UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA

LINKING PERSONALITY AND APPRAISAL MODULATORS IN FISH

MARCO ALEXANDRE CAVACO CERQUEIRA

Tese
Doutoramento em Ciências da Vida, do Mar, da Terra e do Ambiente
Ramo de Aquacultura
(Especialidade Técnicas de produção)

Trabalho efetuado sob a orientação de:

Professora Catedrática Aposentada Maria Teresa Dinis, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Faro, Portugal.
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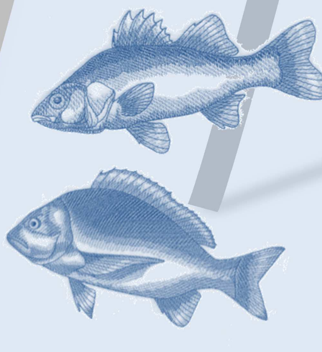
Doutor Simon Mackenzie, University of Stirling, Stirling, UK



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Linking Personality and Appraisal modulators in fish

Declaração de autoria do trabalho

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

Marco Alexandre Cerqueira

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A vocês… *dedico esta tese e o meu amor!*

SUMMARY

The reason why some individuals from the same fish population react so differently under similar aquaculture husbandry practices or to any other stressful situation is at the core of today's fish welfare research. In this context, the large individual variation in the physiological or behavioural response under stressful conditions is now accepted as reflection of distinct personality traits and of divergent cognitive evaluation that the individual makes of the situation, i.e. on the way the stressor is appraised. This thesis aimed to uncover which appraisal criteria fish use, the interplay with their personality traits, the underlying neurophysiological mechanisms and the potential application of psychological modulators of the stress response to improve fish welfare. Thus, the experimental work was oriented towards: i) investigating whether pre-existing inherent traits in behaviour and physiology affect the outcome of exposure to environmental stressors in Gilthead seabream (*Sparus aurata*) and Atlantic seabass (*Dicentrarchus labrax*) (**Chapter II.1 and Chapter II.2**); ii) evaluating the effect of predictability on the onset of action-related responses to aversive and appetitive stimuli (**Chapter III.1**); iii) inferring how predictability stressor modulates the cognitive stress response (**Chapter III.2**); iv) investigating how controllability improves coping ability on both seabream and seabass (**Chapter IV.1**); v) examining the relationship between thermal choice and animal personality using Nile tilapia (*Oreochromis niloticus*) as model (**Chapter IV.2**). Differences in behaviour, physiology and brain states measurements support specific appraisal and motivations in fish, according to the prospect of reward or punishment. This seems to be, nevertheless, highly dependent of both species - and context-specificity. In addition they suggest that predictable stimuli and social support alongside with perception of control can be used as psychological modulators of the stress response to make animals more resilient and empowered under sustainable farming systems. The link between personality and appraisal deserves further investigation as evidences are narrow. The work developed in the core of this thesis has brought new insights on how to manipulate fish´s ability to cope with changes in their environment, ensuring positive outcomes in terms of welfare, fitness and survival.

Keywords: Aquaculture; Environmental Appraisal; Personality; Psychological manipulation; Welfare.

RESUMO

O motivo pelo qual alguns indivíduos pertencentes a uma mesma população de peixes, reagem de modo diferente às rotinas praticadas numa aquacultura, ou em qualquer outra situação motivadora de stress, é hoje o cerne de investigação relacionada com bem-estar animal. Neste contexto, a variabilidade individual encontrada em termos de resposta fisiológica ou comportamental sob condições de stress é presentemente aceite como sendo um reflexo de distintos traços de personalidade e da avaliação cognitiva que o indivíduo faz da mesma, i.e. o modo como o factor de stress é percepcionado/avaliado. Esta tese teve como objectivo investigar quais os critérios que os peixes usam na avaliação do seu meio envolvente, a sua interacção com os seus traços de personalidade, os mecanismos neurofisiológicos subjacentes e o potencial de aplicação de factores psicológicos para orientar a forma como os indivíduos avaliam o seu meio, contribuindo assim para o seu bem-estar. Posto isto, o presente estudo foi desenvolvido para: i) investigar se as disposições intrínsecas pré-existente no comportamento e fisiologia manifestadas pela dourada (*Sparus aurata*) e pelo robalo (*Dicentrarchus labrax*), afectam as suas acções/reacções perante a exposição a factores de stress integrados no seu ambiente (**Capítulo II.1** e **Capítulo II.2**) ; ii) avaliar o efeito da previsibilidade na resposta a estímulos aversivos e apetitivos (**Capítulo III.1**); iii) avaliar de que forma a previsibilidade de um evento aversivo modifica a resposta cognitiva ao stress (**Capítulo III.2**); iv) investigar o efeito da controlabilidade na regulação da resposta de stress a factores aversivos em dourada e robalo de lidar com situações de stress (**Capítulo IV.1**); v) examinar a relação entre temperatura preferencial e personalidade na tilápia do Nilo (*Oreochromis niloticus*) (**Capítulo IV.2**). Diferenças na expressão de comportamento, fisiologia e estados neurais evidenciam motivações e mecanismos específicos para actuar perante o prospecto de recompensa ou de punição. Contudo, isto revela-se ser fortemente influenciado pela especificidade da espécie e do contexto em questão. Mais, sugere que estímulos previsíveis, presença de conspecíficos e controlo podem ser usados como moduladores psicológicos em situações de stress para tornar os animais mais resistentes e adaptados às actividades recorrentes numa aquacultura. A ligação entre personalidade e percepção requer ainda investigação uma vez que as evidências são parcas. O trabalho desenvolvido no âmbito desta tese oferece novas perspectivas em como manipular e melhorar a capacidade dos peixes para lidarem com situações de stress no seu ambiente, o que pode resultar em benefícios para o seu bem-estar, aptidão e sobrevivência.

Palavras-chave: Aquacultura, Percepção/avaliação do ambiente; Personalidade; Manipulação psicológica; Bem-estar animal

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General Introduction

Chapter I

Aquaculture: definition and its importance

The culturing of aquatic organisms is known as aquaculture and covers breeding, rearing and harvesting of both freshwater and marine species (FAO, 2002), which includes a wide variety of animals, from fish to molluscs, crustaceans, amphibians, reptiles and algae, but with finfish representing 67% of all aquaculture production (FAO, 2014). Fish final consumption as food is not however the only purpose of fish farming. Stock management for recreational and commercial fisheries, conserving and saving threatened populations, as well the production of fish for domestic use (as in the case of ornamental fish), are also common objectives of finfish farming. Finfish also became more prominent as a research subject during the past three decades, due to their neural complexity, size and visibility of the industry, being also produced for biomedical research and for environmental impact studies. The range of applications for farmed fish is indeed wide but, ultimately, the major role of aquaculture is to sustain the increasing consumer demand for fish products and the over-exploitation of fisheries (Le Francois et al., 2010).

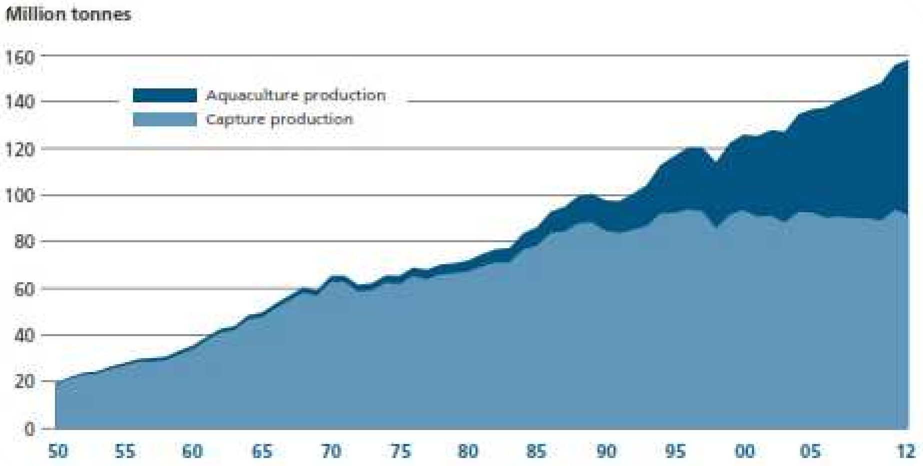


Figure 1.1 |World capture fisheries and aquaculture production (Source: FAO 2014).

Currently, aquaculture products are patently accepted as good replacers of wild aquatic food, since fish culture production has exceeded both fisheries productivity and the rate of human population growth (Fig. 1.1 in FAO, 2014). As the production of fish products moved beyond the provisional rearing of fish for individuals and into product marketing, demand and economics of production led to intensification of aquaculture. In line with this growth, an increasing concern by consumers, researchers and stakeholders regarding how fish are being reared and kept in captivity, their health and welfare has boosted the development of aquaculture-related research areas.

1.1. Research in aquaculture

For all the positive effects aquaculture can have on food supply and conservation of wild fisheries, it can also lead to environmental risks and create sustainability challenges. Research driven by either government or academic laboratories has become essential to avoid such problems and guarantee fish quality and welfare. An assessment of the scope of aquaculture research over the last years shows that subjects such as seafood quality, nutrition, reproduction, genetic improvement, fish health and welfare are prevalent (Fig. 1.2). Nevertheless, all subject areas are related and many often overlapping in the conducted research, with the central goal of adjusting rearing conditions to each species' biology and life cycle. For example, developing specific feeds for specific developmental stages improves the nutritional state of fish, ensuring optimal farming conditions. This topic is particularly important nowadays, given the introduction of alternative ingredient sources in fish feed formulations which, though a requirement to ensure long-term sustainability of the sector, have the potential to undermine fish nutrition, if proper care is not taken. In addition, genetic improvement can be used to develop selective breeding stocks that perform well under certain culture conditions, thus guaranteeing the biological success of the animals in captivity (Beveridge and McAndrew, 2000; Le Francois et al., 2010; Lim and Webster, 2006). Looking at the variety of species used in aquaculture, with their distinct life cycles and habitat preferences, and having in mind the increased public concern about the conditions is which fish are kept in captivity, promoting welfare is undeniably one of the goals of aquaculture-related research.

Overall, effective and sustainable fish culture demands economic production of a product that meets consumer expectations, with minimum negative impacts on fish welfare and on the environment (Le Francois et al., 2010). An interesting study by Kristian et al. (2015), demonstrated how much Norwegian population care about fish welfare. The authors showed that the public is concerned about fish welfare and that they were willing to pay a higher price for welfare-assured fish. However, they agree that the costs of welfare should be shared by the producers and the government. Alongside, fish welfare is not only important for the public perception, marketing or product recognition, but also to enhance production efficiency, quality and quantity (Southgate and Wall, 2001).

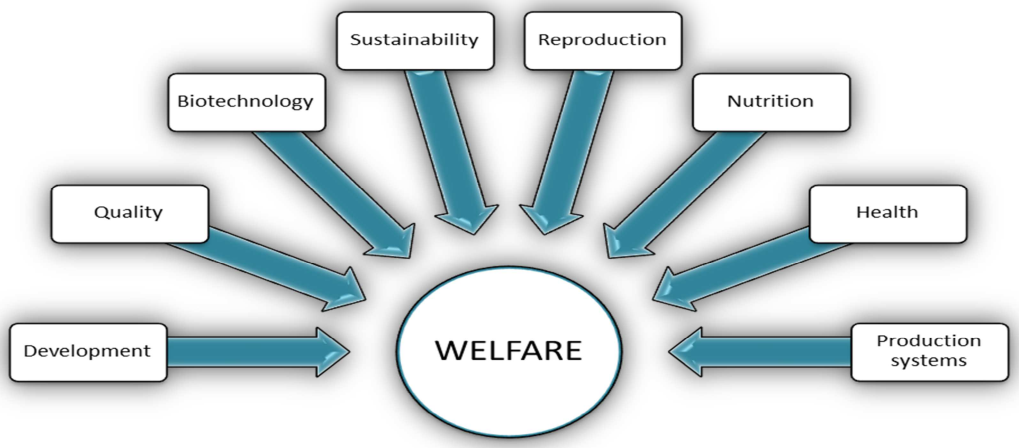


Figure 1.2 |Research-driven areas within aquaculture scientific field with fish welfare as the core goal of those areas

Similar to other countries in southern Europe, research in Portugal has been driven mainly to teleost fish species with high commercial value, such as gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*) and some flatfish, like sole (*Solea senegalensis*) and turbot (*Psetta maxima*) (FAO, 2004). The level of acceptance of cultured fish with higher nutritional value was assessed through a survey on the Portuguese population (Ramalho et al., Unpublished). Although welfare was not the focal point of this survey, positive public preference for such fish points towards the choice of fish being produced under guidelines and standards for good welfare.

1.2. Animal Welfare: origin and definitions

The scientific basis of animal welfare has been established through the “Brambell report” in 1965, in which ideas like “behaviour assessment” or “animal’s needs” were exposed (Rollin, 1989). Since then, many definitions have been proposed, either focusing on animal’s condition, how they subjectively experienced such condition or how it allows them to lead a natural life (FSBI, 2002). These divergent definitions demonstrate that welfare definition is not straightforward, but rather complex and often controversial. The most simplistic and common definition is that animal welfare is consistent with the individual’s quality of life (Appleby, 1999; Miller, 2001). Beyond the simplicity of the definition, different questions can be raised concerning is the definition of “quality of life”, if animals from different taxa experience such condition, or even if it is possible to have it in other conditions than their natural environment. In 1997, the different prevailing definitions were clustered into three categories (Fraser et al., 1997): (1) **feeling-based**: the animal should feel well, being free from negative experiences such as pain or fear, and have access to positive experiences, such as interactions with conspecifics, in the case of social species; (2) **function-based:** focus on the animal's ability to adapt to its present environment, being in good health, with its biological systems functioning properly, including physiological stress responses, and not being forced to respond beyond their capacity; (3) **nature-based** definitions: individuals should express their inherent biological nature, hence the animal should be able to lead a natural life and express its natural behaviour. Indeed, impairing natural behaviour is normally accompanied by health problems and negative experiences, making the different approaches often overlapping (Appleby and Sandøe, 2002). Having this into account, it is evident that no easy and single all-encompassing measure of welfare is available. Despite not being mutually exclusive, these definitions provide a more complete meaning of animal welfare and reflect what we should be focusing on when handling animals. Arguments for and against each approach, with the strengths and weakness of each, were exposed by Appleby and Sandøe (2002), highlighting a necessity for standardization. In the specific case of fish, it has been extensively debated over the last years whether this group of animals has aptitudes to consciously experience the surrounding environment, either by suffering pain or experience positive states, like other vertebrates (Braithwaite and Huntingford, 2004; Braithwaite and Boulcott, 2007; Chandroo et al., 2004; Dawkins, 1998; Rose et al., 2012; Sneddon, 2004), giving "fish welfare" a whole new connotation.

1.3. Fish culture and Welfare standards: where are we?

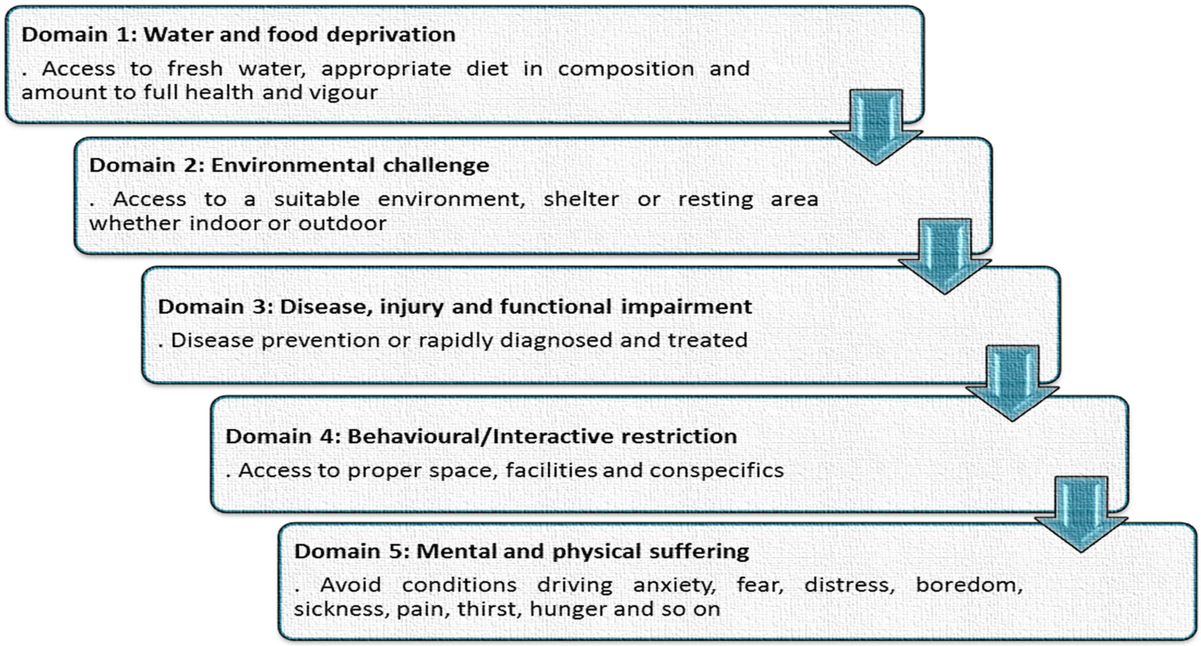
Fish are extremely exploited by humans (e.g. fisheries, intensive aquaculture, pet industry and scientific research); however, recent perception that fish are far more complex organisms than previously thought led this taxa to be considered within national welfare legislation in many world countries (Mejdell et al., 2007). As result, and as any other sector dealing with animals, aquaculture practices are being thoroughly examined to evaluate their impact on both the environment and the welfare of the cultured fish. In the EU, Council Directive 98/58/EC set minimum standards for the protection of animals reared or kept under farming purposes, including fish. But it was mostly over the last two decades that aquaculture industry started to implement Codes of Practice for responsible aquaculture that take fish welfare into consideration (Huntingford, 2008), promoting optimal rearing conditions. The development of guidelines for responsible aquaculture by the Federation of European Aquaculture Producers (FEAP) is just one example of how welfare is a rising concern for farmers. In 2005, the Council of Europe adopted a recommendation on the welfare of farmed fish. In 2008, the World Organization for Animal Health (OIE) adopted guiding principles and policies for fish welfare and, in 2009, adopted an opinion on the general approach to achieve fish welfare and proper killing methods. The involvement of OIE in aquatic animal welfare was noted by Hastein (2007), where the scope and development of the welfare guidelines for aquatic animals were thoroughly described. To adopt these guidelines or protocols all over the aquaculture sector, worldwide and across cultured species, much work has to be undertaken as we are still in the very beginning of understanding what is really good welfare (e.g. depending on the situation, on the production regime, on the species biology or ecology requirements, etc.). Despite the considerable progress made over these last two decades, the knowledge regarding welfare or the proximate mechanisms underlying such concept is still sparse, when compared to mammals or birds (Ashley, 2007; Huntingford et al., 2006; Mejdell et al., 2007; Sneddon, 2007; Turnbull, 2006). In fact, to adopt such guidelines for fish welfare, I share the opinion of Mancuso (2013), which states that research should be the way to address these issues. Efforts should be made to decrease the stress of aquaculture routines, through multidisciplinary approaches that rely on both known and unknown indicators of well-being, behavioural, physiological, neuroendocrine, etc. One good example has been the development of proteomics as a method to obtain unbiased information regarding the impact of stressors on both plasma and organs/tissues (Alves et al., 2010; Rodrigues et al., 2012). Assessment of a wide range of responses under different conditions or situations provides an appropriate and reasonable basis to assist OIE in carrying out its work regarding the protection of fish farming.

1.3.1. Current issues of welfare in fish

Recent studies showing that fish are capable of environmental perception and preference, sense pain in similar ways to mammals and experience aversive states (Braithwaite and Boulcott, 2007a; b; Dunlop et al., 2006; Madaro et al., 2015; Madaro et al., 2016; Millot et al., 2014a; Millot et al., 2014b; Reilly et al., 2008; Sneddon, 2006; Sneddon, 2003; 2009; Vindas et al., 2012; Vindas et al., 2014) have brought to light a whole new perspective in terms of fish welfare, which is now being employed in terms of freedom from hunger, thirst, discomfort, pain, injury, fear, distress, disease, along with freedom to express normal behaviour (Ashley, 2007b). Despite lacking a developed neocortex, self-awareness and cognitive abilities on the same level as mammals, it is suggested that fish can certainly sense noxious stimuli and, to some level, experience both pain and fear (Sneddon, 2009). Recently, in zebrafish (*Danio rerio*), the capacity of expressing emotional fever (e.g. physical reaction similar to fever but triggered by a stressful situation), a trait normally used to identify consciousness in mammals (Rey et al., 2015b), has been described. Such studies, alongside with the extensive evidence of fish behavioural and cognitive talent and pain perception, justifies the need to think about the importance of welfare in aquaculture from an ethical point of view (Brown, 2014; Grigorakis, 2009), rather than only pragmatically (i.e. in terms of quality or performance improvements). Thus, new lines of research focused on understanding the welfare of a more ancient and divergent taxonomic group than mammals are being opened. Still, it is essential to go deeper in safeguarding cultured fish through concession of the same level of protection as that provided to any other vertebrate group.

1.4. Assessing welfare in aquaculture

Though it is true that, in the natural environment, fish can be subjected to many types of stressors (injuries, diseases, parasites, floods or storms, predators or larger conspecifics), they are likely to have adaptive coping mechanisms for dealing with such adverse conditions (up to certain limits, if such conditions are short-term events or otherwise avoidable). On the other hand, various aspects of standard aquaculture practices throughout all production stages can impair considerably their welfare. Often, cultured fish suffer unavoidable and prolonged or repetitive physical constraints, which can undermine their adaptive coping responses, either physiologically or behaviourally. It is thus important to mitigate those effects, minimizing the avoidable stressors during all production cycle and promoting welfare (Conte, 2004; Pickering, 1993). The strategy adopted in fish to “assess or promote welfare” was based on the evaluation of five domains (e.g. conditions) in which welfare might be compromised (Fig. 1.3). Originally edited as the “five freedoms” and defined by UK Farm Animal Welfare Council, was later adapted for fish (FAWC, 1996). Within this framework, in order to achieve good welfare and health of fish in aquaculture, these five conditions should be respected, maintained and improved (Mellor and Stafford, 2001). Fish condition can be assessed by means of their behavioural, physiological, morphological or physical stress responses, which can differ among species. Thus, each species’ biology and environmental requirements must be taken in consideration. In particular, knowledge of species-specific behaviour is critical to safeguarding fish welfare (Conte, 2004). In line with the way welfare definition has been evolving, so has the number of variables used to assess the state of the fish. In addition to physiological and biochemical measures, recent knowledge about behavioural alterations and contiguous molecular states are now seen as important fish responses for welfare assessment. Thus it is reasonable to state that welfare should be viewed as a result of divergent effects (e.g. water quality, tank densities, diet composition, restricted feeding or management procedures as confinement or air exposure) known to affect fish (Branson, 2008) and subsequent individual tolerance to stress, health, aggressiveness or lethargic behaviour.



Domain 1: Water and food deprivation
. Access to fresh water, appropriate diet in composition and amount to full health and vigour

Domain 2: Environmental challenge
. Access to a suitable environment, shelter or resting area whether indoor or outdoor

Domain 3: Disease, injury and functional impairment
. Disease prevention or rapidly diagnosed and treated

Domain 4: Behavioural/Interactive restriction
. Access to proper space, facilities and conspecifics

Domain 5: Mental and physical suffering
. Avoid conditions driving anxiety, fear, distress, boredom, sickness, pain, thirst, hunger and so on

Figure 1.3 |Five domains defined by UK Farm Animal Welfare Council to assure and assess welfare of animal reared and kept under artificial conditions (adapted from FSBI, 2002).

The factors affecting the life of a fish and the indicators used by farmers to assess the general status of their stock have been reviewed by a number of authors (Conte, 2004; Huntingford et al., 2006; Martins et al., 2011a; Schreck et al., 1997) (Table 1.1. adapted from Silva et al., 2013).

"*Welfare indicators that are relevant for inclusion in an operational welfare assessment system should be science-based, should measure welfare over extended time periods, should be measurable on a commercial farm within a realistic framework and should be relevant as a decision support system for the farmer*" (Martins et al., 2011).

Table 1.1 |Main factors affecting fish welfare and most common indicators of impaired fish welfare.

Factors impairing welfare		**Indicators of impaired welfare**	
Environment	Temperature; pH; salinity	**Behavioural**	Food intake; aggressiveness
	O_2; CO_2; NH_3; PO_4^{2-}		Swimming pattern
	Photoperiod		Ventilation rate
	Pollutants		Reaction to novelty
	Xenobiotics		Other changes
Health	Pathogens	**Health and performance**	Injuries; malformations
	Somatic/fin lesions		Diseases; Changes in color
	Disease treatments		Impaired reproduction
	Vaccination side-effects		Growth and FCR
Nutrition	Food deprivation	**Haematological parameters**	Cortisol; Glucose; Lactate;
	Malnutrition		Haematocrit; free fatty acids
	Anti-nutritional factors		Free amino acids
Management practices	Sorting, Handling, Grading	**Post-mortem dynamics**	Rigor mortis; sensorial parameters
	Transporting; harvesting		Energy stores(ATP, glycogen)
	Slauthering; Sampling		Freshness indicators
	Stocking densities		Instrumental parameters
Social dynamics	Sorting/grading	**Cellular stress indicators**	Heat shock proteins
	enforced social contact		Antioxidant potential
	Genetic factors		Reactive oxygen species
	Agonistic behaviors/competition		Apoptosis/necrosis

1.4.1. Stress responses in fish

Stress can be defined as any disturbance of the organism homeostasis as result of an internal or external stressor. Fish respond to external or internal challenges through a series of neurological and endocrine adjustments generically called "stress responses", often used as indicators of impaired welfare (Table 1.1). Stress response has three different stages (Barton, 2002): first an endocrine response is induced through the release of glucocorticoids to the bloodstream, mostly cortisol and epinephrine in teleost fish (Wendelaar Bonga, 1997; 2011); The secondary or metabolic and tertiary or behavioural responses make fish more efficient at overcoming or avoiding the challenge, improving the short-term capacity of fish to cope with it.

Repetitive or long-term activation of endocrine responses can induce chronic stress and eventually motivate maladaptive effects such as decreased growth, low performance and well-being issues, impaired reproductive function and immunosuppression, anorexia, disease and ultimately death (Barton, 2002; Gesto et al., 2008; Iwama, 2007; Pankhurst and Van Der Kraak, 1997; Pankhurst et al., 2008; Pottinger, 2008; Schreck, 2010; Van Weerd and Komen, 1998; Wendelaar Bonga, 1997; 2011). To describe the role of the primary stress response, the concept of allostasis was introduced to support homeostasis. Hence, rather than continuously making every effort towards a static (conceptually optimal) state, organisms have “the ability to achieve stability through change” (McEwen and Wingfield, 2003), which more accurately explains the adaptive and dynamic nature of biological systems. This means that, in a naturally dynamic environment, the internal balance of fish is reshaped to accommodate different requirements and adapt to them. Under an allostatic framework, the absence of any signals or challenges from the envrionment can lead to a state of allostatic underload, which can be as detrimental for the organism as allostatic overload (achieved by the inhability to cope with cumulative disturbing challenges). This implies that insulating fish from all type of challenges would also not be in their best interest, in terms of welfare. Nevertheless, allostatic load can either have an adaptive (load type I, normally referred as “eustress”) or maladaptive value (load type II, normally referred as “distress”). An overview of the relationship between stressors and biological responses within the context of finfish aquaculture can be seen in Fig. 1.4. It should be noted that a stress response is not necessarily a direct indicator of poor welfare. Indeed, lower cortisol levels can mean that fish interregnal tissue was in an overloaded state, hence misleading an interpretation of the effective stressed condition of the individual. Due to this fact, when assessing welfare, different stress responses should be monitored, integrating behaviour, neurophysiology, pathological and molecular indicators to draw robust conclusions about the state of the individual.

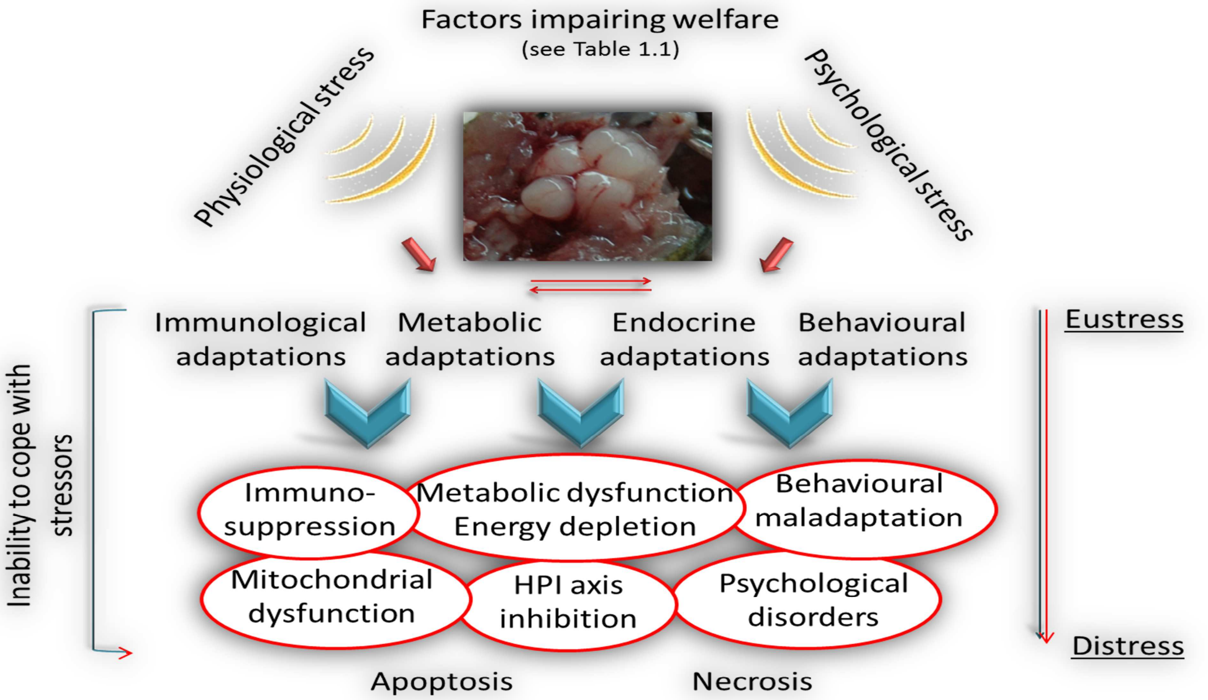


Figure 1.4 |Overview of the relationship between stressors and both physiological and psychological stress, within an allostatic framework.

1.4.1.1. Physiological response to stress

In teleosts, cortisol is the main glucocorticoid released by hypothalamic-pituitary-interregnal tissue axis (HPI) during stress and plasma cortisol concentration is often used as an index of stress response (Barton 2002). Cortisol is a central hormone for the maintenance of allostasis as it supports other hormones during basal conditions and has a stress-induced regulatory role (Mommsen et al., 1999). Other plasma metabolites associated with stress response, such as glucose and lactate, can be also used to assess conditions of impaired welfare, however with some constraints. The response to a stressor is a very dynamic process and physiological measurements can be only a snap-shot and not be representative of the stress experienced (Barton, 2002). Furthermore, cortisol levels are normally biased as blood sampling itself constitutes a source of stress. This hormone is also highly context- and species-dependent, as along with glucose and lactate. More specifically, basal levels measured are known to be affected by environmental conditions, aquaculture procedures, feeding, maturation, season, photoperiod, sex differences and other unknown stressors (Barton, 2002). For instance, in seabass, no differences were found between fish subjected to confinement and non-stimulated fish, and alterations due to external factors was the explanation given for such results (Rotllant et al., 2003). It is generally accepted that cortisol rises after the exposure to a stressor within the first 4-10 minutes and this peak lasts for a few hours (Sumpter, 1997). In seabream, plasma cortisol concentrations increased 50 fold within 30 min after air exposure, while under confinement levels increased 8 fold within the same time (Arends et al., 1999). When fish are subjected to chronic stress, plasma cortisol can be elevated for many days or even weeks, or may return to basal levels due to habituation mechanisms or impairment of the HPI axis e.g. exhaustion of the endocrine stress axis (Madaro et al., 2015). Indeed, the nature of the stressor i.e. intensity, repeatability, predictability, controllability or familiarity, is known to influence the magnitude of cortisol response. In this sense, in the field of psychological modulators of stress response, it was shown that unpredictable, uncontrollable and unfamiliar environmental contexts accentuates stress responses in salmon and rainbow trout respectively, sustained by higher levels of plasma cortisol besides behavioural alterations (Carpenter and Summers, 2009; Madaro et al., 2015; Yue and Duncan, 2006). In addition, inherent features of the individuals promote inter-individual variability in cortisol response under the same challenging situation. Two personality traits have been defined due to their consistent neuroendocrine and behavioural characteristics: the proactive (bold) and reactive (shy) coping response. Psychological stressors and personality will be addressed further down in this chapter (section 1.5).

1.4.1.2. Behavioural responses to stress

Behavioural responses are the first line of defence of the animal against environmental changes, predators or social conflicts and are often caused by the same stimuli that elicited the physiological responses. Within a more welfare functional-based approach, the fish functions that are known to be affected by stressors include foraging behaviour, swimming patterns, shoaling, thermoregulation, orientation, avoidance, ventilator frequency, chemoreception and agonistic behaviour e.g. social interactions or evasion from predators (Conte 2004). Exploratory behaviour, food anticipatory behaviour, environmental preference, learning and reward-related operant behaviour are seen as patterns within a more feeling-based approach, as reviewed by Martins et al. (2011). Due to this extensive list of fish behavioural stress responses and for the sake of briefness, only few behaviours are here briefly described. The behaviours described represent the most important and most used indicators by farmers or researchers:

Freezing behaviour is the most classic response of fish towards conflicting situations by remaining motionless on the bottom and suppressing fin movements (Vilhunen and Hirvonen, 2003), increasing vigilance and arousal over the stressor. Divergent results from several works, often under different contextual situations, showed the dichotomy of this behaviour by being interpreted as either good or poor welfare. Furthermore, as also stated, the divergent fish responses can also be explained by other factors, such as personality traits or individual cognitive appraisal of the challenge. When under rewarding situations, **swimming activity** levels are normally used, rather than freezing behaviour. This behaviour is described as a measure of fish performance, therefore an indicator of the ability of the fish to feed, evade from predators and maintain position in a current (Beaumont et al., 1996). Swimming activity can be related with different swimming behaviours, among them **swimming speed**, which has been used to evaluate the physiological condition of fish (Wolters and Arlinghaus, 2004). As for freezing behaviour, this response can be interpreted in two directions: either signalling underfeeding, thus indicating poor welfare or a foraging strategy, thus indicating good welfare. Indeed, **foraging behaviour** (e.g. the search and exploitation of food resources (Danchin E et al., 2008)) is one of the most used welfare indicators by fish farmers. Teleost fish can exhibit diverse strategies to improve feed intake, in order to satisfy their nutritional requirements. This can be measured by **feeding intake** or **feeding motivation** (e.g. latency to reassume feeding), behaviours which are highly affected by feeding regime: predictable feeding time over random schedules was shown to affect behaviour and physiology (Sánchez et al., 2009). A predictable meal thus seems to help fish readjust themselves for the incoming event and optimize feed intake. It should however be noted that predictable regimes tend to increase “**anticipatory behaviour**”, tuned with increased swimming activity in the feeding area or increased schooling activity (Chen and Purser, 2001). In agreement to what was previously stated, this increase can imply good or poor welfare. Folkedal et al. (2010) stated that a good anticipatory response and feed intake can be signs of high feeding motivation and welfare. Lower latency to reassume feeding was also described as good welfare, demonstrated in Nile tilapia (*Oreochromis niloticus*) and in rainbow trout (Martins et al., 2011b; Øverli et al., 2006b). On the other hand, it can also indicate underfeeding, and here anticipatory responses lead to **aggression** and injuries, with repercussions even for survival. Limited resources can drive fish to agonistic encounters, recognized as responses affecting the dominance rank-based hierarchies (Jobling, 2011). **Agonistic behaviours** refer to a set of fight-or-flight behaviours expressed between at least two social partners in which one exerts dominance over the other (Martins et al., 2011). Fight-or-flight behaviours (onwards referred to as interactions between conspecifics) are a defensive mechanism with the purpose of acting over the stressor or moving away; thus includes attacks, bites or bite attempts, threatening displays such as chasing, and behaviours related to submission such as flight or immobility (Steckler, 2005). Interactions between conspecifics are normally associated with the competition for rewards, thus its absence under punishment situations does not necessarily mean good welfare, but rather a different context and consequently fish expressing other responses. **Shoaling** or group swimming behaviour is another behavioural response that can be used to assess hunger, stress level and health status and it relates to the spatial distribution and swimming activity of the group. The motivational state of the individuals to explore the surrounding environment will vary with their internal affective and physiological states, again resulting from inherent predispositions and from the subjective appraisal of the stressor. If one individual interprets the stressor as more positive (or less negative), or associated with the possibility to escape from an aversive condition (e.g. **operant behaviour** to decrease the impact of the stressor), it will probably increase the level of exploration. In this case, individual personality and subjective appraisal overrides the predisposition of the group swimming behaviour. **Ventilatory frequency** or number of opercular movements provides an index of ventilatory activity, thus offering evidences of physiological stress. Well-balanced ventilatory activity is fundamental to maintain a good oxygen supply to blood and tissues. Increased ventilatory frequency is normally related with poor welfare and can be a consequence of the several aquaculture practices or stressors previously identified. Different authors have shown the effect of such stressors and how they can compromise fish welfare (Barreto and Volpato, 2006; Barreto and Volpato, 2011; Barreto et al., 2003; Brydges et al., 2009; Scott and Sloman, 2004). Nevertheless, this indicator should be combined with other welfare indictors as it does not give any information regarding the nature and intensity of the stressor. In fact, arousal caused by positive experiences can also trigger an increase in ventilatory frequency, hence it should be interpreted with caution.

1.4.1.3. Integrating learning and preference to promote Welfare

Fish natural responses and learned preferences can and should be used by them in order to avoid stressful situations. One example in seabass and seabream is the behavioural response towards a microbial parasite infection known to affect fish gills (e.g. *Amyloodinium ocellatum*), and which compromises oxygen trades and often leads to mortalities (Pereira et al., 2011). Due to the positive correlation of salinity with the occurrence of this parasite, introducing inflow of fresh water to the pond make fish aggregate and adopt a synchronised swimming towards it entrance, this way decreasing the spreading of the infection.

Good welfare is attained when the difference between the current and the expected state is minimized (sensu feeling-based approach). **Learning** is the process by which an animal benefits from existing information from the environment, so that its behaviour becomes fit to the environmental conditions, by maximizing positive and minimizing negative states (Spruijt et al., 2001). This requires that the animals have the ability to learn about predictive stimuli, i.e. which events follows which (classical conditioning), and about the consequences of their own behaviour (operant learning). There is considerable evidence that cultured fish are able to improve performance in order to meet new challenges that are introduced (Fernö et al., 2006). Learning ability allows them to adjust behaviours and has been investigated in a wide range of contexts. In Aquaculture, different strategies are used for that purpose, such as using light to identify where and when the food will be dropped, reducing food waste and improving fish performance and productivity (Karplus et al., 2007). The ability to associate cues with relevant environmental stimuli is crucial for performance, for instance in agonistic interactions and reproduction (Hollis, 1999; Jenkins and Rowland, 1996) and for adapting to new environmental situations such as fish orientation (Braithwaite et al., 1996; Odling-Smee et al., 2006; Vargas et al., 2004), foraging (Warburton, 2003) and predator avoidance (Martins et al., 2011d).

Classical Conditioning learning

In classical conditioning, an association is made between a stimulus and a response. A conditioned stimulus (CS) acquires the ability to trigger a new response by virtue of being paired with an unconditioned stimulus (US), which by definition is biologically important and capable of triggering an innate reflex. For example, carp can learn to associate a 400 Hz pure pulsed sound with food by classical conditioning (Zion et al., 2007). In the same species, known to be a strongly schooling fish, choice of food over social attraction was possible by prior light cues conditioning (Mesquita et al., 2015).

Operant conditioning learning

In operant conditioning, the consequence (positive or negative) of performing a particular behaviour alters the probability of that action being repeated. In this case, the behaviour is spontaneously emitted and not, elicited by a stimulus. For favourable consequences, animals learn to perform it in order to be rewarded. An example, is when Atlantic cod (*Gadus morhua*) were trained to operate a trigger to receive food in an experiment using a self-feeding system (Nilsson and Torgersen, 2010). Under aversive conditions, operant conditioning can take the form of **punishment avoidance conditioning**, in which instead of a reward, the animal receives a punishment (Bolhuis and Giraldeau, 2005): rainbow trout that learned to escape, showed lower cortisol responsiveness and latency to escape over a training period, than no learners (Carpenter and Summers, 2009).

Although learning may occur with a single trial, it often takes gradual training for effective conditioning. However, this can lead to habituation, sometimes described as one of the simpler forms of learning. **Habituation** is a type of non-associative learning leading to a decrement in response intensity repetition of the triggering stimulus. The first conditioning applied is seen as novel and induces a reaction, however, with the repetition such response decreases and may disappear. This can be useful in aquaculture, allowing a better adaption to daily procedures (Fernö et al., 2006). In accordance, social learning also has a pivotal role on that, as organisms are able to learn from others. As expected, this is much more common in social species. The adaptive value of social learning lies in saving time and energy in developing proper coping mechanisms by trial and error. It is also generally assumed that social learning is beneficial, because naïve individuals can acquire adaptive behaviour quickly and efficiently from more knowledgeable individuals (Brown and Laland, 2006; Bshary and Brown, 2014). As referred before, inherent predispositions of the individuals also affect learning, where proactive individuals are quicker learners but often with less awareness of the changes in the environment (Castanheira et al., 2015).

1.5. Intra-individual variability of stress responses

Individual behavioural and physiological variability towards environmental challenges or stressors are today a well-known and accepted phenomenon within the animal kingdom (Carere et al., 2010; Réale et al., 2010). This variation suggests that stress responses do not depend exclusively on the situation to which the individual is exposed, but also on the cognitive evaluation made of the situation, i.e. how the stressor is appraised (Lazarus, 1991). In fish, as often mentioned throughout this chapter, this individual variability is related to differences in learning, adaptation to new environments, growth and metabolism, reproduction, susceptibility to diseases and welfare, health, flesh quality, performance and any other situation that elicits a response from the fish (Ashley, 2007a; Basic et al., 2012; Castanheira et al., 2013b; Herrera et al., 2014; Ibarra Zatarain, 2015; Iguchi et al., 2001; MacKenzie et al., 2009; Rey et al., 2015a; Ruiz-Gomez et al., 2011). Understanding such differences in coping ability and environmental perception, fundamental to fitness and quality of life, is particularly pertinent under aquaculture conditions for an effective understanding of individual behavioural patterns under stressful conditions (Huntingford and Adams, 2005).

Individual response variability to any challenge is not an arbitrary variation around an optimal mean; it is consistent under a diversity of stressful conditions. Behavioural and physiological differences in response to stress, which are consistent throughout time and across situations, are collectively termed as coping styles (Koolhaas et al., 1999a). In the literature, this phenomenon is also referred to as temperament (Francis, 1990), personality (Briffa and Weiss, 2010) or behavioural syndromes (Sih et al., 2004) (see review of Castanheira et al. (2015) for differences between terminology). Despite the different terminology the core concept behind each is based on the fact that individuals consistently differ from one another in behaviour or physiological responses in such a way that these differences can be described as individual traits. The term coping styles is narrowly used and primarily defined as “a coherent set of consistent behavioural and physiological stress responses over time and across situations” (Koolhaas et al., 1999b) and has been considered in a wide range of taxa, such as birds, insects, mammals (including humans) (LaRowe et al., 2006; Reale et al., 2007; van Oers et al., 2005) and fish (Toms et al., 2010; Conrad et al., 2011; Castanheira et al., 2015). When addressing humans or other mammals, the term “personality” is more often used, because it also takes account of the emotional reactiveness of the individuals e.g. feeling, thinking, cognitive appraisal, motivational states (Gosling, 2001). Indeed, several reviews regarding personality in animals in the recent past years have been addressed; non-human primates (Freeman and Gosling, 2010), avian (Groothuis and Carere, 2005), felid (Groothuis and Carere, 2005), canids (Jones and Gosling, 2005) and other species (Gosling, 2001). Furthermore, Martins et., al. (2011d) demonstrated that coping styles in fish are predictive of how stimuli are appraised, hence supporting the inclusion of emotional or affective states as a relevant component of coping styles (see below). In agreement, Coppens et al. (2010a) pinpoints that motivational reflections may explain distinct traits in reaction to challenges. We thus reserve the use of the term “personality traits” in agreement with the objectives described (see below), and will use this terminology through this dissertation, even when mentioning other researchers’ work. It should however be noted that personality in fish does not necessarily imply homology to humans or other animals, as it rather represents a more primitive or simplistic system, likely to be evolutionary conserved, thus working as a raw material for natural selection (Castanheira et al., 2015).

1.5.1 Personality traits in fish

In fish, one of the first references to the existence of distinct behavioural phenotypes was published by Huntingford (1976): three-spined stickleback (*Gasterosteus aculeatus*) were shown to be more aggressive to conspecific intruders during the breeding season, and also more aggressive towards heterospecifics. Moreover, the most aggressive individuals in the breeding season were also the boldest when approached by a predator outside of breeding season. Besides the clear consistency over time (e.g. during and post breeding) and across situations (e.g. conspecifics and heterospecifics), consistency in different behaviours was found. Those results, even though no mention was made, satisfy the description of personality. Since then, several studies have addressed the existence of such traits in fish in different contexts, situations and species that led to specific research focus surrounding this issue. Two distinct stress response patterns have been described, reflected in both behaviour and neuro-endocrines profiles: proactive (active traits) and reactive (passive traits). Behaviourally, proactive individuals are more aggressive, bold when facing potential danger or in exploring novel environments and with a tendency to develop rigid learned routines, hence lower sensitivity to environmental stressors. Physiologically, proactive individuals show lower HPI axis reactivity, hence low production of glucocorticoids (i.e. catecholamines or cortisol) and a high sympathetic activity leading to a high increase of noradrenaline and adrenaline in blood (Overli et al., 2007). In contrast, reactive individuals show lower degrees of aggressiveness and boldness, and are more flexible to changes in the environment. Moreover, reactive individuals show high attack latency and freezing behaviour under stressful situations, higher HPI axis reactivity and lower noradrenaline and adrenaline releases. A number of recent reviews pinpoint the differences between this dichotomous classification (Toms et al., 2010; Conrad et al., 2011; Mittelbach et al., 2014; Castanheira et al., 2015).

Table 1.2 |Summary of the main differences between proactive and reactive individuals (adapted from Castanheira et al., 2015)

Traits	Proactive		Reactive
Aggressiveness	High	⟷	Low
Exploration /risk taking	High	⟷	Low
Feed efficiency	High	⟷	Low
Feeding motivation	High	⟷	Low
Flexibility / plasticity	Low	⟷	High
Activity toward stressors	High	⟷	Low
Sociality	Low	⟷	High
Costs with coping strategy	High	⟷	Low
Standard metabolic demands	High	⟷	Low
Neural plasticity	Low	⟷	High
Immunology	High	⟷	Low

It is also important to note, as reported elsewhere, that the distribution of personality traits throughout captive animals is not expressed as a binomial distribution, it is rather a proactive-intermediate-reactive traits continuum (Réale et al., 2010). The existence of an intermediate group can be due to the lack of environmental demands or pressures and accommodates, in due course, the individuals whose stress response is not consistent over time and contexts (i.e. higher plasticity). In nature, this group is possibly less fit to survive, both in stable and unstable environments (Boersma, 2011).

1.5.1.1. How to assess fish personality in aquaculture

The concept of personalities in fish has gained an increased interest in recent years. The majority of research focused on the most important farmed species, among them Atlantic Salmon (*Salmo salar*) (Kittilsen et al., 2012; Vaz-Serrano et al., 2011), Nile tilapia (Martins et al. 2011b,d), common carp (*Cyprinus carpio*) (Huntingford et al., 2010; MacKenzie et al., 2009), rainbow trout (Laursen et al., 2011; Øverli et al., 2006a; Øverli et al., 2006b), seabass (Ferrari et al., 2014; Millot et al., 2009a; Millot et al., 2009b) and seabream (Castanheira et al., 2013a; b; Herrera et al., 2014). In addition, species with limited expression in these industry have been investigated, such as Senegalese sole (*Solea senegalensis*) (Ibarra Zatarain, 2015; Silva et al., 2010), Atlantic halibut (*Hippoglossus hippoglossus*) (Kristiansen and Fernö, 2007) and turbot (*Psetta maxima*) (Hermann et al., 2016).

Different behavioural screening approaches have become available to assess individual variation in fish, both individual- and group-based (for more details on each test mentioned below see Castanheira et al. 2015). Within the first set of behavioural tests, patterns of feeding behaviour (Barreto and Volpato, 2011), exploration of novel environment (Killen et al., 2011), novel object (Frost et al., 2007), resident-intruder test (Brelin et al., 2005) and net restraining (Castanheira et al., 2013b) are the most commonly used. Hypoxia (Ferrari et al., 2014; Laursen et al., 2011) and risk-taking (Castanheira et al., 2013a) are the behavioural tests normally used for mass-screening. Physiological individual-based approaches can also be used to discriminate distinct adaptive traits, for instance ventilation frequency (Barreto and Volpato, 2011) or metabolic responses (e.g. O_2 consumption measured in metabolic chambers (Herrera et al., 2014), HPI reactivity or sympathetic system activity (Overli et al., 2007)). Recently, it was shown that subtle thermal gradients are likely to impact specific physiological and behavioural processes, which is reflected as a suite of traits described by animal personality (Rey et al., 2015a). As such, thermal preference alongside with hypoxia or risk-taking, are promising tools for farmers, as they can be for mass-screening. Individual tests are often time consuming and demanding (e.g. restraining requires video recording and video analysis afterwards) and highly stressful, sometimes driving even to mortalities; as such, the development of group-based paradigms, adapted to the ecological features of the species, can be highly advantageous since it allows screening large number of fish in a shorter time and with immediate results, being highly attractive for the aquaculture industry. With this, welfare can be attained within production systems, adapting optimal conditions to proactive and reactive traits (Castanheira et al., 2015). The diversity of paradigms to assess coping strategies in fish, which should be fitted to species, shows how challenging it is to assess personality traits. Behavioural responses to changes in the environment can be extremely plastic and most often context-dependent (Coppens et al., 2010b; Wolf and Weissing, 2010),and reported to be shaped by numerous factors, such as the predictability of food regime (Chapman et al. 2010), food density (Dunbrack et al., 1996) or social context (Castanheira et al., 2016). Other factors are also well recognized to affect the consistency of personality: temperature or hypoxia (Biro and Stamps, 2010; Rey et al., 2015a), predation pressure (Brown and Braithwaite, 2004; Archard et al., 2012), learning (Millot et al., 2009b), social interactions (Chapman et al., 2008), environment constancy (Brelin et al., 2008), stress (Ruiz-Gomez et al., 2008) or even the time gap between repeatable tests (Stamps and Groothuis, 2010; Ferrari et al., 2016; Castanheira et al., 2016). Indeed, in seabream, restraining escape performance was shown to be consistent between repeatable tests 14 days and 8 months apart. Nevertheless, after sexual maturation, a loss of consistency was marked. As such, measuring the stress response in different contexts would allow a more accurate characterization of species-specific personality. Analyses of the consistency of behavioural screening results between repeated tests (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) or different challenges (cross-context analyses) are generally carried out over periods of one to eight days (Wilson and Stevens, 2005; Øverli et al., 2007; Wilson and Godin, 2009; Wilson et al., 2010) and are reported to be important to tune the population personality screening; and to effectively discriminate personality (Castanheira et al., 2015, 2016; Ferrari et al., 2016). However, such premise is still one of the major gaps in the literature concerning the characterizations of personalities in animal, including fish, despite the recent works in that direction (Castanheira et al., 2016; Ferrari et al., 2016).

In both seabream and seabass, the net restraining test was shown to be a robust approach to assess divergent personality traits (Castanheira et al., 2013a; Ferrari et al., 2014); it has been developed in the past to evaluate the stress response of seabream to air exposure (Arends et al., 1999) and adapted afterwards as a methodological approach to characterize personalities in Senegalese sole (Martins et al., 2011c; Silva et al., 2010). It is representative of different aquaculture routine procedures (e.g. grading, sampling, sorting, vaccination, transport). The test relies on the escape performance of fish, known to have an ecological implication (interaction between predator and prey) and physiological repercussions (anaerobic recovery capacity of white muscle). In Nile tilapia, latency to reassume feeding and ventilation frequency (Barreto and Volpato et al., 2011) were the most common and efficient tests. In fact, Barreto and Volpato (2011) observed that ventilation frequency of Nile tilapia was correlated with the return to feeding in isolation. By assuming this correlation in stress responses, measuring individual feed intake can be replaced by ventilation frequency, more easily assessed by means of technology, such as using SmartTags. These devices have been developed to measure ventilatory frequency and amplitude of free-swimming fish as an indicator of fish welfare (Damsgard, 2008).

1.5.1.2. Importance of assessing personality

Personality can be an important tool to better understand both ecological and biological dynamics of any organism and hence should be included as an explanatory variable to understand differences in individual survival, reproductive success, species diversity on behavioural interactions, population dynamics (i.e. growth, fecundity and survival), social assemblages patterns, and for improvement on conservation and management of natural resources (Mittelbach et al., 2014). In cultured fish, the advantages of characterizing proactive or reactive traits have now being extensively reviewed (Castanheira et al., 2015; Conrad et al., 2011b; Huntingford and Adams, 2005). As an example, reactive individuals have higher neural plasticity, higher life span and a more robust hormonal regulation (e.g. Castanheira et al., 2015). On the other hand, proactive fish often recover faster from stressful situations (Ward et al., 2004), display lower susceptibility to diseases (Mackenzie et al., 2009), higher growth rates (Basic et al., 2012) and often higher reproductive success (King et al., 2013; Wilson et al., 2010). By accepting the presence of such traits through scientific outputs, fish farmers are provided with tools to understand individual variation in fish for aquaculture practices, in terms of stress responsiveness, fish quality and performance, adaptation, growth, survival, reproduction success and disease resistance, having a positive impact on the productivity, health and welfare of the farmed fish. In this context, selection programmes can be optimized by taking into account the specific behaviour and physiological plasticity of each of the personality groups. In the case of Nile tilapia, for instance, selection for red Nile tilapia (e.g. Homo gold strain) has been undertaken due to high market acceptance in detriment of the common black morph, being then sold at higher prices (Ramírez-Paredes et al., 2012). Nevertheless, it has been shown a low production level due to their lower growth in comparison with wild tilapia. Indeed, effects of water temperature on growth and development of fish have been well documented for many species and, as such, the determination of optimal environmental conditions to each of the distinct personalities can be an advantage to reach optimal growth performance (Azaza et al., 2008). Here, the development of proper group-based tests, such as thermal preference tests, is essential towards selection programmes to screen large number of fish without compromising their prosperity. All of the above could effectively increase the sustainability of the aquaculture industry. Nevertheless, a balanced population regarding distinct coping strategies should be conserved to increase adaptive solutions to uncertain stressors or dynamic environments promoting cooperation and interactions within the fish population (Biro and Stamps, 2008).

1.5.1.3. Personality and Appraisal

Different behavioural phenotypes and their resilience to stressful events have been related not only to inherent predispositions but also to the cognitive appraisal that the individuals take from often complex and dynamic environmental stimuli i.e. the way individuals perceive their surroundings. In order to comprehend the capacity of an organism to regulate its internal state according to the evaluation of the conditions of the surrounding world, rather than using simple stimulus-response processes, is essential to understand individual variability and the adjustment of organisms to changing environments (Faustino et al., 2015). Appraisal theories developed in cognitive psychology have been suggested to provide an operational framework to assess the animals' point of view (Moors et al., 2013) by accounting with both and individuals affective states (Mendl et al., 2009) and both genetic and environmental factors (Enkel et al., 2010) on the evaluation of ambiguous stimuli. Research into affective states or cognition skills of animals have made significant progress over the recent years (Paul et al., 2005; Mendl et al., 2009) and the capacity to scientifically assess animal affective perception should induce the expansion of ground-breaking farming practices based on animals' sentience and their cognitive skills, in order to truly improve their welfare. Several studies have used such a framework to advance to our understanding of the welfare of farmed animals (Désiré et al., 2006; Greiveldinger et al., 2009; 2011; Veissier et al., 2009).

1.5.2. Appraisal concept

Experiencing something unfamiliar or surprising, rather than only physiologically, can also be a source of psychological stress. Stressful experiences are construed as individual-environment transactions and can potentially provide a learning opportunity for adaptation dependant on the meaning of the stimulus to the perceiver (Lazarus, 1999). The basic idea of psychological stress is the hypothesis that the stress response depends on learned expectancies related with the stimuli and the result of the available coping resources. These prospects and the way it influences subjective appraisal were stated to be the major source of individual differences in the stress response (Eriksen et al., 2005).

From Arnold's theory (Arnold, 1960) to the current models (reviewed in Moors et al., 2013), appraisal definition has evolved and adapted to its existence in non-humans animals. **Appraisal** processing can be narrowly separated in two stages: firstly, by the judgement that an individual makes about the psychological relevance of a stimulus to itself to those circumstances and at that point in time (**first appraisal**), and then by the assessment of the resources available to cope with them (**second appraisal**) (Lazarus, 1999; Paul et al., 2005). Thus, it can be considered as a transactional process between the individual and the environment, in which the significance of the event is perceived and evaluated by the individual (Faustino et al., 2015; Moors et al., 2013). By that, it means that such an evaluation assigns the individual's motivational, affective and physiological state, rather than merely by the characteristics of the stimuli or the individual's available resources. Overall, the criteria that contribute to the stressor evaluation process are related to the individual's needs, resources and abilities which often play an integral role in the way individuals cope with these situations and interact with the environment (Scherer, 2009).

According to cognitive theories of emotion, individuals continuously monitor the environment using a set of stimulus evaluation checks (SEC; e.g. intrinsic valence, novelty, prediction error, regularity of the stimuli, capacity for control) in order to evaluate the valence (positive/ negative) and salience (high/ low) of detected stimuli, and also assess the available organismal resources to deal with them (i.e. coping mechanisms) (Faustino et al., 2015; Paul et al., 2005).

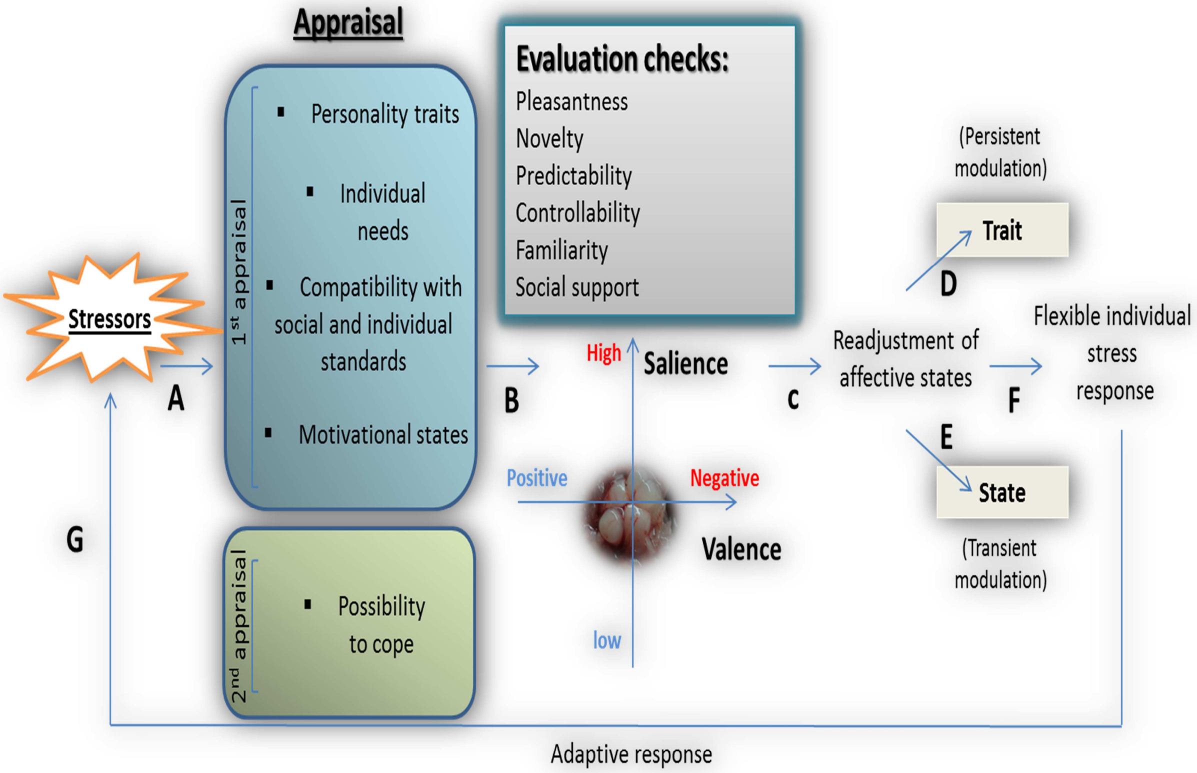


Figure 1.5 |Appraisals processing: (A) animals evaluate environmental changes in an agreement to their needs, inherent dispositions, physiological and motivational states (1st appraisal) and on the available resources to cope with them; (B) they make use of a set of stimulus evaluation checks (SEC) in order to evaluate the valence and salience of the stimuli; (C) readjustment in their affective states; (D) such readjustment become a "trait" if consistent over time with different individuals exhibiting a specific phenotype (between individual variability e.g., optimistic/pessimistic). (E) is a state if readjustment of affective states depends only of the situation or contextual factors at that point in time (within individual variability); (F) outcome of appraisal is translated to the flexible stress response; (E) this flexibility promotes an adaptive response to new environmental changes that may occur (adapted from Faustino et al., 2015).

The outcome of appraisal is translated by an adjustment of the core affective state of the animal to the perceived state of the external environment (see Fig. 1.5 for description of stimuli appraisal processing). Although an integrated study of the different stimulus evaluation checks used by animals is still lacking, empirical evidence for the occurrence of each of these checks has been described in a wide range of animals, from fish to mammals (see Faustino et al., 2015 for a recent review). As stated by Lazarus (1991), the referred criteria became a central tenet of the appraisal concept and implies an interconnection between cognitive and emotional mechanisms (see Fig. 1.6 for detailed information regarding the concepts referred to here and normally used when addressing appraisal). Appraisal is therefore a dynamic process, as it can be modified, altered or adapted to adjust previous evaluations from the environment, making use of relevant and available information to determine the appropriate response (Scherer, 2001, 2009). If individuals are capable of generating flexible responses in accordance with a changing environment, it advocates capacity of mental states, sentience or consciousness, hence cognition. Whether or not all appraisal processes can be regarded as rigorously 'cognitive' is debatable (Lazarus, 1999; Panksepp, 2003) and it depends on the definition employed for the term cognition.

[1]***Sentience*** is the capacity to feel emotions, and involves being conscious of internal and external stimuli, requiring for that some cognitive competences. Sentience shapes motivational states and therefore modulates behavioural and physiological responses of animals. Sentience is a prerequisite for conferring welfare status to animals (Chandroo et al., 2004).

[2]***Cognition*** refers to the processes by which animals capture information about the world and decide to act upon it (Shettleworth, 2001). It includes perception, learning, memory storage, consolidation and retrieval (Dawkins, 2001). Studies on attention, perception, learning and memory are among the most common in cognitive science. The perception of sensations (feelings) can be considered the simplest of the cognitive processes (Duncan and Petherick, 1991).

[3]***Consciousness*** implies the perception of sensations or feelings[5]. It provides behavioural flexibility, efficiency and faster reactions. In evolutionary terms, it offered the organisms an added tool to deal with more complex environments (Panksepp, 2003).

[4]***Emotions*** patterned collection of chemical and neural responses produced by the brain when it detects the presence of an internal or external stimulus with affective relevance. [5]***Feelings*** are the mental representation of these physiological changes i.e. the mental perception of emotions (Damasio, 2001).

[5]***Affective states*** refers to the valence (positive or pleasant/negative or unpleasant) of the subjective mental states i.e. how much an animal wants to have access to a given resource (sense motivational affecting states). This is assumed to be an indirect way of measuring emotions, and thus the subjective states relevant to welfare (Kirkden and Pajor, 2006). Promoting positive affective states would contribute for decreasing the stress endorsed by any environmental changes.

Figure 1.6 | Relevant terms with narrow definitions, commonly used in appraisal and welfare research.

In humans, appraisal cognition relies on high level of conceptual and linguistic processing and counts upon complex cognition-emotion interactions to elicit an appropriate response to the event. Cognitive processes can be considered as a dimension of human emotional states or events, both in the form of appraisals which can trigger the occurrence of particular emotions, and cognitive outputs which can result from emotional states (Paul et al., 2005). In line with this, core affect research has been conceptualized as a dimensional characterization of the emotion experience along two fundamental underlying dimensions: valence (positive/ negative) and intensity (or arousal) (Barrett et al., 2007; Russell, 2003).

1.5.2.1. Appraisal processes in animals

Appraisal processing was proposed to occur at three different levels of consciousness (Leventhal and Scherer, 1987): the **sensorimotor** (automatic triggering of reactions to adaptively significant stimuli); the **schematic** (automatic triggering of learned reactions to previously encountered stimuli, integrates sensory-motor and cues (e.g., visual) signalling specific affective situations); and the **conceptual** (non-automatic, consciously processed reactions. Desiré et al. (2002) suggest that at least both sensorimotor and the schematic levels of appraisal processing are likely to occur in non-human animals, but recent evidence of conceptual processing was shown to occur in lambs (Greiveldinger et al., 2009). We can propose that in order to create internal affective states that support adaptive physiological and behavioural responses towards ecological threats or opportunities, animals must have evolved perceptual and cognitive mechanisms that identify reliable cues in the environment (i.e. aversive vs. appetitive stimuli, respectively). For instance, both flies and crayfish were shown to experience emotion-like states[1] such as fear after repetitive threat stimulus (Fossat et al., 2014; Gibson et al., 2015). Researchers however point out that the research only shows that the individuals experience apparent emotions that have a similar function. It doesn’t mean that these animals necessary have feelings or that they are similar to humans for example. Moreover, animals may express simple basic emotions, such as fear, and those emotion-like reactions were stated to rely on similar non-conscious appraisal processes (Lazarus, 2001). Such primitive `cognitive´ appraisal can then be stimulated on limited perceptual information and it requires only minimal exposure to elicit a response (Paul et al., 2005). Therefore when specific environmental cues deterministically predict an appropriate

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[1] Emotions-like behaviours, affective states or organismic states are used in the core of this dissertation as interchangeably terms, whether to refer to behavioural and physiological responses to stimuli of different valence (pleasant/unpleasant or appetitive/aversive), or of both valence and stimuli salience (intensity/arousal).

response, these responses can be simple reflexes and fixed action patterns elicited by a stimulus in the environment. However, when environmental complexity and variability increase, an environmental cue may no longer be informative and the evolution of appraisal mechanisms that cognitively assess the presence of threats and opportunities in the environment is likely to be projected (Fawcett et al., 2014; McNamara et al., 2013).

1.6. Promoting psychological welfare in cultured fish

1.6.1. Psychological components of the stress response

As stated above, individual variability found in stress responses under the same context may probably be linked with both personality and with the way the stressor is appraised (Ellsworth and Scherer, 2003; Scherer, 2001). This has implications not only for the perception of the stimuli but also for the evaluation of the available coping responses. However, expectancy can be cognitively manipulated by modifying the psychological context in which the stressor is applied, combining both the individual affective state and the surrounding environmental conditions for the appraisal process. This manipulation can be linked to the stimulus appraisal or to the coping response appraisal (see Table 1.2 for examples of appraisal components tested in fish) and intent, in a simplistic way to address this issue, increase the optimism of the individual to respond to any stressor. The lack of knowledge on the precise mechanisms underlying the integration of psychological aspects in the stress physiology of fish remains however unclear and there is disagreement regarding the existence of sentience or consciousness in these taxa.

As presented in Fig. 1.6, sentience can be summed up in an ethical context as the ability to experience pleasure and pain (*i.e.* subjective perceptual experience (Appleby and Sandoe, 2002; Dawkins, 1998). Being increasingly used in animal welfare evaluations and recognised as adaptive products of natural selection (Chandroo et al., 2004), affective states are not directly observable, and behavioural and physiological proxies have to be used in order to probe animal affective states. Indeed, recent studies demonstrated similarity in cognitive complexity between fish and higher vertebrates (Demski, 2013). In this context, Chapter II.1 and Chapter II.2 were developed to address this issue (e.g. evidences of mental capacities to experience discomfort, anxiety, frustration, comfort, boredom, alertness or fear) and concomitantly assess if the target species used in this dissertation are effectively capable of stimuli appraisal processing. Recent reviews of fish cognition suggest fish show a rich repertoire of complex and flexible behaviours. For instance, fish were shown to have functional long-term memories, develop complex traditions, show signs of Machiavellian intelligence, cooperate with and recognise one another and are even capable of tool use (Brown, 2011; Brown, 2014; Bshary et al., 2002). Even with this amount of knowledge, the way fish perceive stimuli and the affective value attributed to them are still poorly understood phenomena.

Table 1.3 |Example of research conducted to test appraisal components in fish

Appraisal component	Level of processing	Function	Fish	Reference
Novelty	Sensorimotor	Alerting	Zebrafish	(Wong et al., 2010)
Pleasantness	Conceptual	Approach/avoidance	Zebrafish	(Xu et al., 2007)
			Zebrafish	(Al-Imari and Gerlai, 2008)
Predictability	Schematic	Anticipation	Cichlid fish	(Galhardo et al., 2011)
			Zebrafish	(Piato et al., 2010)
			Salmon	(Madaro et al., 2015, 2016)
		Prediction error	Salmon	(Vindas et al., 2012, 2014b)
			Trout	(Vindas et al., 2014)
Controllability	Conceptual	Adjustment	Trout	(Carpenter and Summers, 2009)

Faustino et al. (2015) emphasise, and we quote, the importance of using and testing relevant appraisal components performed at different levels of processing and under similar conditions. By investigating if fish can effectively distinguish between and respond to these different checks, the array of emotions may be estimated. The integration of psychological and biological approaches to mechanisms of information processing occurring at different levels has been demonstrated in non-human animals (Désiré et al., 2002; Greiveldinger et al., 2007; 2009; 2011; van den Bos et al., 2003). By subjecting fish to different appraisal checks and measuring their underlying physiological and behavioural responses, direct relationships between presumed appraisals and measurable emotional outcomes can be established. Such possibility offers' the opportunity to assess individual variation in appraisal tendencies and to assess how it affects relevant biological processes, such as learning, environment preference, foraging, reproduction, susceptibility to diseases or disorders and others.

As noted before, the interplay between personality and appraisal in fish is still vague. Within this framework, a case is made in Chapter III and Chapter IV for the possibility of using different psychological components (e.g. predictability, social support and controllability) as modulators of important commercial fish farm stress responses. The development of such paradigms will offer fish farmers' simple and inexpensive tools to reduce the stress of aquaculture practices, by improving the psychological condition of fish, hence the suitability and productivity of the aquaculture system.

1.6.2. Stimuli-related components: predictability, pleasantness and social support

An organism's ability to determine if the stimulus is relevant to elicit further processing relies upon the basis of stimuli-related appraisal components, namely predictability, pleasantness and social support (Ellsworth and Scherer, 2003). By modifying the psychological context in which the stressor is applied, different authors were capable of manipulating the processing of the event and make inferences about the negative versus positive affective states of the individuals. The **predictability** (i.e. expecting the occurrence of a stimulus (Ursin and Eriksen, 2004) of a stressor affects the stress response and this fact has been described in humans and animals, including fish (see Table 1.3 for examples in fish). By offering the possibility to anticipate either rewards or punishment situations, it allows the animal to predict and to activate the proper coping mechanisms to deal with the ambiguity from their environment and consequently increases their welfare (Spruijt et al., 2001). Studies with sheep showed that, by predicting an event in an associative scheme with a light cue, lambs showed reduced startle responses, compared to their counterparts under unpredictable conditions (Greiveldinger, et al., 2007). In rats, different predictable stressors alleviate stress responses and promote place preferences and spatial learning when compared to the same stressors presented in an unpredictable schedule (Orsini et al., 2002; Prior, 2002). Regardless of the valence of the stimulus, it is apparent that animals prefer predictable rather than unpredictable events. Galhardo et al. (2011) using *Oreochromis mossambicus* as fish model, reported that predictable aversive regimes decreased the individuals' cortisol levels. The same author observed that predictable feeding schedules triggered higher levels of anticipatory behaviour and a tendency for higher cortisol levels. The same conclusions were found for seabream on the effects of feeding schedule (Sánchez et al., 2009). In Atlantic salmon, announcing a chasing event by means of a light cue changed the individual's behavioural response reducing the stress, compared to non-announced events (Madaro et al., 2015). Also in zebrafish, signalled aversive events were shown to be less stressful (Piato et al., 2011) and this was determined by the possibility to anticipate the stimulus. In agreement, the relieving effect of predictability also depends upon the stressor's properties (nature, intensity and frequency), the reliability of the signalling system and on the time elapsed between the signal and the onset of the event (Sapolsky, 2004). For instance, studies in halibut showed differences in the stress response depending on whether the CS was overlapped with US (e.g. delay conditioning) or if there was a temporal gap between both (e.g. trace conditioning) (Nilsson et al., 2010). Also, it is highly unlikely that the expectation of a very harmful or a very recurrent event will relieve the stress response. The effect of predictability on the modulation of the stress response is addressed in Chapter III.

Describing how much an animal will, having access, contact, or avoid a given resource with a certain valence (appetitive or aversive), is assumed to be an indirect way of measuring subjective states relevant to welfare (kirkden and Pajor, 2006) and have been used to assess **pleasantness** of events. In humans, current research assessing the effect of pleasantness has been mostly focusing upon the olfactory perception of odours and its preference and on the emotional reaction to the effect of repeated exposure to odours (Delplanque et al., 2015; Ferdenzi et al., 2014). The olfactory system responds faster and more accurately to ecologically-relevant stimuli that signal a potential danger for humans' survival (Boesveldt et al., 2010). It is then arguable that dissociating between pleasant or unpleasant is of crucial value, since unpleasant can have ecological repercussions for fish, either by avoiding aversive situations or approaching rewarding situations. Using a classical conditioning paradigm, zebrafish learned to associate a light/dark compartment (CS) with an electric shock. The individuals learn to swim out the light to the dark compartment to avoid an electric shock, hence pointing it out as unpleasant (Xu et al., 2007). Furthermore, Al-Imari and Gerlai (2008) showed in the same species the value attributed by the individuals to social conspecifics. After a learning period fish associate the presence of conspecifics to a visual cue, and in the absence of conspecifics the fish remain near the source of the cue. Unpaired cue-conspecific groups decreased the time spent by the fish close to the cue, thus signalling the presence of conspecifics as pleasant. Conditioning place preference (CPP) and avoidance (CPA) paradigms used to assess the pleasantness and unpleasantness of stimulus with different valences are discussed in Chapter II. In Chapter III.1 the predictability of both appetitive and aversive stimulus is addressed and distinct responses lined with the pleasantness of the stimulus are discussed.

In agreement with the results from Al-Imari and Gerlai (2008), goldfish were shown to be willing to remain close to conspecifics during low intensity shock stimuli that had been previously shown to elicit avoidance (Dunlop et al., 2006). The presence of a conspecific significantly changed the avoidance response to an electrical shock. These results suggest that shock avoidance in fish is not purely a reflex action, allowing an insight into fish sentience and the value of social support on the modulation of stressful event. Hence, **social support** seems to create a psychological context where the stress response is significantly reduced and where animals display better welfare (DeVries et al., 2003; Sapolsky, 2004). In animals, it is recognized that social interactions could either amend the stress response or aggravate it. The presence of counterparts can provide adaptive value of social buffering or contagion for coping with stress exposure (Beery and Kaufer, 2015; DeVries et al., 2003) but in fish this information is still poorly understood. It is then fundamental to understand how social context influences individual choices and flexibility in highly dynamic environments. One reference to this issue can be found in the work done by Galhardo et al. (2012) in which they showed, even though not being implicit, that social buffering reduced fear and anxiety in a cichlid fish, associated with a novel object paradigm test. Research has been done particularly addressing the effect of familiarity (whether conspecific or not) on the stress response to stressful conditions or even on the stress response of fish kept in isolation under different aversive paradigms. For instance, it was shown that social isolation increases cortisol levels and modifies the feeding and agonistic patterns of interaction in fish (Earley et al., 2006; Martins et al., 2006). How these responses can be ameliorated or worsened by the presence of conspecifics remains unclear. If appraisal depends on the interaction between stimulus characteristics and internal state, an animal expressing a positive affective state created by social support, will tend to evaluate an ambiguous stimulus as more positive (Mendl et al., 2009), thus social support can be used to psychologically decrease the effect of aversive ecological or biological challenges. This issue will be further addressed in Chapter III.2.

1.6.3. Response-related modulators: Controllability

The intrinsic characteristics of an event, such as its **controllability** i.e. the capability to successfully increase the exposure to positive stimuli, decrease/avoid the aversive stimuli (Ellsworth and Scherer, 2003) or adjusting their response when there is no control over the stimulus (Desire et al., 2002) has been proposed to modulate the stress response. Furthermore, it is most likely to shape the affective states of the individuals when offered the possibility to learn how to avoid it or to adapt to it (Fiore et al., 2015). It is common sense that the perception of control can attenuate the effect of daily challenges by increasing coping ability, whereas lack of control or loss of control (after having such possibility) increases stress by projected helplessness states (i.e. perception that cannot avoid or deal with the stressor (Seligman, 1975); have been associated to anxiety-like states suggestive of fearfulness (Landgraf et al., 2016)). In cultured fish, this may be the difference between success or failure. There are few examples found in the literature regarding this issue in fish. To the best of our knowledge, few studies have shown the effect of controllability upon the fish stress response to an aversive situation. Rainbow trout (*Oncorhynchus mykiss*) exhibited a lower cortisol response when given the possibility to escape from a larger counterpart (Carpenter and Summers, 2009). Even though not directly, in salmon, it was shown that behavioural reactions were indicative of the affective state of frustration in response to an omitted expected reward (Vindas et al., 2012). Here, the possibility to increase the exposure to the reward was not measured, rather the omission of an expected a priori predictable reward. Nevertheless, it can be argued that predictable events offer, to some extent at least, some sense of control. Thus the affective states originated by that paradigm can be, to some level, comparable to the effect of losing control. This issue is further discussed in detail in Chapter IV.1 and Chapter IV.2, focusing upon the effects of controllability regarding the enhancement of coping ability and on the ecological repercussions promoted by control over the environment, respectively.

1.7. Fish as a psychological stress model

Fish are highly valuable to ecological and biological research in countless aspects. They are relatively easy to handle and keep and their maintenance is not overly labour intensive. In addition, many fish have short reproductive and developmental stages. Fish are highly sensitive to environmental and social constrains which are aligned with individual variability or divergent individual coping mechanisms thus it make them a significant model for the study of psychological stress and associated studies. This contributes to the study of specific cognitive abilities since, and as described before, fish show consistent behaviour and physiological responses over time and across contexts. The occurrence of cognitive appraisal in fish has been documented in different species and all stimulus evaluation checks involved in appraisal have been described. However, the consistency across species and contexts is still under-explored. For this dissertation, the fish models used here to characterize such appraisal components were Gilthead seabream, Atlantic seabass and Nile tilapia. The first two species are undoubtedly the dominant species in the northern countries from Mediterranean whereas that Egypt, in the southern of Mediterranean, most contributes for Nile tilapia index as the second most produced fish around the world after common carp (FAO, 2014). Traditionally cultured in extensive regime in coastal lagoons and saltwater ponds, these species readily adapted to intensive regimes, which was an important step for large-scale production. With the boom of production in these species an increased public concern regarding their welfare is perceived across the EU. It is recognized that fish are subjected to several major stressors under commercial farming that are known to impair their welfare and their stress response is also highly variable between individuals. The most important criteria regarding aquacultured fish including these species are health value, food safety and quality. Therefore, the study of the psychological components modulating the fish stress response to improve their welfare is not only eligible; it is ethical relying upon the recent evidence for fish sentience. In addition, characterizing and understanding distinct behavioural phenotypes in relation to how they appraise their environment can ultimately safeguard their biological success under culture conditions.

1.8. Objectives

The main purpose of this project was to explore and investigate if the appraisal that fish make of environmental stimuli modifies their behavioural and physiological responses. In addition how distinct personality traits influence the evaluation of such stimuli was characterized. As a complementary objective, the mechanisms underlying the expression of appraisal in seabream and seabass were also characterized. In doing so, this dissertation will contribute toward uncovering the links between brain function, behaviour and adaptive stress physiology in important commercial fish. For this purpose, different goals were stablished:

- ✓ Characterize distinct personality traits in seabream, seabass and Nile tilapia;
- ✓ Validate conditioned place preference/avoidance (CPP/CPA) tests as a method to assess the affective valence of environmental stimuli in teleost fishes, hence showing the capability of perception of fish in respect to their surrounding environment. In this context, how appraisal differs between individuals with different personality traits were assessed (**Chapter II)**;
- ✓ Determine how seabream perceive stimulus salience and valence by using predictability over aversive and appetitive stimulus, how it is being represented in the brain and their behavioural and physiological correlates (**Chapter III.1**). The combination of both social support and predictability were used in **Chapter III.2** to understand how predictability affects the cognitive stress response of seabass in both social isolation and social conditions; and to infer on how social counterparts ameliorates the stress response;
- ✓ Investigate how seabream and seabass appraise their environment when having the possibility to control their behaviour towards an aversive situation. As such, neural states and physiological responses were used to infer if such a possibility increases their coping ability towards such events (**Chapter IV.1**). In addition, the interplay between thermal choices (e.g. by having control over the environment), animal personality and the impact of infection upon this interaction was examined (**Chapter IV.2)**;

In doing so, this dissertation will contribute to uncover the links between brain function, behaviour and adaptive stress physiology in important commercial fish which may give future ideas on how individuals evaluate their environment, hence contributing for sustainability of aquaculture by improving the welfare of farmed fish.

This thesis was conducted within the European project COPEWELL – "A new integrative framework for the study of fish welfare based on the concepts of allostasis, appraisal and coping styles" with seabream and seabass as two main target species. The work presented in the next chapters, despite being totally developed, analysed and written by myself (with the exception of the research papers from chapter II, written as a joint effort between myself and Doctor Sandie Millot, from IFREMER, France), was only made possible by the collaborations, scientific discussions and research developed by the partners integrating this project.

Paradigms to measure cognitive appraisal in fish

Chapter II

CHAPTER II-1

Use of conditioned place preference / avoidance tests to assess affective states in fish

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The following manuscript was attained by the joint effort between myself and Doctor Sandie Millot. Both authors were involved in all the steps of the experiment, data collection and analysis and manuscript preparation.

Use of conditioned place preference / avoidance tests to assess affective states in fish

Abstract: Animal welfare has been defined as the balance between positive and negative experiences or affective states. Despite the growing evidence of complex cognitive abilities and the expression of affective states such as pain and fear, very little is known about ability to experience memory based affective states in non-mammalian animal models. The goal of this study was to validate conditioned place preference/avoidance (CPP/CPA) tests as a method to assess the affective valence of environmental stimuli in teleost fishes. Physiological and behavioural indicators of affective state were used to characterize the response to *a priori* appetitive and aversive stimuli in CPP/CPA tests in gilthead seabream (*Sparus aurata*). Fish were tested individually in a CPP/CPA tank divided into two halves, with one half uniformly white and one half marked by dotted wall patterns. During an initial habituation phase fish were placed in a central alley for 10 min and afterwards allowed to swim freely throughout the whole tank during 20 min in order to determine their initial preferred and non-preferred side (IPS/INPS). During the training phase, fish were presented either with a single aversive stimulus in the IPS (chasing with a dip net) or with a repeated appetitive stimulus (release of pellets) in the INPS. The test phase consisted of the same procedure as the habituation phase. The behaviour of each individual was video-recorded and analysed with video-tracking software. Fish submitted to appetitive stimulus increased significantly the time spent and the distance moved in the stimulation side, while fish exposed to aversive stimulus decreased significantly the time spent in the stimulation side, increased the distance moved in the non-stimulation side and showed an increase in cortisol level. Therefore, the use of behavioural (individual swimming activity) and physiological (plasma cortisol concentration) indicators of affective state during the CPP/CPA test allowed to validate the use of this test as a way to assess the affective valence attributed by fish to different environmental stimuli. Finally, this study also shows that fish are able to retain memories of events with positive/negative valence which are retrieved by environmental cues.

Keywords: *Sparus aurata*; conditioning; swimming activity; cortisol; place learning; welfare

Introduction

We have in recent years witnessed a considerable increase in the public and scientific debate regarding the welfare of animals in human custody. Current concepts of animal welfare acknowledge the fact that welfare incorporates not only physical well-being but also mental well-being and the possibility of animals to live according to their nature and to express their full behavioural repertoire (Brambell, 1965; Korte et al., 2007; Ohl and van der Staay, 2012). This view implies that animals may experience feelings (i.e. affective states *sensu* Mendl et al. 2010), such that they will attempt to minimise negative affective states (e.g. fear) while seeking positive ones (e.g. pleasure; Dawkins, 1983; Duncan, 1996; Boissy et al., 2007). However, affective states are not directly observable, and behavioural and physiological proxies have to be used in order to probe animal affective state. Preference/avoidance and motivation tests have been used for this purpose, based on the assumption that affective states are linked to motivation/preference and ultimately drive behaviour (Kirkden and Pajor, 2006). In these tests the animal is given some control over its environment, so we can observe their choices in preference tests, or how much they are willing to work to access or avoid given resources or threats in operant motivation tests (Endo et al., 2002; Herrero et al., 2005; Kirkden and Pajor, 2006; Yue et al., 2008).

Teleost fishes are currently central in the animal welfare debate, considering the vast numbers of individuals reared in aquaculture (Ashley, 2007; Huntingford and Kadri, 2009). Fish are also gradually introduced as an alternative to small mammals in biomedical and behavioural research (Epstein and Epstein, 2005; Steenbergen et al., 2010; Thorgaard et al., 2002). In this context, it is discussed to what degree fishes possess conscious awareness and mental capacities to experience pain and discomfort (Bekoff, 2007; Browman and Skiftesvik, 2011; Chandroo et al., 2004; Galhardo, 2009; Huntingford et al., 2006; Rose, 2002). Despite the increasing interest in fish welfare and the recent evidence that fish have the capability to experience affective states (Chandroo et al 2004; Galhardo and Oliveira, 2009), the quantification of fish motivation as an indicator of its needs has been only measured using a push-door paradigm test (Galhardo et al., 2011). Generalization of the use of motivation tests across different fish species has been hampered by the fact that the operant task needs to fit the natural behaviour of each species, which varies to a great extent among fish. For example, the successful use of the push-door operant task in tilapia mentioned above took advantage of the robustness of its snout that is used by males in mouth-digging of nests and in mouth-fighting, and would not be appropriate for other species. Therefore, it would be of great value to develop a common motivation test to be used across different species.

The conditioned place preference (CPP) paradigm is a commonly used technique in behavioural neuroscience to evaluate rewarding and aversive effects of addictive drugs (Prus et al., 2009; Tzschentke, 2007). In general, this task involves the establishment of an association between a specific environmental stimulus and a positive or a negative reward. Typically, a positive or negative reward is repeatedly paired with a location marked with a cue so that the animal associates the marked location with the reward, and eventually develops a preference or avoidance (conditioned place avoidance, CPA) even in the absence of the stimulus (Mathur et al., 2011a). Therefore, CPP and CPA paradigms offer the possibility to assess the reward or aversive value that animals attribute to different environmental stimuli in general and can easily be used across different species. In fish, CPP has been used to determine the reinforcing effects of natural rewards, such as food, or social context (Lau et al., 2006; Zala and Määttänen, 2013), but much more frequently of addictive drugs (e.g. Mattioli et al., 1998; Coelho et al., 2001; Darland and Dowling, 2001; Ninkovic and Bally-Cuif, 2006; Mathur et al., 2011a,b; Klee et al., 2011). This test has rarely been used in the field of animal welfare as a tool to evaluate the value that fish attribute to environmental stimuli, hence allowing an indirect assessment of their affective state.

In this study we investigated how Gilthead seabream (*Sparus aurata*), one of the most important commercial species in Europe, evaluated an *a priori* appetitive (food) or aversive (net chasing) stimuli in a CPP/CPA paradigm. In order to validate this paradigm as a measure of valence attributed by the fish to the appetitive/aversive stimuli, we used physiological (cortisol, glucose and lactate levels) and behavioural (distance travelled and swimming activity) variables independent of the CPP/CPA. The broader goal of this work was to validate the use of CPP/CPA test as a method to assess the value that fish attribute to stimuli with different valences by using several physiological and behavioural indicators of internal state.

Material and methods

Experimental procedures 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 Portuguese competent authority for the protection of experimental animals (Direção Geral da Alimentação e Veterinária, Portugal; permit number 0420/000/000-n.99-09/11/2009).

Experimental fish, housing and feeding

Fish were obtained from a commercial fish farming (MARESA Mariscos de Esteros SA, Huelva, Spain) and transported to Ramalhete Research Station (Faro, Portugal). Fish were housed in stock tanks (500L) under standard rearing conditions during 3 months before the start of the experimental procedures (rearing density from 2 kg m^{-3} to 9 kg m^{-3}). Fish were fed a commercial diet (Aquagold 3 mm, Aquasoja, Sorgal SA, Portugal; 44% crude protein, 14% crude fat, 8% ash, 2.5% crude fibres, 1.0% phosphorus) using automatic feeders (1.5% $BWday^{-1}$). Fish were reared in open water circuit tanks, with a temperature of 20 ± 3 °c, salinity of 34 ± 2 ‰ and dissolved oxygen above 95 %, and a 12L: 12D photoperiod was employed with light on at 08:00. One month before the start of experimental procedures, 120 fish were randomly selected, anaesthetised with 2-phenoxyethanol (0.3 ‰, Sigma-Aldrich) and individually identified with a visible implant elastomer tag (VIE; Northwest Marine Technology, USA) in the caudal fin. At the start of the experiment the body mass of the fish was 45 ± 2g (mean ± SE).

Set up and experimental procedures

Two days before the start of the conditioned place preference/avoidance test (CPP/CPA), 4 groups of 6 fish each (with distinct VIE) were placed in 4 different 100L home tanks located in the experimental room. This was done to acclimatize fish to their new environment. The photoperiod and water temperature, salinity and oxygenation were the same as in rearing tanks. This procedure was repeated 5 consecutive times in order to test 30 fish per treatment: appetitive (APP), control appetitive (APPctr), aversive (AVER) and control aversive (AVERctr).

The CPP/CPA test was performed in 6 glass aquaria of 80L (70 cm length x 40 cm width and 30 cm depth). Each aquarium was divided into two halves by a 10 cm wide grey and sheltered central alley (start chamber): one half marked by white and the other half marked by dotted wall patterns (Figure 2.1.1). One infrared LED projector (IR-294S/60, Monacor®) was placed beneath each aquarium.

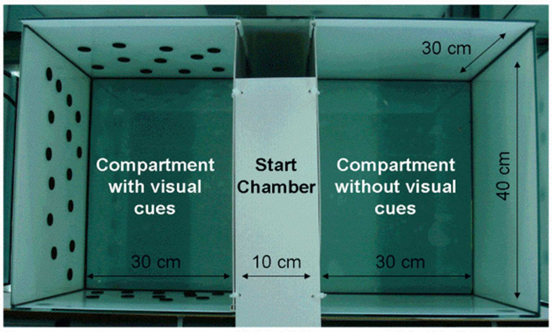

Figure 2.1.1 |Experimental tank: CPP/CPA test glass aquarium (80L) divided in three compartments: one start chamber with grey walls and sheltered and two lateral compartments with white walls and with or without visual cues (black spots).

The CPP/CPA test consisted of three experimental phases conducted over a 3-5 days period. An initial habituation phase was performed on the first day. During this phase, each fish was placed individually in the sheltered start chamber for 10 min. Afterwards, partitions between the start chamber and the lateral compartments were gently removed. Fish were allowed to swim freely throughout the whole tank for 10 min. For each individual the initial preference for white or dotted side (IPS > 50% of the total time) was assessed through a 20 min recording. Then the fish was put back in the home tank. Animals that showed a very strong initial preference (>90% time spent) in either side or strong freezing behaviour (< 500 cm distance moved) were excluded from the study. Therefore, animals that showed an initial preference between 50.1% and 89.9% for either side and which had moved more than 500 cm were used for data analyses. Similarly to Lau et al. (2006), Darland and Dowling (2001) and Kily et al. (2008) we used a single exposure to the CPP/CPA test to establish the fish baseline preference. Mathur et al. (2011a) have shown in zebrafish that repeated exposure to the tank did not significantly alter the baseline preference in a CPP testing.

The habituation phase was followed by a conditioning phase, during which treatments differed between the appetitive and aversive stimulus groups. For the aversive stimulus (AVER), fish was placed in the same aquarium as during the habituation phase but retained in the IPS for 10 min, hereafter termed the stimulation side (SS). Afterwards, the fish was chased with a net during 10 s each 2 min for a period of 10 min. For the appetitive stimulus

(APP), fish (food deprived for 24h) was placed in the non IPS (new SS) for 10 min and then received one food pellet each 30 s during 10 min. Based on preliminary results, the aversive treatment was performed only one day in order to avoid fish habituation to the stimulus contrary to the appetitive treatment which was repeated during three consecutive days for an improved conditioned response. The control fish (AVERctr; APPctr) were handled exactly the same way as the tested animals except that the stimulus was omitted during the training phase. After each treatment, fish was put back in the home tank.

The test phase was performed on the last day of the experiment (the third day for the aversive stimulus or the fifth day for the appetitive one) and consisted exactly of the same procedure as the habituation phase in order to record any behavioural changes. After this last phase, fish were immediately caught and euthanized with an overdose of 2-phenoxyethanol (1 ‰, Sigma-Aldrich). Blood was thus sampled 30 min after fish were transferred to the experimental aquarium (based on Arends et al., 1999). Blood was withdrawn within 3 min from the caudal vein using heparinised syringes and centrifuges at 2000 x g for 20 minutes at room temperature. After centrifugation plasma was frozen in dry ice and stored at -80 °c for glucose (QCA, Spain) and lactate (Spinreact, Spain) kits analysis. Plasma cortisol levels were measured by means of a commercial ELISA kit RE52061 (IBL Hamburg, Germany), with a sensitivity of 2.5 ng ml^{-1}, and intra and inter-assay coefficients of variation (CV) of 2.9 and 3.5 %, respectively. After blood sampling, fish were identified and measured for standard length (cm).

During each phase, individual behaviour was recorded by infrared sensitive video camera (TVCCD-623-COL, Monacor®, Denmark) equipped with infrared filter (dark red, Schneider Optics, USA) and positioned 1m above the tank. The videos were stored in AVI files on a hard drive and analysed afterwards with the Lolitrack 2.0 software (Loligo® Systems, Denmark). Before each video analyses, the background image of each tank was divided into three arenas (Arena 1 = white side, Arena 2 = grey middle alley, Arena 3 = dotted side). For each tank the background was calibrated by marking the length of the Arena 2 in the image and entering its actual value (10 cm). The Lolitrack 2.0 software tracks the fish as a dark object on a light background. By using infrared light beneath the tank we avoid light reflexion on the water surface and optimise the fish tracking by the software. The following parameters were quantified by the software: time spent in each arena (min), distance travelled in each arena

(m) and the swimming speed in each arena (cms^{-1}). In order to remove the influence of fish size in swimming speed data, these values were transformed in body length per second (BLs^{-1}).

Statistics

Statistical analyses were performed using Statistica 7 software (Statsoft, USA). The results were expressed as mean ± standard error (SEM). Data were analysed for normality with a Shapiro–Wilk test and for homoscedasticity with a Bartlett's test. For each treatment (i.e. CPP test for appetitive and CPA test for aversive) repeated-measures ANOVA's were used to analyse: (1) the differences in time spent by the experimental and control fish in the stimulation side (SS) before and after the conditioning phase (2-levels repeated factor: before *vs*. after; categorical variable: experimental *vs*. control fish); and (2) the differences in distance travelled (m) and swimming speed (BLs^{-1}) between experimental and control fish, experimental phases (i.e. before and after conditioning phase) and tank sides (4-levels repeated factor: before SS *vs*. before non-stimulation side (nSS) *vs*. after SS *vs*. after nSS; categorical variables: experimental *vs*. control fish). Planned comparisons were subsequently used to test differences between the habituation and the test phase of each treatment and between the control and the experimental groups both at the habituation and at the test phase of each treatment. One way ANOVA and planned comparisons were used to analyse the differences in plasma concentrations of cortisol ($ngml^{-1}$), glucose ($mmol^{-1}$) and lactate ($mmol^{-1}$) between experimental and control fish for each treatment (i.e. CPP test for appetitive and CPA test for aversive).

Results

From the 120 fish tested in this study, 20 fish did not comply with our acceptance criteria and were thus rejected from the analysis. This resulted in 25 fish being analysed per treatment. A null model of side preference was tested by comparing the observed fish distribution to the theoretical homogeneous distribution in stimulation side and non-stimulation side (50% in each zone) by a Kolmogorov–Smirnov test. No systematic side preference was observed during the habituation phase (stimulus conditioning was performed 44 times in the white side *vs* 56 times in the dotted side; $d= 0.26$; $p > 0.05$).

Time spent in the stimulation side

On average fish subjected to the appetitive stimulus showed a high increase of the time spent in the SS during the test phase (Fig. 2.1.2), whereas fish exposed to the aversive stimulus showed a decrease of the time spent in the SS during the test phase (Fig. 2). Both for the appetitive and for the aversive test the repeated measures ANOVA's revealed no main effect of treatment (overall control *vs*. experimental group) and significant effects of the repeated factor (before *vs*. after exposure to stimulus) and of the interaction between treatment and the repeated factor (Table 2.1.1).

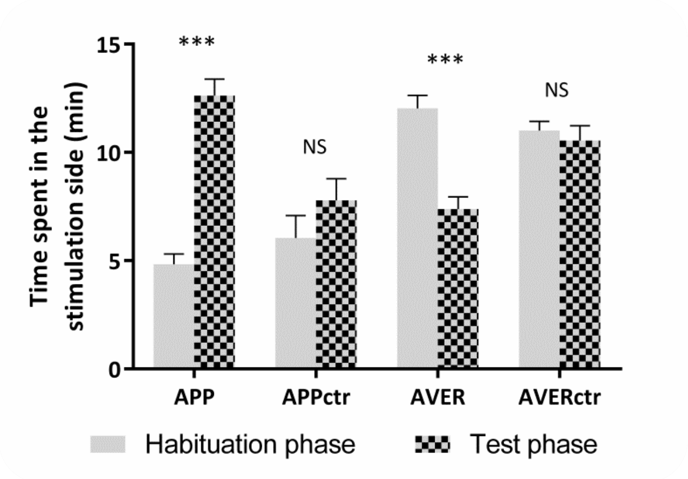

Figure 2.1.2 |Time spent in the stimulation side: time spent (Mean ± SEM; in min) by the fish in the stimulation side during the habituation and test phases for each treatment. Repeated ANOVA, NS = non-significant; *** < 0.001.

Planned comparison analyses showed that in the appetitive test there was a significant increase in the time spent in the SS for experimental group but not for the control group, such that the time spent in the SS, which did not differ between the experimental and control groups before the exposure to the appetitive stimulus, becomes significantly different after the exposure to the stimulus (Table 2.1.1). Conversely, in the aversive test there was a significant decrease in the time spent in the SS for the experimental group but not for the control group, such that the time spent in the SS, which did not differ between the experimental and control groups before the exposure to the aversive stimulus, becomes significantly different after the exposure to the stimulus (Table 2.1.1).

Table 2.1.1 | Results of repeated-measures ANOVA and planned comparisons used to analyse the differences in time spent by the experimental and control fish in the stimulation side (SS) before and after the conditioning phase (2-levels repeated factor: before *vs*. after; categorical variable: experimental (E) *vs*. control (C) fish).

	Appetitive treatment		**Aversive treatment**	
	$F_{1,48}$	***P*-value**	**$F_{1,48}$**	***P*-value**
Treatment (T) Main effect	2.36	0.13	3.17	0.08
Repeated factor (R) main effect	65.55	<0.001*	18.69	<0.001*
T x R interaction	27.93	<0.001*	14.23	<0.001*
Planned comparisons				
C_{before} *vs*. C_{after}	3.95	0.053	0.15	0.698
E_{before} *vs*. E_{after}	89.53	<0.001*	32.77	<0.001*
C_{before} *vs*. E_{before}	1.05	0.31	2.09	0.16
C_{after} *vs*. E_{after}	11.96	<0.001*	11.42	<0.01*

Distance travelled

The main effect of treatment (i.e. overall control *vs*. experimental group) on the distance travelled was not significant, but there was a significant main effect of the repeated measure (i.e. before SS *vs*. before nSS *vs*. after SS *vs*. after nSS) and a significant interaction between treatment and the repeated measure, both for the appetitive and for the aversive experiments (Table 2.1.2). In the appetitive experiment fish increased the distance travelled in the SS in the test phase both in the control and in the experimental group (i.e. exposed to the appetitive stimulus), whereas the distance travelled in the nSS increased only in the control treatment (Table 2.1.2, Fig. 2.1.3).

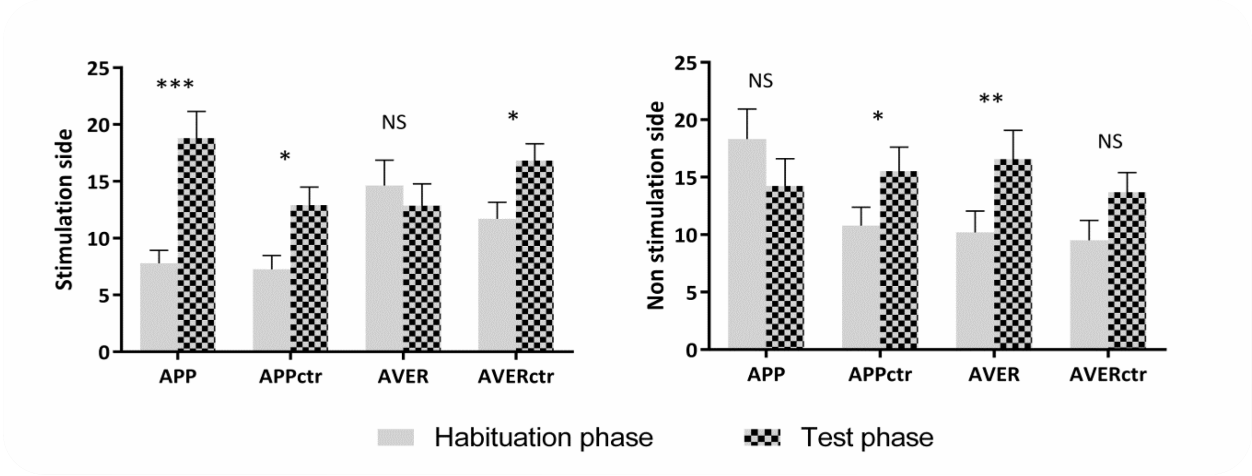

Figure 2.1.3 | Distance travelled (Mean ± SEM; in m) by the fish in the stimulation side and the non-stimulation side during the habituation and test phases for each treatment. Repeated ANOVA, NS = non-significant; * < 0.5; ** < 0.01; *** < 0.001.

Table 2.1.2 | Results of repeated-measures ANOVA and planned comparisons used to analyse the differences in distance travelled (m) between experimental (E) and control (C) fish, experimental phases (before and after conditioning phase) and tank sides (stimulation side (SS) and non-stimulation side (nSS) (4-levels repeated factor: before SS vs. before nSS vs. after SS vs. after nSS; categorical variables: E vs. C fish).

	Appetitive treatment		Aversive treatment	
	F statistic	***P*-value**	**F statistic**	***P*-value**
Treatment (T) Main effect	$F_{1,48}=2.50$	0.12	$F_{1,48}=0.09$	0.77
Repeated factor (R) main effect	$F_{3,144}=9.65$	<0.001*	$F_{3,144}=7.06$	<0.001*
T x R interaction	$F_{3,144}=4.98$	<0.01*	$F_{3,144}=3.13$	<0.05*
Planned comparisons ($F_{1,48}$)				
C_{before} *vs*. C_{after} in SS	5.55	<0.05*	6.67	<0.05*
E_{before} *vs*. E_{after} in SS	25.02	<0.001*	0.79	0.38
C_{before} *vs*. C_{after} in nSS	6.02	<0.05*	4.02	0.051
E_{before} *vs*. E_{after} in nSS	3.72	0.06	9.38	<0.01*
C_{before} *vs*. E_{before} in SS	0.54	0.46	1.21	0.28
C_{after} *vs*. E_{after} in SS	2.19	0.15	2.68	0.11
C_{before} *vs*. E_{before} in nSS	10.54	<0.01*	0.07	0.79
C_{after} *vs*. E_{after} in nSS	0.005	0.95	0.92	0.34

There was a significant difference in the distance travelled between the experimental and the control treatment only in the habituation phase for the nSS (Table 2.1.2, Fig. 2.1.3). In the aversive experiment the distance travelled increased in the test phase for the experimental treatment in the nSS and for the control treatment in SS. (Table 2.1.2, Fig. 2.1.3). Furthermore, there were no significant differences in the distance travelled between the experimental and the control treatments either in the habituation or in the test phase for either the SS or the nSS (Table 2.1.2, Fig. 2.1.3).

Swimming speed

In the appetitive experiment neither the main effect of treatment (i.e. overall control *vs*. experimental group), nor the main effect of the repeated measure (i.e. before SS *vs*. before nSS *vs*. after SS *vs*. after nSS), nor interaction between treatment and the repeated measure, were significant on the swimming speed (Table 2.1.3). However, swimming speed significantly increased from the habituation to the test phase in SS for the control treatment and in the nSS for the experimental treatment (i.e. exposed to the appetitive stimulus; Table 2.1.3, Fig. 2.1.4). There were no significant differences between the control and the experimental treatments either in the habituation or the test phase, either for the SS or for the nSS (Table 2.1.3, Fig. 2.1.4). In the aversive experiment there was a main effect of the repeated factor on swimming speed (i.e. before SS *vs*. before nSS *vs*. after SS *vs*. after nSS), but neither the main effect of the treatment (i.e. overall control *vs*. experimental group) nor the interaction between the treatment and the repeated factor were significant (Table 2.1.3).

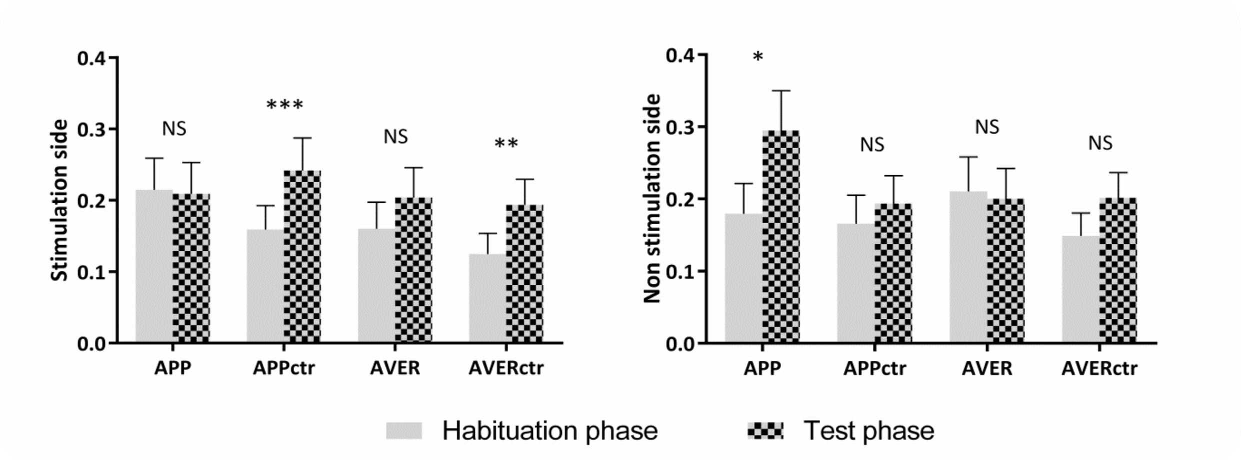

Figure 2.1.4 |Swimming speed (Mean ± SEM; in BLs-1) by the fish in the stimulation side and the non-stimulation side during the habituation and test phases for each treatment. Repeated ANOVA, NS = non-significant; * < 0.5; ** < 0.01; *** < 0.001.

Table 2.1.3 |Results of repeated-measures ANOVA and planned comparisons used to analyse the differences in swimming speed (BLs-1) between experimental (E) and control (C) fish, experimental phases (before and after conditioning phase) and tank sides (stimulation side (SS) and non-stimulation side (nSS) (4-levels repeated factor: before SS vs. before nSS vs. after SS vs. after nSS; categorical variables: E vs. C fish).

	Appetitive treatment		**Aversive treatment**	
	F statistic	***P*-value**	**F statistic**	***P*-value**
Treatment (T) Main effect	$F_{1,48}$=0.83	0.37	$F_{1,48}$=0.73	0.40
Repeated factor (R) main effect	$F_{3,144}$=2.44	0.07	$F_{3,144}$=4.80	<0.01*
T x R interaction	$F_{3,144}$=2.52	0.06	$F_{3,144}$=1.58	0.20
Planned comparisons ($F_{1,48}$)				
C_{before} *vs*. C_{after} in SS	12.79	<0.001*	7.90	<0.01*
E_{before} *vs*. E_{after} in SS	0.05	0.83	2.09	0.16
C_{before} *vs*. C_{after} in nSS	0.03	0.88	4.03	0.05
E_{before} *vs*. E_{after} in nSS	4.21	<0.05*	0.15	0.70
C_{before} *vs*. E_{before} in SS	3.95	0.053	1.22	0.28
C_{after} *vs*. E_{after} in SS	0.67	0.42	0.003	0.96
C_{before} *vs*. E_{before} in nSS	0.005	0.95	2.82	0.10
C_{after} *vs*. E_{after} in nSS	3.98	0.052	0.0009	0.98

Swimming speed only increased from the habituation to the test phase in the control treatment in the SS (Table 2.1.3, Fig. 2.1.4). There were no significant differences between the control and the experimental (i.e. exposed to the aversive stimulus) treatments either in the habituation or the test phase, either for the SS or for the nSS (Table 2.1.3, Fig. 2.1.4).

Blood parameters

The plasmatic concentrations of cortisol were not significantly different between the control and the experimental fish in the appetitive experiment ($F_{1,91}$=0.13, p = 0.72; Fig. 2.1.5). In the aversive experiment fish from the experimental group (i.e. exposed to the aversive stimulus) showed significantly higher plasma cortisol levels than fish from the control group ($F_{1,91}$=5.83, $p < 0.05$). However, cortisol levels of the experimental groups did not differ between the appetitive and aversive experiments ($F_{1,91}$=0.28, p = 0.60), but cortisol levels of the control groups were higher in the appetitive than in the aversive experiment ($F_{1,91}$=5.12, $p < 0.05$; Fig. 2.1.5).

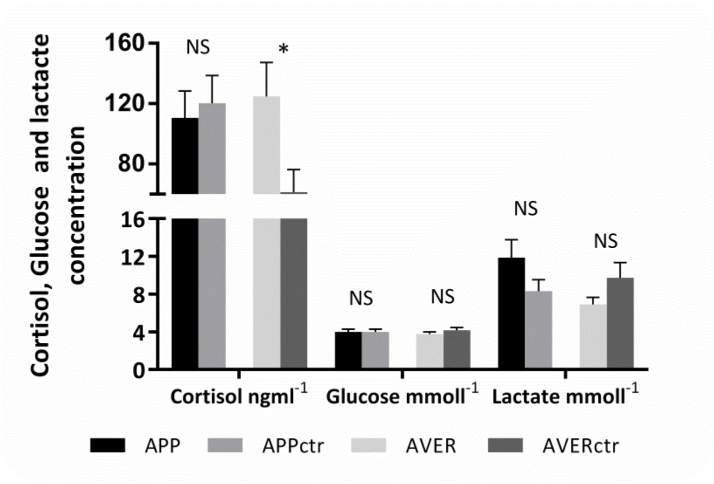

Figure 2.1.5 | Plasma concentration (Mean ± SEM) of cortisol (mg ml^{-1}), glucose (mmol l^{-1}) and lactate (mmol l^{-1}) measured for each treatment. One way ANOVA, NS = non-significant; * < 0.05.

There were no significant differences in plasma concentrations of glucose between control and experimental fish, neither for the appetitive ($F_{1,86}$=0.0006, p = 0.98) nor for the aversive experiment ($F_{1,86}$=1.08, p = 0.30). Furthermore, glucose levels did not differ between the appetitive and the aversive experiments neither for the experimental ($F_{1,86}$=0.31, p = 0.58) nor for the control groups ($F_{1,86}$=0.23, p = 0.63). There were no significant differences in plasma concentrations of lactate between control and experimental fish, neither for the appetitive ($F_{1,86}$=3.19, p = 0.08) nor for the aversive experiment ($F_{1,86}$=2.12, p = 0.15). However, lactate levels were significantly higher in the appetitive than in the aversive experimental group ($F_{1,86}$=6.08, $p < 0.05$), but not between controls ($F_{1,86}$=0.55, p = 0.46).

Discussion

The mechanisms that regulate food reward-associated behaviours have been traditionally studied in rodent models and very seldom in fish. To our knowledge, there are two studies in zebrafish using food as reward in CPP test (Lau et al., 2006; Zala and Määttänen, 2013). Lau et al. (2006) established a CPP paradigm and used it to demonstrate that fish exhibit a robust preference for morphine as well as for food. Zala and Määttänen (2013) used the CPP paradigm to study social learning. However, these authors focused on the change in time spent in the stimulation side, and did not analyse the overall behaviour change and the fish physiological response to the stimulus.

The present study showed that during the test phase fish previously exposed to an appetitive stimulus increased significantly the time spent in the stimulation side. Such place preference suggests that fish attribute a positive valence to the presence of food in this area. In addition, these fish highly increased the distance travelled in the stimulation side. Visual observations clearly revealed that fish explored the bottom of the stimulation side in the test phase as if they were in search of pellets, suggesting a food anticipatory activity (Bassett and Buchanan-Smith, 2007; Galhardo et al., 2011). These fish were also characterized by an increase of swimming speed in the non-stimulation side due to their quick passages in this zone, which could be interpreted as a loss of interest for this area. These results suggest that the changes of behaviour observed for these fish during the test phase are due to their associative learning of the visual cues with the reward, and when placed again in the experimental tank these fish assess the stimulation side as an area of potential food delivery.

No differences in plasma cortisol, glucose and lactate concentrations were observed between fish exposed to the appetitive conditioned treatment and the control ones. The relatively high concentration of plasma cortisol for both experimental and control fish in the appetitive experiment can be explained by the handling stress involved in the experimental procedure (each fish was transferred daily from their home tank to the CPP tank for 5 days in a row). Indeed, the values measured in this study are comparable to those reported by Barton et al. (2005) for seabream exposed to handling stress during 30 sec. Therefore, the plasma cortisol concentrations measured for the appetitive treatment seem to be more representative of the handling procedure, rather than elicited by the intrinsic valence of the stimulus.

Studies based on stress-associated behaviour or avoidance learning are relatively abundant in fish and traditionally used the association between different types of conditioned stimulus (CS; *e.g*. light on, water flow stop, air bubbles) and unconditioned stimulus (US, *e.g*. social aggression, confinement, electric shock). Yue et al. (2004) for instance, revealed that trout are able to learn the association between turning on a light and a net plunging into the water. Trout shuttled from one side of the tank to the other depending on where the light went on. Dunlop et al. (2006) showed that trout and goldfish have the capacity to learn to associate a particular area with a noxious stimulus and retain that learned information. More recently, Martins et al. (2011) showed that Nile tilapia exhibits avoidance learning when exposed to confinement stress. However, fish behavioural and physiological responses toward an aversive stimulus have never been accessed before with a CPA test.

This study demonstrated that a single exposure to a chasing net significantly decreased the time spent by seabream in the stimulation side and increased highly the distance travelled in the non-stimulation side. These behavioural changes clearly suggest a strong avoidance of the stimulation side and a search for an escape in the non-stimulation side. These results propose that the changes of behaviour observed in the aversive treatment during the test phase are due to associative learning of the visual cues with the stressor, and when placed again in the experimental tank these fish still assess the stimulation side as an area of potential aversive event. These results are confirmed by the physiological data. Indeed, even if plasma concentrations of glucose and lactate were not different between experimental and control fish, the plasma cortisol concentration was two times higher for the experimental fish. This result highlights that fish which were previously exposed to the stressor, when placed in the same environment, show a high physiological stress response due to the expectation of an aversive stimulus.

In this study, the control fish provided also interesting findings. As expected control fish in both experiments (appetitive and aversive) did not show any differences in the time spent in both sides during the habituation or test phase. However, fish slightly increased the distance travelled and the swimming speed in both sides during the test phase. The fact that these changes of behaviour happened on both sides of the tank revealed that the control fish did not have any preference for one or the other compartment, and that the increase in swimming activity can be interpreted as a reduction in anxiety during the test phase due to habituation to the experimental set-up. Indeed, during the test phase the control fish of the appetitive experiment had already been exposed 4 times to the set up and the control fish of the aversive experiment 2 times. Consequently it is expectable that the fish would be less stressed during the test phase than during the habituation phase. This highlights the need to minimise as much as possible the fish exposition to the CPP/CPA set-up in order to avoid habituation which may impact fish behaviour and hamper data interpretation.

In conclusion, this study showed that fish exposed to appetitive stimulus in a certain side of the tank induced a preference and higher exploratory behaviour for that side even in the absence of the stimulus (Conditioned Place Preference). In contrary, fish exposed to aversive stimulus (even only one time) in a certain side of the tank exhibit avoidance behaviour for that side and a stress physiological response even in absence of the stimulus (Conditioned Place Avoidance). Thus, this study showed that the CPP/CPA paradigm can be used in fish to assess the valence (positive *vs*. negative) that they attribute to different stimuli.

Acknowledgements

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CHAPTER II-2

Behavioural stress responses predict environmental perception in European seabass (*Dicentrarchus labrax*)

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Behavioural stress responses predict environmental perception in European seabass (*Dicentrarchus labrax*)

Abstract: Individual variation in the response to environmental challenges depends partly on innate reaction norms, partly on experience-based cognitive/emotional evaluations that individuals make of the situation. The goal of this study was to investigate whether pre-existing differences in behaviour predict the outcome of such assessment of environmental cues, using a conditioned place preference/avoidance (CPP/CPA) paradigm. A comparative vertebrate model (European seabass, *Dicentrarchus labrax*) was used, and ninety juvenile individuals were initially screened for behavioural reactivity using a net restraining test. Thereafter each individual was tested in a choice tank using net chasing as aversive stimulus or exposure to familiar conspecifics as appetitive stimulus in the preferred or non-preferred side respectively (called hereafter stimulation side). Locomotor behaviour (*i.e.* time spent, distance travelled and swimming speed in each tank side) of each individual was recorded and analysed with video software. The results showed that fish which were previously exposed to appetitive stimulus increased significantly the time spent on the stimulation side, while aversive stimulus led to a strong decrease in time spent on the stimulation side. Moreover, this study showed clearly that proactive fish were characterised by a stronger preference for the social stimulus and when placed in a putative aversive environment showed a lower physiological stress responses than reactive fish. In conclusion, this study showed for the first time in seabass, that the CPP/CPA paradigm can be used to assess the valence (positive *vs*. negative) that fish attribute to different stimuli and that individual behavioural traits is predictive of how stimuli are perceived and thus of the magnitude of preference or avoidance behaviour.

Keywords: Affective value, Behavioural traits, Perception, *Dicentrarchus labrax*

Introduction

How and for what reasons individuals differ in the way they react to potential risks, handle novelty, or interact with conspecifics remain fascinating questions. Scherer (1987) suggested that the individual evaluates the significance of an event according to a set of stimulus evaluation checks. These evaluate the relevance of the event according to various dimensions (*e.g.* novelty, pleasantness, and importance of the event for the individual), its implication in terms of the individual's needs, the possibility for the individual to cope with the event and the compatibility of the event with social or individual standards. A variety of related concepts have been used to describe individual differences in behaviour that are consistent over time and across situations (see Budaev and Brown, 2011). Wilson et al. (1994) proposed that the shy-bold continuum (propensity to take risk) is a fundamental axis of behavioural variation in many species. Another concept frequently used in the study of animal personality is behavioural syndrome: a suite of correlated behaviours that are expressed either within a given context or across context (Sih, et al., 2004). A third concept frequently used to investigate individual differences in behaviour is personality traits or strategies. Two alternative personality traits are frequently distinguished: proactive and reactive (Benus, et al., 1991; Koolhaas, et al., 1999; Overli, et al., 2007). Proactive individuals are more active, aggressive, bold, tend to form inflexible routines and learn more slowly about small changes in the environment. Reactive individuals, in contrast, are shyer, non-aggressive and more sensitive to environmental changes. The existence such contrasting phenotypes seems to be a widespread phenomenon, with some aspects of this individual variation being reported in invertebrates (e.g. squids, *Euprymna tasmanica* (Sinn, et al., 2008)), lizards (*Anolis carolinensis* (Korzan, et al., 2006)) and in various species of fish (sticklebacks, *Gasterosteus aculeatus*, (Bell, 2005; Bell and Sih, 2007; Huntingford, 1976) rainbow trout, *Oncorhynchus mykiss*, (Øverli, et al., 2005; Sneddon, 2003; Winberg, et al., 2007); Nile tilapia, *Oreochromis niloticus*, (Martins, et al., 2011a; Martins, et al., 2011b); Gilthead sea bream, *Sparus aurata,* (Castanheira, et al., 2013a; Castanheira, et al., 2013b)). Far from being stereotyped and invariant, differences in the behavioural repertoires, learning and memory abilities observed in both phenotypes suggest that fish are curiously plastic (Laland, et al., 2003).

Recent reviews of fish cognition suggest fish show a rich array of sophisticated behaviours. For example they have functional long-term memories, develop complex traditions, show signs of Machiavellian intelligence, cooperate with and recognise one another and are even capable of tool use (Brown, 2014; Brown, et al., 2011; Bshary, et al., 2002). Emerging evidences also suggest that, despite appearances, the fish brain is also more similar to higher vertebrate one than previously thought (Broglio, et al., 2011; Demski, 2013; Rink and Wullimann, 2004). Although this amount of knowledge, the way fish perceive stimuli and the affective value they attribute to them are still poorly known phenomena. For most people, this is either linked to animal sentience or consciousness. Sentience is quite difficult to define or measure, and the meaning is constantly debated by scientists and philosophers alike, but it might be summed up in an ethical context as the ability to experience pleasure and pain (*i.e.* subjective perceptual experiences (Appleby and Sandøe, 2002; Dawkins, 1998)). Being increasingly used in animal welfare evaluations and recognised as adaptive products of natural selection (Chandroo, et al., 2004), affective states are not directly observable, and behavioural and physiological proxies have to be used in order to probe animal affective states. Preference/avoidance and motivation tests have been used for this purpose, based on the assumption that affective states are linked to motivation/preference and ultimately drive behaviour (Kirkden and Pajor, 2006). In these tests the animal is given some control over its environment, so that we can observe their choices in preference tests, or how much they are willing to work to access or avoid given resources or threats in operant motivation tests (Endo, et al., 2002; Herrero, et al., 2007; Kirkden and Pajor, 2006; Yue, et al., 2008). Thus, the ability of fish to express choice according to their preference or avoidance is a well-established phenomenon; however, the extent of intraspecific variation and whether personality traits influence this type of behaviour are still unknown.

In this study we investigated how European seabass (*Dicentrarchus labrax*), one of the most important commercial species in Europe, evaluated putative appetitive (presence of social partners) or putative aversive (net chasing) stimuli in a conditioned place preference/avoidance test (CPP/CPA (Prus, et al., 2009; Tzschentke, 2007)). CPP/CPA is a behavioural paradigm in which a reward or a punishment is paired repeatedly with environmental cues so that the animal associates the cues with the appetitive or aversive stimulus and eventually develops preference or avoidance for the marked location even in the absence of the stimulus (Mathur, et al., 2011). In order to validate this paradigm as a gauge of valence attributed by the fish to the putative appetitive/aversive stimuli, we used physiological (cortisol and glucose) and behavioural (distance travelled and swimming speed) measures. In addition, we investigated whether pre-existing differences in the behavioural response to acute stress (a putative indicator of stress personality traits or animal personality in fish (Backström, et al., 2014; Castanheira, et al., 2013a)) would predict individual variation in the response to putative appetitive and aversive stimuli.

Material and 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. The rules and regulations which protect experimental animals from unnecessary pain and suffering have been strictly followed during the experiment. In preparing the experiment, we have carefully considered the application of the 3R (Replacement, Reduction and Refinement, 2010/63/UE).

Experimental fish, housing and feeding

Fish were hatched and reared at the experimental research station of Ifremer in Palavas-les-Flots (France) until they weighted 0.1 g and then transported to Ramalhete Research Station (Faro, Portugal). Fish were housed in stock tanks (500L) under seabass standard rearing conditions (Chatain, 1994) during 8 months before the start of the experimental procedures (rearing density from 0.3 kg m^{-3} (mean fish weight = 0.1 g) to 10 kg m^{-3} (mean fish weight = 45 g) which are considered as low rearing densities and reach all welfare demand). Fish were fed a commercial diet (Aquagold 3 mm, Aquasoja, Sorgal SA, Portugal; 44% crude protein, 14% crude fat, 8% ash, 2.5% crude fibres, 1.0% phosphorus) using automatic feeders (1.5% BW day^{-1}). Fish were reared in open water circuit tanks, with a temperature of 20 ± 7 °c, salinity of 35 ± 2 ‰ and dissolved oxygen above 95 %, and a 12L:12D photoperiod was employed with light on at 08:00. One month before the start of experimental procedures, 90 fish were randomly selected, anaesthetised with 2-phenoxyethanol (0.3 ‰, Sigma-Aldrich) and individually identified with a PIT-tag (Micro BE, France) injected in the flesh under the dorsal fin and with a visible implant elastomer tag (VIE; Northwest Marine Technology, USA) in the caudal fin. At the start of the experiment the body mass of the fish was 45 ± 1.3g (mean ± SE).

Set up and experimental procedures

Restraining test

Escape behaviour during restraining or confinement has been used to discriminate personality traits as well as physiological correlates of personality traits in different fish species (Backström, et al., 2014; Martins, et al., 2011b; Øverli, et al., 2006; Silva, et al., 2010). More recently, Castanheira et al. (2013) showed in seabream that escape behaviour during restraining was consistent over time and across contexts. Moreover, Ferrari S., Millot S., Leguay D., Chatain B., Bégout ML (unpubl. data) demonstrated in seabass that escape attempts during restraining test were negatively correlated to plasma cortisol concentration. For these reasons, the net restraining test was performed only one time 15 days before the conditioned place preference/avoidance (CPP/CPA) tests. The restraining test consisted of holding each fish individually in an emerged net for three minutes (Arends, et al., 1999; Castanheira, et al., 2013a; Castanheira, et al., 2013b; Martins, et al., 2011a; Martins, et al., 2011b; Silva, et al., 2010). 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 an 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). Behaviours measured were collapsed into first principal component scores using Principal Components Analysis (PCA) in order to obtain a score allowing the characterisation of personality traits. PC1 explained 86% of the variation and the number of escapes was the variable that contributed the most for PC1 (Table 1). Fish presenting a high latency to escape, small number of escape attempts and shorter total time to escape were characterised by a low score and identified as reactive fish. On contrary, fish presenting a lower latency to escape, high number of escape attempts and longer total time to escape were characterised by a high score and identified as proactive (based on Silva et al. 2010;

Martins et al. (2011a,b) and Castanheira et al. 2013a,b). No threshold was applied to separate subjectively the fish in two categories *i.e*. proactive and reactive. Instead these data (personality traits, PT, score) were used as a continuous variable.

Conditioned place preference/ avoidance test

Four days before the start of the CPP/CPA test, 3 groups of 12 fish each (6 focal fish with distinct VIE (Visible Implant Elastomer tag) and 6 familiar conspecifics, *i.e.* all fish coming from the same rearing tank) were placed in 3 different 100L home tanks located in the experimental room. This was done to acclimatize fish to their new environment. The photoperiod and the water temperature, salinity and oxygenation were the same as in rearing tanks. The fish were fed *ad libitum* each morning. This procedure was repeated 5 consecutive times in order to test 30 fish per treatment: appetitive (APP), aversive (AVER) and control (CONT). The CPP/CPA test was performed in 6 glass aquaria of 80L (70 cm length x 40 cm width and 30 cm depth). Each aquarium was divided into two halves by a 10 cm wide grey central alley: one half marked by white walls without dots and the other half marked by white walls with black dots used as visual cues for fish (Fig. 2.2.1). One infrared LED projector (IR-294S/60, Monacor®) was placed beneath each aquarium.

The CPP/CPA test consisted of three experimental phases conducted over a 3 days period. An initial habituation phase was performed on the first day. During this phase, each fish was placed individually in the tank and allowed to swim freely throughout the whole tank for 40 min (this period was determined based on preliminary observations). For each individual the preference for white or dotted side (> 50% time spent) was assessed through a 20 min additional recording. Then the fish was put back in the home tank. Animals that showed a very strong initial preference (> 90% time spent) in either side or strong freezing behaviour (< 500 cm distance moved) were excluded from the study because their initial position during the habituation phase could not be representative of their real preference for that side. Therefore, animals that showed an initial preference between 50.1% and 89.9% for both side and which had moved more than 500 cm were used for data analyses.

The habituation phase was followed by a conditioning phase, during which treatments differed between the appetitive and aversive stimulus groups. For the aversive stimulus (AVER), fish was placed in the same aquarium as during the habituation phase but had only access to the initial preferred side for 20 min, hereafter termed the stimulation side (SS). Afterwards, the fish was chased with a net during 10 s each 4 min for a period of 20 min. For the appetitive stimulus (APP), fish was placed in the non-initial preferred side (new SS) for 20 min and then 2 familiar conspecifics were added in the tank for a period of 20 min. The control fish (CONT) were handled exactly the same way as the tested animals (maintained in the preferred side or in the non-preferred side) except that the stimulus was omitted during the training phase. After each treatment, fish was placed back in the home tank.

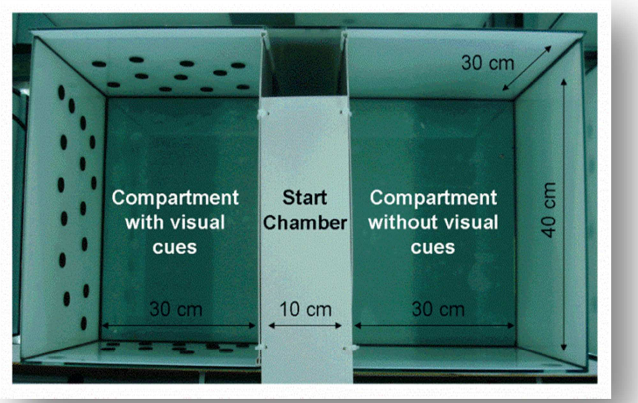

Figure 2.2.1 |Experimental tank: CPP/CPA test glass aquarium (80L) divided in three compartments: one start chamber with grey walls and sheltered and two lateral compartments with white walls and with or without visual cues (black spots).

The test phase was performed on the last day of the experiment (the third day) and consisted exactly of the same procedure as the habituation phase in order to record any behavioural changes. After this last phase, fish were immediately caught and euthanized with an overdose of 2-phenoxyethanol (1 ‰, Sigma-Aldrich). Blood was thus sampled one hour after fish were transferred to the experimental aquarium (based on Fanouraki et al. 2008). Blood was withdrawn within 3 min from the caudal vein using heparinised syringes and centrifuges at 2000 x g for 25 minutes at room temperature. After centrifugation plasma was frozen in dry ice and stored at -80°c for glucose (QCA, Spain) kit analysis. Plasma cortisol levels were measured by means of a commercial ELISA kit RE52061 (IBL Hamburg, Germany), with a sensitivity of 2.5 ng/ml, and intra and inter-assay coefficients of variation (CV) of 2.9 and 3.5%, respectively. After blood sampling, fish were identified and measured for standard length (cm).

During each phase, individual behaviour was recorded by infrared sensitive video camera (TVCCD-623-COL, Monacor®, Denmark) equipped with infrared filter (dark red, Schneider Optics, USA) and positioned 1m above the tank. The videos were stored in AVI files on a hard drive and analysed afterwards with the Lolitrack 2.0 software (Loligo® Systems, Denmark). Before each video analyses, the background image of each tank was divided into three arenas (Arena 1 = white side, Arena 2 = grey middle alley, Arena 3 = dotted side). For each tank the background was calibrated by marking the length of the Arena 2 in the image and entering its actual value (10 cm). The Lolitrack 2.0 software tracks the fish as a dark object on a light background. By using infrared light underneath the tank we avoid light reflexion on the water surface and optimise the fish tracking by the software. The following parameters were quantified by the software: time spent in each arena (min), distance travelled in each arena (m) and the swimming speed in each arena (cms^{-1}). In order to remove the influence of fish size in swimming speed data, these values were transformed in body length per second (BLs^{-1}). To evaluate the fish behavioural changes between the habituation and the test phase, percent change of time spent, distance travelled and swimming speed were calculated as:

[(Test phase value – Habituation phase value) / Habituation phase value] x 100.

Statistics

Behaviours measured in 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 for 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, indicated that correlations between items were sufficiently large for PCA. PCA analyses were performed using SPSS 18.0 for windows. The results are expressed as mean ± standard error of the mean (SEM). All other statistical analyses were performed using Statistica 7 software (Statsoft, USA). The results were expressed as mean ± standard error of the mean (SEM). A null model of side preference was tested by comparing the observed fish distribution to the theoretical homogeneous distribution in the side with or without dots (50% in each side) by a Kolmogorov–Smirnov test. Fish did not show a systematic initial preference for one side or the other one. Consequently, the stimulation was performed 36 times in the side without dots *vs* 42 times in the side with dots ($d= 0.28$; $p > 0.05$). One way ANOVA was used to analyse the differences in percent change of time spent on SS (after arcsine (sqrt (x/100)) transformation) by the experimental (APP or AVER) vs. control fish. Repeated-measures ANOVA's were used to analyse the differences in distance travelled (m) and swimming speed (BLs^{-1}) between experimental and control fish, experimental phases (*i.e.* before and after conditioning phase) and tank sides (4-levels repeated factor: before SS *vs*. before non-stimulation side (nSS) *vs*. after SS *vs*. after nSS; categorical variables: experimental *vs*. control fish). Newman & Keuls tests were subsequently used to test differences between the habituation and the test phase of each treatment and between the control and the experimental groups both at the habituation and at the test phase of each treatment. One way ANOVA followed by Newman & Keuls tests were used to analyse the differences in plasma concentrations of cortisol (ngml^{-1}) and glucose (mmol^{-1}) between experimental and control fish. For both APP and AVER treatments, Pearson correlations matrices between time spent on SS, distance moved in SS and nSS, percent change of time spent on SS, percent change of distance moved in SS and nSS, plasma concentration of glucose and cortisol with personality traits (PT) score were calculated. The significance level of each correlation matrices' was defined according to the table of critical values of Pearson correlation coefficient corrected by the individual number (n) in Scherrer p792 (1984).

Results

From the 90 fish tested in this study, 12 fish did not comply with our CPP acceptance criteria and were thus removed from the analysis. This resulted in the following sample sizes: n=28 for APP, n= 23 for AVER and n= 27 for CONT. During the restraining test, fish waited on average 91s before the first escape attempt and they performed a mean of 5 escape attempts for a total escape time of around 2 s (Table 2.2.1).

Table 2.2.1 |Mean ± SEM, minimum (Min.) and maximum (Max.) values of behavioural variables obtained for the restraining test (N = 90) and PCA loading used to generate a principal component scores (PC1).

Behavioural variables	Mean ± SEM	Min.	Max.	Loadings for PC1	Eigenvalues	% variation explained
Latency escape (s)	90.9 ± 0.75	2	180	-0.855	85.991	85.991
Number escape	5.3 ± 0.06	0	18	0.962	12.481	
Total escape time (s)	1.6 ± 0.02	0	6.8	0.96	1.528	

Restraining test

Time spent on the stimulation side

On average fish subjected to the appetitive stimulus showed a high increase (+ 163%) in the time spent on the SS during the test phase, whereas fish exposed to the aversive stimulus showed a significant decrease (- 42%) in the time spent on the SS during the test phase (Fig. 2.2.2; One way ANOVA $F_{2,75} = 6.60$, $p < 0.01$).

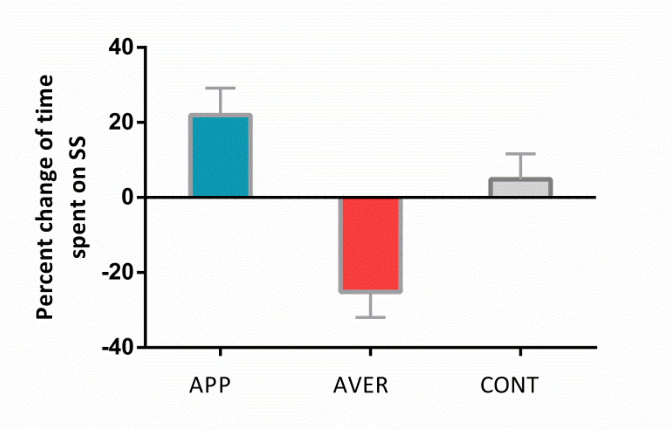

Figure 2.2.2 |Time spent (Mean ± SEM; in min) by the fish in the stimulation side between the habituation and test phases for each treatment. One way ANOVA; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Distance travelled

The main effect of treatment (*i.e.* APP *vs*. AVER *vs.* CONT groups) on the distance travelled was not significant ($F_{2,75} = 0.54$, $p = 0.58$), but there were a significant main effect of the repeated measure (*i.e*. before SS *vs*. before nSS *vs*. after SS *vs*. after nSS; $F_{3,225} = 103.11$, $p < 0.001$) and a significant interaction between treatment and the repeated measure ($F_{3,225} = 7.32$, $p < 0.001$). Thus, regardless of treatment or tank side, fish significantly decreased the distance travelled between the habituation and the test phase (Fig. 2.2.3).

Swimming speed

The main effect of treatment (*i.e.* APP *vs*. AVER *vs.* CONT groups) on swimming speed was not significant ($F_{2,75} = 0.10$, $p = 0.90$), but there were a significant main effect of the repeated measure (*i.e.* before SS *vs*. before nSS *vs*. after SS *vs*. after nSS; $F_{3,225} = 50.47$, $p < 0.001$) and a significant interaction between treatment and the repeated measure ($F_{3,225} = 2.94$, $p < 0.01$). Whatever the treatment and the tank side, fish significantly decrease the swimming speed during the test phase (data not shown).

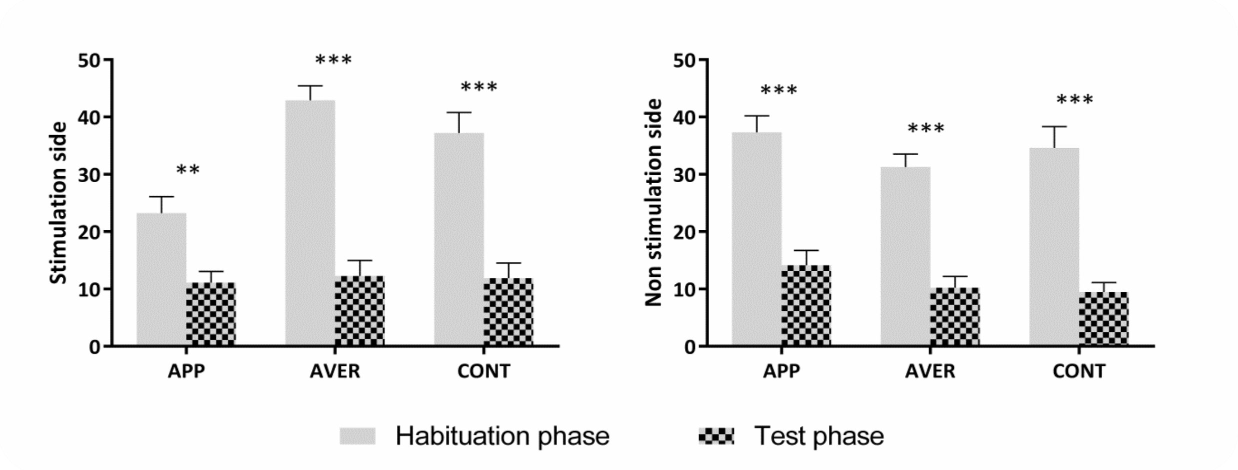

Figure 2.2.3 |Distance travelled (Mean ± SEM; in min) by the fish in the stimulation side and the non-stimulation side during the habituation and test phases for each treatment. Repeated ANOVA, NS = non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Blood plasma analysis

There was no significant difference between APP, AVER and CONT in plasma concentration of cortisol (237 ± 24; 212 ± 25; 270 ± 20 ng ml^{-1} respectively; $F_{2,71} = 1.11$, $p = 0.33$) and glucose (4.32 ± 0.1; 4.33 ± 0.1; 4.26 ± 0.1 mmol l^{-1} respectively; $F_{2,67} = 0.14$, $p = 0.86$).

Correlations between personality traits*, behaviour and physiology parameters*

The correlations matrices for the APP treatment showed that fish that spent the most time on SS during the test phase also had a higher CS score (Table 2.2.2, Fig. 2.2.4). The correlations matrices for the AVER treatment showed that fish characterised by a low CS score increased the distance moved in SS and had higher plasma cortisol concentrations (Table 2.2.2, Fig. 2.2.4).

Discussion

This study demonstrated that a single exposure to an appetitive or an aversive stimulus significantly increased or decreased respectively, the time spent by seabass on the stimulation side of a CPP/CPA setup. This behavioural change clearly suggests that the experimental fish attributed a positive valence to the presence of social partners, and a negative valence to net chasing. Further, it would appear that altered place preference observed during the final test phase (in the absence of stimulus) is due to associative learning of visual cues (black dots) coupled to expected appetitive or aversive stimulus. Notably, both experimental and control fish showed a strong decrease in swimming activity (distance travelled and swimming speed) in both sides of the tank.

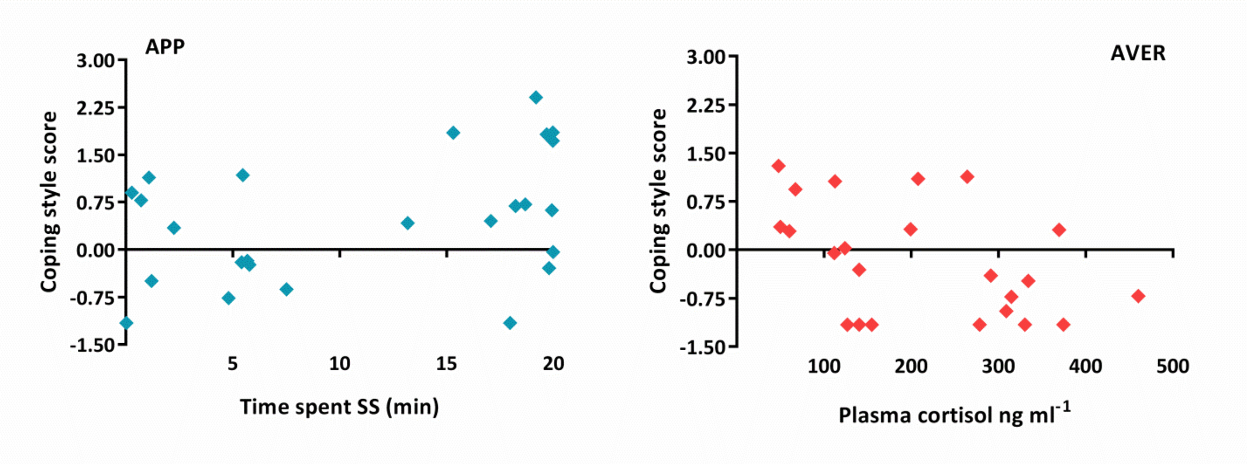

Figure 2.2.4 | Correlations between personality traits score and time spent on SS (min) for appetitive treatment and personality traits score and plasma cortisol concentration (ng ml^{-1}) for aversive treatment. The full black lines represent the linear regressions.

Table 2.2.2 | Pearson correlations matrices and p value for appetitive (APP) and aversive (AVER) treatments between time spent on stimulation side (SS), distance moved in SS and on non-stimulation side (nSS), percent change of time spent on SS, percent change of distance moved on SS and nSS and plasma concentration of cortisol and glucose with personality traits score. The significance level of each correlation matrices was defined according to the table of critical values of Pearson correlation coefficient corrected by the individual number (n) in Scherrer p792 (Scherrer, 1984) (APP: n = 28, |R| > 0.374; AVER: n = 23, |R| > 0.413). The significant results are in bold.

	APP		**AVER**	
Variables measured	**Personality traits score**		**Personality traits score**	
	r	*p*	*r*	*p*
Time spent SS (min)	**0.470**	**0.019**	-0.225	0.340
Distance moved SS (m)	-0.050	0.796	-0.379	0.099
Distance moved nSS (m)	-0.180	0.384	-0.178	0.452
Percent change time spent SS (%)	0.210	0.030	-0.161	0.498
Percent change distance moved SS (%)	-0.130	0.539	**-0.441**	**0.052**
Percent change distance moved nSS (%)	-0.240	0.259	-0.169	0.476
Cortisol (ng ml^{-1})	0.043	0.841	**-0.495**	**0.027**
Glucose (mmol l^{-1})	0.121	0.573	0.009	0.969

This result could be explained either by the fact that fish became habituated to the aquarium and thus reduced exploration or by the experimental procedure which consisted of handling fish each day to transfer them from their home tank to the experimental tank and which could have induced stress (*i.e.* freezing behaviour). Thus, even if fish showed preference or avoidance for the tank zone associated with appetitive or aversive stimulus respectively, they did not express a swimming activity comparable to that observed during the habituation phase. This last interpretation is supported by the similar high plasma concentrations of cortisol and glucose observed across treatments. Thus, the stimulus effect on fish physiology was probably masked by the stress due to the handling procedure. To summarise, even if the experimental set up to assess the affective value that seabass attribute to a stimulus was not optimal due to the handling procedure which has masked part of the behavioural and physiological responses, the results showed nevertheless that fish can learn, remember and make decisions to avoid being exposed to aversive stimulus (net chase) or in contrary to seek for appetitive stimulus (familiar conspecifics).

The consistency of behaviours has not been tested in this study and should be done in the future in order to clearly define if the behavioural stress response measured during the restraining test could be considered as personality traits, behavioural trait or personality. However, restraining test has been shown to be repeatable in sea bream (Castanheira et al., 2013) and correlated with physiological stress in seabass (see method section) and thus the observed behaviour is likely to be a personality trait. More interestingly, this study showed that behavioural stress response or personality modulates the response (*i.e.* appraisal) of appetitive and aversive stimuli. When fish were subjected to an appetitive stimulus, proactive individuals expressed a higher preference (*e.g.* time spent) for the stimulation side than reactive fish. But when fish were submitted to an aversive treatment, reactive fish exhibited an increase of distance moved in the stimulation side (anxiety) and a higher plasma cortisol concentration than proactive fish. These behavioural and physiological changes showed that fish exhibiting proactive behaviour were more responsive to the appetitive stimulus while reactive phenotypes responded to the aversive one. These results suggested also that the proactive phenotype is less fearful when presented with a signal previously associated with an aversive stimulus, as compared to individuals of the reactive type. Previous studies have demonstrated in fish relationship between anxiety or fear behaviour and corticosterone response (Egan, et al., 2009) and limbic neural systems (Chandroo, et al., 2004; Overli, et al., 2007) and also between fearfulness and personality (Martins, et al., 2011b), but it is the first time that a study highlights the link between behavioural stress and fear responses and physiological patterns simultaneously. Even if correspondence between boldness and sociability is not clearly established (Cote et al., 2010; Trompf and Brown, 2014), in our study, proactive fish seemed more responsive to social stimulus than the reactive ones. Pike et al. (2008) showed in three-spined stickleback (*Gasterosteus aculeatus)* that bolder individuals had fewer overall interactions than shy fish, but tended to distribute their interactions more evenly across all group members. Thus, the fact that proactive fish were more attracted by the side where congeners were present did not necessarily means that they are more social but simply that they attribute a higher positive value to this stimulus than reactive fish.

In conclusion, this study showed for the first time in seabass, that the CPP/CPA paradigm can be used (with some set up improvements in order to reduce the fish freeze behaviour due to the handling procedure) to assess the valence (positive *vs*. negative) that fish attribute to environmental stimuli and that the individual's behaviour under stress predicts how stimuli are perceived and thus the subsequent preference or avoidance behaviour.

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Predictability as an appraisal modulator in fish

Chapter III

CHAPTER III-1

Appraisal of stimulus valence and predictability induces emotion-like states in fish

This chapter has been submitted to **Proceeding of Royal Society of London B** as:

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Appraisal of stimulus valence and predictability induces emotion-like states in fish

Abstract: The occurrence of emotions in non-human animals has been the focus of much debate over the years. Although the subjective experience of feelings in animals is not accessible to human observers, other dimensions of emotion, namely the psychophysiological and mental states elicited by emotional stimuli can be studied objectively, and many studies have documented the occurrence of such responses in many tetrapods. More recently, an interest in expanding these studies to other vertebrates and to invertebrates has emerged. Within vertebrates, the study of emotion in teleosts is particularly interesting since they represent a divergent evolutionary radiation from that of tetrapods, and thus they provide an insight into the evolution of the biological mechanisms of emotion. Here we report that seabream exposed to stimuli that vary according to valence (positive, negative) and salience (predictable, unpredictable) exhibit different behavioural, physiological and neuromolecular states. Since according to the dimensional theory of emotion valence and salience define a two-dimensional affective space, our data can be interpreted as evidence for the occurrence of distinctive affective states in fish corresponding to each the four quadrants of the core affective space. Moreover, the fact that the same stimuli presented in a predictable vs. unpredictable way elicited different behavioural, physiological and neuromolecular states suggests that stimulus appraisal by the individual, rather than an intrinsic characteristic of the stimulus, has triggered the observed responses. Therefore, the data presented here supports the occurrence of emotion-like states in fish that are regulated by the individual's perception of environmental stimuli.

Keywords: Emotion, Animal emotion, Affective state, Cognitive appraisal, predictability, Teleosts, Stress, cortisol, gene expression, *Sparus aurata*.

Introduction

The interest in the study of emotion in non-human animals dates back to the publication of Darwin's monograph "The Expression of Emotions in Man and Animals" (Darwin, 1872). However, the fact that human emotions are subjectively experienced as feelings has raised difficulties in defining emotion in animals in objective scientific terms (LeDoux, 2012). Nevertheless, other dimensions of emotion, namely the expression of emotion-specific behaviour and accompanying physiological responses, have been documented in many species and a consensus has emerged that animals should express organismic states that index occurrences of value in the environment, regardless of whether these states are consciously experienced (Anderson and Adolphs, 2014; Dolan, 2002; LeDoux, 2012; Mendl et al., 2010). These organismic states would be triggered by the value, in terms of potential impact in Darwinian fitness, that an animal ascribes to a stimulus and they would instantiate the ability to respond adaptively to environmental threats (e.g. presence of predators; presence of competitors for resources, such as shelters or territories) and opportunities (e.g. mating partners; food availability; possibility of ascending in social hierarchy). Within this framework, these global organismic states, and their behavioural expression, represent the organism's experience of reward and threat, and as such they can be seen as similar to human core affect states (Mendl et al., 2010). The evolution of core affect states (or central emotion states sensu (Anderson and Adolphs, 2014)) in animals is plausible as it would provide a way for animals to maximize the acquisition of fitness-enhancing rewards while, simultaneously, minimizing exposure to fitness-threatening punishers. Moreover, these emotion-like states are characterized by general functional properties (i.e. scalability, valence, persistence and generalization) that apply across species and thus make them recognizable and suitable for phylogenetic studies of emotion (Anderson and Adolphs, 2014). Recently, this approach has been used to describe, at the behavioural level, the occurrence of a core affect state of defensive arousal in fruit flies repeatedly exposed to a threat stimulus (Gibson et al., 2015). Thus, the stage has been set for documenting the occurrence of core affect states across phylogeny and to study how evolutionary conserved are the molecular mechanisms and neural circuits underlying them.

In human research core affect has been conceptualized as a dimensional characterization of the emotion experience along two fundamental underlying dimensions: valence (positive/

negative) and intensity (or arousal) (Barrett et al., 2007; Russell, 2003). Hence, core affect can be represented in a two-dimensional space, which became known as the circumplex model of affect (Russell, 1980), where these two variable define 4 quadrants (Q): Q1 = Positive affect, high arousal (e.g. happiness); Q2 = positive affect, low arousal (e.g. (relaxed mood); Q3 = negative affect, low arousal (e.g. sadness); Q4 = negative affect, high arousal (e.g. fear). The extension of this model to emotion-like states in animals has been proposed by (2010), who suggested that the axis Q3-Q1 defines a reward acquisition system, with Q1 representing appetitive motivational states that facilitate seeking and obtaining rewards and Q3 representing loss or lack of reward and associated low activity states, whereas the axis Q2-Q4 defines a punishment avoidance system, with Q4 associated to active responses to the presence of threat and Q2 to passive responses to low levels of threat. In humans, non-human primates and rodents, where the neural substrates of emotion have been more extensively studied, these two core affect axes have been associated with different neural mechanisms. Reward acquisition relies on the mesolimbic dopaminergic system, in particular the prefrontal cortex and specific hedonic hotspots located in the ventral striatum (e.g. nucleus accumbens) (Berridge and Kringelbach, 2013; 2015), whereas punishment avoidance has been associated either with the fight-or-flight system (in Q4), or with the behavioural inhibition system (in Q2), with the amygdala playing a central role in either case (LeDoux, 2012; Mendl et al., 2010).

In order to create internal emotion-like states that support adaptive physiological and behavioural responses towards ecological threats or opportunities, animals must have evolved perceptual and cognitive mechanisms that identify reliable cues in the environment (i.e. aversive vs. appetitive stimuli, respectively) (Brosch et al., 2010). When specific environmental cues deterministically predict an appropriate response, these responses can be simple reflexes and fixed action patterns elicited by these cues. However, when environmental complexity and variability increase, single environmental cues may no longer be informative and the evolution of appraisal mechanisms that cognitively assess the presence of threats and opportunities in the environment is predicted (Fawcett et al., 2014; McNamara et al., 2013). According to cognitive theories of emotion, individuals continuously monitor the environment using a set of stimulus evaluation checks (e.g. intrinsic valence, novelty, prediction error, capacity for control) in order to evaluate the valence (positive/

negative) and salience (high/ low) of detected stimuli, and also assess the available organismal resources to deal with them (i.e. coping mechanisms) (Faustino et al., 2015; Paul et al., 2005). The outcome of appraisal translates into an adjustment of the core affective state of the animal to the perceived state of the external environment. Although an integrated study of the different stimulus evaluation checks used by animals is still lacking, empirical evidence for the occurrence of each of these checks has been described in a wide range of animals, from fish to mammals (see Faustino et al., (2015) for a recent review).

In this study we have used the Gilthead seabream (*Sparus aurata*) to study if perceived stimulus valence (i.e. appetitive vs. aversive) and salience (i.e. high vs. low) trigger specific behavioural, physiological and brain states, indicative of stimulus-appraisal driven emotion-like states in fish. We have used two stimuli with different intrinsic valences (appetitive: food; aversive: physical constraint) that were presented to the focal individuals in a predictable or unpredictable manner. Predictability was used as a proxy of stimulus salience. The effect of predictability as an appraisal modulator has already been documented in other fish species, both towards aversive and appetitive stimuli (Galhardo et al., 2011). Thus, if emotion-like core affect states are also present in fish we predict that each of the four valence x predictability (salience) treatments will elicit specific internal states and behavioural profiles, which correspond to each of the four quadrants of the circumplex model of affect described above, namely: Q1 = unpredictable appetitive (UnPRDapp); Q2 = predictable appetitive (PRDapp); Q3 = predictable aversive (PRDavr); Q4 = unpredictable aversive (UnPRDavr). The patterns of brain activation for each treatment were characterized using the expression of a set of immediate early genes [early growth response 1 (*egr1*), FBJ osteosarcoma oncogene (*c-fos*), brain-derived neurotrophic factor (*bdnf*) and neuronal PAS domain protein 4a (*npas4*)], as markers of neural activity, in a set of brain regions homologous to those known to be involved in reward and aversion processing in mammals (O'Connell and Hofmann, 2011), namely the medial zone of the dorsal telencephalic area (Dm, putative homologue of the mammalian basolateral amygdala); lateral zone of the dorsal telencephalic area (Dl, hippocampus homologue); ventral nucleus of the ventral telencephalic area (Vv, septum homologue) (Ganz et al., 2012; Ganz et al., 2014) . Circulating cortisol levels were also measured as a proxy for physiological state.

Material and methods

Ethical statement

All experimental procedures were conducted in accordance with the Guidelines of the European Union Council (86/609/EU) and the Portuguese legislation for the use of laboratory animals, and under a "Group-1" license from the Portuguese competent authority for the protection of experimental animals (Direção Geral da Alimentação e Veterinária, Portugal; permit number 0420/000/000-n.99-09/11/2009). Experimental procedures, when necessary, were performed under anaesthesia with 2-phenoxyethanol and every effort was made to minimize suffering.

Subjects and maintenance

One hundred and forty four Gilthead seabream juveniles with an initial body weight of 39.45g ± 10.39 g (mean ± SD) were used for all trials. Fish were obtained from a commercial fish farming (MARESA Mariscos de Esteros SA, Huelva, Spain) and transported to Ramalhete Research Station (University of Algarve, Faro, Portugal). Fish were housed in fibre glass tanks (500L), provided with constant aeration and set in an open water circuit, under standard rearing conditions (temperature = 21 ± 3 °C; salinity = 35 ± 2 ‰; dissolved oxygen > 75 %; 12L: 12D photoperiod, with lights on at 08:00 h) during 3 months before the start of the experimental procedures (initial density of 2 kg m^{-3}). Fish were fed at 3% of body weight with a commercial diet (Aquagold 3 mm, Aquasoja, Sorgal SA, Portugal; 44% crude protein, 14% crude fat, 8% ash, 2.5% crude fibres, 1.0% phosphorus) using automatic feeders.

Experimental procedures

The experiment was conducted between May and June of 2013. Twelve experimental aquaria (70 x 40 x 30 cm) in an open water circuit were used, under the same housing conditions as described above for the stock tanks, with the exception that no air was supplied since it interfered with the quality of the video recording. However, a water flow rate of 2.5 L min-1 ensured oxygen saturation between 75 and 90% in the outlet water. Fish were fed at 1.5 % of BW divided by two meals: 8:30 h and 17:30 h. Water quality was analysed for nitrites (< 0.1 mg.L-1) and ammonia (< 0.1 mg L-1) every three days. Inside each aquaria, covering all the bottom area, there was a blue plastic basket of approximately 60 l (64 cm length x 38 cm width and 25 cm depth), attached to a lifting mechanism (see Fig. 3.1.1). The sides of the experimental aquaria were covered with opaque plastic in order to prevent visual contact between the focal animals and the experimenters.

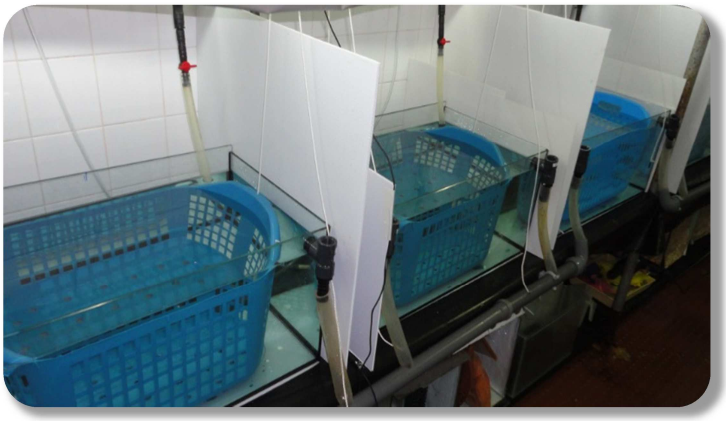

Figure 3.1.1 |Experimental set-up used in the research driven to assess the effect of predictability and stimulus valence on fish stress response.

Immediately after measuring and weighing, 96 fish were divided into groups of 4 individuals each. Individuals were tagged with a multicolour tag (Floy Tag Manufacturing Inc, Seattle, USA) attached behind the dorsal fin. Six groups were tested for each experimental condition (predictable appetitive; predictable aversive; unpredictable appetitive and unpredictable aversive; n=24 fish/ treatment). The experiment lasted 15 days, the first 12 corresponding to the acclimation period and the last 3 to the experimental period. Fish were exposed to 8 training sessions (4 training sessions/day on the first 2 days of the experimental period) followed by 1 test session (morning of day 3), using a delay-conditioning protocol, with light (12 V, 25 W; hanging 10 cm above the tank) as a conditioned stimulus (CS), food as the appetitive unconditioned stimulus, and air exposure as the aversive unconditioned stimulus (USapp and USavr, respectively). Four training treatments were used: (1) Predictable appetitive treatment (PRDapp) - Groups of 4 fish were trained to associate the turn on of the light during 2 min (CS) with a subsequent food reward (USapp: food pellet dropped each 2 sec during the last minute of the light on; see Fig. 3.1.2); the CS overlapped with the US to reinforce the association between stimuli (Nilsson et al., 2008); (2) Predictable aversive treatment (PRDavr) – Similar to PRDapp but the CS used was air exposure (USavr: lifting the basket from the aquarium in approximately 5 sec during the last minute of the light on period; see Fig. 3.1.2); (3) Unpredictable appetitive treatment (UnPRDapp) - Groups of 4 fish were randomly exposed to a food reward (USapp) not coinciding with the CS [i.e. delay of 30 min between the CS and the US, in a random order (i.e. CS presented either before or after US); (3) Unpredictable aversive treatment (UnPRDavr) – Similar to UnPRDapp but the CS used was air exposure (USavr). On the test session all groups were tested in the presence of US+CS. Thus, in the test session individuals were exposed to a predictable aversive or appetitive stimulus, depending on their previous training treatment. Each experimental condition tested is briefly described below and depicted in detail in Table 3.1.1.

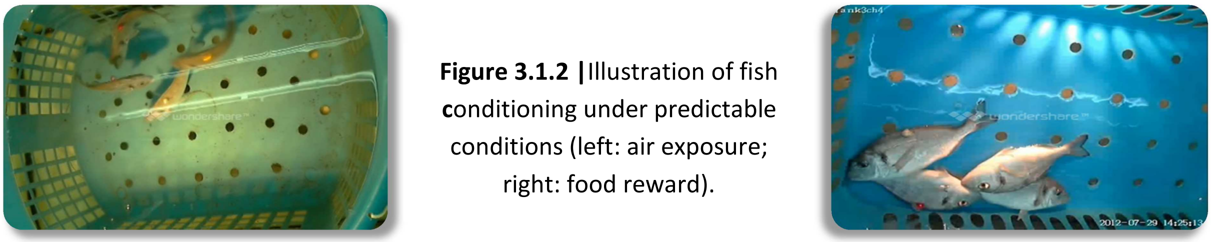

Figure 3.1.2 | Illustration of fish conditioning under predictable conditions (left: air exposure; right: food reward).

Behavioural observations

The behaviour of fish was video recorded both the training and in the test sessions, using a video camera (TVCCD-623-COL, Monacor®, Denmark) and webcams (HD C310 Logitech) positioned 1 m above the tanks. Videos were subsequently analysed using multi-event recorder software (Observer XT® (Noldus, Netherlands). The response of fish to the CS was assessed by: (1) time in freezing, i.e. time fish spent immobile, whether with or without fin movements, either on the bottom or in the water column; (2) social interactions, frequency of frontal and lateral displays, chases or flees; and (3) escape behaviour, i.e. the number of times individuals tried to escape through the holes on the plastic basket.

Table 3.1.1 | Training and trial conditioning procedures for each experimental condition: Predictable appetitive (PRDapp); Predictable aversive (PRDavr); Unpredictable appetitive (UnPRDapp) and Unpredictable aversive (UnPRDavr).

Conditions	**Conditioning procedures (8 training sessions)**	**9th conditioning session**
PRDapp	1 min CS + 1 min CS overlapped with 1 min Usapp	1 min CS + 1 min CS overlapped with 1 min Usapp
PRDavr	1 min CS + 1 min CS overlapped with 1 min Usavr	1 min CS + 1 min CS overlapped with 1 min Usavr
UnPRDapp	1 min CS + 30 min interval + 1 min USapp	1 min CS + 1 min CS overlapped with 1 min Usapp
UnPRDavr	1 min CS + 30 min interval + 1 min USavr	1 min CS + 1 min CS overlapped with 1 min Usavr

Blood Samples and plasma cortisol analysis

Thirty min after the test session, fish were caught and euthanized with an overdose of 2-phenoxyethanol (1 ‰, Sigma-Aldrich). Blood was immediately collected and centrifuged for 15 min at 2000 x g .After centrifugation plasma was frozen in dry ice and stored at -80 ºC until further processing. Plasma cortisol levels were measured using a commercial ELISA kit (RE52061, IBL Hamburg, Germany), with a sensitivity of 2.5 ngml-1, which has been previously validated for seabream (Lopez-Olmeda et al., 2009). Intra and inter-assay coefficients of variation were 2.9% and 3.5 %, respectively.

Brain microdissection and gene expression analysis

Eight individuals from each experimental condition were randomly selected for the assessment of immediate early genes mRNA expression. The fish were sacrificed and the whole skull (with the brain) was removed, immediately embedded in Tissue-Tek® and kept at -80 ºC until mRNA extraction. Brain telencephalon slices were obtained through 150 µm thick cryostat coronal sections. The microdissections were performed with modified 25G steel needles in different regions of the telencephalon i.e. medial part of the dorsal telencephalon (Dm), lateral telencephalon (Dl) and ventral nucleus of the ventral telencephalon (Vv) (see detailed description in Fig. 3.1.3). The brain areas were identified and classified according to Muñoz-Cueto et al. (2001). Tissue was collected directly into lysis buffer from Qiagen Lipid Tissue Mini Kit (#74804; Valencia, CA) and total RNA extracted from the samples, with some adjustments to the manufacturer’s instructions (see Teles et al., 2016 for details). 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 sequences for *18S* (accession # AM490061.1), *eef1a* (accession # AF184170.1), *egr1* (accession # KC442101.1) and *c-fos* (accession #GU108576.1), were obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/nuccore). For *bdnf* and *npas4*, 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;

Untergasser et al., 2012) and synthetized by Sigma-Aldrich (Hamburgo, German). The PCR products were sequenced to confirm the desired primer cDNA amplification. Primer dimers formation was controlled with FastPCR v5.4 software (Kalendar et al., 2014) and optimal annealing temperature was assessed for maximal fluorescence (see Table 3.1.2). 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 $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001).The qRT-PCR was performed using 25 µL reactions including 12.5 µL Light cycler H 480 SYBR Green I Master (Roche diagnostics GmbH, Mannheim, Germany), 0.2 µL of each primer and 1 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, 45 s at primer specific annealing temperature (see table 3.1.2 for details), 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). Two previously established housekeeping genes, *eef1a* and *18S* were used as internal control genes, as its abundance was stable between experimental groups and did not display any treatment effects (data not shown). All reactions were run in duplicate and controls without DNA templates were run to verify the absence of cDNA contamination. Primers efficiency was calculated for each qRT-PCR reaction using Light Cycler 480 II inner software.

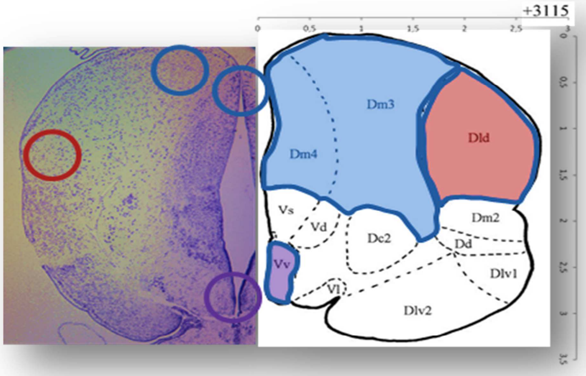

Figure. 3.1.3 |Coronal sections of Gilthead seabream (*Sparus aurata)* telencephalon spaced 400 µm from each other. Nissl staining images (left) and an illustrative section (right) adapted from Muñoz-Cueto et al. (2001) highlighting the areas of interest from the telencephalon. Areas of interest: medial part of the dorsal telencephalon (Dm, Blue), lateral telencephalon (Dl, red), ventral nucleus of the ventral telencephalon (Vv, purple).

Statistical analysis

Descriptive statistics are expressed as mean ± standard error of the mean (SEM). The assumptions of normality and homoscedasticity were confirmed by analysis of the residuals. Homogeneity of variance was checked by Levene's test and log or arcsine transformation was used to achieve homogeneity when required. The effect of conditioning (i.e. learning a cue as a predictor of the appetitive or aversive stimuli) on behavioural variables (i.e. immobility, escape attempts and social interactions) was assessed using a one-way repeated-measures ANOVA (repeated factor = 8 training sessions) followed by Tukey post-hoc tests, for each experimental factor (i.e. stimulus valence and predictability) and for each experimental condition (i.e. PRDapp; UnPRDapp; PRDavr and UnPRDavr).

Table 3.1.2 |List of primers and respective temperature of annealing used for quantitative real-time PCR from the different telencephalon regions studied in Gilthead seabream (*Sparus aurata)* brain.

Gene	Forward Primer	Reverse Primer	Ta*
18S[1]	AGGGTGTTGGCAGACGTTAC	CTTCTGCCTGTTGAGGAACC	57
EeF1a[1]	TGGCTTCAACATCAAGAACG	ATGTGAGCTGTGTGGCAATC	57
c-fos[2]	GGAGAGGAAGCAACAGCAAC	TACAAGTCCGCTCCATAGCC	58
EGR1[2]	ACCTGTGATCGTCGCTTCTC	ATCCTTCTGCCGTAAGTGGA	58
BDNF[2]	GCTCAGCGTGTGTGACAGTA	ACAGGGACCTTTTCCATGAC	59
NPAS4[2]	CAACCAAAGGAGCATCCAAG	AGCCGTGCTTTATCTGCATC	56

*Ta = Temperature of annealing; [1]Reference genes; [2]Target genes.

Planned comparisons were used to test for differences between experimental conditions within the same training trial. A two-way ANOVA was used to assess the main effects of each experimental factor (i.e. stimuli valence: app vs. avr; and predictability: PRD vs. UnPRD) on the behavioural variables in the test trial, on cortisol levels and on IEGs mRNA expression (*egr1*, *c-fos*, *bdnf* and *npas4*) in each brain region (Dm, Dl and Vv). A priori planned comparisons were used to test for specific differences between experimental conditions, namely: PRDapp vs. UnPRDapp; PRDavr vs. UnPRDavr; PRDapp vs. PRDavr; and UnPRDapp vs. UnPRDavr. Pearson and Spearman test, for normal and non-normal data respectively, were used to depict correlations among behavioural variables, between behaviour and cortisol, and between those and gene expression.

Stepwise linear discriminant analyses (LDA), were used to determine which measures of physiological (i.e. cortisol) and central state (i.e. immediate early genes expression in different brain nuclei) are the best predictors of affective states. The F statistic was used as a measure of the contribution of each variable (cortisol concentration and IEG expression in each brain region) to the discriminant functions. An F-value above 3.84 was used as the selection criteria for predictors to enter the model and predictors were removed when the

F-value dropped below 2.71 (e.g. (Maruska et al., 2013)). First, two LDA were run to discriminate between treatments with different valence (i.e. PRDapp and UnPRDapp vs. PRDavr and UnPRDavr) and salience (i.e. PRDapp and PRDavr vs. UnPRDapp and UnPRDavr), independently. Next, the variables that better discriminated valence and salience, independently of each other, were used to feed a third LDA for the four experimental treatments (PRDapp, UnPRDapp, PRDavr, UnPRDavr). Factor loadings above 0.30 were considered important for interpreting discriminant functions. The neurogenomic states, as indicated by the patterns of gene co-expression in each brain region, elicited by each experimental treatment were represented using heatmaps of Pearson correlations matrices, with p-values adjusted following the Benjamini and Hochberg's method (Benjamini and Hochberg, 1995). Differences in gene co-expression patterns between brain areas within each experimental condition, and between experimental conditions within each brain area, were assessed using the quadratic assignment procedure (QAP) correlation test with 5000 permutations (Borgatti et al., 2013). The null hypothesis of the QAP test is that when $p > 0.05$ there is no association between matrices, hence a non-significant p-value indicates that the correlation matrices are different. ANOVA and LDA statistical procedures were run on IBM SPSS® statistics v19.0, statistics performed to evaluate the neurogenomic states and QAP correlations were run on R® (R Development Core Team) and GraphPad Prism® v6.0 for windows was used for chart building and figures layout .

Results

Behavioural validation of predictability treatments

Fish in the predictable treatments (PRDapp and PRDavr) were trained (8 training trials) to associate a conditioned stimulus (CS) with an unconditioned stimulus (US) of either positive (US = food reward) or negative (US = physical confinement) valence. The analysis of the behavioural patterns throughout the training sessions indicates that fish established an association between CS and US, as indicated by a significant increase in social interactions and a decrease of time spent immobile along trials in the PRDapp treatment (Fig. 3.1.4a,b; Repeated measures ANOVA main effects: $F(_{7,322}) = 8.32$; $p < 0.001$ and $F(_{7,322}) = 1.87$; $p = 0.07$, respectively) that became significantly different from the UnPRDapp treatment in the later trials (see planned comparisons results in Figs. 3.1.4a,b). Under PRDavr conditions the association between CS and US was also established, as indicated by a significant increase in escape attempts and by a decrease of time immobile along the training sessions (Fig. 1c,d; Repeated measures ANOVA main effects: $F(_{7,301}) = 4.88$; $p < 0.001$ and $F(_{7,301}) = 19.10$; $p < 0.001$, respectively), that became significantly different from the UnPRDavr treatment in the later sessions (see planned comparisons results in Figs. 3.1.4c,d). Thus, at the end of the training period fish in the predictable treatments have learned to use the CS as a cue signalling exposure to the US, which hence became predictable.

Appraisal-driven behavioural states

After the training period fish from all treatments were exposed to the CS followed by the US in a test trial. The behaviour displayed by fish during exposure to the CS (which is a cue of experimental stimuli only in predictable treatments) was specific of each treatment: expression of social interactions occurred mostly among the individuals of the appetitive treatments, and these were more frequent in PRDapp than UnPRDapp ($F(_{3,89}) = 64.35$; $p < 0.001$; see Fig. 3.1.4e for planned comparisons); on the other hand, escape attempts were expressed mainly among individuals of the aversive treatments, and these were more frequent in PRDavr than in UnPRDavr ($F(_{3,89}) = 53.30$; $p < 0.001$; see Fig. 3.1.4f for planned comparisons). In agreement with these results a significant negative correlation was found between escape events and social interactions (Spearman correlation, $R_s = -0.426$, $n = 86$, $p < 0.001$). Finally, the time spent immobile was also significantly different across treatments ($F(_{3,89}) = 38.89$; $p < 0.001$; see Fig. 1g for planned comparisons). Thus, appraisal of stimulus valence and predictability elicits specific behavioural profiles.

Appraisal-driven physiological states

Plasma cortisol levels were affected by both valence ($F(_{1,80}) = 117.47$; $p < 0.001$) and predictability of the stimulus ($F(_{1,80}) = 64.65$; $p < 0.001$), and an interaction between both experimental factors was also found (valence x predictability: $F(_{1,80}) = 25.26$; $p < 0.001$). Under both valences (i.e. appetitive or aversive) fish in unpredictable treatments had higher cortisol levels than in predictable treatments (see Fig. 3.1.4h for planned comparisons results). Similarly, under both predictability regimens fish exposed to aversive stimulus had higher cortisol levels than fish exposed to appetitive ones (see Fig. 3.1.4h for planned comparisons results). Finally, throughout the training sessions plasma cortisol levels were positively correlated with escape attempts (R_s = 0.309, n = 86, p = 0.004) and negatively correlated with social interactions (R_s = - 0.636, n = 86, p < 0.001).

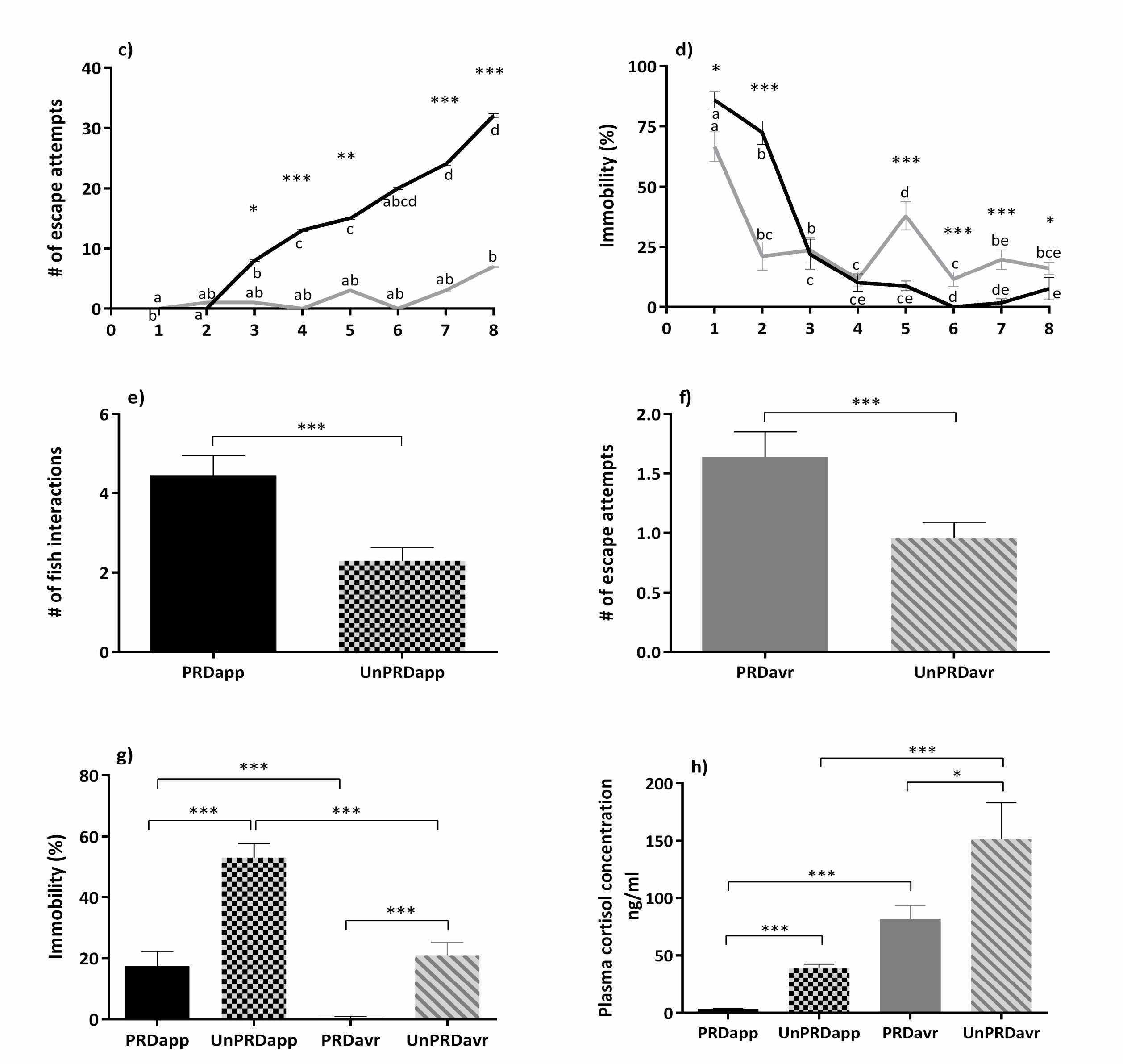

Figure 3.1.4 | Behavioural and physiological responses to stimuli of different valence and salience (i.e. predictability). (a-d) Behaviour expressed by fish during the training sessions for predictability (PRD = predictability treatment; UnPRD = unpredictability treatment): (a) frequency of social interactions in the appetitive treatments; (b) time spent immobile in the appetitive treatments (mean ± SEM); (c) frequency of escape attempts in the aversive treatments; (d) time spent immobile in the aversive treatments (mean ± SEM); significant differences (post-hoc tests) across trials within the same treatment are indicated by different letters; significant differences (planned comparisons) between treatments at each trial are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (e-f) Behaviour expressed by fish during the test session (PRDapp = predictable appetitive treatment; UnPRDapp = unpredictable appetitive treatment; PRDavr = predictable aversive treatment; UnPRDavr = unpredictable aversive treatment): (e) frequency of social interactions in the appetitive treatments; (f) frequency of escape attempts in the aversive treatments; (g) time spent immobile

and (h) plasma cortisol concentrations measured 30 min after the test session (mean ± SEM). Significant differences between treatments (planned comparisons: PRDapp vs. UnPRDapp; PRDavr vs. UnPRDavr; PRDapp vs. PRDavr and UnPRDapp vs. UnPRDavr) in panels (e-h) are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p< 0.001$). All descriptive statistics are mean ± SEM.

Appraisal-driven patterns of brain activation

A univariate analysis of immediate early gene expression at each of the three candidate brain regions sampled shows that Dl did not respond to either stimulus valence or predictability, whereas both Dm and Vv exhibited changes driven by either valence or predictability. The main effects of stimulus valence were restricted to the expression of *bdnf* in Vv and *npas4* in Dm. On the other hand, predictability had a main effect in the expression of all studied genes (*egr1*, *c-fos*, *bdnf*, *npas4*) in Vv, and also of *egr1* and *c-fos* expression in Dm (Table 3.1.3; Fig. 3.1.5). Finally, there was a significant interaction between stimulus valence and predictability in the expression of *bdnf* in Vv (Table 3.1.3).

Appraisal-driven neurogenomic states

Neurogenomic states, as represented by co-expression matrices of the target genes, across the studied brain regions and across experimental treatments are presented in Fig. 3.1.6. The neurogenomic states of Dm and Vv were unique for each of the four experimental treatments (PRDapp, UnPDRapp, PDRavr, UnPRDavr), whereas the neurogenomic state of Dl was similar between PRDapp and UnPRDavr, which were significantly different from either UnPRDapp or PRDavr (Fig. 3.1.6). Regarding the comparison of neurogenomic states across brain regions within each treatment, all treatments presented different gene co-expression patterns across all brain regions, except for UnPRDavr in which case Dm and Vv presented similar neurogenomic states (Fig. 3.1.6).

Correlations between appraisal-driven behavioural, physiological and neuromolecular responses

Positive correlations were found between time spent immobile and the expression of *egr1*, *c-fos* and *npas4* in the Vv telencephalic region ($R_S = 0.415$, $n = 32$, $p = 0.018$; $R_S = 0.540$, $n = 32$, $p = 0.001$; $R_S = 0.346$, $n = 32$, $p = 0.05$, respectively). Also, a positive correlation was found between escape attempts and the expression of *bdnf* in the Vv, whereas social interactions were negatively correlated ($R_s = 0.511$, n = 32, p = 0.003; $R_s = - 0.546$, n = 32, p = 0.001, respectively). Positive correlations were also found between cortisol levels and mRNA expression in the Dm of *egr1* and Vv of *c-fos* ($R_p = 0.434$, n = 32, p = 0.013; $R_p = 0.410$, n = 32, p = 0.020, respectively), in the Dm and Vv of *npas4* ($R_p = 0.356$, n = 32, p = 0.046; $R_p = 0.436$, n = 32, p = 0.013, respectively) and in Vv of *bdnf* ($R_s = 0.687$, n = 32, p < 0.001).

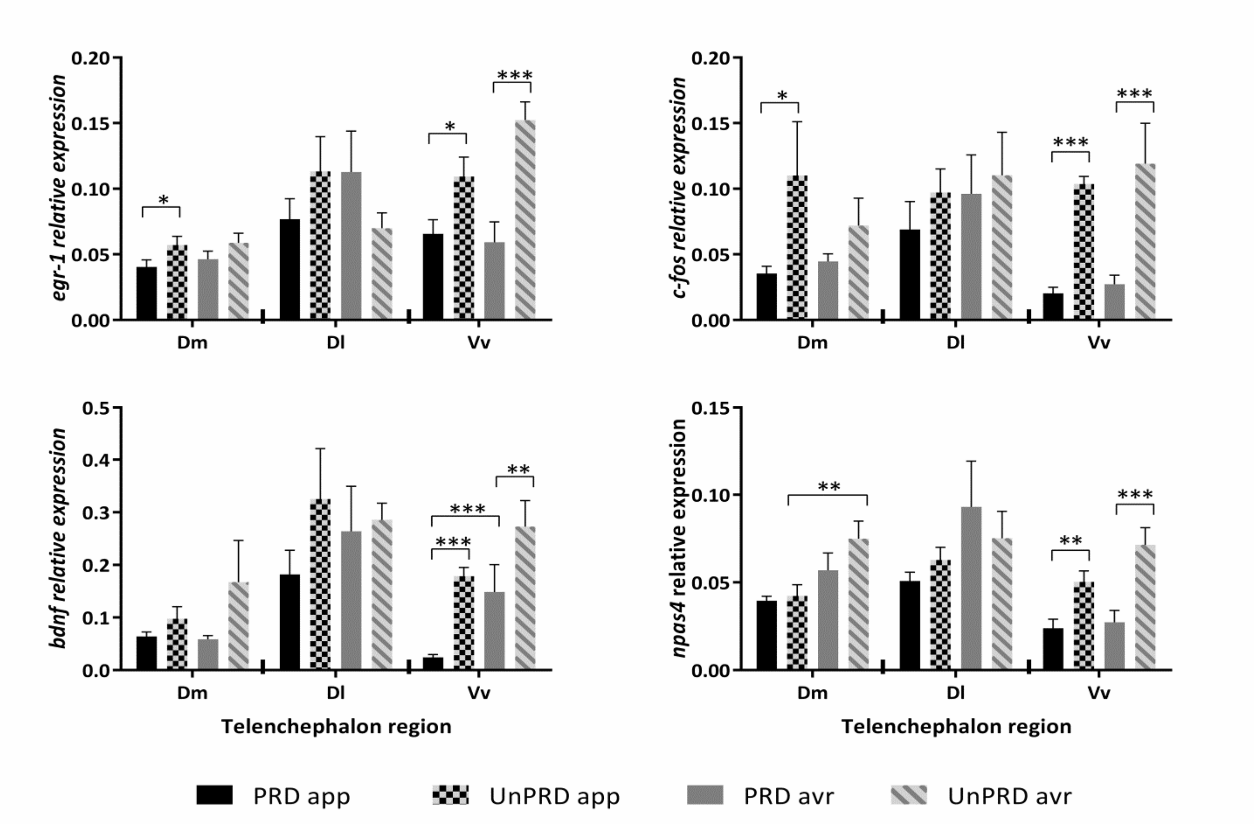

Figure 3.1.5 | Expression (mean ± SEM) of the immediate early genes *egr1*, *c-fos*, *bdnf* and *npas4* in the Dm, Dl and Vv telencephalon regions of seabream in the different experimental conditions. Significant differences (planned comparisons) in expression levels between experimental conditions (i.e. PRDapp vs. UnPRDapp; PRDavr vs. UnPRDavr; PRDapp vs. PRDavr and UnPRDapp vs. UnPRDavr) are indicated by asterisks: * p < 0.05; ** p < 0.01; *** p < 0.001.

Discrimination of appraisal-driven core affective states based on neuromolecular and physiological data

Stepwise linear discriminant function analysis (LDA) was used to investigate if cortisol levels and immediate early gene expression across candidate brain regions can predict to which of the four combinations of stimulus valence and salience (i.e. predictability), which correspond to the four quadrants of the core affective space, the individuals had been exposed to. First, two LDA were performed for stimulus valence and salience (i.e. predictability), independently. Next, the variables that better discriminated valence and salience, independently of each other, were used to feed a third LDA for the four experimental treatments. The LDA for valence (i.e. PRDapp and UnPRDapp vs. PRDavr and UnPRDavr) revealed a single discriminant function (Wilk's lambda = 0.477, chi-square = 21.49, p<0.001), which was significantly loaded with the expression of *npas4* in Dm (0.530) and with cortisol level (0.831), that explained 100% of the variance, hence classifying correctly 100% of the individuals that belong to each valence treatment.

Table 3.1.3 |Two-way ANOVA main effects (stimuli valence and predictability) of the behavioural variables measured (immobility, escape attempts and fish interactions) during the stimulus signalling of the trial session, of cortisol levels measured 30 min after the trial session and of IEG's mRNA expression measured for each telencephalic region. Significant values are highlighted in bold.

Variables		**Stimuli valence**		**Predictability**		**Stimuli valence* Predictability**	
Behaviour	**df**	***F***	***p***	***F***	***p***	***F***	***p***
Immobility (%)	**(1,89)**	45.09	**p < 0.001**	71.81	**p < 0.001**	0.46	p = 0.497
Escape Attempts	**(1,89)**	131.21	**p < 0.001**	6.06	**p = 0.01**	25.27	**p < 0.001**
Fish Interactions	**(1,89)**	173.91	**p < 0.001**	3.41	p = 0.06	15.82	**p < 0.001**
Cortisol							
Cortisol (ng ml^{-1})	**(1,80)**	117.47	**p < 0.001**	64.65	**p < 0.001**	25.26	**p < 0.001**
IEG´s	**Region**						
egr1	**Dm**	0.352	0.558	6.074	**0.02**	0.225	0.639
	Dl	0.063	0.803	0.012	0.915	1.866	0.183
	Vv	0.147	0.704	23.436	**0.001**	3.095	0.089
c-fos	**Dm**	0.198	0.66	4.887	**0.035**	2.06	0.162
	Dl	0.068	0.796	0.63	0.434	1.17	0.289
	Vv	0.282	0.599	46.168	**0.001**	0.618	0.438
bdnf	**Dm**	0.263	0.612	2.757	0.108	0.6	0.445
	Dl	0.882	0.356	1.598	0.217	0.003	0.956
	Vv	24.331	**0.001**	52.526	**0.001**	10.213	**0.003**
npas4	**Dm**	10.52	**0.003**	1.525	0.227	1.346	0.256
	Dl	1.496	0.231	0.184	0.671	0.434	0.515
	Vv	1.334	0.258	28.164	**0.001**	0.447	0.509

Similarly, the LDA for salience (i.e. predictability) (i.e. PRDapp and PRDavr vs. UnPRDapp and UnPRDavr) also revealed a single discriminant function (Wilk's lambda = 0.170, chi-square = 49.58, p<0.001), that explained 100% of the variance, hence correctly classifying all the individuals that belong to each salience treatment. This function was significantly loaded with the expression of *egr1* in Dm (0.580) and of *egr1* (0.635), *c-fos* (0.705), and *bdnf* (0.544) in Vv. When the six variables that were significantly loaded in the valence and salience discriminant functions described above were used to feed a LDA to predict the valence x salience treatment (as a proxy for affective state), this LDA revealed three significant functions (function 1: Wilk's lambda = 0.022, chi-square = 98.98, p<0.0001; function 2: Wilk's lambda = 0.316, chi-square = 29.94, p<0.001; function 3: Wilk's lambda = 0.696, chi-square = 9.43, p=0.05), with functions 1-3 explaining 89%, 8.1% and 2.9% of the variance respectively (Fig. 3.1.7).

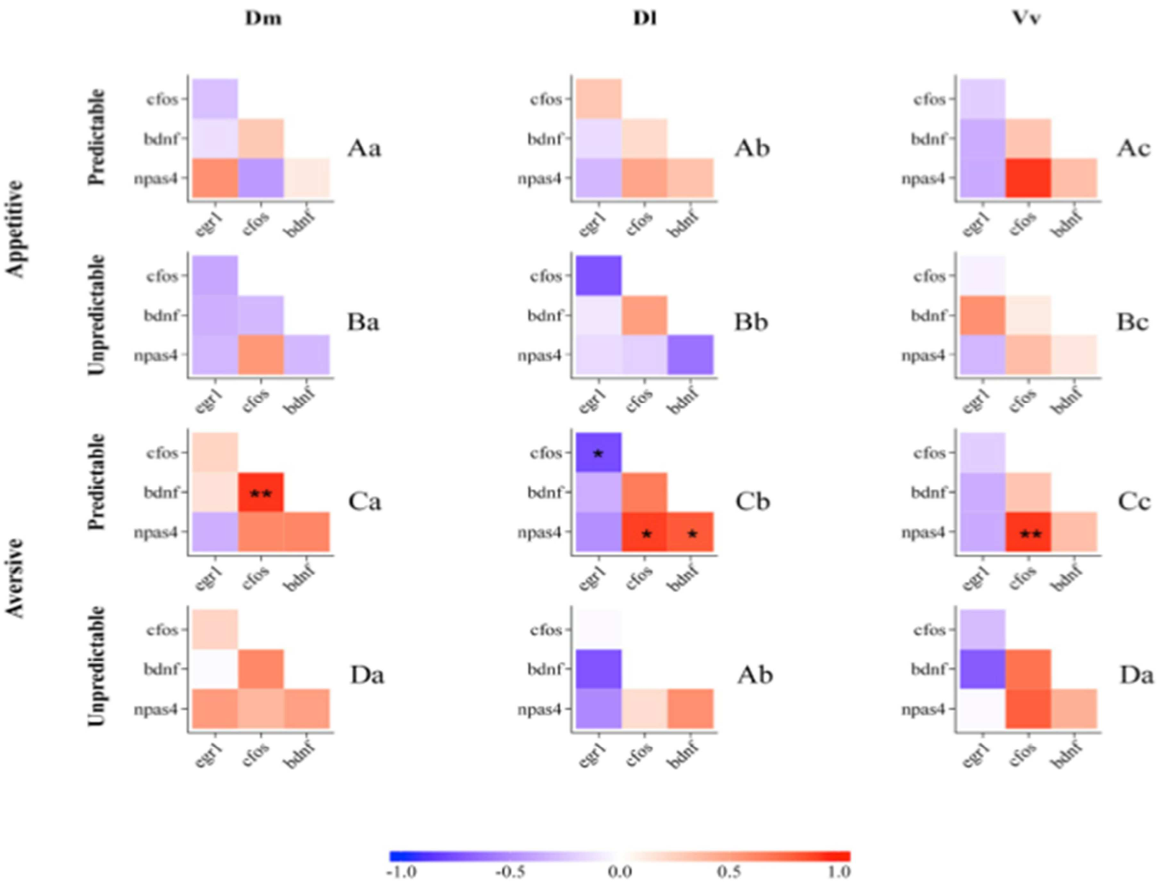

Figure 3.1.6 |Neurogenomic states, as described by correlation (r) matrices of immediate early genes expression in the different brain nuclei (Dm, medial zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area; Vv, ventral nucleus of the ventral telencephalic area) for each affective state (PRDapp = predictable appetitive treatment; UnPRDapp = unpredictable appetitive treatment; PRDavr = predictable aversive treatment; UnPRDavr = unpredictable aversive treatment); Color scheme represents r-values from -1 (blue) to 1 (red); Asterisks indicate significant correlations after p-value adjustment: *p < 0.05; **p < 0.01; ***p < 0.001; different capital letters indicate significantly different co-expression patterns among affective states, and different small letters indicate significantly different co-expression patterns among brain nuclei, using the QAP correlation test.

Analysis of canonical discriminant function coefficients showed that: function 1 was most heavily loaded by *bdnf* and *egr1* expression in Vv (0.856 and 0.646, respectively) and by *egr1* expression in Dm (0.674); function 2 was most heavily loaded by cortisol levels (-0.800) and by *egr1* levels in Dm (0.595); and function 3 was most heavily loaded by *npas4* levels in Dm

(0.713). From Fig. 3.1.7 it can be seen that function 1 discriminated between treatments with different salience (i.e. PRDapp and PRDavr from UnPRDapp and UnPRDavr), whereas functions 2 and 3 jointly discriminate between treatments with different valences (i.e. PRDapp and UnPRDapp vs. PRDavr and UnPRDavr). Together these three functions allowed the correct classification of all individuals according to their treatment (i.e. affective state).

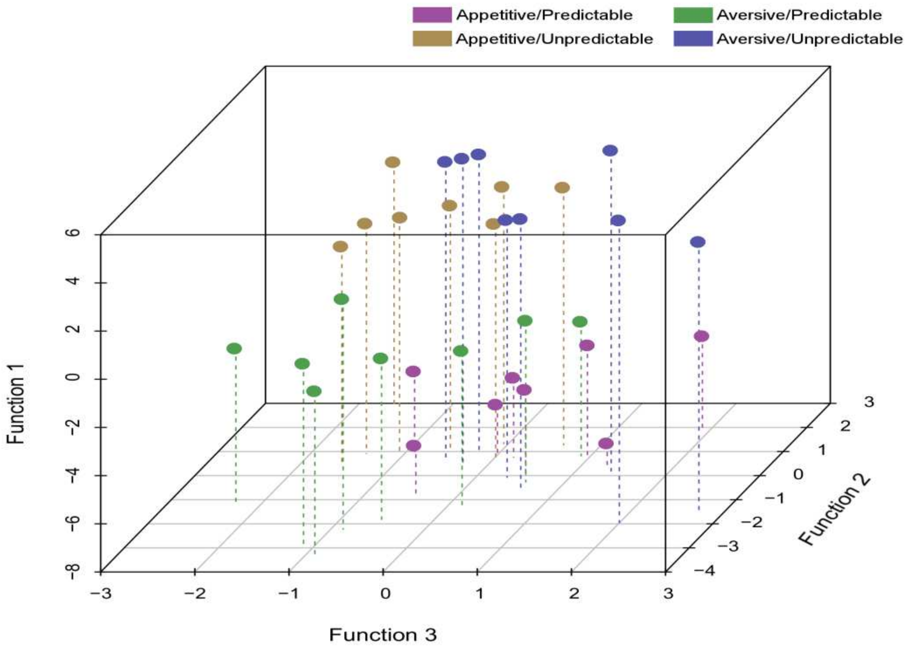

Figure 3.1.7 |Linear discriminant analysis of the four affective states induced by the four experimental treatments (PRDapp = predictable appetitive treatment; UnPRDapp = unpredictable appetitive treatment; PRDavr = predictable aversive treatment; UnPRDavr = unpredictable aversive treatment) as a function of cortisol and immediate early genes levels in all brain regions. Discriminant scores for each individual are plotted.

Discussion

In this study we have shown that seabream exposed to stimuli that vary according to valence (appetitive, aversive) and salience (predictable, unpredictable) exhibit different behavioural, physiological and neuromolecular states that are specific to each combination of valence and salience (i.e. appetitive predictable, appetitive unpredictable, aversive predictable, aversive unpredictable). At the behavioural level fish exposed to each valence by salience combination exhibited specific behavioural profiles, with appetitive stimuli promoting the occurrence of social interactions and aversive stimuli triggering escape attempts. These behaviours were both more frequent in predictable than in unpredictable treatments. Thus, stimulus valence elicited the expression of qualitatively different behaviours, whereas stimulus salience affected quantitatively behavioural expression. At the physiological level circulating cortisol levels were higher in fish exposed to aversive than to appetitive stimuli, and within each valence unpredictable stimuli elicited higher cortisol levels. Again, stimulus of each valence by salience combination elicited distinct cortisol levels. Finally, in order to characterize central states at the level of the central nervous system we have sampled the expression of a set of immediate early genes involved in experience-driven neuroplasticity, namely *egr1*, *c-fos*, *bdnf* and *npas4*, in three brain regions that are homologues in teleost fish to areas known to be involved in reward and aversion processing in mammals (Dm = amygdala, Dl = hippocampus; and Vv = septum). Two (Dm and Vv) out of these three brain regions showed specific responses to emotional stimuli. Stimulus valence triggered different expression profiles of *bdnf* in Vv and of *npas4* in Dm, with aversive stimuli eliciting higher expression levels of both genes than appetitive stimuli. Stimulus salience (predictability) elicited different expression profiles of all four studied genes in Vv (unpredictable > predictable) and of *c-fos* and *egr1* in Dm (unpredictable > predictable). Thus, overall activity of Vv seems to be associated with stimulus valence, a view that is also supported by the positive correlations between time spent immobile (suggestive of freezing behaviour) and mRNA expression of *egr1*, *c-fos* and *npas4* in Vv. Moreover, neurogenomic states, as capture by the co-expression profile (i.e. correlation matrices) of the four studied genes across the three studied brain regions, were unique for each of the four experimental treatments, suggesting that stimulus of each valence by salience combination elicited distinct central states. Finally, we have used linear discriminant function analyses to check if the measured behavioural, physiological and neuromolecular variables could efficiently discriminate the four experimental treatments. A discriminant function that correctly classified 100% of individuals according to stimulus valence that they have been exposed to was significantly loaded with the expression of *npas4* in Dm and with cortisol levels, whereas another discriminant function that classified correctly 100% of the individuals belonging to each salience treatment was loaded with the expression of *egr1* in Dm and of *egr1*, *c-fos*, and *bdnf* in Vv. The variables that were significantly loaded in these two discriminant functions were then used to develop a third discriminant analysis aiming at discriminating the four experimental treatments, which correctly classified 100% of individuals according to treatment. Thus, physiological and neurogenomic state can successfully discriminate the four combinations of valence by salience stimuli. Since according to the dimensional theories of emotion (Mendl et al., 2010; Russell, 2003) valence and salience define a two-dimensional affective space, our data can be interpreted as evidence for the occurrence of distinctive affective states in fish corresponding to each the four quadrants of the core affective space.

Emotions have been described as expressive behaviours associated with internal brain states, which humans experience as feelings (Dolan, 2002). Since animals cannot report the subjective experience of feelings the assessment of emotional states in animals has to rely on the occurrence of stereotypic behaviours associated with internal central states. Thus, from a comparative perspective an emotion can be defined as a brain state, encoded by the activity of specific neural circuits, that is triggered by specific stimuli and that elicits the expression of specific behaviours and other external cues (Anderson and Adolphs, 2014). From this perspective, the results reported here showing that external stimuli of different valence and salience triggers the expression of specific behavioural profiles associated with specific physiological and neuromolecular states supports the occurrence of emotion-like states in fish. Given that emotional states are often associated with human behaviour this result may sound surprising at first. However, the evolution of affective states (i.e. emotions/mood) in animals has been predicted by theoretical models of adaptive decision-making, since it allows an adjustment of the response to cues of reward and punishment according to the autocorrelation of aversive and appetitive events in the environment and internal condition, rather than using a fixed response threshold. Thus, the modulation of decision-making by core affective states would allow animals to give more efficient responses to a wide range of fitness threatening and fitness enhancing events (Ekman, 1992; Nettle and Bateson, 2012; Rolls, 2000; Trimmer et al., 2013). More recently, it has been proposed that these affective states share a number of properties, namely scalability, valence, persistence and generalization, which have been named emotion primitives, that allow their recognition in phylogenetically distant organism, hence opening the study of the biological mechanisms of emotion across different taxa (Anderson and Adolphs, 2014; Gibson et al., 2015). In a previous study using a conditioned place preference/ avoidance paradigm we have shown that seabream exposed to appetitive or aversive stimuli have valence-specific responses (preference vs. avoidance, respectively) that are persistent in time, even when only the CS (i.e. conditioned place) is present (Millot et al., 2014b). Thus, in seabream, at least two of these emotion primitives are present.

The fact that in this study the same stimulus presented in a predictable vs. unpredictable way elicited different behavioural, physiological and neuromolecular states suggests that stimulus appraisal by the individual, rather than an intrinsic characteristic of the stimulus, such as its valence, is triggering the observed responses. Therefore, the occurrence of emotion-like states in fish seems to be regulated by the individual's perception of environmental stimuli. The role of cognitive appraisal in the regulation of stress and emotional states was first proposed in humans and has subsequently been expanded to other animals (Faustino et al., 2015; Mendl et al., 2010; Paul et al., 2005). Thus, animals like humans, are expected to use a set of stimulus evaluation checks (e.g. intrinsic valence, stimulus novelty, violation of expectation) in order to assess the valence and salience of stimuli and select an adaptive response. Many studies have documented the occurrence of such stimulus evaluation checks in animals and their modulation of behavioural and/or physiological responses (see Faustino et al. (2015) for a recent review). In fish the occurrence of cognitive appraisal has been documented in different species and all stimulus evaluation checks involved in primary appraisal have been described (e.g. intrinsic valence of stimuli demonstrated by learned approach/avoidance behaviours, (Millot et al., 2014a; Millot et al., 2014b); predictability, familiarity or controllability as a stimulus novelty cues: Mozambique tilapia, *Oreochromis mossambicus*, (Aires et al., 2015; Galhardo et al., 2011; Galhardo et al., 2012); rainbow trout, *Oncorhynchus mykiss*, (Carpenter and Summers, 2009); violation of expectation: Atlantic salmon (*Salmo salar*, (Vindas et al., 2012; Vindas et al., 2014b); rainbow trout, *Oncorhynchus mykiss*, (Vindas et al., 2014a)). However, the neural bases of these cognitive appraisal processes have not been investigated yet in fish and the present study provided a new insight into these mechanisms.

The understanding of global brain states, as indicated by whole brain activity, has been hampered by technical constraints in recording neural activity across widespread brain regions with high spatial and temporal resolution. In humans and few other animals, this technical challenge has been overcome by functional magnetic resonance (fMRI) using BOLD signal, which provides whole brain analysis of functional activity (e.g. (Van der Linden et al., 2009)). In other animals, molecular markers of neuronal activity, such as the expression of immediate early genes, have been used for this purpose and global patterns of gene expression across brain regions have been related to physiological and behavioural measures (e.g. (Cardoso et al., 2015)). An assumption used in this approach is that behavioural states can be mapped into neuromolecular states of relevant brain regions (aka neurogenomic states, (Cardoso et al., 2015; Zayed and Robinson, 2012)). In this study, a similar approach has been used and the occurrence of specific neurogenomic states induced by emotional stimuli has been investigated in a set of brain regions in seabream that are putative homologues of regions involved in emotional stimuli processing in mammals, namely the amygdala (Dm), the lateral septum (Vv) and the hippocampus (Dl) (Rolls, 2000; Sheehan et al., 2004). In mammals the prefrontal cortex is also known to play an important role in emotional regulation (Quirk and Beer, 2006), but a homologue area in teleost fish is not known. The four immediate early genes used (i.e. *egr1*, *c-fos*, *npas4* and *bdnf*) are involved in different signalling pathways and thus were expected to capture complementary information on neural activation (Leal et al., 2015; Lin et al., 2008; Sun and Lin, 2016). Indeed, these genes exhibited different expression profiles. However, all four genes exhibited a similar pattern of expression in the Vv, with predictability of either appetitive or aversive stimuli heightening their mRNA levels. Moreover, the linear discriminant analysis for predictability identified a significant function loaded with the expression of *egr1*, *c-fos* and *bdnf* in Vv and *egr1* in Dm. Thus, Vv seems to play a role in the appraisal of stimulus predictability in fish. This result is in line with the role of the lateral septum in the perception of stimulus novelty and emotional regulation in other species (Landgraf et al., 1995; Liebsch et al., 1996; Sheehan et al., 2004). In mammals the septum also establishes a circuit with hippocampus (i.e. septo-hippocampal pathway) that modulates memory formation and recall in the hippocampus (Rokers et al., 2002). Thus, the increased activation of Vv in fish of predictable treatments may also reflect associative learning of the CS that signalled the aversive/appetitive stimulus in our experiment. On the other hand, the expression of *bdnf* in Vv and of *npas4* in Dm was higher in the aversive treatments and the discriminant function for stimulus valence was significantly loaded with the expression of *npas4* in Dm and with cortisol levels, hence suggesting an involvement of Dm, eventually modulated by cortisol levels, on the appraisal of stimulus valence. Again this result is in line with results from other studies in mammals that have shown an involvement of the basolateral amygdala in responses to emotional stimuli (LeDoux, 2000) and of *npas4* in fear memory (Ploski et al., 2011; Sun and Lin, 2016). Thus, the neuromolecular data presented here suggest an involvement of both Vv and Dm in the appraisal of emotional stimuli, which supports the occurrence of an evolutionary conserved neural substrate for the processing of emotional stimuli, given the similar role played by the mammalian homologues of these areas.

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CHAPTER III-2

Cognitive appraisal in fish: stressor predictability modulates the physiological and neurobehavioural stress response in seabass

This chapter has been submitted to **Animal Cognition** as:

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Cognitive appraisal in fish: stressor predictability modulates the physiological and neurobehavioural stress response in seabass

Abstract: Much of our understanding of fish's ability to respond to stress comes from studies focused on their physiological responses under stressful situations. There is comparatively little information about their cognitive ability to appraise environmental stimuli and how it modulates the stress response. And, though there are already evidences in fish of the occurrence of each of the stimulus evaluation checks involved in primary appraisal, there is not enough evidence yet to support the occurrence of cognitive appraisal. As such, in this study we addressed the mechanisms underlying this process of how European sea bass (*Dicentrarchus labrax*) cognitively appraise their environment, by measuring the proximate (i.e. neural/physiological) bases of these cognitive appraisal processes and their behavioural correlates. Fish were tested, either in groups or in isolation, under a conditioning associative learning paradigm, to test an aversive stimulus (confinement) given on either a predictable (signalling the onset of a stimulus by a visual cue) or unpredictable way (visual cue dissociated from the stimulus). Though fish under a social context seem to perceive and interpret predictable conditions as less stressful, social isolation seems to mask this stress-mitigating effect of predictability. Proximate states showed that social isolation might have affected the learning ability of the individuals, given no pronounced differences were found between experimental conditions. Moreover, our results suggest that social support has a positive effect on the appraisal that fish do of a stressful situation. These outcomes can have profound implications on the understanding of the regulation of the stress response, as well as on fish welfare and its management.

Keywords: Behavioural contagion, Environmental perception, Psychological components; Stimulus regularity; Social contagion.

Introduction

The literature on stress biology has long established the role of cognitive factors on triggering the stress response, defined as a response of the organism to regain homeostasis when exposed to a homeostasis threatening stimulus or event (aka stressor) (Koolhaas et al., 2011). Since the 1970's it became clear that the cognitive appraisal of stimuli is a key mechanism in the activation of the stress response (Mason, 1968; Weiss, 1972). According to this perspective, it is not the intrinsic physical characteristics of the stimulus that trigger a response but rather the evaluation of what that stimulus or event means to that organism at that moment in time, which depends on stored information in memory about relations between stimuli (i.e. stimulus-stimulus learning or classic conditioning) and about relations between responses and stimuli (i.e. stimulus-response learning or instrumental conditioning) (Ursin and Eriksen, 2004). Therefore, the same stimulus may elicit or not a stress response depending on how it is appraised by the individual. An "alarm" response would occur when expectancies, based on perceived contingencies between stimuli (i.e. stimulus expectancies) and between stimulus and response (i.e. response expectancies), are not met (i.e. when there is a discrepancy between expected situation and perceived situation). Hence, stimulus predictability, which refers to high levels of perceived probability of occurrence of the expected event, and stimulus controllability, which refers to high levels of perceived probability for response outcomes, play a major role on the appraisal of stimuli as aversive or not. Interestingly, the role of cognitive variables in the activation of a stress response was first investigated in laboratory animals, in particular in rodents (e.g. Weiss, 1970), and then extended to humans (e.g. Lazarus, 1999).

In the last decades the role of cognitive variables in the activation of stress responses as well as in triggering responses to appetitive events has been framed under a theory of cognitive appraisal. According to this theory individuals continuously monitor the environment using a set of stimulus evaluation checks (e.g. intrinsic valence, novelty, prediction error, capacity for control) in order to evaluate the valence (positive/ negative) and salience (high/ low) of detected stimuli (primary appraisal), and also assess the available organismal resources to deal with them (secondary appraisal) (Faustino et al., 2015; Mendl et al., 2010; Moors et al., 2013). While the appraisal concept has already been applied to the study of stress and emotional behaviour in animals, mainly in mammals (see Faustino et al. 2015 for a recent review), in fish the whole concept of psychological stress has been rarely addressed (Galhardo et al., 2011; Galhardo, 2009; Madaro et al., 2015; Madaro et al., 2016; Vindas et al., 2014a; Vindas et al., 2014b). However, empirical evidence for the occurrence of each of the stimulus evaluation checks involved in primary appraisal has been described in fish. The appraisal of the intrinsic valence of stimuli can be demonstrated by learned approach/avoidance behaviours, and these have been described in different fish species (Millot et al., 2014a; Millot et al., 2014b). The use of the three cues that signal stimulus novelty have also been documented in fish: the effects of predictability in modulating the behavioural and physiological response to both aversive and appetitive stimuli have been described in the Mozambique tilapia (*Oreochromis mossambicus*) (Galhardo et al., 2011); familiarity with conspecifics has been shown to modulate both exploratory behavior and the response to a territorial intrusion, also in Tilapia (Aires et al., 2015; Galhardo et al., 2012); and the effect of controllability can be illustrated by rainbow trout (*Oncorhynchus mykiss*) that have the chance to actively avoid being defeated by a larger conspecific in a conditioning paradigm exhibiting a lower cortisol response to the conditioned stimulus, than those that cannot escape social defeat (Carpenter and Summers, 2009). Finally, prediction error has recently been documented both in rainbow trout and in Atlantic salmon (*Salmo salar*) using a reward omission paradigm (Vindas et al., 2012; Vindas et al., 2014a; Vindas et al., 2014b). However, this evidence has so far not been explicitly presented as supporting the occurrence of cognitive appraisal in fish and the proximate (i.e. neural/physiological) bases of these cognitive appraisal processes have not been investigated yet in fish. Given the expected universality of stimulus evaluation checks across animals, it is now timely to characterize their occurrence across species and to implement comparative studies on the underlying neural mechanisms. Teleost fish offer an excellent opportunity for such comparative approach, given the divergent evolutionary path between ray-finned fish and tetrapods (Romer, 1966; Venkatesh et al., 2001), and the homologies that have already been established between teleost and mammalian brain regions, that include some of the areas known to be involved in cognitive appraisal in mammals (i.e. amygdala and hippocampus, Broglio et al., 2005; Ganz et al., 2012, 2015). Thus, the study of cognitive appraisal in fish will allow testing if the same cognitive appraisal processes are present in evolutionary divergent vertebrate taxa and if they share homologue neural mechanisms.

In this study we tested the effect of predictability of a stressor on the behavioural and physiological stress response of European sea bass (*Dicentrarchus labrax*). Seabass was used as a model in this study given its wide use in European aquaculture, which makes the results present here not only of importance for the basic biology of fish stress but also to have translational value for the improvement of welfare of farmed fish. We have also characterized the pattern of neuronal activation (using the expression of immediate early genes as markers of neuronal activation) of two brain regions that are homologous to mammalian brain regions known to be involved in cognitive appraisal in mammals, namely the dorsomedial telencephalon (putative teleost homologue of the mammalian amygdala) and the dorsolateral telencephalon (putative teleost homologue of the mammalian hippocampus), in order to test if brain regions involved in cognitive appraisal are evolutionary conserved. Given that predictability is a key stimulus evaluation check in cognitive appraisal theory, its occurrence in fish will also be proof for the occurrence of cognitive appraisal in fish.

Material and methods

Experimental procedures 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 Portuguese competent authority for the protection of experimental animals (Direção Geral da Alimentação e Veterinária, Portugal; permit number 0420/000/000-n.99-09/11/2009). Experimental procedures, when necessary, were performed under anaesthesia with 2-phenoxyethanol and every effort was made to minimize suffering.

Experimental fish and maintenance

A batch of seabass with an initial body weight of 0.5 ± 0.3 g (mean ± SD) hatched at the experimental research station of IFREMER in Palavas-les-Flots (France) were transported to CCMAR Research Station (Faro, Portugal). Fish were reared in 500 L tanks in an open water circuit with constant aeration through air stones (temperature of 21 ± 5 °c, salinity of 35 ± 1 ‰, dissolved oxygen above 75 %, and a 12L:12D photoperiod) during 10 months before the experiments. Fish were initially fed at 10 % of body weight with commercial diets (Aquagold, Aquasoja, Sorgal SA, Portugal), and later food amount was readjusted until 3 % of body weight in accordance with their growth. A total of 96 fish with a body weight of 44.58 ± 6.36 g (mean ± SD) at the start of the experiments were used.

Experimental design and conditioning procedures

Given the fact that seabass are a shoaling species the effects of predictability on the stress response were tested both in isolated fish (Experiment 1) and in fish kept in social groups (Experiment 2). The two experiments occurred between May and June of 2013. Twelve experimental glass aquaria (70 x 40 x 30 cm) were used under the same housing conditions as described above, except for the fact that no air flow was supplied, since the water flow rate of 2.5 L min^{-1} was sufficient to guarantee oxygen saturation. A net, with the same dimension as the lateral wall, was settled in one side of each aquarium at the beginning of the experiment to be used as a confinement net. All aquaria walls were covered with opaque plastic to avert visual contact between the animals and the experimenters. The fish were fed at 3 % Bw^{-1} daily, divided by two meals at 08:00 h and 18:00 h. Water quality was analysed for nitrites (< 0.1 $mg.L^{-1}$) and ammonia (< 0.1 $mg.L^{-1}$) every three days. Temperature, oxygen saturation and pH were daily checked before the cleaning routines performed 1 h after the second meal.

One month before the experiments, 96 fish were tagged under anaesthesia with a 1 cm long floy tag (Floy Tag Manufacturing Inc, Seatle, USA) and with a multicolour pearl attached behind the dorsal fin. Two experimental conditions were tested in each experiment: predictability (PRD) and unpredictability (UnPRD). In Experiment 1 (social isolation), which lasted 3 days, 24 fish were tested in each experimental condition (predictable asocial, PRDa; unpredictable asocial, UnPRDa). In Experiment 2 (social groups), which lasted 14 days, six groups of 4 fish each were used in each experimental condition (predictable social, PRDs; unpredictable social, UnPRDs; n = 24 fish/treatment). The differences in the duration of the two experiments are due to the fact that preliminary trials had shown that seabass kept in small shoals needs between 8 to 12 days to show signals of adaptation to the experimental environment, whether seabass kept in social isolation does not survive that long (e.g. individuals die before resuming feeding). Because of this difference in duration between the two experiments their results cannot be directly compared (see statistical analysis below). After this variable acclimation period, the experimental period occurred in the last 2 days for both experiments and involved 4 training sessions in the first day (at 10:00 h, 12:00 h, 14:00 h and 16:00 h) and 3 training sessions (at 10:00 h, 12:00 h, and 14:00 h) and one test session (at 16:00 h) on the second day (see Fig. 3.2.1 for an overview of experimental procedures). To create the predictable and unpredictable treatments two different training procedures were used. In the predictable treatment a delay conditioning protocol was used for fish to learn to associate a visual cue (CS), which consisted of a yellow and black stripped card with the same size as the lateral wall of the aquaria (40 cm width x 30 cm), with a stressor (US: confinement). The CS was presented on the opposite wall of the confinement net (US), remained in view for 1 min before the occurrence of US, and overlapped 1 min with it. The confinement was obtained by moving the net into the visual cue direction until it reached 15% of the aquarium volume. In the unpredictable treatment fish were presented with the same visual sign but temporally dissociated from the stressor (i.e. 30 min before or after in a random way). The number of conditioning trials used was based on preliminary tests that indicated that 5-6 trials were enough for aversive conditioning in this species. In the test session, fish from both experimental treatments (PRD and UnPRD) were exposed to the visual cue together with the stressor.

Behavioural observations

Fish behaviour was video recorded right before the first training session and during the test session using video cameras (TVCCD-623-COL, Monacor®, Denmark and webcams HD C310 Logitech) positioned 1 m above the tank. Videos were subsequently analysed using multi-event recorder software (Observer XT® from Noldus, Netherlands). The response to the visual cue was assessed using the following behavioural measurements: (1) time spent in freezing behaviour (i.e. time fish spent immobile, with or without fin movements, either on the bottom or in the water column); (2) escape behaviour (i.e. fish swimming strongly, going close to the tank walls or moving the body in a way that looked like escape attempts); (3) shoal cohesion, measured in an arbitrary scale (1 = low, 2 = medium; 3 = high); and (4) exploratory behaviour following the formula:

$$A / t_{maximum}$$

where A is the arithmetic mean of the time fish spent in each one of 3 previously delimited areas of the tank (confinement net area; centre of the tank; and visual cue area), and $t_{maximum}$

is the maximum time found for any of the areas tested. When this ratio is close to 1 it indicates high exploratory behaviour, and when it is close to 0 it indicates low exploratory behaviour.

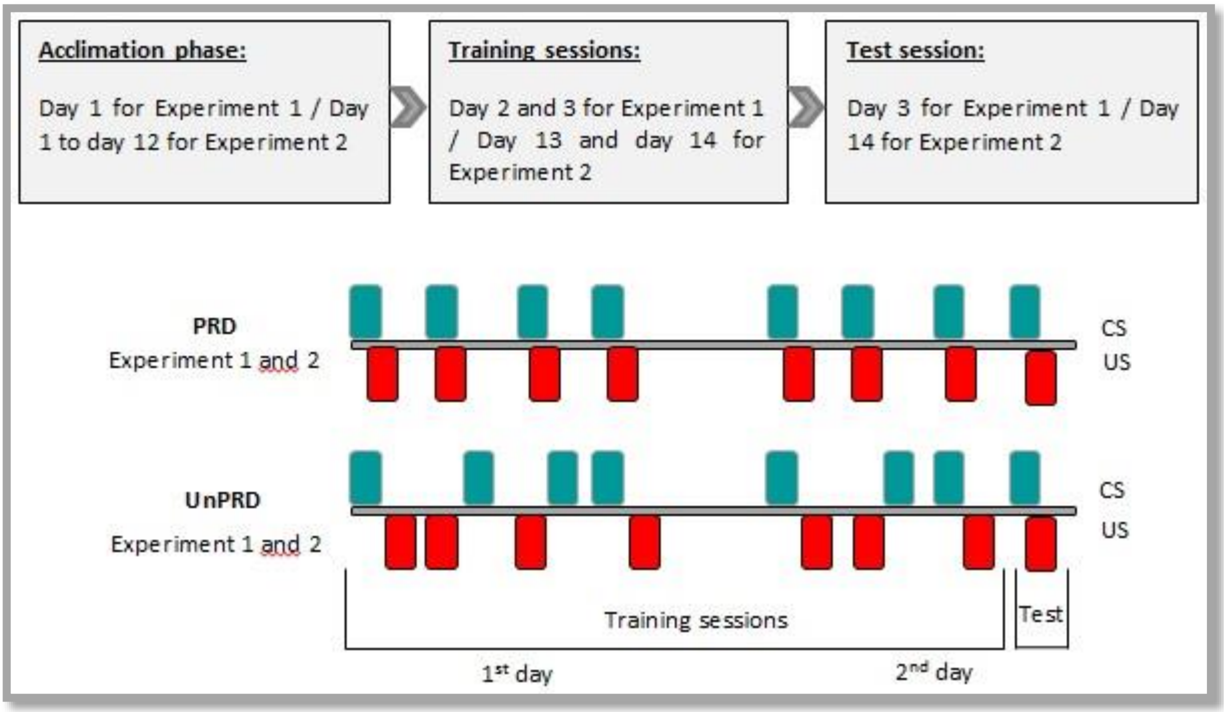

Figure 3.2.1 |Overview of the protocol and experimental conditions used to test predictability as an appraisal modulator of aversive events in seabass *Dicentrarchus labrax*.

Blood Sampling and plasma cortisol analysis

In both experiments, 30 min after the test session, fish were rapidly caught and euthanized with an overdose of 2-phenoxyethanol (1‰, Sigma-Aldrich) and blood was immediately collected from the caudal vein and centrifuged at RT for 25 minutes at 2000 g. Plasma was stored at -80 °C until further processing. Plasma cortisol levels were measured using a commercial ELISA kit (RE52061, IBL Hamburg, Germany), with a sensitivity of 2.5 ng ml^{-1} and intra- and inter-assay coefficients of variation (CV) were 2.9% and 3.5 %, respectively.

Brain microdissection and gene expression analysis

In both experiments 8 individuals from each experimental treatment were randomly selected for the assessment of immediate early genes (IEGs) mRNA expression in brain regions of interest (see below). Fish were sacrificed and the skull with the brain inside was immediately imbedded in Tissue-Tek® and kept at -80ºC until further processing. Brain telencephalon was sliced through 150 µm thick cryostat (Leica, CM 3050S) coronal sections, from which the medial part of the dorsal telencephalon (Dm), the Dorsal division of the lateral telencephalon (Dld) and the Ventral division of the lateral telencephalon (Dlv) (Fig. 3.2.2) were microdissected with modified 25G steel needles using a micropunching technique previously established in the lab (Teles et al., 2015).

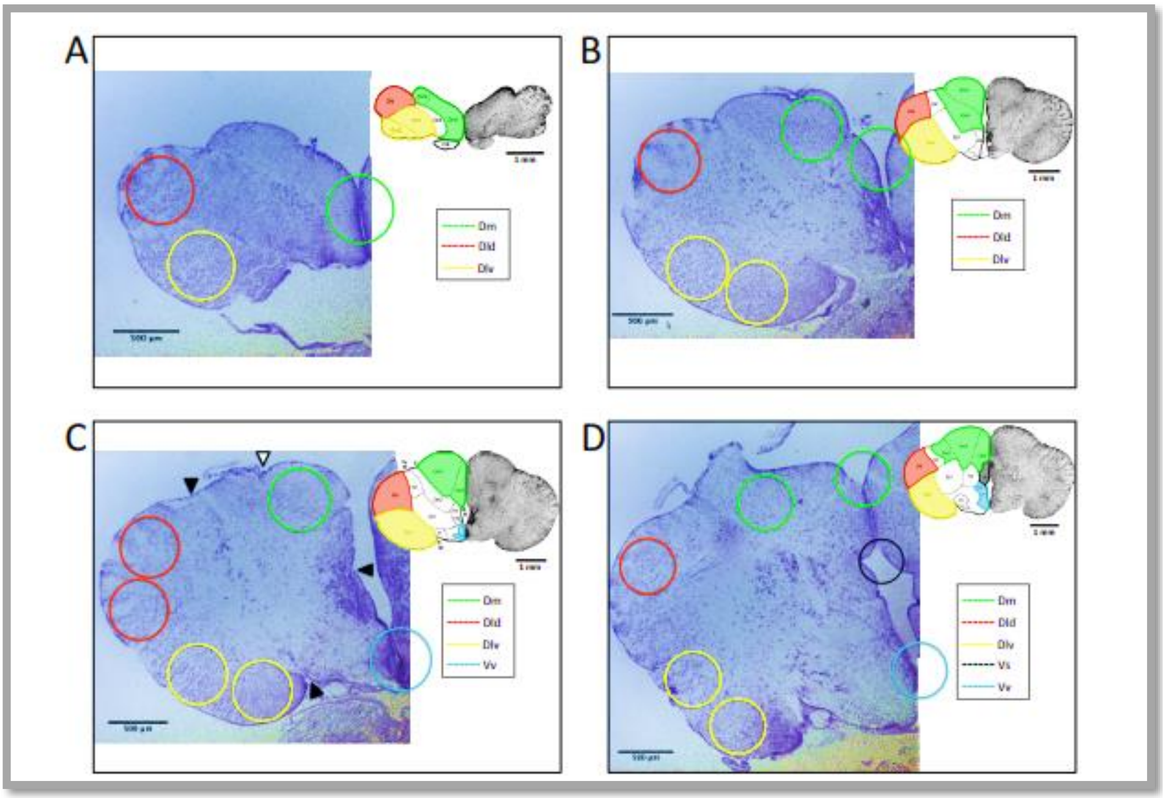

Figure 3.2.2 |Coronal sections of Atlantic seabass (*Dicentrarchus labrax*) telencephalon spaced 400 µm from each other. Nissl staining images and an illustrative section adapted from Cerda-Reverter, et al. (2001) highlighting the areas of interest from the telencephalon. Areas of interest to carry out this research: medial part of the dorsal telencephalon (Dm), lateral dorsal telencephalon (Dld, red) and lateral ventral telencephalon (Dlv, yellow).

These regions of interest in the brain were identified and classified following the available brain atlas for seabass (Cerda-Reverter et al., 2001). Total tissue was collected directly into lysis buffer from Qiagen Lipid Tissue Mini Kit (#74804; Valencia, CA) and total RNA extracted from the samples, with some adjustments to the manufacturer's instructions (see see Teles et al., 2016 for details). RNA from each sample was then reverse transcribed to cDNA (BioRad iScript cDNA Synthesis Kit; Valencia, CA) accordingly to manufacturer´s instructions and used as a template for quantitative polymerase chain reactions (qPCR) of *egr-1*, *c-fos*, *bdnf* and *npas4*, using the geometric mean of the expression of two previously established housekeeping genes, *eef1a* and *18S* (see table 3.2.1 for primer sequences and for qPCR conditions see chapter III.1; topic "*brain microdissection and gene expression analysis*"). The abundance of the internal control genes was stable across experimental treatments. All reactions were run in duplicate and controls without DNA templates were run to verify the absence of cDNA contamination. Fluorescence cycle thresholds (CT) were automatically measured and relative expression of the target genes were calculated using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Primers efficiency was calculated for each qRT-PCR reaction using Light Cycler 480 II inner software.

Table 3.2.1 | List of primers and respective temperature of annealing used for quantitative real-time PCR from the different telencephalon regions studied in Atlantic seabass (*Dicentrarchus labrax*) brain.

Gene	Forward Primer	Reverse Primer	Ta*
18S[1]	ATGCGTGCATTTATCAGACC	CGAAAGTTGATAGGGCAGACA	58
EeF1a[1]	TGGCTTCAACATCAAGAACG	ATGTGAGCTGTGTGGCAATC	57
c-Fos[2]	GCCTGCACCACCTTTACTTC	AGAGGACTGGTCGTTGCTGT	59
EGR-1[2]	GCAGAAGGACAAGAAAGCAGA	GGGGTAAGAAGACACTGGAGA	58
BDNF[2]	GCTCAGCGTGTGTGACAGTA	ACAGGGACCTTTTCCATGAC	57
NPAS4[2]	CAACCAAAGGAGCATCCAAG	AGCCGTGCTTTATCTGCATC	57

*Ta = Temperature of annealing; 1Reference genes; 2Target genes.

Statistical analysis

Given the differences in duration between experiments 1 and 2 the social effects on the stress response were not formally investigated, and the results from the 2 experiments were analysed separately. Parametric assumptions of normality and homoscedasticity of the data were confirmed by analysis of the residuals. Homogeneity of variances was checked by Levene's test. Log, log (X+1) or arcsine transformations were used to match parametric assumptions when required [Experiment 1: time in freezing (arc-sin transformed), escape behaviour and exploratory behaviour (log (X+1) transformed), plasma cortisol concentration and IEGs mRNA expression (log transformed); Experiment 2: time in freezing (arc-sin transformed), exploratory behaviour (log (X+1) transformed, IEGs mRNA expression (log transformed)]. In experiment 1 (social isolation) a T-test was used to compare the two experimental treatments (PRD vs. UnPRD). In experiment 2 (social group), since multiple individuals came from the same social group, a two-way ANOVA with experimental treatment (PRDs vs. UnPRDs) and social group (1-6) as independent variables, was used to assess differences in the behavioural variables and in cortisol. Data on the expression of immediate early genes was also analysed with T-tests in experiment 2 given the fact that only a subset of randomly chose fish was used and therefore most social group only contributed with one fish to the sample. Pearson test was used to assess correlations among variables. Descriptive statistics are expressed as mean ± standard error of the mean (SEM). All statistics were performed using IBM SPSS® statistics v19.0.

Results

Behavioural validation of experimental treatments

Analyses of fish behaviour during the 2 min preceding the first training session (i.e. before any stimulation or manipulation of the fish) showed no significant differences between PRD and UnPRD in any of the two experiments [Experiment 1 (time freezing: t-test = 0.28 (df = 46); p = 0.77; escape events: no events occurred; exploratory behaviour: t-test = -0.09 (df = 46); p = 0.92); Experiment 2 (time freezing: $F_{(1,36)} = 0.43$, p = 0.57; escape events: $F_{(1,36)} = 0.36$, p = 0.55; exploratory behaviour: $F_{(1,36)} = 0.04$, p = 0.88)]. In experiment 2 shoal cohesion before training also did not show differences between PRD and UnPRD ($F_{(1,36)} = 0.11$, p = 0.74).

Effects of stressor predictability on fish behaviour

The behaviour displayed by fish during exposure to the visual cue in the test session was markedly different between experimental treatments in both experiments (Fig. 3a-d). In Experiment 1 (social isolation) there was no significant difference in time spent freezing between the two experimental treatments (t-test = -1.02 (df = 46); p = 0.31), but fish in the unpredictable treatment showed less escape attempts (t-test = 3.91 (df = 46); p < 0.001) and less exploratory behaviour (t-test = 5.78 (df = 46); p < 0.001).

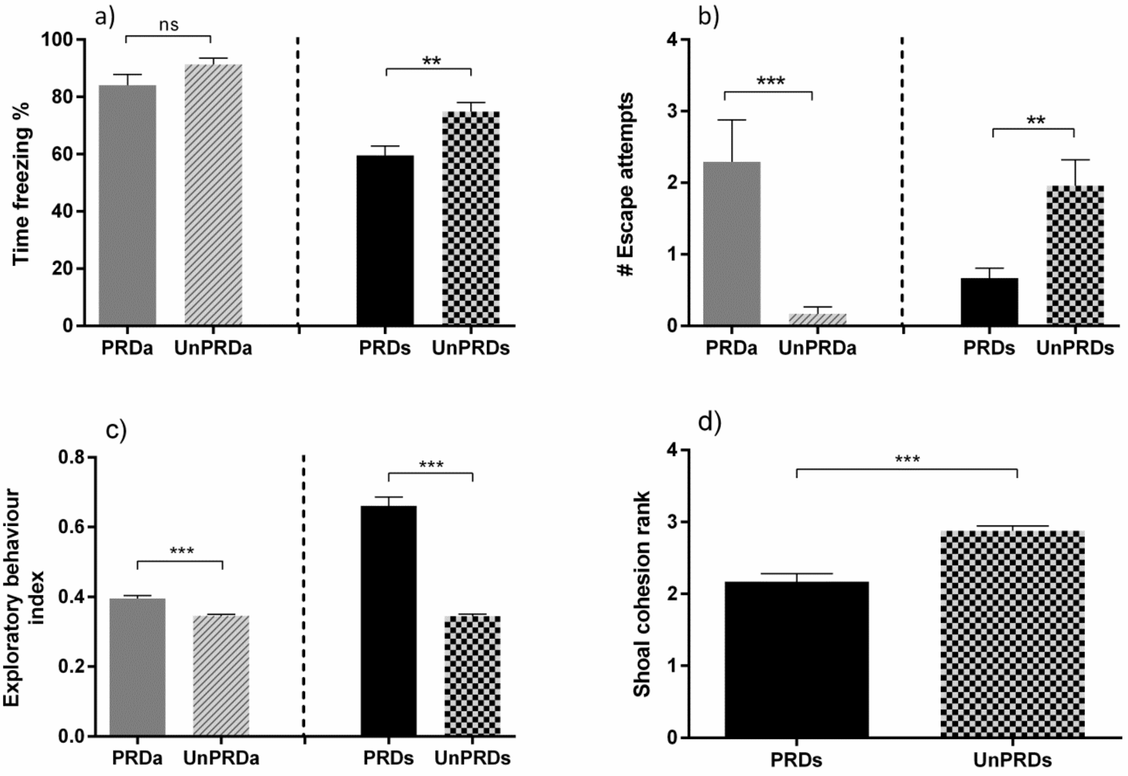

Figure 3.2.3 | Behavioural responses (mean ± SEM) to aversive stimuli. For chart a, b, c, and d, bars on the left side of the dashed line illustrate the fish responses of experiment 1 to both predictable (PRDa) and unpredictable (UnPRDa) conditions; bars on the right side of the dashed line illustrate the fish responses of experiment 2 to both predictable (PRDs) and unpredictable (UnPRDs) conditions. (a) time in freezing; (b) escape attempts; (c) Exploratory behaviour (measured by the arithmetic mean of the time spent in each area of the experimental tank / higher time) and (d) Shoal cohesion rank for fish tested under social conditions (1 – low cohesion; 2 – medium cohesion; 3 – high cohesion). T-test of experiment 1 and Two-way ANOVA of experiment 2 significant differences between treatments are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). All descriptive statistics are mean ± SEM.

Moreover, in this experiment a positive correlation was found between exploratory behaviour and escape attempts ($R = 0.429$, $n = 48$, $p = 0.002$). In Experiment 2 (social group) fish in the predictable treatment spent less time in freezing ($F_{(1,36)} = 11.34$, $p = 0.001$), and showed less escape attempts ($F_{(1,36)} = 10.79$, $p = 0.002$) and more exploratory behaviour ($F_{(1,36)} = 151.54$, $p < 0.001$) than fish in the unpredictable treatment. Moreover, in experiment 2, time in freezing and escape attempts were positively correlated ($R = 0.656$, $n = 45$, $p < 0.001$) and exploratory behaviour and time in freezing were negatively correlated ($R = -0.325$, $n = 48$, $p = 0.024$). Finally, shoal cohesion (measured only for experiment 2) was higher in the unpredictable treatment ($F_{(1,36)} = 27.98$, $p < 0.001$). Moreover, there was a negative correlation between shoal cohesion and exploratory behaviour ($R = -0.491$, $n = 48$, $p < 0.001$).

Effects of stressor predictability on fish physiology

In both experiments fish exposed to unpredictable stressors had higher cortisol levels than fish tested under predictability (Fig. 3.2.4; PRDa vs. UnPRDa: t-test = -2.06, df = 46; p = 0.04; PRDs vs. UnPRDs: $F_{(1,36)} = 24.79$, $p < 0.001$). In Experiment 2 cortisol was positively correlated with both time in freezing and shoal cohesion (Rp = 0.351, n = 45, p = 0.018; R = 0.310, n = 45, p = 0.038), whereas a negative correlation was found with exploratory behaviour (R = -0.491, n = 45, p = 0.001).

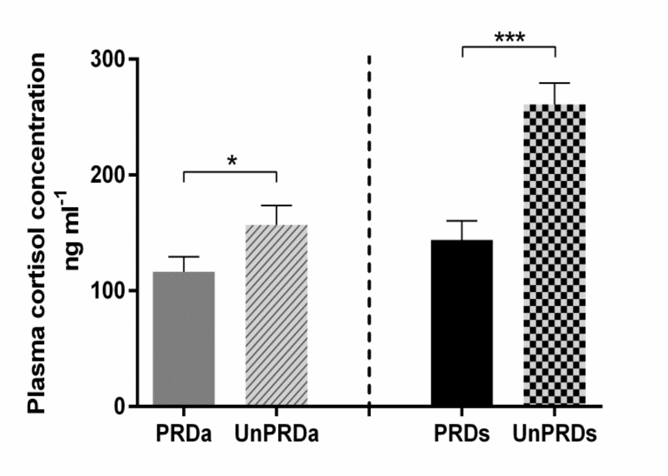

Figure 3.2.4 |Plasma cortisol responses (mean ± SEM) measured 30 min after the test session. Bars on the left side of the dashed line illustrate the fish responses of experiment 1 to both predictable (PRDa) and unpredictable (UnPRDa) conditions; bars on the right side of the dashed line illustrate the fish responses of experiment 2 to both predictable (PRDs) and unpredictable (UnPRDs) conditions. T-test of experiment 1 and Two-way ANOVA of experiment 2 significant differences between treatments are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p< 0.001$). All descriptive statistics are mean ± SEM.

Effects of stressor predictability on brain activation

In experiment 1 (social isolation) both Dm and Dlv exhibited changes driven by predictability, with higher expression levels of IEGs in the unpredictable treatment (i.e. of *c-fos* and *bdnf* at both brain nuclei and of *egr-1* and *npas4* at Dlv; Fig. 3.2.5). In this experiment, positive correlations were found at the Dm between *egr-1* expression and that of either *c-fos*, *bdnf* or *npas4* (Rp = 0.881, n = 16, p < 0.001; R = 0.771, n = 16, p < 0.001; R = 0.648, n = 15, p = 0.009, respectively), and between *c-fos* and both *bdnf* and *npas4* (R = 0.804, n = 16, p < 0.001; R = 0.549, n = 15, p = 0.034, respectively). In the Dlv, positive correlations were also found between *egr-1* expression and that of either *c-fos*, *bdnf* or *npas4* (R = 0.963, n = 15, p < 0.001; R = 0.746, n = 15, p = 0.001; R = 0.594, n = 15, p = 0.019, respectively), and between *c-fos* and both *bdnf* and *npas4* (R = 0.794, n = 16, p < 0.001; R = 0.597, n = 15, p = 0.015, respectively).

In experiment 2 (social groups) only *egr-1* was up-regulated at the Dm under unpredictable conditions (Fig. 3.2.5). Additionally, positive correlations were found between this gene and *c-fos*, *bdnf* and *npas4* at Dm (R = 0.893, n = 16, p < 0.001; R = 0.707, n = 16, p = 0.002; R = 0.567, n = 16, p = 0.022, respectively).

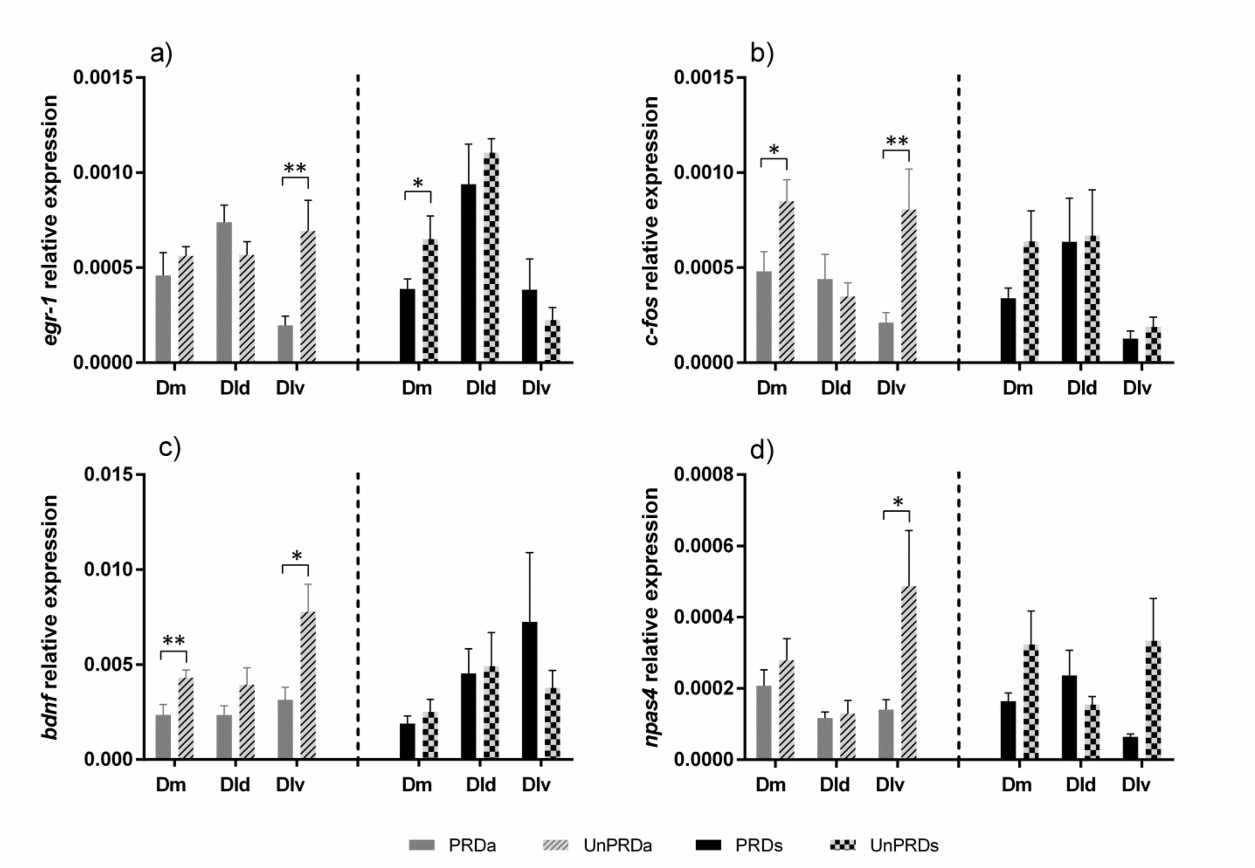

Figure 3.2.5 | Expression (mean ± SEM) of the immediate early genes *egr-1*, *c-fos*, *bdnf* and *npas4* in the Dm, Dld and dlv brain nuclei of seabass measured in the different experimental conditions. For each brain nuclei, bars on the left side of the dashed line illustrate the fish responses from experiment 1; bars on the right side of the dashed line illustrate the fish response from experiment 2. T-test significant differences in expression levels between experimental conditions (i.e. PRDs vs. UnPRDs; PRDa vs. UnPRDa) are indicated by asterisks: * p < 0.05; ** p < 0.01; *** p < 0.001.

Correlations between predictability-driven behavioural, physiological and brain activation measures

In experiment 1 (social isolation), negative correlations were found between cortisol and escape behaviour (R = -0.509, n = 16, p = 0.044) and between escape behaviour and *bdnf* expression in Dm (R = -0.590, n = 16, p = 0.016). Moreover, a positive correlation between escape attempts and exploratory behaviour was found (R = 0.702, n = 16, p = 0.002). In experiment 2 (social groups), a positive correlation was found between time in freezing and escape behaviour (R = 0.850, n = 16, p < 0.001), and a negative correlation was found between exploratory behaviour and shoal cohesion (R = -0.720, n = 16, p = 0.002). Finally, a positive correlation between escape behaviour and the *c-fos* expression in the Dlv was also found (R = 0.584, n = 12, p = 0.046).

Discussion

In this study we have shown that stressor predictability modulates the stress response measured at the behavioural, physiological and neural levels, both in fish exposed to the stressor in social isolation or in social groups. In social isolation fish exposed to an unpredictable stressor exhibited less exploratory and escape behaviours, higher cortisol levels and more neuronal activity in the Dm and the Dlv, as indicated by the expression of IEGs (*egr1, c-fos* and *bdnf* for Dm; and *egr1, c-fos, bdnf* and *npas4* for Dlv), than those exposed to a predictable stressor. When in social groups, fish exposed to the unpredictable stressor showed higher freezing and more escape behaviours, higher shoal cohesion, less exploratory behaviour, higher physiological reactivity and more activation of the Dm as indicated by the expression of *egr1*. Therefore, in both social contexts (i.e. social isolation vs. social group) the exposure to a unpredictable stressor seems to trigger a milder stress response both in terms of the activation of the hypothalamic-pituitary-interrenal axis (HPI) and of the activation of the brain regions putatively involved in the appraisal of the stressor, such as the Dm (fish homologue of the tetrapod pallial amygdala) and the Dlv (fish homologue of the hippocampus). In contrast, at the behavioural level the social context seems to produce antagonic responses, with stressor unpredictability increasing shoal cohesion, freezing and escape responses. Thus, predictability seems to reduce the behavioural response to stress in social groups but not in isolated fish. However, since it was not possible to match the duration of the two experiments, time is a confounding variable and the inference of social effects, albeit plausible, cannot be firmly established here.

The effects of stressor predictability have been extensively described both in humans and in animals (Alvarez et al., 2011; Grillon et al., 2004; Lovallo, 2005; Sapolsky, 2004). The outcomes of such divergent research demonstrate that unpredictable aversive events produce weakening behavioural and cognitive effects, similar to those found in anxiety and mood disorders. However, so far few studies have addressed the effects of predictability in farming fish (e.g. Galhardo, et al., 2011; Madaro, et al., 2015,2016; Vindas, et al., 2014). It is known that anticipation of rewarding and aversive stimuli can change the perception of threatening events, and that as result its manipulation can reduce the negative impact of stressful procedures (Galhardo, et al., 2011; Spruijt, et al., 2001; Vindas, et al., 2014). Our results are sustained by previous research outcomes, which showed that in fish, predictable negative events tend to be less stressful (Galhardo, et al., 2011; Piato, et al., 2010; Weiss, 1970). For example, studies in sheep showed that by predicting an event in an associative scheme with a light cue, lambs showed reduced startle responses than their counterparts under unpredictable conditions (Greiveldinger et al., 2007). In rats, it has also been shown that predictable electric shocks produced less physiological responses and less gastric ulcers (Weiss, 1970) and that unpredictable trauma is a critical factor for later life disorders (Tyler et al., 2007). Since the predictive cue allows the organism to anticipate the income stressor, and therefore to readjust its coping mechanisms to the environment, we can argue that predictable stressors induce short-lasting fear expression concerning imminent threat, while unpredictable threats appears to induce longer-lasting fear elicited by uncertain threat. Such responses were reported to be linked to different states of anxiety (Alvarez et al., 2011). Indeed, unpredictable regimes were shown to increase vigilance and avoidance, which sustain our results regarding social conditions and where it appears to be more detrimental for the individuals. In social isolation, such contradictory patterns in the behavioral expression comparing to social groups, may be representative more of the social condition rather than from the predictability stressor. Nevertheless, by the higher susceptibility to stress reported in this species (Ashley, 2007), such higher escaping and higher exploratory behaviour under predictable conditions, could suggest that fish have perceived the imminent threat. Without social support, fish become more apprehensive which might have triggered the active defensive responses (Fanselow, 1994). It is possible that social support, change the way the individuals experience and evaluates the predictability stressor, by assimilation of how their counterparts are seen to appraise and respond to such event. With some extent at least, the social isolation may have overridden the effect of predictability, but as noted above, such fact deserves further studies.

Interestingly, predictability may have opposite effects if aversive or appetitive events are involved. In tilapia, predictable aversive events elicited lower cortisol responses than unpredictable ones (Galhardo et al., 2011). However, predictable feeding schedules triggered higher levels of anticipatory behaviour and a tendency for higher cortisol levels. Finally, it should be mentioned that the loss of predictability (predictable followed by unpredictable conditions) has also been reported as a stressor by itself, being even more detrimental than unpredictable regimes (Bassett and Buchanan-Smith, 2007; Gilbert-Norton et al., 2009). In fish, a recent work demonstrated that Atlantic salmon, increase aggressive behaviour after a reward deprivation (Vindas et al., 2014a). Thus, predictability of events (e.g. feeding regimes) should be a key factor to take in account in the handling of farmed fish as a way to reduce stress.

The use of IEGs has often been used has molecular markers of neuronal activity to comprehend fish brain states under distinct environmental challenges and it effect on their behavioural and physiological responses (Cardoso et al., 2015; Zayed and Robinson, 2012). If the learned fear is a projection of the appraisal of the conditions in which fish were, and such reactions were underpinned by projections from the Dm and Dlv, it suggests that fish experience some form of emotional processing, therefore of cognition. This outcome is in accordance with the results demonstrated for seabream, where Dm was shown to be differently expressed in the emotional processing of predictability stressor (Cerqueira et al., submitted); and as well as found in mammals, in which the basolateral amygdala contributed for the responses to emotional stimuli (LeDoux, 2000). The involvement of the mammalian homologues brain structures, integrate both emotional processing and memory of aversive event perception (O'Connell and Hofmann, 2011). Therefore, in line with the description above, an up-regulation of *egr1*, *c-fos* and *bdnf* in the Dm under unpredictable conditions, compared to predictable conditions, give the idea that unpredictability was processed as more harmful. In agreement, under social isolation, the involvement of Dlv in the processing of predictability appraisal, suggests that unpredictability evoked a more pronounced "anxiety-like" reactions more fear related, by experiencing such diffuse cue (i.e. decrease vigilance and avoidance under unpredictability). Reinforced by lower levels of exploration and escape attempts, it is apparent that motivation has effectively decreased, again, supportive of longer-lasting fear outcomes. In fish, the Dl´s is reported to be homologous of hippocampus and to play a role in the physiological expression. In mammals, hippocampus modulates memory evoke and formation (Rokers et al., 2002). As such, both physiological reactivity and IEGs upregulation under unpredictability, evidences that the ability of the fish to cope with the situation was more compromised. In addition may suggests that learning or memory have been affected by the unpredictable environment and social condition (Portavella and Vargas, 2005). It is well known that the presence of counterparts it increase the information transfer across the individuals and concomitantly the learning ability (Brown, 2011). Thus, we suggest that isolation may have compromised the predictability appraisal in terms of behaviour, and this condition is foreseen experienced as more negative compared to social conditions. The up-regulation of *bdnf* under unpredictable conditions permits to label such condition as a milder stressor, rather than an acute stress as for omission of expected reward/punishment situations. This is sustained by both the activation of the hypothalamic-pituitary-interrenal axis (HPI) here found and the finding of Vindas et al. (2014b), where the authors shown that *bdnf* expression is inhibited by an acute stressor and by it negative correlation with plasma cortisol levels. The results of these authors yet suggest that the nature and intensity of the stressor (i.e. 30 min of confinement rather than 2 min used in our experiment) are also indicative of the cognitive ability in fish reflected by their perception of the surrounding environmental conditions. Nevertheless, the level of appraisal processing, therefore of their cognition, should only be comparable it the stressor conditions were parallel, which were not. As argued by Faustino et al., (2015) to address fish cognition ability such conditions should be equal among the testing of different stimulus evaluation checks. As such, the neurobehavioural expression demonstrated here put forward the benefit of predictability of stressors, though limited when fish are in situations that do not comply with their biological and ecological features, and suggests the existence of cognition if fish (at some extent at least; relying in the former statement).

Conclusions

Our study disclosed that psychological manipulation created by means of predictability, by either increasing or decreasing the outcome expectancies, modulates the stress response of animals to environmental challenges. As such, using such psychological “tool”, the welfare state of fish can be more easily understood, heightened and possibly sustained. For instance, its application in the aquaculture industry can have added value, as it can improve the quality and performance of fish, hence, its productivity. Further studies of such factors as controllability, which requires a different level of cognitive processing (i.e. the individuals whether choose to increase or decrease their presence to a stressor), may help clear up the mediators linking psychosocial stress to welfare of animals and, additionally, to disclosure how forward-looking are fish cognitive capabilities. Nevertheless, we show here evidences of cognitive ability in fish and still demonstrate the existence of a well conserved neural basis in the processing of emotional stimuli throughout a divergent species from those as humans (evolutionarily speaking).

Acknowledgments

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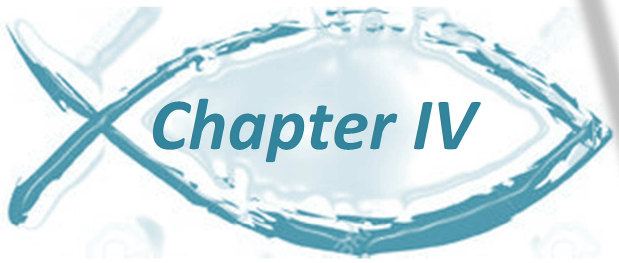
Controllability as an appraisal modulator in fish



Chapter IV

CHAPTER IV-1

Controllability over the environment increases affective stress-coping ability

This chapter is under review as:

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Controllability over the environment increases affective stress-coping ability

Abstract: Offering alternative strategies to deal with stressful situations can improve the individual's ability to cope with environmental challenges. This also enables the possibility to infer about their appraisal capability and to monitor how they perceive such condition, by manipulating the surrounding environmental cues. Within this framework, research was conducted to investigate how gilthead seabream and European seabass appraise their environment, when given the possibility to control the response towards an aversive stimulus. We have looked at changes in behaviour, circulating cortisol and expression of immediate early genes (IEG) in microdissected brain regions known to mediate neural processes of aversion across vertebrates, as a proxy for neural activation. In addition, the effect of loss of control over an aversive stimulus was also assessed. The experimental set-up relied on the principles of operant conditioning of fish, which can learn to associate their own behaviour and the consequences of undertaking a particular action. Both species were subjected to trials where the salience of an aversive stimulus (confinement) was manipulated, by confronting them with either a controllable (possibility to escape from a signalled (light) stimulus) or uncontrollable (impossibility to escape from a signalling event) stimulus. Furthermore, the effect of loss of control (possibility to escape followed by impossibility to escape) was also assessed. Behavioural patterns showed by the fish after learning such association indicate that control over an aversive situation decreases the salience of such condition. Circulating cortisol concentrations support the behavioural findings and also evidenced a higher detrimental effect of losing control, compared to the uncontrollable situation. Each fish species showed a distinct IEG response on each brain region, though fish under controllability conditions displayed consistent lower expression of IEGs, whereas under loss of control conditions, they showed higher expression levels. This study suggests that providing fish with appropriate environmental features, regulated by their neural coordination, can change the way they perceive the environment, with positive outcomes for successful coping ability. Our results explore, for the first time in a non-mammal vertebrate, the proximate neuromolecular basis underlying the cognitive appraisal of controllability.

Keywords: Affective states, Cognitive appraisal, Coping ability, Controllability, Environmental perception; Immediate early genes, Loss of controllability.

Introduction

Due to public and scientific community concern, an incontestable challenge for producers nowadays has been to promote not only the physical, but also the psychological well-being of fish kept in artificial environments, such as in aquaculture conditions. This concern stems from the accepted notion that some form of perception of the surrounding environment takes place in fish. Environmental perception occurs when specific cues trigger reactions from the individual, whether consciously or non-consciously, such as simple reflexes or patterns of regular responses (Lazarus, 2001). However, to identify cues in highly dynamic and unpredictable natural environments, cognitive appraisal mechanisms take place in order to assess the value of the stimulus and act afterwards in conformity to such interpretation (Fawcett et al., 2014; McNamara et al., 2013; Millot et al., 2010; Millot et al., 2014b). The perception of such cues in fish is mediated by a limited number of stimulus evaluation checks (SEC) with different levels of processing (reviewed by Faustino et al. (2015)): firstly, by the individual's judgment about its psychological significance as stressful, positive, predictable, controllable, challenging or irrelevant (primary appraisal) and secondly, by the resources available to cope with them (secondary appraisal) (Lazarus, 1999; Paul et al., 2005). Supplied information or safety cues can be used to modify the psychological context in which the cue is appraised, hence, the processing of the event can be manipulated to elicit negative versus positive emotion-like states (i.e. affective states *sensu* Mendl et al., (2010)) and reduce or increase biological functioning, respectively. Kandel et al. (2012) define affective states as non-conscious and automatic neural responses that can change cognitive interpretation of an event. Despite the increasing evidence of such cognitive ability in fish, this has been assessed mainly by physiological and behavioural outputs (Ashley and Sneddon, 2008). Recently, Cerqueira et al. (submitted_a) showed for seabream (*Sparus aurata*) the neural mechanisms through which this occurs, using predictability as SEC. Such research revealed that the evaluation process and related states is processed in homologue neural structures from those as humans. However, information regarding the neuromolecular processes underlying a different SEC is, to our knowledge, unexplored.

Recent research focusing on learning paradigms showed evidence of complex memory formation and retrieval in emotional and non-emotional contexts in fish (Millot et al., 2014a,b; Portavella et al., 2003; Vargas et al., 2000). Within this framework, avoidance learning conditioning can mimic aversive situations occurring in the natural environment, in which the ability to associate events is essential to modify the individual’s behaviour towards such a dynamic environment. In a first stage, individuals learn to associate a warning or conditioned stimulus (CS) to a negative reinforcer (Unconditioned stimulus: US) through a training period. After such association is established, the CS itself elicits the same response as the US, and becomes a substitute for the US (Pavlov, 1927). The environmental cue acquires a negative valence, eliciting avoidance behaviours such as freezing or withdrawal, which are normally associated to frustration, anxiety, panic or fear, in fish (Fossat et al., 2014; Martins et al., 2011; Sneddon, 2003; Sneddon et al., 2003; Vindas et al., 2012; Vindas et al., 2014a). In stage two, the learner experiences operant conditioning, whereby he realizes that an action by itself can affect the stressful outcome. Adding an operant component to a learning assignment was suggested to increase or even induce fitness (Heisenberg et al., 2001; Moore, 2004; Valente et al., 2012). Moreover, this should emphasize the animal´s ability to determine if the stimulus is relevant to elicit further processing.

Following such premise, we have combined both controllability to an adjusted level of environmental complexity and learning opportunities (i.e. avoidance learning conditioning), stated as contributing for a positive mental attitude from the animal towards their habitat (Galhardo et al., 2009). Controllability is known to successfully decrease the exposure to a perceived stressor, by combining both the individual´s affective state and the environmental conditions for the appraisal process (Ellsworth and Scherer, 2003; Scherer, 2001). Pointed by Fiore et al. (2015), any coping responses specified to solve a perceived operant stressful task (e.g. by escaping, removing, touching, moving or defending) entail highly motivational states. In fish reared in captivity, control or lack of control is particularly relevant, as the adaptive behaviour reactions to such distinct conditions can ultimately be the difference between biological success and failure. The perception of lack of control, relying on an environmental cue preceding an aversive event, was shown to induce stress by both persistency of negative emotional responses (Zvolensky et al., 2000), fear (Armfield, 2007) and health issues (Milde et al., 2005). This effect was confirmed by studies conducted in sheep demonstrating that an aversive event is less stressful when the animal exerts control over it, likely reducing the negative emotional responses (Greiveldinger et al., 2009). In fish, the effect of controllability was demonstrated physiologically in rainbow trout (*Oncorhynchus mykiss*), which showed lower cortisol response when given the possibility to escape from a larger counterpart (Carpenter and Summers, 2009). In seabream, higher cortisol levels were linked with negative emotion-like states (Cerqueira et al., submitted_a). Moreover, perception of losing control after having such possibility was show to interfere with learning under punishment situations in rats, with larger interference effect than even lack of control (Sonoda et al., 1991).

Both seabream and seabass, the most relevant farmed species in southern Europe (FAO, 2012), have shown the capacity to associate different stimuli and retain memories of events (Millot et al., 2014a; Millot et al., 2014b), making them important models to study how environmental stressors impair fish welfare under artificial environments. Still, the proximate mechanisms involved in tuning behavioural and physiological stress responses are scarcely unexplored. Taking this into account, our research proposed to test how controllability over an aversive stimulus affects the coping mechanisms of both seabream and seabass. We have focused on assessing behaviour (immediate responses normally associated with aversive events), physiology (circulating cortisol) and mRNA expression immediately early genes, involved in experience-driven neuroplasticity (*egr-1*, *c-fos*, *bdnf* and *npas4*). The premise that different behavioural patterns elicit different neuromolecular states (Cardoso et al., 2015; Sørensen et al., 2013) was examined in the brain nuclei homologues to those of the mesolimbic reward system from central nervous system (CNS) in mammals: Dm putative homologue from amygdala, Dl from hippocampus (Dld homologues to dorsal pallium and Dlv homologues to hippocampus (Uceda et al., 2015)) and Vv from the lateral septum (Goodson and Kingsbury, 2013; O'Connell and Hofmann, 2011). The existence of emotions-specific behaviour (or organismic states) was recently shown in fish, being regulated by the individual's perception of predictable vs. unpredictable environmental stimuli (Cerqueira et al., submitted_a). Such organismic states showed specific adaptive responses to appetitive and aversive stimulus according to the four emotional quadrants described in humans and extendend for animals' core emotion-like states (Mendl et al., 2010; Russell, 2003). These emotional-like states were elicited by the appraisal value individuals ascribe to environmental stimuli, namely salience (Q1 high / Q3 low) and valence (Q2 positive / Q4 negative). Therefore, by assessing the effect of controllability salience (i.e.

controllability, uncontrollability or loss of controllability), we can infer about the affective subjective appraisal of each condition. Additionally, it provides insight into how individuals perceive their surrounding environment after psychological manipulation. By exploring the positive effect of controllability, along with the mechanisms that mitigate the effect of aversive events, the disparity between animals reared and kept in artificial environments and their natural habitats can be reduced.

Material and methods

Experimental procedures 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 Portuguese competent authority for the protection of experimental animals (Direcão Geral da Alimentacão e Veterinária, Portugal; permit number 0420/000/000-n.9909/11/2009). Experimental procedures, when necessary, were performed under anaesthesia with 2-phenoxyethanol and every effort was made to minimize suffering.

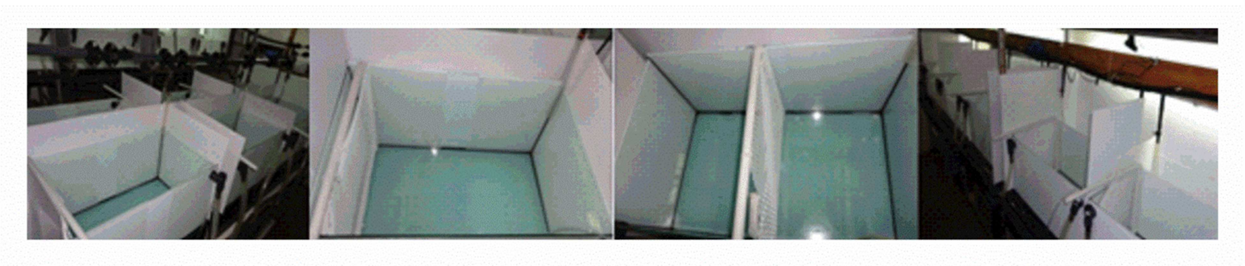

Figure 4.1.1 |Experimental aquaria/setup with the respective confinement net and opaque partitions avoiding the contact between fish and between fish and the operator

*Subjects, housing and feeding*Seabass (0.1 g) hatched at the experimental research station of IFREMER in Palavas-les-Flots (France) and seabream (20 g) from IPMA aquaculture station (Olhão, Portugal) were transported to CCMAR Research facilities (Faro, Portugal). Seabass and seabream were let to acclimate to the new housing conditions, kept in stock tanks of 500 L in an open water circuit during 8 and 3 months, respectively, under species standard rearing conditions until the start of the experiments (temperature 18 ± 3 °c, salinity 35 ± 2

‰, dissolved oxygen above 75 %, and a 12 L : 12 D photoperiod). Fish were hand-fed *ad libitum* with a commercial diet (Aquagold, Aquasoja, Sorgal SA, Portugal). One month before the beginning of the trials, feeding was adjusted to 3 % Biomass day^{-1} and delivered by automatic clock-feeders. At the start of the experiments the body mass of the fish was 47.12 ± 6.80 g for seabass and 56.96 ± 7.95 g for seabream (mean ± SEM).

Experimental procedures

The experiments occurred between February and March of 2014. Seventy-two individuals of each species were tagged with 1 cm long floy tags (Floy Tag Manufacturing Inc, Seatle, USA) with a multicolour pearl attached behind the dorsal fin. Groups of 4 fish were distributed by twelve experimental aquaria of approximately 80 l (70 x 40 x 30 cm depth; N = 48). The experiment was developed in three runs, using both species in a balanced number in each run to reduce bias; N=24 fish/species/run). The rearing conditions were the same as the stocking conditions, with the exception that no air was supplied since it interfered with the video recording quality. Saturation between 75 and 90 % in the outlet water was ensured by the water flow rate of 2.5 L min^{-1}. All aquaria walls were covered with opaque partitions in order to prevent contact between focal animals and the experimenter. Each aquarium was provided with a confinement net attached to a white rigid structure, with the same size as the lateral walls of the tank (Fig. 4.1.1). Each trial run lasted 12 days, including 8 days of acclimation to the experimental tanks and 4 days of training with 2 sessions per day at 10:00 and 15:00 h; the test session of the 4th day i.e. 8th session, was the test session. During this period fish were hand-fed twice a day at 08:00 h and 18:00 h at 3% of biomass day^{-1}. Individual feeding motivation/recovery and online video streaming were used daily to assess abnormal behaviour and health conditions.

Based on delay conditioning, fish were trained to learn an association between a 2 min of light (CS) and a subsequent 5 min confinement (unconditioned stimulus: USavr). The CS overlapped 1 min with the USavr to reinforce the association between stimuli (Nilsson, 2008). The light (12 V, 10 W) was hanged in the top of a lateral wall of the aquarium, on the opposite side of the confinement net. Confinement consisted in moving the net into the light wall direction until reach 15% of the aquarium volume. Fish were tested under different conditions: i) Controllable (CTR) – fish had the choice to escape from USavr (confinement) by a door of 10 cm^2; ii) Uncontrollable (UnCTR) – fish had no choice to escape from USavr; iii) Loss of Controllability (CTRUn) - fish were subjected during 5 conditioning sessions to the same conditions as CTR followed by 2 sessions under UnCTR conditions (Table 4.1.1). In all cases, the event was created by signalling (CS – light) the income aversive stimulus. For the test session the different experimental groups were conditioned by the CS in the absence of the USavr. The absence of USavr legitimates the assessment of differences in physiological and IEG activation responses of fish towards the different experimental conditions tested.

Table 4.1.1 |Training procedures for each experimental condition tested for both seabream and seabass

Trial	Valence	Tanks	Possibility to escape	Training sessions (1st to 7th session)	Test session (8th session)
Controllable CTR	Aversive n=48	6/each species	Yes = 7 sessions	1min CS + 1min CS overlapped with 1 min USavr + 4 min only USavr	1 min CS
Uncontrollable UnCTR			No = 7 sessions		
Loss of Controllability CTRUn			Yes = 1st to 5th sessions No = 6th and 7th session		

Behavioural analysis

Behaviour was video recorded during 10 min before the first training, during the 1st, 5th, 6th and 7th training sessions, and during the test session using video cameras (TVCCD-623-COL, Monacor®, Denmark) and webcams HD C310 Logitech positioned 1m above the tank. Behavioural responses analysis performed during CS was executed with computerized multi-event recorder software (Observer XT®, from Noldus, Wageningen, Netherlands). The conditioned response was determined by the time in freezing (total time without any movement), resting events (total number of freezing events), escape behaviour (i.e. fish swimming strongly, going close to the tank walls or moving in a way that looked like escape attempts), shoal cohesion (1 – low cohesion; 2 – medium cohesion; 3 – high cohesion) and exploratory behaviour (measured by the time fish spent in different areas of the tank: 1 – light side area; 2 – centre of the tank; 3 – confinement net area) summarized by the formula:

$$A / t_{maximum} \qquad [1]$$

Where A is the arithmetic mean of the time fish spent in each one of the areas of the tank, and t the maximum time found for any of the areas tested. When exploratory behaviour is high, this ratio should be close to 1, while it should be close to 0 when exploratory behaviour is low. Previous work with seabream and seabass showed that both species get conditioned after 5 to 6 conditioning sessions (Cerqueira et al., submitted_a,b).

Blood collection and Hormone Analysis

Thirty min after the CS from the trial session, fish were caught and euthanized with an overdose of 2-phenoxyethanol (1 ‰, Sigma-Aldrich). Samples of 400-500 µl of blood were immediately collected from the caudal vein and centrifuged at 2000 x g for 15 min in seabream and 25 min in seabass. After centrifugation plasma was frozen in dry ice and stored at -80 °c until further processing. Levels of free cortisol were then determined by means of a commercial ELISA kit RE52061 (IBL Hamburg, Germany), with a sensitivity of 2.5 ng ml^{-1}, and intra and inter-assay coefficients of variation (CV) of 2.9 and 3.5 %, respectively.

Brain microdissection and immediate early genes (IEGs) expression

Eight individuals of each experimental condition and of each fish species were quickly decapitated by cervical transection and the whole skull (with the brain) removed, immediately embedded in Tissue-Tek® and kept at -80 ºC until further processing. Brains were sliced through 150 µm thick cryostat (Leica, CM 3050S) coronal sections, put onto regular glass slices pre-cleaned with 70% ethanol, and microdissected with modified 25G steel needles. Three nuclei of interest were selected accordingly to homologies between the fish and mammals brain (Goodson and Kingsbury, 2013; O'Connell and Hofmann, 2011) and identified accordingly to the brain atlas of these species (Cerda-Reverter et al., 2001; Munoz-Cueto et al., 2001) i.e Dm, medial zone of the dorsal telencephalic area (basolateral amygdala), Dl, lateral telencephalic area (hippocampus) and ventral nucleus of the ventral telencephalic area (septum) (see detailed description in Fig. 3.1.3 and 3.2.2 from chapter III). In seabass Dl was divided in both dorsal (Dld) and ventral divisions (Dlv); due to morphological reasons, this was not done for seabream. Tissue was collected directly into lysis buffer from Qiagen Lipid Tissue Mini Kit (#74804; Valencia, CA) and stored at -80 ºC until further processing. Using this kit, total RNA was extracted from the samples after thawing, with some adjustments to the manufacturer's instructions (see methods from chapter III.1 for detailed description) and the quality assessed using NanoDrop 1000 spectrophotometer. RNA from each sample was then reverse transcribed to cDNA (BioRad iScript cDNA Synthesis Kit; Valencia, CA) according to manufacturer´s protocol. With cDNA as template, quantitative polymerase chain reactions (qPCR) of *egr-1*, *c-fos*, *bdn*f and *npas4* were performed, using the geometric mean of the expression of two previously established housekeeping genes, eef1a and 18S, as reference (see table 3.1.2 and 3.2.1 for primer sequences and chapter III.1 for qPCR conditions). The abundance of internal control genes was stable across experimental groups. All reactions were run in duplicate and controls without DNA templates were run to verify the absence of cDNA contamination. Fluorescence cycle thresholds (CT) were automatically measured and relative expression of the target genes was calculated using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Primer efficiency values were calculated for each qRT-PCR reaction using Light Cycler 480 II inner software.

Statistical analysis

The assumptions of normality and homoscedasticity were confirmed by analysis of the residuals whereas homogeneity of variance was checked by Levene's test. Log or arcsine transformation was applied to achieve homogeneity, when necessary. Before any stimulation, differences in time freezing (arc-sin transformed) between experimental conditions (CTR vs. UnCTR vs. CTRUn) and for each species were assessed by One-way ANOVA. To validate the effect of conditioning (i.e. if light cue have effectively predicting confinement), variations on the behavioural responses (i.e. time in freezing (arc-sin transformed), resting events, escape attempts, shoal cohesion and exploratory behaviour (log transformed) and escape attempts (log (x+1) transformed)) were assessed using a one-way repeated-measures ANOVA (repeated factor = 4 training sessions) followed by Tukey post-hoc tests for each experimental condition. Planned comparisons were used to assess the differences across experimental conditions within the same training session. For each one of the species, a one-way ANOVA followed by Tukey post-hoc tests was used to test the differences between experimental conditions on the behavioural variables in the test session, on cortisol levels (log transformed) and on mRNA expression of IEGs (*egr-1*, *c-fos*, *bdnf* and *npas4;* log transformed) in each brain region (Dm, Dl and Vv in seabream and Dm, Dld, Dlv and Vv in seabass).

Separate stepwise linear discriminant analyses (LDA) was used to define which brain nuclei state (i.e. immediate early genes expression in different brain nuclei) and cortisol expression better predict the coping responses to aversive events. The F statistic was used as a measure of the contribution of each variable (cortisol concentration and IEGs expression in each brain region) to the discriminant functions. An F-value above 3.84 was used as the selection criteria for predictors to enter the model and predictors were removed when the F-value dropped below 2.71 (e.g. Maruska et al. (2013)). Multiple regression using behavioural parameters as dependent variable and IEGs expression as independent variable, were used to evaluate if any of the behaviours taken were predicted by IEGs mRNA expression from any of the telencephalic regions assessed. The F statistics with the criteria described above was also used. Heatmaps of Pearson correlations matrices, with p-values (Benjamini and Hochberg, 1995) were used to assess the neurogenomic states of fish whereas quadratic assignment procedure (QAP) correlation test with 5000 permutations (Borgatti et al., 2013) test the differences in gene co-expression patterns between brain areas within each experimental condition, and between experimental conditions within each brain area. The null hypothesis of the QAP test is that when $p > 0.05$ there is no association between matrices, hence a non-significant p-value indicates that the correlation matrices are different. ANOVA's, LDA and multiple regressions were performed using IBM SPSS® statistics v19.0, R® (R Development Core Team) were used to assess neurogenomic states and QAP correlations and GraphPad Prism® v6.0 for windows was used for chart building and figures layout. Statistical significance was taken at $p < 0.05$ and the results are expressed as mean ± standard error of the mean (SEM).

Results

Stress Coping-driven behavioural expression

Prior to training, tank and group effect on behaviour was discarded in both species by analysis of time in freezing between experimental conditions (seabream: one-way ANOVA main effects, $F_{(2,69)}= 2.165$; $p = 0.122$; seabass: $F_{(2,69)} = 0.856$; $p = 0.429$). Throughout the training events (see supplementary material Fig. S1 and S2 in the end of this chapter), analysis of distinct behavioural patterns suggests that fish got conditioned to the CS in the absence of USavr (seabream: Repeated measure ANOVA main effects, $F_{(4,276)} = 3.10$, $p = 0.01$; $F_{(4,276)} = 38.31$, $p < 0.001$; $F_{(4,276)} = 1.00$, $p = 0.40$; $F_{(4,276)} = 3.15$, $p = 0.01$; seabass: $F_{(4,276)} = 12.78$, $p < 0.001$; $F_{(4,276)} = 19.25$, $p < 0.001$; $F_{(4,276)} = 2.28$, $p = 0.06$; $F_{(4,276)} = 1.29$, $p = 0.27$ for time freezing, resting events, escape events and shoal behaviour, respectively), thus validating the controllability treatments.

Analysis of the behaviour during the CS of the test session shows a distinct effect of CTR on both species when compared to UnCTR and CTRUn. Between these treatments no clear differences were found (see Table 4.1.2 for one-way ANOVA main effects and Fig. 4.1.2 for Tukey significance differences). The appraised possibility to escape from the USavr seems to increase the coping ability of fish towards aversive events and this appears to be species-specific for time in freezing: seabream increases such response under a controllability scenario, compared to the remaining treatments, whereas seabass decreases it. Unlike for seabream, seabass displayed a significant increase of freezing time on CTRUn compared to UnCTR (Fig. 4.1.2a). Nevertheless, both species displayed on CTR lower number of escape events and lower shoal cohesion and no differences were found between UnCTR and CTRUn (Fig. 4.1.2b,c).

Interestingly, despite the higher time in freezing displayed by seabream on CTR, individuals explored more the experimental tank and showed lower number of resting events compared to CTRUn and UnCTR (Fig. 4.1.2d,e,f). On the other hand, seabass exhibited higher number of resting events, following the changes in freezing time, and no differences were found between CTR and UnCTR for exploratory behaviour. Still, CTRUn showed lower exploration than the former treatments (Fig. 4.1.2d,e,f).

Stress Coping-driven physiological expression

Controllability affected plasmatic cortisol release, as different concentrations were found between treatments in both species (see Fig. 4.1.2f for cortisol concentration measured, Tukey test significance levels for pairwise comparisons and Table 4.1.2 for one-way ANOVA main effects). On both species, control over the incoming challenge decreased the cortisol expression, whereas CTRUn induced a stronger negative effect on individuals, as expressed by higher cortisol levels, than UnCTR itself (Tukey post-hoc: CTR vs UnCTR and CTR vs CTRUn: p < 0.001 on both species; UnCTR vs CTRUn: p = 0.001 and p = 0.018 on seabream and seabass, respectively).

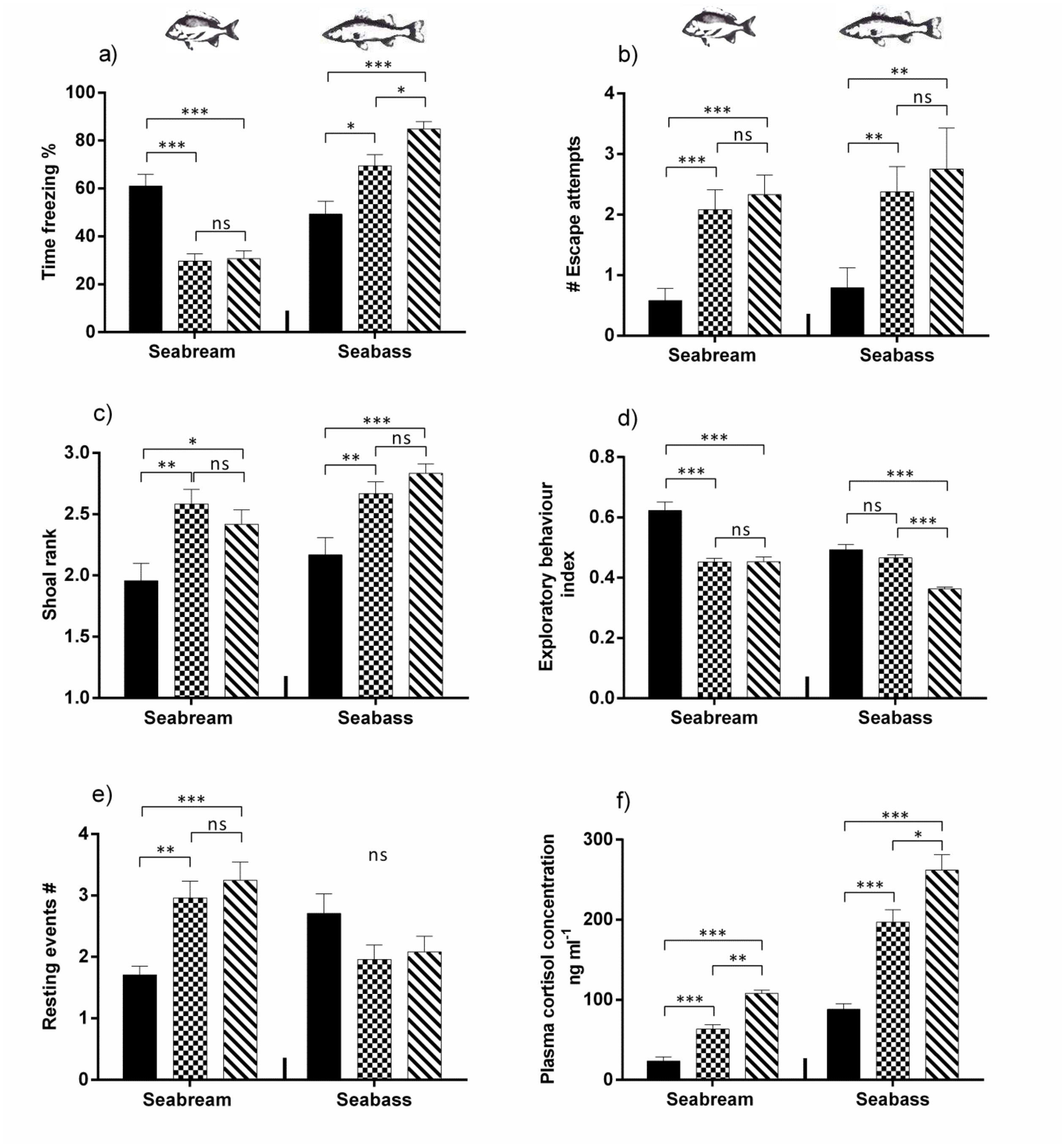

Figure 4.1.2 |Behavioural and physiological responses of seabream and sebass to controllability. (a) Time freezing (%), (b) number of escape attempts, (c) shoal rank, (d) Exploratory behaviour index, (e) resting events (Mean ± SEM) for each experimental factor (i.e. CTR, UnCTR and CTRUn) measured during the CS of the test session and (f) plasma cortisol concentrations measured 30 min after the test session. One-way ANOVA with Tukey post-hoc significance levels are indicated; * p < 0.05; ** p < 0.01; *** p < 0.001.

Table 4.1.2 |One-way ANOVA main effects of the behavioural variables (time in freezing, resting events, escape events, shoal cohesion, and exploratory behaviour), cortisol levels and of IEGs mRNA expression measured in each of the candidate brain nuclei, between experimental conditions (i.e. CTR; UnCTR and CTRUn).

One-Way ANOVA	**df**	**Time freezing**		**Resting events**		**Escape events**		**Shoal cohesion**		**Exploratory behaviour**		**Cortisol**	
		F	***p***	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
Seabream	2,67	20,87	**<0,001**	10,27	**0,001**	12,71	**<0,001**	6,15	**0,003**	3,20	**<0,001**	96,2	**<0,001**
Seabass	2,67	17,55	**<0,001**	1,92	0,154	7,35	**0,001**	9,42	**<0,001**	40,9	**<0,001**	40,2	**<0,001**
Seabream nuclei		**Dm**		**Dl**		**Vv**							
IEGs		***F***	***p***	***F***	***p***	***F***	***p***						
egr-1	1,21	1,8	0,189	7,00	0,005	1,22	0,314						
c-fos	1,21	3,89	**0,037**	0,65	0,531	6,51	**0,007**						
Bdnf	1,21	5,82	**0,010**	1,12	0,343	3,25	0,060						
npas4	1,21	1,94	0,169	0,90	0,421	0,46	0,634						
Seabass nuclei		**Dm**		**Dld**		**Dlv**		**Vv**					
IEGs		***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***				
egr-1	1,21	1,07	0,361	3,15	0,064	5,36	**0,015**	2,31	0,127				
c-fos	1,21	0,93	0,410	2,78	0,084	10,88	**0,001**	0,39	0,681				
Bdnf	1,21	1,41	0,266	3,11	0,066	8,36	**0,002**	6,36	**0,008**				
npas4	1,21	0,93	0,409	2,82	0,083	5,56	**0,012**	0,46	0,637				

Stress Coping-driven patterns of brain activation and neurogenomic states

The expression of the candidate genes on the target brain nuclei demonstrates that both Dm and Vv on seabream and Dlv and Vv on seabass displayed variations determined by controllability. In seabream, both *c-fos* and *bdnf* expressed on Dm and *c-fos* on Vv were affected by controllability. In seabass, on the other hand, all candidate genes expressed on Dlv and *bdnf* on Vv were significantly affected by the experimental conditions (Table 4.1.2 and Fig. 4.1.3). Interestingly, CTRUn genes expression in the Dlv from seabass was consistently higher than those found for CTR or UnCTR, whereas in Vv they show the lowest abundance.

Supporting these findings, neurogenomic states represented by co-expression matrices of the target genes in each brain region, and elicited by each experimental condition, showed that in the Dlv of seabass the expression of the genes were specific for each of the experimental conditions (see the Supplementary Material Fig. S3 and S4 further down in this chapter). Moreover, similar neurogenomic states between Dm and Dld were found for CTR and UnCTR, being the expression found for CTRUn singular of that treatment on these nuclei. In seabream, Dm neurogenomic state showed to be specific only for CTR whereas UnCTR and CTRUn had similar co-expression matrices.

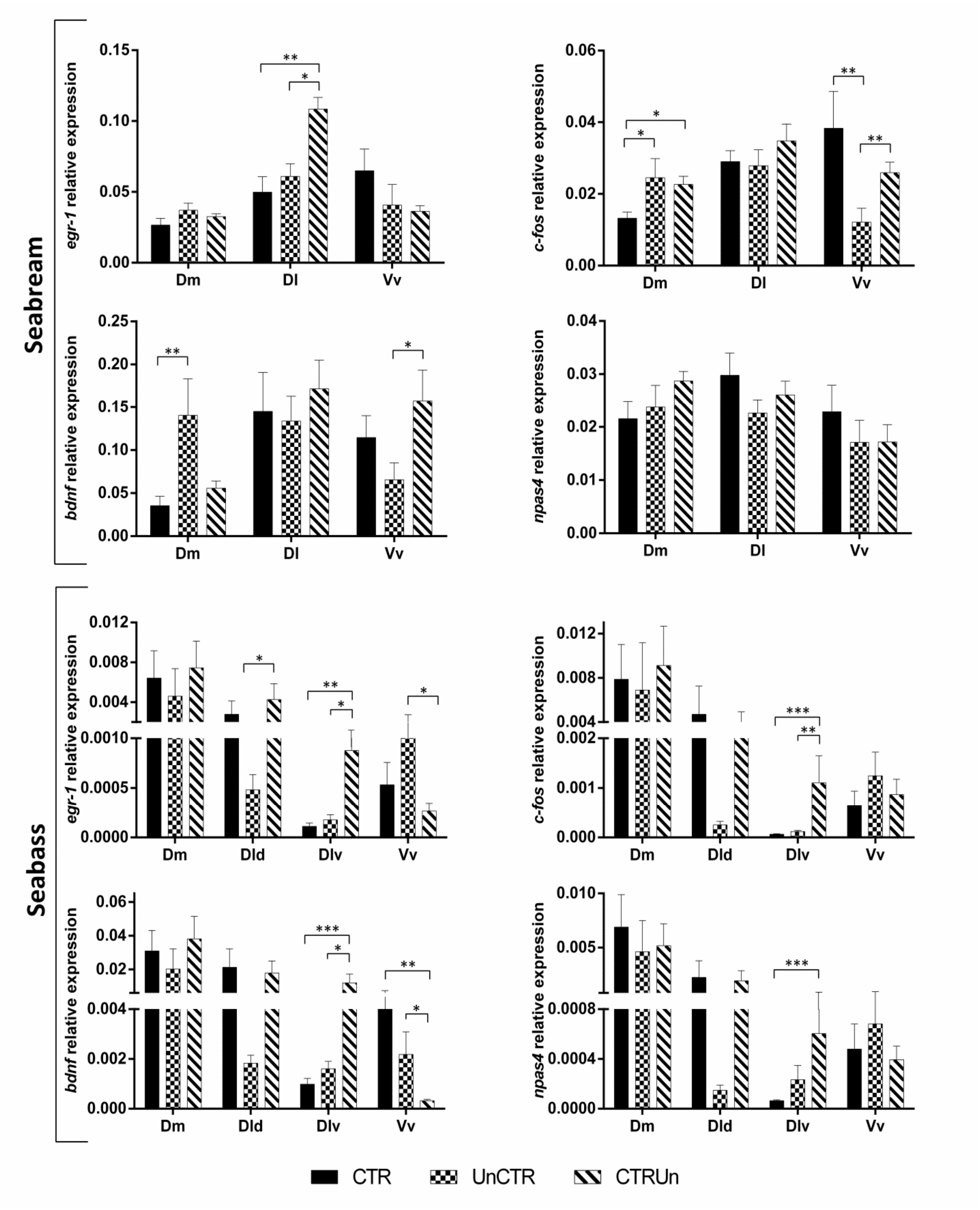

Figure 4.1.3 |E*gr-1*, *c-fos*, *bdnf* and *npas4* relative mRNA expression (mean ± SEM) measured in the Dm, Dl and Vv telencephalic regions of seabream and in the Dm, Dld, Dlv and Vv telencephalic regions of seabass under different experimental conditions (i.e. CTR, UnCTR and CTRUn). One-way ANOVA with Tukey post-hoc indicates significant differences between experimental conditions (i.e. CTR vs UnCTR; CTR vs. CTRUn; UnCTR vs. CTRUn); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Gene expression and physiology predicts coping ability

To predict coping ability towards each experimental condition, a linear discriminant function analysis combining both IEGs expression on the target brain nuclei and cortisol levels was used to reveal the salience with which the stimulus was appraised by the individuals. Two discriminant functions were significantly loaded on both species to predict coping ability over the different treatments (function 1: Wilk's lambda = 0.027, chi-square = 48.76, p < 0.001; Wilk's lambda = 0.171, chi-square = 18.57, p = 0.001; function 2: Wilk's lambda = 0.245, chi-square = 18.99, *p* < 0.001; Wilk's lambda = 0.651, chi-square = 4.50, p = 0.034, for seabream and seabass, respectively) with function 1 and 2 explaining 72.4 % and 27.6 % of variance in seabream and 84 % and 16 % in seabass (Fig. 4.1.4). In seabream, function 1 was heavily loaded by bdnf expression in Dm and *egr-1* in Dl (1.057 and 0.773, respectively); function 2 was most heavily loaded by *c-fos* in Vv 1.365) and by *bdnf* levels in Dm (-0.802). In seabass function 1 was loaded by cortisol and *bdnf* expression in Dld (0.634 and 0.691, respectively) whereas function 2 was loaded by cortisol and *bdnf* expression in Dld (0.786 and -0.736, respectively).

From Fig. 4.1.7 it can be seen that LDA, taking into account gene expression and cortisol levels, predicted 87.5 % of coping ability on CTR, 75 % on UnCTR and 100% on CTRUn (overall 87.5 %) in seabream. In seabass, such variables predicted 87.5 % on CTR and UnCTR and 75 % on CTRUn (overall 83.3 %). Additionally, in seabream, multiple regression analysis with adjusted R square showed that the *egr-1* expressed in the Dl predict 83.7 % of the freezing time behaviour. In seabass, no distinctive areas were found to explain the behavioural expression.

Discussion

The results of this research demonstrates that control, lack of control and loss of control over an aversive event triggers different affective stress-coping responses in two model species very relevant for the Mediterranean aquaculture and welfare research, gilthead seabream and European seabass. Such differences, expressed by changes in behaviour, physiology and neuromolecular states, mirror the subjective appraisal salience ascribed to the stimulus according to the individuals perception and expected consequences of the external environmental condition.

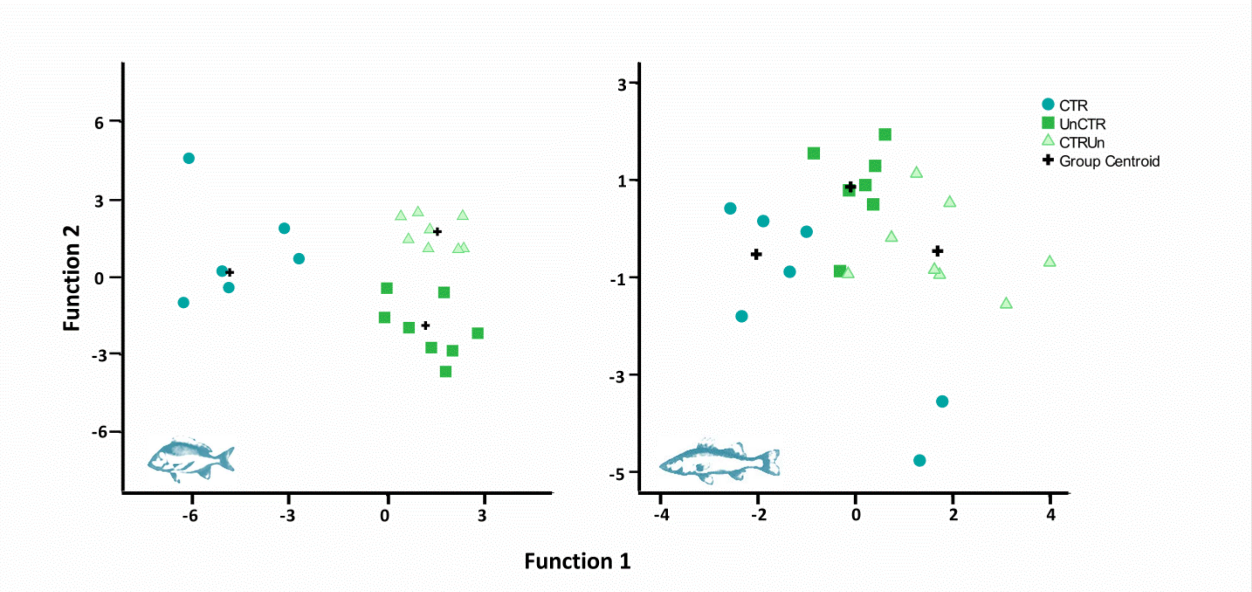

Figure 4.1.4 |Linear discriminant analysis of cortisol and *egr-1*, *c-fos*, *bdnf* and *npas4* expressed in the candidate brain nuclei from seabream (right) and seabass (left). The significant functions 1 and 2 highlight the three coping responses of fish under three experimental conditions i.e. CTR (circles); UnCTR (squares) and CTRUn (triangles). Discriminant scores for each individual are plotted and stars represent the centroid of each classified group.

Stress Coping-driven behavioural expression

Behavioural expression unveiled different strategies between species regarding the effect of controllability where in seabream, the stress-coping responses on CTR are less stress-prominent and there are no clear patterns distinguishing between UnCTR and CTRUn. In the case of seabass, specific behaviours lie behind the different stress-coping responses for the different experimental conditions. Overall, differential patterns of behavioural responses expressed throughout conditioning (see supplementary material Fig. S1 and S2 further down in this chapter), suggest that the CS itself effectively became a substitute for the US, enabling an assessment of expressions of controllability appraisal. Additionally, it shows the capability of both species to recollect memories of the previous aversive events during the punishment-induced training period, as it has been demonstrated elsewhere (Millot et al., 2014a; Millot et al., 2014b). From the examples in the literature (given in the introduction) it is patent that, in any punishment-induced situation, perception of control minimizes the stressor salience, regardless of the stimulus valence. Nevertheless, in the initial state of coping, some degree of stress is essential to activate alertness and metabolism (Moberg, 2000). Here, conditioning allowed individuals to evaluate the regularity of the event, the intrinsic valence of the stimulus, the novelty and the capacity to control it (Faustino et al., 2015; Paul et al., 2005). Then, successful coping suppresses such initial stress reactions and results found in the end session should reflect the stress-coping mechanisms of controllability appraisal.

Seabream, after learning about the possibility to escape, shifted from a more active towards a passive coping strategy to conserve energy and resources i.e. decrease escape attempts and increase freezing. As result, during the effective event CS+US, individuals could focus all resources and energy to escape from it. Such explanation is supported by the time the focal fish took to escape from the 5 min confinement, measured within each species and where seabass took twice the time (mean ± SD of all training sessions analyzed and from all fish/tank; seabream = 105 ± 77 sec; seabass = 202 ± 90 sec; N = 24/species). Such changes, on CTR, might be evocative of a known perceived “distant” danger (i.e. known to be avoidable), rather than of an imminent real danger (i.e. inescapable). Indeed, active coping behaviours have been described as an unconditional response to proximate threats, whereas passive coping strategies are seen as reactions to distal stimuli predictive of danger (Panksepp, 1990). In fish, such adaptive responses were formerly shown to relate to fear reaction or anxiety-like states (Ashley and Sneddon, 2008; Steimer, 2002). In agreement, the shift between active to passive actions indicates a decrease of the individuals’ anxiety-like states or fear over CTR. Moreover, exploratory behaviour has been normally associated with foraging behaviour, thus supporting the motivation increase under CTR i.e. increase the pursuit of their primary needs, such as food, rather than being focusing on the attempt to cope with the know incoming inescapable stressor. In agreement to what was described above, persistent inescapable restraining and loss of escaping possibility seem to induce reduced coping ability (though not completely abolishing it), as the individuals’ escaping reactions were more expressed during the CS signalling. As such, individuals under those conditions were possibly still attempting to gain control (sensu reactant theory Brehm, 1966), rather than showing effects of helplessness, such as passivity or depression-like behaviours (Seligman, 1975). The lack of differences between the latter conditions raises the question of whether the number of training sessions was enough for fish to perceive the shift from CTR to UnCTR. According to Wortman and Brehm (1976), as long as control beliefs are high, individuals would not resort to helplessness behaviours in a situation of loss of control, which seem to be the case as the similarities with UnCTR.

On the other hand, seabass displayed lower time freezing under CTR, whereas CTRUn showed significant higher freezing time even over UnCTR. Inconsistently with freezing patterns, the number of resting events in CTR was higher in this species, whereas in seabream it was lower, despite the higher time in freezing. Such responses suggest species-specific differences in sensitivity to threat or stress, supporting also the possibility that behavioural reactivity may be linked with the individuals' personality traits. The existence of divergent traits in fish is well recognized, including in seabream and seabass (Castanheira et al., 2013; Ferrari et al., 2014), within and between species (see Castanheira et al. (2015) for review). Described whether by consistent behavioural or neuroendocrine patterns (or both) over time and across distinct contexts, different traits were shown to explain individual differences in vulnerability to stress-related diseases in fish (Cerqueira et al., 2016c; Mackenzie et al., 2009; Rey et al., 2015). Indeed, it was reported that seabass is a highly stress-sensitive species (Millot et al., 2009; Rubio et al., 2010). This can explain the lower levels of exploration found under CTRUn compared to UnCTR, since any change in the environment can be markedly stressful. Additionally, seabass is a known gregarious species in their natural environment, which makes it reasonable to consider that the positive effect of social counterparts might have overridden the negative effect of lack of control. In fact, by modifying the effect of unpredictable events, the presence of social counterparts was suggested to be an important modulator of stress response in seabass (Cerqueira et al., submitted_b). The stress due to loss of control in seabass, as demonstrated by higher immobility, seems to go beyond the reactant theory and to represent more proximate helplessness depression-like stress behaviours.

Stress Coping-driven physiological expression

Cortisol levels significantly distinguish between experimental conditions (CTR < UnCTR < CTRUn) on both species. The levels are within those previously reported for both species, even if with divergent protocol conditions (Cerqueira et al., submitted_a,b; Millot et al., 2014a,b). Cortisol release is known to be involved in the modulation of arousal, vigilance, attention and memory formation (van Ast et al., 2013), and to facilitate the encoding of emotion-related memory in fish (Cerqueira et al., submitted_a). Indeed, CTR groups displayed the lowest cortisol concentrations in both species, hence suggesting that the perception of control, rather than non-control or loss of control over a stressful event, is effectively appraised as less harmful. This is compared with our previous findings using the same species, where unpredictable conditions increased the cortisol expression when compared to predictability conditions (Cerqueira et al., submitted_a,b). Predictable environmental cues are known to offer some perception of control to the individuals, this way decreasing their stress response (Bassett and Buchanan-Smith, 2007). In accordance to previous research (Carpenter and Summers, 2009; Galhardo et al., 2011; Øverli et al., 2004), psychological stress increases the synthesis and release of cortisol, in association with fear and anxiety states. With due caution, by measuring cortisol concentration as a proxy of stress salience and supported by behavioural-correlated responses, our results demonstrate evidence of such depression-like acute stress caused by CTRUn even over UnCTR in seabass. Nevertheless, it should be underlined that the coping states of the fish cannot be entirely explained by the cortisol levels as it is not possible to disentangle the levels owing to species-specific inherent predispositions from those promoted by each experimental challenge (e.g. stress reactiveness and social context is known to affect differently each species towards stressful event). Such statement is reinforced by the divergence between cortisol levels and time in freezing patterns, where a negative correlation was found in seabream, while the opposite was seen for seabass.

Affective Coping-driven neuroplasticity profiles

Brain expression of genes known to be involved in experience-driven neuroplasticity displayed specific neural states for each of the distinct experimental conditions tested. Relying on the assumption that neural activation patterns distributed across the brain, triggered by specific stimuli, predict emotions in humans and elicit the expression of specific adaptive behaviours (Anderson and Adolphs, 2014; Chang et al., 2015), our results are suggestive of different organismic states underlying each of the experimental conditions in seabream. Controllability, regardless of the valence of the stimuli, was stated to promote positive emotions in animals (Boissy et al., 2007). In seabream, the expression of *c-fos* and *bdnf* on both Dm and Vv showed specific regulation of appraisal emotional processing in CTR compared to UnCTR and CTRUn, while an up-regulation of *egr-1* on Dl under CTRUn, may be suggestive of a demanding learning processing compared to the former conditions. Discriminatory function 1 loaded by *bdnf* expressed on Dm and *egr-1* on Dl seems to separate experimental conditions by the averseness attributed to the condition, while function 2 loaded by *bdnf* on Dm and *c-fos* on Vv appears to be separating each one by stimulus salience (arousal). Given the similar role played by the mammalian homologues, the involvement of these brain areas is known to integrate both emotional processing and memory of aversive event perception (O'Connell and Hofmann, 2011). Similar results were found in seabream, where both Dm and Vv were shown to display differential neuromolecular expression in the emotional processing of predictability appraisal corresponding to the two dimensions of the core affective space (valence and salience) (Cerqueira et al., submitted_a). In mammals, the role of the lateral septum and amygdala on novelty and emotional processing were shown (Landgraf et al., 1995; LeDoux, 2000; Liebsch et al., 1996; Sheehan et al., 2004). Multiple regression analysis showed that *egr-1* expressed in the Dl was heavily loaded by time in freezing. By the known link between the lateral septum and hippocampus in humans in the modulation of memory formation and recall (Rokers et al., 2002), the up-regulation of *egr-1* over CTRUn on Dl is most likely reflecting the shift from CTR to UnCTR conditions, which resulted in an distinct level of processing from the effective CTR and UnCTR conditions. Given both the Dl's hypothesized role as the homologous structure to the hippocampus and the physiological expression, such result demonstrates that coping ability of fish tested under CTRUn was most likely more compromised, since either learning or memory were probably more affected (Portavella and Vargas, 2005).

On seabass, an up-regulation of all the candidate genes in the Dlv and a down-regulation of *egr-1* and *bdnf* on the Vv upper CTRUn were seen to modulate both behaviour and physiological responses. It is reasonable to argue, on the basis of hodological, developmental and functional comparative evidence from Dlv with the Dl (Uceda et al., 2015), that CTRUn impair spatial learning and memory. Several studies have linked spatial learning with Dlv neuro-activity (Demski, 2013; Portavella et al., 2002; Uceda et al., 2015). Reinforced by lower levels of exploration and increased shoaling cohesion, it is apparent that motivation has effectively decreased under CTRUn, concomitantly increasing the negative emotion-like states underlying the individual's coping ability. In agreement, differences found in *bdnf* expressed on Vv suggest that coping ability was emotionally compromised under CTRUn conditions, since its expression was inhibited. This is in line with Vindas et al. (2014b) outcomes showing that, in Atlantic salmon, unpredictability created by omission of an expected reward inhibited *bdnf* mRNA expression. Discriminant functions loaded by both cortisol levels and *bdnf* expressed in the Dld suggests that similar conditions during the first training sessions, promoted by the sensitivity to stress, have similarly salience-encoded both CTR and CTRUn. Functionally, Dld is considered the fish visual pallium due to its neuroanatomy and electrophysiological evidence (Wullimann and Mueller, 2004), but some evidences indicates a multimodal sensory function of this area (Demski, 2003; Demski, 2013). Vargas et al. (2009) disclosed on goldfish that Dld controls short-term memory processes and plays a role in the performance of activity under stressful situations. In agreement, such similar proximate states on Dld between CTR and CTRUn are probably due to the insufficient sessions to condition the individuals to CTRUn, attenuating the differences between those and CTR. Moreover, fish, knowing to be under inescapable conditions (evidenced by higher time in freezing or shoal cohesion), displayed no apparent motivation, which is most likely linked with the down-regulation found on Dld. Again, the social condition possibly had an additional influence throughout conditioning, moderating the expression of *bdnf* on Dld. Nevertheless, the correlations found between cortisol and the expression of all candidate genes on Dlv suggests a differential integrative emotional regulation within the learning processing. Indeed, physiological expression was shown to reflect emotion-like states in fish (Cerqueira et al., submitted_a). Supporting the coordination of distinct neuromolecular states on behavioural actions (Cardoso et al., 2015), neurogenomic states reveal specific co-expression on CTR on seabream, whereas UnCTR and CTRUn displayed shared similarity of genomic co-expression matrices on Dm. In seabass, each treatment presented a unique neurogenomic state, while between brain nuclei, co-expression was unique only for CTRUn. Such differences, as explained, are possibly related to either increased attention and anxiety-like responses (i.e. CTR) or to those more fear-related (i.e. CTRUn and UnCTR) in seabream, while, in seabass, stress sensitivity probably have accentuated such specificity of CTRUn conditions. Overall analysis of neuromolecular data suggests that the positive effect of controllability is emotionally processed as less harmful and does not impair learning and memory. Additionally, a more severe connotation of losing control even over total lack of control is translated by impairment of learning and accentuated by the species' inherent dispositions and stress sensitivity.

Conclusions

These results extend our knowledge on how the brain from divergent evolutionary vertebrates mediates phenotypically stress coping-behaviours. Accordingly, it suggests that controllability, regulated by their neural organisation, mitigates the effects of aversive events in fish, by changing the way they perceive their environment, with positive outcomes for successful coping ability and ultimately to their welfare and health. As such, in line with research on mammals, our results evidence that fish species show both behavioural and neuroendocrine responses that differentiate between anxiety states and fear adaptive responses, suggestive of well-conserved affective states within vertebrate ancestry.

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Supplementary Material

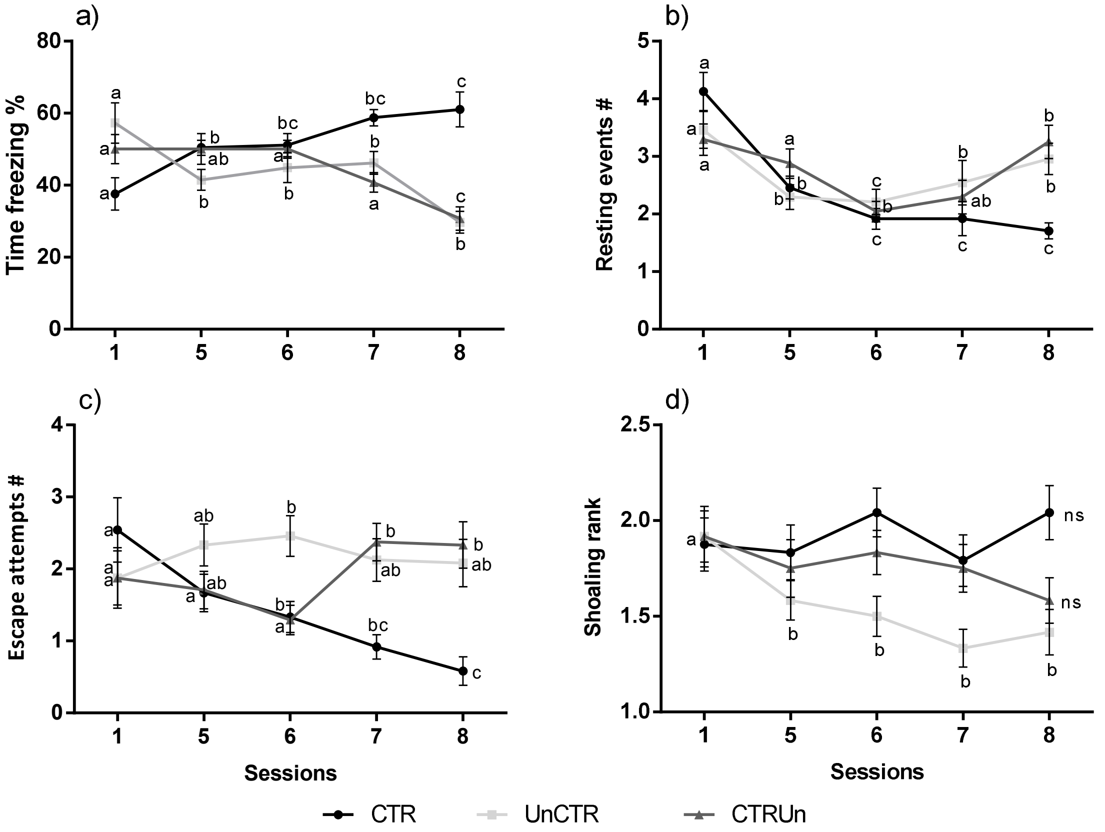

Figure S1 | Seabream a) time freezing, b) resting events, c) number of escape attempts and d) shoal rank (Mean ± SEM) measured under each experimental condition (CTR – controllable; UnCTR – uncontrollable and CTRUn – loss of control) during stimulus signaling throughout the conditioning sessions. Between sessions repeated measures ANOVA with planned multiple comparisons significant levels are indicated by differing letters; ns – non-significant.

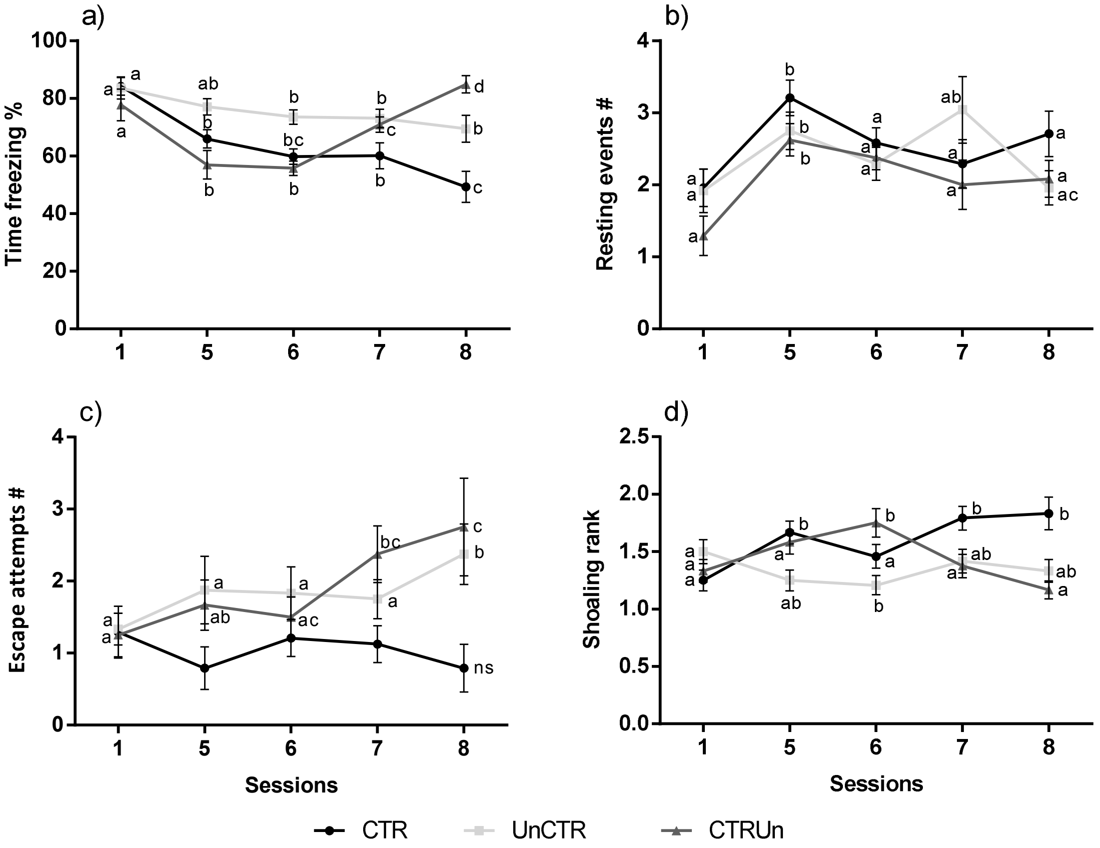

Figure S2 |Seabass a) time freezing, b) resting events, c) number of escape attempts and d) shoal rank (Mean ± SEM) measured under each experimental condition (CTR – controllable; UnCTR – uncontrollable and CTRUn – loss of control) during stimulus signaling throughout the conditioning sessions. Between sessions repeated measures ANOVA with planned multiple comparisons significant levels are indicated by differing letters; ns – non-significant.

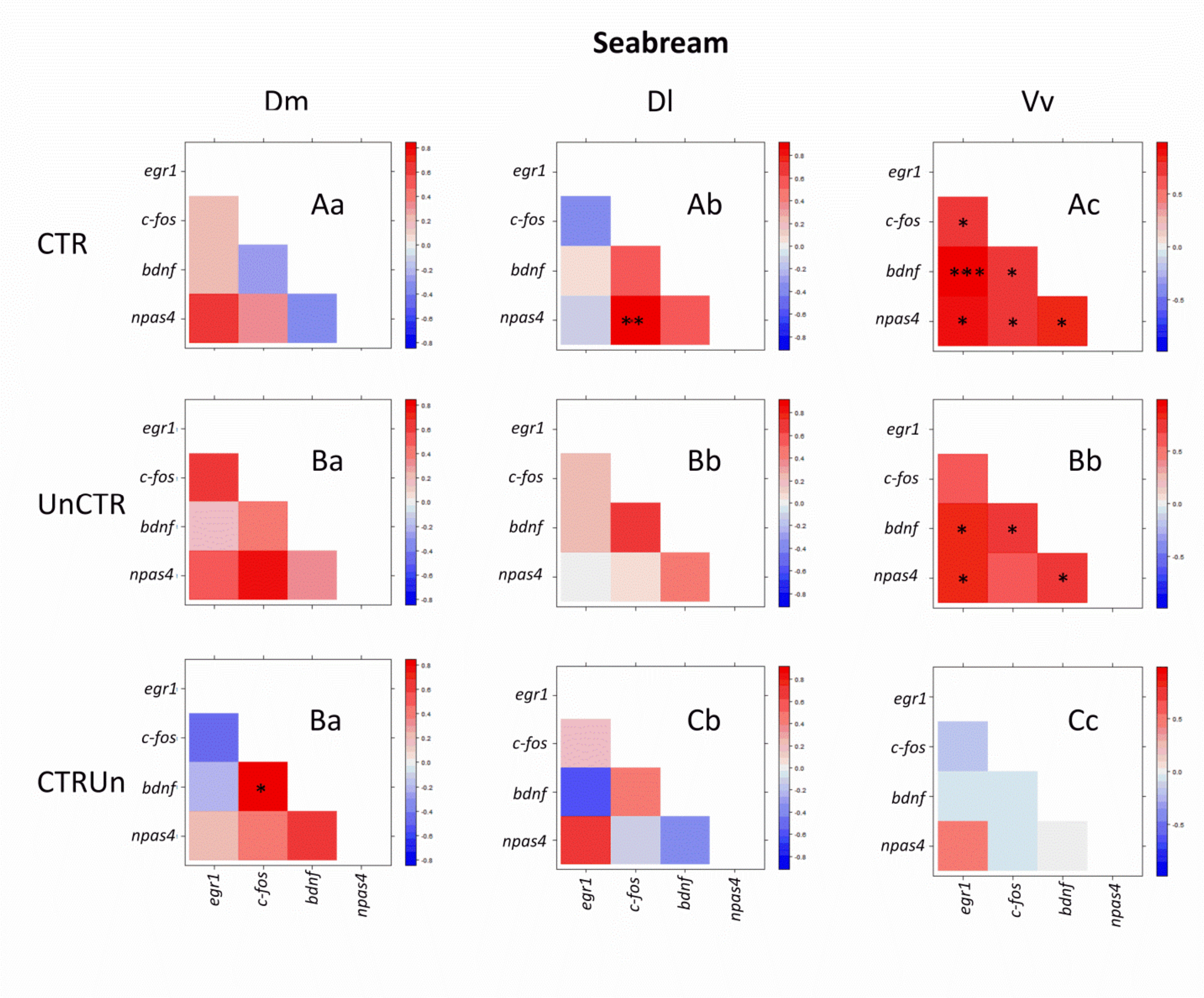

Figure S3 |Neurogenomic states underlying each of the experimental conditions (CTR: controllability; UnCTR: uncontrollability; CTRUn: loss of controllability) and for each of the telencephalic brain region examined (Dm; Dl, and Vv). Asterisks indicate significant correlations after p-value adjustment *p < 0.05; **p < 0.01; ***p < 0.001. Different capital letters indicate significantly different co-expression patterns among experimental conditions, and different small letters indicate significantly different co-expression patterns among brain nuclei, using the QAP correlation test.

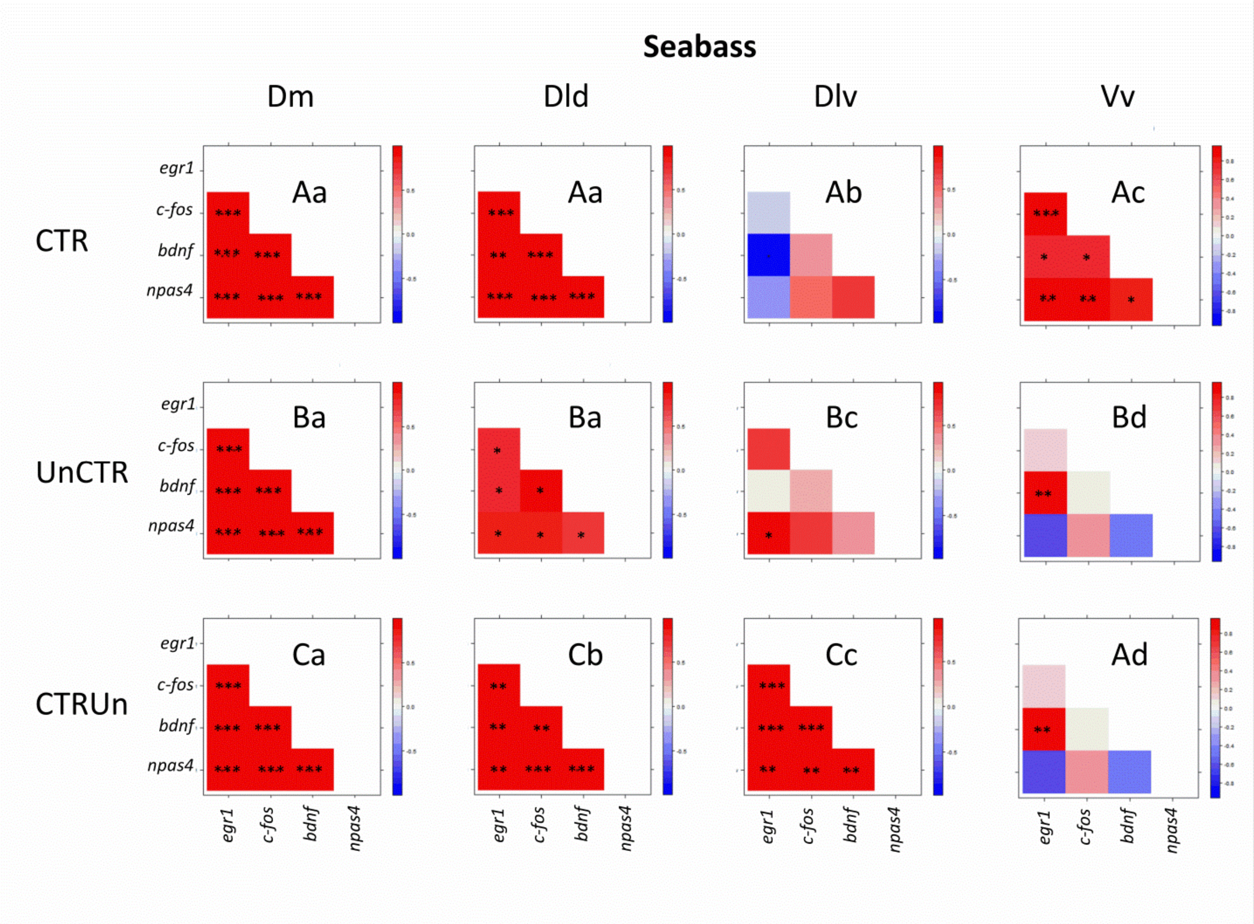

Figure S4 |Neurogenomic states underlying each of the experimental conditions (CTR: controllability; UnCTR: uncontrollability; CTRUn: loss of controllability) and for each of the telencephalic brain region examined (Dm; Dld, Dlv and Vv). Asterisks indicate significant correlations after p-value adjustment *p < 0.05; **p < 0.01; ***p < 0.001. Different capital letters indicate significantly different co-expression patterns among experimental conditions, and different small letters indicate significantly different co-expression patterns among brain nuclei, using the QAP correlation test.

CHAPTER IV-2

Thermal preference predicts animal personality in Nile tilapia *Oreochromis niloticus*

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Thermal preference predicts animal personality in Nile tilapia *Oreochromis niloticus*

Abstract: Environmental temperature gradients provide habitat structure in which fish orientate and individual thermal choice may reflect an essential integrated response to the environment. The use of subtle thermal gradients likely impacts upon specific physiological and behavioural processes reflected as a suite of traits described by animal personality. In this study we examine the relationship between thermal choice, animal personality and the impact of infection upon this interaction. We predicted that thermal choice in Nile tilapia *Oreochromis niloticus* reflects distinct personality traits and that under a challenge individuals exhibit differential thermal distribution. Nile Tilapia were screened following two different protocols: 1) a suite of individual behavioural tests to screen for personality and 2) thermal choice in a custom-built tank with a thermal gradient (T_{CH} tank) ranging from 21 to 33 ºC. A first set of fish were screened for behaviour and then thermal preference and a second set were tested in the opposite fashion; thermal then behaviour. The final thermal distribution of the fish after 48 h was assessed reflecting final thermal preferendum. Additionally, fish were then challenged using a bacterial *Streptococcus iniae* model infection to assess the behavioural fever response of proactive and reactive fish. Results showed that individuals with preference for higher temperatures were also classified as proactive with behavioural tests and reactive contemporaries chose significantly lower water temperatures. All groups exhibited behavioural fever recovering personality-specific thermal preferences after 5 days. Our results show that thermal preference can be used as a proxy to assess personality traits in Nile tilapia and it is a central factor to understand the adaptive meaning of animal personality within a population. Importantly, response to infection by expressing behavioural fever overrides personality related thermal choice.

Keywords: Thermal preference, Animal personality, Environmental choice, Behavioural fever, Nile Tilapia, Physiological regulation.

Introduction

Environmental temperature influences all aspects of an organism's physiology and behaviour, from reproduction to growth, and this dynamic interaction with the environment impacts upon fitness and survival. In mobile ectotherms, such as fish, body temperature closely follows environmental temperature and can only be modified by behavioural means. This behavioural regulation occurs across different temporal scales including daily and seasonal cycles. This impacts upon the basal metabolic rates of ectotherms where small changes in environmental temperature may result in significant metabolic fluctuations (Clarke and Fraser, 2004). For example, fish navigate thermal gradients to attain a preferred thermal optima to improve physiological and in some cases reproductive performance (Angilletta Jr et al., 2002; Huey and Bennett, 1987; Huey and Kingsolver, 1989; Pawson et al., 2000). Recently in *Drosophila* larvae thermosensory neurons where shown to provide the basis for thermotaxis coupled to environmental navigation in a fluctuating thermal environment highlighting the importance of thermal perception (Klein et al., 2015). Thermal optimum is defined as the temperature that maximizes physiological performance at an individual level. Under normal conditions if performance is linked to fitness, natural selection should favour a tight relationship between preferred body temperature and the preferred thermal optima by means of regulatory behaviour (Huey and Bennett, 1987). Such behaviour can be seen as a thermoregulatory response with a positive trade-off between energy demand and physiological requirements (Rey et al., 2015). The thermal optimum for an individual is defined as the final thermal preferendum (Fry, 1947). Calculation of this preferendum is measured for each individual 24-96 h after exposure to an adequate thermal choice. This final temperature represents the interaction between environmental demand and an individual's capacity to respond to such demands. The final thermal preferendum calculated on an individual level has since been used as a mean temperature preference guide in fish (Rey et al., 2015).

Recent progress addressing animal personalities (Castanheira et al., 2013; Herrera et al., 2014; Huntingford et al., 2010; Martins et al., 2011c; Overli et al., 2007; Rey et al., 2013; Silva et al., 2010) has highlighted a central role for individual variation in ecological and environmental challenges (Reale et al., 2007; Sih et al., 2004) and as a tool for resolving variation (MacKenzie et al., 2009). Animal personality traits have been described as underlying tendencies that affect behaviour across contexts, that are stable over time and that vary across individuals (Dingemanse et al., 2010; Reale et al., 2007). Typically, animals categorized as ‘proactive’ (active coping or bold), “intermediate” (more flexible individuals) or ‘reactive’ (passive coping or shy) show distinct differences in behavioural profiles for the same measured variable, such as risk taking and exploration, aggression or feeding (Castanheira et al., 2015; Rey et al., 2013). Among fish, there is scarce information linking personality traits and physiological profiles, particularly with respect to individual differences in adaptability, i.e. optimal conditions created for proactive individuals are likely to be different from those of reactive individuals (Rey et al., 2015) with ecological relevance. On one hand, a proactive individual characterized by increased foraging activity may take more risks to attain more food (Finstad et al., 2007) and therefore is more prone to predation risks (Stamps, 2007) or to being captured (Biro and Dingemanse, 2009), suggesting higher internal energetic requirements with higher metabolic costs. In contrast, a reactive individual can be characterized by low levels of aggression, immobility and avoidance behaviour under aversive events (see review of Castanheira *et al.* (2015)) that may reflect lower metabolic demands. Recently, Rey et al. (2015) using the zebrafish, *Danio rerio,* as a model showed that proactive and reactive animal personalities express different thermal preferendum and activity patterns when a thermal choice is available. Parallel research showed the effect of fluctuations in temperature on personality of two juvenile coral reef fish, reflected by behavioural variation among and within individuals (Biro et al., 2009).

Based on the fact that fish behaviour reflects individual differences in physiological needs, the prospect to use thermal preference as a mass-screening paradigm is attractive. Different experimental screening methods for personality traits along a proactive-reactive continuum have being used with Nile tilapia as a model (Barreto and Volpato, 2011; Barreto et al., 2009; Martins et al., 2011a; b; VeraCruz et al., 2011), with the disadvantage of them being intensive time consuming assays. In this study we aimed to firstly assess if final thermal preferendum played a pivotal role in determining the distribution pattern of Nile tilapia with different personalities and secondly to validate final thermal preferendum as a new physiological paradigm to screen for personality traits. In a final experiment we further explored how a bacterial infection impacts upon the expression of behavioural fever in distinct animal personalities.

Material and Methods

Fish and housing conditions

Nile tilapia (*O. niloticus*; Wild-type and Homo-Gold strains) were obtained at the Institute of Aquaculture, University of Stirling, UK. Prior to the experiments, these fish had been reared together, each family, in the same tanks under normal stocking conditions. Fish were kept in a 500 L fiberglass tank within a RAS system with a continuous water flow. Animals were reared under a 12:12 light-dark cycle and the mean water temperature was of 26.8 ± 1.5 ºC. Aeration was supplied through an air stone and fish were fed with a commercial diet (Skretting® Trout Standard Expanded) twice a day.

Experimental protocol and procedures

Initial trials with *Oreochromis niloticus* (Wild-type, n = 54) were performed to establish the distribution of behavioural phenotypes and behavioural consistency over time. Subsequent experiments using a population of *O. niloticus* (Homo-Gold, n = 350) were carried out due to availability of stocks. The consistency of behavioural responses between these two populations was analysed using DAI data (see below) and is shown in Fig. 4.2.1. Prior to screening, individuals were sorted for size and weight and extremes discarded. Selected animals with mean weight of 8.18 ± 1.55 g were moved to 3 smaller holding tanks, at high density in order to avoid aggression: 20 L white rounded tanks on a recirculation system (37 cm diameter x 28 cm height, with a drainage tube in the centre 3 cm diameter and 20 cm height) and acclimated for 3 weeks prior to behavioural tests (n = 96 / tank; N = 288). A total of 94 animals with mean weight of 10.43 ± 1.33 g were individually tested for DAI latency distribution and net restraining behaviour (n = 94) in parallel tanks to those they were held in (VeraCruz et al., 2011). To test for behavioural consistency, the DAI latency measures test was repeated 24 hours after the first feeding event. After the analysis, the distribution regarding the DAI latency was determined and used as a baseline to discriminate the population by personality and establish the corresponding cut-off DAI latencies (Fig. 4.2.1). Animals were not individually tagged in our experiments and behavioural screening was individually performed before designation of personality.

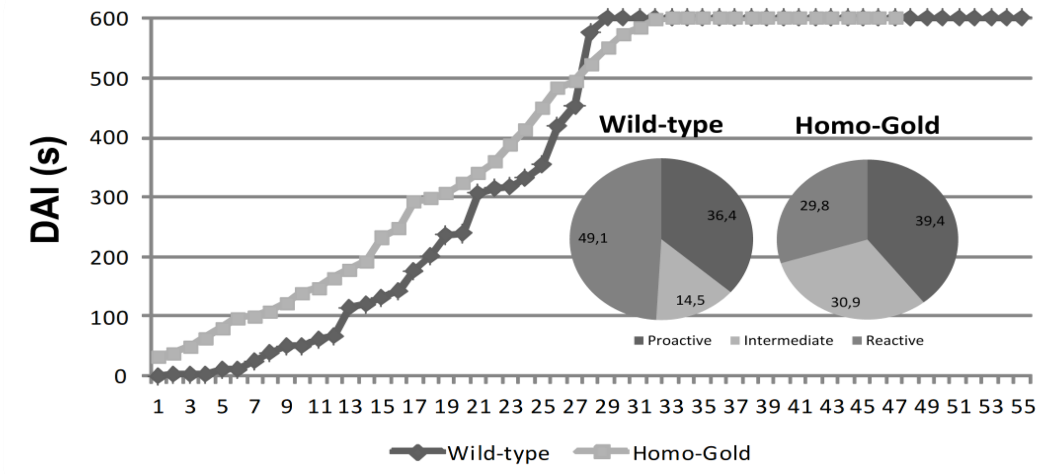

Figure 4.2.1 |Duration of Appetite Inhibition latencies (in seconds) distribution for 54 tilapia from a wild-type background and 94 tilapia with a Homo-Gold genetic background tested (x axis is fish running number and y axis is time in seconds). Homo-Gold individual latency is represented here for illustrative reasons, by the means of DAI_1 and DAI_2 latencies. Fish that spend less than 300 seconds to eat were classified as proactives (Wild type = 36 % and Homo-Gold = 39.4%), between 300 and 600 seconds were intermediates and more than 600 seconds were reactives (Wild type = 49% and Homo-Gold = 29.8 %). Personality percentages plotted as a pie chart for both Wild-type and Homo-Gold.

Personality screening

A further eighty-four fish (from the 288 fish selected stock) with a mean weight of 15.34 ± 2.33 g were individually housed in rows of 12 parallel white rounded tanks of 20 L, as used during stocking. Behaviour was video recorded (HD Webcam C615, Logitech®, USA) for posterior analysis. Cameras were placed above the tanks and used for test 1, 3 and 4. Each fish was subjected to the following individual tests:

1. Novel environment, NE - Screening for novel environment and social isolation was performed immediately after the introduction of the fish into the tank. The first 3 minutes were observed and analysed for swimming activity (% of time the fish took to explore the new environment and described as the fish movement in the water column or at the bottom at any speed or intensity of body movements). Fish were left for one hour to habituate to these new conditions.

2. Ventilation frequency, VF - After one hour of habituation, aeration and water flow were withdrawn to facilitate behavioural measurements. Animals were habituated for 10 min before data recording. Ventilation frequency (opercula movements/minute) was taken by direct observation measuring the time it takes a fish to complete 20 successive opercula or mouth movements within one minute across three consecutive minutes. When time is higher, VF is lower and fish categorized as proactive (Barreto and Volpato, 2011; Barreto et al., 2009). Water flow was then restarted and fish were left to habituate for 30 min.

3. Duration of appetite inhibition, DAI - Latency to reassume feeding after the onset of the food. The test lasted for a maximum of 10 minutes. Latencies to reassume feeding after isolation (DAI latency), latency to first movement (lat2move) and total feed intake (FI) were recorded. According to preliminary trials, fish with DAI latencies shorter than 5 min were categorized as proactive, 5-10 minutes were intermediates and > 10 minutes were categorized as reactive individuals. To check for feeding behaviour consistency, this test was repeated twice (run 1 and run 2: DAI_1 and DAI_2) with an interval between runs of 24 h.

4. Net restraining, NR - The net restraining test consisted of holding each fish individually in an emerged net for 1 min. While in the net, the following behaviours were measured: 1) latency to first escape attempt (lat2escape: 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), 2) number of escape attempts (total escapes) and 3) total time spent on escape attempts (time escape: total time in seconds taken by each fish escaping since the first to the last escape attempts). All animals were initially grouped by their DAI_1 and DAI_2 latency, and then settled in holding tanks separated according to their personality traits (homogeneous groups). The results of the remaining tests were used to assess correlations between different behavioural paradigms and confirm the classifications performed using the DAI latency cut-offs. Fish were kept in groups of 30 before the group test.

Temperature preference chamber test

Thermal preference was assessed in a custom-built multi-chamber tank (adapted from Rey et al. (2015)) (Fig. 4.2.2) firstly under constant temperature and then under a continuous thermal gradient allowing the temperature preference of each group to be recorded over a 48 h time period. The dimension of the tank was adapted to the size of the fish, 126 L (140 x 30 x 30 cm) and divided with 6 transparent glass screens to create 7 equal interconnected chambers. Each screen had a hole at the centre (10 cm diameter; 20 cm from the bottom) to allow connection between chambers and support ease of movement of the fish. Mechanical filters were placed in the 5 central chambers and the bottom gravel covered. Two custom-built multi-chamber tanks were used under different conditions: 1) Thermal gradient (T_{CH})

and 2) Thermal restriction (T_R). Three video cameras (Linksys® webcams) provided continuous monitoring of the tank chambers with automatic recording (Fisheye software, UAB; see Rey et al. (2015) for details).

Figure 4.2.2 |Experimental custom-built multi-chamber tank used for assess thermal preference in Nile tilapia.

Thermal gradient was first tested and optimized (Fig. 4.2.3) to ranges between 20.92 ± 0.04 ºC (chamber 1) to 33.08 ± 0.08 ºC (Chamber 5), with a mean difference in temperature of 3.04 ± 0.10 ºC between each chamber. Extreme lateral chambers were operated as cooling (mean temperature 16 ± 0.02 ºC), by means of a cooler system and a pump, and heating chambers (mean temperature 42 ± 0.02 ºC), by means of a water bath and a pump. Individuals were prevented from entering those chambers by covering the hole in the screens. Thermal restriction conditions were established with the water at rearing temperature 26.58 ± 0.33 ºC for all the tank chambers. During each test dissolved oxygen was recorded at the beginning and at the end of each trial (Handy Polaris, OxyGuard® International, Farum, Denmark). The temperature from each chamber was continuously recorded in the centre of each compartment at the inner wall of the swimming channel for each 15 min with thermocrons iButton (Maxim integrated®, Rio Robles, San Jose, USA). Three groups of fish (n = 8) were used for each personality trait (n = 24) to test for thermal preference. Six groups of naïve fish randomly selected from the stock tanks were used as control groups (n = 48). Three of those groups were tested under T_R conditions and another three under T_{CH} conditions. Fish were settled in the middle chamber (26.58 ± 0.33 ºC and 26.71 ± 0.03 ºC, respectively) with the holes covered until all the fish were deployed and covers then immediately removed. Fish were left for 48 hours and filming began immediately. Water was completely replaced between group tests to guarantee equal conditions throughout the trials.

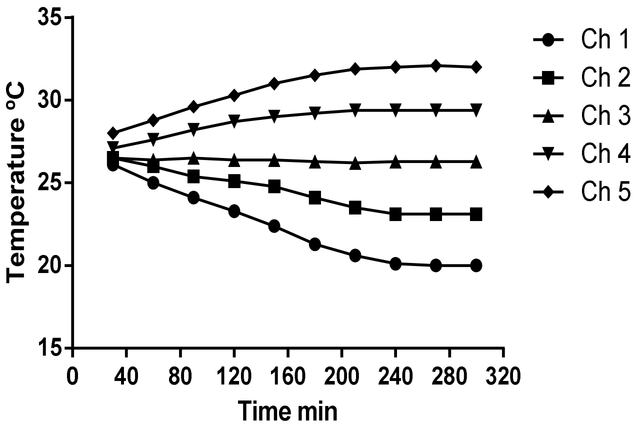

Figure 4.2.3 |Establishment (1) and stabilization (2) of the thermal gradient within each chamber of the experimental aquarium: chamber 1: 20.92 ± 0.04 ºC, chamber 2: 23.66 ± 0.04 ºC, chamber 3: 26.71 ± 0.03 ºC, chamber 4: 29.86 ± 0.02 ºC, chamber 5: 33.08 ± 0.02 ºC.

Validation of thermal preference as an indicator of personality

Eight groups (n = 8) and three control groups of naïve fish randomly selected from stocking tanks (n = 88) with mean weight of 19.23 ± 3.21 g were used to validate thermal preference as a physiological screening proxy without previous behavioural screening. Thermal preference assessment was performed in the same tanks following the same protocol as above with a refined gradient profile of 25.95 ± 0.2 ºC to 33.59 ± 0.3 ºC, with 1.91 ± 0.38 ºC between chambers. After 48 h, fish were transferred to round tanks (same as previously used with the same water recirculation system) and housed as they were distributed in the different chambers in the T_{CH} tank. All individuals were then subjected to the same battery of behaviour tests described above and moved afterwards to the holding tanks as established groups with homogeneous personality traits and left to recover for 12 days.

Data acquisition

Different aspects of fish behaviour were analysed to assess the different personality traits. During the first hour named here as acute thermal period (each min, n=60 events), observations were centred upon 1) distribution of fish in the preference chamber for determination of acute thermal preference, 2) individual latencies to exit from initial chamber to side chambers and 3) the number of transitions between chambers for each group. For the last 8 hours of the test, named here as late thermal period (7 am - 3 pm; n = 33 events), the distribution of fish was recorded for 25 sec, every 15 min and analysed 'a posteriori' for determination of the final thermal preference. The sequence of the trials was alternate between the personality groups to reduce experimental bias.

Infection Challenge

An isolate of *S. iniae* recovered from natural infection in Tilapia was used for the bacterial challenge and had been identified using traditional bacterial methods. The isolate was recovered from frozen stocks onto tryptone soya agar (TSA, Oxoid UK) plates, incubated for 48 h at 28 ºC and identity confirmed using Gram, oxidase, motility, O/F methods (Frerichs 1993) and biochemical profile (API Strep, Biomérieux, Basingstoke, Hampshire, UK). The isolate was passaged through a single fish prior to performing the challenge studies to enhance virulence after long-term storage and the bacterium recovered from the kidney. The challenge inoculum came from a 48 h growth of a single *S. iniae* colony grown in 45 ml of tryptic soy broth (TSB) at 28 ºC, which was centrifuged at 2000 G for 15 min at 4 ºC and the cell pellet re-suspended in sterile physiological saline (0.85 % NaCl) to give an optical density of 1. The bacterial concentration was adjusted using sterile saline to give the challenge inoculate. The virulence and adjusted concentrations of bacteria to the weight of the fish was previously validated (Featherstone et al., 2015). Viable colony counts were performed using the Miles and Misra method (Miles et al., 1938) to check the bacterial concentration and each fish was exposed to 0.1 ml of the bacterial suspension by intraperitoneal injection (i.p.). Homogeneous groups of 8 Tilapia of proactive and reactive individuals were anesthetized with benzocaine and the bacteria administered by i.p. Injection. A control group of naïve fish was used and injected with PBS and were subjected to T_{CH} conditions. Test groups are described in Table 4.2.1. Animals were monitored on a daily basis for 5 days and checked for morbidity/mortality and clinical signs of disease (Shoemaker et al., 2001). The distribution of the fish during the first hour of the first day after deployment and the first 8 hours of the following days was video recorded (7 am - 3 pm; for 25 sec, every 15 min; n = 33 events).

Table 4.2.1 |Experimental infection groups used to test thermal preference of fish with different personality traits to an i.p. Infection with stablished *S. iniae* bacterial model.

Treatment	Conditions	i.p. injection
Naïve Control	T_{CH}	PBS 0.1%
Reactive	T_{CH}	*S.iniae* 2.97 x 10^7 cfu per inoculum
Proactive	T_{CH}	*S.iniae* 2.35 x 10^7 cfu per inoculum

Behavioural and Statistical analysis

Statistics were performed using IBM SPSS® statistics v19.0, R® (R Development Core Team, Vienna, Austria) and GraphPad Prism® v6.0 (San Diego, California, USA) for windows. The assumptions of normality and homoscedasticity were confirmed by analysis of the residuals, with particular attention paid to homogeneity of variances between personality groups (Cleasby & Nakagawa 2011). Arcsin or $\log_{10}$ (x+1) transformation was applied in the case of non-normally distributed variables. DAI_1 and DAI_2 latencies were collapsed into principal component scores prior to clustering. The same procedure was taken with the behaviours measured from NR test. Fish were then segregated in three groups using two distinct forms of unsupervised clustering (k-means clustering and hierarchical clustering using Ward's criterion), which displayed full consistency. Pearson correlation was used to verify the repeatability and consistency of individual DAI latencies between both DAI runs. A one-way ANOVA was used to verify differences between the mean PC1 DAI values for each of the generated clusters and the post hoc Fisher LSD test was performed for specific significant differences. A global Principal component analysis (i.e. using all raw behavioural parameters measured from VF, NE, DAI and NR tests) was used to assess the quality of the separated groups using DAI latencies cut-offs determined during the preliminary test. Using the same variables, Pearson correlation was performed to assess how correlated were the different behaviours measured. To examine the differences between behavioural phenotypes, one-way ANOVA with Fisher LSD post-hoc was used for each of the behavioural paradigms (NE; VF; DAI_1; DAI_2 and NR). For this purpose, behaviours taken during DAI test were collapsed into first component scores using Principal Components Analysis (PCA) for both events. The same approach was used for the behaviours measured from restraining test. The correlation matrix was used to check multicollinearity after varimax rotation. 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. A two-way ANOVA was used to analyse latencies and transitions, after log transformation, as dependent variables with temperature and personality as fixed factors. Both acute and final thermal preference for each of the personality groups was calculated as follows:

$$\frac{n1}{N} \text{ x } T_1 \text{ ºC} + \frac{n2}{N} \text{ x } T_2 \text{ ºC} + \frac{n3}{N} \text{ x } T_3 \text{ ºC} + \frac{n4}{N} \text{ x } T_4 \text{ ºC} + \frac{n5}{N} \text{ x } T_5 \text{ ºC} \quad \text{formula [1]}$$

Where $n_{(i=1,2\ldots5)}$ was the number of fish in the chamber; N the total number of fish in the tank and $T_{(x)}$ the mean temperature of the chamber. Significant differences between thermal preferences of the different personality groups were checked afterwards through Kruskal-Wallis test since this remained non-normal after transformation. For pairwise differences a Mann-Whitney U test was used. GLM mixed model was fitted with number of fish as dependent variable and independent variables (and higher order interactions) were selected for inclusion in the model through a backward stepwise likelihood ratio method and examination of the Wald χ^2 statistic, to reach the minimum suitable model with the lowest Akaike information criterion (AIC) value. Leverage statistics and residual analysis were used to test the validity of model assumptions. The Wald χ^2 was used to test for a significant effect of the temperature or chamber (this last in the case of controls under restriction conditions) and personality on the distribution of the fish over the experimental aquaria, estimated by their coefficients β (negative binomial distribution parameter) T-test or Mann-Whitney U test was used for pairwise comparisons between thermal preference of control groups of both gradients (normal or non-normal data, respectively) and Wilcox rank test for comparisons between thermal preference after 2 and 4 days post infection or dpi (non-normal data). Statistical significance was taken at $p < 0.05$. The results are expressed as mean ± standard error of the mean (SEM).

Results

Behavioural screening for personality traits

Group clustering with fish from the same batch was used to define the cut-offs of DAI latencies as shown (Fig. 4.2.4). PC1 of DAI latencies explained 88% of DAI latency variance, while the PC1 of the restraining test explained 62 % of the restraining test variance, with 75% of the overall behavioural variation being retained in these two components. The consistency over time and across behavioural paradigms of behavioural responses is shown in Table 4.2.2. Interestingly, mostly of the variables were significantly correlated with DAI latency corroborating the use of this variable to separate the groups. Principal component analysis collapsing all behavioural parameters measured is represented in Fig. 4.2.5. The groups exhibited, “driven” by DAI latency, reveal that this behaviour is a robust predictor of distinctive personality groups, as PC1 explained 34 % of such grouping. Behavioural variation unsurprisingly was significant across situations. Nevertheless, 50 % of the grouping was explained by the two first dimensions of the PCA showing that personality is well represented in the behavioural phenotypes.

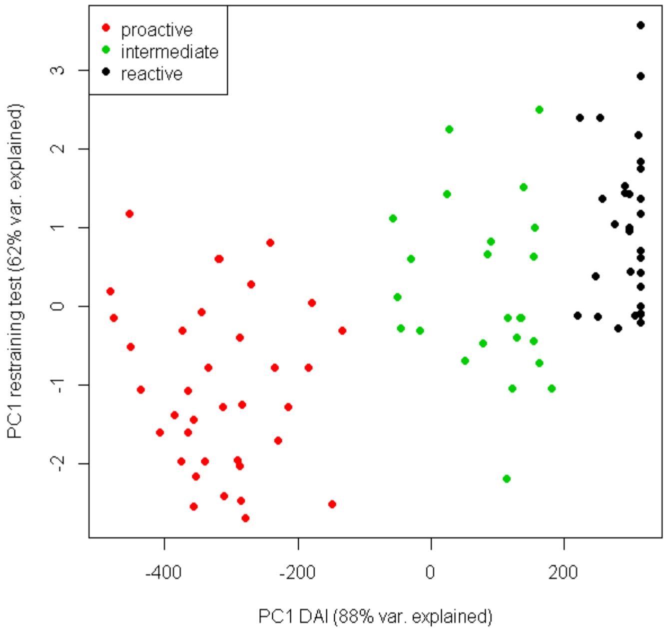

Figure 4.2.4 |Illustration of unsupervised K-means clustering and hierarchical clustering, using Ward's criterion, of the 3 groups segregated by DAI1 and DAI2 latencies, and the behaviours measured from NR collapsed into principal component scores. DAI latencies explained 82% of the fish dispersion, over 62 from the NR test. DAI latencies were chosen as behavioural measure to define the cut-off of the different personality groups. Both DAI latencies events were positively correlated (Pearson correlation r = 0.753, p < 0.001) and the cut-off thresholds were determined for each personality traits (proactive: 0 < DAI < 300; intermediate: 300 < DAI < 600; reactive: DAI > 600; N = 96). One-way ANOVA $F_{(2,91)}$ = 124.33, p < 0.001 (LSD post hoc: proactive vs intermediate, p < 0.001; proactive vs reactive, p < 0.001; reactive vs intermediate, p = 0.009).

Three groups were generated using determined DAI latencies cut-offs (proactive n = 30; intermediate n = 27; reactive n = 26) identifying a balanced population of personality traits based on swimming activity, ventilation frequency, repeated DAI events and the three variables taken from the restraining test. One of the clusters (which we categorized as proactive individuals) took less time to restart feeding (One-way ANOVA; *post hoc* Fisher LSD p = 0.04; p < 0.001) and had a lower number of opercular beats after being deployed in the new environment (*post hoc* Fisher LSD p = 0.02; p < 0.001) when compared with the other 2 clusters (intermediate and reactive, respectively). When compared with reactive individuals, proactive fish showed increased escape behaviour (*post hoc* Fisher LSD p < 0.001), increased swimming and exhibited lower levels of thygmotaxis (*post hoc* Fisher LSD p = 0.02). Differences between personality groups under the different behavioural tests are shown in Fig. 4.2.6. No differences in initial body weight, coefficient of variation of initial body weights and total biomass were observed between individuals ($F_{(2, 71)}$ = 1.215; p = 0.303).

Table 4.2.2 |Consistency of behavioural responses over time (DAI_1 and DAI_2) and across different behavioural paradigms (VF; NE; DAI (DAI latency; lat2move; FI) from both events (DAI_1 and DAI_2); NR (total escapes; time escape and lat2escape) in Nile Tilapia, depicted by Pearson correlation (r).

		VF	NE	DAI_1 lat	lat2 move1	FI_1	DAI_2 lat	lat2 $move_2$	FI_2	lat2 escape	time escape
NE	*r*	-0,26		-0,28	0,15	0,22	-0,17	0,05	0,15	-0,04	0,17
	p	**0,018**		**0,010**	0,185	**0,046**	0,123	0,653	0,165	0,747	0,124
DAI_1 lat	*r*	0,27	-0,28		-0,51	-0,71	0,35	-0,21	-0,47	0,16	-0,28
	p	**0,013**	**0,010**		**0,001**	**0,001**	**0,001**	0,054	**0,001**	0,158	**0,011**
lat2 $move_1$	*r*	-0,09	0,15	-0,51		0,60	-0,19	0,13	0,23	0,01	0,06
	p	0,441	0,185	**0,001**		**0,001**	0,080	0,226	**0,039**	0,919	0,564
FI_1	*r*	-0,19	0,22	-0,71	0,60		-0,30	0,16	0,42	-0,18	0,33
	p	0,080	**0,046**	**0,001**	**0,001**		**0,006**	0,156	**0,001**	0,097	**0,002**
DAI_2 lat	*r*	0,37	-0,17	0,35	-0,19	-0,30		-0,59	-0,71	0,15	-0,22
	p	**0,001**	0,123	**0,001**	**0,080**	**0,006**		**0,000**	**0,001**	0,179	**0,049**
lat2 $move_2$	*r*	-0,29	0,05	-0,21	0,13	0,16	-0,59		0,61	-0,18	0,14
	p	**0,008**	0,653	0,054	0,226	0,156	**0,001**		**0,001**	0,106	0,216
FI_2	*r*	-0,33	0,15	-0,47	0,23	0,42	-0,71	0,61		-0,17	0,17
	p	**0,002**	0,165	**0,001**	**0,039**	**0,001**	**0,001**	**0,001**		0,131	0,125
lat2 escape	*r*	-0,04	-0,04	0,16	0,01	-0,18	0,15	-0,18	-0,17		-0,66
	p	0,728	0,747	0,158	0,919	0,097	0,179	0,106	0,131		0,000
time escape	*r*	-0,19	0,17	-0,28	0,06	0,33	-0,22	0,14	0,17	-0,66	
	p	0,085	0,124	**0,011**	0,564	**0,002**	**0,049**	0,216	0,125	**0,001**	
total escapes	*r*	-0,20	0,27	-0,35	0,11	0,36	-0,20	0,11	0,27	-0,55	0,72
	p	0,073	**0,014**	**0,001**	0,340	**0,001**	0,073	0,333	**0,012**	**0,001**	**0,001**

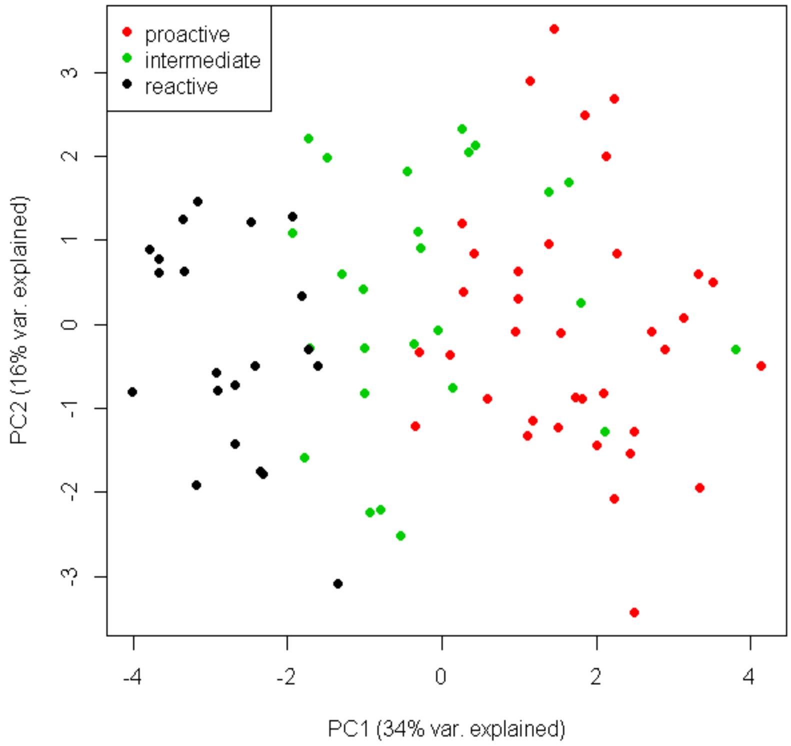

Figure 4.2.5 |Principal component analysis collapsing all behavioural parameters measured within the behavioural paradigms (swimming activity from NE; ventilation frequency from VF, DAI latency, time2move and FI from both DAI_1 and DAI_2; and the three variables taken from the NR [total escapes; time escape and lat2escape]). Fish are classified using predetermined DAI latency cut-offs. Both first and second component scores explained 50 % of the variance.

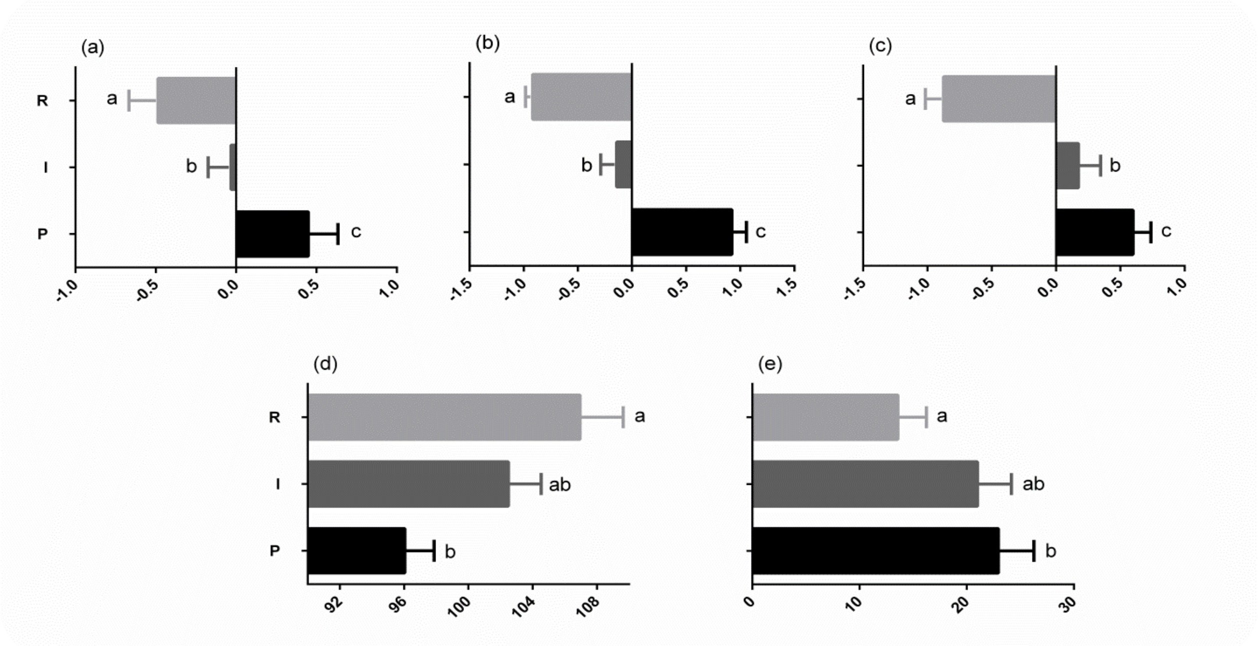

Figure 4.2.6 | Distinct clustered groups (P - proactive (n = 30), I - intermediate (n = 27) and R - reactive (n = 26)) generated after DAI_1 and DAI_2 latency measurements: a) Restraining ($F_{(2,80)} = 7.02$, $p = 0.001$); Individuals with low restraining scores escaped less during net restraining. b) PC1 DAI_1 ($F_{(2,80)} = 13.87$, $p < 0.001$); c) PC1 DAI_2 ($F_{(2,80)} = 15.22$, $p < 0.001$); Individuals with lower DAI scores took longer to restart feeding. d) VF: # opercula beats min^{-1} ($F_{(2,80)} = 7.40$, $p = 0.001$); Individuals with reactive traits showed higher number of opercula beats. e) NE: Swimming Activity % ($F_{(2,80)} = 2.67$, $p = 0.07$); Reactive individuals showed lower swimming activity levels. Fisher LSD post hoc is indicated by differing letters ($p < 0.05$).

Thermal restriction (T_R) vs. thermal choice (T_{CH}) : acute thermal period

Changes in acute thermal preference for each group during the first hour was assessed by counting the number of fish in each chamber at each minute (n = 60 events). The provision of *thermal choice* (T_{CH}) induced an immediate and significant change highlighting preference for the warmest chambers (chamber 4: T = 29.86 ± 0.02 ºC and chamber 5: 33.08 ± 0.02 ºC) in comparison to T_R conditions (Wald $\chi^2_{(4,1497)} = 148.447$ $p < 0.001$; interaction Chamber x Condition). Distribution under *thermal restriction* (T_R) condition was chamber dependent (Wald $\chi^2_{(4,897)} = 142.93$, $p < 0.001$) for all groups tested, indicating that in the absence of a gradient fish remain in the chamber they are introduced into.

Comparing T_R and T_{CH} control groups, latency to exit and number of transitions between chambers shows that fish became more exploratory when in a thermal gradient, with lower latency for chamber transitions and a higher number of transitions between chambers ($F_{(1, 3)} = 9.092$, $p < 0.001$ and $F_{(1,3)} = 193.727$, $p = 0.02$ respectively). For different personalities distributions across chambers under T_{CH} were not significantly different (Wald $\chi^2_{(2,2698)} = 16.996$, $p = 0.590$), however significantly different acute thermal preference were observed

(Kruskal-Wallis $\chi^2_{(2,\ 539)}$ = 7.675, p = 0.022). This is shown as the mean of the individuals at each time point (n = 60) for each chamber temperature (formula [1]). It is worth noting that this latter difference is strongly influenced by the thermal preference found for intermediates (T = 30.28 ± 0.18 ºC) that was lower than the measured proactive preference (T = 31.10 ± 0.15 ºC; Mann-Whitney, U = - 2.579, p = 0.01). No differences were found between intermediate and reactive groups (T = 30.88 ± 0.15 ºC; Mann-Whitney, U = - 1.854, p = 0.06) or between proactive and reactive groups (Mann-Whitney, U = - 1.225, p = 0.22). Latency to exit and number of chamber transitions did not reveal any significant differences ($F_{(2,33)}$ = 0.357; p = 0.702; $F_{(2,33)}$ = 0.142; p = 0.868) during the first hour of acclimation to the thermal gradient.

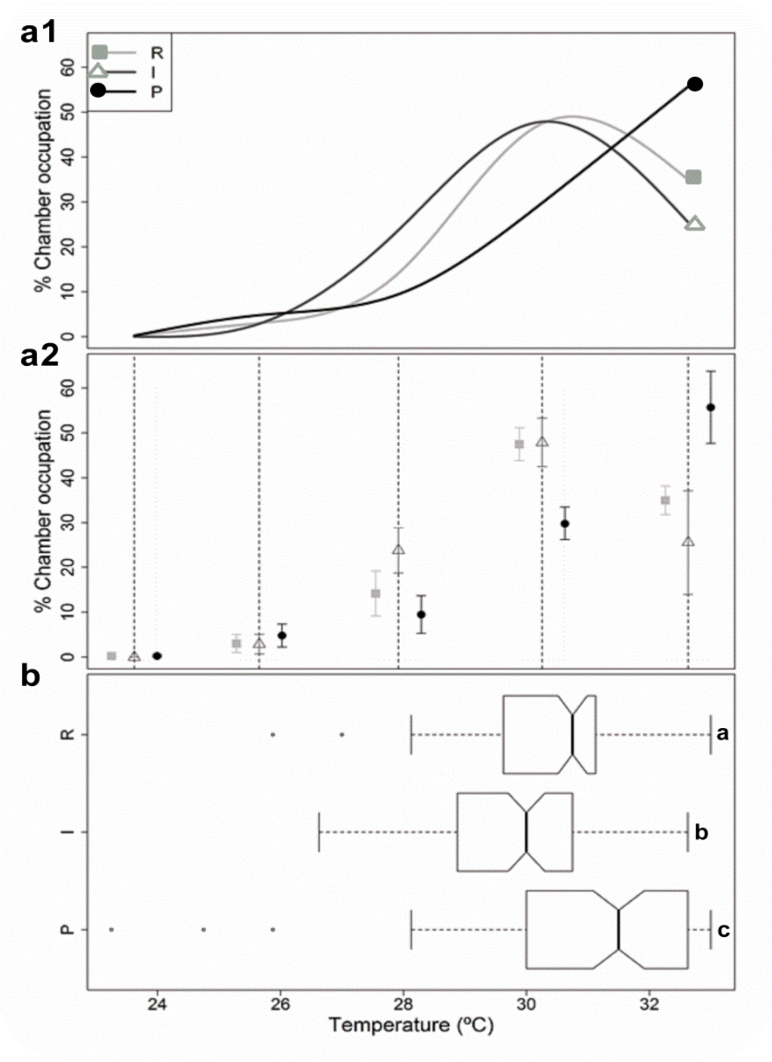

Figure 4.2.7 |a1) Nonlinear fit of fish, proactive, reactive and intermediate, distribution across the thermal gradient, a2) mean + SEM of each group after 48 hours, b) Final thermal preference for each of the personality group tested (mean ± SEM: Proactive, 31.32 ± 0.18 ºC; Intermediate, 30.37 ± 0.14 ºC; Reactive, 29.88 ± 0.12 ºC); Different letters indicate significant pairwise differences (Mann-Whitney U; a,b: $p < 0.05$; a,c: $p < 0.05$; b,c: $p < 0.01$).

Thermal preference and personality

Analysis from the final thermal period showed that proactive, intermediate and reactive groups expressed significantly different final thermal preferences (Kruskal-Wallis, $\chi^2_{(2,296)}$ = 40.779, $p < 0.001$; Fig. 4.2.7a1 and a2) with proactive fish displaying preference for higher temperatures (95 % confidence interval for median (31.1 - 32 ºC) mean T = 31.32 ± 0.18 ºC) and reactive fish lower temperatures (95 % confidence interval for median (30.5 - 31 ºC) mean T = 30.37 ± 0.14 ºC). Interestingly, some intermediate individuals showed preference for even lower temperatures (95% confidence interval for median (29.7 - 30.3 ºC) T = 29.88 ± 0.12 ºC) although the pattern of distribution in the thermal gradient was similar to reactive groups (Wald $\chi^2_{(1,1485)}$ = 3.466 p = 0.065) (Fig. 4.2.7b) and significantly different to proactive fish (Wald $\chi^2_{(1,1485)}$ = 8.292; p = 0.004). Interestingly results show that naïve control groups under T_{CH} conditions preferred temperatures above those experienced during rearing (T_{optima}= 30.54 ºC vs. 27 ºC).

Validation of thermal preference as a screening paradigm

Without any previous behavioural screening 8 groups of naïve individuals (n = 64), were tested under T_{CH} conditions. The population distribution in the last 8h over 48h test was significantly higher for chamber 4 as previously observed (T = 31.19 ± 0.07 ºC) (Wald $\chi^2_{(4,1277)}$ = 26.781; $p < 0.001$)(Fig. 4.2.8a).

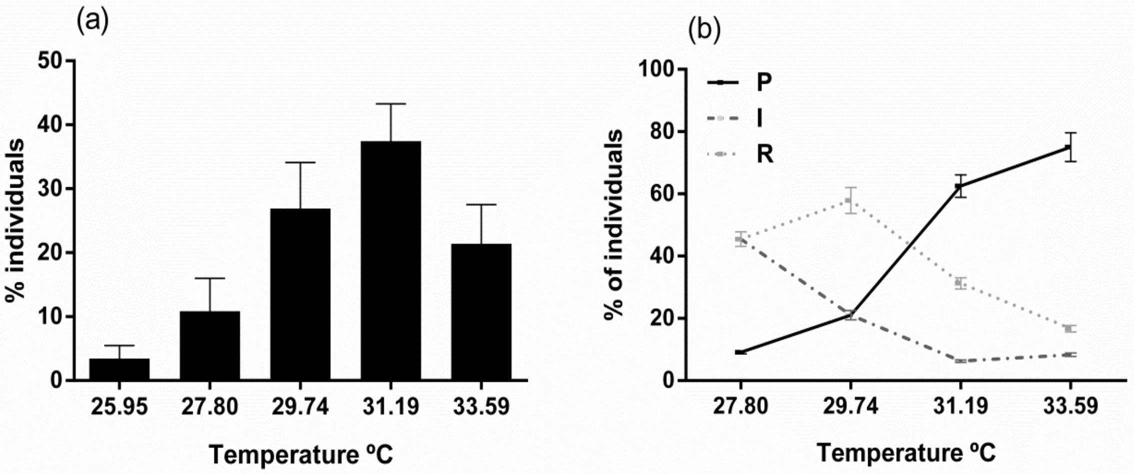

Figure 4.2.8 |a) % of individuals, no behavioural pre-screening, in each chamber of the thermal gradient tank after 48 h (n = 64, % mean ± SEM), b) % of individuals of each personality in each chamber, fish were screened for personality post-thermal distribution.

A posteriori behavioural screening of this population identified that 70% of the fish found in 31.19 ± 0.07 ºC - 33.59 ± 0.10 ºC exhibited proactive traits. Intermediate and reactive individuals were identified in chambers with temperatures ranging between 27.80 ± 0.07 ºC - 29.74 ± 0.05 ºC (Fig. 4.2.8b). In parallel 3 control groups with 8 naïve fish were tested in the T_R environment (T = 26.58 ± 0.35 ºC) to evaluate the possibility of chamber preference independent from temperature. A homogeneous distribution was observed throughout the tank (Wald $\chi^2_{(1,475)} = 65.933$ $p > 0.05$) with no significant differences in preference.

Behavioural fever in animal personalities

Using a previously established *S. iniae* infection model we tested the behavioural fever response (under T_{CH} conditions) of proactive, reactive and randomly selected individuals using an intra-peritoneal route of infection (Table 4.2.1, Fig. 4.2.9). Throughout the 5 days of bacterial challenge no abnormal swimming behaviour was observed in any experimental group. Control, i.p. injected with PBS 0.1 %, individuals did not present any mortality throughout the experimentation. In the proactive group mortalities were observed with 3 dead fish at 2 days post-infection and in reactive fish a single mortality was recorded at 1 day post-infection. In the first hour of acclimation to the T_{CH} conditions differences in thermal preference were found between the groups tested (proactive T = 27.95 ± 0.05 ºC; reactive T = 29.17 ± 0.18 ºC; control T = 31.94 ± 0.58 ºC; Kruskal-Wallis $\chi^2_{(2, 179)} = 129.6$, $p < 0.001$) with both personality groups showing a decrease in acute thermal preference.

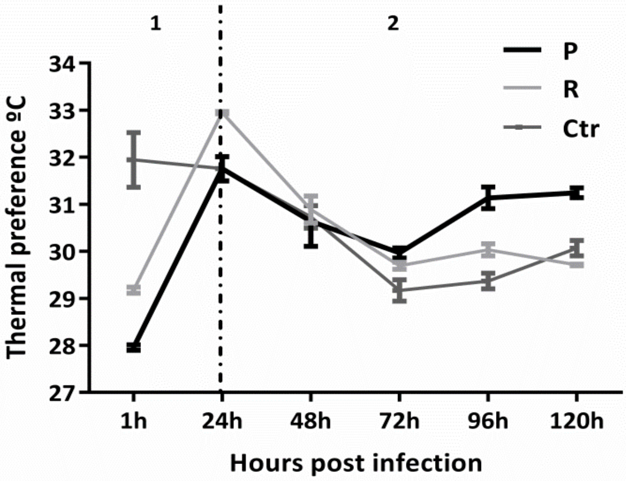

Figure 4.2.9 |Thermal preference (°C) of *S.iniae* challenged individuals after the first hour of deployment post-infection (acute thermal period) and over the first 8 hours of each day throughout the trial (naïve fish (Ctr); reactive fish (R) and proactive fish (P). Final thermal period within infection period are shown between 48 and 72 h post-infection. Final thermal period regarding personality group preference is shown after 120 h post-infection.

At 24 h post infection, a behavioural fever response was evident for both groups with reactive individuals showing a significantly altered temperature preference (proactive T = 31.75 ± 0.25 ºC; reactive T = 32.94 ± 0.06 ºC; Mann-Whitney, U = 43.50; $p < 0.001$). Over the next 48 hours both groups showed a decrease in thermal preference with no significant difference between thermal preferences for the personality groups and the control group (proactive T = 30.63 ± 0.23 ºC; reactive T = 30.88 ± 0.28 ºC; control T = 30.73 ± 0.24 ºC; Kruskal-Wallis $\chi^2_{(2, 98)} = 1.800$, $p = 0.407$). However at 4-5 days thermal preference returned to the previously measured final thermal preference of each group (Fig. 4.2.9: proactive: Wilcoxon rank test: Z = -3.510; $p < 0.001$; T = 31.13 ± 0.10 ºC; reactive: Z = - 4.621; $p < 0.001$; T = 30.03 ± 0.12 ºC; control: Z = -5.012; $p < 0.001$; T = 29.36 ± 0.16 ºC).

Discussion

The linkage between thermal preference and animal personality is a relatively new research paradigm. To the best of our knowledge few studies have used similar approaches to assess personality in fish (Killen, 2014; Rey et al., 2015) or other vertebrate species. In Tilapia housed in a freely accessible thermal gradient, spanning approximately 12 ºC, proactive individuals showed preference for higher temperatures, as compared with reactive individuals. Testing was carried out by both pre-screening the fish for animal personality prior to thermal preference testing and vice versa. To mimic natural conditions, fish should be capable to express their full behavioural repertoire under artificial environments. Giving control or some sense or control can provide to fish the opportunity to activate proper coping mechanisms and minimize the effect of a punishment avoidance situation (normally the effect of standard behavioural paradigms to screen fish) e.g. by escaping, avoiding, moving or defend themselves against it. This allowed us to accurately validate the potential of thermal preference as an indicator of animal personality in mobile aquatic ectotherms. Additional experiments using bacterial infection further highlighted the importance of thermal choice at an individual level. Our results are of significant importance to understand the adaptive meaning of animal personality regarding ecological performance within a population.

Driven by the statement of Bell et al. (2009) in which biologically meaningful variability is conditioned by consistency of individual patterns, we demonstrate the repeatability of individual patterns over time (DAI_1 and DAI_2) and across different situations. Our results show divergent personality traits within our population with proactive individuals being characterized by a faster feed intake recovery after transfer into a novel environment, lower breathing frequency and being more prone to escape from restraining, as compared with reactive individuals. The existence of "intermediate" individuals is common when working with domestic species due to low environmental challenge (Boersma, 2011). Despite the extensive range of behavioural methodologies used, different suites of personality traits have been observed in many animal species (Briffa et al., 2013; Montiglio et al., 2014) and multiple fish species including the olive flounder, *Paralichthys olivaceus* (Rupia et al., 2016), Mulloway Argyrossomus japonicas (Raoult et al., 2012), Gilthead Seabream *Sparus aurata* (Herrera et al., 2014), Senegalense sole *Solea senegalensis* (Castanheira et al., 2011; Martins et al., 2011c), European seabass *Dicentrarchus labrax* (Killen et al., 2012) and Zebrafish *Danio rerio* (Rey et al., 2015). In the case of Tilapia the strong correlations between DAI and personality highlight the usefulness of Duration of Appetite Inhibition as a screening tool for boldness. Our results also support the use of Ventilation frequency (VF) as an indicator of behavioural-physiological traits by showing that VF is a good tool for predicting feeding behaviour in the same context. The same positive correlation between VF and DAI were also found by Barreto and Volpato (2011). In summary, such behavioural responses have also been described in other fish species (MacKenzie et al., 2009; Martins et al., 2011c; Overli et al., 2002; Silva et al., 2010) corroborating the idea that by choosing and tailoring behavioural tests different behavioural profiles can be accurately identified.

Acute thermal period

Fish exhibited significantly different distributions over T_R and T_{CH} in agreement with our predictions based around previous observations in zebrafish (Rey et al., 2015). Fish were able to see between chambers and in the absence of an environmental motivation such as a thermal gradient (T_R) fish displayed strong shoaling behaviour. An environmental enrichment, such as the gravel on the bottom of the tank, might have made individuals inhibit threat-sensitive behaviour such as exploration, risk taking or foraging and to reduce stress (Galhardo et al., 2008). In contrast under TCH conditions control fish distribution was strongly affected and the thermal gradient stimulated increased exploratory activity between chambers. Thus the fish are able to detect environmental thermal variation which could be linked to how individual fish appraise such changes (Martins et al., 2011a). Perception of positive environmental factors combined with the possibility to control phenotypically such environmental condition (e.g. by swimming through the experimental tank) is known to have a positive impact on animal welfare (Greiveldinger et al., 2009). The change in swimming patterns and distribution over the first hour of test might be the result of a trade-off between the costs and benefits associated with thermoregulation (Shine and Madsen, 1996). Differences in swimming activity regarding exploration have previously been reported (Careau et al., 2008) as an intrinsic pattern of personality traits (Reale et al., 2007). Several findings have shown that individual metabolic patterns may be related to different behavioural traits in animals (Biro and Stamps, 2008; Careau et al., 2008; Herrera et al., 2014). This behavioural and physiological covariation has been previously hypothesised in which personality types mirror variation in metabolic patterns (Biro and Stamps, 2010; Careau and Garland, 2012; Metcalfe et al., 2015). Alternatively the impact of the effects of stress on thermal preference due to emotional fever could also explain the change in fish distribution with naïve animals experiencing higher stress responses to the novel tank environment (Rey et al., 2015).

Temperature preference predicts personality – final thermal period

As stated by Jobling (1981) if given enough time fish will congregate at the final thermal preferendum, which is deemed to correspond to the T_{optima} at which fish growth fast. In Nile Tilapia the reported T_{optima} is of 27 - 33 ºC (Azaza et al., 2008; Azaza et al., 2010). Our results agree with this thermal range corresponding to the final thermal preferendum (Fry, 1947). When fish were screened posteriorly for personality our results confirmed an increased final thermal preference in proactive fish in comparison to both other groups tested. Therefore thermal preference can be used as a physiological paradigm to screen fish for personality and provides an important insight into individual variation. Our results are in agreement with previous studies in zebrafish (Rey et al., 2015). Thus, the higher end of temperature preference for the species may reflect increased energetic requirements in proactive individuals with a higher metabolic engine. Interestingly, Blackmer *et al*. (2005) suggested that increased energy requirements in proactive individuals is compensated by swimming behaviour i.e. displacement to warmer places, another component of the energetic repertoire. Such association between metabolic rate and behaviour leading to elevated energy expression in proactive individuals has been previously reported (Biro and Stamps, 2010; Careau et al., 2008). Despite the apparently small difference between thermal preferences for each personality type (about 1 ºC), it has a relevant ecological impact as metabolic rates from proactive individuals increase by >10 % (Clarke and Johnston, 1999) when compared to reactive conspecifics. Thermal preference could be seen as a coadaptation of natural selection being partially explained as a trade-off between inherited behavioural predispositions and physiological demands (Korte et al., 2005). Thus in mobile ectotherms including fish, we propose that thermal preference is correlated to animal personality and understanding how individuals use thermal gradients will aid in our understanding of how individuals in a population optimize and adapt performance and fitness in different environmental scenarios.

Behavioural fever in animal personalities

Under infection, proactive and reactive individuals showed similar thermal strategies to combat bacterial invasion (Fig. 4.2.10). These results extend previous observations describing the impact of thermal choice upon the immune response (Boltana et al., 2013). The implications of animal personality and thermal choice in response to *Streptococcus iniae* infections in Nile tilapia have not been previously reported. *Streptococcus iniae* is a significant pathogen globally affecting Nile Tilapia aquaculture (Eldar et al., 1997; Shoemaker et al., 2001) and has been shown to positively correlate with increased environmental temperature (Bromage and Owens, 2009). Behavioural fever is defined as an acute change in thermal preference driven by pathogen recognition and referred as a synergic signal to improve the immune response of fish (Boltana et al., 2013). To our knowledge these are the first experiments reporting upon the relationship between bacterial infection, behavioural fever and personality in mobile ectotherms. Behavioural fever induced by bacterial infection was originally reported in carp (Reynolds et al., 1976) and few studies have followed upon these original findings. In this experiment we show that infection induces behavioural fever in all fish that were challenged highlighted by the peak thermal response at 24 h post-infection. Initial measures at 1 h post-intra-peritoneal injection highlight differential stress responses where naïve control fish display a strong emotional fever response (Rey et al., 2015). In the other experimental groups this response is not observed likely due to previous habituation to handling during behavioural phenotyping and thermal screening in the gradient environment. Of particular interest is the observation that the host response to infection overrides personality-driven thermal preferences until the infection is cleared. In this experiment no mortalities were observed after 48 h of infection. At 4 days post-infection thermal preferenda were restored as predicted by personality. As stated by Frost *et al.* (2013), natural environments often inflict abiotic challenges on populations. With the threat of climate change and diverse anthropogenic influences, the study of the physiological traits here highlighted by host-pathogen interaction in which thermal choice influences performance, understanding individual response within a population framework becomes critical to predict impact and survival.

Data Accessibility

Data are available on *Figshare*: http://dx.doi.org/10.6084/m9.figshare.3114967 (Cerqueira *et al*. 2015).

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General Discussion, Conclusions and Future perspectives

Chapter V

General Discussion

The aim of the present thesis was to uncover if the appraisal that important farmed fish make of environmental stimuli modifies their behavioural and physiological responses and its implications for their welfare. To pursue this end, different issues were addressed and are further discussed:

I. Characterize distinct personality traits in important commercial farmed fish;
II. Demonstrate the appraisal capability of the target species;
III. Manipulate psychologically the appraisal of environmental stimuli by means of its modulators, such as predictability, pleasantness, social support or controllability;
IV. Assess the influence of personality traits on the appraisal of environmental stimuli;
V. Evaluate the proximate states underlying stimuli appraisal in fish.

From the research conducted on chapter II, it can be taken that fish manifest capability for environmental appraisal, and the use of appraisal components as predictability, pleasantness, social support and controllability (chapter III and IV) are demonstrated to modulate the stress response of fish to environmental challenges. Moreover, such cognitive tools are shown to make animals more resilient to stress by altering how the individuals have experienced their environment; by promoting more positive affective states. Despite the evidences of distinct personalities within the focal population, the link between personality and the appraisal of environmental cues have not been clearly determined. Nevertheless, some evidences are demonstrated in fish to whom have being offered conditions to cope with the stressor (chapter IV), then, indicating the direction to improve fish selection programmes, fish welfare and the sustainability of fish farming.

5.1 Characterization of distinct personality traits in important commercial farmed fish

Studying fish behaviour is both engaging and challenging at the same time, given the large repertoire of fish responses towards the dynamic nature of their surrounding environment. In addition, as previously mentioned, different species have different needs and innate reactions, expressing different behaviours or physiological responses under similar conditions. Such responses, known to be species- and context-specific, are also recognized as being shaped by the individuals' inherent predispositions, which are loaded by their inner biological and ecological features. Distinct behavioural strategies are now well recognized and named as personality traits in fish (see Castanheira et al., 2015 for more information). Knowing and understanding this individual variability in behavioural and physiological strategies within and between species is effectively of extreme importance to understand and maintain fish welfare within aquaculture practice. Within the core of this dissertation, screening the focal fish population by their distinct behavioural strategies was used to better understand the adaptive variation in the stress response to different appraisal modulators (i.e. predictability, pleasantness, social support and controllability).

An important remark is made here, whether by strategic/budget issues*[1] (chapter III-1, III-2 and IV-1) or by lack of evidences of the effect of personality on our data (chapter II-1), the use of personality as an co-factor underlying the behavioural, physiological and neural expression of environmental appraisal was not taken into account on the referred chapters. However, the characterization and identification of distinct personalities of the focal fish was performed in line with the core of this dissertation and are discussed in detail further down.

5.1.1 Behavioural and physiological paradigms to assess personality on the focal fish

As referred, the work described in this dissertation occurred within a European project with seabream and seabass as two of the main target species. In agreement, the establishment of non-invasive methods for a reliable identification of contrasting personality traits in seabream and seabass was already in progress. Briefly, such research was sustained on three features underlying personality identification and characterization: i) inter-individual consistency i.e. variation in behaviour among individuals of a same group or population; ii) intra-individual consistency across context i.e. intra-individual behavioural responses correlations across distinct contexts; iii) intra-individual consistency across time i.e. repeatability of consistent correlated behavioural responses over time (Sih et al., 2015; Wolf

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*[1] Using personality as co-factor would have implied doubling the costs with neuromolecular expression and the time needed to process all the fish brains. Taking that in consideration, it was decided to remove such factor from the research presented on chapter III-1, III-2 and IV-1.

and Weissing, 2010). Taking this in mind, different behavioural paradigms were used in that research, both individual and group-based (e.g. feed intake recovery, novel object and restraining; risk-taking and hypoxia test, respectively) to assess behavioural consistency across contexts and over time. The findings of such research determined that the restraining and risk-taking paradigms on seabream, and restraining and hypoxia on seabass were the tests that showed the most consistent results over time and across contexts (Castanheira et al., 2013; Ferrari et al., 2015). Taking such findings in consideration and the know-how regarding the set-up needed to do such work, the restraining test was chosen to demonstrate and quantify the presence and consistency of individual variation in personality on both seabream and seabass. Furthermore, driven by the absence of the effect of personality on the research conducted on chapter II-1, it was decided to not only repeat the restraining (one week apart from each other), as well as to use a second paradigm to assess distinct behavioural strategies across contexts in seabream.

As such, for the research conducted on chapter III-1, besides repeatable restraining events, also individual feeding motivation (i.e. individual feed intake and latency to start eating within the group) within a social context, was assessed. Those behaviours were taken during the acclimation period of the effective trial, measured from two daily meals and during the for four days after being deployed on the experimental tank and from the four days after being subjected to blood sampling, known to be an acute stress for fish (Arends et al., 2009). On chapter IV-1, only once the restraining test was performed based on the consistency found between events on chapter III-1. Additionally, both feed intake recovery test (i.e. time to restart feeding after being settled in the experimental tank; see chapter IV-2 for the description of this test) and risk-taking (i.e. the time fish took to escape from the net confinement during the training sessions for the fish tested under CTR) were used (see table 5.1 for summary). Current research strategies resort to the use of both individual- and group-based tests to assess fish behavioural strategies; the latter can, besides validating consistency across contexts, offer more realistic outputs of divergent personalities (as fish, as social beings, are normally shoaling individuals).

In seabass tested on chapter III-2 and IV-1, the same approaches were used but feed intake recovery was shown to be unsuitable, most probably due to the species ecological features: seabass is a known gregarious species and highly stress sensitive (e.g. Ferrari et al., 2015). In such cases, unlike seabream, that immediately reacts to food dropping, seabass only reacts after several minutes, sometimes even between 1-2 hours, making this methodological approach inappropriate. Seabass also showed higher levels of fearfulness-reactivity in restraining tests (displaying higher latency to escape, lower number of escape attempts and lower escaping time than seabream of homologous personality), supporting the assertion of high stress sensitiveness for this species. Moreover, higher time needed to recover appetite was evident during and after any manipulation, including anaesthesia, restraining or transport (e.g. seabream immediately restart feeding after the settlement on the experimental tanks, while seabass only respond to the food drop after the third day). In addition to behavioural expression, physiological traits of seabass sustain their relatively high susceptibility to stress, given their basal levels of cortisol are usually higher than those found in other fish species, including seabream (Rotllant et al., 2003). In conformity with the impossibility of using the feed recovery test, activity of fish measured during the first five minutes after being deployed in the experimental tanks (aka open field test) were also measured in the work described in chapter III-2 (see table 5.1 for summary).

The research performed with Nile tilapia, known by their suitable biological and ecological features to behavioural studies, has indirectly benefited from the know-how acquired during the seabream and seabass trials and was used to complement the research findings. Within the research conducted in chapter IV-2, an initial battery of individual-based tests were used (i.e. restraining test, novel environment test (or open field test), feed intake recovery (performed twice 24h apart) and ventilation frequency) to validate thermal choice as group-based test to identify divergent traits (see chapter IV-2 for more information). In this sense, fish were screened and scored according to behaviours expressing fearfulness or exploration (e.g. escaping behaviour, swimming activity, ventilation frequency and feed intake recovery) reported as dimensions of animal personality (Reale et al., 2007; Réale et al., 2010). The existence of distinct personalities in Nile Tilapia wild-type is well acknowledged (Barreto and Volpato, 2011; Martins et al., 2011a; b; VeraCruz et al., 2011), however it has been centred on individual-based tests, and as previously referred, are highly demanding. As far as i know, only recently the characterization of Nile tilapia personality has been performed using group-based tests. Mesquita et al. (2016) performed the screening based on the time, fish, kept in groups, took to leave from a shelter. Nevertheless, the development of a methodology focused on fish physiological preferences can be more attractive and promising to decrease the stress involved and to allow a more accurate distinction between divergent traits (from my point of view, freedom for choice, as for humans (Kim and Sherman, 2007), holds great psychological significance).

Table 5.1 |Summary of the tests used to characterize distinct personality trains in the focal fish; (+) test used; (++) test used twice; (-) test not used.

	Restraining	Risk taking	Feed recovery	Ventilation frequency	Open field test
Chapter II-1	+	-	-	-	-
Chapter II-2	+	-	-	-	-
Chapter III-1	++	-	+	-	-
Chapter III-2	++	-	-	-	+
Chapter IV-1	+	+	+	-	-
Chapter IV-2	+	-	++	+	+

5.1.2 Behavioural and physiological expression characterizing distinct personalities

As expected, divergent behavioural strategies were identified on all the focal species, with individuals being classified as proactive or reactive (described only on chapter II-2 and chapter IV-2 in seabass and Nile tilapia, respectively). As referred above, the existence of such strategies in behaviour were for numerous times shown on the same species. Overall, fish presenting higher risk-taking (lower latency to escape, higher number of escape attempts and time doing it, either from restraining or from confinement), higher willingness to explore and tendency for lower feed intake recovery time, lower ventilation rates and higher thermal preferences were characterized as proactive individuals. On contrary, fish with low predisposition for risk-taking, lower exploration, higher time for feed intake recovery time, higher ventilation rates and lower thermal preferences were characterized as reactive individuals. Individuals with an intermediate behavioural pattern (“intermediates”) were identified and used in these experiments since it allowed to tune the separation of both extremes on behavioural strategies from the focal population. This supports this line of research, enabling a more accurate inference on how personality underlies fish learning and environmental perception (see chapter IV-2 discussion to more information regarding intermediates).

Such differentiation on behavioural and physiological strategies follows a number of studies on different fish species, such as trout, salmon, sticklebacks, catfish , flatfish, zebrafish and other (reviewed in Castanheira et al., 2015 and Sih et al., 2015), where similar individual variability was reported. In conformity, differences in personalities assessed over time and across contexts sustain the principle that strong genetic backgrounds shape how fish interprets, reacts, adapts or adjusts itself to the surrounding environment, based on their beliefs, needs, motivations, affective states and on their environment (Conrad et al., 2011; Reale et al 2007; Sih et al., 2015). Furthermore, some insights regarding the physiology underlying the divergent behavioural expression of different personalities could be obtained from the research conducted on chapter IV-2 (see table 5.2), taking into account the pre-screening made by the restraining and ignoring the effect of each of experimental conditions tested. As such, proactive individuals showed a weaker activation of the HPI axis, resulting in a lesser increase of plasmatic cortisol levels after a stress compared to reactive fish. Such finding is in line with the personality traits model in which individuals with a slow pace of life (reactives: less exploratory, less activity, low risk-taking) express higher blood cortisol levels than those with an active response to stress (proactive: more exploratory, more active and higher predisposition for risk-taking) (Réale et al., 2010). Again, due to their higher stress sensitiveness, the cortisol levels of seabass, between homologous personalities, were higher than those reported for seabream. Indeed, between homologous tests (i.e. net restraining) the same physiological range was found between both species (Castanheira et al., 2013; Ferrari et al., 2015).

Table 5.2 | Proactive, intermediate and reactive plasmatic cortisol concentrations (ng ml^{-1}; mean ± sem) of both seabream and seabass measured 30 min after being subjected to a turning on of a light.

	Seabream	**Seabass**
Proactive	50,46 ± 6,17	168,51 ± 9,75
Intermediate	70,54 ± 7,03	178,17 ± 9,81
Reactive	78,99 ± 6,39	221,06 ± 10,55

5.1.3 Consistency and repeatability across and over time

Taking in consideration the experiments where the consistency in coping strategies was evaluated i.e. where more than one test was used or performed twice (from chapter III-1 onwards), the results showed that both seabream and Nile tilapia demonstrated consistency in the coping strategies adopted across context and over time (see chapter IV-2 for correlations in Nile tilapia). In seabream feeding motivation, individual consistency over time and across stressful events (novel environment and blood sampling) was found. The fish that ate more and that took more time to respond to food, after being settled into a new environment were also the fish that ate more and took more time to respond to food after blood sampling stress (see Fig. 5.1 for the results found on chapter III-1 as an example). Interestingly, individuals exhibiting pre-categorized proactive characteristics (higher restraining scores) also presented longer time to recover feed intake. These results are in contrast with Overli et al. (2006) Martins et al. (2011a) outcomes showing a quicker recovery of feed intake in proactive as compared to reactive fish. Nevertheless, other studies showed the opposite response (LeBlanc et al., 2012; Ruiz-Gomez et al., 2011), including seabream (Castanheira et al., 2013).

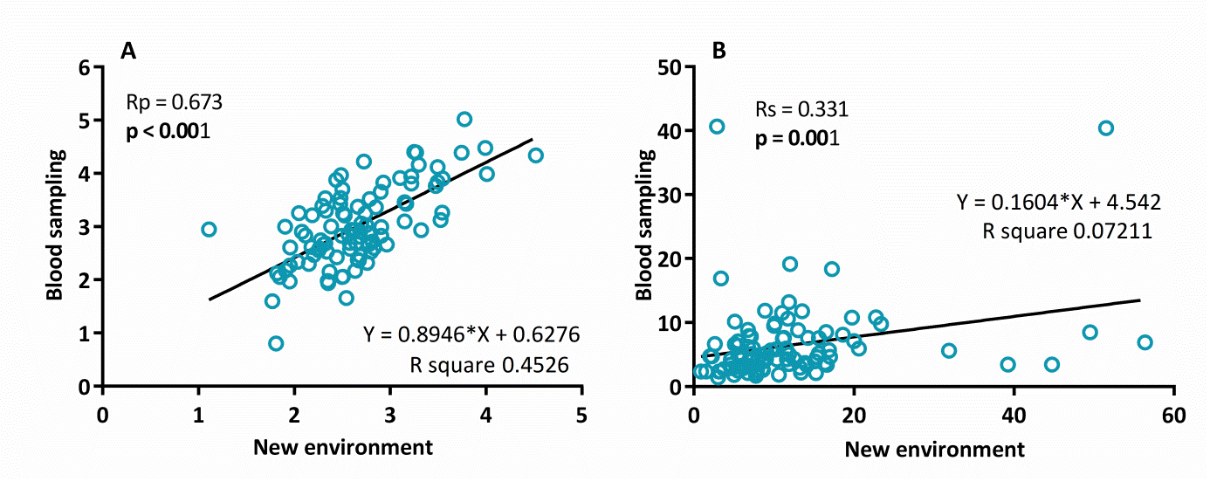

Fig. 5.1 | Consistency of individual fish feeding motivation (N = 96) measured between different stressful events (new environment and after blood sampling): A - Feed intake % Bw day^{-1} (arcsin transformed); B - Latency (s) to recover appetite (log transformed); Rp - Pearson correlation; Rs - Spearman correlation.

We have to point out that in this experiment the groups were organized by homogeneous personalities (i.e. four individuals with the same trait) therefore, it might be possible that reactive fish were compensating the previous social environment on the stock tank (with mixed personalities). Supporting this, a negative correlation was found between restraining and feeding recover on chapter IV-1 where groups with heterogeneous personalities were used. As such, having into account chapter III-1 results on feeding motivation, it can also suggest that reactive, by being more flexible to changes in the environment (Ruiz-Gomez et al., 2011), could have adapted faster after settlement in the experimental tanks. Again, the effect of social context over the expression of personality is apparent. Between repeatable restraining events, a consistency was also found, with fish showing higher restraining scores in the first event also being the fish that showed higher scores in the second event, which is congruent with the results found by Castanheira et al. (2013).

The link between cortisol levels and personality has been reported in several fish species. Nevertheless, the absence of consistency between events in seabass in chapters III-2 and IV-1 suggest that care should be taken when interpreting the results of chapter II-2, since the correlation between restraining scores and cortisol levels is based on only one behavioural test. The lack of consistency over time and across context in seabass (see table 5.3) is in conformity with the study of Ferrari et al., (2015), developed in the same species with homologous behavioural paradigms. Given the gregarious nature of seabass and its susceptibility to stress, social isolation may therefore be highly stressful in this species (Ashley, 2007). It remains unclear how to properly assess personality in seabass as cross-correlations were not found. Further investigations are therefore required to solve such question. In my opinion, to reduce the dichotomy between the test and the species ecological specificity, such way is to address group-based tests as hypoxia, recently reported to be more efficient to differentiate personalities in seabass (Ferrari et al., 2015).

Table 5.3 | Consistency between the behavioural strategies taken by seabream and seabass, across context and over time; Rp – Pearson correlation; Rs – Spearman correlation. Significant correlations are highlighted

	Restraining	**Feed intake PC1scores**	**Latency to eat (s) PC1 scores**	**Risk-taking (s)**
Seabream				
Chapter III-1				
Restraining	Rp = 0,356; n = 96 **p = 0,05**	Rp = -0,075; n = 96 p = 0,470	Rs = 0,273; n = 96 **p < 0,001**	
Chapter IV-1				
Feed recover	Rs = -0,501; n = 72 **p < 0,001**			Rs = 0,215; n = 24 p = 0,142
Risk-taking	Rs = -0,004; n = 24 p = 0,977			
Seabass				
Chapter III-2				
Restraining	Rp = 0,131; n = 71 p = 0,252			
Chapter IV-2				
Open field	Rs = 0,101; n = 71 p = 0,402			

We have seen, in agreement with previous research (Beckmann and Biro, 2013; Biro, 2012; Burns, 2008), that the test chosen should be adapted to the inner traits of each species, which are most likely to differ from each other. Therefore, we argue that standardization, regarding the tests to be used on the characterization of personality, should not be the focus of driven-research; rather it should be given more emphasis to the minimal number of tests considering either the consistency across situations and the repeatability of behavioural strategies over time within the same paradigm. Nevertheless, the conditions in which the tests are executed should be standardized, since it is been reported numerous factors shaping the individuals personality (see chapter I for summary). Such factors affecting personalities lead us to maintain that, to improve the welfare of farmed fish, aquaculture rearing strategies have to be adapted to different coping strategies, adjusted to the different stages of development. Nevertheless, it is clear that the development of proper paradigms to assess personality, known to be highly species-specific should be grounded on suitable research. In this sense, researchers should always be focusing on the development of simple, practical and fast methodological approaches, with straightforward results, to enable farmers with minimal expertise to identify fish with distinct personalities.

5.2 Demonstrating appraisal capability in the target species

Within the field of aquaculture, the recognition that fish are sentient beings and capable of affective experiences (e.g. expressing symptoms of stressful states or of good welfare) is at the core of the driven-research concerning fish farm welfare. Situations normally associated with stress (e.g. in a fish farm: non-adapted housing conditions, pain or injury due to husbandry procedures, handling, constraining, poor social environment or inadequate nutrition) that do not cause obvious physical harm may be affecting the individual’s welfare. If one accepts that fish welfare results from their capability to effectively experience changes in their environment, then understanding how they experience their quality of life based on their affective states become essential. Accordingly, the development of tools to assess and answer how fish appraise their surroundings by assessing their behaviour and physiology is fundamental since affective states or cognition skills are not directly observable.

The research conducted in chapter II addressed such issue and intended to show if the two most important commercial fish species of southern Europe respond in the same manner to the valence of divergent stimuli using CPP/CPA tests to assess motivational states. Stimuli-valence was reported as the core building blocks of emotional experience correlated to specific brain states, supporting adaptive functions as avoidance or approaching behaviours. Nevertheless, it should be highlighted that the authors do not intended to show how conscious are fish motivations, reactions or preferences, but rather to provide insight on how differently they perceive their environment, if more or less comfortable or more or less adequate to their expectations. Indeed, both seabream and seabass have shown valence-specific responses (preference vs. avoidance, respectively), induced by an environmental cue, according to the prospect of reward or punishment. After being exposed to rewarding or aversive conditions in different areas of a fish tank, marked with different background patterns, fish avoided the area experienced as negative and preferred the area experienced as positive, thereby showing their subjective experience. As such, these results suggest that, by increasing the environmental complexity and variability (i.e. context loss and unpredictability), single environmental cues have become no longer informative. By mismatch the mental preferred side of the tank by stimuli presentation on the second trial day, distinct responses between the adaptation day and the test day suggest that the evolution of appraisal mechanisms, that cognitively assess the presence of threats and opportunities in the environment, have been projected (Fawcett et al., 2014; McNamara et al., 2013). Additionally, specific-valence behaviour and physiology (i.e. aversive vs. appetitive stimuli) enable inference about the affective states supporting individual's adaptive responses, since, as stated by Anderson and Adolphs (2014), the occurrence of stereotypic behaviours are associated with internal brain states elicited by specific stimuli and probably encoded by the animal affective states. Such results, despite the differences in the methodological approach, are in accordance with several other studies in zebrafish (Lau et al., 2006; Zala and Määttänen, 2013) , rainbow trout (Yue et al., 2008), goldfish (Dunlop et al., 2006), cichlid fish (Galhardo et al., 2011a; Galhardo et al., 2011b; Martins et al., 2011c), salmon (Madaro et al., 2016; Vindas et al., 2012), cod (Nilsson and Torgersen, 2010; Nilsson et al., 2008) and halibut (Nilsson et al., 2010), where the cognitive skills of fish are evaluated.

Despite using the same approach, it became clearer during the experiments that the CPP/CPA test has to be adapted and refined for each species and for each stimuli valence. Seabream displayed conditioning after one trial to the aversive condition, and needed three trials to get conditioned to the appetitive stimuli (i.e. food), whereas seabass needed one trial to get conditioned to each of the stimulus valence. However, in seabass, food do not works as appetitive stimuli by the reasons already discussed above, where stress sensitiveness and social condition have a large weight. Moreover, by using social context as appetitive stimulus, the conditioning became possible, since the presence of counterparts it increased the information transfer across the individuals and concomitantly the learning ability (Brown, 2011), demonstrating and validating what has been discussed regarding this subject. In seabream, the differences in the number of trials needed are likely to be related to the handling procedure before each step, where fish became stressed before the feeding event and as demonstrated elsewhere, reduced learning ability (Wood et al., 2011).

Overall, both species can associate events that are separated in both time and space (i.e. memory retention), and both display specific responses that reflect explicit expectation of reward or punishment, thereby demonstrating to be potential good models for Pavlovian learning and to assess fish cognitive ability regarding environmental perception. Additionally, such findings suggest that both species, most likely expanded on all fish taxa, are able to make decisions based on subjective experience, fulfilling the requirements to be incorporated in the sphere of sentient animals, concerning thus to a feeling-based approach (Fraser et al., 1997) (see chapter I, topic 1.2). These findings highlight the benefits of using frameworks and methods derived from cognitive sciences to assess the animal point of view, thereby improving the assessment of their welfare. Therefore, such findings validate CPP/CPA tests as a resourceful methodology to assess motivational states in teleost fish regarding either aversive or appetitive stimulus, also providing information on the affective appraisal perception of fish over their surrounding environment (food as a reward and net chasing as punishment). I can argue, though, that to understand entirely the motivation of the individuals, these results should have been supported by the assessment of the neural states and additionally, by different reward/punishment stimuli salience, because, although stimuli-valence are emotionally processed, emotional experiences also vary in reported activation or arousal (Mendl et al., 2010).

5.3 Psychological manipulation of environmental appraisal

Despite the extended knowledge on psychological factors affecting the stress response in other vertebrates, very little is known in fish, since a higher emphasis has been put on physiological stress processes. Thus, the integration of psychological and biological approaches to mechanisms of animal information processing only recently has been demonstrated in fish (see Faustino et al., 2015 for review and chapter III-1 for specific references). Relying on the knowledge that repeated exposure to novelty, suddenness, unpredictability, social isolation and loss of expectancies or control affects animal welfare, the research conducted on chapter III and IV, sought to demonstrate how such appraisal modulators affects “emotionally” the stress responses of both seabream and seabass. The research was driven on the basis that, in their natural environment, fish are frequently confronted with uncertainty and the need to make a decision about the actions when faced with it. These decisions are known to be influenced by the individual’s prior experiences and the consequences of having taken such decisions, which will directly influence their coping ability (relying on their affective states), and consequently their welfare.

5.3.1 Predictability, pleasantness and social support modulates fish appraisal

Predictability was used as a proxy of stimulus salience and distinct stimuli (aversive vs. appetitive) as a proxy of pleasantness in seabream, whereas in seabass only the salience was tested; in seabass, some insights were taken regarding the effect of social support. By modifying the psychological context in which the stressor was applied, the authors were able to i) manipulate the processing of the event, ii) evaluate the level of challenge set by their environmental situation and iii) determine the negative vs. positive affective states of the individuals underlying predictability and expressed by the individuals’ behaviour and physiology. So, as referred, fish probably make use of the projected affective states and memories to guide their individual choices. Thus, generic knowledge of cognition (i.e. how they appraise the environment), can improve our understanding of the animals’ welfare.

Taking this in mind, and supported by the learning ability evidence from the results of chapter II, the continuous information collecting offered by the classical conditioning performed, has worked as a key player to depict how predictability increases the aptness of fish to modulate the stress response, and to refine the differences within and between saliences and valences. Indeed, it is well accepted that fish, as in mammals, learn to escape, approach, evade or defend themselves against challenges from the environment. Nevertheless, the hypothesis that fish behavioural reactions are modulated by the predictability of the event is supported by these research findings. The results from chapter III show that fish attribute different valences (i.e. appetitive vs. aversive) and different predictability saliences (i.e. high arousal vs. low arousal) according to their perception of the surrounding environment. Such perception elicited specific measurements of behavioural, physiological and brain states, indicative of the fish affective states and of how each condition was processed i.e. indexing the stressfulness of the event (i.e. predictable appetitive < unpredictable appetitive < predictable aversive < unpredictable aversive). It is patent an increase in fish activity under predictable situations, expressed by lower time in freezing and cortisol, in seabream accompanied with an increase of social interactions and escape events, specific for rewarding and aversive conditions, respectively; in seabass accompanied with higher exploration. Such behavioural responses of seabream to food rewards have been associated with positive emotions in pigs (Dudink et al., 2006), rats (van der Harst et al., 2003), minks (Vinke et al., 2004) and sheep (Greiveldinger et al., 2007). In fish, the response patterns were described as signalling lower stress levels (Galhardo et al., 2011b; Sánchez et al., 2009). Indeed, during the signalling anticipating food reward, manifestation of an increase of fish activity was most likely to occur in places where the food had been previously dropped during training. In such situation, if the delay between signalling and reward is short, welfare is not compromised (Galhardo et al., 2011b), while a longer delay period can lead to anxiety or frustration (Gilbert-Norton et al., 2009). The anticipatory behaviour expressed by the individuals under putative predictable aversive conditions can be confusing, since such anticipatory behaviour is suggestive of fearful states. Nevertheless, such response could be interpreted as a signal of boredom (see Fig 5.2). Boredom was stated to increase arousal by encouraging the behavioural pursuit of alternative goals (Bench and Lench, 2013) which probably induced fish to explore and escape more. In this sense, the value attributed to the aversive challenge seemed to be reduced by the mental state of the fish due to its anticipation (supported by lower cortisol releases). This is consistent with the findings from Bassett and Buchanan-Smith (2007), which state that predictability of aversive events is less detrimental because it signals the safety periods in the absence of a cue (i.e. low degree of discrepancy between internal expectancies and the reality created by predictability decreases the stress response (Ursin and Eriksen, 2004)). The same conclusions were found in fish (Galhardo et al., 2011b). On the other hand, unpredictable regimes may have driven animals to sense a loss of control after failing to anticipate both appetitive and aversive events, evidenced by the higher time spent on the bottom, close to the tank walls (data not reported). This behaviour is comparable to thygmotaxis described in rats (Simon et al., 1994) and coyotes (Gilbert-Norton et al., 2009) as anxiety. In addition, in different studies using stressful contexts, inhibitory response expressed by freeze behaviour (a component of fear responses (LeDoux, 2000)) was coupled to a reduction of the coping ability (Galhardo et al., 2011b; Yue et al., 2004) and represented canonical anxiety-like states (Fossat et al., 2014; Gibson et al., 2015) possibly analogous to fear in mammals.

Additionally, the findings of the research performed with seabass, rather than showing only the positive effect of predictable regimes under aversive stimulus, provide support for the hypothesis that an individuals' affective state is shaped by the affective actions of others, when exposed to the same "emotional" event, as so often demonstrated in humans (van der Schalk et al., 2015). This study suggests that seabass experience and evaluate differently a predictable vs. unpredictable stressor by assimilation of how their counterparts are seen to appraise and respond to such event. It must be said that to evaluate the effect of social support was not the purpose of the research; nevertheless such results (higher exploration levels, lower freezing and lower escapes) point towards the importance of social support on seabass, which possibly also applies to many other social species. The impossibility to strictly follow the same protocol between group-based and individual-based tests (see chapter III-2 for more explanations) prevents taking firm conclusions regarding this issue. However, from what have been described in this species in terms of stress sensitivity and sociality in some way sustain such findings. These results are in agreement to other studies performed in guppies (Bhat and Magurran, 2006), goldfish (Laudien et al., 1986), cichlid fish (Galhardo et al., 2012) and African catfish (Martins et al., 2006) demonstrating the positive effect of social support.

Physiological outputs show higher levels of circulating cortisol in fish exposed to aversive than those of appetitive stimuli, and within each valence, unpredictable stimuli elicited higher cortisol levels. Again, stimulus of each valence by salience combination elicited distinct cortisol levels. If cortisol is an indicator of the level of discomfort that fish experience, then differences in the perceived stimulus would predict increased cortisol levels as the averseness of the stimulus increases (Key, 2015). Such results thus endorse predictability as a positive stress modulator. Indeed, outcome expectancies, novelty, lack of control or of social context are psychological factors known to enhance hypothalamic-pituitary-adrenal (HPA) axis responses to challenge in animals (Boissy and Lee, 2014; Boissy et al., 2007). The HPA axis is a known key biological system for the individual to maintain an integrated behavioural response to environmental challenges and an important mediator between stress and welfare in fish farms (Schreck, 2010). Despite the cortisol response to increased stress being highly variable (Fatira et al., 2014; Quillet et al., 2014) and context specific (Manek et al., 2014), the physiological response of the fish reported here support how differently fish have experienced each of the experimental conditions.

Neurogenomic states underlying predictability of stimuli with different valences and saliences has been demonstrated for the first time in fish, and surprisingly (or not) was seen to be specific to each combination of salience and valence. Complementing the research conclusions described above regarding chapter II, the data can be interpreted as evidence for the occurrence of distinctive affective states in fish corresponding to different organismic states (see introduction of chapter III-1 for review).

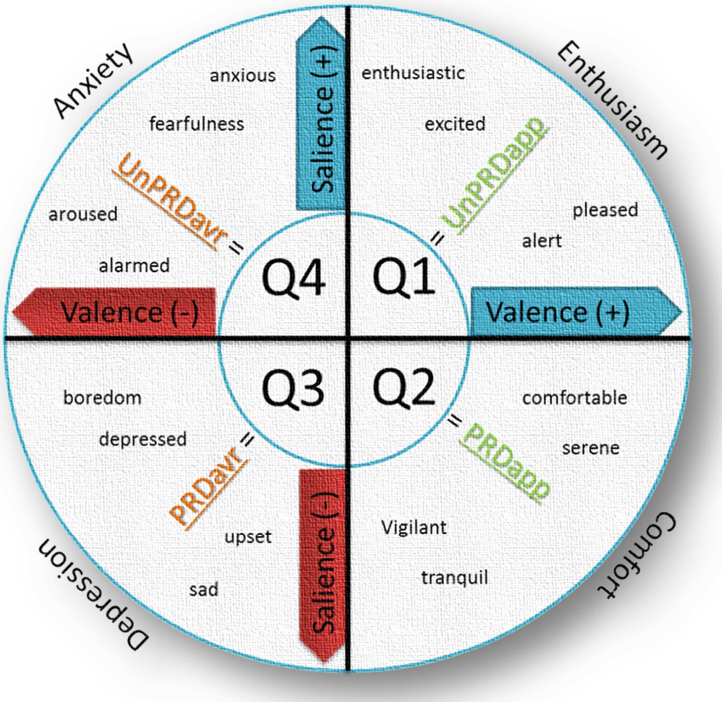

Figure 5.2 | Core affect represented in two-dimensional space (adapted from Mendl et al., (2010) underwriting fish organismic states. Positive affective states are in quadrants Q1 and Q2, and negative states in quadrants Q3 and Q4. Within each quadrant are examples of emotions-like states respective of each experimental condition (Q1 = predictable appetitive; Q2 = predictable appetitive; Q3 = predictable aversive; Q4 = unpredictable aversive).

Such organismic states evidence the emotions-specific behaviours that have catalogued each of the experimental conditions, regardless of being conscious (Anderson and Adolphs, 2014; Dolan, 2002; LeDoux, 2012; Mendl et al., 2010); see Fig. 5.2 and chapter III-1 for review. In agreement, fish under putative predictable rewarding situation most likely feels vigilant but comfortable with the situation; fish under unpredictable rewarding situation are excited but alert, given failure to predict the feeding time; fish under predictable aversive conditions exhibits boredom (despite being aversive, individuals are driven to react to other goals); fish under unpredictable aversive conditions most likely feel anxiety and fearfulness evidenced by higher freezing levels. In accordance to such findings, flies were shown to express internal states exhibiting emotions-specific behaviours in response to repetitive visual threat stimuli whereas crayfish demonstrate behaviours representing anxiety-like states (Fossat et al., 2014, Gibson et al., 2015), response that has been associated with unpredictable environments (Grillon et al., 2009; Grupe and Nitschke, 2013). Assessing such behaviours driven by specific organismic states would help to interpret the welfare state of the fish, by comprehend the way they perceive their environment.

These studies suggest that predictability can be used as a stress management tool for fish subjected to either appetitive or aversive stimulus. Predictability seems to promote more positive appraisals of the environment with concomitant better coping response to stress. In addition, propose that cognitive/emotional manipulation created by means of social support affects how predictability modulates the stress response to environmental challenges, but further studies should be performed to clarify these findings. Additionally, it supports the existence of a well-conserved neural basis in the processing of emotional stimuli throughout divergent species in evolution. However, it should be mentioned that the extent to which predictability is positive, under rewarding situations, could depend on the delay between the signalling and the event, since it can drive to an increase of aggressiveness if the delay is long (with welfare repercussions). As such, the intensity of the event and the delay between the signal and the event should be taken into consideration and it should be adapted for each species. In the same way, under aversive situations, the increase of activity can reach states of anxiety-like behaviours if the fish realize that they have no possibility to cope with it; it has been claimed that the benefit of predictability of stressors is limited when the fish have no way to avoid the stressor (Madaro et al., 2016).

5.3.2 Control over the environment increases fish fitness towards environmental challenges

Following Madaro et al., (2016) statement (referred above), the research conducted in chapter IV was focused on determining if controllability, associated with predictable conditions, increases coping ability. The underlying hypothesis is that coping responses, described as an outcome of a complex set of appraisals of stressful events (Aldwin, 2007; Lazarus, 1991; Lazarus, 1999; Taylor and Stanton, 2007; Zimmer-Gembeck et al., 2009), when lined with perception of control, would most likely reflect positive emotions (Boissy et al., 2007). The prediction that positive emotions facilitate adaptive coping and adjustment to acute and chronic stress is indeed demonstrated in this studies: from the research described on chapter IV-1 and relying on the previous research showing specific organismic states underpinning the stress response, it can be seen that perceived control diminishes the stress response by increasing coping ability, whereas lack of control and loss of control induced helplessness states. It should be noted, though, that integration of appraisal controllability is species-specific, as demonstrated for predictability. The effect of controllability was similar to the effect of predictability in seabass, with fish expressing lower freezing time after recognizing the possibility of escaping from confinement, supporting the stress sensitiveness already referred and demonstrated in the previous studies. In seabream a decrease in freezing time under perceived control is seen, unlike the effect seen for predictability. Such difference between stimulus evaluation check (SEC) might rely on the different nature of the aversive stimulus used (i.e. air exposure in chapter III-1 and confinement in chapter IV-1). Therefore, we cannot clearly state whether different behavioural patterns expressed between distinct SEC are indicative of species-specificity or of stimulus-specificity (or both). According to Faustino et al. (2015), to fully understand appraisal ability in fish, different SEC acting at different levels of processing should be evaluated, using parallel methodological conditions (i.e. nature, intensity and frequency of the stimuli). For seabream, we have unfortunately not followed such premise, which makes explanations for differences between SEC to be of speculative nature. Nevertheless, based on physiological and neural states (see chapter IV-1, Fig. 4.1.4), perception of control induces better coping strategies, which, manifested behaviourally, support what is described in chapter IV-1 for seabream (i.e. increased freezing is most likely a reflection of this species’ coping strategy to safeguard energy to deal with the perceived onset of confinement). In accordance, the study conducted in chapter IV-2 demonstrates how environmental choice influences performance and also suggests how it can affect fitness and survival of individuals. Nile tilapia was shown to swim up a gradient of temperatures to alleviate the effect of a bacterial infection, and such choice was seen even to override the individuals' genetic predispositions (i.e. personality traits). Such coping strategy supports the eliciting of positive organismic states when the individuals have possibility to act toward an environmental challenge. Indeed, according to classic theories of stress and coping (e.g., Lazarus 1991; Taylor and Stanton 2007), it is emotional reactions that partly signal whether coping responses are required to deal with one's internal state, to manage the stressful situation, or both (Barrett, 2006; Zimmer-Gembeck et al., 2009). Overall, these results show the positive adaptive value of controllability for successful coping with stress. Perceived control over a stressful event is a positive psychological experience, which has been often studied as relevant to understanding animal' coping responses (Ellsworth and Scherer, 2003; Scherer, 2001). Parallel finding have been demonstrated in other animals such as sheep (Greiveldinger et al., 2009); rats (Sonoda and Hirai, 1993), pigs (Ernst et al., 2005) or even spiders (Armfield, 2007).

To my knowledge, the effect of losing control over a stressful situation after having such possibility is shown for the first time in fish. In both seabream and seabass, the behavioural coping strategy adopted do not allow a clear distinction between lack of control or loss of control, in terms of which condition induces less stress. On the other hand, measurements of physiological and brain states indicate that loss of control has been appraised as more detrimental for fish coping; that is, the salience of loss of control has most likely elicited more negative emotions due to low motivation to pursue a goal. Moreover, in my view, the methodological approach to evaluate the effect of loss of control can also be refined; fish were subjected to few training sessions of lack of control over several ones having the possibility to control the exposure to the stressor. It is likely that fish have retained the prospect of escaping and still having the expectation to recover such possibility. Such condition can have led the individuals to aversive arousal, stimulated by the violation of expectation, and leading to compensatory behaviours i.e. individuals redoubled their efforts and put more energy to pursuit their goal. As such, an increase in the number of sessions with enforced lack of control (after having control) would probably induce differences between these conditions. This perspective is consistent with recent theorizing on threat compensation (e.g., Jonas et al., 2014). Findings have demonstrated that loss of control can stimulate approach motivation as part of an adaptive motivational system aimed at coping with perceived lack of control (Greenaway et al., 2015). This is supported with seabass data where higher freezing and lower exploration suggests that the species' inherent social predispositions have led social counterparts to acquire its behaviour (and its perception of the stimulus). We, thus reserve the right to argue that loss of control, if lack of control be properly extended, would instigate higher aversive arousal therefore, higher anxiety or stressfulness, indicative of poor welfare.

Such findings illustrate how the coping ability of fish is connected to controllability appraisal of stressful events, including how much control fish perceive and the emotions elicited. Additionally, as demonstrated during training, these results suggest that coping skills become stronger the more the individual make use of them to overcome challenging experiences. In general, the stronger the perception of control, the better are organismic states, which promote adequate coping strategies and, thus, improved welfare; these efficient adjustment mechanisms require the fine coordination of underpinning processes in the brain. On the contrary, perception of lack of control or loss of control induces negative organismic states, which lead to helplessness states and poor welfare. Between lack and loss of control, the latter is speculated to be more negative for fish, but further studies are needed to clarification. Thus, good welfare means an increased ability to cope with environmental challenges, and it is defined by not only to the actual situation (incoming stressors) the animals are living but, but most importantly, how they appraise it.

5.4 The link between personality and appraisal

In general, these results are not clear in determining whether personality affects environmental appraisal or vice-versa. Nevertheless, some evidences are pinpointed here suggesting such relation.

On chapter II-1, there was effectively (as it was assessed) no effect of personality on both behavioural and physiological responses of seabream: both proactive and reactive individuals have expressing these responses as if having attributed the same valence "rank" to the stimulus to which was subjected. Based on what have been already described before, it can be speculated that, if the salience of the stimulus have also been tested, rather than only the valence, perhaps could have refined how the different traits react to the stimuli. In this sense, the methodological approach may have limited the expression of divergent responses between personalities. Moreover, the identification of the divergent traits was performed without checking for consistency over time and across context, which may also have contributed to a suboptimal characterization of the fish population.

In seabass, we found evidences that personality traits modulate the response (*i.e.* appraisal) to appetitive and aversive stimuli. When fish were subjected to an appetitive stimulus, proactive individuals expressed a higher preference (*e.g.* time spent) for the stimulation side than reactive fish, but when fish were submitted to an aversive treatment, reactive fish exhibited an increase of distance moved in the stimulation side (anxiety) and a higher plasma cortisol concentration than proactive fish. Such exploration-activity has been documented as a distinctive trait between animals' personalities (Castanheira et al., 2013; Conrad et al., 2011; Ferrari et al., 2015; Martins et al., 2011c; Silva et al., 2010). For seabass, and comparing with the parallel seabream experiment, the higher time of adaptation to the tank may have created the conditions necessary to pinpoint differences in personality regarding the appraisal of the stimuli, by decreasing the stress of handling. However, as stated, behavioural consistency over time was not evaluated, which makes the link between personality and appraisal shallow, from my point of view. This is supported by side work, not reported in this thesis, conducted to measure the link between personality and appraisal. I have evaluated if seabass pre-categorized by distinct personalities differ in their appraisal of the environmental conditions to which were being subjected. Indeed, no relationship was found between personality and both predictability and controllability appraisal. By its sensitiveness to stress, the training sessions 2-4 h apart from each other may have been interpreted as highly stressful, and again, supressing the expression of personality.

Similarly, no differences were found for seabream within the research from chapter III-1. It is reasonable to say that one shortcoming of such research might have been the use of groups of fish with homogeneous personalities, as referred in the beginning of this chapter. The establishment of social hierarchies in the natural environment occurs due to the presence of different personalities within a population, so it is likely that the focal fish had to shape their personality according to their individual position within the group. Castanheira et al. (2016) showed in seabream that only intermediate traits, rather than proactives and reactives, display consistency over time; Intermediate traits are characteristic of the most flexible fish; such population are thus organized ”more proactive” and “more reactive” fish and the establishment of the group hierarchy is performed “naturally”. Accordingly, the lack of differences between personalities in the way they perceived the stimuli salience and valence might have been masked by such methodological condition. Nevertheless, from the research developed in chapter IV-1, we have seen that proactive seabream expressed lower cortisol levels than reactive individuals for each of the conditions tested, supporting an effect of personality on the way seabream appraise their environment (see Fig 5.3).

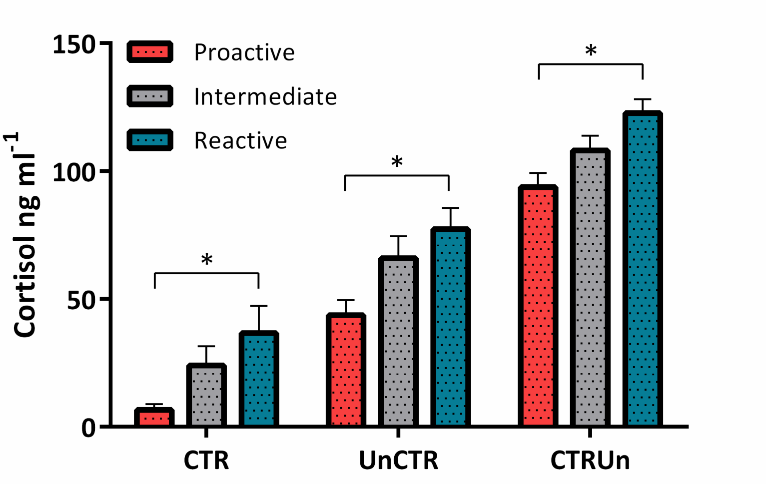

Figure 5.3 |Proactive, intermediate and reactive plasmatic cortisol concentrations (ng ml^{-1}; mean ± SEM) of seabream measured 30 min after being subjected to controllability (CTR), uncontrollability (UnCTR) and loss of controllability (CTRUn) learning association; CTR: $F_{(2,21)} = 3.863$, p = 0.039 * $p < 0.05$; UnCTR: $F_{(2,23)} = 4.380$, p = 0.026 * $p < 0.05$; CTRUn: $F_{(2,22)} = 5.136$, p = 0.016; Tukey post hoc: * $p < 0.05$.

On the other hand, no link was found between personality and the behavioural patterns expressed, suggesting that, even though each personality perceived each condition differently, the artificial environment and specificity of the trial may have constrained individuals from freely expressing their behaviour.

To solve this issue, promoting some environmental enrichment, which has been reported to soften the stress response in fish (Galhardo et al., 2008), can be significant. Indeed, to study appraisal and take reliable outputs, we need to psychologically manipulate fish. As such, we need to ensure that fish are in a good psychological state to avoid bias (e.g.

pessimistic/optimistic), otherwise results may not be representative of the real appraisal of the situation involved.

In Nile tilapia, the research was supported by environmental enrichment (i.e. gravel on the bottom of the tank) and a wider range of freedom (allowing them to select their preferred temperature to fight back a bacterial infection). This experiment was the one closest to fish natural environments and, therefore, these results are possibly the most reliable concerning the link between appraisal and personality. Proactive fish have higher metabolic demands so, given the possibility of choice, would select higher temperature environments, in line with their higher activity. Such premise was demonstrated with the research described in chapter IV-2, where proactives preferred warmer environments assessed by both chamber preference (i.e. thermal environments) and latency to cross between chambers, in comparison with reactive individuals (see chapter IV-2 for comparative studies). Additionally, higher activity was seen for this category. Moreover, individuals exhibited emotional fever as reaction to the effects of stress and behavioural fever in reaction to the infection, both states supportive of emotional states underlying fish preferences, reactions, needs or choices (Rey et al., 2015a; Rey et al., 2015b). One approach that could have complemented our behavioural results, as referred before, would have been to concurrently assess both physiological and neural expression, which would contribute to a better understanding of behavioural results and possibly lead to an identification of the neural states underlying this link between personality and appraisal. Additionally, the use of replicates of each personality trait and possibly the use of heterogeneous populations (personality-wise) in the bacterial infection challenge could have enhanced the reliability of such results on the effects of personality and appraisal on fish fitness and, ultimately, survival.

5.5 Proximate states uncover appraisal in fish

The goal of the research undertaken is not to replace mammalian behavioural paradigms with farm fish models. However, the evidence of such organismic states underlying fish environmental appraisal and their coping mechanisms (from research III-1 and IV-1) shows that translational neuroscience research can decidedly benefits from a complementary use of fish models to understand evolutionarily conserved pathways and circuits of appraisal processing and most probably of different phenotypes. The research from chapter III-1 suggests that fish are capable to project different states according to the interaction salience-valence of the stimuli (see topic 5.3.1 regarding neurogenomic states discussion). Alongside, also different coping mechanisms were mapped differently in the brain of seabream and seabass whether having or not the possibility to cope with an aversive event (see topic 5.3.2). Thus, there are evidences here of how appraisal is mediated by brain regions known to be involved in reward and aversion processing in mammals (O'Connell and Hofmann, 2011) (see chapter III-1 in introduction). The use of other components of appraisal generalization of the response to different contexts and trans-situationally (i.e. the same response to unrelated stimulus) (Gibson et al., 2015) would have allowed to take more outcomes regarding this issue, and is still needed more research to address clearly the outputs of this research.

Nevertheless, this research raised one fundamental question; by the species-specificity to the modulation of stress, it justifies the need to assess behaviour, along with both physiology and brain states and driven by research, to accurately characterize the fish stress response in a first instance, rather than only looking at behaviour (i.e. the same behaviour might indicate different perception of the environment, and thus different stress states), which is the usual approach. Only then, correlated behaviour should be used as a “measure” of stress.

Conclusions

The objectives of the work pointed out in the beginning of this chapter were attained:

i) Seabream, seabass and Nile tilapia show divergent personality traits on a proactive-intermediate-reactive continuum. In general, extreme proactive individuals were characterized by higher risk-taking, higher escaping performance, and by being more exploratory and active. On the opposite extreme are reactive individuals, characterized by lower risk-taking, lower escaping performance, and by being less exploratory and active, displaying lower latency to reassume feeding. The results regarding Nile tilapia research display the usefulness of using both individual- and group-based tests to characterize personality. Such results show a high degree of repeatability over time and consistency across context in terms of behavioural responses, which supported the characterization of distinct personalities by thermal preference. Therefore, it seems reasonable to assume that, for a thorough and proper characterization of personality, there are no short-cuts: it must be managed case-by-case regarding the species' ecology, biology, and the context of their surrounding environment.

ii) The focal species displayed the ability to make decisions on subjective experiences, thus fulfilling the requirements to be integrated in the domain of sentient animals (within a feeling-based approach). Both seabream and seabass expressed specific appraisal and motivations, coordinated by their behaviour and physiology, according to the prospect of reward or punishment. Hence, it suggests that fish leverage previous memories and learning actions towards environmental cues, to readjust their responses, improving their coping ability, to deal with environmental challenges. Further, Nile tilapia made use of thermal sensitiveness to cope with a bacterial infection according to their inherent ecological predispositions. Therefore, we demonstrate that the evolution of appraisal mechanisms occurred in fish, enabling them to cognitively assess the presence of threats and opportunities in the environment, with associated influence on their biological success or failure.

iii) Differences in behaviour, physiology and brain states measurements support first, the ability of environmental appraisal by fish; secondly, the use of psychological modulators to manipulate the perception that fish do from their environment; thirdly, that regular stimuli and social context alongside with perception of control can be used as psychological modulators of the stress response to improve the welfare, whether under stressful situation or not, both in fish farms and elsewhere. Moreover, measurements of neural states and cortisol levels, when assessing the effects of both stimuli valence and salience, evidenced differences in environmental perception according to their emotional state. Understanding the evolution of the biological mechanisms that generate emotions (which are ultimately expressed by behaviour) in an older vertebrate, such as fish, will improve our understanding of animal´s ability to cope with changes in their environment, therefore with positive outcomes for their welfare, fitness and survival. As for personality characterization, these results suggest that the appraisal SEC in fish should be adapted on a case-by-case basis, as the same behaviour in different species could mean different perception of the stimuli. Overall, I endorse the use of these SEC to be included in husbandry and experimental practices of fish produced in captivity.

iv) The link between personality and appraisal deserves further investigation due to the impossibility of clearly assessing the neural states of fish underlying environmental appraisal according to distinct personality traits. However, from the personality-dependent differences in cortisol expression levels found in seabream under aversive conditions (Fig. 5.3) and from the thermal preference differences in Nile tilapia, under bacterial infection, we can speculate that individuals with distinct traits appraise an aversive event differently. If such is the case, different personalities will have different range of biological success depending on the context. Regarding rewarding situations, further studies are needed to draw any conclusion.

v) The prospect that positive emotions improve welfare in fish is still debated due to the lack of evidence for either emotions and/or sentience in fish. Given the phylogenetic position of fish within the vertebrate lineage, these results have considerable implications for the acceptance and understanding of the evolution of the biological mechanisms that generate emotions. Along with the development of proper cognitive SEC methodologies adapted for each fish species, this work provides a significant contribution to multidisciplinary, cross-species neurobehavioral research. Therefore, it is time to move beyond the discussions of whether or not fish have emotions, if they are capable of appraisal, if they are sentient or not, and to address more relevant fundamental questions of fish affective behaviours and their proximate mechanisms. Doing so is essential to improve our understanding of how fish can cope with changes in their environment.

Future perspectives

The characterization of divergent personalities in seabass remains inconclusive, and by its value for the Mediterranean countries, within an aquaculture perspective, such issue should be addressed to enhance the production systems by their adaption for each of the personality traits. Such issue is therefore, in my view, a major constraint for fish farmers, as we know that divergent personalities have different possibilities of success depending on their environment e.g. proactive fish are less flexible to changes in the environment (Basic et al., 2012). Its production should, thus, be performed in intensive production systems, where conditions are more stable, rather than using semi-extensive or extensive production systems. Additionally, multidisciplinary efforts accommodating behaviour, physiology and neurogenomic expression should be undertaken to refine the characterization of divergent traits in fish. From the literature review made within the core of this dissertation, it is reasonable to state that there are no optimal methodological approaches to characterize personalities, since results depend on the environmental context in which the individual is, along with its needs, motivations and adaptability capacity. Therefore, standardization of general strategy to identify distinct personalities is not, from my point of view, feasible; hence, strategies should be adapted to each fish species, for each developmental stage, context, gender, stress susceptibility and ecological dispositions.

This research outcomes demonstrate fish appraisal ability, but to which level such appraisal occurs is still arguable, as few appraisal modulators were tested, and the proximate states underlying appraisal seems to be disconnected from behavioural expression (at least to some extent). Environmental appraisal, which determines fish behaviour expression, is difficult to explain, as an environmental challenge usually elicits different responses and these are highly species- and context-dependent: thus, assessing the affective states of the individuals can be the most appropriable path to determine such appraisal level. In that sense, an approach as follow by Gibson et al. (2015) with fruit flies, in which emotions-like states were evoked by combining different components of appraisal as valence, persistence of the response to the stimuli valence, scalability (i.e. intensity of the response to different salience of the stimulus), generalization of the response to different contexts and trans-situationally is attractive to use in fish (see chapter III-1 for further clarification); not to demonstrate if experience fear, happiness, anger, sadness or any other emotion, rather to get to the bottleneck about the mechanisms and questions underling fish appraisal. By understanding such states, the animal “point of view” regarding conditions in artificial environments can be more easily determined and, consequently, their welfare can be effortlessly assessed.

To assess how environmental choice predicts personality and appraisal in fish, further studies contemplating other species and strains of the same species should be used to determine at what point such possibility of choice increases fitness towards environmental changes, such as the threat of climate change or from any anthropomorphic influences. In addition, other factors beyond temperature should be addressed (e.g. salinity, organic matter concentration, dissolved oxygen, ammonia, etc.).

The research conducted in this dissertation was planned and performed in a small scale, but to fully understand how appraisal SEC modulates fish stress responses within aquaculture context, parallel research should be up-scaled. For instance, in offshore sea cages, predictability can be easily achieved e.g. using the noise of the boat approaching the cage, will enable evaluation (through video recording), of fish reaction to feeding or sampling anticipation.

Implications for aquaculture

This dissertation describes how studying psychological manipulation and respective cognitive functions in farm animals and the emotion-induced alterations in such functions can help us to better assess how comfortable fish feel in their environment. The outcomes of this research suggest that environmental comfort really matters to them. Fish farm management procedures, and of animal in general, designed to create cognitive challenges incorporating screening for personalities, positive anticipation and the possibility to cope with stressful events, are promising and may provide practical approaches for making animals more resilient and empowered under sustainable farming systems. These changes in management practices adapted to the way animals' experiences environment could easily be introduced into farming systems (e.g. by signalling a rewarding situation with an aversive event with low impact, would allow individuals, after a learning period, to better cope with further stressful routines from standard husbandry practices) because they are simple, harmless and inexpensive, with the prospect to positively impact animal welfare and fish farming sustainability.

There are around 30000 fish species around the globe with history of evolutionary success, therefore it is reasonable to say that each species diverge on their needs, their coping ability and their motivations; conscious or not, fish relies on their mechanistic perception of the environment to adapt and evolve. Though such fact makes it impossible to generalize our results to all fish species, they nevertheless, greatly contribute to explore the usefulness of psychological components to improve the welfare of farmed fish and the sustainability of aquaculture activities.

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