecific were the bolder when approaching predators outside the breeding season.

A distinction is often made between the two strategies of coping: the proactive and reactive. Proactive individuals are aggressive, bold, with a tendency for routine forming and low attack latency. Reactive individuals show lower degrees of aggressiveness and boldness, and are more flexible to changes in the environment than proactive individuals. Moreover, reactive individuals show high attack latency
and freezing behaviour in response to stressful situations (Koolhaas et al., 1999).

**Table 1 - Main differences between proactive and reactive individuals**

<table>
<thead>
<tr>
<th></th>
<th>Proactive</th>
<th>Reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Behavioural Traits</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High levels of aggressiveness</td>
<td>Low levels of aggressiveness</td>
<td></td>
</tr>
<tr>
<td>Bold</td>
<td>Shy</td>
<td></td>
</tr>
<tr>
<td>Low attack latency</td>
<td>High attack latency</td>
<td></td>
</tr>
<tr>
<td>Rigid routine forming</td>
<td>Low levels of routine forming</td>
<td></td>
</tr>
<tr>
<td><strong>Physiological Traits</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low HPA axis reactivity</td>
<td>High HPA axis reactivity</td>
<td></td>
</tr>
<tr>
<td>High sympathetic activity</td>
<td>Low sympathetic activity</td>
<td></td>
</tr>
<tr>
<td>Low parasympathetic activity</td>
<td>High parasympathetic activity</td>
<td></td>
</tr>
<tr>
<td>High post-stress corticosteroid production</td>
<td>Low post-stress corticosteroid production</td>
<td></td>
</tr>
<tr>
<td>Low density of AVP fibers projections to lateral septum</td>
<td>High density of AVP fibers projections to lateral septum</td>
<td></td>
</tr>
<tr>
<td>Low AVP septal release under stress*</td>
<td>High AVP septal release under stress*</td>
<td></td>
</tr>
<tr>
<td>High levels of 5-HT_{1A} receptor mRNA expression in hippocampus</td>
<td>Low levels of 5-HT_{1A} receptor mRNA expression in hippocampus</td>
<td></td>
</tr>
</tbody>
</table>

*Only in mammals. See text to further information.

Besides behaviour, the definition of coping style also comprehends differences in physiological traits (behavioural and physiological traits are resumed in Table 1). Actually, proactive and reactive individuals show differences in their neuroendocrinology, immunity resistance and neural characteristics, as detailed below. Although most of the literature of physiological characteristics of coping styles refers to mammalian examples, whenever possible fish examples are presented and compared with mammalian data.

Reactive individuals have a high reactivity to stressors in one of the major stress systems, the hypothalamic-pituitary-adrenocortical (HPA) axis, when compared to proactive individuals. This characteristic has been documented in mammals (mouse: Veenema et al., 2004; rat: Steimer et al., 1997; rhesus monkey: Suomi, 1997; pig: Hessing et al., 1994) and birds (great tit: Carere and van Oers, 2004) and teleost fish (Øverli et al., 2002; Øverli et al., 2005). In the last example, rainbow trout (*Oncorhynchus mykiss*), were selected for high (HR) and low (LR) plasma cortisol response to stress (Pottinger and Carrick, 1999), i.e. higher or lower reactivity of the HPI axis, the fish homologue of the (HPA) axis (Mommsen et al., 1999). When these selected lines were kept in isolation, which is a stressful situation for them, the authors observed that HR expressed reactive-like behaviours whereas LR expressed
proactive-like behaviours (Øverli et al., 2002; Øverli et al., 2005). Likewise, as in other taxa, proactive individuals showed lower plasma cortisol levels than reactive ones.

Proactive individuals present a strong sympathetic activation (e.g. enhanced heart rate) in response to stressors, whereas reactive individuals present a weak response of the sympathetic nervous system. Once more, these differential activations were found in several taxa, such as mammals (mouse: Henry and Stephens, 1977; rat: Sgoifo et al., 1997; rhesus monkey: Suomi, 1997; pigs: Hessing et al., 1994) birds (chicken: Korte et al., 1998) and teleost fishes (van Raaij et al., 1996). In farmed rainbow trout subject to hypoxic stress, sympathetic nervous system activation was higher in proactive-like individuals in comparison to reactive-like individuals (van Raaij et al., 1996).

Differences in coping behaviour have also been described to have significant impact in disease vulnerability. Kavelaars and colleagues have shown that feral rats with low latency to attack an intruder (i.e. proactive-like behaviour) are more susceptible to contract an experimentally induced autoimmune disease EAE (Experimental Allergic Encephalomyelitis) than individuals that do not attack the intruder (i.e. reactive-like behaviour) (Kavelaars et al., 1999). However, when examining other diseases, proactive individuals are not always the more susceptible coping style. In another study, non-aggressive mice, with low non-social exploration levels and high levels of subordination (i.e. reactive-like behaviours) developed most pulmonary metastases when compared with proactive-like individuals (Vegas et al., 2006). Therefore, some diseases may have greater incidence in proactive individuals whereas other type of diseases has greater effect in reactive ones.

In fish, so far, no evidence has been found relating coping styles to disease vulnerability. However, there is a study that shows indirect evidence of this link since it relates cortisol levels with disease vulnerability. Kittilsen et al. (2012) reported that densely pigmented male rainbow trout present a lower parasitic load than sparsely pigmented individuals and, in a parallel experiment, that densely pigmented fish had lower cortisol levels after a confinement test. These authors suggested that steroid hormones, in particular cortisol, have a role in parasite resistance. Since proactive individuals are characterized by a low HPI reactivity, and therefore, have a lower cortisol response to stress, these results may implicate that proactive rainbow trout had a higher parasite resistance than reactive trout. However, it is important to emphasize that no coping styles were ascertain in this study and therefore the extrapolation between the results and coping styles are merely speculative.
Differences related to coping styles are also observed at the neural level. The neuropeptide vasopressin (AVP), which has been proven to have important roles in the regulation of various emotional and social behaviours (Ferris, 1992), shows differences between proactive and reactive individuals. Proactive individuals show lower density of AVP fibers projecting to lateral septum than reactive individuals in both mice (Compaan et al., 1993) and rats (Everts et al., 1997). In rats, Beiderbeck et al., (2007) showed that when confronted with an intruder, the highly aggressive rats had a significant decrease in septal AVP release. However, when analysing the levels of arginine vasotocin (AVT), the teleost homologue of mammalian arginine vasopressin (AVP), in the forebrain of the rainbow trout, no differences were found between proactive and reactive individuals (Backström et al., 2011). One must note that AVP levels were measured in whole forebrain and not in the ventral part of the ventral telencephalon, which is the putative homologue of the lateral septum in fish (O’Connell and Hofmann, 2011; O’Connell and Hofmann, 2012).

Also at a neural level, different coping styles show differences in the serotonin (5-hydroxytryptamine, 5-HT) system. 5-HT is a neurotransmitter that is also related with regulation of emotions and behaviours. When analysing postsynaptic 5-HT\textsubscript{1A} receptor mRNA expression, Korte et al. (1996) showed that proactive mice expressing more postsynaptic 5-HT\textsubscript{1A} receptor mRNA in the hippocampus than reactive mice (Korte et al., 1996). Also in rats, proactive individuals show higher hippocampal 5-HT\textsubscript{1A} receptor mRNA expression in both pre- and postsynaptic level (Keck et al., 2005).

As far as it is known, no evidence has been found relating 5-HT levels in the dorsolateral telencephalon (the fish homologue to the mammalian hippocampus (O’Connell and Hofmann, 2011; O’Connell and Hofmann, 2012)) and coping styles in fish. However, Øverli et al. (2001) using the HR-LR rainbow trout selected lines mentioned above, showed that LR individuals had higher levels of 5-hydroxyindoleacetic acid (5-HIAA, a serotonin metabolite) than HR in the telencephalon in baseline but not in stressed conditions. Although not comparable with the mammal’s examples, it is interesting that higher basal levels of 5-HIAA were observed in proactive-like individuals (LR), the same coping style where higher levels of 5-HT\textsubscript{1A} receptor mRNA were also found in mammals.

Considering all these differences, especially at neural level, it is expected that individuals of different coping strategies may present dissimilar patterns of brain activation in response to stressors. In the next section we will further address this hypothesis and specify which brain areas are more expected to be relevant for the study of coping styles.
1.2. Regions of Interest in the Brain

Cognitive appraisal of a stimulus, rather than its physical characteristics, is a major determinant of the aversive value of that stimulus to the individual (Koolhaas et al., 1999). In fact, it has been shown that stimulus appraisal varies with personality in humans (Tong, 2010), avoidance reactivity in rats (Steimer et al., 1997) and coping styles in fish (Martins et al., 2011). In the last example, Nile tilapia (Oreochromis niloticus) were conditioned using avoidance learning. Fish previously screened for coping style were placed in a tank divided into two parts by a divider, which were linked by an opening in the divider. The unconditioned stimulus (US) consisted in a grid that would go down the tank, confining the fish to the bottom of the tank, and the conditioned stimulus (CS) consisted in stopping of the water flow to the tank. When the CS was presented, fish had the opportunity to escape from the confinement side of the tank to the safe side of the tank. Reactive individuals spent more time in the safe side of the tank after the CS was presented than proactive ones. The authors suggested that the perception (appraisal) of CS aversiveness was coping style dependent (Martins et al., 2011).

The mesolimbic reward system has been pointed as the neural circuitry where the appraisal of a stimulus occurs (Deco and Rolls, 2005; O’Connell and Hofmann, 2011; Wickens et al., 2007). It has been recently proposed (O’Connell and Hofmann, 2011; O’Connell and Hofmann, 2012) that the mesolimbic reward system is part of a more complex network – the social decision making network (SDMN) – composed of both the reward system and the social behaviour network (SBN) (sensu Newman, 1999). The SDMN takes the idea presented by Newman (1999) that a social behaviour is better explained by the network activation pattern, than by the activation of specific nodes of the network, and provides an adaptive value to the activation pattern of the SBN by merging it with the mesolimbic reward system. Thus, a social behaviour (coddled by the SBN) could be reinforced (by the mesolimbic reward system), and therefore would gain adaptive value (O’Connell and Hofmann, 2011; O’Connell and Hofmann, 2012).

Considering that differences in stimulus appraisal between individuals with different coping styles maybe the basis for the emergence of these behavioural and physiological syndromes, and that the mesolimbic reward system is the main network where stimulus appraisal occurs, this study focused on the activation pattern in response to stressors of selected nodes from this network, namely: the lateral part of the dorsal telencephalon [Dl; divided in the dorsal and ventral divisions of the Dl (Dld
and Dlv, respectively)], the medial part of the dorsal telencephalon (Dm), the ventral nucleus of the ventral telencephalon (Vv), the supracommissural nucleus of the ventral telencephalon (Vs). Although not included in the mesolimbic reward system, the preoptic area (POA) was also sampled, given its key role in the stress response (see below).

In the present study, Dld and Dlv were sampled separately, due to citoarchitectural and functional reasons. Dld is composed of small cells compacted in bundles (Fig S1) and Dlv is composed of larger cells disposed in columns in a more sparse way (Fig S1) (Cerdá-Reverter et al., 2001). Functionally, Dld is considered the fish visual pallium due to its neuroanatomy and electrophysiological evidence (Demski, 2003; Demski, 2013). However, some hodological evidences indicate a multisensory function of this region (Demski, 2013). Dlv is considered the homologue of the mammalian hippocampus (Broglio et al., 2011; Demski, 2013). In a study using two blenniid fish species (Lipophrys pholis and Parablennius parvicornis), females from both species presented greater home ranges and larger Dlv, but not Dld, than males. The authors suggested that this size difference might result from the need for greater spatial ability in females than males, since the former have larger home ranges (Costa et al., 2011). Furthermore, lesions in the Dlv caused impairment of spatial learning but not emotional learning in goldfish (Portavella et al., 2002), similarly to what occurs in hippocampal lesions in mammals (Broadbent et al., 2004; Morris et al., 1982).

Dm is considered to be the fish homologue of the mammalian basolateral amygdala (O’Connell and Hofmann, 2011). This area is functionally homologue of basolateral amygdala both in sensory and emotional processing. Various studies have shown that Dm is involved in the sensory processing of various types of stimuli, such as visual, olfactory, and auditory signals (see Demski, 2013), as it occurs in the mammalian basolateral amygdala (LeDoux, 1995; O’Connell and Hofmann, 2011). Regarding emotional processing, lesions in Dm impairs emotional learning in fish (Broglio et al., 2011; Portavella et al., 2002), such as avoidance learning and pairing between a certain taste and visceral discomfort (Broglio et al., 2011). Lesions in the mammalian basolateral amygdala achieve the same result, i.e. impairment of emotional learning, such as fear conditioning (LeDoux, 1995).

Regarding Vv, it is considered the putative homologue of mammalian lateral septum due to its neurochemical and hodological patterns (O’Connell and Hofmann, 2011; for a more complete review see: Wullimann and Mueller, 2004). Further, in a study with seven species of butterflyfishes (Chaetodontidae), Dewan et al. (2011) demonstrated that aggressive species presented a greater density of AVT-ir axonal
varicosities in Vv than non-aggressive species. In mammals and birds’ species, infusion of AVP in lateral septum resulted in an increase of aggression (Ferris and Delville, 1994; Goodson and Adkins-Regan, 1999). These results may indicate that Vv and lateral septum are functionally homologues as well.

Vs is considered the putative fish homologue of the mammalian extended amygdala (bed nucleus of the stria terminalis and medial amygdala), because of its genetic profile, hodological patterns, and functionality (O’Connell and Hofmann, 2011). In both fish and mammals these areas are associated with aggressive and courtship behaviours. In the bluegill fish (Lepomis macrochirus) there is an increase of aggression after electrical stimulation of Vs (Demski and Knigge, 1971), whereas lesions in the medial amygdala result in decrease of aggressive behaviour in rats (Vochteloo and Koolhaas, 1987).

POA is not part of the mesolimbic reward system. However, it plays a major role in the stress response. In response to a stressful condition, the fish HPI axis is activated and neurons in a sub-region of the POA, the nucleus preopticus (NPO; according to Metz et al., (2004)), release corticotropin-releasing hormone (CRH) that is known to regulate the secretion of ACTH in teleosts (Lederis et al., 1994). ACTH acts on fish interrenal cells that produce cortisol. Cortisol acts as an inhibitor of CRH and ACTH synthesis in NPO cells and pituitary, respectively, leading fish to reach its basal state (for a complete review on fish stress response see Bonga, 1997). Fish POA is the putative homologue of mammalian POA, since they share neurochemical and hodological patterns (O’Connell and Hofmann, 2011). These areas are also thought to be functionally homologues since both areas play a role on courtship and aggressive behaviours. For example, POA lesions decrease aggressive behaviour in Crenilabrus and Diplodus fish species (Bruin, 1980) as it occurs in lesions in mammalian (e.g. rat) POA (Albert et al., 1986). Furthermore, lesions in NPO of killifish (Fundulus heteroclitus) resulted in decrease of spawning behaviour (Macey et al., 1974), and a similar situation is observed in damaged POA male rats that exhibits a decrease sexual behaviours, such as mounts and ejaculations (Arendash and Gorsk, 1983).

Additionally to POA, some of the area that belong to the mesolimbic reward system are also involved in the regulation of HPA axis activity by the central nervous system, the so called central stress circuitry in mammals (Cullinan et al., 2008; Herman and Cullinan, 1997; Senba and Ueyama, 1997). If this circuitry also exists in fish, the areas chosen are relevant not only in assessing the salience and valence of a stimulus, but also in the regulation of the stress response by the central nervous system. Likewise, these areas are good candidate areas where differences brain...
activity would occur between coping styles. In this study immediate early genes (IEG’s) were used as a proxy of brain activation, hence differential IEG’s expression in the above candidate areas was assessed.

1.3. Immediate Early Genes

In eukaryote cells there are at least three pathways through which extracellular stimuli can influence gene expression in a cell nucleus.

Some molecules, such as steroids, can pass through membrane and bind to cytoplasmic receptors. This receptor-ligand complex can directly act on DNA, regulating gene expression (Fig.1). Other molecules that cannot cross the membrane, bind to membrane receptors and activate a second messenger system that will trigger an intracellular pathway that leads to phosphorylation of constitutive transcription factors (e.g. CREB). These transcription factors can directly act on DNA and change expression of primary target genes (Fig.1) or they can regulate gene expression in an “indirect” way (Fig.1). To do so, they transcribe primary response genes, best known as immediate early genes, that code proteins that will act as transcription factors for secondary target genes, or late genes (Hughes and Dragunow, 1995; Morgan and Curran, 1989).

Therefore, immediate early genes (IEGs) can be defined as a subset of genes that are rapidly induced and transiently expressed in response to extracellular stimuli. IEG transcription occurs even in the presence of protein synthesis inhibitors, indicating that de novo protein synthesis is not necessary for the process (Clayton, 2000; Hughes and Dragunow, 1995). For this reason, IEGs form the first genomic response to extracellular stimuli.

The IEG response depends on the gene that is translated. IEG proteins may act as transcription factors (e.g. c-fos and egr-1) or as effector proteins (e.g. arc and homer1). Transcription factors regulate the expression of target genes, denominated late response genes, affecting downstream pathways. In contrast, effector proteins act directly in the cell changing its function and/or structure (Clayton, 2000).

The IEG response is observed in various types of cells (Clayton, 2000). In neurons of the central nervous system (CNS), IEG induction can be caused by membrane depolarization, chemical or electrically induced seizure, brain injury or sensory signals and/or cues (reviewed in Hughes and Dragunow, 1995). After synaptic transmission, an alteration of IEGs basal levels is observed (Sheng and Greenberg, 1990). Hence, IEG expression in the brain has been used as a reliable method of mapping the anatomical regions that are activated given a certain
stimulus.

Figure 1 – Influence of cellular gene expression by extracellular stimuli. Gene expression can be regulated by exogenous stimuli by three different pathways: (1) steroid-receptor complex act as a transcription factor (TF), (2) constitutive transcription factors can act directly as a TF in a target gene or (3) induce IEG expression that will act as a TF of a target gene, called late gene. PK – Protein Kinase (adapted from Hughes and Dragunow, 1995)

The IEGs c-fos and egr-1 (also known as krox-24, ngfi-a, zenk, and zif-268) are the most commonly used genes in brain activation studies (Clayton, 2000; Terleph and Tremere, 2006). Both genes have a peak of expression 30 minutes after stimulation (Bisler et al., 2002; Burmeister and Fernald, 2005; Mello and Ribeiro, 1998). This is an advantage considering that IEGs have a rapid and transient expression and a non-coincident peak of expression could compromise measurement of multiple IEGs.

C-fos belongs to the Fos family composed by C-Fos, FosB, Fra-1 and Fra-2. By itself, c-Fos protein cannot bind to DNA, and so does not act as a transcription factor (Chiu et al., 1988). These proteins need to form heterodimers with members of
Jun family (c-Jun, JunB and JunD), through leucine zipper interactions, forming AP-1 (activator protein-1) that will act as a transcription factor (Chiu et al., 1988; Herdegen and Leah, 1998). The AP-1 can promote both up- and down-regulation of target genes, depending on which member of Jun family C-Fos heterodimerizes with: c-Fos/c-Jun transcription factor enhances target genes expression, whereas c-Fos/JunB complex inhibits gene expression (Schütte et al., 1989; Sheng and Greenberg, 1990).

egr-1 belongs to Egr family (Egr-1, Egr-2, Egr-3 and Egr-4), characterized by a three zinc-fingers' DNA-binding domain (O'Donovan et al., 1999). Unlike c-fos, egr-1 does not need to form dimers to act as a transcription factor and is able to regulate its own transcription (Cao et al., 1993).

1.3.1 c-fos and egr-1 as markers of neural activity

Detection of IEGs expression in the brain has been used as a method of mapping the anatomical regions that are activated in response to specific stimuli. For example, using in situ hybridization and/or immunohistochemistry, IEG expression revealed the anatomical areas activated by sensory perception of stimuli such as odor (Asok et al., 2013; Koistinaho and Sagar, 1995; Zangenehpour and Chaudhuri, 2002; Zhu et al., 1995), light perception (Bepari et al., 2012; Guthrie et al., 1993; Hess et al., 1995), or flavor (DiNardo and Travers, 1997; Harrer and Travers, 1996) and as the result of social stimuli (O’Connell et al., 2013). Variation of IEGs expression is also detected in more complex processes such as learning and memory (Dragunow, 1996). Although these studies have been done primarily in mammals, mapping of brain regions responses to exterior stimuli have also been performed in other taxa, such as birds (Mello et al., 1992), amphibians (Hoke et al., 2004) and teleost fishes (O’Connell et al., 2013).

Differences in IEG expression have also been seen after stress induction. Some brain areas such as the dorsomedial telencephalon (putative functional equivalent of amygdala in mammals), the dorsolateral telencephalon (putative functional equivalent of hippocampus in mammals) and the preoptic area show such differences. c-fos and egr-1 (or zif26) play important roles in this process (Cullinan et al., 1995a; Malkani and Rosen, 2000; Rosen et al., 1998; Senba and Ueyama, 1997; Stamp and Herbert, 1999). Furthermore, these two genes encode transcription factors that regulate downstream effector genes, denoted as late response genes, some of them responsible for changing neural physiology (Herdegen and Leah, 1998).
1.4. *Dicentrarchus labrax*

The European seabass (*Dicentrarchus labrax*), hereafter seabass, is a common demersal species of temperate coastal waters. It is distributed all around Europe and North Africa, namely in the Mediterranean Sea, the Black Sea, and the western coast of the Europe and north of Africa, from the British Isles and Norway to Morocco and Canaries (FAO website\(^a\)).

Over the last century, the production of seabass acquired more importance. Since the 1950s until 2011, the capture of seabass had an increase of more than thirty-three-times fold, with 9305 tonnes of fish caught in 2011 (Fig. 2A; FAO website\(^a\)). Fish farming of this species started in the 1960s in France and Italy, and in the late 1970s it was practiced by most of the Mediterranean countries (FAO website\(^b\)). In 2011, aquaculture annual production of seabass reached a new record, with 144365 tonnes of fish produced (Fig. 2B; FAO website\(^a\)). For these reasons, seabass is seen nowadays as a leading species of Mediterranean fish trade.

![Figure 2](image)

**Figure 2 – Consumption of seabass throughout XX and XXI centuries.** (A) Consumption of wild captured seabass and (B) Consumption of aquaculture farmed seabass. (FAO website\(^a\))

One of the major concerns about aquaculture is the welfare of the farmed fishes. Several studies demonstrated differences between domesticated and wild-derived individuals. Overall, domesticated fish tend to be more aggressive and more likely to take risks than wild-derived fishes (Huntingford and Adams, 2005). As mentioned above, this proactive-like coping type is suitable for stable and predictable environments, as is the case of fish farms. Therefore, domesticated fishes tend to perform better in a farm tank than wild-derived fishes. However, not all the domesticated fishes are aggressive and bold. Reactive-like fish have more difficulties to reach food, facing longer periods of fasting (Huntingford and Adams, 2005). This can potentially result in the compromise of fish welfare.

Detection and identification of coping styles in farmed fishes can, therefore, be an advantageous procedure both to fishes and to the producers. By choosing the
right proportions of proactive and reactive fish in the tanks, producers may assure the welfare of the animals and, with well-fed fish, investing in big fishes to sell.

1.5. Main Hypothesis

In the present study, we are interested to ascertain if different coping styles have dissimilar brain activity patterns in both control and stress conditions. To do so, immediate early gene mRNA expression levels were used as a proxy of brain activation.
Materials and Methods

2.1. Animals

A total of fifty-six European seabasses (Dicentrarchus labrax) breed and reared at the experimental research station of Ifremer (Palavas-les-Flots, France) were used in this experiment. The fish were from both sexes (females: n=16; males: n=37), with a wide range of length and weight (length: mean=180.983, σ=17.544; weight: mean=86.302, σ=25.926). Fish derived from a second generation of fishes selected for food deprivation (J) resistance were used in this experiment. J- animals (n=27) lose less weight at feed deprivation and J+ (n=26) lose more weight at feed deprivation (Daulé et al., 2014).

2.1.1. Behavioural Experiment

The behavioural experiment was performed at the laboratory of Prof. Marie-Laure Bégout (Sebastien Ferrari and Marie-Laure Bégout, unpublished data) and consisted in: coping style screening and stress treatment (fish were sampled at 215 and 342dph, respectively).

The hypoxia avoidance test was used for coping style screening, since, from a battery of behavioural tests, it has been shown to be the most reliable to predict coping style (Sebastien Ferrari and Marie-Laure Bégout, unpublished data). The test was performed in an apparatus consisting of two identical fish tanks connected by an acrylic pipe. One of the tanks was normally oxygenated (normoxic tank), whereas the other tank was supplied with nitrogen (hypoxic tank), inducing hypoxia in the tank water by decrease oxygen saturation. Sixty fish were placed in hypoxic tank and had to escape to normoxic tank, where they were immediately netted and rested in a separate tank. Fish were characterized as proactive, intermediate and reactive. The first twenty fish to pass to normoxic tank were considered as proactive, the last fish that did not escaped from hypoxia were referred as reactive, and the remaining fish that escaped from hypoxia after the 20th fish were considered intermediate. This procedure was repeated six times, with a total of 360 fish screened (for this study 20 proactive, 17 intermediate, and 19 reactive fish were used).

For the stress treatment, fish were confined in a net for 30min and then allowed to recover during 30min. Then they were anesthetized (benzocaine, 200ppm), a blood sample was collected, and they were sacrificed with an overdose of benzocaine. Fish from the control group were immediately anesthetized for blood collection and then sacrificed as in the stress group (stress: n=26; control: n=27).
After euthanasia, fish were kept on ice until beheaded, and heads were immediately imbedded in OCT and kept at -80°C until further processing.

2.2. Micropunch dissections

Brain slices were obtained through 150µm-thick cryostat coronal sections. The sections were mounted on pre-frozen microscope slides and then immediately transported to a magnifier on dry ice. The slide was rested in an inverted Petri dish filled with dry ice.

The regions of interest were collected by punching them out of the brain slices with a cannula made from 25G steel needles for Dld, Dlv, Dm and Vs, and 27G steel needles for Vv and POA. To avoid contamination and RNA degradation, needles were cleaned with RNaseZAP™ (Sigma-Aldrich, Hamburg, Germany), followed by ethanol 70%, and finishing with RNase-free water, and dried overnight in a stove. Single needles were used for each brain area, and a new needle set (composed of four 25G cannulae and two 27G cannulae) was used for each fish. The brain areas were identified and classified as in Cerdá-Reverter et al. (2001) (see Fig. S1 for the exact location of punched areas). Punches were blown out of the needle to an aluminium-sheet-covered 1.5mL microtube with 50µL quiazo. Punches of the same brain area were placed together in the same microtube. Microtubes were kept on ice while punching the same brain area and then transferred to dry ice when finished. The samples were stored at -80°C until further use.

2.3. mRNA extraction and cDNA synthesis

Micropunches from the brain tissue were disrupted and homogenized in 100µL QIAzol® Lysis Reagent (1.5mL microtube) using a vortex. The homogenate was incubated at room temperature for 7min and then 50µL chloroform was added. The solution was vigorously shaken for 15s and incubated at room temperature for 5 minutes. After this time the sample was centrifuged at 13000g for 20min at 4°C in order to deposit the cell debris. The supernatant was transferred to a new tube, where 1 volume of 70% ethanol was added and the tube was vortexed for 3 sec.

The sample was then transferred to a RNeasy® column in a 2mL tube and left for 5min at room temperature, after which a series of washes were made in the centrifuge (Table 2).
Table 2 – Washes performed for mRNA extraction in RNeasy® column

<table>
<thead>
<tr>
<th>Washer</th>
<th>Quantity</th>
<th>Velocity</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>non</td>
<td>n.a.</td>
<td>9000g</td>
<td>1 min</td>
</tr>
<tr>
<td>Buffer RW1</td>
<td>700 µl</td>
<td>9000g</td>
<td>1 min</td>
</tr>
<tr>
<td>Buffer RPE</td>
<td>500 µl</td>
<td>9000g</td>
<td>1 min</td>
</tr>
<tr>
<td>Buffer RPE</td>
<td>500 µl</td>
<td>9000g</td>
<td>2 min</td>
</tr>
<tr>
<td>non</td>
<td>n.a.</td>
<td>13000g</td>
<td>3 min</td>
</tr>
<tr>
<td>RNAse-free water</td>
<td>25 µl</td>
<td>9000g</td>
<td>2 min</td>
</tr>
</tbody>
</table>

The flow-through obtained in the last wash (see Table 2) was collected and placed in RNeasy® column again to centrifuge for 2 min at ≥ 9000g. The solution collected was measured in Nanodrop to access mRNA concentration and then was stored at -80°C. Random samples were chosen to check RNA integrity through 2100 Bioanalyzer (Agilent Technologies).

cDNA synthesis was performed with iScript™ cDNA Synthesis Kit from Bio-Rad. For each sample, 4µL of 5x iScript™ reaction mix and 1µL of iScript™ reverse transcriptase was added in a microtube and vortexed. Afterwards, 15µL of RNA template was added to each sample. Samples were incubated in a termocycler in accordance with manufacturer’s instructions (5min at 25°C, 60min at 42°C and 5min at 85°C) and stored at -20°C.

2.4. Primers Design

Primer sets were designed using Primer3 software (Koressaar and Remm, 2007; Untergasser et al., 2012). Primer dimers formation was controlled with FastPCR v5.4 software (Kalendar et al., 2011; Kalendar et al., 2014) and optimal annealing temperature was assessed for maximal fluorescence.

To ensure that primer sets were amplifying the sequence of interest an agarose gel electrophoresis was conducted in order to ascertain if the PCR product had the expected size (data not shown). Furthermore, to confirm the proper DNA sequences were amplified, samples were sequenced at the Genomic Unit of Instituto Gulbenkian de Ciência.

In RT qPCR, an accurate quantification of samples is critical regardless of DNA template concentration. To validate that initial template concentration correlates with the Ct value obtained during amplification, a ten-fold dilution series efficiency test was performed. RT-qPCR assays were performed as described in RT-qPCR section of Materials and Methods (see p. 16) with the following DNA template dilutions: 1:1, 1:10, 1:10², 1:10³, 1:10⁴, and 1:10⁵.
Regression lines for each primer set was obtained through the plotting of $\log_{10}(\text{dilution ratio})$ against the cycle threshold (Ct) value obtained in the qPCR. Efficiency (E) was calculated using the regression line's slope resulting from the plot through equation 1:

$$E = 10^{\left(\frac{-1}{\text{slope}}\right)} - 1$$  \hspace{1cm} (1)

Primers were optimized when regression plot had a high coefficient of determination ($R^2 > 0.98$), and when the RT-qPCR dilution series assay had high amplification efficiency (0.9 < E < 1.1) (Taylor et al., 2009).

2.5. Real-time qPCR

To determine gene expression levels in the different brain areas punched, real-time qPCR were performed. For this purpose, primers were designed to measure c-fos, egr-1 (Early growth response gene 1), and a reference gene (the ribosomal gene 18S; Table 3). All primers were commercially synthesized (Sigma-Aldrich, Hamburg, Germany).

### Table 3 - List of primers used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Ta*</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-fos</td>
<td>GCCTGCACCACCTTTTACTTC</td>
<td>AGAGGACTGGTGTTCGCTGT</td>
<td>58°C</td>
</tr>
<tr>
<td>egr-1</td>
<td>GCAGAAGGACAAGAAAGCAGA</td>
<td>GGGTAAGAAGAAGACTGGAGA</td>
<td>57°C</td>
</tr>
<tr>
<td>18S</td>
<td>ATGCGTGCATTATCAGACC</td>
<td>CGAAAGTTGATAGGGCAGACA</td>
<td>62°C</td>
</tr>
</tbody>
</table>

*Ta - Annealing Temperature. GenBank reference: (1)DQ838581.1; (2)FM007218.1; (3)AY831388.1

Real-Time qPCR assays were performed with Maxima SYBR Green/ROX qPCR Master Mix 2X (Thermo Scientific, Waltham, MA, United States of America), prepared as follows: 12.5µL SYBR Green/ROX qPCR Master Mix, 0.2µL forward primer (50pmol/µL), and 0.2µL reverse primer (50pmol/µL) were used. Each reaction consisted of 24µL of master mix and 1µL of cDNA, functioning as template. Quantification of gene expression was measured on Strategene Mx3000p QPCR system, programmed with the following PCR conditions: (i) denaturation (5min at 95°C); (ii) amplification and quantification (40 cycles; 30s at 95°C, 30s at primer-specific annealing temperature, 30s at 72°C with a single fluorescence measurement); and (iii) melting curve assessment (30s at 95°C; 30s at 55°C, followed by an 55–95°C with a heating rate of 0.5°C/s and a continuous fluorescence measurement; 30s at 95°C).
Miner software (Zhao and Fernald, 2005) was used to estimate individual samples Cts and efficiencies.

2.5.1. Relative Quantification of Genes

Relative quantification of mRNA amount for each sample was normalized to reference gene (18S) through equation 2:

\[
(1+E_{\text{Ref}})^{C_{\text{Ref}}}/(1+E_{\text{gene}})^{C_{\text{gene}}}
\]

(2)

where \(E_{\text{Ref}}\) is the efficiency of the reference gene, \(E_{\text{gene}}\) is the efficiency of the gene of interest, \(C_{\text{Ref}}\) is the cycle threshold for reference gene and \(C_{\text{gene}}\) is the cycle threshold for the gene of interest. As suggested, efficiencies of the genes, as well as samples’ Cts, were used when relative quantification was inferred (Zhao and Fernald, 2005).

2.6. Statistical Analyses

In this study, several independent variables were assessed (sex, strain, treatment, and coping style), and some of them were not balanced, and for this reason, there were some variants of the interception of all these variables that was not filled. Therefore, and taking into account the question of this study focus on the effect of treatment in the coping style, one-way MANOVAs were used to assess if there were main effects of sex and strain in the dependent variables studied, and if there were none these two variables would be removed of further analyses. Factorial ANOVAs were used to assess if there were a relationship between treatment and coping styles for the dependent variables studied.

Hence, to test if there were sex or strain differences in fish length or weight, a one-way MANOVA was used for each of these factors, with length and weight as dependent variables and sex (male and female) or strain (J+ and J-) as independent categorical variables. Factorial MANOVA was used for the same purpose for the independent categorical variables treatment (control and stress) and coping style (proactive, intermediate, and reactive). Planned comparisons of least square means were used to assess if there were differences in length and weight between control and stressed individuals for each coping style. The same analyses were performed to evaluate if IEG’s gene expression in the brain could explain the coping styles observed in fish after a stressful situation. Thus, one-way ANOVAs were used to assess if there were differences in gene expression in sex and strain for each area,
with brain areas (Dld, Dlv, Dm, Vv, Vs, and POA) as dependent variables, and sex or strain as independent categorical variables. Factorial MANOVA was also performed to ascertain if IEG’s expression in the brain could explain the behavioural differences observed in fish with different coping styles after a stressful situation, with brain areas (Dld, Dlv, Dm, Vv, Vs, and POA) as dependent variables, and treatment (control and stress) and coping style (proactive, intermediate, and reactive) as independent categorical variables. Planned comparisons of least square means were used to assess the effect of stress (control vs. stress) in each coping style for each brain area. Planned comparisons were used over post hoc analyses, since there were specific comparisons established a priori to be done as well as other combinations that would not be compared (e.g. control proactive vs. stress reactive).

No comparisons were made between areas, either for the same treatment or between treatments, since different amounts of tissue were sampled for each area. Even though target genes were normalized to a reference gene, there was no validation that the reference gene was expressed in the same amount in all brain areas and for this reason normalization of target genes cannot be extended to comparisons between areas of the same phenotype. Comparisons between brain areas of the same coping style but different treatment (e.g. Dld of control proactive vs. Dlv of stress proactive) were not performed since the results from these analyses may not be due to the effect of stress, but come from endogenous differences referred above. This type of comparisons would be only possible if the control and stressed fish were the same.

The neurogenomic state of each composed phenotype (i.e. combination between treatment and coping style) was determined through correlation analysis. Pearson r correlation between gene expression of different brain areas was used as a representation of co-activation of the areas, such that the greater the correlation coefficient, the greater the co-activation between areas. Co-activation patterns were visually represented using heatmaps of the correlation matrices.

To determine if two correlation matrices were related, quadratic assignment procedure (QAP) was used. QAP technique was selected instead of Mantel matrix permutation test, since the former randomly permutes the rows and equivalent columns to create numerous independent matrices where the proprieties of the original matrix are preserved (Borgatti et al., 2013). The significance is determined by comparing the correlation in the observed matrix with the correlations in the generated matrices to ascertain where, in the distribution of generated correlations, the observed correlation is found (Borgatti et al., 2013). P values lower than 0.05
indicate the two matrices compared were related. QAPs were conducted with 5000 permutations and using UCINET 6 software.

Structural analyses of the brain network were conducted using two social network analysis measures: centrality and cohesion. *Eigenvector centrality* was chosen as a centrality measure because, besides taking into account how many connections a node has, it also gives different weights to each connection depending on how well connected the node, from which the connection derives, is (Borgatti et al., 2013). *Density* was the cohesion measure chosen and is the proportion between the number of connections in a network and all the possible connections that network could have (Borgatti et al., 2013). Comparisons of densities between phenotypes were made using a bootstrap t-test with 5,000 sub-samples.

All statistical analyses for gene expression were performed on ln-transformed data in order to fit parametric assumptions. Normality was verified using Shapiro-Wilk’s W test, and skewness and kurtosis values were used to claim normality through the central limit theorem. Homoscedasticity was verified through Bartlett and Levene tests.

Sample sizes varied due to non-amplification of some samples during qPCR or Miner failed to fit exponential phase in some samples.

Statistical analyses were performed using STATISTICA 12 (StatSoft), and network analyses were performed on UCINET 6 (Borgatti et al., 2002). Heatmaps were produced using R, and network representations were produced on Python.
Results

3.1. Body Size and Weight

3.1.1. Sex and Strain

No multivariate main effect was found for all the variables analysed for both fork length and weight (one-way MANOVA; sex: Wilk's $\lambda = 0.895$, $F_{(2,50)} = 2.930$, $p = 0.063$; strain: Wilk's $\lambda = 0.999$, $F_{(2,50)} = 0.034$, $p = 0.955$), although there is a tendency for length and weight to be different depending on the sex. In fact, sex is the only variable in which univariate main effects were either significant or marginally non-significant for fork length and weight, respectively (fork length: $F_{(1,51)} = 5.377$, $p = 0.024$; weight: $F_{(1,51)} = 3.902$, $p = 0.054$; see Table S1-2 for all univariate results). These results suggest that females are larger than males (Fig 3A). These differences may be due to sexual dimorphism in this species. In fact, previous studies demonstrated that, for this species, females are both larger and heavier than males even before 10 months old (Saillant et al., 2001).

3.1.2 Treatment and Coping Style

There was neither a multivariate main effect of fork length and weight for both treatment and coping style (Factorial ANOVA; treatment: Wilk's $\lambda = 0.977$, $F_{(2,46)} = 0.551$, $p = 0.580$; coping style: Wilk's $\lambda = 0.915$, $F_{(4,92)} = 1.039$, $p = 0.391$), nor a significant interaction between the two main factors (Wilk's $\lambda = 0.899$, $F_{(4,92)} = 1.254$, $p = 0.294$). Additionally, no significant univariate main effect was found for all the variables studied (Table S3; Fig. 3C-D). Planned comparisons were used to assess if, within each coping style, there were differences in body length and weight between control and stressed individuals. No differences were observed for each phenotype analysed (Table S4; Fig. 3E); however reactive individuals presented marginally non-significant differences for both length and weight between control and stressed groups.

Taking together, these results suggest that fishes from different lengths and weights are distributed across treatments, and for this reason these two variables may be discarded as relevant variables for further analyses.
Figure 3 – Fork length and weight distribution in the seabass population. These variables were compared between (A) sexes, (B) strains, (C) treatment, (D) coping style, and (E) composed phenotype. Values are means ± SEM. * Significantly different from males (p<0.05).
3.2. Sex and Strain

The distribution of sex and strain across treatments was also incomplete, hence an initial analysis was made to ascertain if there was a relationship between these two variables and the gene expression in each area. For this purpose, a one-way MANOVA test was performed. No significant multivariate main effect was found in any genes, either for sex (c-fos: Wilk's $\lambda=0.884$, $F_{(6,37)}=0.807$, $p=0.571$; egr-1: Wilk's $\lambda=0.956$, $F_{(6,33)}=0.251$, $p=0.955$) or strain (c-fos: Wilk's $\lambda=0.758$, $F_{(6,37)}=1.968$, $p=0.095$; egr-1: Wilk's $\lambda=0.751$, $F_{(6,33)}=1.825$, $p=0.124$). No significant univariate main effect for brain area was found for either sex or strain (Table 4 and 5; Fig 4A and 4B). Therefore, these two variables were discarded in further analyses.

### Table 4 – Univariate main effects of one-way MANOVA for IEGs expression between sexes on each brain area

<table>
<thead>
<tr>
<th></th>
<th>c-fos</th>
<th></th>
<th>egr-1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_{(1,42)}$</td>
<td>$p$</td>
<td>$F_{(1,38)}$</td>
<td>$p$</td>
</tr>
<tr>
<td>Dld</td>
<td>0.611</td>
<td>0.439</td>
<td>0.007</td>
<td>0.934</td>
</tr>
<tr>
<td>Dlv</td>
<td>0.253</td>
<td>0.618</td>
<td>0.013</td>
<td>0.910</td>
</tr>
<tr>
<td>Dm</td>
<td>0.343</td>
<td>0.561</td>
<td>0.197</td>
<td>0.659</td>
</tr>
<tr>
<td>Vv</td>
<td>1.470</td>
<td>0.232</td>
<td>1.173</td>
<td>0.286</td>
</tr>
<tr>
<td>Vs</td>
<td>0.847</td>
<td>0.363</td>
<td>0.397</td>
<td>0.532</td>
</tr>
<tr>
<td>POA</td>
<td>2.603</td>
<td>0.114</td>
<td>0.153</td>
<td>0.698</td>
</tr>
</tbody>
</table>

### Table 5 – Univariate main effects of one-way MANOVA for IEGs expression between strains on each brain area

<table>
<thead>
<tr>
<th></th>
<th>c-fos</th>
<th></th>
<th>egr-1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_{(1,42)}$</td>
<td>$p$</td>
<td>$F_{(1,38)}$</td>
<td>$p$</td>
</tr>
<tr>
<td>Dld</td>
<td>0.192</td>
<td>0.663</td>
<td>0.348</td>
<td>0.559</td>
</tr>
<tr>
<td>Dlv</td>
<td>1.245</td>
<td>0.271</td>
<td>0.544</td>
<td>0.465</td>
</tr>
<tr>
<td>Dm</td>
<td>0.009</td>
<td>0.927</td>
<td>0.020</td>
<td>0.887</td>
</tr>
<tr>
<td>Vv</td>
<td>2.350</td>
<td>0.133</td>
<td>2.443</td>
<td>0.126</td>
</tr>
<tr>
<td>Vs</td>
<td>2.194</td>
<td>0.146</td>
<td>1.673</td>
<td>0.204</td>
</tr>
<tr>
<td>POA</td>
<td>2.373</td>
<td>0.131</td>
<td>1.415</td>
<td>0.242</td>
</tr>
</tbody>
</table>
3.3. Treatment and Coping Style

To determine if different coping styles had differential gene expression, both in a control and in a stressed state, a Factorial MANOVA was used. The stress treatment had a significant and a close-to-significant multivariate main effect for c-fos and egr-1, respectively (c-fos: Wilk’s $\lambda=0.456$, $F_{(6,33)}=5.827$, p<0.001; egr-1: Wilk’s $\lambda=0.684$, $F_{(6,29)}=2.230$, p=0.069). This effect was not found either in coping style (c-fos: Wilk’s $\lambda=0.688$, $F_{(12,66)}=1.131$, p=0.351; egr-1: Wilk’s $\lambda=0.689$, $F_{(12,58)}=0.988$, p=0.472) or in the interaction between the two factors (c-fos: Wilk’s $\lambda=0.827$, $F_{(12,66)}=0.549$, p=0.874; egr-1: Wilk’s $\lambda=0.673$, $F_{(12,58)}=1.058$, p=0.411). These results indicate that c-fos expression varies between different stress states but not between coping styles.

3.3.1. Treatment

There was an increase in c-fos mRNA expression for Dld ($F_{(1,38)}=4.350$, p=0.044) and a decrease for Vv ($F_{(1,38)}=5.088$, p=0.030), Vs ($F_{(1,38)}=4.820$, p=0.034), and POA ($F_{(1,38)}=13.489$, p=0.001) in stressed fish when compared to control fish (Fig 5A), suggesting an increase in Dld neural activity a decreased neural activity in Vv, Vs, and POA in response to stress. Variation in mRNA expression levels were not significant for Dlv ($F_{(1,38)}=0.554$, p=0.461), and Dm ($F_{(1,38)}=1.158$, p=0.289) (Fig 5A),
suggested the neural activity in these areas was not different in stressed vs. non-stressed individuals.

**egr-1** mRNA expression decreased in Vs (F(1,34)=6.191, p=0.018) and POA (F(1,34)=5.198, p=0.029) in stressed fish (Fig 5B), suggesting a decreased neural activity in these areas in response to stress. Dld (F(1,34)=0.280, p=0.600), Dlv (F(1,34)=0.159, p=0.693), Dm (F(1,34)=0.389, p=0.537), and Vv (F(1,34)=2.312, p=0.138) showed no significantly differences between treatments (Fig 5B), indicating that these areas have similar neural activity both in stress and control individuals.

3.3.2. Coping Style

There was no significant difference in gene expression either for **c-fos** (Dld: F(2,38)=2.432, p=0.101; Dlv: F(2,38)=1.991, p=0.151; Dm: F(2,38)=1.180, p=0.318; Vv: F(2,38)=2.198, p=0.125; Vs: F(2,38)=1.763, p=0.185; POA: F(2,38)=0.499, p=0.611) or for **egr-1** (Dld: F(2,34)=2.370, p=0.109; Dlv: F(2,34)=1.740, p=0.191; Dm: F(2,34)=1.187, p=0.317; Vv: F(2,34)=0.893, p=0.419; Vs: F(2,34)=0.253, p=0.778; POA: F(2,34)=1.042, p=0.364) in relation to coping style (Fig 6A), suggesting that any coping style shows differential gene expression in each brain area.

![Figure 5](image-url) – IEG mRNA expression in control and stressed individuals for each brain area studied. (A) **c-fos** mRNA expression (B) **egr-1** mRNA expression. Values are means ± SEM. *p<0.05.

![Figure 6](image-url) – IEG mRNA expression in individuals with different coping styles for each brain area studied. (A) **c-fos** mRNA expression; (B) **egr-1** mRNA expression. Values are means ± SEM. No significant differences were found.
3.3.3. Treatment*Coping Style

The interaction between the two factors showed no significant univariate main effect for both c-fos (Dld: $F_{(2,38)}=0.242$, $p=0.787$; Dlv: $F_{(2,38)}=0.337$, $p=0.716$; Dm: $F_{(2,38)}=0.098$, $p=0.907$; Vv: $F_{(2,38)}=0.386$, $p=0.682$; Vs: $F_{(2,38)}=0.376$, $p=0.689$; POA: $F_{(2,38)}=1.047$, $p=0.361$) and egr-1 (Dld: $F_{(2,34)}=0.259$, $p=0.774$; Dlv: $F_{(2,34)}=0.060$, $p=0.942$; Dm: $F_{(2,34)}=0.100$, $p=0.905$; Vv: $F_{(2,34)}=1.182$, $p=0.319$; Vs: $F_{(2,34)}=0.032$, $p=0.968$; POA: $F_{(2,34)}=0.678$, $p=0.514$). These results indicate that gene expression does not vary for each coping style either in control or stress conditions, suggesting that the differences in behaviour of fish with different coping styles during a stressful condition are not explained by IEG expression in the brain areas sampled. Planned comparisons were performed to confirm these results.
3.3.4. Planned Comparisons

For c-fos, after a stressful stimulus, both proactive and reactive fish exhibited no differences in gene expression for all brain areas studied, whereas intermediate individuals showed a decrease of gene expression in POA ($\text{Wilk's } \lambda = 0.798$, $F_{(1,38)}=9.626, p=0.004$) (Fig 7A). All univariate main effects are presented in Table 6.

$egr-1$ differences in gene expression after a stressful situation are identical to c-fos, with proactive and reactive individuals presenting no significant differences and with intermediate fish showing a decrease in gene expression in POA ($\text{Wilk's } \lambda=0.879; F_{(1,34)}=4.679; p=0.038$) (Fig 7B). All univariate main effects are presented in Table 7.

3.4. Neurogenomic States

Neurogenomic states were defined as the number and pattern of co-activations between brain areas. For this purpose Pearson’s $r$ correlations of IEG expression between pairs of brain regions were used as a measurement of co-activation, and the value of IEG expression at each region as a measurement of how active that area was. The neurogenomic states for each treatment are represented as heatmaps in Fig. 8 and 9, for c-fos and $egr-1$, respectively.

In c-fos, co-activations between brain areas were found in almost all neurogenomic states (Fig. 8). In control, non-stressful, condition, proactives showed positive correlation between Vv and Vs ($r=0.845, p=0.008$), and close-to-significant positive correlation between Vs and POA ($r=0.707, p=0.050$), and close-to-significant negative correlations between Dld and Dlv ($r=-0.653, p=0.079$), and Dld and POA ($r=-0.647, p=0.083$). Intermediate fish presented positive correlations between Dld and Dlv ($r=0.923, p=0.003$), Dld and Vv ($r=0.918, p=0.003$), Dld and POA ($r=0.842, p=0.018$), Dlv and Vv ($r=0.838, p=0.019$), Dlv and POA ($r=0.803, p=0.030$), Dm and Vv ($r=0.862, p=0.012$), Dm and Vs ($r=0.899, p=0.006$), Dm and POA ($r=0.808, p=0.028$), Vv and POA ($r=0.765, p=0.045$), and a close-to-significant positive correlation between Dld and Dm ($r=0.724, p=0.066$), and between Vv and Vs ($r=0.728, p=0.064$). In reactive individuals positive correlations were found between Dld and Dlv ($r=0.892, p=0.003$), Dld and POA ($r=0.786, p=0.021$), and Dm and Vv ($r=0.960, p<0.001$), and close-to-significant positive correlations between Dlv and POA ($r=0.641, p=0.087$), and Dm and POA ($r=0.651, p=0.080$).
Table 6 – Planned comparisons results between control and stress individuals’ c-fos mRNA expression of each coping style. Significant values are highlighted in bold.

<table>
<thead>
<tr>
<th></th>
<th>Proactive</th>
<th>Intermediate</th>
<th>Reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wilk's λ</td>
<td>F(1,38)  p</td>
<td>Wilk's λ</td>
</tr>
<tr>
<td>Dld</td>
<td>0.978</td>
<td>0.853 0.362</td>
<td>0.978</td>
</tr>
<tr>
<td>Dlv</td>
<td>0.979</td>
<td>0.811 0.373</td>
<td>0.999</td>
</tr>
<tr>
<td>Dm</td>
<td>0.997</td>
<td>0.112 0.740</td>
<td>0.979</td>
</tr>
<tr>
<td>Vv</td>
<td>0.981</td>
<td>0.731 0.398</td>
<td>0.972</td>
</tr>
<tr>
<td>Vs</td>
<td>0.975</td>
<td>0.989 0.326</td>
<td>0.915</td>
</tr>
<tr>
<td>POA</td>
<td>0.955</td>
<td>1.784 0.190</td>
<td><strong>0.798</strong></td>
</tr>
</tbody>
</table>

Table 7 – Planned comparisons results between control and stress individuals’ egr-1 mRNA expression of each coping style. Significant values are highlighted in bold.

<table>
<thead>
<tr>
<th></th>
<th>Proactive</th>
<th>Intermediate</th>
<th>Reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wilk's λ</td>
<td>F(1,34)  p</td>
<td>Wilk's λ</td>
</tr>
<tr>
<td>Dld</td>
<td>0.999</td>
<td>0.046 0.832</td>
<td>0.997</td>
</tr>
<tr>
<td>Dlv</td>
<td>0.998</td>
<td>0.064 0.802</td>
<td>1.000</td>
</tr>
<tr>
<td>Dm</td>
<td>1.000</td>
<td>0.001 0.973</td>
<td>0.996</td>
</tr>
<tr>
<td>Vv</td>
<td>0.978</td>
<td>0.753 0.392</td>
<td>0.999</td>
</tr>
<tr>
<td>Vs</td>
<td>0.918</td>
<td>3.034 0.091</td>
<td>0.953</td>
</tr>
<tr>
<td>POA</td>
<td>0.984</td>
<td>0.552 0.463</td>
<td><strong>0.879</strong></td>
</tr>
</tbody>
</table>
Regarding stressed individuals, proactives had positive correlations between Dld and Dlv (r=0.862, p=0.006), Vv and Vs (r=0.734, p=0.038), and Vs and POA (r=0.719, p=0.045), and close-to-significant positive correlations between Dm and Vv (r=0.636, p=0.090); intermediates showed positive correlations between Dld and POA (r=0.936, p=0.006), Dlv and Dm (r=0.886, p=0.019), Dlv and Vs (r=0.914, p=0.011), Vv and Vs (r=0.852, p=0.031), and close-to-significant positive correlations between Dlv and Vv (r=0.789, p=0.062), Dm and Vs (r=0.748, p=0.087), and Vv and POA (r=0.789, p=0.062). No significant correlations were observed between areas in reactive individuals.

Figure 8 – Heatmap of correlations between c-fos expression of brain areas in each composed phenotype: (A) control proactive, (B) stress proactive, (C) control intermediate, (D) stress intermediate, (E) control reactive, and (F) stress reactive. *0.05<p<0.1, **0.01<p<0.05, ***0.001<p<0.01, and ****p<0.001
Analysing the entire matrices pattern, in the control matrices (Fig 8A,C,E), proactive had the less number of correlations between areas, and intermediate stands out with a larger number of correlations. In stress matrices (Fig 8B,D,F), there were no correlations in reactive matrix and intermediate matrix had, once again, the major number of correlations.

Concerning egr-1, the majority of neurogenomic states presented co-activations between brain areas (Fig 9). In the control condition, proactive fish presented a positive correlation between Vv and Vs (r=0.937, p=0.001), and close-to-significant positive correlations between Dld and Dlv (r=0.634, p=0.091), and Dlv and POA (r=0.698, p=0.054). In intermediate individuals positive correlations were found between Dld and Dlv (r=0.963, p=0.002), Dld and Dm (r=0.861, p=0.027), Dld and Vv (r=0.911, p=0.012), Dld and POA (r=0.913, p=0.011), Dlv and Vv (r=0.844, p=0.035), Dlv and POA (r=0.843, p=0.035), Dm and Vv (r=0.927, p=0.008), Dm and Vs (r=0.958, p=0.003), Dm and POA (r=0.837, p=0.038), Vv and Vs (r=0.912, p=0.011), and Vv and POA (r=0.834, p=0.039), and close-to-significant positive correlations between Dld and Vs (r=0.761, p=0.079), Dlv and Dm (r=0.794, p=0.059), and Vs and POA (r=0.784, p=0.065). Reactive fish showed positive correlations between Dld and Dlv (r=0.853, p=0.015), Dld and Dm (r=0.774, p=0.041), Dld and POA (r=0.859, p=0.013), and Dm and Vv (r=0.941, p=0.002), and close-to-significant positive correlations between Dld and Vv (r=0.752, p=0.051), and Dlv and POA (r=0.736, p=0.059).

After a stress situation, proactive individuals showed positive correlations between Dld and Dlv (r=0.910, p=0.004), and Vs and POA (r=0.774, p=0.041), and close-to-significant positive correlation between Dlv and Vs (r=0.709, p=0.075).

Intermediates presented positive correlations between Dlv and Dm (r=0.941, p=0.005), Vv and POA (r=0.857, p=0.029), and Vs and POA (r=0.826, p=0.043), and close-to-significant positive correlation between Dld and Dlv (r=0.751, p=0.085), and Vv and Vs (r=0.778, p=0.068). In reactive fish no significant correlation was found, however there were close-to-significant positive correlations between Dm and Vv (r=0.742, p=0.091), and Vs and POA (r=0.764, p=0.077).

Taking into account the entire matrices, in the control condition the proactive fish matrix has the less number of correlations, and intermediate fish matrix has more correlations than the proactive fish matrix. Regarding stress matrices, the intermediate fish matrix has the major number of correlations and reactive fish the lowest number of correlations.
Figure 9 – Heatmap of correlations between egr-1 expression of brain areas in each composed phenotype: (A) control proactive, (B) stress proactive, (C) control intermediate, (D) stress intermediate, (E) control reactive, and (F) stress reactive. *0.05<p<0.1, **0.01<p<0.05, ***0.001<p<0.01, and ****p<0.001

When comparing the matrices of c-fos and egr-1, there are common aspects to be noted. In the control condition, intermediate fish have the largest number of correlations and proactive fish have the lowest number of correlations. In the stress condition, intermediate fish have, again, more correlations and reactive fish are the ones with fewer correlations. Furthermore, for both genes, there is a correlation between Vv and Vs in proactive fish in the control condition (Figs. 8A and 9A); correlations between Dld and Dlv, Dld and Vv, Dld and POA, Dlv and Vv, Dlv and POA, Dm and Vv, Dm and Vs, Dm and POA, Vv and POA in intermediate fish in the
control condition; and correlations between Dld and Dlv, Dld and POA, and Dm and Vv in reactive fish in the control condition (Fig. 8E and 9E). Regarding the stressed condition correlations were found between Dld and Dlv, and Vs and POA in the proactive phenotype (Figs. 8B and 9B); and between Dlv and Dm in the intermediate phenotype (Fig. 8D and 9D).

QAP correlations were used to ascertain if neurogenomic states were significantly different between coping styles within the same treatment, or for the same coping style within different treatments. The neurogenomic states for c-fos presented differences in all comparisons that were made (Table 8), suggesting that each coping style has its own neurogenomic pattern, both in control and stressed conditions, and that different stress states have dissimilar neurogenomic patterns for the same coping style. For egr-1, QAP correlations were significant in two comparisons: control proactive vs. control intermediate (p=0.014), and stress intermediate vs. stress reactive (p=0.037). These results suggest that proactive and intermediate fish in the control condition have neurogenomic states for egr-1 which are not different from each other, and that stressed intermediate and reactive fish also share similar neurogenomic states for egr-1.

Table 8 – Comparison between two composed phenotype through QAP Correlations for c-fos and egr-1. Significant values are highlighted in bold. CP: control proactive, CI: control intermediate, CR: control reactive, SP: stress proactive, SI: stress intermediate, SR: stress reactive.

<table>
<thead>
<tr>
<th></th>
<th>c-fos</th>
<th></th>
<th>egr-1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QAP</td>
<td>p</td>
<td>QAP</td>
<td>p</td>
</tr>
<tr>
<td>CP vs. CI</td>
<td>-0.0976</td>
<td>0.4183</td>
<td>0.6295</td>
<td>0.0142</td>
</tr>
<tr>
<td>CP vs. CR</td>
<td>-0.3057</td>
<td>0.1394</td>
<td>0.2221</td>
<td>0.1784</td>
</tr>
<tr>
<td>CI vs. CR</td>
<td>0.3325</td>
<td>0.1384</td>
<td>0.212</td>
<td>0.2188</td>
</tr>
<tr>
<td>SP vs. SI</td>
<td>-0.1915</td>
<td>0.2835</td>
<td>0.0772</td>
<td>0.3851</td>
</tr>
<tr>
<td>SP vs. SR</td>
<td>0.0581</td>
<td>0.4313</td>
<td>0.0914</td>
<td>0.3969</td>
</tr>
<tr>
<td>SI vs. SR</td>
<td>0.2746</td>
<td>0.1344</td>
<td>0.4901</td>
<td>0.0366</td>
</tr>
<tr>
<td>CP vs. SP</td>
<td>0.3235</td>
<td>0.1212</td>
<td>0.3068</td>
<td>0.11</td>
</tr>
<tr>
<td>CI vs. SI</td>
<td>0.0585</td>
<td>0.3647</td>
<td>0.0731</td>
<td>0.3093</td>
</tr>
<tr>
<td>CR vs. SR</td>
<td>-0.089</td>
<td>0.3913</td>
<td>0.1526</td>
<td>0.2727</td>
</tr>
</tbody>
</table>

3.4.1. Centrality

Eigenvector centrality was used as a measure of centrality to ascertain which brain areas were more or less connected in each brain network (Tables S5 and S6).
| Table 9 – Eigenvector centrality for c-fos expression in each brain area for each composed phenotype |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Control                                         | Proactive       | Intermediate    | Reactive        | Proactive       | Intermediate    | Reactive        |
| Dld                                             | 0.390           | 0.423           | 0.474           | 0.372           | 0.394           | 0.244           |
| Dlv                                             | 0.383           | 0.404           | 0.410           | 0.344           | 0.444           | 0.451           |
| Dm                                               | 0.390           | 0.418           | 0.435           | 0.385           | 0.379           | 0.333           |
| Vv                                               | 0.414           | 0.435           | 0.375           | 0.404           | 0.435           | 0.486           |
| Vs                                               | 0.452           | 0.358           | 0.313           | 0.515           | 0.413           | 0.439           |
| POA                                             | 0.415           | 0.407           | 0.423           | 0.409           | 0.379           | 0.443           |

| Table 10 – Eigenvector centrality for egr-1 expression in each brain area for each composed phenotype |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Control                                         | Proactive       | Intermediate    | Reactive        | Proactive       | Intermediate    | Reactive        |
| Dld                                             | 0.418           | 0.420           | 0.450           | 0.430           | 0.356           | 0.259           |
| Dlv                                             | 0.352           | 0.394           | 0.428           | 0.447           | 0.457           | 0.403           |
| Dm                                               | 0.363           | 0.417           | 0.410           | 0.216           | 0.399           | 0.300           |
| Vv                                               | 0.464           | 0.421           | 0.423           | 0.299           | 0.448           | 0.538           |
| Vs                                               | 0.398           | 0.392           | 0.302           | 0.508           | 0.355           | 0.422           |
| POA                                             | 0.443           | 0.404           | 0.419           | 0.470           | 0.422           | 0.462           |
Regarding c-fos, in the control situation, Vs seems to be the central node in the proactive fish network, since this brain area was the one with higher eigenvector value (0.452); in intermediate fish there was no evident central area, but Vs was the most peripheral area, since it had the lowest eigenvector value (0.358); and in reactive fish Dld was the most central area (0.474) and Vs the most peripheral one (0.313). In stressed individuals, Dld was the most central area in proactive fish (0.515), and the most peripheral area in reactive ones (0.244); in stressed intermediate fish, no clear central and peripheral areas were found.

For egr-1, in control condition, Vv was the most central area (0.464) for proactive fish; no evident central area was found for intermediates; and in reactive fish, Dld was the most central area and Vs the most peripheral. Concerning stressful condition, Vs and Dm were the most central (0.508) and peripheral (0.216) areas, respectively, in proactive individuals; Dld and Vs were the most peripheral areas in intermediates (0.356 and 0.355, respectively); and Vv was the most central area (0.538) in reactive fish, whereas Dld was the most peripheral one (0.259).

3.4.2. Cohesion

Density was used as a measure of cohesion. The results for c-fos and egr-1 were very similar in this respect. For both genes, intermediate coping style presented the highest density in both treatments (Tables 9 and 10), suggesting that intermediate individuals have more areas co-activating than the other coping styles for both treatments.

<table>
<thead>
<tr>
<th>Table 11 – Density of connections for each composed phenotype for c-fos mRNA expression</th>
<th>Table 12 – Density of connections for each composed phenotype for egr-1 mRNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td><strong>Stress</strong></td>
</tr>
<tr>
<td>Proactive</td>
<td>0.429</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.759</td>
</tr>
<tr>
<td>Reactive</td>
<td>0.547</td>
</tr>
</tbody>
</table>

Comparisons between the densities also presented very similar results for both genes. Both c-fos and egr-1 showed that in control conditions all coping styles were significantly different from each other (Table 11), and in a stressful situation intermediates were significantly different from proactive and reactive fish, but proactive and reactive individuals were not significantly different from each other (Table 11). When comparing different treatments for the same coping style,
intermediate and reactive fish were significantly different, but proactive individuals showed no significant differences between control and treatment (Table 11).

Table 13 – Comparison of densities of connections between groups of two composed phenotypes. Bootstrap t-test with 5000 sub-samples was used. Significant values are highlighted in bold. CP: control proactive, CI: control intermediate, CR: control reactive, SP: stress proactive, SI: stress intermediate, SR: stress reactive.

<table>
<thead>
<tr>
<th></th>
<th>c-fos</th>
<th>egr-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t</td>
<td>p</td>
</tr>
<tr>
<td>CP vs. CI</td>
<td>-5.904</td>
<td>0.0002</td>
</tr>
<tr>
<td>CP vs. CR</td>
<td>-1.9405</td>
<td>0.0134</td>
</tr>
<tr>
<td>CI vs. CR</td>
<td>4.9089</td>
<td>0.0002</td>
</tr>
<tr>
<td>SP vs. SI</td>
<td>-4.3111</td>
<td>0.0002</td>
</tr>
<tr>
<td>SP vs. SR</td>
<td>0.6137</td>
<td>0.2769</td>
</tr>
<tr>
<td>SI vs. SR</td>
<td>6.0861</td>
<td>0.0002</td>
</tr>
<tr>
<td>CP vs. SP</td>
<td>0.4546</td>
<td>0.3121</td>
</tr>
<tr>
<td>CI vs. SI</td>
<td>1.9165</td>
<td>0.022</td>
</tr>
<tr>
<td>CR vs. SR</td>
<td>2.1278</td>
<td>0.015</td>
</tr>
</tbody>
</table>

These results suggest that, for both genes, in a control, non-stressful situation all coping styles have dissimilar numbers of connections between the brain areas, but in a stressful situation proactive and reactive fish have similar number of connections, that were different from the intermediates. Furthermore, intermediate and reactive individuals had different number of connections between areas depending on their stress state, but proactive individuals have similar number of connections among brain areas both in control and stressful situations.
Discussion

In this experiment, we aim to ascertain if different coping styles presented unique brain activation patterns in response to a stressor. Expression of the immediate early genes *c-fos* and *egr-1* were used as proxies of neuronal activation in specific brain areas with important roles in the mesolimbic reward system and in the stress response.

The results in the current study demonstrate that stressed individuals have differential gene expression levels when compared with control, non-stressed, individuals, namely an increase of IEG expression in Dld and a decrease in Vv, Vs, and POA. These results may suggest that stress promotes an increase of Dld neural activity and a decrease in Vv, Vs, and POA neural activities. Dld is related primarily with visual processing but some evidences indicate a multimodal sensory function of this area (Demski, 2003; Demski, 2013). The increase of IEG expression in Dld is in accordance with the literature, where studies in mammals showed an increase of IEG expression in sensory-related areas in response to stress. In rat, *c-fos* and *egr-1* expressions increase in the occipital cortex after 30min of a restraint or forced-swim stress (Cullinan et al., 1995b) and in another study restraint stress induced an increase of *c-fos* levels in the somatosensory cortex (Mohammad et al., 2000).

It was expected that IEG expression would increase in POA, since there is an increase in expression of CRF in this area after a stressful situation (Bernier and Craig, 2005; Doyon et al., 2005; Huising et al., 2004). Nevertheless, both *c-fos* and *egr-1* expression decreased after stress. It is extremely unlikely that stress cause an inhibition in CRF releasing neurons in seabass, since this hormone is critical for HPI axis activation, and, subsequently, for the stress response itself. Furthermore, HPI or HPA axis activation is a widespread phenomenon, observed in several taxa of animals (Bonga, 1997; Moore and Jessop, 2003; Siegel, 1980). One more parsimonious explanation is that the tissue collected in POA is not only composed of CRF releasing neurons but also by other neurons with inhibitory function. As mentioned in Introduction, only NPO, a sub-region of POA, is known to release CRF. In the present study all POA was sampled, and due to its dimensions it is possible that peripheral tissue was also sampled. In mammals, both the immediate vicinity (peri-PVN) and a more distal vicinity (other sub-regions of the POA, namely medial POA (Herman and Cullinan, 1997)) of the paraventricular hypothalamic nucleus (PVN) neurons – CRF-releasing neurons known to be activated after a stressful episode (Ulrich-Lai and Herman, 2009) – are known to have inhibitory, GABAergic, projections to PVN (Cullinan et al., 2008; Herman and Cullinan, 1997; Senba and
If these inhibitory vicinity is also present in the fish brain, it is almost certain that the non-NPO, inhibitory neurons collected in the POA were greater than NPO tissue.

In mammals, various studies also demonstrate an inhibitory effect of lateral septum (Vv), and extended amygdala (Vs) on HPA activity through GABAergic extensions to the CRF-releasing neurons of PVN (Cullinan et al., 2008; Herman and Cullinan, 1997; Senba and Ueyama, 1997). If these connections were conserved within taxa and found in fish, the decrease of activity observed in Vv, Vs and POA may result in an ease of the inhibition of NPO, which, together with excitatory projections, may contribute to an enhancement of HPI axis activity.

The decrease of IEG expression after a stress is contradicted by the literature, where several studies in mammals, mostly in rats, showed an increase of IEG expression levels after an acute stress (Cullinan et al., 1995b; Huang et al., 2004; Knapska and Kaczmarek, 2004; Melia et al., 1994; Mohammad et al., 2000). Even in the teleost Astatotilapia burtoni, individuals with higher levels of plasma cortisol, presumably more stressed, presented higher levels of egr-1 in Dm than individuals with lower levels of cortisol (Wood et al., 2011). However, these results may not be comparable with the ones in the present experiment, since the behavioural paradigms were not the same, which may imply differential activation of brain areas. A previous study in Dicentrarchus labrax, where CRF and urotensin I (UI) expression in the POA and caudal neurosecretory system (CNSS) were measured after different stressors, demonstrated that stress response was dependent on the stressor applied (Bernier et al., 2008). Since IEG are used as a proxy of neural activation and CRF expression is stressor-dependent, different behavioural paradigms, i.e. different stressors, may have differential IEG expression.

When analysing each coping style, POA in Intermediate individuals is the only area that responded to stress. One reason for this generalized lack of response may be the fact that the fish were sacrificed 1h after the beginning of the stressor has been applied. The consequences of this time lag are discussed below.

No response was found for proactive and reactive individuals. These individuals represent two extremes of a continuous spectrum of phenotypes, and therefore with a higher level of correlated traits. This lack of potency may result in rapid, almost predetermined responses, so rapid that when animals were sacrificed, IEG levels had returned to basal levels. Conversely, intermediates represent all the other phenotypes of the phenotypic spectrum and therefore include various possible responses. This may result in a more delayed IEG expression. Consequently, this intermediate group was the only one where differences in IEG expression between
stress and control individuals were observed. There is no evidence to point out that POA is the only area differentially expressed depending on the coping style, since the time lag proposed above. However, these results may suggest that POA may be the last area to reach its basal state after a stressful stimulus.

4.1. Neurogenomic States

The patterns of the neurogenomic states for c-fos were unique for each phenotype. Each coping style had its own neurogenomic pattern both in control conditions and after a stressful stimulus. Furthermore, the control and stress neurogenomic patterns for the same coping style were different from each other. These results suggest that the c-fos neurogenomic pattern is predictive not only of the coping style but also of the stressful state of the individual. However, egr-1 neurogenomic patterns were not so informative. Control proactive neurogenomic state was not different from control intermediate, and stress intermediate neurogenomic state was not different from stress reactive. These results, together with the fact that, when analysing the main effect of treatment, c-fos presented the same and even more areas responding to the treatment than egr-1, suggest that c-fos is a better reporter of neural activity under confinement stress for this population of seabass than egr-1. Furthermore, no differences were observed between coping styles when each area was analysed by itself, however when comparing all areas as a network revealed differences between coping styles, as in accordance with the network theory that a phenotype is better understood by the interaction of the brain areas network, rather than by specific brain areas individually. Once again, these differences were more robust for c-fos neurogenomic patterns, reinforcing the suggestion that this gene is a better reporter of neural activity than egr-1.

Another interesting result in the study of neurogenomic states was the fact that Intermediate coping style had the larger number of co-activation between the areas studied for both IEG, compared with proactive and reactive neurogenomic states. Proactive and reactive individuals are characterized by phenotypes with strongly correlated traits, whereas in intermediate individuals the correlations between traits are not so well defined. It is proposed that a positive correlation may exist between the degree of phenotypic plasticity of an individual and the number of co-activations between brain areas.
4.2. Final Remarks

Taken together the results obtained in this study suggest that stress induce a decrease in immediate early gene expression in areas related with the mammalian central stress circuitry followed by a disruption of co-activation of areas that were co-activated in control, non-stressed, situations (Figs. 10 and 11). Furthermore, to our knowledge this study is the first demonstration that a phenotype is better understood by the interaction of the brain areas network, than by the brain areas individually, as it is proposed by the brain networks theory (O’Connell and Hofmann, 2011; O’Connell and Hofmann, 2012).

Fish sampled were very heterogeneous in terms of body length and weight. In future experiments animals of the same size should be used. Animals with different body size and weight usually have different brain size. For example, the teleost brown ghost knifefish (*Apteronotus leptorhynchus*) showed positive correlations between brain weight and body weight, and brain weight and total length. Further, estimated number of brain cells was also positively correlated with both body weight and total length (Zupanc and Horschke, 1995). The results in the supra-cited article reveal the importance of an homogeneous sample regarding weight and length of the fishes used in brain microdissections experiments. In the present study efforts were made to standardize how micropunches were collected. However, these efforts were only successful in the x-axis, but not in the y-axis of the coronal sections, since only one row of punches were collected in the outermost surface of the slices (see Fig S1). Another question for future studies is to ascertain if the variables introduced in the behavioural test had influence in both coping style and immediate early gene expression. For example, Øverli et al. (2006) showed that female rainbow trout move less in a confinement test and start feeding faster than males in a novel environment, suggesting sex differences in response to stressful situations. In the current experiment, such differences were not observed. One reason for this inconsistency may be the fact that the number of males and females wasn’t balanced, neither in each experimental phenotype, nor in the total number of individuals in the current experiment. Finally, the present study was conducted on brain areas presumably relevant to stress coping style. However, other areas of interest may have been neglect. In future studies, techniques such as *in situ hybridization* should be used to identify all the brain areas that are responding differently between coping styles and posteriorly use quantitative techniques, such as qPCR, to accurately measure the IEG expression.
Figure 10 – Co-activation patterns of c-fos mRNA expression in a subset nuclei of the SDMN for each composed phenotype: (A) control proactive, (B) stress proactive, (C) control intermediate, (D) stress intermediate, (E) control reactive, and (F) stress reactive. Circumferences diameters are proportional to the relative gene expression. Connections thicknesses are representative of Pearson’s r results. Green lines are representative of positive correlations (r>0), and red lines represent negative correlations (r<0). Asterisks and plus represent significant and close-to-significant results (‘0.05<p<0.1, *0.01<p<0.05, **0.001<p<0.01, and ***p<0.001).
Figure 11 – Co-activation patterns of egr-1 mRNA expression in a subset nuclei of the SDMN for each composed phenotype: (A) control proactive, (B) stress proactive, (C) control intermediate, (D) stress intermediate, (E) control reactive, and (F) stress reactive. Circumferences diameters are proportional to the relative gene expression. Connections thicknesses are representative of Pearson’s r results. Green lines are representative of positive correlations (r>0), and red lines represent negative correlations (r<0). Asterisks and plus represent significant and close-to-significant results (\*0.05<p<0.1, \*0.01<p<0.05, **0.001<p<0.01, and ***p<0.001).
References


Supplementary Information

Figure S1 – Telecphalic regions in the brain of seabass (Dicentrarchus labrax) and the location of micropunches dissections. (A-G) Coronal sections of the telencephalon of seabass, spaced 400µm from each other. In the right side, illustrative sections adapted from Cerdá-Reverter et al., (2001) highlights the areas of interest. The left side, the specific ranges where each area is sample is represented on Nissl staining images. Larger circles correspond to 25G needles diameter (500µm) and smaller circles correspond to 27G needles diameter (400µm). Areas of interest: dorsal division of the dorsal telencephalon (Dld, red), ventral division of the dorsal telencephalon (Dlv, yellow), ventral nucleus of the ventral telencephalon (Vv, blue), supracommissural nucleus of the ventral telencephalon (Vs, black), preoptic area (POA, orange).
Table S1 – Univariate main effects of one-way MANOVA for fork length and weight between sexes

<table>
<thead>
<tr>
<th></th>
<th>$F_{(1,51)}$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fork Length</td>
<td>5.377</td>
<td>0.024</td>
</tr>
<tr>
<td>Weight</td>
<td>3.902</td>
<td>0.054</td>
</tr>
</tbody>
</table>

Table S2 – Univariate main effects of one-way MANOVA for fork length and weight between strains

<table>
<thead>
<tr>
<th></th>
<th>$F_{(1,51)}$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fork Length</td>
<td>0.069</td>
<td>0.794</td>
</tr>
<tr>
<td>Weight</td>
<td>0.062</td>
<td>0.805</td>
</tr>
</tbody>
</table>

Table S3 – Univariate main effects of factorial MANOVA for fork length and weight between treatments and coping styles

<table>
<thead>
<tr>
<th></th>
<th>Treatment</th>
<th>Coping Style</th>
<th>Treatment*Coping Style</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_{(1,47)}$</td>
<td>$p$</td>
<td>$F_{(2,47)}$</td>
</tr>
<tr>
<td>Fork Length</td>
<td>0.668</td>
<td>0.418</td>
<td>1.215</td>
</tr>
<tr>
<td>Weight</td>
<td>0.976</td>
<td>0.328</td>
<td>1.759</td>
</tr>
</tbody>
</table>

Table S4 – Planned comparisons results between control and stress individuals’ fork length and weight of each coping style

<table>
<thead>
<tr>
<th></th>
<th>Proactive</th>
<th>Intermediate</th>
<th>Reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wilk’s $\lambda$</td>
<td>$F_{(1,47)}$</td>
<td>$p$</td>
</tr>
<tr>
<td>Fork Length</td>
<td>0.974</td>
<td>1.231</td>
<td>0.273</td>
</tr>
<tr>
<td>Weight</td>
<td>0.977</td>
<td>1.127</td>
<td>0.294</td>
</tr>
</tbody>
</table>