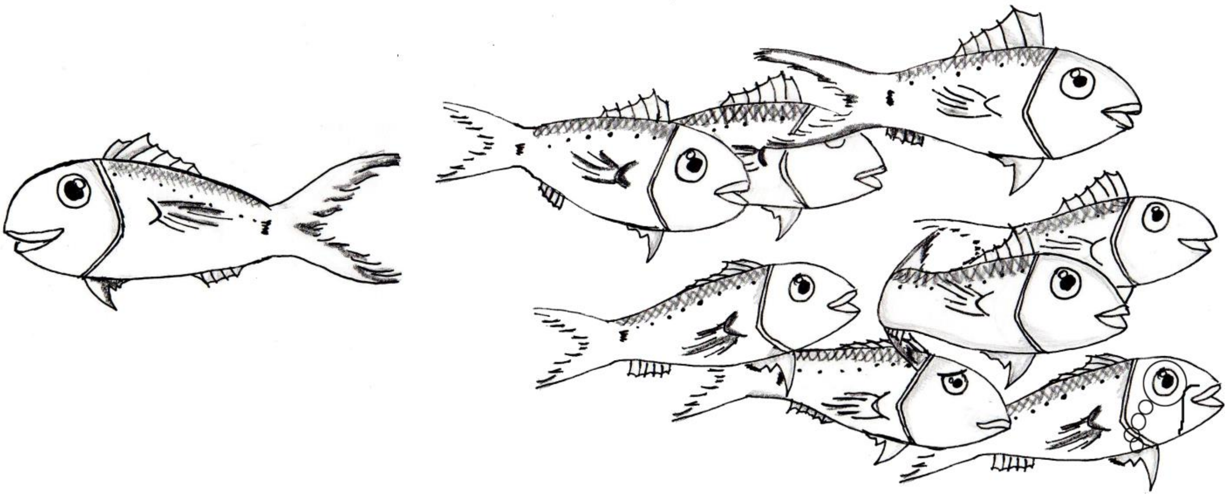
Plasticity of coping styles in farmed fish: Behavioural and Neuro-endocrine profiling



Maria Filipa Bento de Oliveira Falcão Castanheira

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UNIVERSIDADE DO ALGARVE

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Doutoramento em Ciências Biologicas

(Especialidade em Biologia Marinha)

Trabalho efetuado sob a orientação de:

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Declaração de autoria do trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

F Castanheira

Aos meus amores,

Rui e Eva

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SUMMARY

Intensive aquaculture practices frequently expose fish to a range of stressors. Gilthead seabream (*Sparus aurata*) is the most important farmed species in the Mediterranean, and like in other vertebrates, exhibit pronounced individual differences in stress responsiveness, however to which extent such variability is part of coping styles remains unclear. As such, this Thesis aimed to provide methods and tools to analyse coping styles in seabream, with specific reference to the presence, variability and consistency of individual trait correlations relevant to fish welfare. Using an evolutionary approach, it integrated and explored the adaptive links between behaviour, physiology and brain function, especially aiming to explore individual variation in adaptive responses of seabream when exposed to the same stressful situation. The background and consequences of coping styles in aquaculture is introduced in **Chapter 2**. **Chapter 3** aims to quantify individual differences in cortisol response in seabream and to assess whether it can predict aggressive behaviour. Results provided the first evidence for a link between HPI responsiveness and aggressive behaviour in seabream. This suggests that individual differences in aggression are part of coping styles and therefore can be predictive of and predicted from other traits. In **Chapter 4**, it was investigated, for the first time in seabream, whether individual differences in behavioural responses to a variety of challenges are consistent over time and across contexts using both individual and grouped-based tests. Results suggest consistency over time and across-context in behavioural responses to challenges both using individual and grouped-based tests. This study highlights the possibility to predict behaviour in groups from individual coping traits.

One of the traits that has been shown to be consistent over time and across context in the **Chapter 4** was the escape response under a restraining test. Using this trait as a proxy of coping styles in seabream the consistency of escape behaviour was investigated in **Chapter 5** and **6**. In **Chapter 5** we investigated the effect of avoidance in gilthead seabream kept under different social contexts, i.e. the influence of other group members on individual avoidance behaviour consistency. The results demonstrate that grouping individuals with similar coping styles induces changes in coping styles whereas grouping individuals with different coping styles favours coping styles to remain the same. These findings suggest an influence of the social environment in seabream coping styles. In **Chapter 6** we investigate the long term consistency of coping styles both, over time and during different life history stages. Our results show consistent behaviour traits in seabream when juveniles, and a loss of these behavioural traits when adults. Therefore, these results underline that adding a life history approach to data interpretation is as an essential step forward towards understanding coping styles. **Chapter 7** aimed to characterize the stress coping ability and brain function in seabream, by investigating the behavioural and forebrain physiological responses of fish displaying contrasting coping styles to the same stimulus (escape response under a restraining test). Results show differences in activation of region-specific telencephalic regions between seabream displaying contrasting coping styles. It confirms the hypothesis that in seabream, inhibitory and excitatory markers of neural function appear to be associated with reactive and proactive coping styles, respectively. The main findings of this thesis are discussed and the main conclusions are presented in **Chapter 8**. It is concluded that fish with contrasting stress coping styles show clear differences in behavioural and physiological parameters. Moreover, these differences can change according to social environment and life history.

In general this Thesis has generated new knowledge of the mechanisms underlying individual responses of fish to stress providing new insights on the interrelations between different relevant husbandry practices, fish performance and welfare.

Keywords: *Sparus aurata*, aquaculture, behaviour, neural activity, stress response, welfare, personality, individual variation; behavioural syndromes.

RESUMO

A prática de aquacultura intensiva expõe os peixes com frequência a uma variedade de stresses. A dourada (*Sparus aurata*) é, hoje em dia, a espécie mediterrânica de maior importância para o sector da aquacultura, e como outros vertebrados, apresenta evidentes diferenças individuais na resposta ao stress no entanto, a informação que existe a respeito da contribuição destas diferenças individuais na capacidade de resposta ao stress é pouco evidente. Assim, os objectivos da presente Tese consistem na criação de ferramentas e indicadores para analisar e caracterizar a existência de variabilidade individual na dourada, com referência específica à variação e consistência de características individuais relevantes para o bem-estar dos peixes. Para tal usamos uma abordagem evolutiva, e tentamos integrar e explorar as ligações adaptativas entre comportamento, fisiologia e regulação cerebral, com o objectivo de explorar a variabilidade individual na dourada nas respostas adaptativas quando expostas às mesmas situações de stress. A contextualização e consequências da presença de variabilidade individual nos peixes de aquacultura são introduzidas no Capítulo 2. O Capitulo 3 tem como objectivo quantificar as diferenças individuais na produção de cortisol após um *stress* na dourada e avaliar se essa produção é indicativa da variabilidade existente no comportamento agressivo destes peixes que não foram isolados previamente. Estes resultados mostram pela primeira vez uma associação entre comportamentos agressivos e respostas fisiológicas ao stress. Isto sugere que as diferenças no comportamento agressivo fazem parte da variação individual e que podem ser usadas para prever outras características individuais nesta espécie. No Capítulo 4, foi avaliado, pela primeira vez em dourada, se as diferenças individuais nas respostas comportamentais a uma variedade de desafios são constantes ao longo do tempo e em diferentes contextos usando testes individuais e de grupo. Estes resultados mostram características comportamentais constantes nas respostas aos testes individuais e em grupo ao longo do tempo e em diferentes contextos. Este estudo é particularmente interessante porque destaca a possibilidade de prever as características individuais de comportamento em grupo através de diferenças individuais medidas em isolamento.

Uma das características comportamentais individuais que demonstraram ser constantes ao longo do tempo e em diferentes contextos no Capítulo 4 foram as tentativas de fuga ao confinamento dos peixes num camaroeiro fora de água.

Estas marcantes características comportamentais foram usadas como um factor indicativo da existência de variabilidade individual na dourada nos Capítulos 5, 6 e 7. No Capítulo 5, foi avaliado, a tentativa de fuga ao confinamento na dourada estabulada em diferentes contextos sociais, ou seja, foi avaliado a influencia do grupo social em que o individuo esta inserido na manutenção da resposta comportamental de fuga. Os resultados demonstram que o agrupamento de indivíduos com características comportamentais semelhantes resulta numa mudança na resposta comportamental de fuga no entanto o agrupamento de indivíduos com características comportamentais diferentes resulta numa consistência do comportamento de fuga. Estes resultados sugerem que o contexto social parece desempenhar um papel importante na explicação da variação individual de comportamento na dourada. O Capítulo 6 investiga a consistência da variação individual de comportamento ao longo do tempo e durante distintas fases do ciclo de vida da dourada. Os nossos resultados apresentaram evidencias de traços de comportamento consistentes na dourada quando juvenis, e uma perda dessa consistência comportamental quando adultos. Estes resultados salientam a importância da adição de uma abordagem temporal alargada para a interpretação da variabilidade individual na capacidade de resposta ao stess.

O Capítulo 7 tem como objectivo caracterizar a variação individual de comportamento e regulação cerebral na dourada, por meio da investigação das respostas fisiológicas do prosencéfalo dos peixes que apresentam distintos comportamentos quando submetidos ao mesmo stress (tentativas de fuga ao confinamento). Os resultados apresentaram diferenças na activação das regiões telencefálicas específicas da dourada que apresentam comportamentos distintos ao confinamento. Este facto confirma a hipótese de que na dourada, marcadores inibitórios e excitatórios da função neural parecem estar associados com indivíduos reactivos e proactivos, respectivamente. As conclusões desta tese são discutidos e as principais conclusões são apresentadas no Capítulo 8.

No geral, a presente Tese permitiu gerar novo conhecimento em relação aos mecanismos subjacentes a variação individual de respostas ao stress fornecendo uma nova percepção sobre as consequências da variação individual e as diferentes práticas de produção, desempenho e bem-estar dos peixes.

Palavras-chave: *Sparus aurata*, aquacultura, comportamento, activação neural, resposta ao stress, bem-estar, personalidade, variação individual.

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Neural
Activity
on
Sparrus
Auratus

CHAPTER

1

General Introduction

1. Introduction

1.1 Aquaculture

The trends of global demographics show that the world population is growing and will continue to grow. We are around seven billion today and by 2050 we will reach nine billion: two billion more mouths to feed. The protein demand for all of those hungry mouths is expected to increase, however animal farming requires arable land and fresh water, both of which are in short supply. Increased fisheries do not seem to be a viable option as wild fish populations are already overexploited. Aquaculture as a resource-efficient method to produce protein-rich food from animals is a strong candidate to meet this protein demand. According to FAO 2012 projections, it is estimated that global aquaculture production will need to reach 80 million tonnes by 2050. Nevertheless, doubling aquaculture production without further increasing the industry's sustainability could lead to a doubling or more of environmental impacts. Alongside, and perhaps partly due to this rapid expansion of aquaculture, the welfare of farmed fish has received increasing attention. Fish welfare is an important issue for the industry, not just for public perception, marketing and product acceptance, but also often in terms of production efficiency, quality and quantity (Broom 1998; Southgate and Wall 2001). Stressors in aquaculture are unavoidable and reducing stress is a fundamental goal for successful growth and production as well as welfare. However, fundamental insights are lacking on whether and how fish are coping with acute and chronic stressors in aquaculture rearing conditions.

1.2 Coping styles

The study of consistent individual variation in behaviour, physiology and cognitive/emotional patterns has become a "hot topic" in a wide range of biological disciplines; ranging from evolutionary ecology to health sciences and cultured-systems biology (Cavigelli 2005; Favati et al. 2014; Gosling 2001; Koolhaas et al. 1999, 2010; Korte et al. 2005; Réale et al. 2007; Sih et al. 2004).

In animals, including fish, consistent individual differences in several aspects of stress responsiveness have been associated with differences in behaviour and physiology (Øverli et al. 2007; Réale et al. 2010ab; Silva et al. 2010).

These individual differences may reflect distinct coping styles (often also referred as personalities, temperament, behaviour syndromes, bold/shy continuum). One of the main challenges when addressing the topic of coping styles in fish is the confusion in the literature regarding terminology. Many authors use terms such as behavioural syndromes, personality and temperament as synonyms of coping styles. Despite all these terms share common grounds, such as the recognition that individual differences are biological meaningful, they differ mainly on the range of biological parameters that are included. Coping styles often include both behavioural and physiological responses to stressors (Koolhaas et al. 1999), behavioural syndromes (Sih et al. 2004) include, most of the times, only behavioural differences (not necessarily under stress conditions), while personality include not only consistency in single behavioural traits, but also correlations between multiple traits (Gosling 2001).

Coping styles are shaped by evolution and are adaptive response patterns to challenges in the natural environment. Each individual seems to adopt a certain coping style and the different coping types are present in all kinds of animals (Carere et al. 2005; Koolhaas et al. 1999; Øverli et al. 2004a). In animals, including fish, two main coping styles categories types are recognised: proactive (active coping or bold or 'fight-flight') and reactive (passive coping or shy or 'non-aggressive'). Proactive individuals are behaviourally characterised by territorial control, active avoidance and aggression, and physiologically by low hypothalamus-pituitary-adrenal (HPA axis) reactivity to stress and low parasympathetic reactivity, while sympathetic reactivity is high (Koolhaas et al. 1999). In contrast, reactive individuals are behaviourally characterised by immobility, passive avoidance and low levels of aggression, and physiologically by an increase in HPA reactivity, high parasympathetic reactivity and low sympathetic reactivity (Koolhaas et al. 1999). Furthermore, in a stable environment, proactive individuals might be in advantage, because they are characterised by easily developing routines and a rigid type of behaviour, while reactive individuals might be better equipped to cope with a changing or unpredictable environment, because they are more flexible and react more adequately to environmental stimuli (Benus et al. 1991a; Bolhuis et al. 2004; Ruiz-Gomez et al. 2011; Verbeek et al. 1994).

This thesis will focus on behavioural and neuro-endocrine profiling of coping styles in farmed fish (see review of this thematic in chapter 2) using Gilthead seabream as a model species. This species will be introduced in the next section.

1.3 Seabream

Gilthead seabream (*Sparus aurata* Linnaeus 1758) is a teleost fish from the Sparidae family and Perciformes order constituted by 29 gender and 100 species. Seabream are commonly found in the Mediterranean Sea and along the Eastern Atlantic coast, from Great Britain to Senegal (Moretti et al. 1999). Since it is an euryhaline and eurythermal species, seabream can be found from shallow waters up to depths of 90m. Adult seabream spend most of the year in shallow coastal lagoons, but when autumn approaches mature fish begin migrating towards the open sea into deeper water (25-50m) to spawn (Sanchez-Lamadrid 2004). Spawning occurs in late autumn, and the pelagic eggs are released into the sea (Zohar 1978). During the spring and summer, juveniles migrate to protected coastal waters, where they find better feeding conditions and finally they travel back into lagoons and shallow water, where they form schools (Sanchez-Lamadrid 2004). Their natural diet is preferably carnivorous, predator of benthic species, especially shellfish (bivalves and gastropods), crustaceans, worms and small fish (Arias 1976, 1980; Francescon et al. 1987). Major efforts to breed and mass-produce the Gilthead seabream finally succeeded in the 1980’s (Moretti et al. 1999). Seabream was proved to be a relatively fast growing for a Mediterranean species, and soon was cultured throughout much of the Mediterranean coast. Subsequently, the annual production of gilthead seabream increased regularly until 2000, when it reached a peak of over 87,000 tonnes (FAO 2005). The hatchery production and farming of gilthead seabream is considered success stories of the aquaculture business and currently, it is a major aquaculture commodity in Europe. Seabream is a protandric hermaphrodite that achieves sexual maturity in functional males during the first year of life and in females between 2 and 3 years (FAO 2005). In the nature, the breeding season of this species ranges from October to December. In culture conditions, maturation of the broodstock can be controlled by photoperiod and temperature manipulation (Moretti et al. 1999), making eggs available throughout most of the year.

1.4 Challenges in seabream farming and coping styles

It is well documented that various husbandry conditions during all stages of the life cycle of seabream in intensive culture, can lead to stress, increased disease susceptibility and impaired performance. Handling and high stocking density (>10kg m-^{3}) cause stress, measured as increased plasma cortisol levels (Arends et al. 1999; Laiz-Carrion et al. 2005; Montero et al. 1999; Rotllant et al. 2001; Tort et al. 1996), decreased immune condition (Ortuno et al. 2001) and/or earlier onset and resolution of rigor mortis (Matos et al. 2010). Also, food competition and dominance hierarchies induce high plasma cortisol levels (Cammarata et al. 2012) and immunosuppression in subordinate fish (Montero et al. 2009). Random feeding cause arrhythmic and constantly increased swimming behaviour (Andrew et al. 2004; Sanchez et al. 2009) as well as high plasma cortisol levels, indicating stress (Sanchez et al. 2009). Other factors resulting in stress are noise (Wysocki et al. 2013) and colour of glass gravel (Batzina and Karakatsouli et al. 2012).

However, all of the previous studies, for seabream and also for most of the farmed fish, have been orientated to the study of stress response without considering individual variation in coping styles.

Response to stress can also be correlated to variation in behaviour, as mentioned in the coping styles section. The importance of stress in fish welfare is well known. Stress is an adaptive response to changes, but the detrimental effects of stress are only evident when the sources of stress are unavoidable, prolonged and repeated. Therefore, differences in how animals respond to stress in aquaculture are important because proactive and reactive fish differ in their coping strategies and perception of external events (Korte et al. 2005; Koolhaas 2007, 2008). Therefore, while one individual may interpret a situation as being highly stressful, another may interpret it as mildly stressful or even not at all stressful.

Many of the aquaculture practices such as transport, handling, feeding techniques, human presence, and stocking densities, potentially elicit chronic stress responses. Chronic stress has implications in resistance to disease; it has been found that stress can suppress immune function (Koolhaas 2008); stress also leads to reduction in growth rates and consequently decrease production (Strand et al. 2007).

In seabream, coping styles could be one of the most important factors to explain stress responsiveness in rearing conditions and can be crucial for a better assessment of fish welfare and its future improvement in the industry.

1.5 General aim and research questions

Alongside, and perhaps partly due to this rapid expansion of aquaculture, the welfare of farmed fish has received increasing attention. Gilthead seabream (*Sparus aurata*) is the most important farmed fish species in the Mediterranean, and yet our knowledge on how individuals cope with farming practices is still scarce. Coping styles may be one of the most important factors to explain individual differences in adaptability and stress responsiveness in rearing conditions. Therefore, a general aim of this thesis is to provide methods and tools to analyse coping styles in Gilthead seabream. Such information will provide an increase contribution on the understanding of individual differences in stress responsiveness and comprehension of the underpinning mechanisms involved in coping styles in Seabream. This information may be useful for the industry in the future and will bring the possibility to use farmed fish as simpler models to understand underlying mechanism of coping styles in vertebrates.

This thesis aims at answering the following research questions:

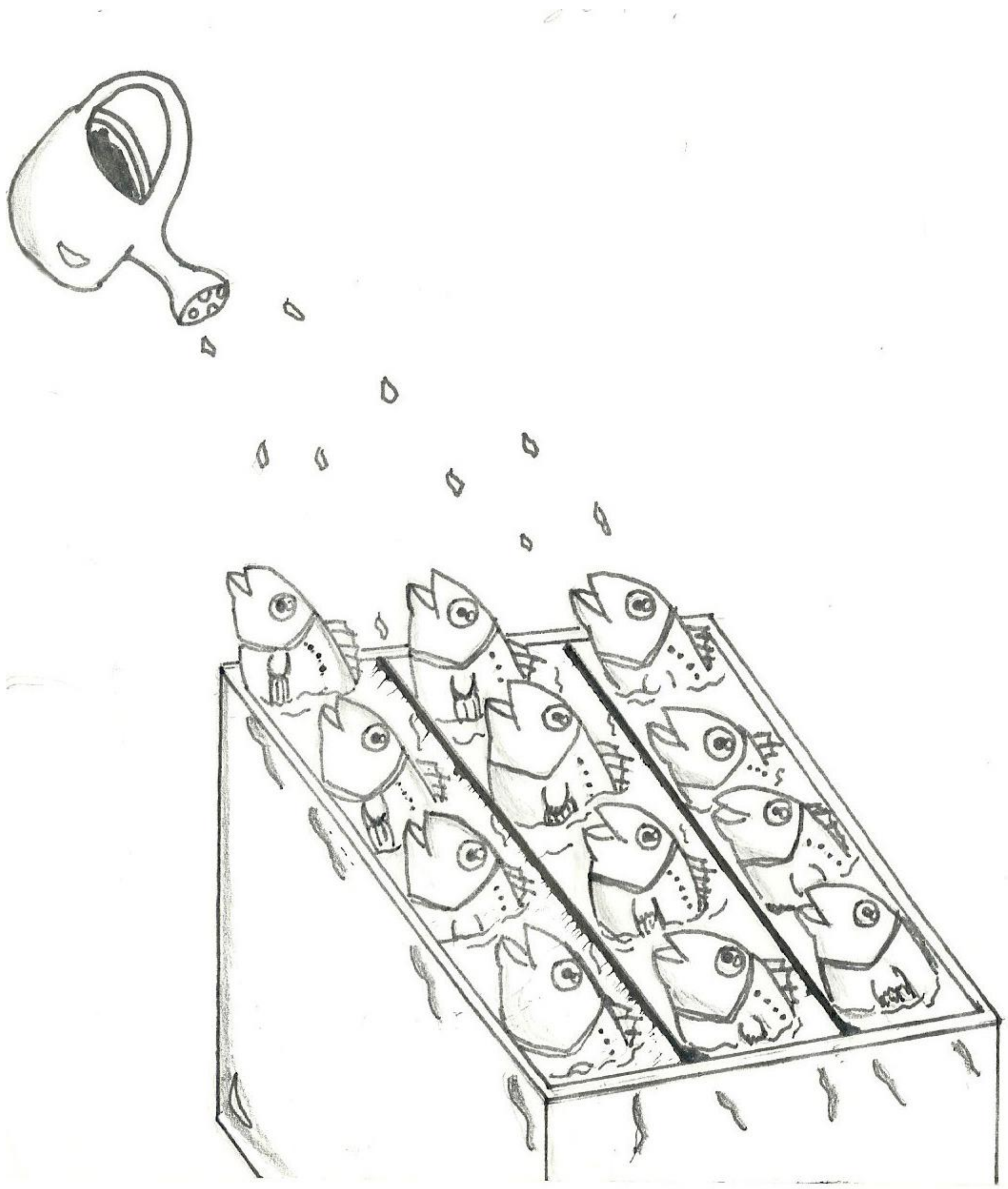
1. Are coping styles present in gilthead seabream?

2. Are coping styles consistent in gilthead seabream?

3. Are coping styles and brain function linked in gilthead seabream?

1.6 Thesis outline

This thesis is composed of a general introduction (**chapter 1**), a review of coping styles in farmed fish (**chapter 2**), five experimental chapters (**chapter 3,4,5,6 and 7**) and a general discussion (**chapter 8**). **Chapter 3** provides the first evidence for a link between HPI responsiveness and aggressive behaviour in seabream. This suggests that individual differences in aggression are part of coping styles. The next step was to determine whether individual differences in behavioural responses to a variety of challenges are consistent over time and across contexts using both individual and grouped-based tests (**chapter 4**). Moreover, one of the bottlenecks in coping styles investigation is related with the consistency of divergent coping styles when exposed to distinct environmental conditions (e.g. social environment, environmental changes). Therefore, in **chapter 5**, we established the consistency of coping styles in fish kept under different social contexts. Moreover, the long term consistency of coping styles in seabream was also investigated (**chapter 6)**, namely the consistency over time (short-term consistency and long-term consistency) of behavioural and physiological responses of fish with divergent coping styles. In addition, the influence of maturation and sex inversion on coping strategies in seabream was evaluated. Once the presence and consistency of coping styles in gilthead seabream were determined, we focused on understanding the underlying mechanism of coping styles in Seabream. Therefore, in **chapter 7** we attempted to establish a link between stress coping ability and brain function, investigating brain differences in fish displaying divergent coping styles. Finally in **chapter 8**, the main findings of this thesis are integrated and discussed in a wider context.



CHAPTER

2

Consequences of coping styles for aquaculture

Coping styles in farmed fish: consequences for aquaculture

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Abstract

Individual differences in physiological and behavioural responses to stressors are increasingly recognised as adaptive variation and thus, raw material for evolution and fish farming improvements including selective breeding. Such individual variation has been evolutionarily conserved and is present in all vertebrate taxa including fish. In farmed animals, the interest in consistent trait associations, i.e. coping styles, has increased dramatically over the last years because many studies have demonstrated links to performance traits, health and disease susceptibility and welfare. This study will review: 1) the main behavioural, neuroendocrine, cognitive and emotional differences between reactive and proactive coping styles in farmed fish, 2) the methodological approaches used to identify coping styles in farmed fish; including individual (group) mass-screening tests and 3) how knowledge on coping styles may contribute to improved sustainability of the aquaculture industry, including welfare and performance of farmed fish. Moreover, we will suggest areas for future research, where genetic basis (heritability/epigenetic) of coping styles, and the neuroendocrine mechanisms behind consistent as well as flexible behavioural patterns are pinpointed as central themes. In addition, the ontogeny of coping styles and the influence of age, social context and environmental change in coping styles will also be discussed.

Keywords: Individual variation, Farm animals, Stress response; Personality; Behavioural syndromes

Introduction

In animals, including fish, individual differences in response to challenges are associated with differences in behaviour (Øverli et al. 2007; Réale et al. 2010a). Many of these associations have been shown to be consistent under stressful conditions and thus to represent coping styles in accordance with the definition by Koolhaas et al. (1999), as 'a coherent set of behavioural and physiological stress responses, which is consistent over time and which is characteristic to a certain group of individuals'. Terminology remains one of the main challenges when addressing the topic of consistent individual variation in physiology and behaviour. Other authors use terms such as behavioural syndromes (Sih et al. 2004), personality (Gosling 2001) and temperament (Francis 1990) more or less synonymously, while physiologists tend to refer to coping styles. These terms and designated definitions, share common grounds such as the recognition that individual variation may be consistent and biologically meaningful, and individual differences in certain behavioural traits are consistent and predictive of other behaviours or physiological responses shown in another context. Typically, in biomedical research and agricultural sciences the term "coping styles" is preferred, while in behavioural ecology the terms behavioural syndromes are more common. The range of biological parameters considered also differs between the different terms. Coping styles often include both behavioural and physiological responses to unfavourable environments and stress (Koolhaas et al. 1999) while behavioural syndromes include only behavioural differences and not necessarily under stress conditions. Personality and temperament, in humans, include essentially emotional reactivity traits. However, when applied to animals, the term personality often ignores the emotional component. Table 2.1 summarises the terminology concerning individual variation. Recognising that both physiological and behavioural traits are important, throughout this review the term "Coping styles" will be used in accordance with the definition by Koolhaas et al. (1999, see above).

In fish, the importance of understanding mechanisms involved in coping styles have gained increasing attention. Especially since, conditions that are well tolerated by some individuals may be detrimental to others, the concept of coping styles are important for their welfare (Huntingford and Adams 2005; Huntingford et al. 2006), health and diseases resistance (Fevolden et al. 1992, 1993; MacKenzie et al. 2009, Kittilsen et al. 2012), performance traits (Martins 2005, Martins et al. 2011ab; Øverli et al. 2006 ab) and interpretations of molecular data (Johansen et al. 2012; MacKenzie et al. 2009; Rey et al. 2013).

Moreover, Martins et al (2011c) showed that coping styles are predictive of how stimuli are appraised, supporting the inclusion of emotional or affective states (in this case fear) as essential component of coping styles in fish. Also Millot et al. (2014a) shown that fish are able to retain memories of events with positive/negative valence which are retrieved by environmental cues.

Table 2.2 summarises the main behavioural and physiological differences between reactive and proactive individuals. Nevertheless, it is imperative to note that the differentiation in coping styles may not be expressed as a binomial distribution in most of the species but rather as a continuous distribution with the majority of individuals expressing intermediate characteristics. In addition, Boersma (2011) suggested that the relative occurrence of contrasting coping styles depends on the type of population (i.e. wild or domesticated). This author showed that in wild populations of rats, the coping strategies of the individuals within a population display a binomial distribution: with extremes proactive vs. reactive individuals. Rats with an intermediate coping style are generally not present in a population in the wild since they have a lower fitness in both stable and new or instable environments. In contrast, Réale et al. (2007, 2010ab) demonstrated a normal distribution of coping styles in the wild in several species. Moreover, in laboratory or domestic settings there is less environmental pressure pushing the population into a bimodal distribution of coping styles. This means that in domesticated population a normal distribution in coping styles is usually observed (Spoolder et al. 1996).

Table.2.1 Summary of the terminology used concerning individual variation

	Terminology	Definition	References
Individual variation and Terminology			
Consistency in behaviour	Personality (temperament)	Those characteristics of individuals that describe and account for consistent patterns in feeling, thinking and behaving.	Francis 1990 Gosling 2001
Correlation between behaviours	Behavioural syndromes	A suite of correlated behaviours reflecting individual consistency in behaviour across multiple situations	Shi et al. 2004
Correlation between behaviour and physiology	Stress coping styles	A coherent set of behavioural and physiological stress responses which is consistent over time and which is characteristic of a certain group of individuals	Koolhaas et al. 1999

Table.2.2 Behavioural and physiological differences between proactive and reactive fish

	Proactive	Reactive	References
Behavioural characteristics			
Actively escape to stressor	High	Low	Silva et al. 2010; Martins et al. 2011c; Brelin et al. 2005; Laursen et al. 2011
Feed efficiency	High	Low	Martins et al. 2005abc, 2006ab; van de Nieuwegiessen et al. 2008
Feeding motivation	High	Low	Øverli et al. 2007; Kristiansen and Fernö 2007
Risk taking and exploration	High	Low	Huntingford et al. 2010; Øverli et al. 2006ab; MacKenzie et al. 2009; Millot et al. 2009ab
Aggressiveness	High	Low	Øverli et al. 2004a, 2005; Castanheira et al. 2013a
Social influence	Low	High	Magnhagen 2007; Magnhagen and Staffan 2005; Magnhagen and Bunnefeld 2009
Sensitive to environmental stressors	Low	High	Höglund et al. 2008
Plasticity/Flexibility/Routine formation	Low	High	Chapman et al. 2010; Ruiz-Gomez et al. 2011
Physiological characteristics			
HPI reactivity	Low	High	Castanheira et al. 2013a; Øverli et al. 2006ab; Trenzado et al. 2003
Sympathetic reactivity	High	Low	Schjolden et al. 2006; Verbeek et al. 2008; Barreto and Volpato 2011
Parasympathetic reactivity	Low	High	Verbeek et al. 2008; Barreto and Volpato 2011
Hormonal modulation	Low	High	LeBlanc et al. 2012
Oxygen consumption	High	Low	Herrera et al. 2014; Killen et al. 2011, Martins et al. 2011d
Myocardial dysfunction	Low	High	Johansen et al. 2011
Neural plasticity	Low	High	Johansen et al. 2012
Immunity	High	Low	Kittilsen et al. 2012

Nowadays, stress coping styles are clearly identified in fish and have contributed to the understanding of individual variation in the ability to cope with stressful events. A consensus is emerging that increased understanding of the consequences of stress coping styles in aquaculture is important to safeguard a sustainable development of this industry.

This study will review: 1) the main behavioural, neuroendocrine, cognitive and emotional differences between reactive and proactive coping styles in farmed fish, 2) the methodological approaches used to identify coping styles in farmed fish; including individual (group) mass-screening tests and 3) how knowledge on coping styles may contribute to improved sustainability of the aquaculture industry, including welfare and performance of farmed fish.

Assessment of coping styles in farmed animals

Land farm animals

Assessment of coping styles in farm animals gained momentum in the late 1980´s. In those studies researchers applied the concept of coping styles to domestic livestock and started to understand how distinct individual traits were related with stress coping under common rearing conditions. For instance, in piglets, the most common test is the “back test” (Hessing et al. 1993, 1994) which consists of restraining each piglet in a supine position for 1 min and classification of pigs is then based on the number of escape attempts made. Other tests commonly used in pigs and other farm animals like cows, cattle and sheep are the open field test (Spoolder et al. 1996; Magnani et al. 2012; van Reenen et al. 2005), the novel object test (Spoolder et al. 1996; Magnani et al. 2012; Spake et al. 2012; Van Reenen et al. 2005), the novel environment test (Hopster 1998) and the resident intruder test (Bolhuis et al. 2005a; Spake et al. 2012). Along with behavioural responses also physiological responses are measured including cortisol responsiveness, heart rate (Korte et al. 1999), gastric ulceration and vocalisation (Hessing et al. 1993; Ruis et al. 2001; van Reenen et al. 2002; van Erp-van der Kooij et al. 2003; Van Reenen et al. 2005; Hopster 1998; Spake et al. 2012).

Several traits attributed to proactive and reactive individuals in land farmed animals have also been identified in fish suggesting that many of such traits have been evolutionary conserved in vertebrates (see references below).

Identifying coping styles in farmed fish

Over the last years, the number of papers addressing coping styles in fish has raised rapidly. Many of these studies address farmed fish including common carp (*Cyprinus carpio*) (MacKenzie et al. 2009; Huntingford et al. 2010), Nile tilapia (Barreto and Volpato 2011; Martins et al. 2011ab), Atlantic salmon (*Salmo salar*) (Kittilsen et al. 2009a, 2012; Vaz-Serrano et al. 2011), Atlantic halibut (*Hippoglossus hippoglossus*) (Kristiansen and Fernö 2007), rainbow trout (Øverli et al. 2005, 2006ab; Schjolden et al. 2005; Ruiz-Gomez et al. 2008, 2011; Höglund et et al. 2008; Laursen et al. 2011), sea bass (Millot et al. 2009ab) and gilthead seabream (Castanheira et al. 2013ab; Herrera et al. 2014) Table 2.2. Like in mammals, two main coping styles are typically recognised: proactive (active coping or bold or 'fight-flight') and reactive (passive coping or shy or 'non-aggressive'). Typically, proactive individuals are behaviourally characterized by: 1) active escape from a stressor (Brelin et al. 2005; Laursen et al. 2011; Silva et al. 2010; Martins et al. 2011c); 2) high feed efficiency (Martins et al. 2005ab, 2006ab; van de Nieuwegiessen et al. 2008); 3) high feeding motivation after transfer to a new environment (Øverli et al. 2007) or food type (Kristiansen and Fernö 2007); 4) high risk taking and exploratory when exposed to novelty (Huntingford et al. 2010; Øverli et al. 2006a; MacKenzie et al. 2009; Millot et al. 2009a); 5) high social rank (dominant) during aggressive encounters (Øverli et al. 2004ab, 2005; Castanheira et al. 2013a); 6) low social influence (Magnhagen 2007; Magnhagen and Staffan 2005; Magnhagen and Bunnefeld 2009); 7) low sensitive to environmental stressors (Höglund et al. 2008); 8) establishment of routines and have less behavioural flexibility (Chapman et al. 2010; Ruiz-Gomez et al. 2011) when compared to reactive individuals. Proactive individuals exhibit typical physiological and neuroendocrine characteristics such as: 1) lower hypothalamus-pituitary-interrenal (HPI) activity (Silva et al. 2010), as measured by basal cortisol levels; 2) lower HPI reactivity (Castanheira et al. 2013a; Øverli et al. 2007; Trenzado et al. 2003), as measured by increase in cortisol over basal levels when stressed; 3) higher sympathetic reactivity and lower parasympathetic reactivity (Verbeek et al. 2008; Barreto and Volpato 2011), measured as opercular beat rate;

4) low hormonal modulation (LeBlanc et al. 2012); 5) higher oxygen consumption during stress (Herrera et al. 2014; Killen et al. 2011; Martins et al. 2011d); 6) lower myocardial dysfunction (Johansen et al. 2011); 7) lower neural plasticity (Johansen et al. 2012) and 8) high immunity (Kittilsen et al. 2012) when compared to reactive individuals.

In addition to behaviour, physiological and neuroendocrine characteristics, proactive and reactive fish have also been reported to differ in cognitive and emotional traits. One of the best examples of the characterization of coping styles in fish comes from studies using selected lines of rainbow trout (*Oncorhynchus mykiss*). These lines were segregated into high- and low-responding individuals (HR, LR) on the basis of their plasma cortisol response after confinement test (Pottinger and Carrick 1999). Studies in these lines demonstrated a link between cognition and coping styles: Moreira et al. (2004) showed that HR-LR individuals differed in memory retention in addition to cortisol responsiveness. The extinction of a conditioned response (i.e. how quickly the conditioned response was lost after the end of reinforcement) was greater among LR individuals.

In accordance with this, Ruiz-Gomez et al. (2011) showed a higher propensity to develop and follow routines (reversal learning) in LR trout. They continue to perform a learned pattern even if the conditions change. LR fish showed slower reversal learning when finding relocated feed, and it was suggested that this reflects a cognitive difference, where LR fish have a stronger tendency to develop and follow routines. This is in accordance with what have been suggested as general differences between proactive and reactive individuals, where reactive individuals react to environmental changes while proactive individuals follow predictions of the actual environment (Coppens et al. 2010).

Various methodologies used to characterise coping styles in fish have been adapted from those used in land farmed animals, an example is the restraining test, which is very similar to the back-test commonly used in pigs. The restraining test in fish consists of holding each individual in an emerged net for a certain limited period depending on the species (Arends et al. 1999; Silva et al. 2010; Castanheira et al. 2013ab). While in the net, the following behaviours have been measured: latency to escape, number of escape attempts and total time spent on escape attempts. Proactive individuals have been shown to exhibit more and longer escape attempts as compared to reactive individuals (Silva et al. 2010; Martins et al. 2011cd). Other tests used in land farmed animals that have been adapted and applied to fish include the novel object test (Frost et al. 2007; Basic et al. 2012), the exploration test (Chapman et al. 2010; Killen et al. 2011; Magnhagen and Staffan 2005; Magnhagen and Bunnefeld 2009) and the resident intruder test (Øverli et al. 2002ab; Brelin et al. 2005).

Recent studies using farmed fish as models have suggested the possibility to discriminate coping styles using grouped-based test (e.g. hypoxia test developed in rainbow trout by Laursen et al. (2011) and adapted to gilthead seabream by Castanheira et al. 2013b). Briefly, the hypoxia test consists of reducing the oxygen levels in one side of a two chambers tank and measuring the escape behaviour from the hypoxia to the normoxia side. Another group-based test is the risk- taking test (or exploration test) which consists of a tank separated in two distinct areas: safe and risk areas. Fish are placed in the safe area (darkened settling chamber), connected by a plastic tunnel or an opening to a risk area (open field). The risk area is usually associated to feed delivery zone to stimulate fish going to the non-familiar area (Millot et al. 2009a; Huntingford et al. 2010; Castanheira et al. 2013b).

Finally, it should be noted that an increasing number of studies also report that contrasting coping styles in fish are reflected in somatic and morphological traits such as developmental rate (Andersson et al. 2011, 2013ab) and pigmentation patterns (Kittilsen et al. 2009ab, 2012; Bäckström et al. 2014). Genetic markers for variable stress resistance are also increasingly explored (Rexroad et al. 2012). Thus, tools to characterise coping styles and personality traits in fish are becoming increasingly available which fulfils an important prerequisite for the effort towards understanding both the biological background and applied potential of this type of individual variation. The presence of coping styles in the most important farmed fish species and the common tests used are presented in Table 2.3.

Table 2.3 A. Summary of the evidence of coping styles in farmed fish and common tests used- Freshwater Fish

Fish Species	Tests	Screening	Observations	References
Freshwater Fish				
Common carp (*Cyprinus carpio*)	Risk-taking, competitive ability	Group	Rate of exploration and competitive ability are consistent over time and related to risk-taking behaviour: individuals that explored more quickly the novel environment were the first to gain access to restricted feed.	Huntingford et al. (2010)
	Risk-taking	Group	Individual differences in behavioural responses, immune condition and baseline gene expression.	MacKenzie et al. (2009)
Nile tilapia (*Oreochromis niloticus)*	Feed intake recovery	Individual	Individual differences in ventilation rate and correlate with the rate of feeding recovery in isolation.	Barreto and Volpato (2011)
	Feed intake recovery	Individual	Proactive individuals seem to exhibit a faster recovery of feed intake after transfer into a novel environment and use feed resources more efficiently.	Martins et al. (2011 ab)
	Feed intake recovery, novel object, restraining	Individual	Inclusion of emotional reactivity (fearfulness) and appraisal as discriminating variables between reactive and proactive individuals.	Martins et al. (2011c)
	Feed intake recovery, feeding behaviour	Individual + Group	Proactive individuals seem to exhibit a faster recovery of feed intake after transfer into a novel environment and use feed resources more efficiently. Feeding behaviour could be used as a predictor of feed efficiency.	Martins et al. (2011ab)
African catfish (*Clarias gariepinus)*	Feed intake, aggression	Individual + Pairwise	Individual differences in residual feed intake are related with differences in aggressive behaviour: more efficient individuals are more aggressive.	Martins et al. (2008)
	Alarm cues, feeding behaviour	Individual	Feeding efficiency (residual feed intake) related with opposite behavioural responses to conspecific skin extract.	van de Nieuwegiessen et al. (2008)
	Escape test	Individual + Group	Behavioural responses to the escape test (after a group-housed period) changed according to the group composition.	van de Nieuwegiessen et al. (2010)
Perch (*Perca fluviatilis*)	Habitat utilisation and feeding activity in visual contact with a potential predator, Risk-taking	Individual + Group	Proactive individuals spent more time in the open field and tended to be faster to enter in unknown environments. Modulation of individual behaviours by other group members.	Magnhagen and Staffan (2005) Magnhagen (2007) Magnhagen and Bunnefeld (2009)

Table 2.3 (continued)

Fish Species	Tests	Screening	Observations	References
Diadromous Fish				
Atlantic salmon (Salmo salar)	High/Low stress response	-	Individual differences in disease resistance in lines selected for high and low post stress plasma cortisol levels.	Fevolden et al. (1993)
	Feeding in isolation, confinement	Individual	HR (more reactive) fish showed increased susceptibility to infectious. Pigmentation profiles are correlated with stress cortisol response. Distinct vulnerability to parasites correlates with pigmentation (high/low black skin spots).	Kittilsen et al. (2009ab, 2012)
	Resume feeding in isolation	Individual	Early emerging individuals showed a shorter time to resume feeding after transfer to rearing in isolation.	Vaz-Serrano et al. (2011)
Rainbow trout (*Oncorhynchus mykiss*)	Aggression	Pairwise	Lower brain serotonergic activity in socially naïve fry with big yolk and higher propensity for social dominance and aggression.	Andersson and Höglund (2012)
	Emergence from spawning gravel	Group	Relationship between characteristics expressed in early development and stress coping styles. The LR fish line has bigger eggs, yolk reserves and faster developmental rate.	Andersson et al. (2013ab)
	Novel object , resident intruder, confinement	Individual	Individual differences were behavioural constant, but no differences were found between LR-HR lines.	Basic et al. (2012)
	Confinement	Group	Inflammatory challenge with bacterial pathogens reported distinct disease resistance between coping styles.	Fevolden et al. (1992)
	Novel object	Individual	Social context is an important modulator of coping styles. Bold fish may be more flexible to changing conditions as opposed to shy individuals.	Frost et al. (2007)
	Initiation of avoidance swimming (larvae)	Individual	Yolk-sac fry originating from the HR strain were more sensitive to environmental stressors, and have shown a shorter reaction time to low oxygen levels.	Höglund et al. (2008)
	Confinement	Individual	HR fish seem to be associated with cardiac remodeling and altered gene expression.	Johansen et al. (2011)
	Confinement, social stress (dominant resident fish)	Individual + Group	Neurobiological mechanism underpinning differences in plasticity associated with distinct coping styles.	Johansen et al. (2012)
	Confinement	Individual	Differences between the HR-LR fish strain in the degree of pigmentation.	Kittilsen et al. (2009ab)
	Hypoxia	Group	Behavioural responses to hypoxia can be used as a non-invasive method for sorting fish according to stress coping styles.	Laursen et al. (2011)

Table 2.3 (continued)

Fish Species	Tests	Screening	Observations	References
Rainbow trout (*Oncorhynchus mykiss*)	Exploratory behaviour, Risk-taking	Individual + Group	The importance of the genetic regulation: isogenic lines with contrasted behavioural responses to a set of environmental stimuli.	Millot et al. (2014b)
	Emersion, confinement	Group	HR-LR individuals differed in memory retention.	Moreira et al. (2004)
	Locomotor activity, smaller conspecific intruder, feed intake	Individual + Pairwise	Behavioural and physiological differences between HR and LR fish established differences in performance.	Øverli et al. (2002ab)
	Aggressive behaviour , feed intake, confinement	Individual + Pairwise	LR fish were more aggressive when placed in a dominant social position.	Øverli et al. (2004a)
	Confinement, locomotor activity, feed intake	Individual	Individual differences in behavioural responses. Synthesis and metabolism of monoamine neurotransmitters and their metabolites were elevated after stress to a larger degree in HR fish.	Øverli et al. (2004b)
	Feed intake recovery, confinement	Individual	Behavioural indicators of stress-coping styles related with sex difference. Immature males resumed feeding after transfer to social isolation quicker than males. Females settling down and ceasing to move in a panic-like manner quicker than males during the confinement.	Øverli et al. (2006a)
	Crowded/ uncrowded conditions	Group	Distinct susceptibility under crowded condition and more feed waste in units containing HR when transported.	Øverli et al. (2006b)
	Feeding behaviour	Individual	Differences in responsiveness to environmental change: LR fish shown to develop routines more easily.	Ruiz-Gomez et al. (2011)
	Feed intake recovery, conspecific intruder	Individual + Pairwise	Behavioural plasticity is limited by genetic factors determining social position in early life. Some behavioural differences can be modified by experience.	Ruiz-Gomez et al. (2008)
	Confinement	Group	Differences between the HR-LR fish in plasma amino acids and liver glycogen concentration.	Trenzado et al. (2003)
	Crowded/ uncrowded conditions	Group	Performance discrepancy between the HR-LR fish related with competitiveness/ aggressiveness. Differences in plasma glucose levels and glycogen levels.	Trenzado et al. (2006)
Brown Trout (*Salmo trutta*)	Feed intake recovery, resident-intruder, hypoxia, confinement	Individual	Individual differences in behavioural responses on resident-intruder, hypoxia and confinement. No differences in feed intake recovery.	Brelin et al. (2005)

Table 2.3 (continued)

Fish Species	Tests	Screening	Observations	References
Marine Fish				
Gilthead Sea bream (*Sparus aurata*)	Restraining, aggression	Individual + Pairwise	Fish with lower cortisol levels (proactive) when exposed to stress are more aggressive.	Castanheira et al. (2013a)
	Feed intake recovery, novel object, restraining, risk-taking	Individual + Group	Behavioural differences are consistent over time and predictable based on other behaviours. Possibility to predict behaviour in groups from individual personality traits.	Castanheira et al. (2013b)
	Risk-taking, hypoxia	Individual + Group	Risk-avoiders (reactive) behaviours were negatively correlated to movement and oxygen consumption rates in metabolic chambers.	Herrera et al. (2014)
	Feed intake recovery, exploration, restraining, risk-taking, hypoxia	Individual + Group	Behavioural differences were not consistent over time or across context in individual-based tests. In contrast, strong individual consistency was observed for all variables measured in group based tests. Hypoxia-avoiders had lower cortisol rate, higher activity and were higher risk-takers: the 3 characteristics of proactive coping style	Ferrari et al. (2014)
Sea bass (*Dicentrarchus labrax*)	Exploration + swimming activities after a stimulation	Individual	Whatever the level of domestication and selection for growth fish presented the same flight response and stimulus exposure induced a significant decrease in exploratory behaviour and swimming activity. Only one generation of captivity could be sufficient to obtain fish presenting the same coping style characteristics (bolder) than fish reared for at least two generations.	Millot et al. (2009a)
	Risk-taking	Group	Wild fish were generally bolder than selected fish during two first days of test but showed a decrease in risk taking behaviour during a third day test. Selected fish showed a constant increase in their risk-taking behaviour over time.	Millot et al. (2009b)
Senegalese sole (*Solea senegalensis*)	Feed intake recovery, restraining	Individual	Proactive fish exhibit shorter feeding latency, higher duration of escape attempts and lower undisturbed cortisol levels than passive individuals.	Silva et al. (2010)
	Restraining	Individual	Individual differences in metabolism are predictive of distinct coping styles.	Martins et al. (2011d)
Sole (*Solea solea*)	Novel environment, light avoidance, feeding efficiency	Group + Individual	Proactive fish (high swimming activity) were most feed efficient and grew faster.	Mas-Muñoz et al. (2011)
Halibut (*Hippoglossus hippoglossus*)	Swimming behaviour, feed intake	Group	Reactive individuals were unable to adapt, or adapted very slowly, to floating feed showed decreased feed intake and increased stereotypic (surface swimming) activity – reflects high routine formation.	Kristiansen and Fernö (2007)

Consistency and plasticity of coping styles in farmed fish

One of the major gaps in the literature concerning the characterization of coping styles in animals, including fish, is the lack of knowledge on the consistency of individual differences. This includes knowledge on both contextual consistency i.e. 'the extent to which scores for behaviour expressed in one context are correlated across individuals with scores for behaviour expressed in one or more other contexts, when behaviour in all of the contexts is measured at the same age and time' and temporal consistency i.e. 'the extent to which scores for behaviour in a given context at a given time are correlated across individuals with scores for the same behaviour in the same context at a later time' (Stamps and Groothuis 2010).

Studies in the HR/LR rainbow trout lines showed that proactive and reactive individuals exhibit consistent traits. Over a period of 7 days, feeding responses after transfer into a novel environment, responses to a novel object, aggressiveness and responses to confinement were behaviourally constant, but no differences between lines were apparent (Basic et al. 2012). The ontogenic consistency of these traits where also demonstrate by Höglund et al. (2008) and Andersson et al. (2011, 2013ab).

However, most of the studies on coping styles characterization have been done on selected HR -LR fish lines (Øverli et al. 2005, 2007) which raises the question whether similar consistency responses can be observed in non-selected populations. In line with the previous information, recent studies on non-selected populations seem to support the consistency of behavioural responses both over time and across-context. Castanheira et al. (2013b) using a non-selected population of gilthead seabream (*Sparus aurata*) showed that individual differences in risk-taking behaviour and escaping behaviour in response to stressors are consistent over a period of 15 days. Moreover the same authors also showed that some behaviour can be used to predict other behaviours expressed in a different context (e.g. individuals that took longer to recover feed intake after transfer into a novel environment, exhibited higher escape attempts during a restraining test and escaped faster from hypoxia conditions).

Consistency of individual differences is a key element to identify coping styles in fish. However, this does not exclude the possibility that individuals change their coping style over time and context. In fact individual plasticity i.e. 'the extent to which the behaviour expressed by individuals with a given genotype in a given context at a given age and time varies as a function of the set of conditions experienced by those individuals before the behaviour was expressed' (Stamps and Groothuis 2010), is very important because it allows individuals to adjust their behaviour to novel or instable environments. Few studies have addressed how plastic, proactive and reactive coping styles are. Frost et al. (2007) suggested that social context is an important modulator of coping styles in rainbow trout. These authors showed that bold individuals observing another's losing fights or with lower responses to novelty (novel objects and novel prey) reduced their boldness. However, shy individuals just alter their behaviour (increase their boldness responsiveness) when their relative competitive ability was similar or higher than their conspecifics. These results suggest that bold individuals may be more flexible to changing conditions as opposed to shy individuals (Frost et al. 2007). Similar differences in behavioural plasticity have been documented during feeding response in presence of the novel object. Basic et al. (2012) showed that proactive individuals adopt a more flexible behaviour by suppressing feed intake in presence of the novel object. In contrast, Ruiz-Gomez et al. (2011) have reported opposite results, i.e. LR (proactive) individuals seem to be more fixed in responses (relocated feed) when confronted with a new situation in contrast with HR individuals.

Individuals differ in how the environmental stimuli are appraised and how they are able to adjust and adapt their physiology and behaviour to help them cope more effectively. Part of this plasticity is supported and influenced by cognition and neural plasticity. The underlying neurobiological mechanism underpinning differences in plasticity between reactive and proactive individuals have been recently studied by Johansen et al. (2012) in the HR/LR rainbow trout lines. These authors measured genes involved in neural plasticity and neurogenesis (PCNA, BDNF, NeuroD and DCX) using quantitative PCR in brains of rainbow trout under baseline conditions and in response to short-term confinement and long-term social stress. They showed that a higher degree of neural plasticity in reactive individuals might provide the ideal conditions to support their higher behavioural flexibility as opposed to proactive individuals.

Furthermore, Ebbesson and Braithwaite (2012) reviewed the influence of neural plasticity and cognition shaped by the environmental experiences in several fish species. These authors agree that, neural plasticity aids in the adaptation and flexibility, demanding by the diverse environments in which fishes live. These, make the brain more sensitive to the surrounding environment moulding the adaptive responses to the environment both over the individual life and over evolutionary time.

However, there is still a long way to go in understanding plasticity of coping styles in order to improve the management and welfare of aquaculture populations.

Aspects of how coping styles change with age, social context and new environmental conditions should be explored in the future. Environmental changes might be particularly relevant during this era of an ongoing global climate change. Global warming could cause changes in species behaviour and life history (Kling et al. 2003). The impacts of climate change in aquaculture can be direct e.g. changes in water temperature, or indirect such as the increase of fishmeal costs and its consequences for aquaculture feeds. The recent approach by Dingemanse et al. (2009) offers a theoretical framework to help understanding plasticity of coping styles. They proposed the concept of behavioural reaction norms, i.e. measuring individual behavioural response over an environmental gradient (e.g. social environment, environmental changes). According to the same authors the same behaviour can be measured over multiple environmental gradients and individual behaviour can be described as a linear regression line linking the response with the environmental conditions. In the linear regression the intercept of the line describes the average individual level of the behaviour and the slope represents the individual degree of plasticity.

Using temperature as an environmental gradient, on a recent work with zebrafish, Rey et al. 2015a showed differences in thermal preferences for proactive and reactive fish under a thermal gradient. Proactive individuals preferred higher temperatures than reactive reflecting differences already detected on basal metabolic rates and different acclimation and environmental adaptation capacities between both coping styles.

Proxies for measuring coping styles in fish

Coping styles characterization in fish can be time consuming, especially when individual-based tests are used. Therefore, several proxies have been suggested in the literature to characterize coping styles without the need to undertake complex behavioural tests.

Ventilation rate has been shown to be a sensitive indicator of fish physiological responses to stress. Barreto and Volpato (2011) observed that ventilation rates of Nile tilapia were correlated with the feeding resumption in isolation. Individuals with high ventilation rates resumed feeding later than fish with low ventilation rates.

Skin pigmentation has also been suggested to predict coping styles in fish (Kittilsen et al. 2009ab; 2012). High spotted Salmonids showed lower cortisol levels than lower spotted conspecifics (Figure 2.1). Visual markers provide a suitable tool that can be easily combined with other common procedures, such as size-grading or vaccination. Furthermore, Kittilsen et al. (2012) provided evidence for individual variation in parasites incidences while screening distinct coping styles. Individuals with high incidence of black skin spots harboured fewer ectoparasites (sea lice) as compared to less pigmented fish.

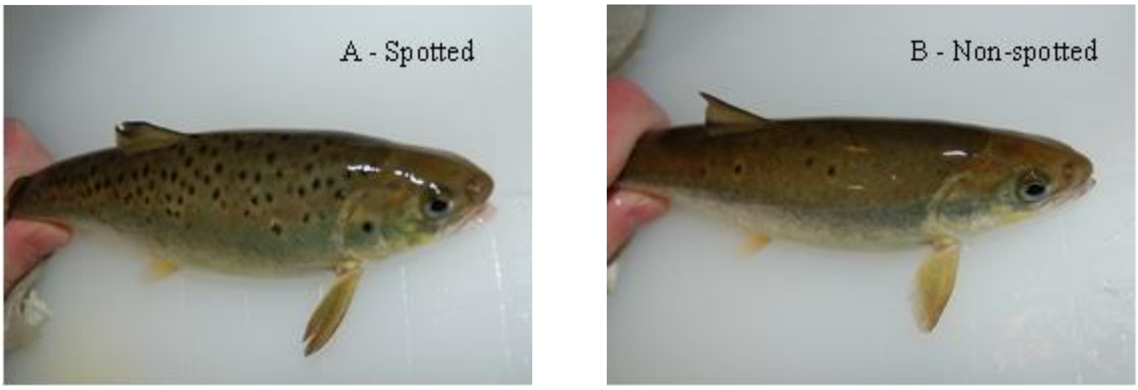

Figure 2.1 Distinct pigmentation profiles in Atlantic salmon defined as (a) 'spotted', that is, stress resistant and proactive and (b) 'non-spotted', that is, stress sensitive and reactive. Reproduced with permission from Kittilsen et al. (2009b).

Observations of ear and tail postures are reliable non-invasive method for assessing emotional reactivity in pigs (Reimert et al. 2013) and sheep (Reefmann et al. 2009) and have been suggested as proxies for coping styles screening. In fish very little is known about the link between body postures and coping styles. Recently, Martins et al. (2012) used fin spreads (defined as a sudden elevation of the dorsal fin) to distinguish bold and shy individuals of the colonial fish, Neolamprologus caudopunctatus. Results showed that reactive individuals exhibited a higher number of fin spreads in response to novelty.

The time to reach the first feeding in Salmonids has also been suggested to predict coping styles. Recently, Andersson et al. (2013b) reported a coupling between stress coping styles and the time to reach first feeding (low cortisol responders had larger yolk reserves at emergence time) which can be used as a proxy.

What are the consequences of different stress coping styles in farmed fish for Aquaculture?

The presence of coping styles is now well recognised in farmed fish and its implication for aquaculture can be widespread. Individual fish within a population often differ in how strongly they respond, behaviourally and physiologically, under stress conditions. A failure to accommodate the coping styles of fish under farming conditions can lead to problems linked with production (e.g. aggression, growth and disease resistance).

Growth performance and energetics

One of the best examples of the implications of coping styles in performance traits comes from studies with African catfish (Martins 2005). By studying individual differences in growth and how these relate with individual differences in feed intake, feeding behaviour and feed efficiency, Martins (2005) showed that the most efficient individuals were those reacting quicker to the presence of pellets and consuming their meals faster after transfer into a novel environment. These individuals were also those that exhibited a lower cortisol response after acute stress. All these characteristics (better feed efficiency and lower stress responsiveness) are clearly beneficial under aquaculture conditions.

Several studies revealed that coping styles play an important role in growth performance and feed conversion. In common carp the competitive ability (success in gaining access to a spatially restricted feed source) was shown to be consistent over time and related to risk-taking behaviour (Huntingford et al. 2010). The same behavioural characteristics have been observed on sea bass (Millot et al. 2009b). Data from Martins et al. (2011 abd) have shown that proactive individuals (Nile tilapia) seem to exhibit a faster recovery of feed intake after transfer and to use feed resources more efficiently. In Atlantic salmon conditions that normally prevail in intensive rearing systems (e.g. restricted feeding regimes, high density) may favour proactive individuals (Huntingford 2004; Huntingford and Adams 2005).

Coping styles have also been linked with differences in metabolism (Huntingford et al. 2010; Martins et al. 2011d). In nature, the metabolic rate of an animal is linked to the willingness of risk-taking while foraging (Careau et al. 2008). Hence, increased energetic requirements in individuals with a higher metabolic demand could require them to forage more often or take more risks to achieve a higher rate of feed intake (Abrahams and Sutterlin 1999; Finstad et al. 2007). Huntingford et al. (2010) and Herrera et al. (2014) reported that in carp and seabream respectively the risk-taking behavioural phenotype is associated with a relatively high metabolic rate, while the risk-avoiding phenotype is associated with a lower rate. Killen et al. (2011) reported in sea bass that the amount of risk-taking among individuals was positively correlated with their routine metabolic rate. However, Martins et al. (2011d) have reported opposite results in metabolic rate (oxygen consumption) measured when Senegalese sole were housed in respirometry chambers. These authors suggested that different individuals reacted differently when housed in the metabolic chambers that functioned as confinement chambers. Individuals that consumed less oxygen in a respirometry chamber were also the individuals that reacted sooner to a confinement stress (typical from proactive coppers). This apparent contradiction may have to do with the passive benthic life-style sole, compared to other more active fish species.

In addition, yolk-sac fry originating from the HR strain were more sensitive to environmental stressors, and have shown a shorter reaction time to low oxygen levels (Höglund et al. 2008). This suggests that differences in coping styles are expressed at

early developmental stages before social or environmental interference. Proactive individuals seem to have a “fast” development strategy (or fast pace of life) as demonstrated by an earlier hatching and consumption of egg yolk reserves as compared to reactive (Andersson and Höglund 2012). Such life strategy has an impact on metabolic needs and most likely on the nutritional requirements. For instance, optimal dietary lipid content could depend on coping styles because metabolic rates are different and hence energy requirements could vary.

In rats metabolic differences between coping styles have been associated with metabolic diseases (Boersma 2011). Using selected Roman Low Avoidance (RLA) and Roman High avoidance (RHA) rats Boersma (2011) showed that different strains differ in plasma insulin levels, both in baseline conditions and during the intravenous glucose tolerance tests. Reactive RLA individuals were associated with insulin resistance and elevated levels of plasma leptin, free fatty acids levels, liver triglycerides, and an increased visceral fat content, especially when over feeding a high fat diet. Proactive RHA individuals were extremely resistant to diet-induced insulin resistance. Thus, coping styles of an individual seems to be associated with particular metabolic and (patho-) physiological characteristics.

Selection programmes

Selection programmes in farmed fish focus essentially on growth performance (Gjedrem 2005). As shown by Martins et al. (2005c) individuals exhibiting fast growth are often included in a proactive coping style. However, proactive individuals have also been shown to be more aggressive (Øverli et al. 2004ab; Castanheira et al. 2013a). Selection for fast growing individuals may results in co-selection of undesirable traits such as aggression. Aggressiveness has been linked with a diversity of aquaculture problems including decreased feed intake, growth dispersion, chronic stress and disease vulnerability (Ashley 2007). Furthermore, fighting brings a significant cost in terms of increased energy expenditure that may promote inefficient growth. In addition, aggression among fish in production systems can be a cause of skin and fin damage. This damage can directly reduce the value of the farmed product and increase the vulnerability to diseases. Moreover, proactive individuals have also been shown to develop routines more easily (Ruiz-Gomez et al. 2008, 2011; Basic et al. 2012; Frost et al. 2007). Such characteristic may be more advantageous under stable conditions provided by intensive husbandry systems but prejudicial in extensive or semi-intensive husbandry systems with lower standardized conditions.

Disease resistance and parasites

Another important implication of coping styles in farmed fish is the different disease susceptibility exhibited by proactive and reactive individuals. Diseases are one of the main challenges in aquaculture and can represent a considerable financial burden to the farmer. Studies on inflammatory challenge with bacterial pathogens reported distinct disease resistance between coping styles (Fevolden et al. 1992, 1993; MacKenzie et al. 2009).

Fevolden et al. (1993) suggested selection targeting distinct coping styles rather than for specific immune traits, selecting for a broad spectrum of defence mechanisms and hence affecting resistance to several diseases.

Moreover, MacKenzie et al. (2009) showed distinct regulation of proinflammatory gene expression suggesting that fundamental differences in cytokine regulation exist in fish with distinct coping styles. In particular, tumor necrosis factor-alpha (TNFα) and interleukin 1-beta (IL1β), putative cytokines involved in the development of inflammation in fish, differed between proactive and reactive individuals.

Among the diseases, Salmon lice are considered a major threat to marine Salmonids farming (Johnson et al. 2004) the evidence that salmon with higher black skin spots harboured fewer mature female lice carrying egg sacs suggests that individual host traits may decrease parasite infestation. Moreover (Øverli et al. 2014) demonstrate that the presence of sea lice affect behaviour and brain serotonergic activity in Atlantic salmon. Still, further studies should address the biology behind coping styles and resistance to parasites, bacteria and viruses.

Furthermore, (Kittilsen et al. 2009b) established that distinct pigmentation profiles are correlated with stress cortisol response in Salmonids (Figure 2.1). Low cortisol responders were found to be consistently more spotted than high cortisol responders. Another study by the same authors Kittilsen et al. (2012) provided evidence for individual variation in parasites resistance to sea lice particularly, salmon louse (*Lapeophtheiras salmonis*) carrying egg sacs.

Fish welfare

In most fish species, chronic or acute stress is considered as the main factor reducing animal welfare in intensive husbandry productions (Ashley 2007; Huntingford et al. 2006). However, despite the link between acute response to challenges and coping styles very little information is available about chronic stressors and coping styles.

One of the best examples used to discriminate distinct susceptibility to chronic stressors was performed using selected lines of wild house-mice. Strains of mice have been created through selective breeding for divergent hypothalamic-pituitary-adrenal axis responses to a standardized aggressiveness test: Short Attack Latency, high aggressive/ proactive (SAL) and Long Attack Latency, low to non-aggressive/ reactive (LAL) (Benus et al. 1991a). Using these lines, Veenema et al. (2003) showed that response to a chronic stressor resulted in symptoms in LAL (proactive) mice characterized by decreased body weight, elevated plasma adrenocorticotropic hormone (ACTH) and corticosterone levels and a lower hippocampal mineralocorticoid receptor (MR): glucocorticoid receptor (GR) ratio.

Korte et al. (2005) mention that adaptive processes, actively maintain stability through change (allostasis) are dependent on the personality type and associated stress responses. The benefits of allostasis and the costs of adaptation (allostatic load) lead to different trade-off in health and stress related diseases, reinforcing that both coping styles (proactive/reactive) can be successful, under different environmental conditions.

Furthermore, van de Nieuwegiessen et al. (2010) showed that chronic stressors (stocking density) affect the performance traits in African catfish differently according to coping strategies. Fish housed at high density showed an increase in activity and decrease in aggression levels. In addition, at high density, reactive individuals reared in mixed groups showed a comparable growth rate to intermediate and proactive individuals. It seems that the presence of intermediate and proactive individuals stimulates the feeding motivation of reactive individuals.

Undoubtedly, coping styles play an important role in how different individuals appraise the housing environment and thereby their welfare status. Huntingford and Adams (2005) reviewed the welfare consequences of coping strategies in Salmonids. They suggest that when fish are housed at high densities and with a predictable feed source, as is usually the case in intensive husbandry systems, reactive individuals may fail to flourish. Another interesting question related with high densities, is the difference on how proactive and reactive individuals react to the suppression of aggressive behaviour induced by crowding i.e. the propensity for higher aggression in proactive individuals suggests that they will suffer most in high densities.

In contrast to Huntingford and Adams (2005), no indications were found for welfare consequences of different coping strategies in intensive husbandry systems in African catfish (van de Nieuwegiessen et al. 2010). Although an impaired growth performance of reactive fish housed in reactive groups was shown, no effects were detected in reactive fish housed in mixed groups, which is the common rearing practice.

Based on these results, individual coping styles should not be used as a welfare indicator, but one may infer a welfare problem when the behaviour identified under the proactive/reactive continuum changes. Even though, the housing environments may have profound effects on behaviour and welfare. For example, in pigs the environmental enrichment effects were shown to be much higher in LR than in HR and were reflected in more time on play behaviour and more oral manipulation of pen mates (Bolhuis et al. 2005b). In addition, the same authors showed that the effect of environmental enrichment on weight gain may differ for pigs with divergent coping styles. In fish, the effect of environmental enrichment (i.e. substrate availability) as behavioural and physiological indicators of welfare was study by Galhardo et al. (2008) whom showed that the absence of substrate decreased territorial behaviour, increase aggression levels, cortisol and glucose; all of which are suggestive of a stress-related context. This suggests that the welfare of at least some fish species may be negatively affected by the absence of substrate or other environmental enrichment, and this effect may change in distinct fish coping styles.

Furthermore, aggressiveness level is one of the differences between proactive and reactive individuals. Literature suggests that proactive individuals show high levels of aggressiveness (Øverli et al. 2004ab; Castanheira et al. 2013a). Aggression has been linked with a diversity of aquaculture-relevant problems including decreased feed intake, growth dispersion, chronic stress and disease vulnerability (Huntingford and Adams 2005; Martins et al. 2011e) which as a consequence can impair fish welfare.

Moreover, Vindas et al. (2012, 2014ab) showed good evidence that Atlantic salmon possess a nervous system and a brain sufficiently complex to demonstrate individual responses to frustrations conditions when an omission of an expected reward occurs. Deviation from routine feeding practices, in intensive farming conditions, could have negative consequences, in terms of both production and welfare as a consequence of frustration-induced agonistic behaviours.

Knowing that farmed fish have coping styles and that coping styles differ in how they appraise their environment may help designing farming environments that are more diverse and could improve the welfare of individuals with different coping styles. In turn, this may increase production output.

Flesh quality

Nowadays there is evidence showing that inadequate fish husbandry results in lower meat quality (Ribas et al. 2007; Robb et al. 2000; Matos et al. 2010, 2011). Studies show that fish subjected to stress prior to and during slaughter, in particular salmonids, display a softer texture and lower flesh quality (Bahuaud et al. 2010; Kiessling et al. 2004). Some studies suggest that the production of low cortisol-responsive fish could benefit commercial parameters such as flesh quality (Pottinger 2001). High fillet quality (e.g. textural characteristics, freshness and health value) is a requirement for feed production and coping styles can attenuate or aggravate the effect of stressors on filet quality. However, knowledge on the mechanisms responsible for individual differences in flesh quality is still largely unknown.

Production systems

It is also important to understand how divergent coping styles perform in different aquaculture production systems. Recirculating Aquaculture Systems (RAS), for example are expected to expand in the future as they offer the possibility to have a high production with a minimum ecological impact (Martins et al. 2010).

Mota et al. (2014) showed that steroids (glucocorticoids, androgens and a progestin) in their free and conjugated forms tend to accumulate in the rearing water of commercial RAS at levels that can potentially be detected by some fish species. However, we still do not know how sensitive the different coping styles are to the re-uptake of steroids and olfactory cues present in the water and how such sensitivity can induce different welfare levels.

Furthermore, the range of the coping styled spectrum that leads to maximum growth performance, highest welfare condition and disease resistance, may change depending on the husbandry system, once different types of intensive, semi-intensive or extensive systems present very different social and environmental conditions to fish.

Future perspectives

Coping styles are present in a variety of farmed fish and may impact aquaculture in different ways. However, one of the main difficulties in understanding the implications of coping styles under farming conditions is the methodology available that relies heavily on individually-based tests. Screening in isolation may induce significant stress in social species. Consequently, the development of grouped-based tests (Figure 2.2) may in the future facilitate mass screening of fish stocked at high densities and therefore may be more easily applied under farming conditions. Examples of potential mass screening tests are the hypoxia and the risk-taking tests (Millot et al. 2009b; Huntingford et al. 2010; Laursen et al. 2011; Castanheira et al. 2013b). Additionally, further studies should be considered to validate the temporal consistency over time of the distinct traits. One of the limitations of the available knowledge regarding the temporal consistency is that it refers always to short term consistency (usually a few weeks) (Basic et al. 2012; Castanheira et al. 2013b). However, van Reenen (2012) demonstrated long-term consistency of individual differences in behavioural and adrenocortical responses of dairy cattle to acute stressors. The observations were recorded in rearing period (6 - 7 months), gestation (22 - 24 months) and first lactation (25 - 29 months). They showed that individual differences in struggling in a restraint test at 7 months of age predicted those in open field locomotion during first pregnancy. In addition, individuals with high cortisol responses and reactive behaviour measured as high avoidance and less exploration to open field and novel object tests at 6 months of age, also exhibited high cortisol responses to both tests at 29 months of age. Similar studies, over longer periods of time should be undertaken also in fish.

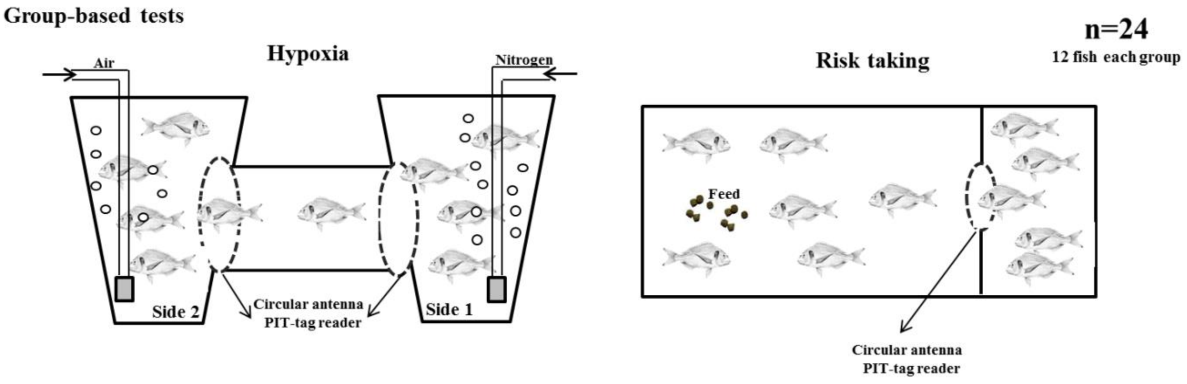

Figure 2.2 Schematic representation of the group-based tests used to determine coping styles in Gilthead seabream Sparus aurata. Reproduced with permission from Castanheira et al. (2013b).

Measures of HPA axis reactivity, locomotion, vocalisation and adrenocortical and behavioural responses to novelty contributed to the understanding of ability to cope with stress and supporting the idea that stress responsiveness may be mediated by multiple independent underlying traits. Some authors have suggested that cortisol and behavioural responses to stressors are linked to two independent dimensions of stable trait characteristics (Koolhaas et al. 2010). These authors suggested that the quality of the response to a challenging condition (coping style) is independent from the quantity of that response (stress reactivity). According to the same authors, the physiological responses to stress such as the HPI axis reactivity (one of the most significant differences between proactive and reactive individuals) is more related to an emotional response to stress than to coping styles. Eventually a decoupling of these axis, coping styles and emotional, could bring new light to understand the pronounced individual variation in plasma cortisol response observed. It is also important to perform studies regarding the influence of age, environmental conditions, nutrition and social group in coping styles. In other comparative models (e.g. cows, pigs) coping styles can change partly according to the social environment (van Reenen 2012; van Erp-van der Kooij et al. 2003). In addition, van Erp-van der Kooij et al. (2003) showed that coping styles in piglets can change according to the social environment although at an older age, this ability was lost.

In addition, different coping styles also differ in their adaptability towards shifts in environmental conditions. In mice Benus et al. (1988) showed that individual differences in aggressiveness (a component trait of coping styles) explain differences in adaptation to external factors. The adaptation to a new photoperiod cycle took two fold long in the aggressive mice. However, in farmed fish there are no similar studies in literature.

Still, studies in farmed fish such as the selected trout lines can open the possibility to use fish as simpler models to understand underlying mechanism of coping styles in vertebrates such as those related to neural activity and their implications in behaviour.

The knowledge of coping styles can help to improve the sustainability of production through the establishment of more fine-tuned culture strategies. In this way the feed waste can be minimized since each coping style is related to particular physiological and behavioural responses and some culture variables could be adjusted.

Moreover, the genetic basis (heritability/epigenetics) of coping styles, disease susceptibility as well the neuroendocrine mechanisms behind consistent as well as flexible behavioural patterns are here pinpointed as central themes and open research lines on application of coping styles to aquaculture.

Conclusions

The presence of coping styles is now well recognised in farmed fish and its implication for aquaculture can be wide as here reviewed. Taken together, the fairly extensive literature on coping styles in fish shows that screening for coping styles is species-specific. The recent development of group-based tests and the use of proxies may provide an opportunity for mass screening in the future. Mass screening into different coping styles may help optimizing the production systems as optimal conditions for proactive individuals are likely to be different from those of reactive individuals.

In addition, the recognition that farmed fish exhibit coping styles means that a number of behavioural and physiological responses will vary as part of a common "package" that should be taken into consideration when designing selection programs.

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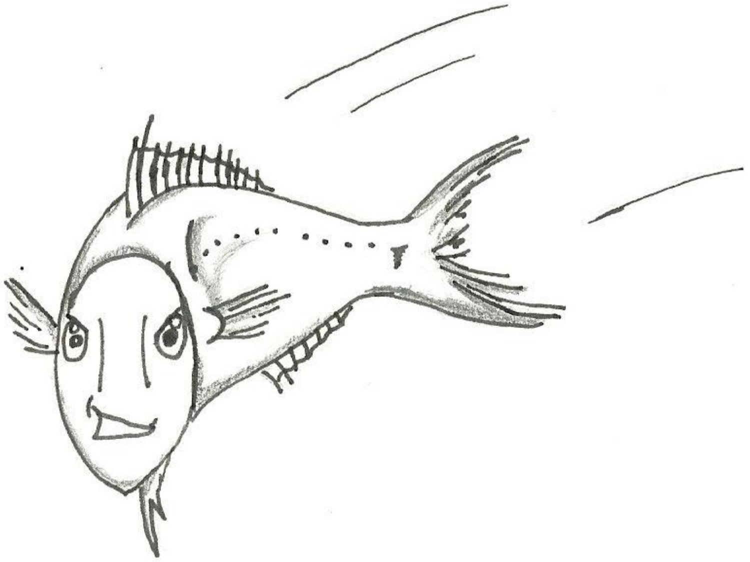

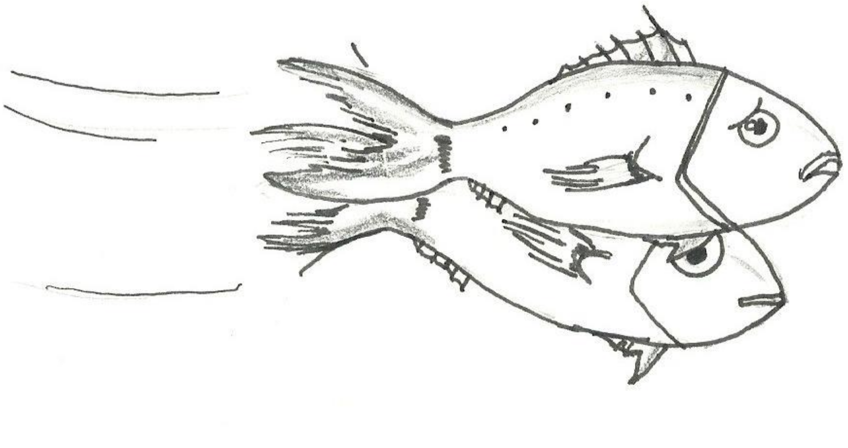

CHAPTER

3

Cortisol responsiveness and aggressive behaviour: Indicators of divergent coping styles

Linking cortisol responsiveness and aggressive behaviour in gilthead seabream *Sparus aurata*: indication of divergent coping styles

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Cortisol responsiveness and aggressive behaviour: Indicators of divergent coping styles

Chapter 3

Abstract

Farmed animals, including fish, often exhibit a pronounced individual variation in stress coping styles with proactive and reactive individuals differing in a variety of neuroendocrine and behavioural responses. In this study we disclosed that individual differences in cortisol responsiveness after a restraining test were predictive of aggressive behaviour in gilthead seabream *Sparus aurata*, one of the most important Mediterranean farmed fish. Seabream juveniles (n=24, initial weight: 49.3±7.3 g) were exposed to a restraining test that consisted of keeping each fish in an emerged net for three minutes. Afterwards fish returned to their home tank and 30 min after were rapidly caught and anaesthetised for blood sampling. Blood was collected from the caudal vein and analysed for cortisol (radioimmunoassay). After 3 months the same individuals were exposed to an aggression test: fish were allowed to interact with a naïve fish of similar size (max 10 % weight difference) for 20 min. Aggressive behaviour was determined as follows: latency to start chasing (time taken until the first chase; chase was defined as a sudden change in swimming direction and speed as a response to an approach by the opponent); number of chases; latency to fighting (time taken until the first fight; fight was defined when fish engaged in a circle movement around each other) and number of fights. Results showed that individuals exhibiting lower cortisol responsiveness after a restraining test were more aggressive (cortisol vs number of chases: rs=-0.479, p=0.021) In conclusion, seabream juveniles exhibited pronounced individual differences in cortisol responsiveness and aggression that are interrelated and likely to be distinctive traits of coping styles.

Keywords: Stress response, Aggression, Individual variation, Resident-intruder test

Introduction

In animals, including fish, individual differences in stress responsiveness have been associated with differences in behaviour (Øverli et al. 2007). These individual differences may reflect distinct coping styles (personalities, temperament, behaviour syndromes, bold/shy continuum). The concept of coping styles can be defined as 'a coherent set of behavioural and physiological stress responses, which is consistent over time and which is characteristic to a certain group of individuals' (Koolhaas et al. 1999; p 925). Distinctive aggressiveness and hypothalamus–pituitary–interrenal (HPI) axis reactivity are two of the most important differences between proactive and reactive individuals. Literature suggests that proactive individuals, show high levels of aggression (Benus et al. 1988; Øverli et al. 2004ab; Bolhuis et al. 2005a; Millot et al. 2009a) and lower HPI axis reactivity (Benus et al. 1991b; Øverli et al. 2004ab; Silva et al. 2010). In addition the same studies suggest that reactive individuals, as opposed to proactive individuals can be characterised by low levels of aggression and high HPI axis reactivity However, few studies are available, exploring the relation between aggressive behaviour and cortisol post-stress in fish. For instance, studies in rainbow trout (Oncorhynchus mykiss) showed that individual variation in plasma cortisol levels are negatively correlated with aggressive behaviour (Øverli et al. 2002; 2004ab). In these studies, fish were kept in isolation for a period of time to induce territoriality and motivation to express aggressive behaviour (Øverli et al. 1999; Pottinger and Carrick 2001). However, isolation per se may have carry-over effects that could influence the ability to engage in aggressive behaviour with other conspecifics (Gómez-Laplaza and Morgan 2000). For instance, it has been reported for several fish species that isolation can impair feeding motivation (Martins et al. 2006abc), induce depressive behaviour (Price et al. 1994) and increase territoriality (Øverli et al. 2004a). In addition, sociability has been shown to be a personality trait in fish (Conrad et al. 2011). Therefore it is likely that the absence of social interaction affect differently proactive and reactive individuals. Reactive individuals have been shown to be more attracted to other conspecifics as compared to proactive (Pike et al. 2008). This could suggest differential effects of isolation in individuals with distinct coping styles (Webster et al. 2007). Isolation affects the individuals differently and consequently may reflect distinct motivation states such as motivation to fight. However, to the best of our knowledge, in fish, no study has ever addressed aggression tests without a previous period of isolation. In some fish species a period of isolation may not be required to the expression of aggressive behaviour.

Several farmed fish species have been shown to have large individual differences in cortisol responsiveness (Pottinger et al. 1998; Pottinger and Carrick 2001; Øverli et al. 2004b; Martins et al. 2006a; Trenzado et al. 2006), including gilthead seabream (*Sparus aurata*) (Arends et al. 1999; Rotllant et al. 2000; Tort et al. 2001; Barton et al. 2005; Tintos et al. 2006; Sánchez et al. 2009). In addition to cortisol responsiveness, differences in aggressiveness are amongst the most distinctive traits between coping styles (Koolhaas et al. 1999). Thus, while the importance of these distinctive traits is recognised, few studies e.g. (Reyes-Tomassini 2009) have focused on aggressive behaviour in seabream. Aggression has been linked with a diversity of aquaculture-relevant problems including decreased feed intake, growth dispersion, chronic stress and disease vulnerability (Huntingford and Adams 2005; Martins et al. 2011e) which as a consequence can impair fish welfare. Understanding the magnitude of variability in aggression of farmed fish is fundamental if remediation measures are to be taken. It is also very important to understand whether the variability of aggression is linked with other traits such as stress responsiveness as this can suggest that individual differences in aggression are part of coping styles and therefore can be predictive of and predicted from other traits. The existence of individual variation in stress responsiveness and aggressiveness is likely to have an impact on how individual fish perform in culture and on their welfare. This study aims to quantify individual differences in cortisol post-stress in seabream and to assess whether it can predict aggressive behaviour in fish not previously isolated. Seabream is the most farmed fish among Mediterranean countries (Barazi-Yeroulanos 2010), however there is still a lack in knowledge concerning individual variation in stress responsiveness and aggressiveness.

Material and methods

Experimental animals, housing and feeding

Twenty-four gilthead seabream juveniles, with an initial body weight of 49.3±7.2 g (mean±SD) were used as experimental animals. The fish used in our experiment were not sexually mature. The body weight at the start of the experiment was 49.3±7.2 g (mean±SD). All animals were obtained from a commercial fish farm (MARESA Mariscos de Esteros SA, Huelva, Spain). Upon arrival at Ramalhete Research Station (Faro, Portugal), three months before the start of the experiment, fish were housed in a stock with standard rearing conditions until the start of the experimental procedures. From these, 24 individuals, randomly selected, were individually PIT-tagged (Trovan®, Netherlands) and stocked in two groups of 12 individuals on 70 L tanks in an open water circuit. Water temperature (19.8±2.1 ºC), salinity (33.8±2.4 ‰), dissolved oxygen (98.4±2.8 %), NO2-N (0.0±0.0 mg L-1) and NO3-N (0.0±0.0 mg L-1) were checked daily. A 12L: 12D photoperiod was maintained with day break set at 8:00 h. Fish were fed with automatic feeders, with a commercial diet (Aquagold 2mm, Aquasoja, Sorgal SA, Portugal; 44 % crude protein, 14 % crude fat, 8 % ash, 2.5 % crude fibres, 1.0 % phosphorus).

Experimental procedures

Restraining test

Fish were housed individually in a 40 L glass aquarium (37 cm length x 40 cm width x 40 cm depths) in an open water circuit during 9 days. The net restraining test was done in day 9, after onset of isolation. Individually housed fish were kept visually isolated from one another by white plastic partitions around tanks, except for the front side which allowed daily visual observations of the fish. After blood sampling fish were stocked in two groups of 12 individuals on 70 L tanks in an open water circuit. The order of restraining test was randomized each time. The net restraining test consisted of holding each fish individually in an emerged net for three minutes, as previously performed in other studies (Arends et al. 1999; Silva et al. 2010; Martins et al. 2011cd). Blood samples were collected 30 minutes after the start of net restraining, according to

Arends et al. (1999). Therefore, fish were quickly taken out from each tank at the same time and anaesthetized with 2-phenoxyethanol (1000 ppm, Sigma-Aldrich). Blood was withdrawn within 3 min from caudal the vein using heparinised syringes and centrifuged at 2000 × g for 20 minutes at room temperature. After centrifugation plasma was frozen in liquid nitrogen and stored at -80 °C for cortisol analysis. After blood sampling individuals were weighed and identified.

Aggression test

After 3 months of the restraining test, the same individuals were exposed to an aggression test. The aggression test consisted of allowing each fish to interact with a naïve fish with similar size (max 10 % weight difference). Individuals (focal and naïve) were previously weight (one week before the aggression test) to avoid stress susceptibility near the aggression test. The aggression test was performed in the same day and in the same part of the day (morning) for all individuals. The water was changed between tests and the naïve fish was used just one time. Fish (focal and naïve) were rapidly netted and placed at the same time on the, 150 L fibreglass tank (50 cm length x 30 cm width x 25 cm depths). The observation period lasted 20 minutes; after each fish (focal and naïve) return to their home tank. Before transferring the fish to the experimental tank, food (0.5 % BW) was introduced in one corner. Food was used as an available resource to motivate fighting. Aggressive behaviour was determined as following: latency to start chasing (time taken until the first chase; chase was defined as a sudden change in swimming direction and speed as a response to an approach by the opponent); number of chases; latency to fighting (time taken until the first fight; fight was defined when fish engaged in a circle movement around each other) and number of fights, adapted from Reyes-Tomassini (2009). None of the fish suffered any physical injury during the fight. During the encounter, aggressive behaviours were video recorded (MicroVideo™ camera MCV2120-WP-LED, Canada) for posterior analysis. The behaviour of focal and naïve fish were analysed to evaluate the influence of the naïve aggressive behaviour on the aggressive behaviour of our focal fish. In our study it should be noted that the type of aggressive displayed did not result in skin lesions, fin damage or physical injuries. In addition visual observation after the aggression test revealed that all fish recovered well from the test and did not show signs of distress such as abnormal swimming behaviour or lack of feeding.

Cortisol analyses

Plasma cortisol levels were measured with a commercially available competitive binding Coat-A-Count® Cortisol kit (SIEMENS Medical Solutions Diagnostics, USA) adapted from Irwin et al. (1999). Briefly, 50 ml of each sample to be assayed was transferred into an Ab-Coated tube and 1 ml of 125I Cortisol added. The tubes were then incubated for 45 min at 37 ºC in a water bath. The contents of all tubes were decanted, and allowed to drain for 5 min before being read on a gamma counter (2470 WIZARD2TM, PerkinElmer TM, Inc., Belgium) for 1 min. A calibration curve was used to convert results from percent binding cortisol to concentration (ng ml-1). The Coat-A-Count cortisol antiserum cross-reacts 100 % with cortisol, 11.4 % with 11-deoxycortisol, 0.98 % with cortisone, 0.94 % with corticosterone and 0.02 % with progesterone.

Data analysis

Statistical analyses were performed using SPSS 18.0 for windows. The results were expressed as mean±standard deviation (SD). Relationships between post-stress cortisol and behaviour traits were investigated using Spearman correlation after data failed to pass the normality Kolmogorov-Smirnov test. This test was also used to verify whether the order of blood sampling affected cortisol levels. A nonparametric test, Mann Whitney U test, was used to verify differences in weight between reactive and proactive individuals. Since a high number of individuals didn´t fight this test was also used to verify differences in cortisol levels between fighters and non-fighters. Regression analyse was also performed to examine associations between aggressive behaviour and cortisol post-stress. Statistical significance was taken at $p<0.05$.

Results

Figures 3.1, 3.2, 3.3, 3.4 and 3.5 depict the pronounced individual variation in cortisol levels post-stress levels and aggressive behaviour obtained during the aggression test: latency to chase, latency to fighting, number of chase and number of fights. Number of chases showed the highest variability ranging from 0 to 103. After the restraining test, cortisol varied between 6.2 ng ml-1 and 117.3 ng ml-1. The order of restraining was randomized and no effect of order was detected on cortisol levels (*p*>0.05). In addition, no differences in weight were observed between proactive and reactive individuals (*p*>0.05).

The correlation between the plasma cortisol and aggressive behaviour is shown in Figure 3.6. Plasma cortisol was significantly correlated with number of chases ($r_s = -0.479$, $p = 0.021$) but not with latency to chase ($p > 0.05$). Figure 3.7 depicts the post-stress cortisol levels between fighters and non-fighters, ($p = 0.034$); fighters had significantly low post-stress cortisol levels than non-fighters individuals. The regression analyse showed that 21% of the variation in post-stress cortisol levels can be explained by differences in aggressive behaviour.

No correlation did not find between chasing behaviour of the naïve and focal fish (latency to chase: $r_s = -0.332$, $p = 0.112$; number of chase: $r_s = -0.225$, $p = 0.303$) suggesting that the aggressive behaviour of the focal fish is not simply a response to the aggressive behaviour exhibited by the naïve fish.

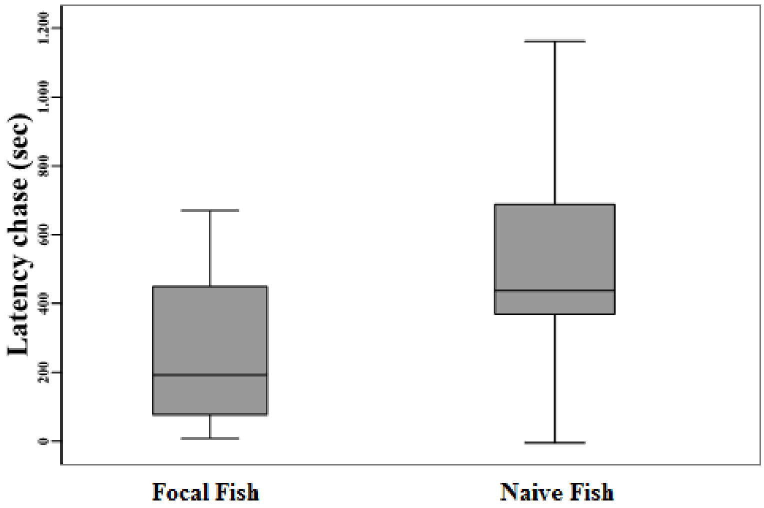

Figure 3.1. Box plot of latency to chase for focal and naïve individuals (n = 24). The box includes observations from the 25th to the 75th percentile; the horizontal line within the box represents the median value. Lines outside the box represent the 10th and the 90th percentiles.

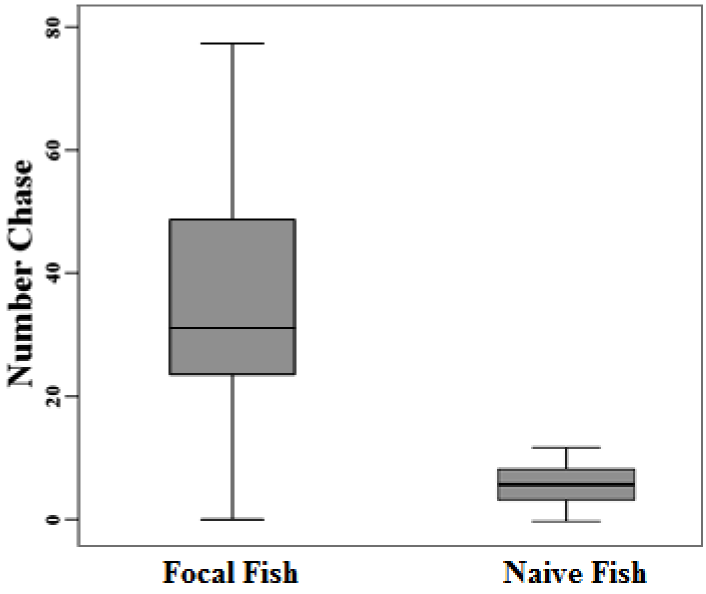

Figure 3.2. Box plot of number of chase for focal and naïve individuals (n = 24). The box includes observations from the 25th to the 75th to (n percentile, the horizontal line within the box represents the median value. Lines outside the box represent the 10th and the 90th percentiles

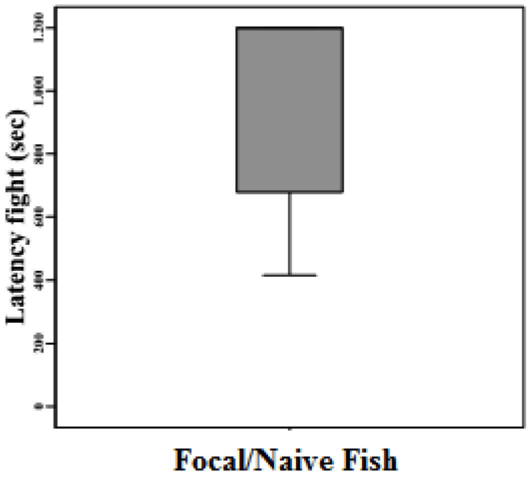

Figure 3.3. Box plot of latency to fight focal/naïve individuals (n = 24). The box includes observations from the 25th to the 75th percentile, the horizontal line within the box represents the median value. Lines outside the box represent the 10th and the 90th percentiles

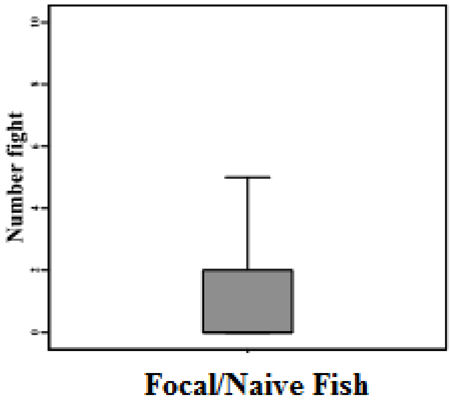

Figure 3.4. Box plot of number of fight focal/naïve individuals (n=24). The box includes observations from the 25th to the 75th percentile, the horizontal line within the box represents the median value. Lines outside the box represent the 10th and the 90th percentiles.

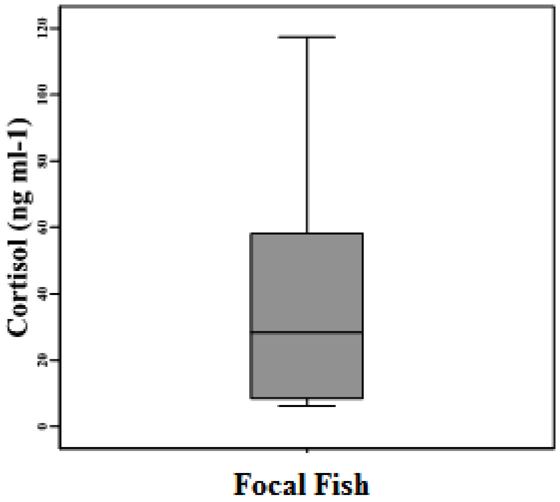

Figure 3.5. Box plot of post-stress cortisol levels for focal individuals (n = 24). The box includes observations from the 25th to the 75th percentile, the horizontal line within the box represents the median value. Lines outside the box represent the 10th and the 90th percentiles.

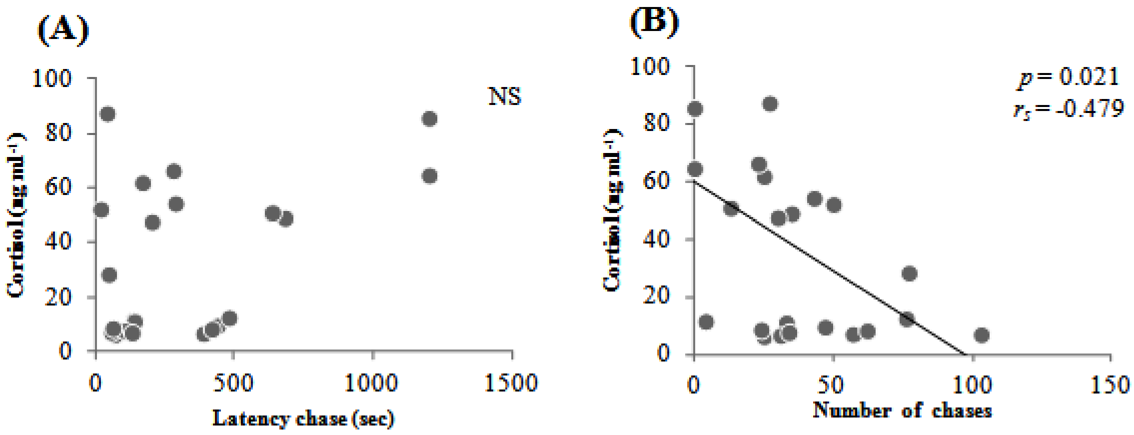

Figure 3.6. Relationship between plasma cortisol levels obtained after a restraining test and aggressive behaviour (latency (A) and number of chases (B)) on gilthead seabream Sparus aurata juveniles (n = 24).

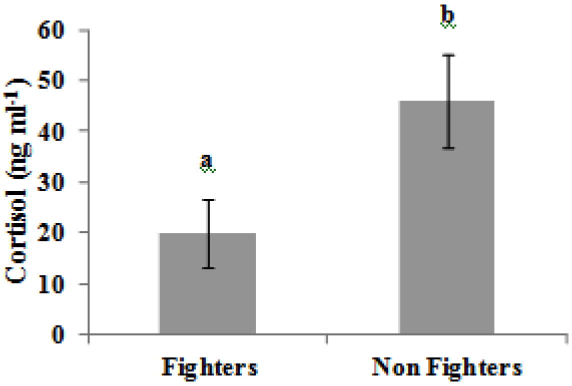

Figure 3.7. Cortisol post-stress responsiveness of Fighters and Non fighters gilthead seabream Sparus aurata juveniles (n = 24). Data are presented as means ± SD. Different letters indicate significant differences (nonparametric test, Mann Whitney U test: p = 0.034).

Discussion

This study showed that gilthead seabream with lower post-stress cortisol levels are more aggressive. These results are in agreement with other studies showing that lower cortisol responders after acute stress are markedly more aggressive (Pottinger and Carrick 2001; Øverli et al. 2004a). In addition, the values of plasma cortisol after stress were similar to those reported in other studies for this species (Arends et al. 1999; Rotllant et al. 2001; Barton et al. 2005; Ellis et al. 2012). However in seabream there is no knowledge related to aggression behaviour during dyadic interaction.

In the present study, a high number of individuals presented agonistic interactions (chases and fights), even without a period of isolation to establish territoriality. Both focal and naïve individuals were exposed to the same conditions prior to the start of the aggression test. On one hand, the lack of isolation prior to testing offers several advantages such as a reduced experimental duration and the avoidance of isolation associated stress, especially in social species (Gómez-Laplaza and Morgan 2000). Previous studies suggest that the reactive type seems to be more affected by isolation as compared to proactive individuals (Benus et al. 1991b; Ruis et al. 2001). Such influence of isolation on reactive individuals may place them in a disadvantage point at the start of an aggression test as compared to individuals that are not so affected by isolation. Avoiding a period of isolation prior to an aggression test has already been used in other vertebrates (pigs) to establish a link between restraining stress and aggressiveness (Bolhuis et al. 2005a). On the other hand, the lack of isolation may strengthen the possible influence of previous social interactions on the outcome of the aggressive encounter. Nevertheless it is interesting to notice that previous studies using 1 week of isolation prior to a dyadic encounter found similar results to the ones showed here (Øverli et al. 2004a), i.e. more aggressive individuals have lower cortisol levels after acute stress.

The results from this study suggest that the variability in aggressive behaviour found in the focal fish is not dependent of the aggressive behaviour exhibited by the naïve fish but rather an individual characteristic of aggressiveness. In this study the naïve fish exhibited lower levels of aggression (both latency and number of chases). The reason for this difference is not clear. The major difference between the focal and naïve fish prior to the start of the aggression test was that the focal fish were handled 3 months before the start of the aggression test while the naïve fish were not (only focal fish were exposed to the restraining test). Whether more frequent handling results in fish that are more robust to future handlings is not known. If this was the case then our focal fish would have been less affected by the transfer to the aggression arena as compared to the naïve fish.

In this study, post-stress cortisol explained 21 % of the variation in aggressive behaviour. Previous studies performed with fish selected for cortisol responsiveness that also differed in aggressiveness may help understanding the link between cortisol and aggression (Øverli et al. 2002; Øverli et al. 2004b; Brelin et al. 2005). In fish, cortisol release is mainly controlled by HPI axis (Barton 2002). When the HPI axis is activated, corticosteroids are secreted into the plasma in order to maintain the homeostatic control of stress responsiveness (Vijayan et al. 1994). Corticosteroids also affect behaviour through genomic and non-genomic mechanisms in the central nervous system as they easily penetrate the blood-brain barrier, in fact it has been reported, in several species an enhanced aggression as a result of increased corticosteroids levels (Sapolsky 1990; Øverli et al. 1999; DiBattista et al. 2005). Schjolden et al. (2009) reported the involvement of both genomic and non-genomic mechanisms underlying the effect of cortisol through mineralocorticoid (MR) and glucocorticoid receptors (GR) on fish aggressiveness. Individuals differing in coping styles differ in MR and GR activity (Veenema et al. 2003), which mediates the action of cortisol on the initiation and termination of the stress response but also on the activation of several behaviours that facilitate overcoming a stressor. Aggressiveness is likely to be activated when facing a social stressor and underlying differences in brain MR/GR activity may contribute to individual differences in aggressiveness.

In the current study, 79 % of the variation in aggressiveness remains unexplained. Factors such as underlying metabolic differences (Brown et al. 2011), winner and loser effect (Hsu and Wolf 1999) and the effect of nutritional status (Damsgird and Dill 1998) could contribute to explain such variability. For instance, previous studies showed that fish with divergent coping styles differ in resting metabolic rate (RMR) with proactive individuals exhibiting higher RMR (Huntingford et al. 2010; Martins et al. 2011d).

Another possibility to explain differences in aggressiveness other than inherent differences in cortisol responsiveness could be prior fighting experiences. Previous winners are more likely to win again future aggressive encounters as compared to losers (Oliveira et al. 2011). Prior to the aggression test in this study fish were stocked in groups, therefore we cannot exclude the possibility that preceding fighting experiences could have influenced aggressiveness. Additionally, it is likely that the effect of prior fighting experiences on future aggression is also mediated by cortisol, as in many fish species losers exhibit significantly higher cortisol concentrations than winners (Øverli et al. 1999; Sloman et al. 2001).

An alternative explanation for the individual differences observed in the present study could be related to differences in hunger levels between individuals (Neat et al. 1998; Briffa and Sneddon 2007). Hungrier fish could have fought more for the food placed in the arena. However, this seems unlikely as individuals were fasted for 24h before the start of the aggression test, suggesting similar hunger level in all fish.

In summary, this study provides the first evidence for a link between HPI responsiveness and aggressive behaviour in seabream. This suggests that individual differences in aggression are part of coping styles and therefore can be predictive of and predicted from other traits. Further studies should be considered to assess the temporal consistency of these distinct traits. Extensions of this study could be the investigation of the underlying metabolic and neurological mechanisms that explain aggressive behaviour.

Acknowledgements

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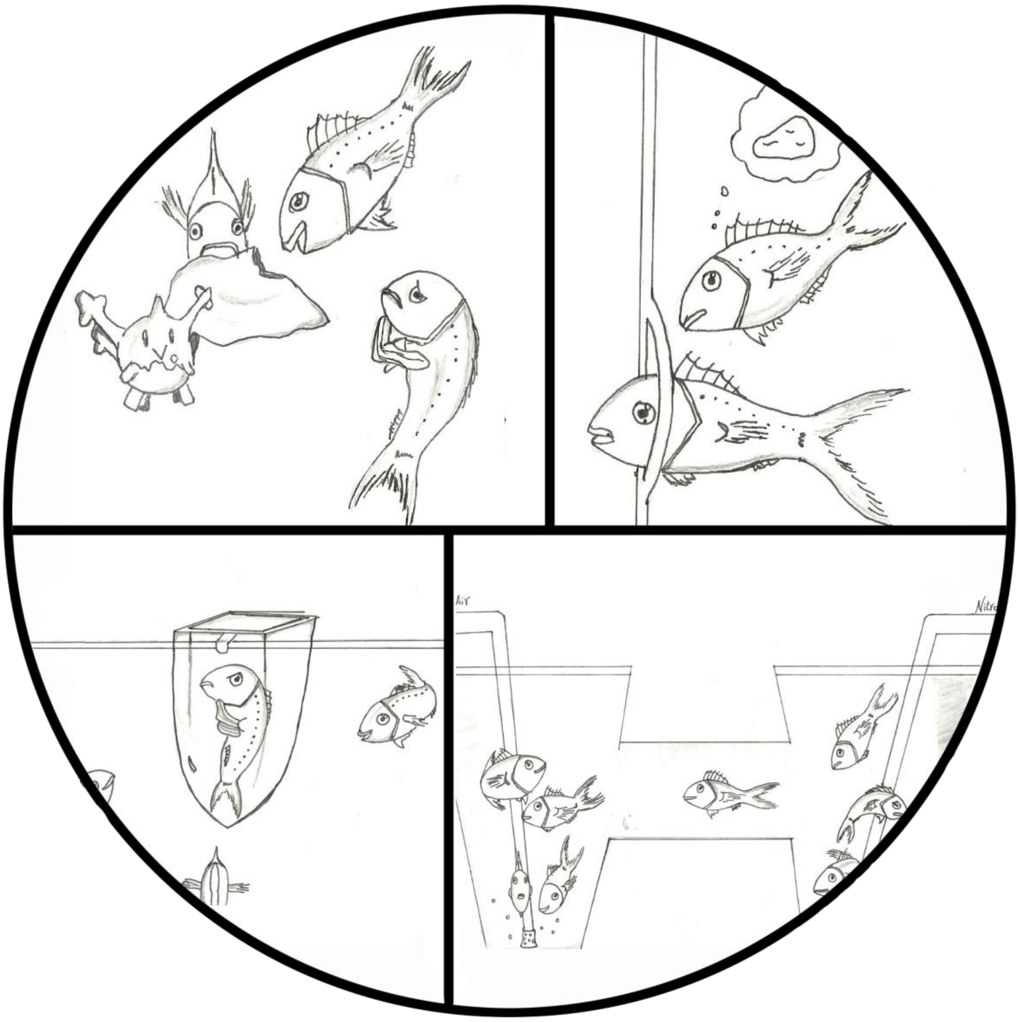
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CHAPTER

4

Can we predict personality in fish?

Can we predict personality in fish? –

searching for consistency over time and across contexts

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Can we predict personality in fish?

Abstract

The interest in animal personality, broadly defined as consistency of individual behavioural traits over time and across contexts, has increased dramatically over the last years. Individual differences in behaviour are no longer recognised as noise around a mean but rather as adaptive variation and thus, essentially, raw material for evolution. Animal personality has been considered evolutionary conserved and has been shown to be present in all vertebrates including fish. Despite the importance of evolutionary and comparative aspects in this field, few studies have actually documented consistency across situations in fish. In addition, most studies are done with individually housed fish which may pose additional challenges when interpreting data from social species. Here, we investigate, for the first time in fish, whether individual differences in behavioural responses to a variety of challenges are consistent over time and across contexts using both individual and grouped-based tests. Twenty-four juveniles of Gilthead seabream Sparus aurata were subjected to three individual-based tests: feed intake recovery in a novel environment, novel object and restraining and to two group-based tests: risk-taking and hypoxia. Each test was repeated twice to assess consistency of behavioural responses over time. Risk taking and escape behaviours during restraining were shown to be significantly consistent over time. In addition, consistency across contexts was also observed: individuals that took longer to recover feed intake after transfer into a novel environment exhibited higher escape attempts during a restraining test and escaped faster from hypoxia conditions. These results highlight the possibility to predict behaviour in groups from individual personality traits.

Keywords: Behaviour syndromes, shy; bold; reactive; proactive; personality; temperament.

Introduction

In animals, individuals differ consistently in several aspects of their behaviour (Budaev and Zworkykin 2002; Réale et al. 2010a; Sih et al. 2004). These individual differences may reflect distinct coping styles, behavioural syndromes, personalities or temperament. All these concepts embrace a similar definition which is a suite of correlated traits that are consistent across time and context (Koolhaas et al. 1999). In fish, two major personality types are recognised: proactive (active coping or bold or 'fight-flight') and reactive (passive coping or shy or 'non-aggressive'). Proactive individuals create routines and seem to have a high level of active avoidance, locomotor activity and low flexibility in behavioural responses when faced with challenges, this pattern being the opposite for reactive individuals (Benus et al. 1991ab; Koolhaas et al. 1999; Ruiz-Gomez et al. 2011). In addition, proactive individuals exhibit typical physiological and neuroendocrine characteristics such as lower hypothalamus-pituitary-interrenal (HPI) activity (Silva et al. 2010) and lower HPI reactivity (Øverli et al. 2007) as compared to reactive individuals. In this paper personality traits are defined as physiological and behavioural responses to environmental changes which manifest as correlated trait-clusters (Sørensen et al. 2013).

The importance of understanding individual variation in fish has been shown to have implications in a wide range of fields including behavioural ecology (Budaev and Zworkykin 2002; Réale et al. 2010ab; Sih et al. 2004), neurosciences (Johansen et al. 2012) aquaculture (Huntingford and Adams 2005; Martins et al. 2011ab), welfare (Martins et al. 2012; Øverli et al. 2007), health and diseases susceptibility (Fevolden et al. 1992, 1993), performance traits (Martins et al. 2011ab; Øverli et al. 2007) and interpretations of molecular data (Alves et al. 2010; Johansen et al. 2012; MacKenzie et al. 2009).

Fish are increasingly used as comparative models to uncover many of the fundamental question underlying the origin and implications of coping styles. Consequently, there is a growing interest on studying fish personality. Thus, while the importance of comparative studies to animal coping styles research is recognised (Réale et al. 2007), there is a lack of basic information that underlines the existence of personality in a particular species. Such information includes to which extent observed individual differences are consistent over time and predictive of other behaviours measured in different contexts. Consistency is used to describe a behavioural measure that is predictable across time and/or contexts. Even if the intensity of the behaviour changes, the rank position in relation to others, remains the same (Budaev and Zworkykin 2002; Toms et al. 2010). A recent study using selected lines of rainbow trout (*Oncorhynchus mykiss*), proactive and reactive individuals were shown to exhibit consistency over a period of 7 days in traits associated to coping styles, feeding responses, presence of a novel object, aggressiveness and confinement (Basic et al. 2012). Most of the studies on coping styles characterization have been done on selected fish lines which raises the question whether similar consistency responses can be observed in non-selected populations.

Another drawback of fish personality studies is the fact that the majority of tests developed are based on individually-housed animals (Barreto and Volpato 2011; Martins et al. 2011c; Øverli et al. 2006; Silva et al. 2010). Individuals may differ in the interpretation of housing condition and consequently present distinct motivational states (Galhardo and Oliveira 2009). In addition, sociability has been shown to be a personality dimension, also in fish, suggesting that the effect of isolation can differ between individuals with different personality. Grouped-based tests may therefore have an added value when characterizing personality traits in fish. However, personality traits may also vary with social context (Galhardo et al. 2012) and phenomena such as facilitation may influence the results (Reebs 2000). To the best of our knowledge no study has ever addressed personality traits in fish using both individual and group based screening tests.

Here, we investigate whether individual differences in behavioural responses to a variety of challenges can be used to assess personality in fish. Several tests were developed and repeated twice: feed intake recovery in a novel environment, novel object, restraining, risk-taking and hypoxia. These tests focus on one personality dimension: the exploration-avoidance (Champagne et al. 2010; Réale et al. 2007) also as a review of the other personality dimensions in fish]. Gilthead seabream (*Sparus aurata*) was used as our model specie, as it is widely used in research due to its robustness and well known biology and behaviour. It is also ranked second as the most important European farmed fish (Barazi-Yeroulanos 2010).

Material and methods

All experiments described were conducted in accordance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals, and under a "Group-1" licence from the Veterinary Medicines Directorate, the Portuguese competent authority for the protection of animals, Ministry of Agriculture, Rural Development and Fisheries, Portugal. Permit number 0420/000/000-n.99-09/11/2009. At the end of the experimental procedures, individuals used in this study were kept under group conditions (11.2 kg m^{-3}) and optimal water and feeding conditions as they will be used in another study that aims at looking at consistency of personality over longer time periods.

Experimental animals, housing and feeding

Twenty-four juveniles of Seabream, Sparus aurata, with an initial body weight of 49.31±7.25 g (means±SD) were used as experimental animals. All animals were obtained from a seabream producer (MARESA Mariscos de Esteros SA, Huelva, Spain) and were kept in stock groups until the start of the experiment. Individuals were individually PIT-tagged (Trovan®, Netherlands) one week before the start of the experimental procedures. Water temperature (19.8±2.1 ºC), salinity (33.8±2.4 ‰), dissolved oxygen (98.4±2.8 %), NO2-N (0.0±0.0 mg L-1) and NO3-N (0.0±0.0 mg L-1) were checked daily. A 12L: 12D photoperiod was maintained with day break set at 8:00 h. Fish were fed with automatic feeders, with commercial diet (Aquagold 2mm, Sorgal SA, Portugal; 44 % crude protein, 14 % crude fat, 8 % ash, 2.5 % crude fibres, 1.0 % phosphorus). The same feed and photoperiod was used during the experimental procedures.

Personality screening

Each fish was subjected to the following tests: 1) Feeding recovery in a novel environment (adapted from Øverli et al. 2006b), 2) Novel object (adapted from Frost et al. 2007), 3) Restraining (adapted from Arends et al. 1999, Silva et al. 2010 and Martins et al. 2011cd 4) Hypoxia (adapted from Laursen et al. 2011) and 5) Risk-taking (adapted from Huntingford et al. 2010). Tests 1 – 3 were individually-based while tests 4 and 5 were grouped-based (see Figure 4.1). Each test was repeated twice (run 1 and run 2) with an interval between runs of 14 days. Individually-based tests were carried out first (both run 1 and 2) followed by the grouped-based tests. Between individual and groups-based tests, fish were kept in groups of 12 fish. These groups were maintained during the group testing.

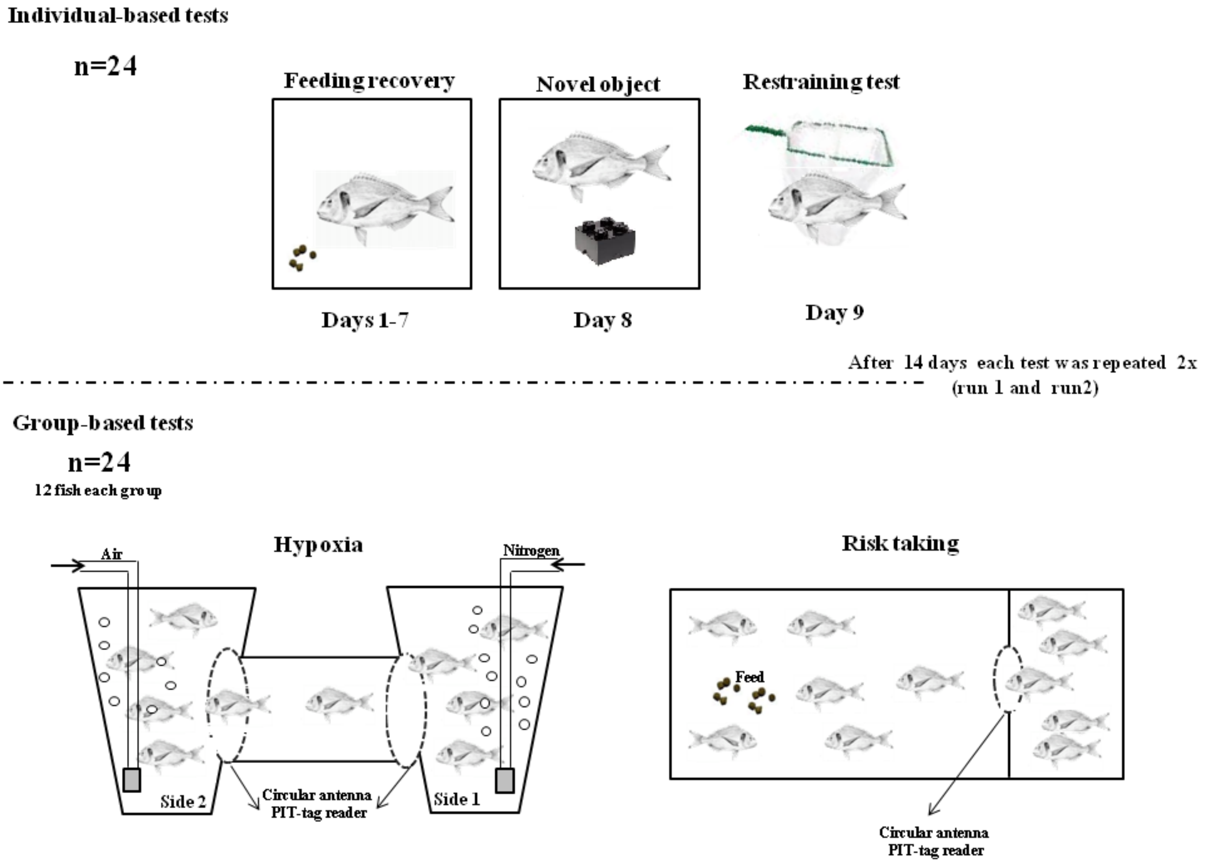

Figure 4.1. Schematic representation of the experimental set-up used to determine personality in Gilthead seabream Sparus aurata. Daily feed intake recovery on isolated fish (n=24), was recorded during 7 days. On day 8 and 9the same fish were submitted to novel object test and net restraining test, respectively. Each test was repeated twice (run 1 and run 2) with an interval between runs of 14 days. Individually-based tests were run first (both run 1 and 2) followed by the grouped-based tests.

Individual-based tests

Fish were housed individually in a 40 L glass aquarium (37 cm length x 40 cm width x 40 cm depths) in an open water circuit during 9 days. The water flow rate was

60 L.h-1, nearly 1.5 renovations per hour. Water temperature (19.3±2.1 ºC), salinity (33.8±2.4 ‰), dissolved oxygen (98.1±1.3 %), NO2-N (0.0±0.0 mg L-1) and NO3-N (0.0±0.0 mg L-1) were checked daily.

Feeding recovery test

The feeding recovery test consisted of following daily feed intake recovery in fish housed in isolation for 7 days. Fish (n =24) were fed ad libitum, by hand, twice per day (09:30 and 15:30) using the same diet mentioned before. The order of feeding was randomized every meal. Five pellets were added at the start of feeding and the number of pellets eaten by each fish was noted and replaced by new ones as soon as they were consumed. Feeding continued for a maximum of 1 h, after which the remaining pellets were collected and counted. Feeding recovery was determined as following: feeding latency (time in seconds taken by each fish to consume the first pellet); total feeding time (total time in seconds taken by each fish to consume all pellets until apparent satiation); number of feeding acts (number of times an individual approached the pellets resulting in feed consumption), number of feeding days (number of days that result on feed intake) and feed intake (% BW^{-1}).

Novel object test

The novel object test (day 8, after onset of isolation) consisted of a Lego® brick (3cm length x 3 cm width x 2.3 cm height – used during the 1st run) or a table tennis ball filled with sand (2 cm radius – used during the 2nd run) that were dropped suddenly in the middle of the tank. The bottom of the test tanks were divided into three distinct zones: 5 and 10 cm radius around the novel object and the remaining area, which were marked with a text marker on the bottom of the tank. Fish behaviour was video recorded (SONY, DCR-SR190E, Japan) for posterior analyses. Cameras were placed above the tanks. The observation period lasted 15 minutes and started immediately after the novel object was dropped in the tank. During the 15 min observation period the following parameters were measured: latency to enter the 5 cm and 10 cm radius areas (time in seconds taken by each fish to enter in each area) and the number of times fish entered in each area. The entrance in the area was defined when the snout of the fish was inside the area.

Restraining test

The net restraining test (day 9, after onset of isolation, last day of individually-based tests) consisted of holding each fish individually in an emerged net for three minutes (Arends et al. 1999; Silva et al. 2010; Martins et al. 2011cd). While in the net the following behaviours were measured: latency to escape (time in seconds taken by each fish to show an escape attempt; escape attempt was defined as a elevation of the body from the net; number of escape attempts and total time spent on escape attempts (total time in seconds taken by each fish escaping since the first to the last escape attempts).

Blood samples were collected 30 minutes after the start of net restraining, according to Arends et al. (1999). Therefore, fish were quickly taken out from each tank at the same time and anaesthetized with 2-phenoxyethanol (1000 ppm, Sigma-Aldrich). Blood was withdrawn within 3 min from caudal vein using heparinised syringes and centrifuged at 2000 × g for 20 minutes at room temperature. After centrifugation plasma was frozen in liquid nitrogen and stored at -80 °C for cortisol analysis. After blood sampling individuals were weighed and identified.

Group-based tests

Hypoxia test

The hypoxia test consisted of reducing the oxygen levels in one side of a two-chamber tank and measuring the escape behaviour from the hypoxia to the normoxia side. The tank was composed of two similar circular tanks (40 L) that were connected with a transparent plastic pipe (40 cm length x 6 cm radius). In the extremes of the connection pipe two circular antennas were placed, (diameter 100/125 x 20 mm Trovan®, Netherlands), to allow individual tracking of the fish passing through the pipe. Each side of the tank was equipped with water inflow, outflow and air stone supply. The connection pipe was closed with a removable door (13 cm length x 13 cm width) before the start of the test. Each group of fish (n=12) were allowed to settle overnight in one side (side 1) before the start of the experiment. At the beginning of the experiment the water supply was stopped on both sides. Aeration on side 1 was turned off and replaced by nitrogen which leads to a gradual decrease in oxygen concentration (Figure 4.2). Afterwards, the door blocking the connection tube was removed and the circular antennas started to register the fish movement between sides. The dissolved oxygen in the water (DO) was measured by an Oxyguard handy probe (Handy Delta, USA). Figure 4.1 shows the DO decrease over time. During the hypoxia test, fish behaviour was video recorded (MicroVideo™ camera MCV2120-WP-LED, Canada) for posterior analyses. The following behaviours were measured: latency to escape hypoxia (time in seconds taken by each fish to escape hypoxia conditions); order of escape and number of returns (number of times an individual returns to the hypoxia side after being in the normoxia side). The hypoxia test was finalised when half of the fish escaped from the hypoxia side or when a concentration of 3 mg.L -1 DO was reached.

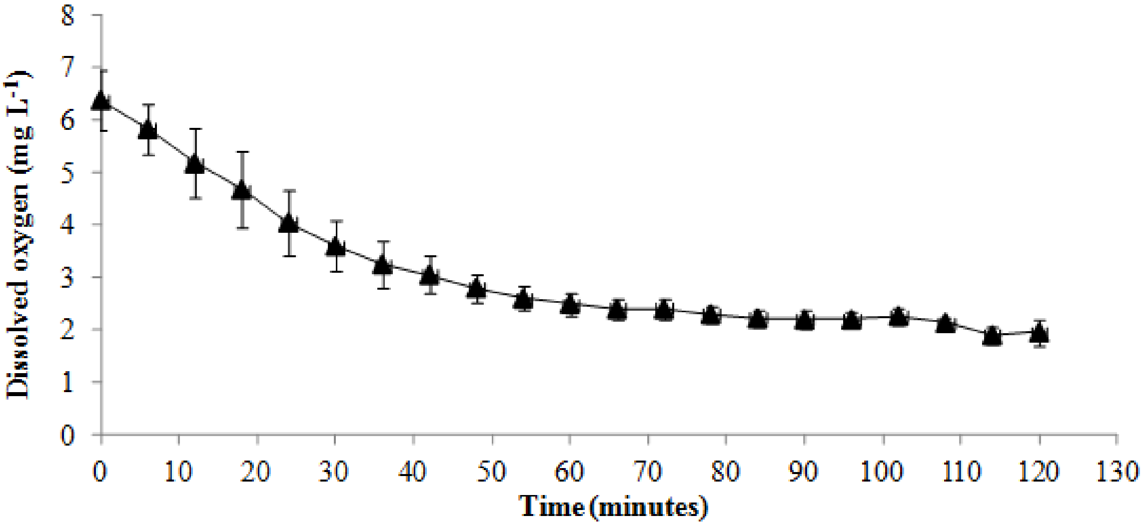

Figure 4.2. Decrease of dissolved oxygen in the water (DO) over a period of hypoxia test. Values are the mean of two runs for all the individuals.

Risk-taking test

The risk-taking test was done on a 300L fibreglass tank (100 cm length x 60 cm width x 50 cm depth) separated in two distinct areas: safe and risk areas. The areas were divided using a solid plastic partition (2 mm thickness) with a hole (6 cm radius), connected to a circular antenna, diameter 100/125 x 20 mm (Trovan®, Netherlands) that allowed the identification of which fish passed through the hole and the time of each passage. The connection hole was closed with a removable door (13 cm length x 13 cm width). Each group of fish (n=12) were allowed to settle overnight in the safe area before the start of the experiment. At the beginning of the experiment the door was removed and 10 pellets (6 % BW-1) were released into the risk area every 5 minutes to stimulate fish going to the risk area. Fish behaviour was video recorded (MicroVideo™ camera MCV2120-WP-LED, Canada) for posterior measurement of: latency for risk-taking (time in seconds taken by each fish to enter the risk area); order of risk-taking and number of returns (number of times an individual returns to the safe area after being in the risk area). The risk-taking test was finalised when half of the fish entered in the risk area or 4.5 hour after the beginning of the experiment.

Cortisol analyses

Plasma cortisol levels were measured with a commercially available competitive binding Coat-A-Count® Cortisol kit (SIEMENS Medical Solutions Diagnostics, USA) adapted from (Irwin et al. 1999). Briefly, 50 ml of each sample to be assayed was transferred into an Ab-Coated tube and 1 ml of 125I Cortisol added. The tubes were then incubated for 45 min at 37 ºC in a water bath. The contents of all tubes were decanted, and allowed to drain for 5 min before being read on a gamma counter (2470 WIZARD2TM, PerkinElmer TM, Inc., Belgium) for 1 min. A calibration curve was used to convert results from percent binding cortisol to concentration (ng ml-1). The Coat-A-Count cortisol antiserum cross-reacts: 100 % with cortisol, 11.4 % with 11-deoxycortisol, 0.98 % with cortisone, 0.94 % with corticosterone and 0.02 % with progesterone.

Data analysis

Statistical analyses were performed using SPSS 18.0 for windows. The results are expressed as mean±standard deviation (SD). Behaviours measured in each test were collapsed into first principal component scores using Principal Components Analysis (PCA). The correlation matrix was used to check multicollinearity, i.e., to identify variables that did not correlate with any other variable, or correlate very highly ($r=0.9$) with one or more variables. Kaiser–Meyer–Olkin (KMO) test for sample adequacy was always greater than 0.5 and the Bartlett's test of sphericity was significant for all tests. The PC1 for run 1 and run 2 for each test was averaged and used to investigate cross-context relationships. Spearman correlation analyses were used after data failed to pass the normality Kolmogorov-Smirnov test. In addition, a two-step cluster analyses was performed using the PC1 average (from run 1 and 2) of the tests that revealed consistent responses over time (risk-taking and net restraining). An independent-samples T test, was used to verify differences between the generated clusters. Statistical significance was taken at $p<0.05$.

Results

Individual variation

Table 4.1 depicts the pronounced individual variation in different behavioural variables obtained for each test in Gilthead seabream *Sparus aurata* ($n=24$).

Consistency over time

The consistency over time in behavioural responses is shown in Table 4.2. There was a strong positive correlation between the behaviour in run 1 and 2 of the restraining ($r_S=0.36$, $p=0.01$) and risk taking ($r_S=0.53$, $p<0.001$) tests. Feeding recovery ($r_S=0.28$, $p=0.06$) and hypoxia ($r_S=0.40$, $p=0.06$) showed a strong trend ($p=0.06$) towards consistency over time while the novel object test ($r_S=-0.98$, $p=0.66$) did not result in consistent behavioural responses.

After the restraining test, the cortisol values were 36.17±32.54 ng ml^{-1} (means±SD) and varied between 6.2 ng ml^{-1} and 117.33 ng ml^{-1} in run 1 and were 40.87±27.52 ng ml^{-1} (means±SD) and varied between 9.9 ng ml^{-1} and 87.41 ng ml^{-1} in run 2. Cortisol responsiveness was not consistent over time ($p>0.05$). Behavioural responses during the restraining test were not correlated with cortisol responsiveness.

Table.4.1. Mean ± SD, minimum (Min.) and maximum (Max.) values of behavioural variables obtained for each test in Gilthead seabream Sparus aurata during all the experimental procedures (n=24).

		Run 1			Run 2		
Behavioural context	**Behaviours within each context**	**Mean ± SD**	**Max.**	**Min.**	**Mean ± SD**	**Max.**	**Min.**
Feeding recovery	Lat feeding (sec)	2622.35 ± 828.08	3600.00	898.71	2159.99 ± 923.78	3567.21	852.43
	Total feeding time (sec)	480.49 ± 559.93	1821.86	0	825.77 ± 629.73	1996.14	0
	# feeding sessions	1.50 ± 1.58	4.86	0	3.11 ± 2.53	9	0
	Feed intake (% BW)	0.16 ± 0.17	0.55	0	0.26 ± 0.22	0.67	0
	# feeding days	3 ± 2	6	0	3 ± 2	7	1
Novel object	Lat 5 cm radius area (sec)	387.50 ± 370.30	900.00	19.00	489.23 ± 345.26	900.00	10.00
	#5 cm radius area	8 ± 10	36	0	3 ± 4	13	0
	Lat 10 cm radius area (sec)	207.08 ± 282.57	900.00	19.00	298.27 ± 285.03	900.00	2.00
	#10 cm radius area	13 ± 11	43	0	8 ± 8	26	0
Restraining	Lat escape (sec)	99.96 ± 65.98	180.00	1.00	41.96 ± 33.20	124.00	1.00
	# escapes	8 ± 8	24	0	17 ± 8	35	6
	Total escape time (sec)	8.71 ± 10.25	38.00	0.00	15.65 ± 10.05	43.00	2.00
Hypoxia	Hypoxia lat (sec)	7048.00 ± 7378.00	16200.00	0.00	4167.00 ± 4842.00	16200.00	1020.00
	# returns	4 ± 7	24	0	7 ± 8	23	0
	Hypoxia escape order	8 ± 5	15	1	6 ± 4	15	1
Risk taking	Risk latency (sec)	9323.00 ± 6869.00	16200.00	300.00	7553.00 ± 7897.00	16200.00	0.00
	# returns	1 ± 2	11	0	4 ± 8	29	0
	Risk escape order	8 ± 5	15	1	8 ± 5	15	1

Table 4.2. Consistency over time (run 1 and run 2) of behavioural responses in Gilthead seabream Sparus aurata obtained during transfer into a novel environment, novel object, restraining, risk-taking and hypoxia tests (n=24).

Consistency over time	**Feeding Recovery Run2**	**Novel Object Run2**	**Restraining Run2**	**Risk taking Run2**	**Hypoxia Run2**
Feeding Recovery Run1	$r_s = 0.28$ $p = 0.06$				
Novel Object Run1		$r_s = -0.98$ $p = 0.66$			
Restraining Run1			$r_s = 0.36$ $p = 0.01$		
Risk taking Run1				$r_s = 0.53$ $p = 0.00$	
Hypoxia Run1					$r_s = 0.40$ $p = 0.06$

Cross-context consistency: correlations between tests

The PCA loadings of each test used to generate a principal component score (PC1) to assess cross-context correlations are shown in Table 4.3. Figure 4.3 depicts the relationship between the average PC1 (run1 and run 2) for the behavioural responses observed during feeding recovery, restraining, hypoxia and risk taking test. Individuals that escaped faster from hypoxia, tried to escape more in a restraining test (r_s=-0.53, p=0.01), were more risk-takers (r_s=0.40, p=0.05) and took longer to recover feed intake (r_s=0.51, p=0.01) while in isolation.

Two groups were generated with the cluster analysis (proactive, n=20 and; reactive, n=4) based on restraining and risk-taking PC1 average. Figure 4.4 depicts the differences between proactive and reactive individuals showing that one of the clusters (which we call- Proactive individuals) escaped significantly more during restraining (p=0.05) and were more risk-takers (p=0.01) as opposed to the other cluster (Reactive individuals).

Table 4.3. PCA loadings of within-context behavioural variables used to generate a principal component scores (PC1) in run 1 and run 2.

Behavioural context	Behaviours within each context	Loadings for PC1- RUN 1(component matrix)	% Variation explained	Loadings for PC1- RUN 2(component matrix)	% Variation explained
Feeding recovery	Latency feeding	-0.981	95.458	-0.959	88.058
	Total feeding time	0.978		0.948	
	Number feeding sessions	0.965		0.932	
	Feed intake	0.975		0.928	
	Number feeding days	0.986		0.926	
Restraining	Latency escape	-0.835	83.041	-0.773	59.431
	Number escapes	0.964		0.655	
	Total escape time	0.929		0.870	
Hypoxia	Hypoxia latency	0.963	76.208	0.904	74.598
	Number returns	-0.666		-0.751	
	Hypoxia escape order	0.957		0.925	
Risk taking	Risk latency	0.941	77.311	0.957	80.174
	Number returns	-0.729		-0.744	
	Risk escape order	0.950		0.967	

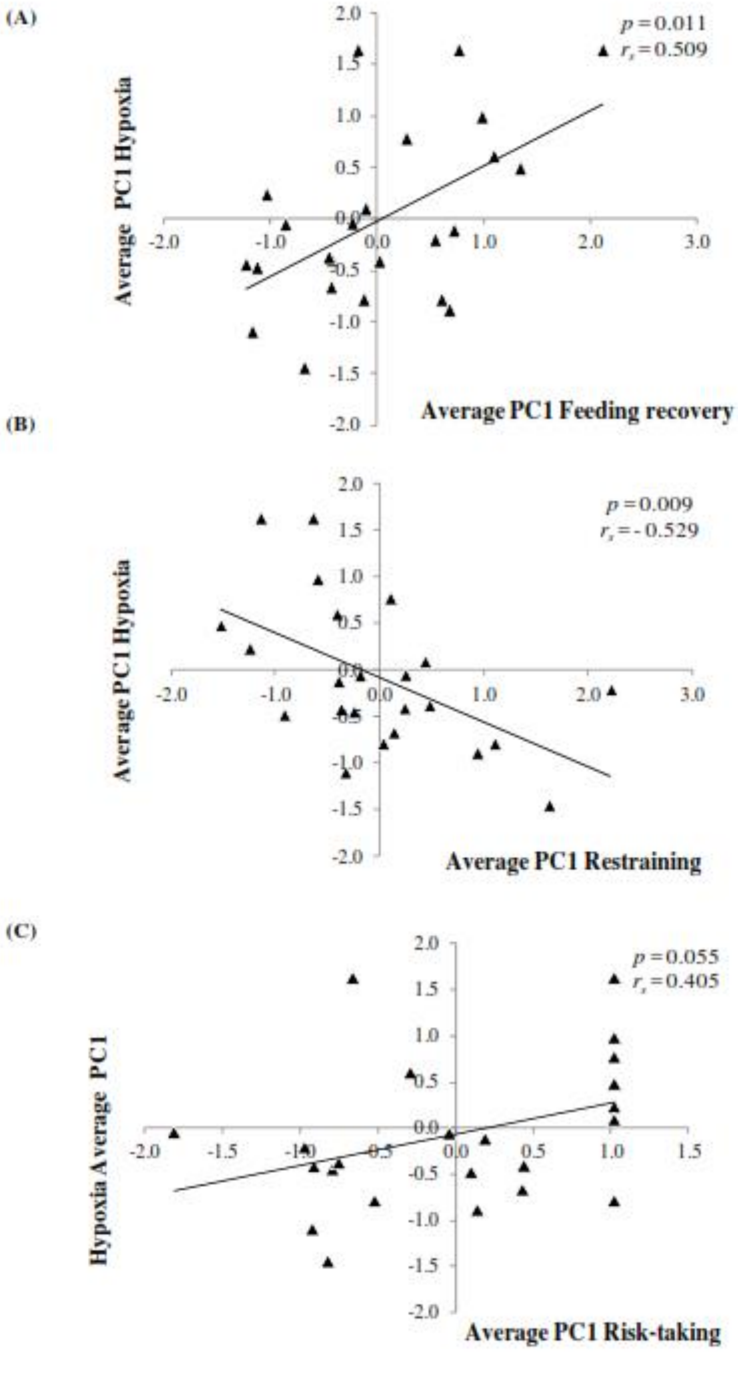

Figure 4.3. Relationship between the average PC1 behavioural score (from run1 and run2) during the hypoxia and feeding recovery (A – individuals with high hypoxia scores took longer to escape hypoxia conditions and resumed feed intake faster, net restraining (B - individuals with high hypoxia scores took longer to escape hypoxia conditions and escaped less during net restraining) and risk taking (C - individuals with high hypoxia scores took longer to escape hypoxia conditions and longer to take risks) tests on seabream Sparus aurata juveniles (n=24).

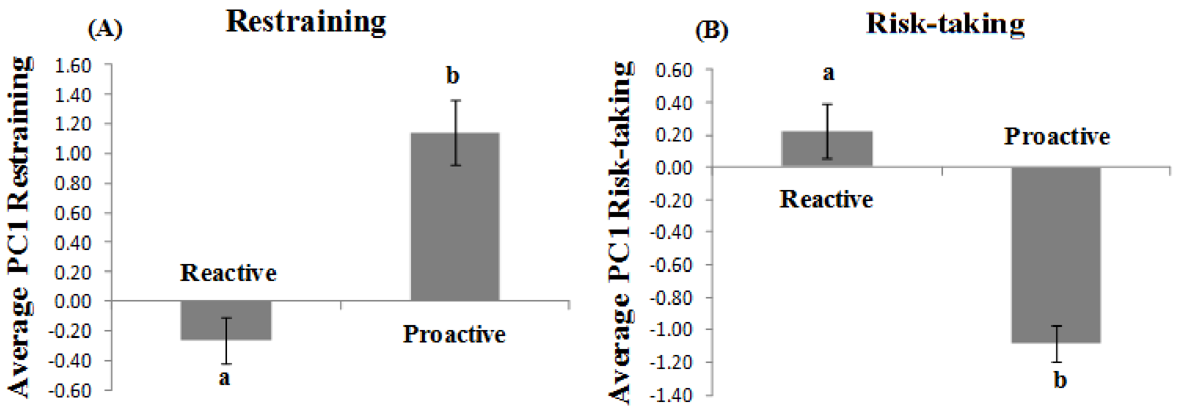

Figure 4.4. Distinct groups (Proactive (n=20) and Reactive (n=4)) generated after cluster analysis, based on restraining and risk-taking PC1 average. Different letters indicate significant differences (independent T-test): restraining (p=0.05); risk-taking (p=0.01)). A- Individuals with high restraining scores escaped more during net restraining. B- Individuals with high risks scores took longer to take risk

Discussion

This study characterized for the first time fish personality considering both the consistency of behavioural differences over time and across contexts using a battery of individual and grouped-based tests. Among the different tests used, the escape behaviour during restraining and the risk taking behaviour showed the most consistent results. In addition, a relationship across contexts was found between hypoxia and feeding recovery, net restraining and risk taking tests.

Considering the consistency of behavioural responses over time, the escape response during a restraining test was shown to be the most repeatable: individuals showing lower latency to escape, higher number of escape attempts and spending more time escaping in run 1 showed a similar behaviour after 14 days when the test was repeated. Escaping behaviour during restraining or confinement has been used to discriminate coping styles in other animals, e.g pigs (Bolhuis et al. 2005a) and also in fish (Martins et al. 2011cd; Øverli et al. 2006ab; Silva et al. 2010). However, previous studies performed in fish showed contradictory results. On one hand, several studies showed that the proactive coping style is behaviourally characterised by a high level of locomotor activity during confinement or restraining as opposed to reactive individuals (Brelin et al. 2005; Martins et al. 2011cd; Silva et al. 2010). On the other hand, higher locomotor activity during confinement or restraining has been observed more in reactive as opposed to proactive individuals (Øverli et al. 2002, 2006b). It is interesting to notice that these latter studies showing higher locomotor activity during confinement in reactive animals used fish selected lines. In addition, proactive individuals usually exhibit a lower hypothalamus–pituitary–interrenal (HPI) axis reactivity. In the present study, no correlation between escape behaviour and plasma cortisol was found. Several studies have documented the lack of correlation between plasma cortisol levels obtained after stress and behavioural responses (Silva et al. 2010; van Erp-van der Kooij et al. 2003; van de Nieuwegiessen et al. 2008). Some authors have suggested that cortisol and behavioural responses to stressors are linked to two independent dimensions of stable trait characteristics (Koolhaas et al. 2010). These authors suggested that the quality of the response to a challenging condition (coping style) is independent from the quantity of that response (stress reactivity). According to the same authors, the physiological responses to stress such as the HPI axis reactivity (one of the most significant differences between proactive and reactive individuals) is more related to an emotional response to stress than to coping styles. Eventually a decoupling of these axis, coping styles and emotional, could bring new light to understand the pronounced individual variation in plasma cortisol response observed in seabream after stress.

The other test that revealed consistent behavioural responses was the risk-taking test. Certain individuals were consistently the first to take the risk to venture into an unknown environment where food was present. One may wonder what the main driving force leading fish to cross the opening into a new environment was: 1) the willingness to explore a new environment; or 2) the motivation to eat, since food was only available in the new area. Toms et al. (2010) suggested that hunger levels may influence risk-taking instead of proactive traits. In our study fish were fed ad libitum prior to the transfer to the risk-taking tank which could have minimized the differences in hunger level between proactive and reactive. On the other hand, proactive and reactive individuals differ in their metabolism (Careau et al. 2008; Martins et al. 2011d), consequently we cannot exclude that proactive individuals were hungrier and probably take more risks like going into a potentially dangerous or unknown environment, to get food.

Considering the consistency across contexts, individuals escaping more during the restraining test also escaped faster from hypoxia conditions. These results are in contrast to the findings of Laursen et al. (2011), who reported that reactive fish escaped faster to hypoxic conditions. This suggests that reactive fish exhibit higher levels of behavioural flexibility. However, another study using the same selected trout lines suggested that depending on the context, proactive individual may adopt a more flexible behaviour (Basic et al. 2012). One possibility to explain the differences found in the present study using seabream as compared to Laursen et al. (2011) is the existence of species-specific differences in sub-lethal effects of reduced levels of dissolved oxygen; around 3 mg L^{-1} in trout (Raleigh et al. 1984) and 1 mg L^{-1} in seabream (Reig 2001). Therefore the propensity to escape could be expected to be different between these species. In Laursen et al. (2011) individuals exhibit escape behaviour when exposed to decreased oxygen levels varying from 90 to 30 % saturation. In seabream, however, individuals start escaping hypoxia only when oxygen concentrations reach level close to 30 % saturation (3 mg L^{-1}). These differences in responsiveness may suggest that in trout, reactive individuals known to be more sensitive to changes in environmental conditions (Ruiz-Gomes et al. 2011) are the first to escape hypoxia. However in seabream, escape behaviour starts only when oxygen concentrations reach to sub-lethal levels. In such situation, proactive individuals known to exhibit active attempts to counteract stressors (Benus et al. 1991b) could be the first to escape hypoxia. To which extent the onset of responses of proactive and reactive individuals is dependent of how strong the stress is (or is interpreted to be) close to life-threatening conditions needs to be further investigated.

In this study, individuals exhibiting typical proactive characteristics (higher risk taking, higher escaping behaviour) were individuals taking longer to recover their feed intake while in isolation. These results are in contrast with (Øverli et al. 2006b) and (Martins et al. 2011ab) who showed a quicker recovery of feed intake in proactive as compared to reactive fish. However, other studies (LeBlanc et al. 2012; Ruiz-Gomes et al. 2011), showed opposite results, i.e. proactive individual take longer to recover feed intake. Such inconsistency of results may be due to species-specific behaviour and/or to previous experiences (e.g. social experiences, nutritional background) that fish were exposed prior to the start of the experiments. In our study, reactive individuals recover feed intake faster and this can be due to showing some kind of compensatory feed intake as a result of previous social environment. Alternatively, reactive individuals by being more flexible (Ruiz-Gomes et al. 2011) could have adapted faster when placed in a new environment.

In the present study the novel object test did not result in consistent behavioural responses. In contrast, Frost et al. (2007) screened bold and shy individuals using their latency to come within close proximity of a novel object. However, Galhardo et al. (2012) found a lack of consistency in exploration–avoidance traits as measured by the novel object test in Mozambique tilapia (*Oreochromis mossambicus*). In highly social species, personality traits may vary with social context and when this happens, it is crucial to take in account the social setting when assessing personality traits. Another possible explanation, for the absence of significant results in the novel object test could be related to differences in the size of the experimental glass aquarium. The experimental glass aquarium used in our study was square compared with rectangular tanks used by (Frost et al. 2007) and consequently in our study individuals could have had more difficulty to express exploration-avoidance behaviour towards the novel object, once they had less space available between aquarium walls and the object.

The present study shows for the first time a link between individually- and grouped- based test in fish personality characterization. Nearly all studies developed to study fish personality were based on individually-based tests (Frost et al. 2007; Martins et al. 2011cd; Øverli et al. 2006; Silva et al. 2010). A few examples are available using grouped-based tests (Huntingford et al. 2010; Laursen et al. 2011). However, to the best of our knowledge no study has used both approaches to assess personality in fish. One of the main criticisms with individually based tests is that they do not reflect what is happening in a group. On one hand different personalities could exhibit a different degree of sensitization to isolation. On the other hand group testing may lead to individuals modulating their own behaviour based on other´s behaviours (e.g. facilitation (Reebs 2000)). An interesting extension of the previous study would be to repeat with the same individuals the same test both in individual and grouped-based contexts and compare the behavioural responses.

In summary, this study suggests that individual differences in behavioural responses towards challenges reflect the presence of personality in fish. Using a non-selected fish line we found consistency over time and across-context in behavioural responses to challenges using individual and grouped-based tests. This study highlights the possibility to predict behaviour in groups from individual personality traits. Therefore, these findings may contribute to understand the pronounced individual variation in stress responses observed in this species. Furthermore, this study highlights the possibility to develop mass-screening methods to assess personality in fish that are grouped-based and therefore less time consuming as compared to individual-based tests.

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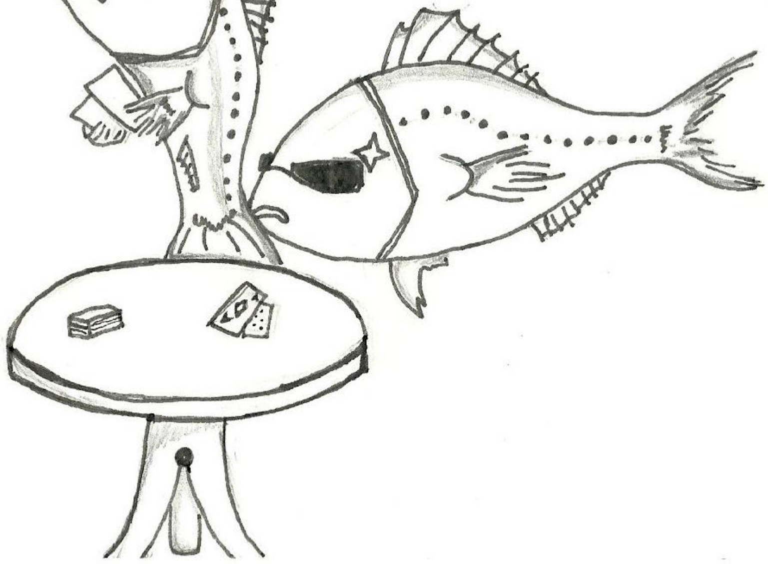

CHAPTER

5

Are personality traits consistent in fish?

The influence of social context

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The influence of social context

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Are personality traits consistent in fish? Social context

Chapter 5

Abstract

Individual differences in behavioural and physiological responses to challenges are progressively accepted as adaptive variation and reveal a strong degree of evolutionary conservation throughout the vertebrate taxa. Previous studies in gilthead seabream (*Sparus aurata)* suggested that individual differences in behaviour reflect distinct coping styles or personality, contrasting consistent traits associations. One of the traits that have been shown to be consistent over time and across context is the escape response under a restraining test. Using this trait as a proxy of personality in seabream the influence of social context in the consistency of escape behaviour was investigated. Individually tagged juvenile seabream (n=360; 70.18 ± 11.44 g; mean ± SD) were subjected to a restraining test that consisted of keeping each fish in an emerged net for one minute. Behaviours measured in the net (latency to escape; number of escape attempts and total time spent on escaping) were collapsed into first principal component scores using Principal Components Analysis (PCA). Using the PCA scores the individuals were distributed into homogeneous groups (n=30 each group) of proactive, reactive and intermediate. Control groups consisted of mixed groups with 1/3 of each coping style. After one month the same individuals were exposed to the same test (restraining test) to assess consistency of behavioural responses. Results indicate that homogenous groups of proactive (p=0.086) and reactive (p=0.159) individuals did not exhibit consistent behavioural responses as opposed to the intermediate (p=0.028) and control groups (p<0.001). This study thus confirms that the social context in which fish are kept significantly influence personality traits.

Keywords: Individual variation, *Sparus aurata*, Coping styles, Behavioural syndromes, Group composition, Social information

Introduction

In recent years the study of individual differences in behavioural and physiological responses to challenges (i.e. animal personality or coping style) has been increasing considerably. The adaptive importance of individual variability has become a central subject in a wide range of different biological disciplines ranging from behavioural ecology to biomedical research (Francis 1990; Gosling 2001; Koolhaas et al. 1999; Sih et al. 2004).

Despite the diversity of terminology and designated definitions (Francis 1990; Gosling 2001; Koolhaas et al. 1999; Sih et al. 2004), there seems to be a consensus that individual variation may be consistent and biologically meaningful, and individual differences in certain behavioural traits are consistent and predictive of other behaviours or physiological responses shown in another context (Koolhaas et al. 1999).

Several studies in fish have provided early documentation on individual consistency in behaviour (Brelin et al. 2005; Castanheira et al. 2013ab; Huntingford 1976; Martins et al. 2012; Millot et al. 2009ab; Øverli et al. 2004) that reflects distinct personality traits usually categorized in two contrasting personality types, proactive (active coping or bold or 'fight-flight') and reactive (passive coping or shy or 'non-aggressive' (Koolhaas et al. 1999; Øverli et al. 2007). Behaviourally, proactive individuals are characterised by active avoidance, low flexibility, high levels of aggression, territorial control, and other behavioural responses that suggest active efforts to counteract a negative stimulus, this pattern being the opposite for reactive individuals (Koolhaas et al. 1999, 2010, Ruiz-Gomes et al. 2011).

Seabream (*Sparus aurata*) is one of the most important farmed species in the Mediterranean. Recently, the presence of personality types in seabream has been shown based on individual differences in cortisol responsiveness after a restraining test (Castanheira et al. 2013a), together with individual differences in behavioural responses to a variety of challenges that are consistent over time and across contexts using both individual and grouped-based tests (Castanheira et al. 2013b). Using the previous results we can hypothesize that some dimensions of personality, more specifically the escape response (avoidance) can be influenced by the group where the fish are reared. Avoidance is the tendency or absence of the tendency to engage with novelty, and is accepted as one of the main dimensions of personality in animals (Réale et al. 2007). The existence of individual variation in the escape response and the influence of social context on that response are likely to have an impact in adaptability and welfare in aquaculture rearing conditions. Despite the ecological significance (predator-prey-interaction) and physiological implications (anaerobic recovery capacity of white muscle) of the escape response in aquaculture rearing condition that trait can be easily accessed during normal rearing procedures (e.g. grading, vaccination, transport).

Indeed, it is well documented that social context exerts considerable influence on the individual personality (Webster and Ward 2011). Social processes, such as conformity (the tendency of individuals to adopt the behaviour of the majority of their group mates) and facilitation (the presence of group mates affects the behaviour of an individual, allowing individuals to perform behaviours that they would not do if they were alone) exert a known influence on the behaviour of grouping animals and hence isolated animals could behave differently (Magnhagen and Staffan 2005; Magnhagen 2007; Magnhagen and Bunnefeld 2009). The importance of social context in fish is also illustrated by its role in social familiarity (Galhardo et al. 2012), social dominance (Montero et al. 2009), social plasticity (Oliveira 2009, 2012) and social learning (Brown et al. 2003). Therefore, it is expected that personality traits are flexible when exposed to distinct environmental conditions (e.g. social group), dependent of the social relationship and personality of the individuals group members.

Thus, while the importance of sociability in personality is recognised, the study of social context in fish typically address the effect of group size or composition, and potential effects of social context (group composition) on stress response have been so far largely ignored.

With all this in mind, the present research investigates the effect of avoidance in gilthead seabream kept under different social contexts, i.e. the influence of other group members on an individual avoidance behaviour consistency.

Methods

The experiment described was conducted in accordance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals, and approved by the ethics committee from the Veterinary Medicines Directorate, the Portuguese competent authority for the protection of animals, Ministry of Agriculture, Rural Development and Fisheries, Portugal. Permit number 0420/000/000-n.99-09/11/2009.

Experimental animals, housing and feeding

The experiment was carried out at the Ramalhete Research Station from CCMAR (Faro, Portugal). All animals used were randomly selected from a population, housed in two fibreglass stock tanks (500L) n=250 per tank under standard rearing conditions (Morales 1983). All animals were obtained from a seabream producer (MARESA Mariscos de Esteros SA, Huelva, Spain) and were kept in stock groups until the start of the experiment. Individuals were anaesthetised with 2-phenoxyethanol (0.5 ‰, Sigma-Aldrich) which rendered them completely motionless within 10 s of immersion and individually PIT-tagged (Trovan®, Netherlands) in the muscle under the dorsal fin. After tagging fish were placed in a bucket with clear water and aeration to recovery from the anaesthetic before laid in the rearing tanks. All the individuals were recovery within 30 s in maximum from the anaesthetic procedure. This procedure were done two weeks before the start of the experimental procedures. During rearing water temperature (22.3±1.2 ºC), salinity (35.9±1.4 ‰), dissolved oxygen (98.1±1.8 %), NO_2-N (0.0±0.0 mg L^{-1}) and NO_3-N (0.0±0.0 mg L^{-1}) were checked daily and a natural photoperiod was provided. Fish were fed 2% BW day^{-1} , by hand, twice per day (09:30 and 14:30), with a commercial diet (Aquagold 3mm, Sorgal SA, Portugal; 44 % crude protein, 14 % crude fat, 8 % ash, 2.5 % crude fibres, 1.0 % phosphorus). The same feed and photoperiod was used during all experimental procedures.

Experimental procedures

Individually tagged Seabream juveniles (n=360; 70.18 ± 11.44 g; mean ± SD) were subjected to a net restraining test for personality screening. The escape behaviour under a restraining test is one of the traits that have been shown to be consistent over time and across context in previous studies (Castanheira et al. 2013b). Briefly, the net restraining test consists of holding each fish individually in an emerged net for three minutes. While in the net the following behaviours were measured: i) latency to escape (time in seconds taken by each fish to show an escape attempt; escape attempt was defined as an elevation of the body from the net; ii) number of escape attempts and iii) total time spent on escape attempts (total time in seconds taken by each fish escaping since the first to the last escape attempts) (for details see Castanheira et al. 2013b). Behaviours measured in the net were video recorded, analysed and collapsed into first principal component scores using Principal Components Analysis (PCA) in order to obtain a score allowing the individual characterization of personality. Individuals presenting a high latency to escape, low number of escape attempts and shorter total time escaping were characterized by a low score and identified as reactive fish. Individuals presenting a lower latency to escape, high number of escape attempts and longer total time escaping were characterized by a high score and identified as proactive. These scores were used as a continuous variable with a range from -1.07 to 1.08. Using the PCA scores the individuals were distributed into homogeneous groups (n=30 each group; in triplicate) of proactive, reactive and intermediate. Control groups consisted of mixed groups with 1/3 of each coping style (10 proactive, 10 reactive and 10 intermediate animals). Experimental groups were kept in plastic tanks (100 L) during one month. After this period the same individuals were exposed to the same test (restraining test final) to assess the consistency of behavioural responses.

Data analysis

Statistical analyses were performed using SPSS 19.0 for Windows. The results are expressed as mean±standard deviation (SD). Behaviours measured in the net restraining test were collapsed into first principal component scores (PC1) with orthogonal rotation (varimax) using Principal Components Analysis (PCA). The correlation matrix was used to check multicollinearity, i.e., to identify variables that did not correlate with any other variable, or correlate very highly (r=0.9) with one or more other variables. Kaiser–Meyer–Olkin (KMO) test for sample adequacy was always greater than 0.5 and the Bartlett's test of sphericity was significant for all tests.

Spearman correlation analyses were used when data failed to pass the Kolmogorov-Smirnov test for normality. Statistical significance was taken at $p<0.05$.

Results

Table 5.1 depicts the pronounced individual variation during the restraining test: latency to escape, number of escape attempts and total time escaping. Individuals performed on average 10 escape movements for a total escape time of around 14 seconds and a latency to escape of 18 seconds. Latency to escape had the higher variation of the measured variables, with a range from 1 to 180 seconds.

Table 5.1. Mean ± SD, minimum (Min.) and maximum (Max.) values of behavioural variables obtained for the initial restraining test (N = 360) and PCA loading used to generate a principal component scores (PC1).

Behavioural variables	Mean ± SEM	Min.	Max.	Loadings for PC1	% variation explained
Latency escape (s)	18.5 ± 28.0	1	180	-0.709	73.757
Number escape	10.5 ± 7.6	0	35	0.925	
Total escape time (s)	14.2 ± 9.9	0	42	0.924	

The order of restraining was randomized and no effect of order was detected ($p=0.615$). In addition, no significant differences in body weight were observed between individuals with different coping styles ($p=0.785$).

The correlation between the personality scores (initial and final) to assess consistency of behavioural responses is shown in Figure 5.1. Personality scores were significantly correlated with final restraining scores in intermediate ($r_s=0.290$, $p=0.028$) and control groups ($r_s=0.458$, $p<0.001$). No correlation was found between the homogenous groups of proactive ($r_s=0.209$, $p=0.086$) and reactive ($r_s=0.175$, $p=0.159$) individuals, demonstrate that proactive and reactive individuals were more likely to re-adjust their individual escape behaviour as opposed to the intermediate and control individuals.

Some fish lost the PIT-tags during the experiment thus, it was not possible to analyse the behaviours of all individuals. However the sample size used was still very robust: (n=69 proactive, n=57 intermediate, n=66 reactive and n=79 control individuals.

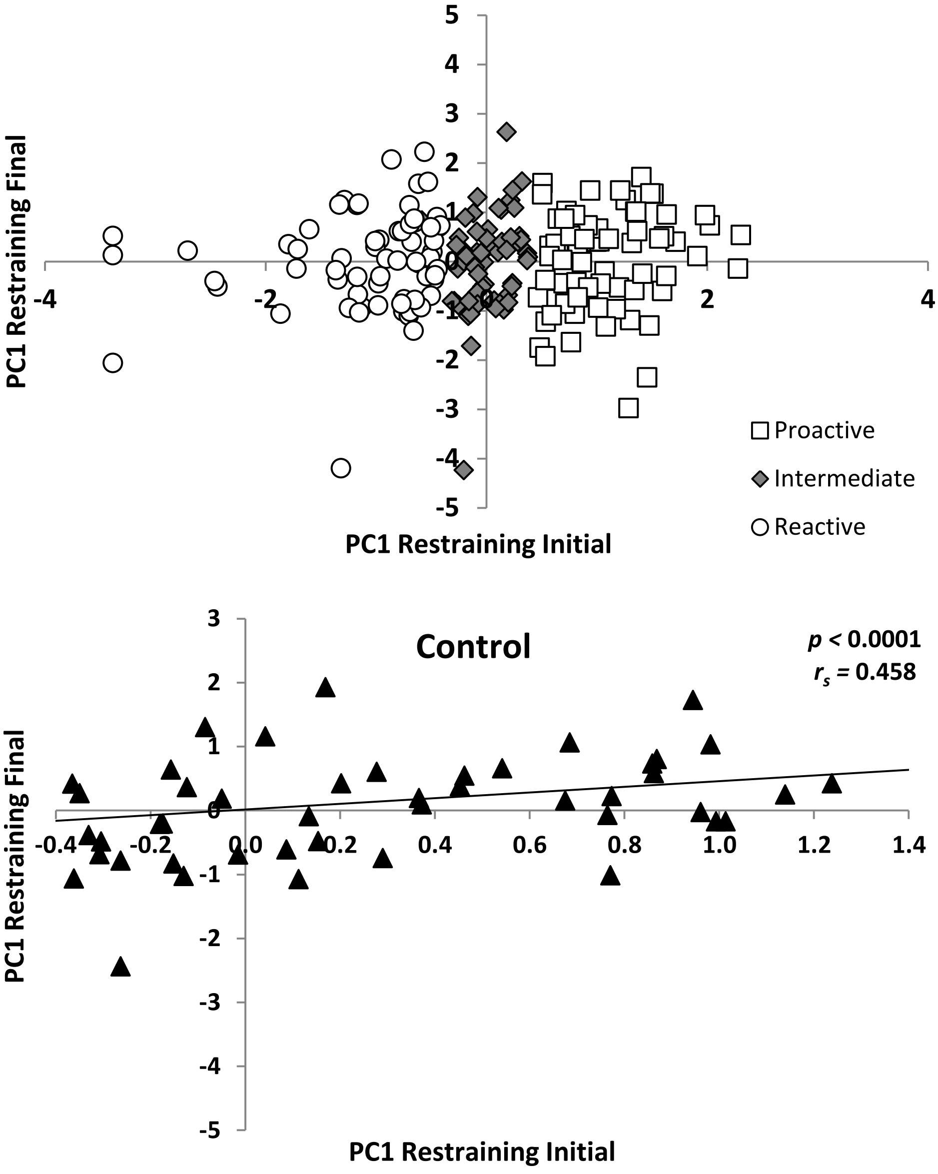

Figure 5.1. Relationship between the PC1 behavioural personality scores (initial and final) during net restraining test (A-Proactive; Intermediate; Reactive; B-Control individuals).

The correlation between the escape behaviour during three minutes and the first minute of the restraining test is shown in Figure 5.2. The first minute of restraining scores was significantly correlated with the three minutes for the initial (r_S=0.680, p<0.001) and final (r_S=0.775, p<0.001) screening.

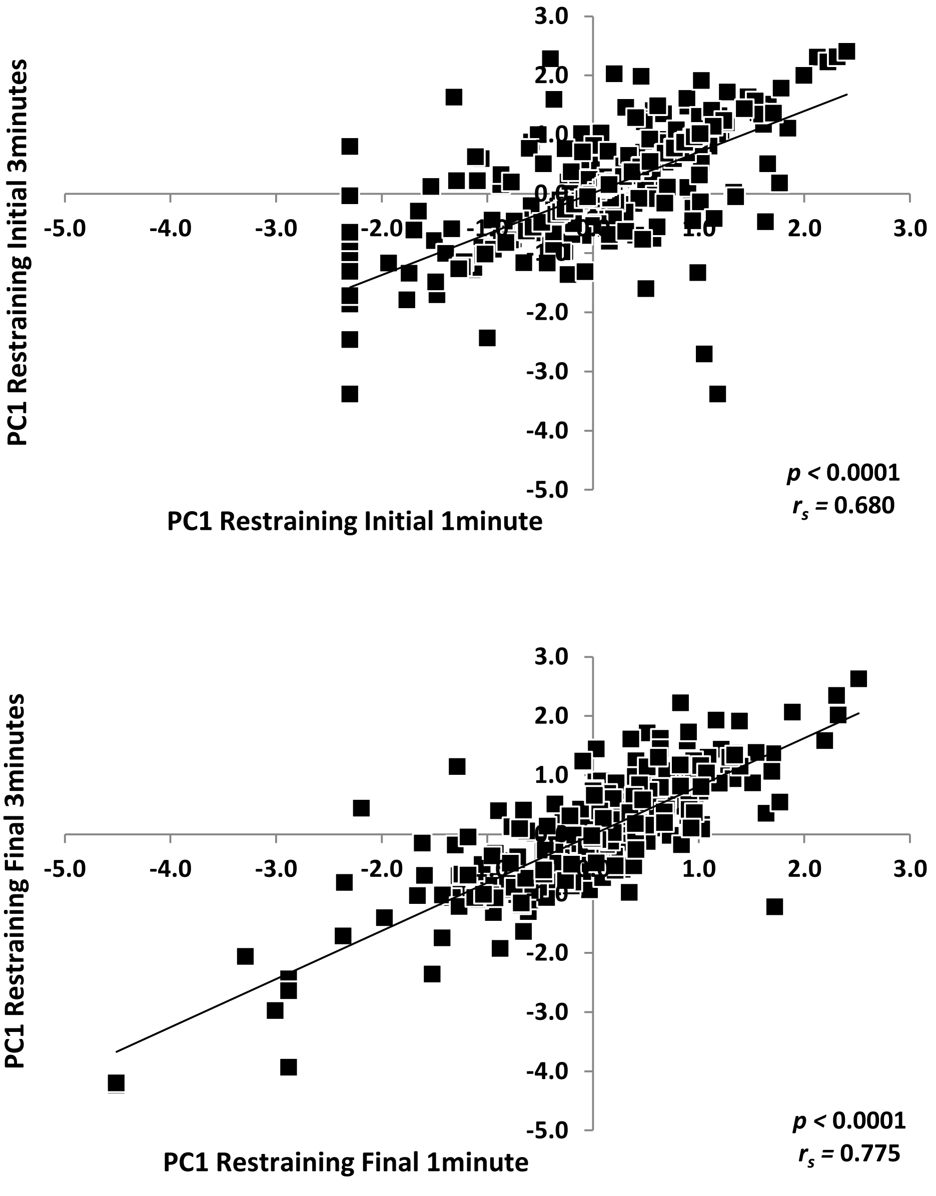

Figure 5.2. Relationship between the PC1 behavioural personality scores during three minutes and the PC1 personality scores during the first minute of the restraining test (A- Initial; B-Final).

Discussion

The main objective of the current study was to investigate the consistency of escape behaviour; one of the main dimensions of personality in animals, in fish kept under different social contexts i.e. the influence of other group members on the consistency of individual avoidance behaviour. Here, the escape/avoidance response during the restraining test indicated a consistent personality trait in intermediate and control groups: individuals showing lower latency to escape, higher number of escape attempts and spending more time escaping during the initial screening, showed a similar behaviour after one month when the test was repeated. In proactive and reactive groups, no correlation was found.

Available evidence suggests that social context strongly influence the individual personality (Webster and Ward 2011). Social context is involved in the regulation of numerous characteristic behaviours such as social facilitation (Webster et al. 2007), social familiarity (Galhardo et al. 2012), social dominance (Montero et al. 2009), social plasticity (Oliveira 2009, 2012) and social learning (Brown et al. 2003). The presence of conspecifics may cause individuals to enhance or suppress threat-sensitive behaviour such as activity, exploration/risk, foraging, feeding rate and courting opportunities (Schuett et al. 2010, 2011; Cote et al. 2010, 2011). Furthermore, individuals with extreme personality types may be affected in a different way. We expected that reactive individuals are more sensitive to isolation and try to adjust/follow more often the behaviour of other group members than proactive individuals. In perch (*Perca fluviatilis*), a modulation of individual behaviours by other group members was suggested and bolder individuals changed the behaviour less when alone than shyer, and had a higher influence in the group. (Magnhagen and Staffan 2005; Magnhagen 2007; Magnhagen and Bunnefeld 2009) The same studies showed that even though the presence of conspecifics significantly affected individual behaviour compared to when alone, the individual behavioural responses remained predictable in isolation or under social contexts. Therefore, the degree of change induced by the presence of conspecifics on the individuals responses, compared to when alone, is also influenced by the initial responsiveness of the individual (Magnhagen and Staffan 2005; Magnhagen 2007; Magnhagen and Bunnefeld 2009).

Moreover, Webster et al. (2007) showed a link between boldness and social facilitation in three spine sticklebacks (*Gasterosteus aculeatus*). When bolder individuals (more active) were tested alone, those that were more active resumed foraging sooner when subjected to a simulated predator attack, and also consumed a higher number of preys in foraging competition trials. However, this relationship was not observed when additional conspecifics were present, demonstrating significant effects of group size upon boldness.

Our results therefore suggest the potential influence of the social context in fish. A finding that can be explained by the fact that individuals tending to adjust their social behaviour according to the available social information in the group, in order to adjust and optimize their own personality type. These adjustments could have an ecological and evolutionary significance related with adaptation to new environmental conditions. Social support might be an advantage to allow individuals to work in cooperation to enhance investment in more profitable activities such as foraging, exploration and mating. The heterogeneity in avoidance response of the group may support some individuals to flourish when the environment change. In addition, the understanding of those differences may have several practical implications. One example is the possibility to takes advantage of this social behaviour and develops rearing conditions accordingly. For example the aquaculture industry may takes advantage of this group heterogeneity in semi-intensive and extensive conditions where the individuals are more susceptible to environmental changes (i.e. in a changing environment the social support may result in a potential boost of the production and the performance of some individuals may be reflected in a faster growth. On one hand, our results comply with previous studies indicating the presence of personality types that seemed based on innate traits (Brelin et al. 2005; Castanheira et al. 2013b; Huntingford 1976; Martins et al. 2012; Millot et al. 2009ab; Øverli et al. 2004ab). On the other hand, personality types can be modified by the influence of other group members (Magnhagen and Staffan 2005; Magnhagen 2007; Magnhagen and Bunnefeld 2009).

Such disparity of results may be due to species-specific behaviour and/or to previous experiences (e.g. social experiences) that fish were exposed prior to the start of the experiments. Frost et al. (2007) suggested that social context is an important modulator of coping styles in rainbow trout. These authors showed in which, previous positive and negative experiences affect personality and modify boldness. However, shy individuals just alter their behaviour (increase their boldness responsiveness) when their relative competitive ability was similar or higher than their conspecifics. In addition, Ruiz-Gomez et al. (2008) suggest that genetic differences determine social position in early life, whereas some behavioural components of coping styles can be modified by social experience, Moreover, we can also assume that this lack of behaviour consistency measured on proactive and reactive groups, may not represent a "loss" of personality in some individuals, but are instead the reflection of stressors such as social stress. Some species develop strong social hierarchies (Barreto Volpato 2006; Ejike and Schreck 1980; Fox et al. 1997) that may cause changes in personality types according to the available social information in the group. Koolhaas and Boer (2008) showed that groups of proactive individuals may encourage a higher level of aggressiveness and fights so that dominant individuals can keep their position in the group. Taking this in account, we can hypothesise that, after some time it will be difficult to maintain the initial rank position in all individuals, and some of them need to adjust their one personality type in order to balance the social group composition. In reactive groups we can expect that some individual have similar adjustments of behaviour, but in an opposite way. If we think about the adaptive value of aggressiveness such adjustments are logical (e.g. less fight promotes a better welfare of the group). Nevertheless, what are the advantages of these adjustments in alignment with the dimension measure in the present study (the escape response- avoidance)?

It is very likely that some reactive individuals in a group will take this opportunity to express a proactive behaviour in order to have some benefits in the group e.g. a proactive position can promote a major role in the group or greater access to feed, high explorative behaviour and more "creative" mating rituals. According to this, intermediate groups and groups with 1/3 of each personality type could be better balanced, promoting a consistent behaviour shown in this experiment (Figure 5.1).

Based on our results we could wonder if the individuals that changed the initial personality type (groups of proactive and reactive) by the influence of other group members, might go back to the innate traits when place in intermediate groups or groups with 1/3 of each personality type. Further experiments are needed to determine the plasticity of each personality type under distinct social group compositions.

An alternative/complementary explanation could be that individual differences depend on the social regulation of gene expression, so that different brain genomic and epigenetic states may match with distinct social regulation in behavioural responses, reflecting a higher or lower social plasticity according to the group composition. In fact, Oliveira (2012) proposed an integrative framework for understanding the proximate mechanisms and ultimate consequences of social plasticity. According to this framework, social plasticity is related with biochemically switching of the neural network underlying social behaviour in response to perceived social information. However, the present data set focused on behavioural responses alone. To which extend such mechanisms (brain genomic and epigenetic) are present in fish and contribute to explain behavioural differences in proactive and reactive individuals related with the social group composition still need to be investigated.

This study also showed that the first minute of the restraining test is representative and enough to characterise the three minutes of the restraining test (Figure 5.2) which may facilitate its use in further personality screening. This finding allows screening in an emerged net during one minute that maybe is less demanding for individuals and researchers with the possibility to screen a large number of individuals in a shorter time period.

The knowledge of personality can help to improve the sustainability and welfare of the aquaculture industry through the establishment of more fine-tuned rearing strategies. Moreover, culture variables could be adjusted in relation to specific group behavioural responses, when designing selection programs.

Conclusions

In summary, the results of the present study indicate that homogenous groups of proactive and reactive individuals did not exhibit consistent behavioural responses as opposed to the intermediate and control groups. These results underline the idea that the social contexts in which fish are kept significantly influence personality traits of individuals, which can be modified by the influence of other group members.

Further studies should be addressed to cover both the behavioural and the physiologic mechanisms underlying these differences. A possible extension of this study would be the investigation of the underlying neurological mechanisms that explain distinct social differences related with distinct personality types.

In addition, these results may open up new perspectives for breeding programmes in this species. The traits to be selected deserve further investigation but the social context certainly has influence in the breeding selection and optimization of rearing conditions.

Acknowledgments

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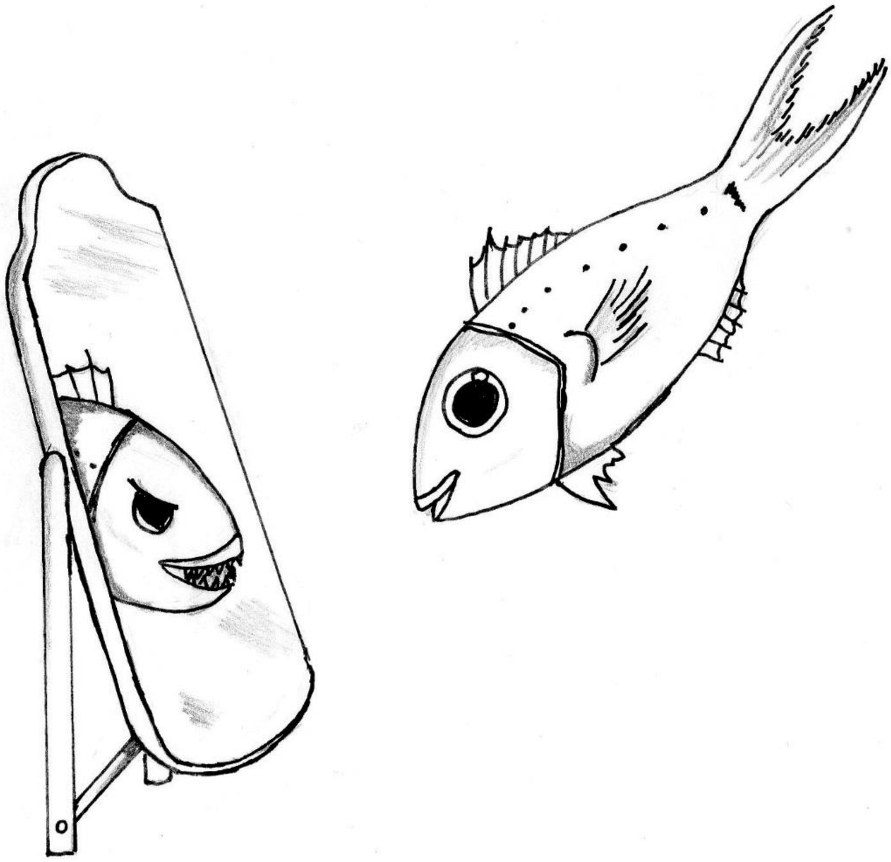

CHAPTER

6

Are coping styles consistent in fish?

A life history approach

Are coping styles consistent in the teleost fish *Sparus aurata* through sexual maturation and sex reversal?

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A life history approach

Chapter 6

Abstract

Individual differences in behaviour and physiological responses to stress are associated with evolutionary adaptive variation and thus, raw material for evolution. In farmed animals, the interest in consistent trait associations, i.e. coping styles, has increased dramatically over the last years. However, one of limitations of the available knowledge, regarding the temporal consistency, is that it refers always to short term consistency (usually few weeks). The present study used an escape response during a net restraining test, previously shown to be an indicative of coping styles in seabream, to investigate long term consistency of coping styles both, over time and during different life history stages. Results showed both short-term (14 days) and long-term (8 months) consistency of escape response. However, we did not found consistency in the same behaviour after sexual maturation when the restraining test was repeated 16, 22 and 23 months after the first test was performed. In conclusion, this study showed consistent behaviour traits in seabream when juveniles, and a loss of this behavioural traits when adults. Therefore, these results underline that adding a life history approach to data interpretation as an essential step forward towards coping styles foreground.

Keywords: Individual variation, Personality, Farm animals, Production, Sperm quality, Stress response

Are coping styles consistent in fish? A life history approach

Chapter 6

Introduction

The study of consistent trait associations in behaviour and physiological responses to challenges (i.e. animal personality or coping style) are associated with adaptive variation and thus, become a raw material for evolution in a wide range of biological disciplines (Francis 1990; Gosling 2001; Koolhaas et al. 1999; Korte et al. 2005; Réale et al. 2007; Sih et al. 2004). An understanding of this inter-individual variation is essential to improve our knowledge of the adaptive value of behaviour and physiological traits (Koolhaas et al. 1999). In the scientific literature, these consistent individual differences are alternatively referred to as personality (Gosling, 2001), temperament (Réale et al. 2007), behavioural syndromes (Sih et al. 2004) or coping styles (Koolhaas et al. 1999). The previous terminology stand for different phenomena: personality defines consistency of at last one single behavioural trait, but can also include correlations between multiple traits (Gosling, 2001); temperament describes the idea that individual behavioural differences are repeatable situations and should be studied within an evolutionary ecology framework (Réale et al. 2007); behavioural syndromes demark a set of correlated behavioural traits (Sih et al. 2004) and coping styles defines a set of behavioural patterns correlated with consistent physiological traits (Koolhaas et al. 1999).While, despite the diversity of terminology and designated definitions (Francis 1990; Gosling 2001; Koolhaas et al. 1999; Sih et al. 2004), there seems to be a consensus that individual differences in certain traits biologically meaningful and are consistent and predictive of other behavioural patterns or physiological responses shown in another context.

Several studies in fish have provided early documentation on individual consistency (Castanheira et al. 2013ab; Coppens et al. 2010; Koolhaas et al. 1999; Martins et al. 2012; Millot et al. 2014ab; Øverli et al. 2004ab, 2007; Ruiz-Gomez et al. 2011; Schjolden and Winberg 2007) that reflects distinct behavioural and physiological patterns usually categorized in two contrasting personality types, proactive (active coping or bold or 'fight-flight') and reactive (passive coping or shy or 'non-aggressive') (Koolhaas et al. 1999; Øverli et al. 2007). Behaviourally, proactive individuals are characterised by active avoidance (Brelin et al. 2005; Castanheira et al. 2013ab; Laursen et al. 2011; Martins et al. 2011cd; Silva et al. 2010), low flexibility (Chapman et al. 2010; Ruiz-Gomez et al. 2011), high levels of aggression (Castanheira et al. 2013a; Øverli et al. 2004, 2005), territorial control (Øverli et al. 2004, 2005), and other behavioural responses that suggest active efforts to counteract a negative stimulus, this pattern being the opposite for reactive individuals (reviewed *in* Castanheira et al. 2016; Koolhaas et al. 1999; Øverli et al. 2007). Physiologically, proactive individuals exhibit typical physiological and neuroendocrine characteristics such as lower hypothalamus-pituitary-interrenal (HPI) activity and lower HPI reactivityas compared to reactive individuals (reviewed *in* Castanheira et al. 2016; Koolhaas et al. 1999; Øverli et al. 2007). Nowadays, stress-coping styles are clearly identified in fish and have contributed to the understanding of individual variation in the capacity to cope with stressful events (i.e. conditions that are well tolerated by some individuals may be detrimental to others) (Huntingford and Adams 2005).

In farmed fish, the interest in consistent trait associations, i.e. coping styles, has increased dramatically over the last years because many studies have demonstrated links to performance traits (Øverli et al. 2007; Martins et al. 2011ab), health and diseases resistance (Fevolden et al. 1992, 1993; MacKenzie et al. 2009; Kittilsen et al. 2012) and welfare (Huntingford and Adams 2005; Huntingford et al. 2006). For example, in common carp (Huntingford et al. 2010) and seabass (Millot et al. 2009ab) the feed competition was shown to be related with distinct risk-taking behaviour. Data from Martins et al. (2011ab) shown that proactive tilapias were more feed efficient and has a faster recovery of feed intake after transfer. Another important implication of coping styles in farmed fish is the distinct disease resistance between coping styles. MacKenzie et al. 2009 showed that proactive and reactive common carp responded differently to inflammatory challenge with bacterial pathogens.

A failure to accommodate the coping styles of fish under farming conditions can lead to problems linked with production (e.g. aggression, growth and disease resistance).

Thus, a consensus is emerging that increased understanding of the consequences of stress-coping styles in aquaculture is important to safeguard a sustainable development of this industry and increase the production output.

However, there is still a long way to completely understand the coping styles thematic in order to improve the management and welfare of farmed fish.

One of the major gaps in the literature concerning the characterisation of coping styles in fish, is related with the temporal consistency, once published work so far refers to short-term (usually a few weeks) consistency (Basic et al. 2012; Castanheira et al. 2013b).

Although behavioural ecologists begun to consider potential links between life history trade-offs, related with productivity (i.e. growth and/or fecundity) and personality traits in animals (Biro and Stamps 2008). For instance, in rainbow trout *Oncorhynchus mykiss,* shy individuals consistently showed strong anti-predatory responses than bold individuals, both as juveniles and as adults stages (Biro et al. 2006, 2004). In Atlantic salmon *Salmo salar*, consistent differences in growth trajectories that appear at an early age were correlated with migration variation later in life (McCarthy 2000; Metcalfe et al. 1998). According the previous studies, a life-history approach of these individual differences is essential to increase our knowledge on the adaptive value of coping styles in farming fish. In fact, Seebacher et al. (2015) proposed an integrative framework for underlying the physiological mechanisms and ultimate consequences of locomotion in personality traits. According to this framework, locomotion is a mechanistic performance related with muscle contractile function and differences in voluntary speed could explain behavioural differences between individuals within populations.

In agreement with the previous study, in seabream, one of the traits that have been shown to be consistent over time and across context is the escape response under a restraining test (Castanheira et al. 2013b). In addition, escape performance has ecological significance (predator-prey-interaction), physiological implications (anaerobic recovery capacity of white muscle) and can be considered as a parallel to the forced swimming test, which is widely used in rodents for biomedical studies to coping styles screening. Moreover, the escape performance is very representative of conditions that the fish have to deal in practical aquaculture conditions (e.g. grading, vaccination, transport). Thus, we used the escape response in a net to validate the temporal consistency over time in seabream.

Moreover, very little is known about the influence of maturation or sex change on the coping strategies of fish species that undergo sex inversion such as the protandrous hermaphrodite teleost, seabream *Sparus aurata*. In farming conditions, sex inversion usually occurs around the end of the second year (Brusléa-Sicard and Fourcault 1997) when males undergo sex reversal into females. In fish, it is well documented that gender has a factor influencing coping strategies (Øverli et al. 2006b). Øverli et al. (2006b) addressed changes in the locomotor response to an acute confinement stress between male and female rainbow trout (*Oncorhynchus mykiss*), with females decreasing and stopping to move faster than males. Sex inversion may have important consequences for differences that can be found in coping strategies between fish at early life stages of development and adults.

In addition, stressful events (e.g. being chased by a net, being in a more crowded environment) are linked to a decrease in sperm motility and eggs fertilization (Schreck 2010). According to this we expected that proactive individuals are more successful breeders. In fish, few studies indicate that coping styles could be linked to sperm motility. Ibarra-Zatarain et al. (2013) did not found a significant effect of coping styles in gamete quality in Senegalese sole (*Solea senegalensis*). However, Clement et al (2005ab) found that dominant African cichlid fish (*Astatotilapia burtoni*) are more effective breeders. The lack of information and distinct results found in sperm motility and coping styles, highlights the needs for further investigation in this topic.

The objective of the current study was to investigate the consistency of coping styles over time and during life history using the behavioural responses during a net restraining test and cortisol responsiveness at distinct developmental stages. This approach made it possible to assess the influence of maturation and sex inversion on coping strategies in gilthead seabream, a fish species that undergoes sex inversion. We predicted that both age and life experience would influence the individual behavioural consistency of coping styles thus, the individual adaptation capacity may be different according life history. Therefore, a life history approach of coping styles could represent a new key to enhance, fish welfare, improve disease resistance and performance at distinct stages of fish development reflected in aquaculture sustainability.

Methods

The experiment was conducted in accordance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals, and under a “Group-1” license approved by the ethics committee from the Veterinary Medicines Directorate, the Portuguese competent authority for the protection of animals, Ministry of Agriculture, Rural Development and Fisheries, Portugal. Permit number 0420/000/000-n.99-09/11/2009.

Experimental animals, housing and feeding

The study was performed at the Ramalhete Research Station (CCMAR, Faro, Portugal). Fish were acquired from a seabream breeder without selection programme based on behavioural profile (MARESA Mariscos de Esteros SA, Huelva, Spain). All individuals used were housed in fibreglass stock tanks (500L) under standard rearing conditions (Morales 1983) until the experiment start. Throughout all the experimental period (24 months) water parameters were daily analysed with an average water temperature of 19.5 ± 2.1 ºC, a salinity of 34.3 ± 2.4 ‰ dissolved oxygen concentration of 96.5 ± 3.3 %, nitrites (< 0.1 mg.L-1) and ammonia (< 0.1 mg.L-1) and a natural photoperiod was provided (37° 0' 22.35" N 7° 58' 3.35" W). Fish were fed 0.5 to 2% BW day-1, by hand, twice per day (09:30 and 14:30), with a commercial diet (Aquagold 2, 3 and 5 mm, Sorgal SA, Portugal) according to the body weight. The same diet and photoperiod were used through the experiment.

Fish were PIT-tagged (ID100 Implantable Transponder, Trovan®, Netherlands; Dimensions: 2.12 x 11.5mm), two weeks before the start of the experimental procedures. PIT-tag was inserted through an injection device (ID100/Disposable Implantable Transponder, Trovan®, Netherlands), under the skin on the left side of the dorsal fin. All animals behaved normally after PIT-tagged (i.e. without changes in swimming speed, manoeuvre swimming complexity and feed intake).

Experimental procedures

Coping styles screening

Individually tagged juvenile fish (n=60, 22.7±3.9 g; mean ± SD) were initial screening using a consecutive series of behavioural tests: (1) feeding recovery after transfer to a novel environment; (2) behaviour towards a novel object; (3) escape response and cortisol responsiveness in a restraining test, 4) avoidance response towards hypoxia conditions and 5) risk taking. Each test was repeated twice (run 1 and run 2) 14 days apart to assess short-term consistency of behavioural responses over time (for details see Castanheira et al. 2013b).

Using previous results (Castanheira et al. 2013ab), the escape behaviour during the restraining test was applied to validate if the coping styles found in this species are stable along life stages, i.e. long term consistency. Briefly, the net restraining test consists of holding each fish individually in an emerged net for one minute. While in the net the following variables were measured: i) latency to escape (time in seconds taken by each fish to show an escape attempt; escape attempt was defined as an elevation of the body from the net; ii) number of escape attempts and iii) total time spent on escape attempts (total time in seconds taken by each fish escaping since the first to the last escape attempts). Without any change in light conditions (i.e. using the natural light) behaviours measured in the net were video recorded with a camera (MicroVideo™ camera MCV2120-WP-LED, Canada) previously placed over the restraining test setup, analysed using a stopwatch and collapsed into first principal component scores using Principal Components Analysis (PCA) (for details of statistical analyses see subsection 2.6). Individuals presenting a high latency to escape, low number of escape attempts and shorter total time escaping were characterized by a low score and identified as reactive fish. On opposite, individuals presenting a lower latency to escape, high number of escape attempts and longer total time escaping were characterized by a high score and identified as proactive (based on Castanheira *et al.* 2013ab). No threshold was applied to separate subjectively the fish in two categories i.e. proactive and reactive. These data (PC1) were used as a continuous variable. Experimental groups were kept in plastic tanks (100L) during 8 months and submitted to two series of behavioural tests

14 days apart, previously described. After this period (run 3 of restraining test) individuals were randomize in three groups, housed in fibreglass stock tanks (500L) and on-grown until adult stage. Runs 1, 2 and 3 were done before and 4, 5 and 6 after sexual maturation. Details of behavioural test and sampling time points (runs) are given in the Table 6.1.

Blood sampling and cortisol analysis

Blood samples were collected 30 minutes after the start of the net restraining test, according to Arends et al. (1999). Therefore, fish were quickly taken out from each tank at the same time and anaesthetized with 2-phenoxyethanol (1000ppm, Sigma–Aldrich).Using heparinised syringes blood was withdrawn within 5min from the caudal vein to avoid cortisol increase due to manipulation during sampling (Rotland and Tort 1997). After sampling, blood was centrifuged at 2000×g for 20 minutes at room temperature, frozen in liquid nitrogen and stored at −80˚C for posterior cortisol analysis.

Table 6.1. Overview of analyses and sampling time points (runs) during the experiment.

Restraining							
Run	**Date 1**	**Date 2**	**Weight (mean ± SD)**	**N**	**Racio males:females**	**Notes**	**Analyses**
1	13-09-2011	-------	(22.7 ± 3.9 g)	56	-------	Before sexual maturation (undifferentiated gonads)	Restraining, cortisol
2	27-09-2011	14 days after run1	(39.2 ± 8.0 g)	56	-------	Before sexual maturation (undifferentiated gonads)	Restraining, cortisol
3	24-05-2012	8 months after run1	(98.9 ± 15.3 g)	56	-------	Before sexual maturation (undifferentiated gonads)	Restraining
4	12-01-2013	16 months after run1	(454.1 ± 69.0 g)	53	53:0	After sexual maturation (all males produced sperm)	Restraining, sperm production
5	04-11-2013	22 months after run1	(856.8 ± 127.0 g)	53	38:15	After sexual maturation sex change (38 males)	Restraining, cortisol, sperm production
6	13-12-2013	23 months after run1	(832.9 ± 127.7 g)	51	39:14	After sexual maturation sex change (39 males)	Restraining, cortisol, sperm production and sperm quality parameters

Plasma cortisol levels were determined using a commercial available ELISA kit (RE52611, IBL International, Hamburg), with a sensitivity of 0.05ng ml^{-1} and precision intra- and inter-assay coefficients of variation (CV) of 7.5 and 17%, respectively. This kit has been previously validated for seabream (López-Olmeda et al. 2009).

Sperm quality analyses

Thirty minutes after the restraining test sperm was collected by stripping (n=39 males). For this the anesthetized fish were placed on a holder with the belly facing up, and gentle pressure was applied from each side of the belly toward the genital pore while sperm were collected using a syringe without needle., After collection sperm was maintained at 4◦C for motility analyses. Sperm motility was analysed in duplicate using a computer-assisted sperm analysis (CASA) software. The CASA software integrates the successive positions of the heads of moving spermatozoa in consecutive frames of video records to calculate their trajectories and characteristics. Sperm placed in a Makler chamber (0.5µl of diluted sperm; 1:6, v/v in 1%NaCl) was activated with 20 µl of sea water, and immediately, digitalized images obtained using 10x negative phase contrast objective in a light microscope (Nikon E200, Tokyo, Japan) were recorded with a Basler camera (Basler Afc, Ahrensburg, Germany) at 15, 30, 45 and 60 seconds post-activation. Images were processed with CASA software to determine total spermatozoa motility, which refers to the fraction of sperm that display any type of movement (TM, %), progressive motility, fraction of sperm moving in a straight line (PM, %), curvilinear velocity, the actual velocity along the trajectory (VCL, m/s),straight line velocities, the straight line distance between the start and the end points of the track divided by the time of the trace (VSL, m/s) and linearity index, the ratio of the net distance moved to total path distance(LIN, %).

Data and Statistical analysis

All statistical analyses were performed in SPSS 19.0 *for Windows* (IBM, USA). The results are presented as mean±standard deviation (SD). Behaviours measured in the net restraining test (i.e. latency to escape, number of escape attempts and total time spent on escape attempts) were collapsed into first principal component scores (PC1) using Principal Components Analysis (PCA), with orthogonal rotation (varimax). The correlation matrix was used to check multicollinearity, i.e., to identify variables that did not correlate with any other variable, or correlate very highly (r=0.9) with one or more other variables. Kaiser–Meyer–Olkin (KMO) test for sample adequacy was greater than 0.5 and Bartlett's test of sphericity was significant, indicating that correlation between items was sufficiently robust for PCA.

Spearman correlation analyses were used when data failed to pass the normality Kolmogorov-Smirnov test. A two-step cluster analyses was performed using the PC1 net restraining. Kruskal-Wallis test was used to verify differences between the generated clusters. Sperm motility parameters of proactive, intermediate and reactive males were compared using general linear models with the Bonferroni correction. Statistical significance was accepted at $p<0.05$.

Results

Coping styles plasticity: Short and long-term consistency

The PCA loadings of the net restraining test variables used to generate the principal component score (PC1) to assess consistency over time between runs are shown in Table 6.2.

There was a significant correlation between the escape behaviour in runs 1 and 2 (14 days apart) of the restraining test Figure 6.1 (r_S=0.354, p=0.009). The escape response during the restraining test was shown to be repeatable over a period of 14 days: individuals showing lower latency to escape, higher number of escape attempts and spending more time escaping in run 1 showed a similar behaviour after 14 days when the test was repeated to check short-term consistency. In addition, a long-term consistency run3 (8 months after run1) was also observed. Figure 6.2 shows a significant correlation between the escape behaviour in runs 1 and 3 (r_S=0.286, p=0.036), runs 2 and 3 (r_S=0.675, p<0.001) of the restraining test. No consistency could be found in run 4 (16 months, r_s=0.147, p=0.302); run 5 (22 months r_s=-0.211, p=0.146) and run 6 (39 months, r_s=-0.243, p=0.092) in relation to run1, or run 4 (16 months, r_s=0.270, p=0.062); run 5 (22 months r_s=-0.168, p=0.249) and run 6 (39 months, r_s=-0.095, p=0.515) in relation to run2, or run 4 (16 months, r_s=0.302, p=0.031); run 5 (22 months r_s=-0.118, p=0.418) and run 6 (39 months, r_s=-0.097, p=0.506) in relation to run3, or between run 4 and 5 (r_s=-0.032, p=0. 826) and runs 4 and 6 (r_s=-0.118, p=0.419) or 5 and 6 (r_s=0.220, p=0.129).

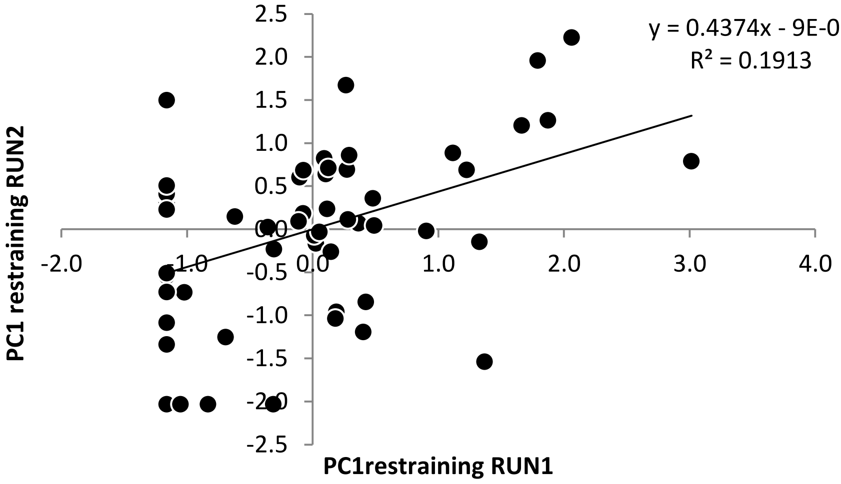
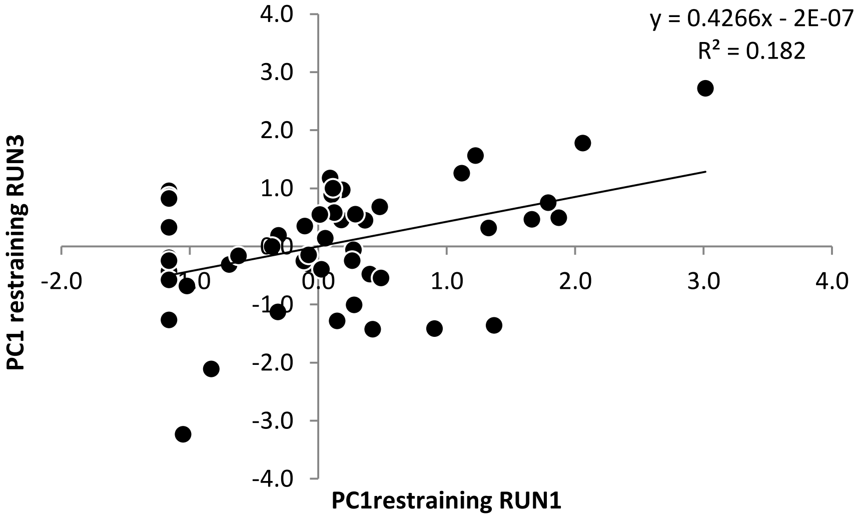


Figure 6.1. Relationship between the PC1 behavioural personality score (from run1 and run2) during the net restraining test on seabream *Sparus aurata* (n = 60) – short-term consistency trial.



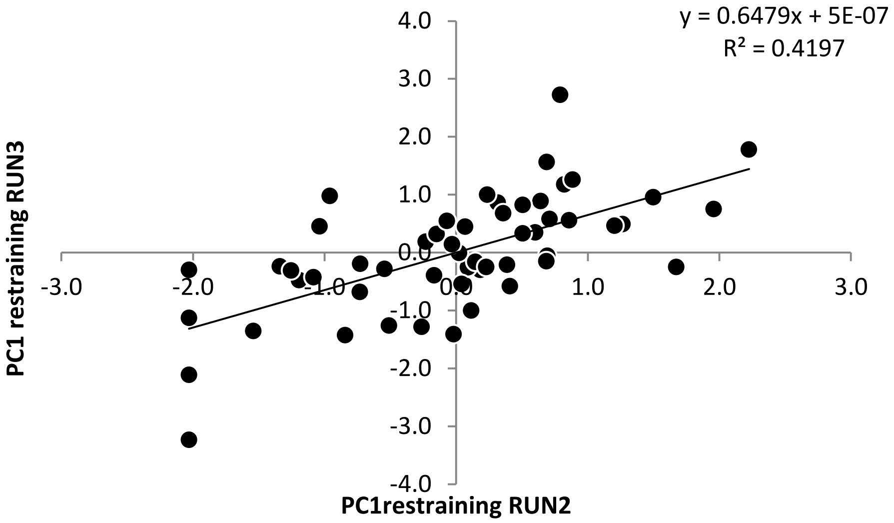


Figure 6.2. Relationship between the PC1 behavioural personality score (run1 and run3; run2 and run3 respectively for graphs on the top and bottom) during the net restraining test on seabream *Sparus aurata* (n = 60) - long term-consistency trial.

Table 6.2. PCA loadings of the net restraining test variables used to generate a principal component scores (PC1) in runs 1, 2, 3, 4, 5 and 6.

Behavioural test: Restraining	**Behavioural variables**		
	Latency to escape	**Number of escapes**	**Total escaping time**
Loadings for PC1- RUN 1(component matrix)	-0.809	0.933	0.877
% Variation explained		76.468	
Loadings for PC1- RUN 2 (component matrix)	-0.81	0.868	0.879
% Variation explained		72.771	
Loadings for PC1- RUN 3 (component matrix)	-0.6	0.846	0.778
% Variation explained		56.051	
Loadings for PC1- RUN 4 (component matrix)	-0.554	0.892	0.856
% Variation explained		61.193	
Loadings for PC1- RUN 5 (component matrix)	-0.846	0.945	0.904
% Variation explained		80.881	
Loadings for PC1- RUN 6 (component matrix)	-0.838	0.893	0.857
% Variation explained		74.49	

Plasma cortisol concentrations after restraining test

Figure 6.3 depicts the post-stress cortisol levels over time, F (3,199) = 387.146, $p<0.001$, with a mean rank of 44.62 for run 1, 47.89 for run 2, 294.69 for run 5 and 443.91 for run 6. Plasma cortisol was not significantly correlated with the escape behaviour during the restraining test run1 ($r_s = -0.207$, $p = 0.133$), run2 ($r_s = 0.012$, $p = 0.933$), run5 ($r_s = 0.220$, $p = 0.129$) and run6 ($r_s = 0.136$, $p = 0.350$).

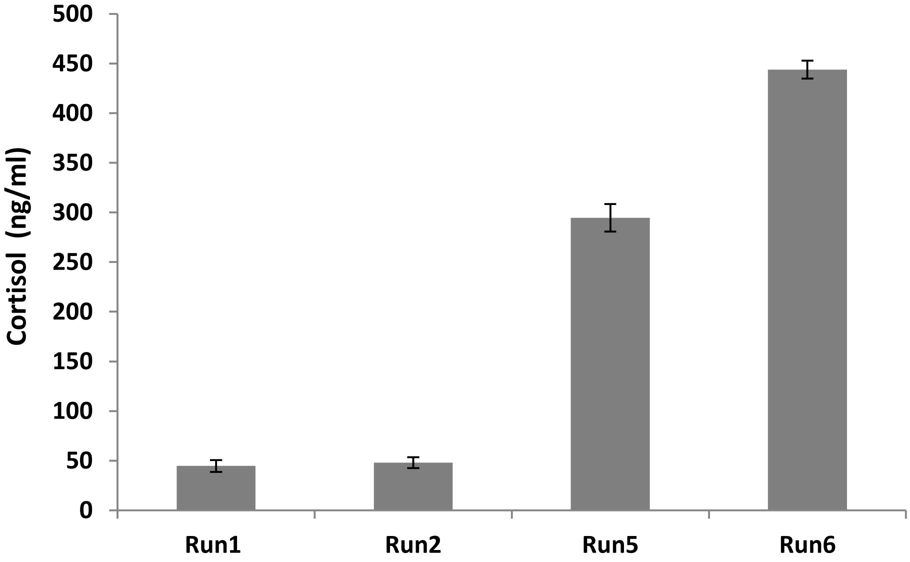


Figure 6.3. Cortisol post-stress responsiveness over time (run1, run2, run5 and run6) on seabream *Sparus aurata* juveniles (n = 60). Data are presented as means ± SD. Different letters indicate significant differences (nonparametric test, Kruskal-Wallis test: $p < 0.001$).

Relationship between sperm motility and coping styles

Motility sperm parameters decreased linearly from 15 to 60 seconds post-activation. No significant differences were found in sperm motility related with the behavioural responses during run6 of the restraining $p>0.05$.

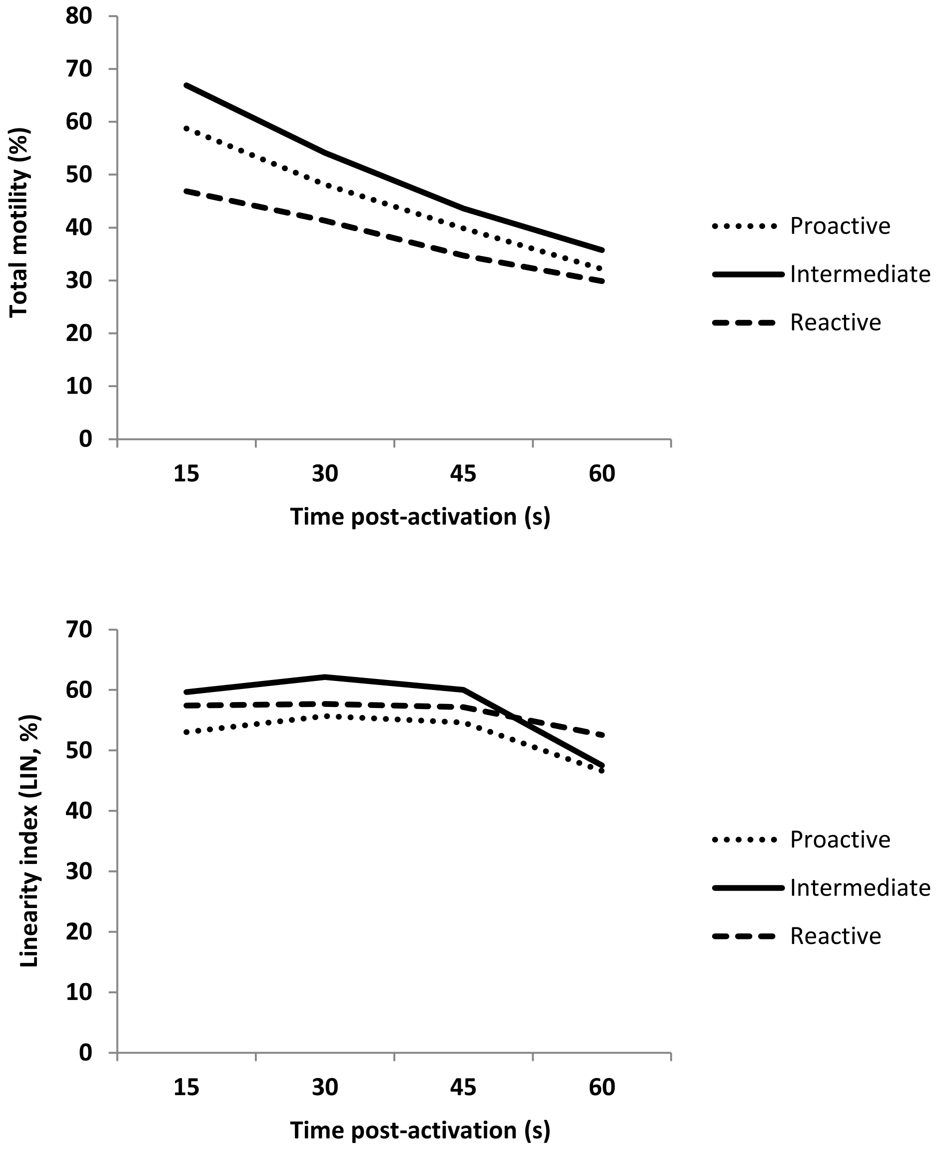


Figure 6.4. Sperm motility parameters (A-total spermatozoa motility; B-linearity index, LIN) in proactive, intermediate and reactive males at 15, 30, 45 and 60 s post-activation. Statistical analyses were performed using general linear models with the Bonferroni correction ($p < 0.05$).

Discussion

The main objective of the current study was to characterize fish coping styles considering the consistency of behavioural responses over time and during life history using the behavioural responses during a net restraining test and cortisol responsiveness at distinct life history stages. This study showed consistency in behavioural responses (over time) during the restraining test until 8 months after the first screening. More specifically, individual escape response in run 1 was similar to the escape response observed after 14 days (short-term consistency) and 8 months (long term-consistency) when the test was repeated. However, in the runs after (run 3) the onset of sexual maturation this escape behaviour consistency during restraining was lost. In our study, the short-time consistency results is in accordance with previous studies indicating the presence of coping styles that seems to be based on innate traits (Brelin et al. 2005; Castanheira et al. 2013b; Huntingford 1976; Martins et al. 2012; Millot et al. 2014ab; Øverli et al. 2004ab) and do not add any novelity to the previous findings in fish coping styles. However, the lack of consistency found in coping styles along life stages is a new important finding. These results are expected and suggest that differences in coping styles expressed at early developmental stages may change according age and life history experiences. This highlighting that coping styles are not fixed and this can be reflected in distinct behavioural strategies to cope with the same stressful condition Thus, different life stages could mean a paradigm shift in coping styles field and following hypotheses may be considered to contribute to this lack of consistency in escape behaviour responses: (1) the sexual maturation and the sex inversion process compromise the consistency of coping styles abilities or/and (2) the contextual importance of the net restraining response may differ according to age and fish development.

Sex has been identified as a factor influencing coping strategies in fish (Øverli et al. 2006b; Reyes-Tomassini 2009). In fact, Øverli et al. (2006b) demonstrated sex-specific variations in behavioural responses to an acute confinement stress in rainbow trout, with females decreasing and stopping to move faster than males. In addition, Reyes-Tomassini (2009) established that proactive individuals play a role in seabream sex change, but to ascertain their exact influence needs more accurate investigation in order to predict a model of that contribution. The coping styles screening using the behaviour of the net restraining test in run 4 of our experiment was done in sexually mature fish (i.e. all fish were males with active sperm production) however, in run 5 the screening were done in other stage of fish life (i.e. after sex inversion) which means that some of the fish that we have screening before were now females. Taking the previous studies into account, we can hypothesise that males and females will behave differently when confronted with the same challenge (i.e. net restraining) and sex maturation and sex inversion processes could have played a role on the results found in this work (i.e. the absence of behaviour consistency in the net restraining test found after sex reversion). However, very little is known about the influence of sex change on the coping strategies of fish species that experience sex inversion.

In contrast, it is well known that changes of context may induce divergent behaviour responses in fish with distinct coping styles. In Artic charr, Magnhagen et al. (2015) found a lack of consistency in behavioural responses (i.e. per cent time struggling) between two runs related with the different experiences which fish were subjected before the runs. The first run was performed with fish immediately taken from their home tank, but the second run was preceded by a feeding and a resident-intruder test. Similarly, in our study the run 3 and following runs were performed with fish immediately taken from their home tank, however the first two runs were preceded by feeding observation and a novel object test, giving another experience to the individuals compared to the last runs. Hence, the results found may be also a reflection of contextual changes. Similarly, Frost et al. (2007) found that positive and negative life events can predict changes in personality in rainbow trout. Once the individual internalizes those experiences they are said to be a part of that individual personality. In addition, individuals also receive feedback from other individuals or groups about their own personality and this can be a driving force of change in personality in our study. Moreover, our previous results suggest that social context affect stability of coping styles for gilthead seabream before sexual maturation (Castanheira et al. 2016). Thus, individual experiences vary as a function of age, social context and major life events (e.g. sex maturation and sex inversion) and can lead to changes in personality. In the present study the change in response to the restraining test (escape response from the net) after sexual maturation (run3) may reflect a plasticity of coping styles. This change in coping styles of individual fish could be associated with species adaptation/survival strategies and concomitant modulation of fish physiology. While juveniles allocate almost of the energy in survival (e.g. foraging, escape), allocation of energy in adults is more focused in the reproductive success and offspring viability. The biological significance of this observation remains unclear, but it should be noted that can be related with the allostatic ability to respond to challenges.

In addition, no relationship was found between escape behaviour and plasma cortisol levels. Several studies have documented the lack of correlation between plasma cortisol levels obtained after stress and behavioural responses (Silva et al. 2010; van Erp-van der Kooij et al. 2003; van de Nieuwegiessen et al. 2008). Some authors have suggested that cortisol and behavioural responses to stressors are linked to two independent dimensions of stable trait characteristics (Koolhaas et al. 2010). These authors suggested that the quality of the response to a challenging condition (coping style) is independent from the quantity of that response (stress reactivity). According to the same authors, the physiological responses to stress such as the HPI axis reactivity (one of the most significant differences between proactive and reactive individuals) is more related to an emotional response to stress than to coping styles. Eventually a decoupling of these axis, coping styles and emotional, could bring new light to understand the pronounced individual variation in plasma cortisol response observed in seabream after stress. However, in this study we observed a significant increase in the magnitude of the cortisol response, more than fourfold, from juveniles to adults. This difference maybe related with the developmental stage of the fish that can affect its responsiveness to a stressor (Barton 2002). However our results are in contrast with previous studies such as Pottinger et al. (1995) who found a reduction of stress response in adult rainbow trout as a result of a reduced regulatory feedback with the onset of maturity. The possible influence of age on the stress responsiveness in seabream remains to be investigated. Such discrepancy of results may be due to species-specific behaviour and/or age influence.

An alternative explanation could be that the cortisol responses found as a response of repeated stressors (i.e. at adult stage fish exhibit a cumulative response of repeated net restraining tests). Several studies demonstrate that fish can exhibit a cumulative response to repeated stressors (Carmichael et al. 1983; Flos et al. 1988; Maule et al. 1988) found that when juvenile chinook salmon were given multiple handling stressors, the peak cortisol responses after the final disturbance were cumulative.

Based on our results we could wonder whether the cortisol levels measured do in fact represent the immediate response to stressful conditions or are instead the reflection of other factors such as, genetic factors, developmental factors, environmental factors and repeated stressors that mask the correlation between behaviour and cortisol responses.

An alternative explanation is that cortisol levels were measured in the peak of cortisol level for seabream (i.e. 30 min after the net restraining test). Recent evidence suggests a correlation between cortisol and behavioural responses during the recovery from stress over time and not is one specific time point (Tudorache et al. 2013, 2014). According to this, the absence of correlation found can be a reflection of using a static point of cortisol measure since a time dependent parameters from peak to baseline.

In what refers to the results in sperm motility, no significant differences were found related with the net behavioural responses. In aquaculture, the relation between stress and reproduction (e.g. sperm motility) has been largely studied (Cosson et al. 2008; Schreck 2010). Stressful life events may have negative effects in semen quality and reproductive processes (Schreck 2010). Also, in many cases, a correlation between sperm motility and the ability to fertilize eggs has been established. However, less attention has been given to the possible correlation between fish stress coping styles and reproductive performance and/or gamete quality. The few studies found in relation to this thematic have distinct results. Clement et al (2005ab) found that dominant African cichlid fish (*Astatotilapia burtoni*) are more successful breeders. In accordance with our study, Ibarra-Zatarain et al (2013), did not found significant responses between coping styles and gamete quality in Senegalese sole (*Solea senegalensis*). Further research is needed in order to confirm or reject the existence of a relationship between coping styles and reproductive success. This would be important to improve both broodstock husbandry and the design of aquaculture breeding programs.

Conclusions

In summary this study provides the first evidence of life history impact in the assessment of coping styles consistency. This study showed behaviour consistency responses in fish during a net restraining test only before sexual maturation. Therefore, this finding emphasizes the value of taking a life history approach into account to improve knowledge in the consistency of coping styles. This suggests that behavioural aspect of coping styles are not fixed and may change according to life history events. This reflects an adaptive response to physiological, behavioural and social differences along life.

Acknowledgments

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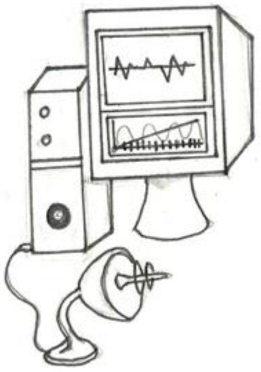

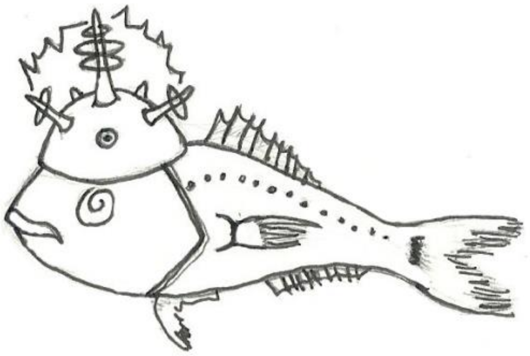

CHAPTER

7

Coping styles and forebrain neural activity

Coping styles and forebrain neural activity in Seabream *Sparus aurata*

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Abstract

Individual differences in behaviour and neuro-endocrine responses are associated with evolutionary adaptive variation, which is across all vertebrate taxa. To establish a link between stress coping and brain function, we investigated differences in forebrain gene expression of the immediate early gene (*c-fos*) and brain derived neurotrophic factor (*bdnf*), in fish displaying divergent coping styles at basal and acute stress conditions. Results on situ hybridisation and gene expression analysis showed that proactive fish displayed post-stress decreased *c-fos* and increased *bdnf* transcript levels compared to reactive individuals in the subpallial supracommisural (Vs) and ventrolateral (Vv) areas, respectively. We here submit novel evidence that inhibitory and excitatory markers of neural function are associated with reactive and proactive coping styles in seabream, respectively.

Introduction

A significant number of studies have recently been addressing neurobiological, cognitive and emotional processes in fish (Allen 2013; Ebbesson and Braithwaite 2012; Millot et al. 2014ac; Rey et al. 2015b; Vindas et al. 2012, 2014ab). This is both due to applied aspects, such as the increasing attention to animal welfare and ethical food production (Broom 2007; Galhardo and Oliveira 2009; Huntingford et al. 2006; Papoutsoglou 2012), and to a fundamental interest in the evolution of complex neurobiological mechanisms, such as learning and memory (Cabanac et al. 2009; Rodríguez et al. 2007; Salas et al. 2006). Furthermore, under stressful conditions individual variation in responsiveness is a key to understanding biological adaptation and may help to elucidate why some conditions are detrimental for some individuals but not others. Consequently, the study of consistent over-time and across-situations trait associations in behavioural and physiological responses to challenges (i.e. animal personality or coping style) has become a "hot topic" in a wide range of biological disciplines; ranging from evolutionary ecology to health sciences and cultured-systems biology (Cavigelli 2005; Favati et al. 2014; Gosling 2001; Koolhaas et al. 1999, 2010; Korte et al. 2005; Réale et al. 2007; Sih et al. 2004). In this context, animals are usually divided in two contrasting coping styles a proactive or reactive type. Proactive animals are behaviourally characterised by active avoidance, low flexibility, high levels of aggression and dominance, as well as physiologically by lower hypothalamus-pituitary-adrenal/interrenal (HPA/HPI) axis activity and lower parasympathetic reactivity. Meanwhile reactive animals exhibit an opposite behavioural and physiological phenotype to proactive ones (Koolhaas et al. 1999, 2010; Øverli et al. 2007; Ruiz-Gomes et al. 2011).

From a behavioural and neurobiological perspective it has been proposed that differing coping styles reflects underlying neural causal mechanisms (Øverli et al. 2007; Coppens et al. 2010). Forebrain telencephalic areas, such as the hippocampus and amygdala have been reported to play a key role in arousal and emotional control in mammals (Carter 1996; Maren 2001) and its believed that functional homologous structures play the same role in other vertebrates (O´Connell and Hofmann 2012; Goodson and Kingsbury 2013). For example, it has been suggested that the pallial dorsolateral (Dl) and dorsomedial (Dm) areas in fish correspond to the mammalian hippocampus and amygdala, respectively, while the subpallial supracommissural (Vs) and ventral (Vv) areas are equivalent to the mammalian, extended amygdala and lateral septum (LS), respectively (Goodson and Kingsbury 2013; O´Connell and Hofmann 2011).

This study aims to characterize stress coping ability and brain function in seabream, by investigation the behavioural and forebrain physiological responses of fish displaying contrasting coping styles to the same stimulus. The transcript abundance of the immediate early gene c-fos and the neuronal plasticity marker brain derived neurotrophic factor (bdnf), were studied by both in situ hybridisation and qPCR. We hypothesised that differences in behaviour during net restraining test may be associated with differential region-specific telencephalic regulation. Better understanding of neurobiological mechanisms involved in behavioural responses, learning and memory will provide novel tools for both the aquaculture industry and for fish model systems focusing on central nervous system function, such as neuropsycopathological disorders.

Methods

The experiment was conducted in accordance with the Guidelines of the European Union Council (86/609/EU) and Portuguese legislation for the use of laboratory animals. The experimental protocol was approved by the ethics committee from the Veterinary Medicines Directorate, the Portuguese competent authority for the protection of animals, Ministry of Agriculture, Rural Development and Fisheries, Portugal. Permit number 0420/000/000-n.99-09/11/2009.

Experimental animals, housing and feeding

The experiment was carried out at the Ramalhete Research Station from Centre of Marine Sciences - CCMAR (Faro, Portugal). All experimental fish were obtained from a seabream producer (MARESA Mariscos de Esteros SA, Huelva, Spain) and were kept in stock groups until the start of the experiment in fibreglass stock tanks (500 L) under standard rearing conditions (for further details see Morales 1983). Two hundred and sixteen individuals were individually PIT-tagged (Trovan®, Netherlands) two weeks before the start of the experimental procedures. Throughout all the experimental period (1.5 months) fish were kept on a natural photoperiod (at 37° 0' 22.35" N 7° 58' 3.35" W), with an average water temperature of 19.8 ± 1.1 ºC, a salinity of 33.9 ± 2.1 ‰ and a dissolved oxygen concentration of 98.5 ± 2.8 %. Water quality was daily analysed for nitrites (< 0.1 mg.L-1) and ammonia (< 0.1 mg.L-1). Fish were fed 2% BW day-1, using automatic feeders, with a commercial diet (Aquagold 3mm, Sorgal SA, Portugal; 44 % crude protein, 14 % crude fat, 8 % ash, 2.5 % crude fibres, 1.0 % phosphorus).

Experimental procedures

All tagged Seabream juveniles (n = 216; 49.14 ± 7.9 g; mean ± SD) were subjected to a net restraining test in order to determine their coping style (following previous described methodology by Castanheira et al. 2013b). Briefly, each fish is restrained in a handling net and exposed to air for 1 minute. Fish behaviour was video recorded (MicroVideoTM camera MCV2120-WP-LED, Canada) for later quantification of: i) latency to escape (time in seconds taken by each fish to show an escape attempt; i.e. an elevation of the body from the net; ii) number of escape attempts and iii) total time in seconds from first to last escape attempt. Immediately after the restraining test all individuals were placed back into their respective holding tanks. Following statistical analyses (for details see subsection 2.6), all fish were categorized into proactive, reactive or intermediate coping styles, which were randomly mixed (n = 36; 12 individuals per style) in 6 tanks (100 L) and left undisturbed for 1 month before sampling.

Sampling protocol

In order to characterize clear differences between opposite coping styles, only proactive and reactive individuals were further analysed. Sampling was conducted by either catching fish directly from their holding tank (basal conditions; n = 20, 10 reactive and 10 proactive), or by sampling 30 min after an acute stress test (i.e.

restraining, done as previously described; n = 28, 14 reactive and 14 proactive). Immediately after collection, individuals were euthanized with an overdose of 2-phenoxyethanol (Sigma-Aldrich), which rendered them completely motionless (no opercular movement) within 10 s of immersion. Fish were rapidly weighed, fork length measured and brain samples were processed in 2 different ways: 1. Six fish were fixed by vascular perfusion with 4% paraformaldehyde (PF) in 0.1 M Sørensens phosphate buffer (PB; 28 mM NaH2PO4, 71 mM Na2HPO4, pH 7.2). The brains were dissected out and posteriorly fixed in the same fixative for 16 h at 4 °C. The brain tissue was washed three times 20 min in PB, cryopreserved overnight in 25% sucrose in PB at 4 °C, embedded in Tissue-Tek OCT-Compound (Sakura Fintek, USA) and stored at -80 °C until sectioning for in situ hybridization. 2. Twenty fish were decapitated and whole heads were placed in containers with Tissue-Tek O.C.T compound (Sakura Fintek, USA) and immediately frozen with dry ice. Frozen containers were then placed in individually labelled tubes and stored at –80 °C for posterior analysis of gene expression in microdissected areas.

In-situ hybridization (ISH)

In order to pinpoint interest areas and tendencies of bdnf and c-fos transcript abundance, we conducted an ISH (sense and antisense) on parallel sections of 3 seabream per coping style after acute stress. Adjacent transversal 12 μm sections were cut using a Leica CM 1850 cryostat (Leica Microsystems, Wetzlar, Germany), collected on SuperFrost Ultra Plus glasses (Menzel Glaser) and dried at 65 °C for 10 min. Digoxigenin-labeled riboprobes were prepared using a digoxigenin (DIG)-RNA labeling mix in accordance with the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The ISH probes for c-fos and bdnf were 542 and 611 nucleotides long, respectively. Forward GGCTCGAGTTCATTCTCGCT and reverse GTCGTTGCTGTTGCTTCCTC and forward GGACTCCACAGAGTGGTGGT and CCAGCCAATCTTCTTTTTGC reverse primers were used to clone the c-fos and bdnf probe primers, respectively. The quality and quantity of the synthesized riboprobes were assessed by agarose gel electrophoresis. Pre-treatment and treatment of sample for ISH was conducted as specified by Ebbesson et al. 2011. Before ISH, the tissue was air dried at room temperature for 1 h and at 65 °C for 10 min, rehydrated in ethanol (95–50%), washed 1 min with 2 X standard saline citrate (SSC), then permeabilised with proteinase K (10 µg ml-1 in 0.1 M Tris-HCl pH 8.0) for 3.5 min, post-fixed in 4% PF in KPBS (137 mM NaCl, 1.4 mM KH2PO4, 2.7 mM KCl, 4.3 mM Na2HPO4; pH 7.3) for 5 min, followed by rinsing two times for 2 min in KPBS. Tissue was then treated with 0.1 M triethanolamine (TEA, pH 8.0; Sigma-Aldrich) for 3 min and then with 0.25% acetic anhydride (Sigma-Aldrich) in 0.1 M TEA for 10 min. Finally, tissue was dehydrated in ethanol (50–100%) and air dried for 1 h. For hybridization, 100 ng digoxigenin-labeled probe in 100 µl of hybridization solution was applied to each slide. The composition of the hybridization solution was: 10 mM Tris-HCl, 300 mM NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.2% tween-20, 1% blocking solution (Roche Diagnostics), 0.1% dextransulphate (Sigma-Aldrich) and 50% deionized formamide (Sigma-Aldrich). Incubation was carried out at 65 °C for 18 h, using humidity chambers and hybri-slips (Sigma-Aldrich) to prevent evaporation. Sense probe was applied as a control for nonspecific staining. After hybridization, tissue was washed two times for 30 min in 2 X SSC, 30 min in 50% deionized formamide in 2 X SSC at 65 °C, and two times for 10 min in 2 X SSC at 37 °C. The tissue was treated for 20 min with RNase A (0.02 mg / ml; Sigma-Aldrich) at 37 °C, and washed 20 min at 65 °C. The sections were incubated 1 h with 2% blocking solution in 2 X SSC with 0.05% Triton X-100 and then overnight with alkaline phosphatase-conjugated sheep anti-DIG goat antibody (dilution 1:2000; Roche Diagnostic). The tissue was washed two times 10 min in 1 X maleate buffer and then for 10 min in visualization buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5). The staining reaction with chromogen substrate (3.4 µl of nitroblue tetrazolium, 3.5 µl of 5-bromo-4-chloro-3-indoylphosphate (Roche Diagnostics) and 0.24 mg ml-1 levamisole in visualization buffer) was carried out for 3 to 24 h in darkness at room temperature (samples were routinely checked to avoid overexpression). The reaction was terminated with stop solution 10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 8.0) and tissue was mounted in ProLong Gold (Invitrogen, Carlsbad, CA, USA). Photographs were taken using a digital camera (Leica DFC 320, Leica 350 FX) attached to a Leica DM 6000B microscope using the LEICA APPLICATION SUITE, version 3.0.0 image acquisition and processing software. Adobe Photoshop CS5 (Adobe Systems Inc., San Jose, CA, USA) was used for the adjustment of contrast and brightness.

Microdissections and gene expression analysis

Frozen whole heads were sliced in 150 μm thick cryostat coronal sections using a Microm HM500 cryostat (Microm GmbH, Walldorf, Germany), set for -25oC. The sliced tissue was thaw mounted on glass slides, for microdissection. The microdissections were performed with modified 25G steel needles in four different regions of the telencephalon; the pallial Dm and Dl, as well as the subpallial Vs and Vv, following procedures described by Vindas et al. 2014a. The brain areas were identified following the stereotaxic Atlas by Muñoz-Cueto et al. (2001). Tissue was collected directly into qiazol lysis buffer from Qiagen Lipid Tissue Mini Kit (#74804; Valencia, CA) and manufacturer's instructions (with some adjustments; for complete details please refer to supplementary materials) were followed in order to extract total RNA. RNA from each sample was then reverse transcribed to cDNA (BioRad iScript cDNA Synthesis Kit; Valencia, CA) accordingly to manufacturer´s instructions. The qRT-PCR protocol was based and adapted on procedures previously performed by Desjardins and Fernald (2010). Partial sequence for the control gene 18S (accession # AM490061.1) was retrieved from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/nuccore) and c-fos sequence (isotig05571) is available in Garcia de la Serrana (2012). The bdnf primers were designed using NCBI sequences from several fish species and then aligned with ClustalW to select the most conserved regions (www.genome.jp/tools/clustalw) (Thompson et al. 1994). Primers for all target mRNA were designed using Primer3 software (Koressaar and Remm, 2007; Untergasse et al. 2012) and synthetized by Sigma-Aldrich-Aldrich (Hamburgo, German). The PCR products were sequenced to confirm the desired primer cDNA amplification. Amplification products were 175 pb for c-fos, 105 pb for bdnf and 164 pb for 18s. Primer dimers formation was controlled with FastPCR v5.4 software (Kalendar, et al. 2014) and optimal annealing temperature was assessed for maximal fluorescence (table 7.1). Fluorescence cycle thresholds (CT) were automatically measured using a Roche

Light Cycler 480 II (Roche Diagnostics, Penzberg, Germany), and relative expression of the target genes were calculated using the 2-ΔCt method (Livak and Schmittgen, 2001). The qtRT-PCR was carried out using 8 µL reactions including 4 µL Power SYBR® Green PCR Master Mix (Biosystems, Foster City, USA), 0.2 µL of each primer (50 pmol/µl) and 2 µL of cDNA template (RNA equivalent). Cycling conditions were as follows: (i) denaturation (5 min at 95 °C); (ii) amplification and quantification (40 cycles; 30 s at 95 °C, 30 s at primer specific annealing temperature, 30 s at 72 °C with a single fluorescence measurement); and (iii) melting curve assessment (30 s at 95 °C; 30 s at 55 °C, followed by an 55–95 °C with a heating rate of 0.5 °C/s and a continuous fluorescence measurement; 30 s at 95 °C). A previously established housekeeping gene 18S was used as an internal control gene, as its abundance was stable between experimental groups and did not display any treatment effects (data not shown). All reactions were run in triplicate and controls without DNA templates were run to verify the absence of cDNA contamination. Primers efficiency was calculated for each qtRT-PCR reaction using a ten-fold dilution series efficiency test (amplification efficiency > 0.9) (Taylor et al. 2010).

Table 7.1. Primer sequences and annealing temperatures (Ta) for the genes studied (Fw: forward primer, Rv: reverse primer).

Gene	Primer sequences	Ta (ºC)
c-fos	Fw: 5’-GAAGGAGAGGCTCGAGTTCA-3’ Rv: 5’-TGGCTGGCTGGAAGTGATAG -3’	57
bdnf	Fw: 5’-GCTCAGCGTGTGTGACAGTA -3’ Rv: 5’- ACAGGGACCTTTTCCATGAC-3’	59
18S	Fw: 5’-AGGGTGTTGGCAGACGTTAC-3’ Rv: 5’-CTTCTGCCTGTTGAGGAACC -3’	57

Data analysis

Statistical analyses were performed using SPSS 19.0 for Windows. The results are expressed as mean ± standard error of mean (SEM). Behaviours measured in the net restraining test were collapsed into first principal component scores (PC1) with orthogonal rotation (varimax) using Principal Components Analysis (PCA). The correlation matrix was used to check multicollinearity, i.e., to identify how much variable correlated with each other (where a high correlation is $r = 0.9$). Kaiser–Meyer–Olkin (KMO) test for sample adequacy was always greater than 0.5 and the Bartlett's test of sphericity was significant for all tests. Two-way analysis of variance (ANOVA) was used to compare gene expression data for microdissected areas, with coping style (reactive vs. proactive) and treatment (basal conditions vs. acute stress) as independent variables. Models were assessed by their capacity to explain the variability and interaction effects and were kept or dropped according to total model "lack of fit" probabilities. In addition, contrast effect tests were conducted in order to ascertain differences between groups dependent upon significant ANOVA effects. A corrected α was used to establish significance, dependent on the amount of comparisons conducted. Before final acceptance of the model, diagnostic residual plots were examined to ensure that no systematic patterns occurred in the errors (e.g. fitted values vs. observed values and q-q plots), when necessary, values were arcsine-transformed. Statistical significance was taken at $p < 0.05$.

Results

Coping styles selection

Individuals characterized by high latency to escape, low number of escape attempts and shorter total time escaping had PCA low scores and were classified as reactive fish (Castanheira et al. 2013ab). Meanwhile, fish characterized by low latency to escape, high number of escape attempts and longer total time escaping had high PCA scores and were classified as proactive (scores ranged from -2.68 to 2.05). Please refer to Table 7.2 for a full overview of measured behavioural parameters.

From the total of 216 fish screened, 72 were classified as proactive and 72 were reactive. Intermediates represented a 66 % of the total and were discarded.

Table 7.2. Mean ± SD of the behavioural parameters measured: latency to escape, number of escapes attempts and total escaping time, as well as principal component analysis (PCA) loading factors of the net restraining test variables used to generate the principal component scores (PC1).

Net restraining test	Behavior Mean (± SD)	Min.	Max.	Loadings for PC1	% Variation explained
Latency to escape (sec)	14 ± 14	1	60	-0.775	65.697
Number of escapes attempts	11 ± 6	0	31	0.872	
Total escaping time (sec)	6 ± 5	0	45	0.781	

In situ hybridization

ISH results show that the mRNA abundance of the early activity gene c-fos were higher expressed in both the Vs and the Vv of reactive fish at acute stress levels, in comparison to proactive individuals (Figure 7.1).

Meanwhile even though both coping styles displayed bdnf expression in the Vv, proactive fish had a tendency to display slightly higher transcript abundance compared to reactive individuals (Figure 7.1).

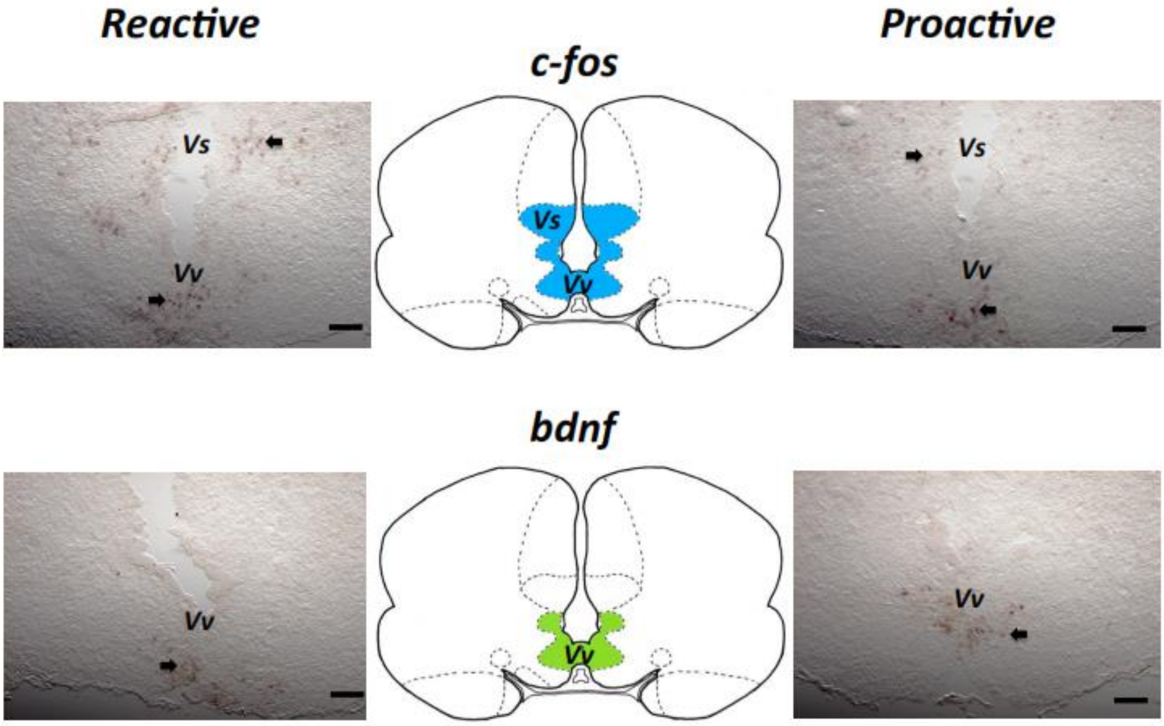

Figure 7.1. *In situ* hybridization (ISH) of the immediate early gene *c-fos* (A) and brain derived neurotrophic factor *bdnf*, (B) after an acute stress challenge in the supracommisural nucleus of the ventral telencephalon (Vs) and ventral part of the ventral telencephalon (Vv). Blue and green coloring indicates an apparent higher expression of transcript levels in reactive and proactive fish, respectively. Marked areas in dashed lines show approximate limits for region-specific studied areas. Arrows in pictures indicate stained cells. The scale bars represent 100 µm.

Gene expression

On c-fos mRNA expression (Fig 7.2) there was a significant effect of coping style in the Vs area (F(2,25) = 6.5, $p < 0.05$), with reactive fish showing higher abundance compared to proactive and treatment effect (F(2,25) = 11.6, $p < 0.005$), with higher mRNA levels post-stress, compared to basal conditions (Fig 7.2).However, there was no interaction effect between stress and coping style. No other significant effects were found in studied areas for c-fos mRNA transcript levels.

Regarding bdnf mRNA transcript expression, we found a significant interaction effect (F(3,22) = 4.3, $p = 0.05$) in the Vv in which there was a strong tendency for proactive fish to have increased levels at acute stress conditions ($p < 0.05$, not significant due to corrected $\alpha = 0.0125$), compared to all other groups (Fig 7.1). No other significant effects on bdnf mRNA levels were found for this or any of the other studied regions. An overview of the mean (± SEM) and statistical analyses for all transcript abundance levels can be found in Table 7.3.

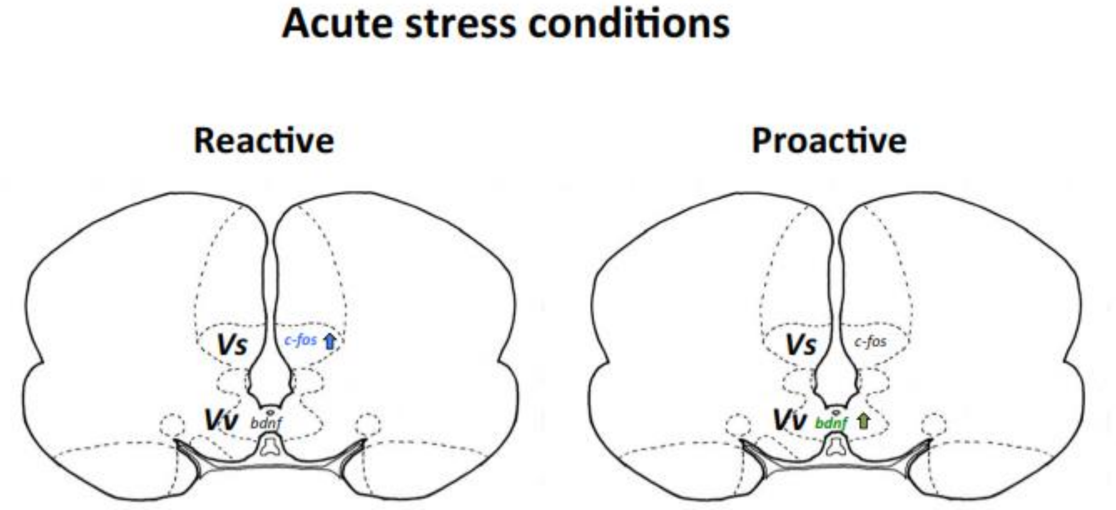

Figure 7.2. Schematic representation of transverse sections of a Gilthead Seabream´s telencephalon illustrating the regulation of the immediate early gene *c-fos* and the neuroplasticity maker *brain derived neurotrophic factor* (*bdnf*), in the supracommisural nucleus of the ventral telencephalon (Vs) and ventral part of the ventral telencephalon (Vv) after an acute stress challenge in reactive and proactive individuals. Green (in proactive) and blue (in reactive) arrows indicate higher expression of transcript levels in response to stress.

Table 7.3. Mean (± SEM) gene expression of the early activity gene *c-fos* and the neuroplasticity marker *brain derived neurotrophic factor* (*bdnf*) in reactive and proactive Gilthead seabream at basal and acute stress conditions in the dorsolateral pallium (Dl), dorsomedial pallium (Dm), supracommisural nucleus of the ventral telencephalon (Vs) and ventral part of the ventral telencephalon (Vv). Two-Way ANOVA statistics for effect of coping style, stress and the interaction between style and stress (if it was maintained in the model which was indicated by "lack of fit" analysis), are given for each variable.

	Reactive		**Proactive**		**ANOVA**		
	Control	**Stress**	**Control**	**Stress**	**Style**	**Stress**	**Interaction**
Dl							
c-fos	0.15 ± 0.06	0.14 ± 0.05	0.09 ± 0.02	0.35 ± 0.14	$F_{(2,24)} = 0.81$, $p = 0.38$	$F_{(2,24)} = 2.33$, $p = 0.14$	------
bdnf	0.07 ± 0.03	0.08 ± 0.02	0.14 ± 0.03	0.07 ± 0.01	$F_{(2,26)} = 2.27$, $p = 0.14$	$F_{(2,26)} = 1.17$, $p = 0.29$	------
Dm							
c-fos	1.23 ± 0.85	2.99 ± 1.53	0.61 ± 0.3	0.45 ± 0.18	$F_{(2,21)} = 2.54$, $p = 0.13$	$F_{(2,21)} = 0.83$, $p = 0.37$	------
bdnf	0.6 ± 0.38	1.31 ± 0.61	8.55 ± 4.29	0.67 ± 0.28	$F_{(2,26)} = 1.72$, $p = 0.2$	$F_{(2,26)} = 2.02$, $p = 0.17$	------
Vs							
c-fos	0.08 ± 0.02	0.27 ± 0.07	0.04 ± 0.01	0.1 ± 0.02	$F_{(2,25)} = 6.5$, $\mathbf{p = 0.02}$	$F_{(2,25)} = 11.6$, $\mathbf{p = 0.002}$	------
bdnf	0.02 ± 0.006	0.03 ± 0.006	0.03 ± 0.007	0.02 ± 0.003	$F_{(2,24)} = 0.16$, $p = 0.69$	$F_{(2,24)} = 0.1$, $p = 0.76$	------
Vv							
c-fos	0.03 ± 0.01	0.07 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	$F_{(2,22)} = 0.64$, $p = 0.43$	$F_{(2,22)} = 1.39$, $p = 0.25$	------
bdnf	0.01 ± 0.005	0.01 ± 0.003	0.01 ± 0.002	0.02 ± 0.007	$F_{(3,22)} = 0.77$, $p = 0.39$	$F_{(3,22)} = 1.42$, $p = 0.24$	$F_{(3,22)} = 4.31$, $\mathbf{p = 0.05}$

Discussion

We here for the first time submit evidence of differences in activation/regulation of region-specific telencephalic regions between seabream displaying contrasting coping styles. Our results show that proactive fish display post-stress decreased c-fos and increased bdnf transcript levels compared to reactive individuals in the subpallial supracommisural (Vs) and ventrolateral (Vv) areas, respectively. Notably these 2 areas and homologous have been associated with goal-oriented behaviour and emotional reactivity in both fish and mammals respectively (Goodson and Kingsbury 2013; O´Connell and Hofmann 2011; Silva et al. 2015; Vindas et al. 2014a).

Gene expression has been proven to be a reliable approach to identify how specific neuronal networks respond differentially to environmental inputs and how these are linked to alterations in cognitive ability. In this context, the study of immediate early genes (IEGs), that are rapidly and transiently induced upon neuronal stimulation, combined with expression of markers associated with neural plasticity has provided fundamental insights into the neurobiology of cognition (Wood et al. 2011, Burmeister and Fernald 2005). Their accumulation in cells (mRNA or protein) is thus widely used as a marker of neural activity (e.g. Burmeister and Fernald 2005). Notably, the IEG c-fos has important regulatory functions during cell proliferation and its use has permitted the functional neuroanatomical mapping of neural systems in response to specific environmental stimuli (Hoffman et al. 1993; VanElzakker et al. 2008). In our study we found that fish displaying opposed stress coping styles displayed clear telencephalic region-specific differences in c-fos expression when subjected to the same stressful stimuli. Interestingly, Lau et al. (2011) reported that subjecting zebrafish to stress leads to an increase in anxiogenic behaviour, which in turn is characterized by a higher expression of c-fos mRNA in the Vs area. In agreement with these results, we found that the more stress sensitive individuals (i.e. reactive fish), which are more prone to anxiety-like behaviour (Øverli et al. 2007), also exhibited an increase expression in c-fos mRNA in the Vs after stress. Moreover, lesions of the Vs area decrease defensive behaviour in *Macropodus opercularis* and goldfish (Davis et al. 1976, 1978; Rooney and Laming 1986) suggesting that this area has an important excitatory role in proactive behavioural outputs. Taking this into account, we speculate that the increased neural activity in the Vs after stress in reactive fish may be associated with their passive behavioural coping strategy. That is, increased activity of the Vs may be part of a regulatory neural mechanism for the inhibition of active behaviour, particularly after experiencing an acute stress situation. However, further studies targeting activation and regulation of telencephalic forebrain regions in opposite coping styles will be fundamental in elucidating activation and inhibition of neural networks associated with these strategies.

Regarding the expression of the plasticity marker brain derived neurotrophic factor (bdnf), we found that proactive individuals responded to acute stress with higher bdnf mRNA abundance in the Vv. Recent studies have reported changes in markers for structural plasticity in the fish brain in response to a range of factors including acute and chronic stress (Johansen et al. 2012; Sørensen et al. 2013). In addition, bdnf has also recently been linked with differences in brain activation under unpredictable environments in Atlantic salmon (Vindas et al. 2014a). Notably the fish´s Vv area has been proposed to be functionally homologous to the mammalian lateral septum (LS) which is associated with the modulation of social behaviour as well as the evaluation of stimulus novelty (Luo et al. 2011; Maeda and Mogenson 1981; Singewald et al. 2011; Swanson 1998; Swanson and Petrovich 2000). Proactive individuals are behaviourally characterised by a higher propensity to develop and follow routines and have been reported to exhibit less behavioural flexibility (Chapman et al. 2010; Ruiz-Gomez et al. 2011). Therefore, in this context, an increase in neural plasticity in proactive fish could be considered surprising. However, in our study these differences only arise after stress (restraining), specifically in the Vv. Hence, it could be hypothesized that after acute stressful events the higher flexibility of proactive individual's associated with their active behavioural coping, may be characterized by increased bdnf expression in specific telencephalic areas, such as the Vv. Interestingly, in agreement with these results, Vindas et al. (under review), report that proactive Atlantic salmon subjected to an acute stressor show an increase of bdnf transcript levels in both the Vv and the dorsolateral pallium (Dl). Consequently, taking this into consideration, it appears that the Vv may be an important area associated with active behavioural coping to acute stress in fish, but future research into neural regulation of behaviour under different environmental conditions is needed in order to confirm the role of this and other forebrain areas.

Conclusions

We here report differences in activation of region-specific telencephalic regions between seabream displaying contrasting coping styles. This work confirms the hypothesis that in seabream, inhibitory and excitatory markers of neural function appear to be associated with reactive and proactive coping styles, respectively.

Importantly, increased understanding of the neurobiological mechanisms underpinning individual behavioural profiles will help to improve welfare and husbandry practices in the aquaculture industry; for example by applying it to selective breeding in order to minimize vulnerable phenotypes. In addition, by characterising the neural regulation of individuals expressing alternate coping styles, we will gain further insight into central nervous system function and dysfunction.

Acknowledgments

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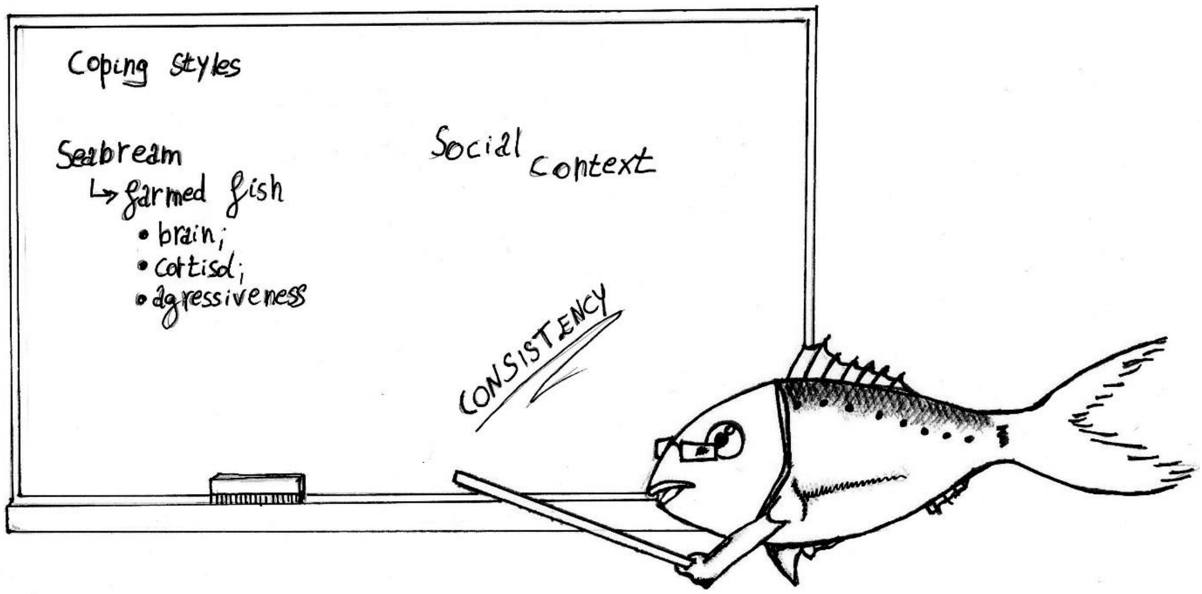

Coping styles
Seabream
↳ farmed fish
• brain;
• cortisol;
• agressiveness
Social context
CONSISTENCY

CHAPTER

8

General discussion

8. General discussion

The general aim of this study was to understand differences in the way individual Gilthead seabream (*Sparus aurata*) are coping with social and environmental challenges in aquaculture rearing conditions. This thesis has shown for the first time a link between HPI responsiveness and aggressive behaviour in seabream, demonstrating the existence of coping styles, and particularly we did show that aggression is part of coping styles (**Chapter 3)**. In **Chapter 4 we** tested individual differences in behavioural responses to a variety of challenges by subjecting fish to a battery of both individual and grouped-based tests. The escape response during a net restraining test was shown to be consistent over time and across context and was used as an indicator of coping styles in seabream in the following chapters. **Chapter 5** and **chapter 6** tested the consistency of divergent coping styles. The influence of social context in the consistency of escape response during a net restraining test was investigated in **chapter 5** and the long-term consistency of coping styles in seabream was investigated in **chapter 6**. Lastly, **chapter 7** investigated differences in forebrain neural activity on individual coping characteristics of Gilthead seabream.

This final chapter explores the results of the previous chapters and the impact of individual coping styles on the performance, as well the possible implications for fish welfare. Coping styles implications to research and aquaculture industry are also discussed together with suggestions for future research. Finally, this chapter summarizes the main conclusions drawn from this thesis.

8.1. Are coping styles present in Gilthead seabream?

8.1.1. Individual differences in aggressiveness and stress response: an indication of coping styles in Sparus aurata

This thesis suggests a relationship between aggressive behaviour and cortisol responsiveness in Gilthead seabream measured after a net restraining test (chapter 3). Proactive individuals were more likely to initiate fights, spent more time on fighting and fought more often than reactive individuals. The results of chapter 3 are in line with other studies reporting that proactive individuals behave more aggressively than reactive individuals (Benus et al. 1988; Bolhuis et al. 2005a; Koolhaas et al. 1999; Millot et al. 2009a; Øverli et al. 2004ab). However a novel finding was described in chapter 3. Contrary to the conventional studies done in fish (Øverli et al. 1999; Pottinger and Carrick 2001), here we saw that without a period of time in isolation to induce territoriality and motivate aggressiveness, proactive fish displayed more aggressive behaviour than reactive fish. Thus, in some fish species an isolation period may not be required for the expression of aggressive behaviour. The consequences of aggression in aquaculture are various. For example, during feeding, agonistic interactions between fish increase. This may result in heterogeneous growth, since bold-aggressive fish could potentially get most of the food. It also could lead to the development of infectious diseases as a result of injury (Ashley 2007).

In chapter 3, it is also shown that aggressiveness was correlated with cortisol responsiveness after stress (i.e. after a net restraining test). Gilthead seabream with lower post-stress cortisol levels are mostly more aggressive, these patterns were opposite to fish with high post-stress cortisol levels and are the first indication of the presence of coping styles. However, we found a high variability in aggressiveness in low cortisol responders that may be related with unmeasured factors as for example the coping style of the naïve fish. Parallel to the current thesis, several studies have shown that lower cortisol responders after stress are more aggressive (Pottinger and Carrick, 2001; Øverli et al. 2004ab). Thus, suggesting that individual differences in aggression and cortisol are part of coping styles. After these conclusions we moved to determine and characterize more distinct traits in Gilthead seabream (chapter 4).

8.1.2. Coping styles traits in Sparus aurata: behavioural and physiological responses

Different aspects of behaviour and physiology (cortisol production) were approached in chapter 4 to characterize seabream coping styles in a variety of challenges using both individual and grouped-based tests. Fish were subjected to three individual-based tests (feed intake recovery in a novel environment, novel object and restraining) and to two group-based tests (risk-taking and hypoxia). All the tests were adapted from tests that have been used previously as coping styles characterization in others species: Feeding recovery in a novel environment (adapted from Øverli et al. 2002a); Novel object (adapted from Frost et al. 2007); Restraining (adapted from

Arends et al. 1999, Silva et al. 2010 and Martins et al. 2011cd); Hypoxia (adapted from Laursen et al. 2011) and Risk-taking (adapted from Huntingford et al. 2010). This study showed for the first time in seabream that both risk taking and escape behaviours during restraining were consistent over a period of 14 days. In addition, consistency across contexts was also observed: individuals that took longer to recover feed intake after transfer into a novel environment, exhibited higher escape attempts during a restraining test and escaped faster from hypoxia conditions.

Considering the consistency of behavioural responses over time, our results are in agreement with previous studies (Brelin et al. 2005; Martins et al. 2011c) indicating the tendency to show a proactive (i.e. escape) or reactive (i.e. immobility or less escape) behaviour response over a period of 14 days. In addition, proactive individuals has been related to a lower hypothalamus–pituitary–adrenal/interrenal (HPA/I) axis reactivity (Koolhaas et al. 1999; Veenema et al. 2003). Although data on fish are not always consistent, e.g. Silva et al. (2010) studies point to a higher HPI-axis reactivity for proactive individuals after stressful conditions. Some authors have suggested that cortisol and behavioural responses to stressors are linked to two independent dimensions of stable trait characteristics (Steimer et al. 1997; Koolhaas et al. 2010). These authors suggested that the quality of the behavioural response may be a dimension that is independent from the magnitude of the response. According to these authors, the physiological responses to stress such as the HPI axis reactivity is more related to an emotional response to stress than to coping styles. This model may explain why we did not find correlations between plasma cortisol levels after the net restraining test and behavioural data (chapter 4).

Considering the consistency across contexts, individuals escaping more during the restraining test in general also escaped faster from hypoxia conditions and took longer to recover feed intake. Our results are in line with findings from others authors where, proactive individuals escape more and faster during the restraining test (Brelin et al. 2005; Martins et al. 2011c; Silva et al. 2010). However, in relation to hypoxia and feeding recovery in isolation these results are opposite to the previous findings of Laursen et al. 2011 and Øverli et al. 2002a, respectively. Laursen et al. (2011) presented that reactive trout escaped faster from hypoxic conditions. One possible explanation for the differences found in our study is the presence of species-specificity differences in sub-lethal effects of reduced levels of dissolved oxygen (Raleigh et al. 1984; Reig 2001). In trout, fish escaped when exposed to decreased oxygen levels varying from 90 to 30% saturation. In seabream, however, individuals start escaping hypoxia only when oxygen concentrations reach level close to 30% saturation. This suggests that proactive fish that develop and follow routines faster (Ruiz-Gomez et al. 2011) are the last to escape from hypoxia conditions in Laursen et al. (2011) study. Nevertheless in seabream, individuals started to escape when oxygen concentrations reached sub-lethal levels and proactive individuals, known to exhibit active attempts to counteract stressors (Benus et al. 1991ab; Øverli et al. 2007), were the firsts to escape from hypoxia conditions. Other possible explanation may be related with the fact that Laursen et al. (2011) used selected trout lines with contrasting (high vs low) post-stress cortisol: the high-responding (HR) and the low-responding (LR) (Pottinger and Carrick 1999). Seabream used in all the experiment of this thesis were not submitted to such selection.

In relation to feeding recovery in isolation our results are in opposition with previous studies indicating that proactive individuals recover feed intake faster than reactive (Øverli et al. 2002a). Such contradiction of results may be due to species-specific behaviour and/or to previous experiences (e.g. social experiences, nutritional background) that fish were exposed prior to the start of the experiments. Furthermore, Ruiz-Gomez et al. (2011) demonstrated that proactive individuals develop and follow routines more often and this could promote a slower adaptation in a new environment, being this a possible explanation for the differences found.

Despite evidence for species-specificity in coping styles characterization, one of the most important findings in this thesis was the demonstration that it is possible to predict behaviour in groups from personality traits measured in individual seabream in isolation.

Overall, results of chapter 4 demonstrate that escape response under a restraining test is a consistent trait over time and across context. Using these results, this trait was used as a proxy of coping styles in seabream in chapter 5, chapter 6 and chapter 7. In addition, escape performance has ecological significance (predator-prey-interaction), has physiological implications (anaerobic recovery capacity of white muscle) and can be considered as a parallel to the forced swimming test, which is widely used in rodents for biomedical studies. Moreover, the escape performance is very representative of conditions that the fish have to deal in practical aquaculture conditions (e.g. grading, vaccination, transport).

8.2. Are coping styles consistent in the teleost fish Sparus aurata?

Social environment

Distinct aspects of coping styles characterisation were accomplished in chapter 3 and chapter 4. However, data on fish are not always consistent (e.g. David et al. 2012; Ferrari et al. 2015), and point to different factors (e.g. Archard et al. 2012; Brelin et al. 2008; Chapman et al. 2010) that can shape or influence coping styles. Different aspects of consistency in coping styles were studied in this thesis for seabream (chapters 5 and 6). In chapter 5, results showed that social context in which fish are kept significantly influences personality traits. Furthermore, these findings also showed that proactive and reactive individuals adopt different social strategies that cannot just be explained as differences in coping styles *per se*, but are rather related to the consistency of coping styles according to the social group composition. The social group in which fish live, is probably one of the most challenging and complex aspects of their environment. In some species the group structure is based in the development of strong social hierarchies (Barreto and Volpato 2006; Ejike and Schreck 1980; Fox et al. 1997). Thus, the social environment can be a source of social support, i.e. stable, positive social bonds can supply animals with an improved capacity to cope with environmental demands (Schuett et al. 2010, 2011; Cote et al. 2010, 2011). On the other hand, we can hypothesise that long-term instability in social relationships may lead to an aversive situation, in particular for submissive individuals. The results of chapter 5 suggest that individual coping characteristics of fish may influence the consistency of coping styles, that may be reflected in a change and regrouping of coping styles to adopt different social strategies. In addition, these findings indicate that mixing groups of seabream with 1/3 of each (proactive, reactive and intermediate) coping style appears to be favourable for both welfare and productivity.

Long and short term consistency

In chapter 6, we tested the consistency of coping styles in seabream, both, over time and during life history. The consistency of coping styles is still hugely debated and the results of chapter 6 could shed some more light on it. Most behavioural studies assessing the consistency of coping styles over time are based on the use of different tests over a relatively short period (e.g. chapter 4 and 5). Few studies have investigated the consistency of coping styles over both short and long time intervals (David et al. 2012; Ferrrari et al. 2015).Furthermore, Bell et al. (2009) reported a higher consistency in tests separated by short intervals than for those separated by longer intervals. This is not surprising, because several studies have indicated a role for various factors influencing coping styles. Moreover, the results of chapter 5 suggest that social context affects the stability of coping styles in gilthead seabream juveniles.

In chapter 6, the assessment of the short- and long-term consistency of behavioural responses was done using fish behaviour during the net restraining test. Data in chapter 6 showed that individual escape responses are consistent after 14 days (short-term consistency) and 8 months (long term-consistency) after the first test was performed. Nevertheless, after the onset of sexual maturation (16, 22 and 23 months after the first test) this behaviour consistency during restraining was lost. This finding suggests that part of the consistency in behaviour may be affected by the sexual maturation or/and sex inversion process. We also hypothesized in Chapter 6 that the contextual importance of the net restraining response may differ according to fish development.

Magnhagen et al. (2015) demonstrated that changes of context may induce divergent behaviour responses in fish with distinct coping styles. In chapter 6, the first two runs of a net restraining test, conducted two weeks apart, were highly correlated. The test was in both runs preceded by the same events, including feeding observations and a novel object test but after the second run the restraining was performed immediately after taking the fish from their home tank.

Also the increase in fish weight may have an influence the way it perceives the restraining test. This means that the impulse needed to counteract gravity should be higher in adults than in juveniles. A possible explanation may be related with the net restraining test *per se* and how fish, perceive this test. Thus, in adult fish caution is needed when restraining test is used and the method may need to be refined. Therefore, this preliminary finding is very important and more information should be brought into the foreground of coping styles consistency. Moreover, further studies should be considered to assess behaviour long-term consistency associated with divergent coping styles, and also using other dimensions of personality (e.g. aggressiveness.) Other tests (e.g. risk-taking) will likely help to map the specific response of each species studied.

In addition, it may be necessary to include a combination of neurobiological, neuroendocrine and/or genetic proxies to be able to fully answer this question of long-term consistency of coping styles.

8.3. Do fish with distinct coping styles show differences in neuronal regulation?

The behavioural results presented and discuss in chapters 4 and 6 strongly suggest that different behavioural responses to stress are indeed associated with seabream characterized by opposing coping styles. Notably, since behavioural outputs may have a common neurobiological component, it is then reasonable to expect that a different activation/regulation of neural genes will be associated with the regulation of coping styles.

In chapter 6 we assessed a link between stress coping ability and brain function, by investigating the behavioural and forebrain physiological responses of fish displaying opposed coping styles during a net restraining test. For that, we conducted in situ hybridisation and quantified gene expression of the immediate early genes *c-fos* and the neuronal plasticity marker brain derived neurotrophic factor (*bdnf*) in specific activated telencephalic regions: the pallial dorsolateral (Dl) and dorsomedial (Dm) areas, as well as the subpallial supracommisural (Vs) and ventrolateral (Vv) areas.

In fish species, it has been now shown that there is a high level of conserved brain function, specifically in forebrain areas, which have been associated with arousal and emotional control in vertebrates (Goodson and Kingsbury 2013; O´Connell and

Hofmann 2011). Results obtained in Chapter 6 indicate that proactive fish show post-stress decreased *c-fos* (a marker for early gene activity, associated with neural activation, Hoffman et al. 1993; VanElzakker et al. 2008) and increased *bdnf* (associated with neural plasticity, learning and memory, Johansen et al. 2012; Sørensen et al. 2013) transcript levels compared to reactive individuals in the subpallial supracommisural (Vs) and ventrolateral (Vv) areas, respectively. The Vs and Vv areas, along with their putative mammalian homolog structures have been associated with both goal-oriented behaviour and emotional reactivity in mammals and fish species (Goodson and Kingsbury 2013; O´Connell and Hofmann 2011; Silva et al. 2015; Vindas et al. 2014a). This thesis suggests that fundamental cognitive differences between proactive and reactive seabream are an intrinsic component of inter-individual variation in this fish species. Importantly, an increased understanding of the basic mechanisms associated with individual behavioural coping styles across vertebrates may help to further establish the use of fish models, instead of mammalian ones, in biomedical research in order to better understand the vertebrate nervous system.

8.4. Practical implications and future directions

The results of the present thesis clearly indicate individual variation in behaviour and physiological responses in Gilthead seabream (*Sparus aurata*). These findings significantly strengthen the idea that stress responsiveness in seabream is dependent not only on the type of stressor but also on the coping style that characterises an individual.

Many of the aquaculture practices such as transport, handling, feeding techniques, management procedures and stocking densities all have strong effects on stress responses, subsequent stress tolerance, health and the occurrence of aggressive behaviour. Therefore, individual differences in how fish respond to stress are important in aquaculture. The present findings provide possibilities on a more applied level as to use coping styles traits as a strategy to reduce disease susceptibility, minimise stress responses, and avoid aggression.

The use of coping styles traits in breeding programmes in addition to “classical” fitness traits, such as growth performance, feed efficiency and disease resistance may represent additional selection criteria that should be included in breeding programmes aimed at the improvement of adaptive capacity and welfare. Such knowledge might enable the breeding of fish that are optimally adapted to their environment, including alternative husbandry systems that are intended to improve animal welfare and sustainability. Furthermore, a number of studies, including results from this thesis, have shown that proactive individuals are low cortisol responders but also more aggressive individuals.

Thus, selection of stress resistant individuals leads to unintentional selection of other undesirable traits that might create basis for new challenges in rearing conditions. However, previous information of fish coping styles may help to reduce aggression in production systems through changes of how feed is delivered or excluding extremely high responders in the selection process for example.

Furthermore, and as demonstrated in this thesis, coping styles should interpreted with care. We provide evidence that sociality, age and context may also be relevant to the fish coping styles adaptation in farming conditions.

In addition, this thesis shows that individual coping styles have distinct underlying neural mechanism and that can open the possibility to use farmed fish as models to understand mechanism of coping styles in vertebrates, such as those related to neural activity and their implications in behaviour.

The results of the present thesis clearly provide tools to reliably identify individuals with contrasting coping style, and provide a causal framework for this variation, and may therefore considerably underpin efforts to improve animal welfare in aquaculture. The next challenge is to define and implement farming environments that are more diverse and could improve the welfare of individuals with different coping styles. Moreover, the genetic basis (heritability/epigenetics) of coping styles, disease susceptibility as well the neuroendocrine mechanisms behind consistent as well as flexible behavioural patterns, are here pinpointed as central themes and open research lines on application of coping styles to aquaculture.

Therefore, future research should incorporate concepts such as stability through change, i.e. allostasis (Korte et al. 2007), appraisal and cognition (Galhardo et al. 2009; Millot et al. 2014ac) in farmed fish production. That will certainly allow a more comprehensive picture of how to improve the welfare of individuals with different coping styles, as well as it may increase the production output.

However, we are aware that some limitations may exist in this study. For example some of the limitations on this study may be related with the isolation *per se,* novel environment, small size of the units or even the handling of the animals inherent of this type of experiments in laboratorial conditions. For example, it would be interesting to investigate in detail patterns of behaviours that act as displacement activities and the possible occurrence of stereotypes. Thus, the occurrences of stereotypic behaviours on specific conditions deserve further investigation.

Another limitation of the study may be related with the industry approach. Based on the results of this Thesis further research should focus on the flexibility of coping styles using simulating farming conditions (e.g. large-scale studies).

8.5. Main conclusions

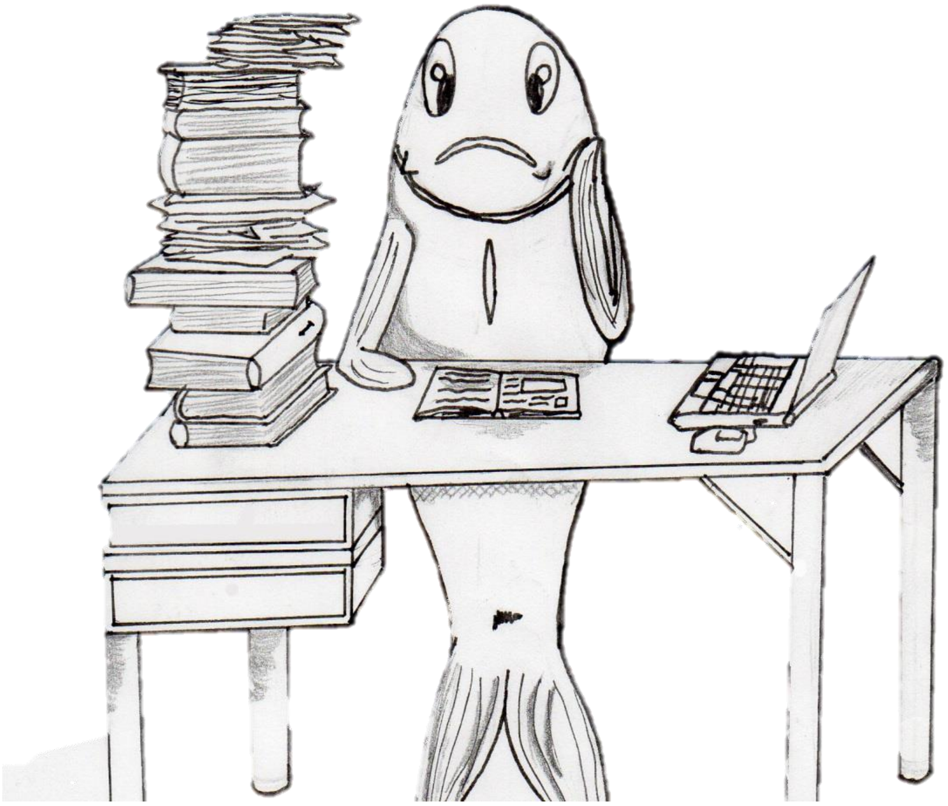
The following main conclusions were drawn from this thesis in what concerns of coping styles in Gilthead seabream (*Sparus aurata*):

- The combination of behavioural and physiological measures demonstrates and quantifies the presence and consistency of individual variation in coping style in *Seabream.*

- The results of the net restraining test were consistent over time and between contexts, at least until sexual maturation. In addition, behaviour measured in groups can predict individual coping styles.

- Coping styles can change according to social environment and life history.

- Brain function differs between proactive and reactive fish individuals and these differences appear to be conserved through the vertebrate lineage.



References

References

Abrahams MV, Sutterlin A (1999) The foraging and antipredator behavior of growth enhanced transgenic Atlantic salmon. *Animal Behavior* 58: 933–942.

Allen C (2013) Fish Cognition and Consciousness. Journal of Agricultural and Environmental Ethics 26: 25-39.

Alves RN, Cordeiro O, Silva TS, Richard N, de Vareilles M, Marino G, DiMarco P, Rodrigues PM, Conceição LEC (2010) Metabolic molecular indicators of chronic stress in gilthead seabream (*Sparus aurata*) using comparative proteomics. *Aquaculture* 299: 57-66.

Andersson MÅ, Silva PIM, Steffensen JF, Höglund E (2011) Effects of maternal stress coping style on offspring characteristics in rainbow trout (*Oncorhynchus mykiss*). *Hormones & Behavior* 60: 699-705.

Andersson MÅ, Höglund E (2012) Linking personality to larval energy reserves in rainbow trout (*Oncorhynchus mykiss*). PLoS ONE 7(11): e49247.

Andersson MÅ, Khan UW, Øverli O, Gjoen HM, Höglund E (2013a) Coupling between stress coping style and time of emergence from spawning nests in salmonid fishes: Evidence from selected rainbow trout strains (*Oncorhynchus mykiss*). *Physiology & Behaviour* 117: 30-34.

Andersson MÅ, Laursen DC, Silva PIM, Höglund E (2013b) The relationship between emergence from spawning gravel and growth in farmed rainbow trout *Oncorhynchus mykiss*. *Journal of Fish Biology* 83(1): 214-219.

Andrew JE, Holm J, Kadri S, Huntingford FA (2004) The effect of competition on the feeding efficiency and feed handling behaviour in Gilthead Sea Bream (*Sparus Aurata* L.) held in tanks. *Aquaculture* 232: 317–31.

Archard GA, Earley RL, Hanninen AF, Braithwaite VA (2012) Correlated behaviour and stress physiology in fish exposed to different levels of predation pressure. *Functional Ecology* 26: 637-645.

Arends RJ, Mancera JM, Munoz JL, Wendelaar Bonga SE, Flik G (1999) The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *Journal of Endocrinology* 163: 149-157.

Arias A (1976) Sobre la biología de la dorada, *Sparus aurata* L., de los esteros de la provincia de Cádiz. *Investigación Pesquera* 40: 201-222.

Arias AM (1980) Crecimiento, régimen alimentario y reproducción de la dorada (*Sparus aurata* L.) y dl robalo (*Dicentrarchus labrax* L.) en los esteros de Cádiz. *Investigación Pesquera* 44(1): 59-83.

Ashley PJ (2007) Fish welfare: Current issues in aquaculture. *Applied Animal Behaviour Science* 104: 199-235.

Bahuaud D, Gaarder M, Veiseth-Kent E, Thomassen M (2010) Fillet texture and protease activities in different families of farmed Atlantic salmon (Salmo salar L.). *Aquaculture* 310: 213-220*.*

Barazi-Yeroulanos L (2010) Synthesis of Mediterranean marine finfish aquaculture – a marketing and promotion strategy. General Fisheries Commission for the Mediterranea., Food and Agriculture Organization of the United Nations (FAO) Rome, 198p.

Barreto RE, Volpato GL (2006) Stress responses of the fish Nile tilapia subjected to electroshock and social stressors. *Brazilian Journal of Medical and Biological Research* 39: 1605-1612.

Barreto RE, Volpato GL (2011) Ventilation rates indicate stress-coping styles in Nile tilapia. *Journal of Bioscience* 36: 851-855.

References

Barton BA, Schreck CB, Sigismondi LA (1986) Multiple acute disturbances evoke cumulative physiological stress responses in juvenile Chinook Salmon. *Transactions of the American Fisheries Society* 115: 245-251.

Barton BA (2002) Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology* 42: 517-525.

Barton BA, Ribas L, Acerete L, Tort L (2005) Effects of chronic confinement on physiological responses of juvenile gilthead sea bream, *Sparus aurata* L., to acute handling. *Aquaculture Research* 36: 172-179.

Basic D, Winberg S, Schjolden J, Krogdahl Å, Höglund E (2012) Context-dependent responses to novelty in Rainbow trout (*Oncorhynchus mykiss*), selected for high and low post-stress cortisol responsiveness. *Physiology and Behaviour* 105: 1175– 1181.

Batzina A, Nafsika K (2012)The presence of substrate as a means of environmental enrichment in intensively reared Gilthead Seabream *Sparus Aurata*: growth and behavioral effects. *Aquaculture* 370–371: 54–60.

Benus RF, Koolhaas JM, Van Oortmerssen GA (1988) Aggression and adaptation to the light-dark cycle: Role of intrinsic and extrinsic control. Physiology & Behavior 43: 131-137.

Benus RF, Bohus B, Koolhaas JM, van Oortmerssen GA (1991) Behavioural differences between artificially selected aggressive and non-aggressive mice: response to apomorphine. *Behavioural Brain Research* 43: 203-208.

Benus RF, Bohus B, Koolhaas JM, van Oortmerssen GA (1991) Heritable variation for aggression as a reflection of individual coping strategies. *Cellular and Molecular Life Sciences* 47: 1008-1019.

Biro PA, Abrahams MV, Post JR, Parkinson EA (2004) Predators select against high growth rates and risk-taking behaviour in domestic trout populations*. Proceedings of the Royal Society B*: Biological Sciences 271: 2233–2237.

Biro PA, Abrahams MV, Post JR, Parkinson EA (2006) Behavioural trade-offs between growth and mortality explain evolution of submaximal growth rates. *Journal of Animal Ecology* 75: 1165–1171.

Biro PA, Stamps JA (2008) Are animal personality traits linked to life history productivity? *Trends in Ecology and Evolution* 23: 361–368.

Boersma G (2011) Personality and the pathophysiology of energy metabolism In: *Department of neuroendocrinology*. University of Groningen the Netherlands, pp. 1-242.

Bolhuis EJ, Schouten WGP, de Leeuw JA, Schrama JW, Wiegant VM (2004) Individual coping characteristics, rearing conditions and behavioural flexibility in pigs. *Behavioural Brain Research* 152: 351–360.

Bolhuis EJ, Schouten WGP, Schrama JW, Wiegant VM (2005a) Individual coping characteristics, aggressiveness and fighting strategies in pigs. *Animal Behaviour* 69: 1085-1091.

Bolhuis JE, Schouten WGP, Schrama JW, Wiegant VM (2005b) Behavioural development of pigs with different coping characteristics in barren and substrate enriched housing conditions. *Applied Animal Behaviour Science* 93: 213–228.

Brelin D, Petersson E, Winberg S (2005) Divergent Stress Coping Styles in Juvenile Brown Trout (*Salmo trutta*). *Annals of the New York Academy of Sciences* 1040: 239-245.

Brelin D, Petersson E, Dannewitz J, Dahl J, Winberg S (2008) Frequency distribution of coping strategies in four populations of brown trout (*Salmo trutta*). *Hormones and Behavior* 53: 546-556.

References

Briffa M, Sneddon LU (2007) Physiological constraints on contest behaviour. *Functional Ecology* 21: 627-637.

Broom DM (1991) Animal welfare—concepts and measurement. *Journal of Animal Science* 69: 4167–4175.

Broom DM (2010) Animal welfare: an aspect of care, sustainability, and food quality required by the public. *Journal of Veterinary Medical Education* 2010 Spring 37: 83-8.

Brown C, Laland KN (2003) Social learning in fishes: a review. *Fish and Fisheries* 4: 280-288.

Brown C, Laland K, Krause J (2011) Fish Cognition and Behavior. John Wiley & Sons.

Brusléa-Sicard S, Fourcault B (1997) Recognition of sex-inverting protandric *Sparus aurata*: ultrastructural aspects. *Journal of Fish Biology* 50: 1094-1103.

Budaev SV, Zworykin DD (2002) Individuality in fish behavior: Ecology and comparative psychology. *Journal of Ichthyology* 42: 189-195.

Burmeister SS, Fernald RD (2005) Evolutionary conservation of the egr-1 immediate-early gene response in a teleost. *Journal of Comparative Neurology* 481: 220-232.

Cabanac M, Cabanac AJ, Parent A (2009) The emergence of consciousness in phylogeny. *Behavioural Brain Research* 198: 267-272.

Cammarata M, Vazzana M, Accardi D, Parrinello N (2012) Seabream (*Sparus Aurata*) Long-Term Dominant-Subordinate Interplay Affects Phagocytosis by Peritoneal Cavity Cells. *Brain, Behavior, and Immunity* 26: 580–587.

Cañon Jones HA, Noble C, Damsgård B, Pearce GP (2011) Social network analysis of the behavioural interactions that influence the development of fin damage in Atlantic salmon parr (*Salmo salar*) held at different stocking densities. *Applied Animal Behaviour Science* 133: 117-126.

Careau V, Thomas D, Humphries MM, Réale D (2008) Energy metabolism and animal personality. *Oikos* 117: 641-653.

Carere C, Drent PJ, Koolhaas JM, Groothuis TGG (2005). Epigenetic effects on personality traits: early food provisioning and sibling competition. *Behaviour* 142: 1335-1361.

Carmichael GJ, Wedemeyer GA, McCraren JP, Millard JL (1983) Physiological effects of handling and hauling stress on Smallmouth Bass. *The Progressive Fish-Culturist* 45: 110-113.

Carter R (1996) Mapping the Mind. Phoenix Press, London.

Castanheira MF, Herrera M, Costas B, Conceição LEC, Martins CIM (2013a) Linking cortisol responsiveness and aggressive behaviour in gilthead seabream Sparus aurata: Indication of divergent coping styles. *Applied Animal Behaviour Science* 143: 75-81.

Castanheira MF, Herrera M, Costas B, Conceição LEC, Martins CIM (2013b) Can we predict personality in fish? – searching for consistency over time and across contexts. *PLoS ONE* 8(4): e62037

Castanheira MF, Cerqueira M, Millot S, Gonçalves RA, Oliveira C, Conceição LEC, Martins CIM (2016) Are personality traits consistent in fish? - The influence of social context. *Applied Animal Behaviour Science* 178: 96-101.

Cavigelli SA (2005) Animal personality and health. *Behaviour* 142: 1223-1244.

References

Champagne DL, Hoefnagels CCM, de Kloet RE, Richardson MK (2010) Translating rodent behavioral repertoire to zebrafish (*Danio rerio*): Relevance for stress research. *Behavioural Brain Research* 214: 332-342.

Chapman BB, Morrell LJ, Krause J (2010) Unpredictability in food supply during early life influences boldness in fish. *Behavioral Ecology* 21: 501-506

Clement TS, Grens KE, Fernald RD (2005a) Female affiliative preference depends on reproductive state in the African cichlid fish, *Astatotilapia burtoni*. *Behavioral Ecology* 16: 83-88.

Clement TS, Parikh V, Schrumpf M, Fernald RD (2005b) Behavioral coping strategies in a cichlid fish: the role of social status and acute stress response in direct and displaced aggression. *Hormones and Behavior* 47: 336-342.

Conrad JL, Weinersmith KL, Brodin T, Saltz JB, Sih A (2011) Behavioural syndromes in fishes: A review with implications for ecology and fisheries management. *Journal of Fish Biology* 78: 395-435.

Coppens CM, De Boer SF, Koolhaas JM (2010) Coping styles and behavioural flexibility: Towards underlying mechanisms. *Philosophical Transactions of the Royal Society B: Biological Sciences* 365: 4021-4028.

Cosson C, Groison A-L, Suquet M, Fauvel C, Dreanno C, Billard R (2008) Studying sperm motility in marine fish: an overview on the state of the art. *Journal of Applied Ichthyology* 24: 460-486.

Cote J, Fogarty S, Weinersmith K, Brodin T, Sih A (2010) Personality traits and dispersal tendency in the invasive mosquitofish (*Gambusia affinis*). *Proceedings of the Royal Society B: Biological Sciences* 277: 1571-1579.

Cote J, Fogarty S, Brodin T, Weinersmith K, Sih A (2011) Personality-dependent dispersal in the invasive mosquitofish: group composition matters. *Proceedings of the Royal Society B: Biological Sciences* 278: 1670-1678.

Damsgird B, Dill LM (1998) Risk-taking behavior in weight-compensating coho salmon, *Oncorhynchus kisutch*. *Behavioral Ecology* 9: 26-32.

David M, Auclair Y, Cézilly F (2012) Assessing short- and long-term repeatability and stability of personality in captive zebra finches using longitudinal data. *Ethology* 118: 932-942.

Davis RE, Kassel J, Schwagmeyer P (1976) Telencephalic lesions and behavior in the teleost, *macropodus opercularis*: Reproduction, startle reaction, and operant behavior in the male. *Behavioral Biology* 18: 165-177.

Desjardins JK, Fernald RD (2010) What do fish make of mirror images? *Biology Letters* 6: 744-747.

DiBattista JD, Anisman H, Whitehead M, Gilmour KM (2005) The effects of cortisol administration on social status and brain monoaminergic activity in rainbow trout *Oncorhynchus mykiss*. *Journal of Experimental Biology* 208: 2707-2718.

Dingemanse NJ, Van der Plas F, Wright J, Réale D, Schrama M, Roff DA, Van der Zee E, Barber I (2009) Individual experience and evolutionary history of predation affect expression of heritable variation in fish personality and morphology. *Proceedings of the Royal Society B: Biological Sciences* 276: 1285-1293.

Ebbesson LO, Nilsen TO, Helvik JV, Tronci V, Stefansson SO (2011) Corticotropin-releasing factor neurogenesis during midlife development in salmon: genetic, environmental and thyroid hormone regulation. *Journal of Neuroendocrinology* 23: 733-741.

Ebbesson LO, Braithwaite VA (2012) Environmental effects on fish neural plasticity and cognition. *Journal of Fish Biology* 81 (7): 2151-2174.

Ejike C, Schreck B (1980) Stress and social hierarchy rank in coho salmon. *Transactions of the American Fisheries Society* 109: 423-426.

Ellis T, Yildiz H, López-Olmeda J, Spedicato M, Tort L, Øverli Ø, Martins CI (2012) Cortisol and finfish welfare. *Fish Physiology and Biochemistry* 38: 163-188.

FAO (2005) Cultured aquatic species information programme: *Sparus aurata*, Fisheries and Aquaculture Department [online], Rome. http://www.fao.org/fishery/culturedspecies/Sparus_aurata/en.

FAO (2012) The State of World Fisheries and Aquaculture 2012 (Rome), p. 209.

Favati A, Leimar O, Radesäter T, Løvlie H (2014) Social status and personality: stability in social state can promote consistency of behavioural responses. *Proceedings of the Royal Society of London B: Biological Sciences* 281: 20132531.

Ferrari S, Millot S, Leguay D, Chatain B, Bégout M-L (2015) Consistency in European seabass coping styles: A life history approach. *Applied Animal Behaviour Science* 167: 74-88.

Fevolden SE, Refstie T, Røed KH (1992) Disease resistance in rainbow trout (*Oncorhynchus mykiss*) selected for stress response. *Aquaculture* 104: 19-29.

Fevolden SE, Nordmo R, Refstie T, Røed KH (1993) Disease resistance in Atlantic salmon (Salmo salar) selected for high or low responses to stress. Aquaculture 109: 215-224.

Finstad AG, Forseth T, Ugendal O, Naese TF (2007) Metabolic rate, behaviour and winter performance in juvenile Atlantic salmon. *Functional Ecology* 21: 905–912.

Flos R, Reig L, Torres P, Tort L (1988) Primary and secondary stress responses to grading and hauling in rainbow trout, *Salmo gairdneri*. *Aquaculture* 71: 99-106.

Fox HE, White SA, Kao MH, Fernald RD (1997) Stress and dominance in a social fish. *Journal of Neuroscience* 17: 6463-6469.

Francis RC (1990) Temperament in a Fish: A Longitudinal Study of the Development of Individual Differences in Aggression and Social Rank in the Midas Cichlid. *Ethology* 86: 311-325

Frost AJ, Winrow-Giffen A, Ashley PJ, Sneddon LU (2007) Plasticity in animal personality traits: does prior experience alter the degree of boldness? *Proceedings of the Royal Society B: Biological Sciences* 274: 333-339.

Galhardo L, Correia J, Oliveira RF (2008) The effect of substrate availability on behavioural and physiological indicators of welfare in the African cichlid (*Oreochromis mossambicus*). *Animal Welfare* 17, 239-254.

Galhardo L, Oliveira RF (2009) Psychological stress and welfare in fish. *Annual Review of Biomedical Sciences* 11: 1-20.

Galhardo L, Vitorino A, Oliveira RF (2012) Social familiarity modulates personality trait in a cichlid fish. *Biology Letters* 8: 936–938.

Garcia de la Serrana D, Estévez A, Andree K & Johnston IA (2012) Fast skeletal muscle transcriptome of the gilthead sea bream (*Sparus aurata*) determined by next generation sequencing. *BMC Genomics* 13: 181.

Gjedrem T (2005) *Selection and Breeding Programs in Aquaculture*, 364 p.

Gómez-Laplaza LM, Morgan E (2000) Laboratory studies of the effects of short-term isolation on aggressive behaviour in fish. *Marine and Freshwater Behaviour and Physiology* 33: 63-102.

References

Goodson JL, Kingsbury MA (2013) What's in a name? Considerations of homologies and nomenclature for vertebrate social behaviour networks. *Hormones and Behaviour* 64: 103-112.

Gosling SD (2001) From mice to men: what can we learn about personality from animal research? *Psychological Bulletin* 127: 45-86.

Gray JD, Milner TA, McEwen BS (2013) Dynamic plasticity: the role of glucocorticoids, brain-derived neurotrophic factor and other trophic factors. *Neuroscience* 239: 214-227.

Greenberg ME, Xu B, Lu B, Hempstead BL (2009) New insights in the biology of BDNF synthesis and release: implications in CNS function. *Journal of Neurosciences* 29: 12764-12767.

Herrera M, Castanheira MF, Martins CIM, Conceição LEC (2014). Link between risk taking and the behavioral and physiological responses to confinement stress in seabream Sparus aurata. *Applied Animal Behaviour Science* 155: 101-108.

Hessing MJC, Hagelsø AM, van Beek JAM, Wiepkema RP, Schouten WGP, Krukow R (1993) Individual behavioural characteristics in pigs. Applied Animal Behaviour Science 37: 285-295.

Hessing MJC, Hagelsø AM, Schouten WGP, Wiepkema PR, Van Beek JAM (1994) Individual behavioral and physiological strategies in pigs. *Physiology & Behavior* 55: 39-46.

Hoffman GE, Smith MS, Verbalis JG (1993) c-Fos and Related Immediate Early Gene Products as Markers of Activity in Neuroendocrine Systems. *Frontiers in Neuroendocrinology* 14: 173-213.

Höglund E, Gjøen HM, Pottinger TG, Øverli Ø (2008) Parental stress-coping styles affect the behaviour of rainbow trout *Oncorhynchus mykiss* at early developmental stages. *Journal of Fish Biology* 73: 1764-1769.

Hopster H (1998) Coping strategies in dairy cows. *In:* Institute of Animal Sciences.Wageningen University, The Netherlands, pp. 1-152.

Hsu Y, Wolf LL (1999) The winner and loser effect: integrating multiple experiences. *Animal Behaviour* 57: 903-910.

Huntingford FA (1976) The relationship between anti-predator behaviour and aggression among conspecifics in the three-spined stickleback, *Gasterosteus Aculeatus*. *Animal Behaviour* 24: 245-260.

Huntingford FA (2004) Implications of domestication and rearing conditions for the behaviour of cultivated fishes. *Journal of Fish Biology* 65: 122-142.

Huntingford FA, Adams C (2005) Behavioural syndromes in farmed fish: implications for production and welfare. *Behaviour* 142: 1213-1227.

Huntingford FA, Adams C, Braithwaite VA, Kadri S, Pottinger TG, Sandøe P, Turnbull JF (2006) Current issues in fish welfare. *Journal of Fish Biology* 68: 332-372.

Huntingford FA, Andrew G, Mackenzie S, Morera D, Coyle SM, Pilarczyk M and Kadri S (2010) Coping strategies in a strongly schooling fish, the common carp *Cyprinus carpio*. *Journal of Fish Biology* 76: 1576–1591.

Ibarra-Zatarain Z, Fatsini E, Rey Planellas S, Cherenguini O, Rasines I, Neil D (2013) Preliminary results on the influence of stress coping styles on spawning success in Senegalese sole (*Solea senegalensis*) broodstock held in captivity, Conference paper, September 2013, 4th Workshop on the Biology of Fish Gametes, Faro, Algarve, Portugal.

Irwin S, Kenny AP, O'Halloran J, FitzGerald RD, Duggan, PF (1999) Adaptation and validation of a radioimmunoassay kit for measuring plasma cortisol in turbot. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 124: 27-31.

Johansen IB, Lunde IG, Røsjø H, Christensen G, Nilsson GE, Bakken M, Øverli Ø (2011) Cortisol response to stress is associated with myocardial remodeling in salmonid fishes. *The Journal of Experimental Biology* 214: 1313-1321.

Johansen IB, Sørensen C, Sandvik GK, Nilsson GE, Höglund E, Bakken M, Øverli Ø (2012) Neural plasticity is affected by stress and heritable variation in stress coping style. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* 7: 161-171.

Johnson SC, Treasurer JW, Bravo S, Nagasawa K, Kabata Z (2004) A review of the impact of parasitic copepods on marine aquaculture. *Zoological Studies* 43 (2): 229–243.

Jong IC, Ruis MAW, Blokhuis HJ (1999) Coping styles in animals: current in behavior and stress-physiology. *Neuroscience & Biobehavioral Reviews* 23: 925-935.

Laiz-Carrión R, Sangiao-Alvarellos S, Guzmán JM, Martín del Río MP, Soengas JL, Mancera JM (2005) Growth performance of gilthead sea bream *Sparus Aurata* in different osmotic conditions: Implications for osmoregulation and energy metabolism. *Aquaculture* 250: 849–861.

Kalendar R, Lee D, Schulman AH (2014) FastPCR software for PCR, in silico PCR, and oligonucleotide assembly and analysis. *Methods in Molecular Biology* 1116: 271-302.

Kiessling A, Espe M, Ruohonen K, Mørkøre T (2004) Texture, gaping and colour of fresh and frozen Atlantic salmon flesh as affected by pre-slaugher iso-eugenol or CO_2 anaesthesia. *Aquaculture* 236: 645-657.

Killen SS, Marras S, McKenzie DJ (2011) Fuel, fasting, fear: routine metabolic rate and feed deprivation exert synergistic effects on risk-taking in individual juvenile European sea bass. *Journal of Animal Ecology* 80(5): 1024-33.

Kittilsen S, Ellis T, Schjolden J, Braastad BO, Øverli Ø (2009a) Determining stress-responsiveness in family groups of Atlantic salmon (*Salmo salar*) using non-invasive measures. *Aquaculture* 298: 146-152.

Kittilsen S, Schjolden J, Beitnes-Johansen I, Shaw JC, Pottinger TG, Sørensen C, Braastad BO, Bakken M, Øverli Ø (2009b) Melanin-based skin spots reflect stress responsiveness in salmonid fish. *Hormones and Behavior* 56: 292-298.

Kittilsen S, Johansen IB, Braastad BO, Øverli Ø (2012) Pigments, parasites and personalitiy: Towards a unifying role for steroid hormones? *PLoS ONE* 7 (4): e34281.

Kling GW, Hayhoe K, Johnson LB, Magnuson JJ, Polasky S, Robinson SK, Shuter BJ, Wander MW, Wuebbles DJ, Zak DR, Lindroth RL, Moser SC, Wilson ML (2003) Confronting Climate Change in the Great Lakes Region. Impacts on our Communities and Ecosystems. Union of concerned scientists, Cambridge, Massachusetts, and Ecological Society of America, Washington, D.C. (http://www.ucsusa.org/greatlakes).

Kondo Y, Shinoda A, Yamanouchi K, Arai Y (1990) Role of septum and preoptic area in regulating masculine and feminine sexual behavior in male rats. *Hormones and Behaviour* 24: 421-434.

Koolhaas JM, Korte SM, De Boer SF, Van Der Vegt BJ, Van Reenen CG, Hopster H, De Jong IC, Ruis MAW, Blokhuis HJ (1999) Coping styles in animals: current in behavior and stress-physiology. Neuroscience and Biobehavioral Reviews 23: 925-935.

Koolhaas JM (2008) Coping style and immunity in animals: Making sense of individual variation. *Brain Behavior and Immunity* 22: 662-667.

Koolhaas JM, de Boer SF, Coppens CM, Buwalda B (2010) Neuroendocrinology of coping styles: Towards understanding the biology of individual variation. *Frontiers in Neuroendocrinology* 31: 307-321.

Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23: 1289-1291.

Korte SM, Ruesink W, Blokhuis HJ (1999) Heart rate variability during manual restraint in chicks from high- and low-feather pecking lines of laying hens. *Physiology & Behavior* 65 (4-5): 649-652.

Korte SM, Koolhaas JM, Wingfield JC, McEwen BS (2005) The Darwinian concept of stress: Benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. *Neuroscience and Biobehavioral Reviews* 29: 3-38.

Kristiansen TS, Fernö A (2007) Individual behaviour and growth af halibut (*Hippoglossus hippoglossus* L.) fed sinking and floating: Evidence of different coping styles. *Applied Animal Behaviour Science* 104: 236-250.

Kurvers R, Prins HHT, van Wieren SE, van Oers K, Nolet BA, Ydenberg RC (2010) The effect of personality on social foraging: shy barnacle geese scrounge more. *Proceedings of the Royal Society B-Biological Sciences* 277: 601-608.

Kyle AL, Peter RE (1982) Effects of forebrain lesions on spawning behaviour in the male goldfish. *Physiology and Behaviour* 28: 1103–1109.

Lau BY, Mathur P, Gould GG, Guo S (2011) Identification of a brain center whose activity discriminates a choice behavior in zebrafish. *Proceedings of the National Academy of Sciences of the USA* 108: 2581-2586.

Laursen DC, Olsén HL, Ruiz-Gomez MdL, Winberg S, Höglund E (2011) Behavioural responses to hypoxia provide a non-invasive method for distinguishing between stress coping styles in fish. *Applied Animal Behaviour Science* 132: 211-216.

LeBlanc S, Höglund E, Gilmour KM, Currie S (2012) Hormonal modulation of the heat shock response: insights from fish with divergent cortisol stress responses. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 302: R184-R192.

Livak K, Schmittgen T (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-DeltaDeltaCT method. *Methods* 25: 402 - 408.

López-Olmeda JF, Montoya A, Oliveira C, Sánchez-Vázquez FJ (2009) Synchronization to light and restricted-feeding schedules of behavioral and humoral daily rhythms in gilthead sea bream (*Sparus aurata*). *Chronobiology International* 26: 1389-1408.

Luo AH, Tahsili-Fahadan P, Wise, R.A., Lupica, C.R., Aston-Jones, G. 2011. Linking context with reward: A functional circuit from hippocampal CA3 to ventral tegmental area. *Science* 333: 353-357.

MacKenzie S, Ribas L, Pilarczyk M, Capdevila DM, Kadri S, Huntingford FA (2009) Screening for Coping Style Increases the Power of Gene Expression Studies. *PLoS ONE* 4: e5314.

Maeda H, Mogenson GJ (1981) Electrophysiological responses of neurons of the ventral tegmental area to electrical stimulation of amygdala and lateral septum. *Neuroscience* 6: 367-376.

Magnani D, Cafazzo S, Calà P, Costa LN (2012) Searching for differences in the behavioural response of piglet groups subjected to novel situations. *Behavioural Processes* 89: 68-73.

Magnhagen C, Staffan F (2005) Is boldness affected by group composition in young-of-the-year perch (*Perca fluviatilis*)? *Behavioral Ecology and Sociobiology* 57: 295-303.

Magnhagen C (2007) Social influence on the correlation between behaviours in young-of-the-year perch. *Behavioral Ecology and Sociobiology* 61: 525-531.

References

Magnhagen C, Bunnefeld N (2009) Express your personality or go along with the group: what determines the behaviour of shoaling perch? *Proceedings of the Royal Society B: Biological Sciences* 276: 3369-3375.

Magnhagen C, Backström T, Øverli Ø, Winberg S, Nilsson J, Vindas MA, Brännäs E (2015) Behavioural responses in a net restraint test predict interrenal reactivity in Arctic charr *Salvelinus alpinus*. *Journal of Fish Biology* 87: 88-99.

Mainwaring MC, Beal JL, Hartley IR (2011) Zebra finches are bolder in an asocial, rather than social, context. *Behavioural Processes* 87: 171-175.

Maren S (2001) Neurobiology of Pavlovian fear conditioning. Annual Review of Neuroscience 24: 897-931.

Martins CIM (2005) Individual variation in growth of African catfish Clarias gariepinus: a search for explanatory factors (Phd thesis). Wageningen University, The Netherlands.

Martins CIM, Aanyu M, Schrama JW, Verreth JAJ (2005a) Size distribution in African catfish (*Clarias gariepinus*) affects feeding behaviour but not growth. *Aquaculture* 250: 300-307.

Martins CIM, Schrama JW, Verreth JAJ (2005b) Inherent variation in growth efficiency of Africain catfish *Clarias gariepinus* (Burchell, 1822) juveniles. *Aquaculture Research* 36: 868-875.

Martins CIM, Schrama JW, Verreth JAJ (2005c) The consistency of individual differences in growth, feed efficiency and feeding behaviour in African catfish *Clarias gariepinus* (Burchell 1822) housed individually. *Aquaculture Research* 36: 1509-1516.

Martins CIM, Schrama JW, Verreth JAJ (2006a) The relationship between individual differences in feed efficiency and stress response in African catfish *Clarias gariepinus*. *Aquaculture* 256: 588-595.

Martins CIM, Trenovski M, Schrama JW, Verreth JAJ (2006b) Comparison of feed intake behaviour and stress response in isolated and non-isolated African catfish. *Journal of Fish Biology* 69: 629-636.

Martins CIM, Schrama JW, Verreth JAJ (2006c) The effect of group composition on the welfare of African catfish (*Clarias gariepinus*). *Applied Animal Behaviour Science* 97: 323-334.

Martins CIM, Hillen B, Schrama JW, Verreth JAJ (2008) A brief note on the relationship between residual feed intake and aggression behaviour in juveniles of African catfish *Clarias gariepinus*. *Applied Animal Behaviour Science* 111: 408-413.

Martins CIM, Eding EH, Verdegem MCJ, Heinsbroek LTN, Schneider O, Blancheton JP, d'Orbcastel ER, Verreth JAJ (2010) New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. *Aquacultural Engineering* 43: 83-93.

Martins CIM, Conceição LEC, Schrama JW (2011a) Consistency of individual variation in feeding behaviour and its relationship with performance traits in Nile tilapia *Oreochromis niloticus*. *Applied Animal Behaviour Science* 133: 109-116.

Martins CIM, Conceição LEC, Schrama JW (2011b) Feeding behavior and stress response explain individual differences in feed efficiency in juveniles of Nile tilapia *Oreochromis niloticus*. *Aquaculture* 312: 192-197.

Martins CIM, Silva PIM, Conceição LEC, Costas B, Höglund E, Øverli Ø, Schrama JW (2011c) Linking Fearfulness and Coping Styles in Fish. *PLoS ONE* 6: e28084.

Martins CIM, Castanheira MF, Engrola S, Costas B, Conceição LEC (2011d) Individual differences in metabolism predict coping styles in fish. *Applied Animal Behaviour Science* 130: 135-143.

References

Martins CI, Galhardo L, Damsgård B, Spedicato MT, Zupa W, Beauchaud M, Kulczykowska E, Massabuau JC, Carter T, Planellas SR, Kristiansen T (2011e) Behavioural indicators of welfare in farmed fish. *Fish Physiology and Biochemistry* 38: 17-41.

Martins CIM, Schaedelin FC, Mann M, Blum C, Mandl I, Urban D, Grill J, Schößwender J, Wagner RH (2012) Exploring novelty: a component trait of behavioural syndromes in a colonial fish. *Behaviour* 149: 215-231.

Mas-Muñoz J, Komen H, Schneider O, Visch SW, Schrama JW (2011) Feeding Behaviour, Swimming Activity and Boldness Explain Variation in Feed Intake and Growth of Sole (*Solea solea*) Reared in Captivity. *PLoS ONE* 6: e21393.

Matos E, Gonçalves A, Nunes ML, Dinis MT, Dias J (2010) Effect of harvesting stress and slaughter conditions on selected flesh quality criteria of gilthead seabream (*Sparus aurata*). *Aquaculture* 305: 66-72.

Matos E, Silva TS, Tiago T, Aureliano M, Dinis MT, Dias J (2011) Effect of harvesting stress and storage conditions on protein degradation in fillets of farmed gilthead seabream (*Sparus aurata*): A differential scanning calorimetry study. *Feed Chemistry* 126: 270-276.

Maule AG, Schreck CB, Bradford CS, Barton BA (1988) Physiological effects of collecting and transporting emigrating juvenile Chinook Salmon past dams on the Columbia river. *Transactions of the American Fisheries Society* 117: 245-261.

McCarthy ID (2001) Competitive ability is related to metabolic asymmetry in juvenile rainbow trout. *Journal of Fish Biology* 59: 1002–1014.

Metcalfe NB (1998) The interaction between behavior and physiology in determining life history patterns in Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences* 55: 93-104.

Millot S, Bégout ML, Chatain B (2009a) Exploration behaviour and flight response toward a stimulus in three sea bass strains (*Dicentrarchus labrax* L.). *Applied Animal Behaviour Science* 119: 108-114.

Millot S, Bégout ML, Chatain B (2009b) Risk-taking behaviour variation over time in sea bass *Dicentrarchus labrax*: effects od day-night alterations, fish phenotipic characteristics and selection for growth. *Journal of Fish Biology* 75: 1733-1749.

Millot S, Cerqueira M, Castanheira MF, Øverli Ø, Martins CIM, Oliveira RF (2014a) Use of conditioned place preference/avoidance tests to assess affective states in fish. *Applied Animal Behaviour Science* 154: 104-111.

Millot S, Péan S, Labbé L, Kerneis T, Quillet E, Dupont-Nivet M, Bégout ML (2014b) Assessment of Genetic Variability of Fish Personality Traits using Rainbow Trout Isogenic Lines. *Behavior Genetics* 44(4):383-393.

Millot S, Cerqueira M, Castanheira MF, Øverli Ø, Martins CIM, Oliveira RF (2014c) How coping style influences environmental perception in European sea bass (*Dicentrarchus labrax*)? *PloS ONE* 9(9): e108800.

Montero D, Izquierdo MS, Tort L, Robaina L,Vergara JM (1999) High Stocking Density Produces Crowding Stress Altering Some Physiological and Biochemical Parameters in Gilthead Seabream, *Sparus Aurata*, Juveniles. *Fish Physiology and Biochemistry* 20 (1):53–60.

Montero D, Lalumera G, Izquierdo MS, Caballero MJ, Saroglia M, Tort L (2009) Establishment of dominance relationships in gilthead sea bream *Sparus aurata* juveniles during feeding: effects on feeding behaviour, feed utilization and fish health. *Journal of Fish Biology* 74: 790-805.

Morales JC (1983) Acuicultura marina animal, ed. Mundi-Prensa, Madri. 1.423p.

References

Moreira PSA, Pulman KGT, Pottinger TG (2004) Extinction of a conditioned response in rainbow trout selected for high or low responsiveness to stress. *Hormones and Behavior* 46: 450-457.

Moretti A, Criado MPF, Cittolin G, Guidastri R (1999) Manual on hatchery production of seabass and gilthead seabream. Food and Agriculture Organization of the United Nations, Rome, Italy 194 pp.

Mota VC, Martins CIM, Eding E, Canário AV, Verreth JA (2014) Steroids accumulate in the rearing water of commercial recirculating aquaculture systems. *Aquacultural Engineering* 62: 9-16.

Neat FC, Huntingford FA, Beveridge MMC (1998) Fighting and assessment in male cichlid fish: The effects of asymmetries in gonadal state and body size. *Animal Behaviour* 55: 883-891.

Noble C, Mizusawa K, Suzuki K, Tabata M (2007) The effects of differing self-feeding regimes on the grouwth, behaviour and fin damage of rainbow trout held in groups. *Aquaculture* 264: 214-222.

O'Connell LA, Hofmann HA (2011) The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *Journal of Comparative Neurology* 519: 3599-3639.

Öhman A, Flykt A, Lundqvist D (2000) Unconscious emotion: Evolutionary perspectives, psychophysiological data and neuropsychological mechanisms. In R. D. Lane & L. Nadel (Eds.), Cognitive neuroscience of emotion (pp. 296–327). New York: Oxford University Press.

Oliveira RF (2009) Social behavior in context: Hormonal modulation of behavioral plasticity and social competence. *Integrative and Comparative Biology* 49: 423–440.

Oliveira RF, Silva JF, Simões JM (2011) Fighting zebrafish: characterization of aggressive behavior and winner-loser effects. *Zebrafish* 8: 73-81.

Oliveira RF (2012) Social plasticity in fish: Integrating mechanisms and function. Journal of Fish Biology 81: 2127-2150.

Ortuño J, Esteban MA, Meseguer J (2001) Effects of Short-Term Crowding Stress on the Gilthead Seabream (*Sparus Aurata* L.) Innate Immune Response. *Fish & Shellfish Immunology* 11: 187–197.

Øverli Ø, Harris CA, Winberg S (1999) Short-term effects of fights for social dominance and the establishment of dominant subordinate relationship on brain monoamines and cortisol in rainbow trout. *Brain Behavior and Evolution* 54: 263–275.

Øverli Ø, Kotzian S, Winberg S (2002a) Effects of Cortisol on Aggression and Locomotor Activity in Rainbow Trout. *Hormones and Behavior* 42: 53-61.

Øverli Ø, Pottinger TG, Carrick TR, Øverli E, Winberg S (2002b) Differences in behaviour between rainbow trout selected for high- and low-stress responsiveness. *Journal of Experimental Biology* 205: 391-395.

Øverli Ø, Korzan WJ, Höglund E, Winberg S, Bollig H, Watt M, Forster GL, Barton BA, Øverli E, Renner KJ, Summersa CH (2004a) Stress coping style predicts aggression and social dominance in rainbow trout. *Hormones and Behavior* 45: 235- 241.

Øverli Ø, Korzan WJ, Larson ET, Winberg S, Lepage O, Pottinger TG, Renner KJ, Summersa CH (2004b) Behavioral and neuroendocrine correlates of displaced aggression in trout. *Hormones and Behavior* 45: 324- 329.

Øverli Ø, Winberg S, Pottinger TG (2005) Behavioral and neuroendocrine correlates of selection for stress responsiveness in rainbow trout - A review. *Integrative and Comparative Biology* 45: 463-474.

Øverli Ø, Sørensen C, Kiessling A, Pottinger TG, Gjøen HM (2006a) Selection for improved stress tolerance in rainbow trout (*Oncorhynchus mykiss*) leads to reduced feed waste. *Aquaculture* 261(2): 776-781.

Øverli Ø, Sørensen C, Nilsson GE (2006b) Behavioral indicators of stress-coping style in rainbow trout: Do males and females react differently to novelty? *Physiology & Behavior* 87: 506-512.

Øverli Ø, Sørensen C, Pulman KGT, Pottinger TG, Korzan W, Summers CH, Nilsson GE (2007) Evolutionary background for stress-coping styles: Relationships between physiological, behavioral, and cognitive traits in non-mammalian vertebrates. *Neuroscience and Biobehavioral Reviews* 31: 396-412.

Øverli Ø, Nordgreen J, Mejdell CM, Janczak AM, Kittilsen S, Johansen IB, Horsberg TE (2014) Ectoparasitic sea lice (*Lepeophtheirus salmonis*) affect behaviour and brain serotonergic activity in Atlantic salmon (*Salmo salar* L.): Perspectives on animal welfare. Physiology & Behavior 132: 44-50.

Papoutsoglou SE (2012) The role of the brain in farmed fish. *Reviews in Aquaculture* 4: 1-10.

Pike TW, Samanta M, Lindström J, Royle NJ (2008) Behavioural phenotype affects social interactions in an animal network. *Proceedings of the Royal Society of London B: Biological Sciences* 275: 2515-2520.

Pottinger TG, Balm PH, Pickering AD (1995) Sexual maturity modifies the responsiveness of the pituitary-interrenal axis to stress in male rainbow trout. *General and Comparative Endocrinology* 98: 311-320.

Pottinger TG, De La Higuera Gonzales M, Fevolden SE, Roed KH, Izquierdo Lopez, MS (1998) Selective breeding for stress tolerance in aquacultured fish, In: European Commission DG 12 Science, R.a.D. (Ed.), Luwembourg, pp. 25-29.

Pottinger TG, Carrick TR (1999) Modification of the Plasma Cortisol Response to Stress in Rainbow Trout by Selective Breeding. *General and Comparative Endocrinology* 116: 122-132.

Pottinger TG (2001) Effects of husbandry stress on flesh quality indicators in fish. In: Kestin SC, Warriss PD (Eds.), Farmed fish quality. Blackwell Oxford, 145-160.

Pottinger TG, Carrick TR (2001) Stress Responsiveness Affects Dominant–Subordinate Relationships in Rainbow Trout. *Hormones and Behavior* 40: 419-427.

Pottinger TG (2006) Context dependent differences in growth of two rainbow trout (*Oncorhynchus mykiss*) lines selected for divergent stress responsiveness. *Aquaculture* 256: 140-147.

Price J, Sloman L, Gardner R, Gilbert P, Rohde P (1994) The social competition hypothesis of depression. *The British Journal of Psychiatry* 164: 309-315.

Raleigh RF, Hickman T, Solomon RC, Nelson PC (1984) Habitat suitability information: rainbow trout U.S. Fish Wildl. Serv. FWS/OBS-82/10.60. 64 p.

Réale D, Reader SM, Sol D, McDougall PT, Dingemanse NJ (2007) Integrating animal temperament within ecology and evolution. *Biological reviews of the Cambridge Philosophical Society* 82 (2): 291-318.

Réale D, Dingemanse NJ, Kazem AJN, Wright J (2010a) Evolutionary and ecological approaches to the study of personality. *Philosophical Transactions of the Royal Society B: Biological Sciences* 365: 3937-3946.

Réale D, Garant D, Humphries MM, Bergeron P, Careau V, Montiglio PO (2010b) Personality and the emergence of the pace-of-life syndrome concept at the population level. *Philosophical Transactions of the Royal Society B-Biological Sciences* 365: 4051-4063.

Reebs SG (2000) Can a minority of informed leaders determine the foraging movements of a fish shoal? *Animal Behaviour* 59: 403-409.

Reefmann N, Bütikofer Kaszàs F, Wechsler B, Gygax L (2009) Ear and tail postures as indicators of emotional valence in sheep. *Applied Animal Behaviour Science* 118: 199-207.

Reig AC (2001) Influencia de la temperatura y la salinidad sobre el crescimiento y consumo de oxígeno de la Dorada (*Sparua aurata* L.). Barcelona: Universitat de Barcelona 206 p.

Reimert I, Bolhuis JE, Kemp B, Rodenburg TB (2013) Indicators of positive and negative emotions and emotional contagion in pigs. *Physiology & Behavior* 109: 42-50.

Rexroad C, Vallejo R, Liu S, Palti Y, Weber G (2012) QTL affecting stress response to crowding in a rainbow trout broodstock population. *BioMed Central Genetics* 13: 97.

Rey S, Boltana S, Vargas R, Roher N, MacKenzie S (2013) Combining animal personalities with transcriptomics resolves individual variation within a wild-type zebrafish population and identifies underpinning molecular differences in brain function. *Molecular Ecology* 22 (24): 6100-6115.

Rey S, Huntingford FA, Boltaña S, Vargas R, Knowles TG, Mackenzie S (2015a) Fish can show emotional fever: stress-induced hyperthermia in zebrafish. *Proceedings of the Royal Society B: Biological Sciences* 282: 20152266.

Rey S, MacKenzie S (2015b) Linking stress coping styles with the brain gene expression across three different fish species: Atlantic salmon, European seabass and Gilthead seabream (*Salmo salar, Dicentrarchus labrax and Sparus aurata*). Book of abstracts of the Association for the Study of Animal Behaviour (ASAB) 2015, 18th-20th March, Durham, UK

Reyes-Tomassini JJ (2009) Behavioral and neuroendocrine correlates of sex change in the Gilthead Seabream *Sparus aurata* (PhD Thesis) Biotechnology Institute, Faculty of the Graduate School of the University of Maryland, College Park,USA, p. 229.

Ribas L, Flos R, Reig L, MacKenzie S, Barton BA, Tort L (2007) Comparison of methods for anaesthetizing Senegal sole (*Solea senegalensis*) before slaughter: Stress responses and final product quality. *Aquaculture* 269: 250-258.

Robb DHF, Kestin SC, Warriss PD (2000) Muscle activity at slaughter: I. Changes in flesh colour and gaping in rainbow trout. *Aquaculture* 182: 261-269.

Rodríguez F, Broglio C, Durán E, Gómez A, Salas C (2007) Neural mechanisms of learning in Teleost fish in *Fish Cognition and Behavior* (eds C. Brown, K. Laland and J. Krause), Blackwell Publishing Ltd, Oxford, UK.

Rooney DJ, Laming PR (1986) Localization of telencephalic regions concerned with habituation of cardiac and ventilatory responses associated with arousal in the goldfish (*Carassius auratus*). *Behavioral Neuroscience* 100: 45-50.

Rotllant J, Tort L (1997) Cortisol and glucose responses after acute stress by net handling in the sparid red porgy previously subjected to crowding stress. *Journal of Fish Biology* 51: 21-28.

Rotllant J, Balm PHM, Ruane NM, Pérez-Sanchez J, Wendelaar Bonga SE, Tort L (2000) Pituitary proopiomelanocortin-derived peptides and hypothalamus-pituitary-interrenal axis activity in gilthead sea bream (*Sparus aurata*) during prolonged crowding stress: differential regulation of adrenocorticotropin hormone and α-melanocyte-stimulating hormone release by corticotropin-releasing hormone and thyrotropin-releasing hormone. *General and Comparative Endocrinology* 119: 152-163.

Rotllant J, Balm PHM, Pérez-Sánchez J, Wendelaar-Bonga SE, Tort L (2001) Pituitary and interrenal function in gilthead sea bream (Sparus aurata L., Teleostei) after handling and confinement stress. *General and Comparative Endocrinology* 121: 333-342.

Ruis MAW, te Brake JHA, Engel B, Buist WG, Blokhuis HJ, Koolhaas JM (2001) Adaptation to social isolation: Acute and long-term stress responses of growing gilts with different coping characteristics. *Physiology & Behavior* 73: 541-551.

Ruiz-Gomez ML, Kittilsen S, Höglund E, Huntingford FA, Sørensen C, Pottinger TG, Bakken M, Winberg S, Korzan WJ, Øverli Ø (2008) Behavioral plasticity in rainbow trout (*Oncorhynchus mykiss*) with divergent coping styles: When doves become hawks. *Hormones and Behavior* 54: 534-538.

Ruiz-Gomez ML, Huntingford FA, Øverli Ø, Thörnqvist P-O, Höglund E (2011) Response to environmental change in rainbow trout selected for divergent stress coping styles. Physiology & Behavior 102: 317-322.

Sánchez JA, López-Olmeda JF, Blanco-Vives B, Sánchez-Vázquez FJ (2009) Effects of feeding schedule on locomotor activity rhythms and stress response in sea bream. *Physiology & Behavior* 98: 125-129.

Sanchez-Lamadrid A. (2004) Effectiveness of releasing gilthead sea bream (*Sparus aurata*, L.) for stock enhancement in the bay of Cadiz. *Aquaculture* 231: 135-148.

Sapolsky RM (1990) Adrenocortical function, social rank, and personality among wild baboons. *Biological Psychiatry* 28: 862-878.

Satou M, Oka Y, Kusunoki M, Matsushima T, Kato M, Fujita I, Ueda K (1984) Telencephalic and preoptic areas integrate sexual behavior in hime salmon (landlocked red salmon, *Oncorhynchus nerka*): results of electrical brain stimulation experiments. *Physiology & Behavior* 33: 441-447.

Schjolden J, Backström T, Pulman KGT, Pottinger TG, Winberg S (2005) Divergence in behavioural responses to stress in two strains of rainbow trout (*Oncorhynchus mykiss*) with contrasting stress responsiveness. *Hormones and Behavior* 48: 537-544.

Schjolden J, Winberg S (2007) Genetically determined variation in stress responsiveness in rainbow trout: behavior and neurobiology. *Brain, Behavior and Evolution* 70: 227-238.

Schjolden J, Basic D, Winberg S (2009) Aggression in rainbow trout is inhibited by both MR and GR antagonists. *Physiology & Behavior* 98: 625-630.

Schreck CB (2010) Stress and fish reproduction: The roles of allostasis and hormesis. *General and Comparative Endocrinology* 165: 549-556.

Schuett W, Godin JGJ, Dall SRX (2011) Do female zebra finches, *Taeniopygia guttata*, choose their mates based on their 'personality'? *Ethology* 117: 908-917.

Schuett W, Tregenza T, Dall SRX (2010) Sexual selection and animal personality. *Biological Reviews* 85: 217-246.

Seebacher F, Little AG, James RS (2015) Skeletal muscle contractile function predicts activity and behaviour in zebrafish. Journal of Experimental Biology 218: 3878-3884.

Sheng M, Greenberg ME (1990) The regulation and function of *c-fos* and other immediate early genes in the nervous system. *Neuron* 4: 477-485.

Sih A, Bell A, Johnson JC, Ziemba RE (2004) Behavioral syndromes: an ecological and evolutionary overview. *The Quarterly Review of Biology* 19: 372-378.

Silva PIM, Martins CIM, Engrola S, Marino G, Øverli Ø, Conceição LEC (2010) Individual differences in cortisol levels and behaviour of Senegalese sole (*Solea senegalensis*) juveniles: Evidence for coping styles. *Applied Animal Behaviour Science* 124: 75-81.

Silva PIM, Martins CIM, Khan UW, Gjøen HM, Øverli Ø, Höglund E (2015) Stress and fear responses in the teleost pallium. *Physiology & Behavior* 141: 17-22.

Singewald GM, Rjabokon A, Singewald N, Ebner K (2011) The modulatory role of the lateral septum on neuroendocrine and behavioural stress responses. *Neuropsychopharmacology* 36: 793-804.

Sloman KA, Metcalfe NB, Taylor AC, Gilmour KM (2001) Plasma cortisol concentrations before and after social stress in rainbow trout and brown trout. *Physiological and Biochemical Zoology* 74: 383-389.

Sørensen C, Johansen IB, Øverli Ø (2013) Neural plasticity and stress coping in teleost fishes. *General and Comparative Endocrinology* 181: 25-34.

Southgate P, Wall T (2001) Welfare of farmed fish at slaughter. *In Practice* 23: 277-284.

Spake JR, Gray KA, Cassady JP (2012) Relationship between backtest and coping styles in pigs. *Applied Animal Behaviour Science* 140: 146-153.

Spoolder HAM, Burbidge JA, Lawrence AB, Simmins PH, Edwards SA (1996) Individual behavioural differences in pigs: intra-and inter-test consistency. *Applied Animal Behaviour Science* 49: 185-198.

Stamps JA and Groothuis TGG (2010) The development of animal personality: relevance, concepts and perspectives. *Biological Reviews* 85: 301–325.

Steimer T, la Fleur S, Schulz PE (1997) Neuroendocrine correlates of emotional reactivity and coping in male rats from the Roman high (RHA/Verh)- and low (RLA/Verh)- avoidance lines. *Behavior Genetics* 27: 503–512.

Strand A, Magnhagen C, Alanara A (2007) Effects of repeated disturbances on feed intake, growth rates and energy expenditures of juvenile perch, *Perca fluviatilis*. *Aquaculture* 265: 163-168.

Suau P and López J (1976) Contribución al estudio de la dorada, *Sparus aurata* L. *Investigación Pesquera* 40: 169-199.

Suri D and Vaidya VA (2013) Glucocorticoid regulation of brain-derived neurotrophic factor: relevance to hippocampal structural and functional plasticity. *Neuroscience* 239: 196-213.

Swanson LW, Petrovich GD (1998) What is the amygdala? *Trends Neuroscience* 21: 323-331.

Swanson LW (2000) Cerebral hemisphere regulation of motivated behaviour. *Brain Research* 886: 113-164.

Taylor S, Wakem M, Dijkman G, Alsarraj M, & Nguyen M (2010) A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines. *Methods* 50(4): S1-S5.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673-4680.

Thorgaard GH, Bailey GS, Williams D, Buhler DR, Kaattari SL, Ristow SS, Hansen JD, Winton JR, Bartholomew JL, Nagler JJ, Walsh PJ, Vijayan MM, Devlin RH, Hardy RW, Overturf KE, Young WP, Robison, BD, Rexroad C, Palti Y (2002) Status and opportunities for genomics research with rainbow trout. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 133: 609-646.

Tintos A, Míguez JM, Mancera JM, Soengas JL (2006) Development of a microtitre plate indirect ELISA for measuring cortisol in teleosts, and evaluation of stress responses in rainbow trout and gilthead sea bream. *Journal of Fish Biology* 68: 251-263.

Toms CN, Echevarria DJ, Jouandot DJ (2010) A methodological review of personality-related studies in fish: Focus on the Shy-Bold axis of behavior. *International Journal of Comparative Psychology* 23: 1-25.

Tort L, Sunyer JO, Gómez E, Molinero A (1996) Crowding Stress Induces Changes in Serum Haemolytic and Agglutinating Activity in the Gilthead Sea Bream *Sparus Aurata*. *Veterinary Immunology and Immunopathology* 51: 179–188.

Tort L, Montero D, Robaina L, Fernández-Palacios H, Izquierdo MS (2001) Consistency of stress response to repeated handling in the gilthead sea bream *Sparus aurata* Linnaeus, 1758. *Aquaculture Research* 32: 593-598.

Trenzado CE, Carrick TR, Pottinger TG (2003) Divergence of endocrine and metabolic responses to stress in two rainbow trout lines selected for differing cortisol responsiveness to stress. *General and Comparative Endocrinology* 133: 332-340.

Trenzado CE, Morales AE, Higuera M (2006) Physiological effects of crowding in rainbow trout, *Oncorhynchus mykiss*, selected for low and high stress responsiveness. *Aquaculture* 258: 583-593.

Tudorache C, Schaaf MJM, Slabbekoorn H (2013) Endocrine stress response varies with coping style in zebrafish. *Journal of Endocrinology* 219: 251–258.

Tudorache C, ter Braake A, Tromp M, Slabbekoorn H, Schaaf MJM (2015) Behavioral and physiological indicators of stress coping styles in larval zebrafish. *Stress* 18: 121-128.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3--new capabilities and interfaces. *Nucleic Acids Research* 40 (15): e115.

van de Nieuwegiessen PG, Boerlage AS, Verreth JAJ, Schrama JW (2008) Assessing the effects of a chronic stressor, stocking density, on welfare indicators of juvenile African catfish, *Clarias gariepinus* Burchell. *Applied Animal Behaviour Science* 113: 270-275.

van de Nieuwegiessen PG, Schrama JW, Verreth JAJ (2008) A note on alarm cues in juvenile African catfish, Clarias gariepinus Burchell: Indications for opposing behavioural strategies. *Applied Animal Behaviour Science* 113: 270-275.

van de Nieuwegiessen PG, Ramli NM, Knegtel BP, Verreth JA, Schrama JW (2010) Coping strategies in farmed African catfish Clarias gariepinus. Does it affect their welfare? *Journal of Fish Biology,* 76, 2486-2501.

van Erp-van der Kooij E, Kuijpers AH, van Eerdenburg FJ, Dieleman SJ, Blankenstein DM, Tielen MJ (2003) Individual behavioural characteristics in pigs—influences of group composition but no differences in cortisol responses. *Physiology & Behavior* 78: 479-488.

van Reenen CG, Van der Werf JT, Bruckmaier RM, Hopster H, Engel B, Noordhuizen JP, Blokhuis HJ (2002) Individual Differences in Behavioral and Physiological Responsiveness of Primiparous Dairy Cows to Machine Milking. *Journal of Dairy Science* 85: 2551-2561.

van Reenen CG, O'Connell NE, Van der Werf JT, Korte SM, Hopster H, Jones RB, Blokhuis HJ (2005) Responses of calves to acute stress: Individual consistency and relations between behavioral and physiological measures. *Physiology & Behavior* 85: 557-570.

van Reenen CG (2012) Identifying temperament in dairy cows In: *Department of Livestock Research*. Wageningen University, The Netherlands, pp. 1-217.

VanElzakker M, Fevurly RD, Breindel T, Spencer RL (2008) Environmental novelty is associated with a selective increase in Fos expression in the output elements of the hippocampal formation and the perirhinal cortex. *Learning & Memory* 15(12): 899–908.

Vaz-Serrano J, Ruiz-Gomez ML, Gjøen HM, Skov PV, Huntingford FA, Øverli Ø, Höglund E (2011) Consistent boldness behaviour in early emerging fry of domesticated Atlantic salmon (*Salmo salar*): Decoupling of behavioural and physiological traits of the proactive stress coping style. *Physiology & Behavior* 103: 359-364.

Veenema AH, Meijer OC, De Kloet ER, Koolhaas JM (2003) Genetic Selection For Coping Style Predicts Stressor Susceptibility. *Journal of Neuroendocrinology* 15: 256-267.

Verbeek ME, Drent MPJ, Wiepkema PR (1994) Consistent individual differences in early exploratory behaviour of male great tits. *Animal Behaviour* 48: 1113-1121.

Verbeek P, Iwamoto T, Murakami N (2008) Variable stress-responsiveness in wild type and domesticated fighting fish. *Physiology & Behavior* 93: 83-88.

Vijayan MM, Reddy PK, Leatherland JF, Moon TW (1994) The Effects of Cortisol on Hepatocyte Metabolism in Rainbow Trout: A Study Using the Steroid Analogue RU486. *General and Comparative Endocrinology* 96: 75-84.

Vindas M, Folkedal O, Kristiansen T, Stien L, Braastad B, Mayer I, Øverli Ø (2012) Omission of expected reward agitates Atlantic salmon (*Salmo salar*). *Animal Cognition* 15: 903-911.

Vindas M, Sørensen C, Johansen IB, Folkedal O, Höglund E, Khan UW, Stien L, Kristiansen T, Braastad B, Øverli Ø (2014a) Coping with Unpredictability: Dopaminergic and Neurotrophic Responses to Omission of Expected Reward in Atlantic Salmon (*Salmo salar*). *PLoS ONE* 9: e85543.

Vindas MA, Johansen IB, Vela-Avitua S, Nørstrud KS, Aalgaard M, Braastad BO, Höglund E, Øverli Ø (2014b) Frustrative reward omission increases aggressive behaviour of inferior fighters. *Proceedings of the Royal Society of London B: Biological Sciences* 281: 20140300.

Webster MM, Ward AJW, Hart PJB (2007) Boldness is influenced by social context in threespine sticklebacks (*Gasterosteus aculeatus*). *Behaviour* 144: 351-371.

Webster MM, Ward AJ (2011) Personality and social context. *Biological Reviews* 86: 759-773.

Wood LS, Desjardins JK, Fernald RD (2011) Effects of stress and motivation on performing a spatial task. *Neurobiology of Learning and Memory* 95: 277-285.

Wysocki LE, Davidson III JW, Smith ME, Frankel AS, Ellison WT, Mazik PM, Popper AN, Bebak J (2007) Effects of aquaculture production noise on hearing, growth, and disease resistance of rainbow trout *Oncorhynchus mykiss*. *Aquaculture* 272: 687–697.

Zohar Y, Abraham M, Gordin H (1978) The gonadal cycle of the captivity reared hermaphroditic teleost Sparus aurata (L.) during the first two years of life. *Annales Biologie Animale Biochimie Biophysique* 18: 877-882.

Presentations and publications from the project

Presentations and publications from the project

Scientific publications in peer-reviewed journals

- Castanheira, M.F.,Martínez Páramo, Sonia; Figueiredo, F; Cerqueira, Marco, Millot, Sandie; Oliveira, Catarina, Martins, C.I.M.; Conceição, L.E.C. (Accepted) Are coping styles consistent in the teleost fish Sparus aurata through sexual maturation and sex reversal? Fish Biology and Biochemistry.

- Castanheira, M.F.,Cerqueira, Marco, Millot, Sandie, Gonçalves, Rui A., Oliveira, Catarina, Conceição, L.E.C., Martins, C.I.M. (2016) Are personality traits consistent in fish? - The influence of social context. Applied Animal Behaviour Science 178: 96-101.

- Castanheira, M.F., Conceição, L.E.C., Millot, Sandie, Rey, Sonia, Bégout, Marie-Laure, Damsgård, Børge, Kristiansen, Tore, Höglund, Erik, Øverli Øyvind, Martins, C.I.M. (2015) Reviews in aquaculture Coping styles in farmed fish: consequences for Aquaculture. Reviews in Aquaculture 7:1-19.

- S.Millot, M. Cerqueira, M.F. Castanheira, Ø. Øverli, C.I.M. Martins, R. F. Oliveira (2014). How coping style influences environmental perception in European sea bass (Dicentrarchus labrax)? PLoS ONE 9(9):e108800.

- Herrera, M., Castanheira, Maria F., Martins, C.I.M., Conceição, L.E.C. (2014). Link between risk taking and the behavioral and physiological responses to confinement stress in seabream Sparus aurata. Applied Animal Behaviour Science 155: 101-108.

- S. Millot, M. Cerqueira, M.F. Castanheira, Ø. Øverli, C.I.M. Martins, R. F. Oliveira (2014).Use of conditioned place preference/avoidance tests to assess affective states in fish. Applied Animal Behaviour Science 154:104-111.

- Castanheira, Maria F., Herrera, M., Costas, B., Conceição, L.E.C., Martins, C.I.M., (2013). Can we predict personality in fish? – searching for consistency over time and across contexts. PLoS ONE 8(4): e62037.

- Castanheira, Maria F., Herrera, M., Costas, B., Conceição, L.E.C., Martins, C.I.M. (2013). Linking cortisol responsiveness and aggressive behaviour in gilthead seabream Sparus aurata: indication of divergent coping styles. Applied Animal Behaviour Science 143: 75-81

Presentations and publications from the project

Oral presentations in congresses

- Castanheira, M.F.,Cerqueira, M.,Millot, S., Gonçalves, R.A., Oliveira, C., Conceição, L.E.C., Martins, C.I.M., (2015). Are personality traits consistent in fish? - The influence of social context. Book of abstracts Netherlands society for behavioural biology 2015, 25th-27th November, Soesterberg, The Netherlands

- Ferrari, S., Castanheira, M.F., Evensen,T.H., Martins, C.I.M., Oliveira C, Conceição, L.E.C., Chatain B., B. Damsgård, B., Bégout, M.L. (2014). Comparative characterization of coping styles in three european farmed fish species. EAS Aquaculture Europe 2014 Conference, 14-17 October, Donostia-San Sebastián, Spain.

- Cerqueira, M., Millot, S., Castanheira, M.F., Oliveira, R.F., Martins, C.I.M. (2014). Modulating the way fish appraises their world: How predictable events affect behaviour and physiological responses in fish. EAS Aquaculture Europe 2014 Conference, 14-17 October, Donostia-San Sebastián, Spain.

- Millot, S., Cerqueira, M., Castanheira, Maria F., Martins, C.I.M. (2013). How fish appraise their environment: comparative study between different stimuli valences and personalities in European sea bass (Dicentrarchus labrax). Book of abstracts Behaviour 2013, 4th-8th August, Newcastle, UK.

- Castanheira, Maria F., Herrera, M., Costas, B., Conceição, L.E.C., Martins, C.I.M. (2012). Linking cortisol responsiveness and aggressive behaviour in gilthead seabream Sparus aurata: evidence of divergent coping styles. Book of abstracts 46th Congress International Society Applied Ethology, 31July-4August, Vienna, Austria. Pag 17.

- Castanheira, Maria F., Herrera, M., Costas, B., Conceição, L.E.C., Martins, C.I.M. (2012). Individual behavioural responses towards challenges in gilthead Sea bream Sparus aurata: evidence of behavioural syndromes. Book of abstracts the XV Symposium The Physiology of Fish Behaviour, 9-13July, Norwich, UK. Pag 16.

Poster presentations in congresses

- Castanheira, M.F., Millot, S., Cerqueira, M., Conceição, L.E.C., Martins, C.I.M., (2013). Coping styles and welfare in farmed fish: current understanding and future directions.SPE- Congresso Nacional de Etologia, 24,25 October, Lisbon, Portugal.

- Cerqueira, M., Millot, S., Castanheira, M.F., Martins, C.I.M., (2013). Feed intake recovery after stressful events in Sea Bream (Sparus aurata): influence of coping styles.SPE- Congresso Nacional de Etologia, 24,25 October, Lisbon, Portugal.

Presentations and publications from the project

- Castanheira, M.F.,Cerqueira, M.,Millot, S., Gonçalves, R.A., Oliveira, C., Conceição, L.E.C., Martins, C.I.M., (2012). Are personality traits consistent in fish? - The influence of social context. Book of abstracts Behaviour 2013, 4th-8th August, Newcastle, UK.

- Cerqueira, M., Millot, S., Castanheira, M.F., Gonçalves, R.A., Oliveira, R.F., Martins, C.I.M. (2012). Modulating the way fish appraise their environment: the role of predictability on behavioural responses to appetitive and aversive stimuli in Gilthead sea bream (Sparus aurata). Behaviour 2013, 4th-8th August, Newcastle, UK.

- Castanheira, M.F., Herrera, M., Costas, B., Conceição, L.E.C., Martins, C.I.M., (2012). Uso del concepto de coping styles como herramienta para la identificación de comportamientos agresivos en la dorada (Sparus aurata). VI Jornadas de Acuicultura, 09-10 Maio, Cartaya, Spain.

- Herrera, M., Castanheira, M.F., Conceição, L.E.C., Martins, C.I.M., (2012). Relaciones entre metabolismo y comportamiento en la dorada (Sparus aurata). VI Jornadas de Acuicultura, 09-10 Maio, Cartaya, Spain.

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