The neural basis of threat-sensitive behaviour in fish

Nevronale mekanismer for fryktatferd hos fisk

Philosophiae Doctor (PhD) Thesis

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Preface

The studies presented in this thesis were carried out at the Department of Animal and Aquacultural Sciences at the Norwegian University of Life Sciences and at the Institute of Aquatic Resources, Section for Aquaculture, at the Danish Technical University (DTU Aqua) under the supervision of Dr. Øyvind Øverli, Dr. Erik Höglund, Dr. Catarina I.M. Martins and Professor Hans Magnus Gjøen. These studies benefited from funding by the Project SFRH/BD/44103/2008 from “Fundação para a Ciência e Tecnologia” (Portugal).

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Finally, to all of you who are not mentioned, you definitely not forgotten. Thank you so much to all of you!!!

Patrícia I.M. Silva

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Papers I-IV

Abbreviations

5-HIAA	5-indoleacetic acid
5-HT	5-hydroxytryptamine, serotonin
CRH	Corticotropin releasing hormone
CS	Conditioned stimulus
DA	Dopamine
Dl	Dorsolateral pallium
Dm	Dorsomedial pallium
HPA-axis	Hypothalamic-pituitary-adrenal axis
HPI-axis	Hypothalamic-pituitary-interrenal axis
HR	High responding
LR	Low responding
NE	Norepinephrine
US	Unconditioned stimulus

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Paper I

Feeding motivation as a personality trait in Nile tilapia (*Oreochromis niloticus*): Role of serotonergic neurotransmission

Silva, P.I.M., Martins, C.I.M., Höglund, E., Gjøen, H.M., Øverli, Ø., 2014. Fish Phisiology and Biochemistry 40: 1547-1557. DOI: 10.1007/s10695-014-9947-2

Paper II

Stress and fear responses in the teleost pallium

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In press in Physiology and Behavior.

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Silva, P.I.M., Øverli, Ø., Martins, C.I.M., Vindas, M.A., Gjøen, H.M., Höglund, E. *Manuscript.*

Abstract

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Fear, or threat-sensitive behaviour, is an emotionally guided response allowing animals to minimize exposure to potential danger. Stress is a set of physiological/behavioural responses to such exposure. Among other functions, stress responses prompt learning, and as a result, similar circumstances are later recognised and avoided (feared). In parallel with higher vertebrates, fish show a strong and consistent individual variation in behaviour exhibiting two primary types of personalities: proactive and reactive response patterns. The aim of this thesis is to study the neural basis of threat-sensitive behaviour in teleost fish, by focusing on brain monoaminergic activity and individual differences in threat-sensitive behaviour. Results indicate that fish with contrasting stress coping styles show clear differences in behavioural output when exposed to a fearful situation, with reactive individuals displaying a more fearful behaviour. Proactive individuals appear to respond less to changes in the environment; suggesting that their behaviour is to a larger degree guided by previously learned routines rather than environmental cues. When exposed to fear and/or stress-inducing stimuli, experimental fish showed regionally discrete changes in limbic monoamine neurotransmission, supporting the view that homologues to the mammalian hippocampus and amygdala control individual differences in behaviour and stimulus perception even in non-mammalian vertebrates.

Sammendrag

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Frykt og fryktatferd er emosjonelt styrte responser, utviklet gjennom evolusjon hos dyr og mennesker for å unngå farer i omgivelsene. Fysiologiske og atferdsmessige stressresponser, eller stressmestestringsstrategier, er tett koblet til frykt, og disse responsene kan gjensidig forsterke hverandre. Opplevelse av akutt stress og frykt leder blant annet til en ekstraordinær innlæring av hendelsesforløpet, noe som fører til at tilsvarende omstendigheter senere gjennkjennes og unngås (fryktes). I likhet med hos pattedyr og mennesker vil en hos fisk og andre modelldyr gjenkjenne to grunnleggende forskjellige stressmestringsstrategier i slike situasjoner, proaktive (preget av aktiv respons) og reaktive (preget av passiv unngåelse). Denne avhandlingen har undersøkt underliggende nevrobiologiske mekanismer bak individuell variasjon i fryktatferd hos fisk, med fokus på de monoaminerge signalsystemene serotonin, dopamin og noradrenalin. Det ble observert at fisk med ulik stressmestringsstrategier også hadde ulik fryktatferd, der reaktive individer var mer fryktsomme. En proaktiv mestringsstrategi innebærer altså at individet reagerer mindre på endringer i miljøet, og opprettholder innlærte rutiner i større grad. Mikroddisseksjon av spesifikke områder i hjernen som antas tilsvare pattedyrenes limbiske system (inkludert hippocampus og amygdala), viste at ulikheter i disse hjernefunksjonene kan forklare utviklingen av ulike personlighetstyper også hos fisk.

1. Introduction

Fish are currently rising as an interesting alternative to small laboratory mammals in biomedical and behavioural research because of easy maintenance, short generation intervals and increasing numbers of mapped genomes (Darland and Dowling, 2001; Guo, 2004; Epstein and Epstein, 2005; Lieschke and Currie, 2007; Terzibasi et al., 2007; Sørensen et al., 2013; Kalueff et al., 2014). Furthermore, particular attention is being given to possible cognitive and emotional processes in fish (Rose, 2002; Chandroo et al., 2004a; Huntingford et al., 2006; Braithwait and Boulcott, 2007; Cotee, 2012; Millot et al., 2014a), due to both a rising interest in animal welfare and the fundamental scientific enquiry into the evolution of complex neurobiological phenomena. Within this context, this thesis will focus on a set of rarely discussed phenotypic traits with high adaptive value, namely threat sensitive behaviour guided by fear and stress responses in teleost fish. I will initially review central concepts, and identify knowledge gaps to be addressed experimentally.

Fear, or threat-sensitive behaviour, is an emotional response to guide behaviour away from potential danger. Stress is a set of physiological/behavioural responses to such danger. Among other functions, stress responses prompt learning, and as a result, later circumstances are recognised and avoided (feared). As such, expectancy of future circumstances (fear) may in itself be enough to release a stress response.

In parallel with higher vertebrates, fish exhibit a strong and consistent individual variation in behaviour. Therefore, it is likely that the individual perception of threat will also differ between individuals. The link between personality and fear has been addressed in humans. For example, Tong (2010) has identified one personality trait (neuroticism - a fundamental personality trait characterized by anxiety, moodiness, worry, envy and jealousy) that can explain individual differences in appraisal-emotion relationships for anger, sadness, fear, and guilt. This study showed that individuals displaying higher neuroticism were more likely to experience negative emotions such as fear. These findings imply that personality traits affect not only how people appraise their environments but also the reactivity of their negative emotions to appraisals. However, the magnitude of individual variation in cognitive appraisal of fear/threat in non mammalian vertebrates (and thus the biological background for the evolution of this ability in humans) has yet to be described.

1.1.Threat sensitive behaviour - an evolutionary perspective

The ability to respond to threat allows organisms to anticipate/avoid danger, and therefore increase their chances of survival. Threat-sensitive behaviour, for instance to escape from a predator or from a dominant individual, brings benefits such as survival or avoiding injuries. On the other hand, the necessary measures to respond to a threat can be costly, as an individual must also allocate time and energy for other essential activities such as foraging, reproduction and territorial defence (Godin and Smith, 1988; Chivers et al., 2001). However, it is believed that if the intensity of such responses can be adjusted respectively to the level of perceived risk, then such behaviour should be a selective advantage (Helfman, 1989; Lima and Dill, 1990).

Threat-sensitive behaviour falls under the definition of fear. Fear can be defined as the activation of a defensive emotional and behavioural system that defends animals or humans against possible environmental threats (Fendt and Fanselow, 1999). Emotions are a set of neural responses that occur unconsciously when the brain detects challenging or rewarding situations. These responses are automatic and occur within the brain and involve changes in arousal levels, cognitive functions and in endocrine, autonomic and musculoskeletal responses (Kandel et al., 2012). Therefore, in this context, emotions are not similar or indicative of subjective feelings. In vertebrates, behaviours associated with fear and anxiety have been suggested to have a common phylogenetic ancestry (Höglund et al., 2005a; Cachat et al., 2011; Okamoto and Aizawa, 2013). This common phylogenetic ancestry is also reflected in various neuroendocrine responses to fear and stress (Winberg and Nilsson, 1993a; Mok and Munro, 1998; Eriksen et al., 2005; Medeiros et al., 2014, and see discussion in the following). However, in the present thesis both terms (i.e. threat-sensitive behaviour and fear) are used interchangeably but should not be confounded with subjective feeling.

1.2. Importance of threat sensitive behaviour in aquaculture and biological research

Under aquaculture conditions, fish are subjected to several potential harmful activities, such as handling, grading, vaccination, agonistic behaviour or high stocking densities. Such conditions may cause skin damage and result in the release of alarm cues into the surrounding environment, leading to threat-sensitive behaviour of individuals (see the chapter on "Risk assessment" below). Fish exhibiting threat-sensitive behaviour may allocate energy resources to non-foraging activities and therefore show reduced growth rates, resulting in economic loss. Hence, understanding the underlying mechanisms of threat-sensitive behaviour in fish is of paramount importance, not only in fundamental research but also for practical applications in commercial activities, such as the aquaculture industry. Chemical cue induced threat-sensitive behaviour may for instance occur in recirculation aquaculture systems, a type of production system that is foreseen to increase in the coming years due to its environmental advantages (Martins et al., 2010; Dalsgaard et al., 2013). In these production systems, the water is re-used and over time alarm substances released from fish due to handling/grading may accumulate in the systems and potentiate the perception of threat.

If the neural basis for threat-sensitive behaviour in fish is homologous to fear and anxiety behaviours found in humans, the use of fish in drug screening could be applied (Cachat et al. 2011; Clark et al., 2011). For example, threat-sensitive behaviour could be shaped by manipulating the serotonin (5-HT) system by changing the levels of the amino acid precursor of 5-HT, tryptophan, in feed (Cools et al., 2008, and see chapter on "Neurobiology of threat sensitive-behaviour" below, reviewing the involvement of 5-HT in threat-sensitive behaviour in mammals). Therefore, investigating if and how 5-HT and other monoaminergic signalling systems are activated within the fish brain when exposed to potential dangerous situations is motivated.

1.3.Risk assessment

In aquatic ecosystems, risk assessment is often adjudicated via chemosensory cues (Kats and Dill, 1998; Wisenden and Chivers, 2006). These cues include predator odours, damage-released chemical alarm cues and disturbance cues (Chivers and Smith, 1998). Predator odours can give information about the intensity of local threats (Kusch et al., 2004; Ferrari et al. 2006a). For instance, fathead minnows (*Pimephales promelas*) are known to discriminate predator size, proximity and density based on predator odours (Kusch et al., 2004; Ferrari et al. 2006b).

Damage-released cues, also known as chemical alarm cues or “Schreckstoff” substance, can be found in several prey fish and are produced and/or stored in the epidermis (Brown et al., 2006a). These cues are released after a mechanical damage to the skin, and evoke short-term increases in anti-predator behaviours (Chivers and Smith, 1998; Wisenden and Chivers, 2006). However, alarm-cues can also be released voluntarily, as shown for example in Iowa darter, *Etheostoma exile* (Wisenden et al., 1995). When detected by nearby conspecifics or sympatric heterospecifics, alarm cues usually evoke species-specific anti-predator responses; like increased shoaling, freezing, and refuge seeking (Brown and Godin, 1999; Brown et al., 1995; Chivers and Smith, 1998; Mathis and Smith, 1993). Some studies propose that a strong relationship exists between the concentration of alarm-cues and the intensity of the anti-predator responses (Jachner and Rydz, 2002; Dupuch et al., 2004; Brown et al., 2006b, 2009). Nevertheless, prey can react to alarm cues at very low concentrations by increasing vigilance towards secondary risk assessment cues (Brown et al., 2004) or by adjusting their foraging tactics (Foam et al., 2005). Alarm cues have been demonstrated in a number of fish taxa including Ostariophysans, darters, gobies, sculpins, sticklebacks, poecilids, cichlids (like Nile tilapia), and salmonids (Chivers and Smith, 1998). In the Ostariophysan group, the alarm substance is enclosed in large epidermal cells called club cells (Smith, 1992). Club cells lack an outlet to the surface, indicating that the alarm cue is only released when the skin is damaged. It is suggested that alarm cues are hypoxanthine 3-N-oxide or a similar molecule with a nitrogen-oxide functional group (Brown et al., 2000).

From an ethological point of view, it is important to note that both the production of and response to alarm cues is context and life-stage dependent. Lastein et al. (2008) demonstrated that fish not only stop producing but also stop responding to alarm cues during breeding season (the majority of non-reacting individuals were ovulated or spermiated). This suggests that the timing of the loss of alarm cue cells in nature corresponds with the development of androgen-induced secondary sexual characters and with high levels of testosterone. Furthermore, Lawrence and Smith

(1989) showed that each club cell of the fathead minnow contained enough alarm cues to create an active space of 80 litres. One square centimetre of skin would consequently give an active signal in about 58,000 litres. Moreover, Wisenden and Smith (1998) showed that fish adjust their synthesis of alarm cues based on the level of perceived risk. This suggests that close physiological control of phenotypic responses is mandatory, and threat-sensitive behaviour in fish is likely mediated by limbic neural systems (Chandroo et al., 2004b). Because of these key properties (strong stimulus, well investigated physiology, and individual variation), chemical alarm cues are a good model system to study threat sensitive behaviour in fish, and the underlying neurobiological control mechanisms. This model will be addressed in paper II of the present thesis using Nile tilapia.

1.4.Learning about threat versus innate responses and retention of acquired threat information

Some researchers focused on the importance of previous experience, rather than genetic factors, in threat sensitive behaviour. While some prey fish seem not to respond to predators unless they had previous experience (Chivers and Smith, 1994a, 1994b; Chivers et al., 1995; Mirza and Chivers, 2000, 2001; Alvarez and Nicieza, 2003; Kristensen and Closs, 2004; Bass and Gerlai, 2008) others appear to react to predators even without experience (Berejikian et al., 2003; Vilhunen and Hirvonen, 2003; Hawkins et al., 2004; Scheurer et al., 2007). One example of a prey fish that reacts to predators even without previous experience is predator naïve Nile tilapia, which increases opercular movements during visual exposure to a predator (Barreto et al., 2003). This supposed innate recognition may also be present in the absence of genetic fixation (Brown et al., 2006a). Prey can show strong avoidance responses to any novel cue, a tendency also known as neophobia (Sneddon et al., 2003). Genetically fixed responses to predator cues and neophobia are two very different situations, nonetheless, it has been argued that being capable to react to a predator upon a first encounter should eliminate the cost of learning (Blumstein, 2006; Ferrari et al., 2007).

It is known that several fish can learn to recognize unknown predators through conditioning with alarm cues. Experiments conducted in order to investigate these paired stimuli associations, utilize damage-released alarm cues paired with either visual or chemical cues of a novel predator, which results in learned recognition of the predator stimulus (Chivers and Smith, 1998; Brown and Chivers, 2005; Brown et al., 2006a). It has also been investigated whether the strength of the learned response to a predator odour is related to the intensity of the initial conditioning. For example, Ferrari et al. (2005) exposed predator-naïve fathead minnows to low, intermediate and high concentrations of conspecific alarm cues together with the odour of a novel predator. In the initial conditioning, as well as 24 hours later, minnows showed stronger antipredator responses if initially exposed to a high concentration of cues. In the following experiments, Ferrari and Chivers (2006) tested if recent experience regulates threat-sensitive learning by exposing fathead minnows to either a high or low concentration of conspecific alarm cues paired with a predator odour. The results of this study suggested that minnows adjusted their level of antipredator response to the most recent experience instead of responding with an average intensity to all the learning opportunities. The concentration of predator odour during conditioning can also give useful information about the threat of the predator. Ferrari et al. (2006c) conditioned fathead minnows with conspecific alarm cues and different concentrations of predator odour. Interestingly, no differences were found in the antipredator response despite the different concentrations of predator odour. These results are expected, since the initial intensity of antipredator response is controlled by the concentration of detected alarm cues (which in this case was the same). If the intensity of antipredator response is controlled by the concentration of detected alarm cues, then the production of alarm cues per individual may, consequently, affect the magnitude of antipredator response in conspecifics.

Under natural conditions, prey is exposed to diverse sources of information simultaneously. Therefore, it is hypothesised that prey that are able to acquire multiple risk cues should be more capable of receiving reliable information concerning local threats (Smith and Belk, 2001; Brown and Magnavacca, 2003; Blanchet et al., 2007) and that multiple cues should interact in an additive or cooperative manner (Lima and Steury, 2005). This is referred to as the sensory complementation hypothesis. For example, it was discovered that under laboratory conditions, stream dwelling young-of-the-year (YOY) Atlantic salmon (*Salmo salar*) responded in an additive manner to multiple stimuli. That is, fish reacted stronger to the presentation of predator odour and a predator visual cue in comparison to when fish were exposed to only one of the predator cues (Blanchet et al., 2007). However, Kim et al. (2009) have shown that sensory complementary effects may diverge depending on age and/or experience. Elvidge et al. (2013) proposed that wild juvenile Atlantic salmon living under weakly acidic conditions demonstrate significantly greater or hypersensitive antipredator responses to visual cues when compared to fish under neutral conditions. Differences in antipredator responses between neutral and weakly acidic streams result from the loss of chemical information on predation risk.

Very little is known about the retention time of acquired threat information. Brown and Smith (1998) demonstrated that hatchery-reared rainbow trout (*Oncorhynchus mykiss*) could retain a learned response to a single pairing of alarm cues and predator odour for up to 21 days. Chivers and Smith (1994a) suggested that depending on the initial conditioning, retention may be differential. These authors conditioned fathead minnows to recognize visual cues of either Northern pike or goldfish (*Carassius auratus auratus*) as potential predators. Initially fathead minnows showed similar learned predator recognition to both potential predators. However, after almost 2 months of the conditioning the response was different. Fathead minnows showed a more intense response to Northern pike than to goldfish. Some state that, optimally, prey should only respond to learned predator cues which display an actual threat (Gonzalo et al., 2009). Furthermore, Brown et al. (2011) tested if different growth rates were related to different learned retention periods. In that study, juvenile rainbow trout were fed for 7 days at either high or low food rations (which induced different growth rates) and were afterwards conditioned to recognise pumpkinseed odour as a threat. Both groups exhibited similar learned responses 24 hours after conditioning. However, only the group fed at a low food ration exhibited an antipredator response, 8 days after conditioning. This suggested that growth rate influences the retention time of the learned response.

As predation risk is not constant over time, one may expect that prey would not retain information that is no longer needed. However, under constant predation risk one would expect this information to be continuously reinforced (Brown et al., 2006a). As the telencephalon in fish has been related to spatial, relational and emotional memory (Broglio et al., 2005), this brain region may play a key role in the retention of such information.

1.5.Neurobiology of threat sensitive-behaviour

It is improbable that animals with a different brain structure to humans would experience the concept of threat like we do. Nonetheless, if an animal experiences suffering or discomfort, the nature of the perceived threat is no less important (Ashley and Sneddon, 2008). The neural system underlying threat-sensitive behaviour in higher vertebrates is well reported and a large quantity of this work relates to the behavioural paradigm called fear conditioning. Some researchers have debated that fish do not possess brain regions or any functional equivalent that allow fish to able to experience fear (Rose, 2002). However, other researchers suggest that there is anatomical, physiological, and behavioural evidence that makes fish capable to experience fear (Chandroo et al., 2004b). Even though it is debatable, the most recent data are often interpreted as showing that fish are indeed able to experience fear, or at least show behaviors and brain activation patterns consistent with such an experience. This thesis will scrutinize this interpretation in several novel contexts, thus expanding on existing studies.

The vertebrate brain can be considered to be composed of three main divisions; the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (Butler and Hodos, 2005). The forebrain comprises two areas, the telencephalon and the diencephalon (Butler and Hodos, 2005). The grey matter that covers the telencephalon, also called pallium, has thickened throughout evolution to various extents in different classes of vertebrates, and in mammals it consists of a laminated structure, the cerebral cortex (Striedter, 1997). Humans and primates have the most developed cortex with the evolution of the neocortex (Butler and Hodos, 2005). The majority of modern fish species possesses an unlaminated pallium, but evidence suggests that it has developed into a differentiated structure with respect to the processing of sensory information (Bradford, 1995; Butler, 2000).

Some authors argue that in fish the telencephalon is the location of several brain structures that are homologous to those associated with fear in higher vertebrates (Bradford, 1995; Chandroo et al., 2004b; Portavella et al., 2004). The amygdala is recognised for having an important role in arousal and emotions including fear in mammals (Carter, 1996; Maren, 2001). The amygdaloid complex in fish is located in the telencephalon (Butler, 2000). Specifically, the dorsomedial (Dm) telencephalon in fish has been implicated in emotional learning and is thought to be homologous to the amygdala in mammals (Bradford, 1995; Butler, 2000; Portavella et al., 2004) while the dorsolateral (Dl) telencephalon is thought to be homologous to the hippocampus of higher vertebrates (Figure 1). The comparative study of homologies in the fish forebrain is complicated due to the process of telencephalic eversion during development in comparison to the process of evagination in mammals (Portavella et al., 2002). In the face of such difficulties it is necessary to compile all findings of anatomical and functional studies in order to comprehend brain evolution. In fish, lesions on the Dm produces changes in aggressive behaviour, and similar results were observed after amygdalar lesion or stimulation in higher vertebrates (Portavella et al., 2002). Furthermore a study using a two-way active avoidance learning with overlapping stimuli demonstrated that lesions on the Dm in goldfish impaired the avoidance response acquired previously (Portavella et al., 1998). However, Dl lesions did not have an influence on the response.

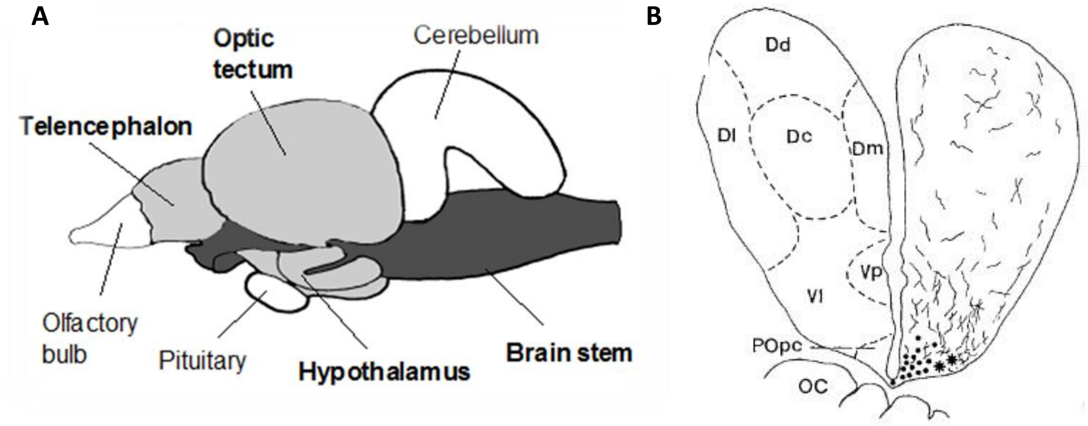

Figure 1. A - Schematic figure of a generalised fish brain. Structures generally used for the analysis of monoaminergic activity are in bold font. Figure from Øverli (2001). **B** - Schematic figure of a transverse section of the rainbow trout telencephalon. Forebrain areas presented and discussed above are: the dorsomedial pallium (Dm), the dorsolateral pallium (Dl). Figure from Navas et al. (1995).

Collectively, the above results demonstrate that Dm lesions impaired emotional learning, and, importantly, similar effects were observed with pallial amygdala lesions in mammals (Ambrogi-Lorenzini et al., 1991; Killcross et al., 1997). In another study using the active avoidance learning paradigm, an interstimuli gap of 5 seconds between CS off and US on was used. In this situation, both Dm and Dl lesions impaired the retention of the avoidance response in goldfish (Portavella et al., 1998). In this study, both emotional and temporal factors were important to solve the task. In studies of fear conditioning in mammals, hippocampus lesions disrupted the conditioned response when a temporal interstimuli gap separated the CS and the US (Olton et al., 1987; Kesner, 1998). These studies demonstrate that Dm impaired acquisition and retention of a conditioned avoidance response. Dl lesions impaired specifically spatial learning as well as conditioned avoidance response in the presence of a temporal factor. Likewise, in mammals, lesions of the pallial amygdala affected emotional conditioning, while hippocampal lesions affected spatial memory and timing tasks (Olton et al., 1987;

Ambrogi-Lorenzini et al., 1991; Eichenbaum et al., 1992; LeDoux, 1995; Killcross et al., 1997; Kesner, 1998). These results suggest the existence of differentiated memory and learning systems in the forebrain of teleosts. Dm areas may be implicated in emotional learning, and Dl areas may be implicated in spatial or temporal memory.

Furthermore, in higher vertebrates, threat sensitive behaviour is often followed by a range of autonomic changes (LeDoux, 2000) like increased heart rate, endogenous analgesia and the release of hormones like cortisol (Ashley and Sneddon, 2008) and adrenaline (Nijsen et al., 1998).

Within the brain, the dopamine (DA), serotonin (5-Hydroxytryptamine, 5-HT), and norepinephrine (NE) monoaminergic signalling and neuromodulatory systems are activated in response to a threatening stimulus in mammals (Goldstein et al., 1996; Millan, 2003). Early investigations demonstrated that exposure of rats to uncontrollable footshocks resulted in decreased cortical levels of tissue NE and in increased levels of noradrenergic metabolites. The same stimuli accelerated the metabolism of DA and 5-HT without changing the absolute level of these amines (Bliss, 1968). In fish, exposure to skin extract/alarm cues increased DAergic activity in the telencephalon (Höglund et al., 2005a). The authors state that these results suggest that the telencephalic DA systems are important for threat sensitive behaviour. In this study, the observed active and passive avoidance responses appear to be related to the increase in telencephalic DAergic activity. Further studies will need to be carried out to conclude which structure of the telencephalon was involved in this response.

Still very little is known about the relationship between threat-sensitive behaviour and monoaminergic function in fish. 5-HT is a monoamine neurotransmitter involved in a wide range of behaviours in humans and experimental animals (Cools et al., 2008). The serotonergic system seems to be highly conserved among vertebrates

(Krek and Dietrich, 2008) and low serotonergic activity has been implicated in a general behaviour inhibition such as after exposure to predator threat (Winberg et al., 1993). In humans, low levels of 5-HT are linked with enhanced brain response to threat stimuli such as fearful faces (Cools et al., 2008). Also, in lower vertebrates such as fish, higher 5-HT activation has been linked to low ranks in a social hierarchy (Winberg and Nilsson, 1993b), reduced feed intake (Øverli et al., 1998) and reduced aggression (Höglund et al., 2005b). Furthermore, brain serotonergic activity was negatively correlated with risk-taking behaviour in sticklebacks (Bell et al., 2007). These authors showed that risk-taking behaviour under predation risk was positively correlated with 5-HT and negatively associated with 5-HT turnover. Also, higher 5-HT turnover has been shown in fish exposed to alarm cues without having acces to hiding substrate (Höglund et al., 2005a). However, even if the latter studies suggest an involvement of central 5-HT in threat sensitive behaviour, the actual role of 5-HT in this behaviour remains unclear. 5-HT signalling is studied in this context using a brain punch technique to discriminate regional brain patterns of activation in paper II and IV of the present thesis.

1.6.Animal personality

The study of consistent individual variation in behaviour, physiology and cognitive/emotional patterns has become a central subject in a wide range of different biological disciplines; ranging from evolutionary ecology to health sciences (Koolhaas et al., 1999, 2008, 2010; Gosling, 2001; Sih et al., 2004; Cavigelli, 2005; Korte et al., 2005; Réale et al., 2007; Favati et al., 2014) and has even been used to explain political and social phenomena (Anderson and Summers, 2007). In the scientific literature, these consistent individual behavioural differences are alternatively referred to as personality (Gosling, 2001), temperament (Réale et al., 2007) or shyness/boldness (Wilson et al., 1994), when consistent over time and across situations. Some researchers go further in the conceptualisation of personality to include not only consistency in single behavioural traits, but also correlations between multiple traits. This approach has yielded definitions such as behavioural syndromes (Sih et al., 2004) and coping styles (used when behavioural patterns correlate to consistent physiological traits, see Koolhaas et al., 1999).

Sih et al. (2004) defines behavioural syndromes as sets of correlated behaviours which are consistent over time and across situations, while Koolhaas et al. (1999) defines coping styles as a set of behavioural and physiological stress responses which are consistent over time and characteristic to a certain group of individuals. A basic characteristic behind all terminology and underlying definitions is that they reflect differences that are consistent over time. From a behavioural neuroscience perspective, it is reasonable to propose that such consistency also reflects individual variation in underlying causal mechanisms (Øverli et al., 2007; Coppens et al., 2010).

Although possibly an oversimplification, consistent and correlated behavioural, physiological and cognitive/emotional patterns tend to be categorized in two contrasting adaptive strategies, proactive and reactive (Koolhaas et al., 1999; Øverli et al., 2007). Such coherent variation in physiology and behaviour, i.e. coping styles, are categorized by the discrepancy between two primarily different types of behavioural and physiological stress responses (proactive and reactive individuals), and a series of common patterns have been identified throughout the vertebrate subphylum (Koolhaas et al., 1999; Groothuis and Carere, 2005; Øverli et al., 2004a; 2007; Schjolden and Winberg, 2007; Coppens et al., 2010; Ruiz-Gomez et al., 2011; Castanheira et al., 2013). Behaviorally, proactive individuals are categorised by high levels of aggression, territorial control, active avoidance and other behavioural responses that suggest active efforts to counteract a negative stimulus (Koolhaas et al., 1999 and 2010). Reactive individuals on the other hand are usually categorised by low levels of aggression and passive avoidance of a negative stimulus. Furthermore, proactive individuals typically show low levels of behavioural flexibility and seem to be best adapted to stable environments (Benus et al., 1991; Verbeek et al., 1994; Bolhuis et al., 2004; Ruiz-Gomez et al., 2011). Physiologically the proactive individuals are characterized by low hypothalamus-pituitary-adrenal (HPA axis) reactivity to stress and low parasympathetic reactivity, while sympathetic reactivity is high. In contrast, animals with a reactive coping style exhibit high HPA reactivity, high parasympathetic reactivity and low sympathetic reactivity (Koolhaas et al., 1999; Table 1). Furthermore, proactive individuals seem to be more susceptible to develop hypertension and arthrosclerosis (Koolhaas et al., 1999) and reactive individuals more susceptible to develop infectious diseases (Korte et al., 2005).

Table 1. Summary of the behavioural and physiological differences between proactive and reactive coping styles.

Behavioural characteristics	**Reactive**	**Proactive**
Attack latency	High	Low
Active avoidance	Low	High
Routine formation	Low	High
Immobility	High	Low
Behavioural flexibility	High	Low
Physiological characteristics		
HPA/I-axis reactivity	High	Low
Sympathetic reactivity	Low	High
Parasymphathetic reactivity	High	Low

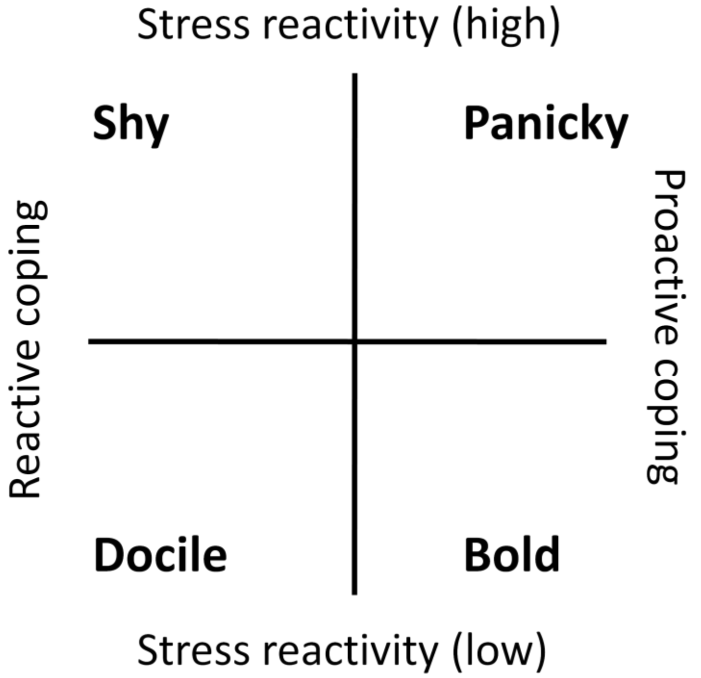

Figure 2. Two-tier model with coping style and stress reactivity as two independent dimensions. (Based on Koolhaas et al., 2010).

Steimer et al. (1997) suggested for the first time, that stress reactivity and coping-styles may be two independent dimensions. These authors presented a two-tier model (Figure 2) in which stress reactivity is independent of the coping style dimension defined as the way in which emotion is expressed behaviourally. The interpretation is that the quality of the behavioural response may be a dimension that is independent from the magnitude of the response (Koolhaas et al., 2010). This model may explain why some authors did not find correlations between plasma cortisol levels in stressed individuals and behavioural data (e.g. Silva et al., 2010).

The presense of contrasting coping styles appears to facilitate phenotypic diversification in populations confronted with fluctuations in the environment over time. For instance, proactive individuals display a set of behaviours that increase survival and reproductive success in stable environments, while reactive individuals perform better in unstable environments (Sih et al., 2004). Several conserved patterns support the adaptive theory of coping styles, and comparative research on fish provided early documentation on both individual consistency in behaviour (Huntingford, 1976; Wilson et al., 1993) and underlying proximate physiological mechanisms (Øverli et al., 2005; Schjolden et al., 2005a).

Individual variation in several traits like growth, stress response and behaviour has been described in several fish species (Jobling and Reinsnes, 1986; Jobling and Koskela, 1996; Carter et al., 1998; Carter and Bransden, 2001; Martins et al., 2005, 2006; Øverli et al., 2006a). Like in other species, for some time this variation was considered as a consequence of the establishment of social hierarchies or was even interpreted as the result of inaccurate measurements or as a non-adaptive variation around an adaptive mean (Groothuis and Carere, 2005). However, further studies demonstrated that individual variation is also a consequence of inherent genetic factors (Øverli et al., 2002; Martins et al., 2005; Schjolden and Winberg, 2007). In the absence of social hierarchies, fish still show individual variation in growth, stress response and behaviour, and such variation is consistent over time (Martins et al., 2005; van de Nieuwegiessen et al., 2008) and generations (Pottinger and Carrick, 1999).

One of the most used parameters to assess different coping styles in fish is the resumption of feed intake in novel environments or after stress (Øverli et al., 2006a; 2007; Kittilsen et al., 2009a and b; Silva et al., 2010; Martins et al., 2011a; Basic et al., 2012). Novel object test, latency reaction time to introduced food (Silva et al., 2010) were other behavioural methods previously used to assess personalities in fish. The link between coping styles and emotions, such as fear, has been addressed in humans, non-human mammals and birds. Different personality types have been shown to differ in emotional reactivity (Steimer et al., 1997) and the reactivity to negative appraisals (Tong, 2010). Fear reactivity has been shown to be a dimension of temperament in humans (Rothbart and Jones, 1998; McCrae and Costa, 1997) influencing the susceptibility to depression and anxiety (Shin and Liberzon, 2010). Therefore understanding coping styles is necessary for the study of fear/threat responses as fear-inducing stimuli may be perceived diffently between individuals. Furthermore, understanding the link between coping styles and threat sensitive behaviour in fish may unravel whether emotions are an essential component of coping styles in species distributed throughout the vertebrate subphylum. This topic will be addressed in paper III.

1.7.Contrasting coping styles as a tool to study the neurobiology of fear

As stated before threat-sensitive behaviour or fear can be defined as the activation of a defensive emotional and behavioural system that allows animals or humans to counteract or minimize the effect of possible environmental threats. As mentioned above, even in fishes proactive individuals tend to form routines and behave consistently in changing environments (Ruiz-Gomez et al., 2011). Whether these differences rely on how environmental stimuli are perceived or interpreted remains largely unknown in fishes (but see Millot et al., 2014b), and it would therefore be of interest to study how contrasting coping styles react when presented with a threatening stimulus, by which some kind of behavioural reaction is provoked.

In 1996, Dr. Tom Pottinger at the Windermere Laboratory, Natural Environment Research Council Institute of Freshwater Technology, UK started a selection program to develop two lines of rainbow trout with contrasting (high vs low) post-stress cortisol levels. The two developed lines were the high-responding (HR) rainbow trout and the low-responding (LR) rainbow trout (Pottinger and Carrick, 1999). As their names suggest, HR fish were selected for having a consistent high cortisol response to stress while the LR fish were selected for having a consistent low cortisol response to stress. Initially, the selection program was carried out for aquaculture purposes, however, HR and LR lines have demonstrated to differ in a number of behavioural aspects indicating that behavioral traits were co-selected with cortisol stress responsiveness (Pottinger and Carrick, 2001; Øverli et al., 2002). Throughout the past years HR/LR rainbow trout lines have been a subject of interest and a great deal of research has been conducted using these lines (Øverli et al., 2004a, 2006b, 2007; Schjolden et al., 2005b, 2006; Pottinger, 2006; Höglund et al., 2008; Ruiz-Gomez et al., 2008, 2011; Basic et al., 2012).

Behaviourally, HR fish tend to be socially subordinate when compared to LR individuals and subjected to size matched contests (Pottinger and Carrick, 2001; Øverli et al., 2004a, 2005, 2007; Scholden and Winberg, 2007), although such behaviour can be context dependent (Ruiz-Gomez et al., 2008). Furthermore, HR fish resume feeding slower than LR fish (Øverli et al., 2002; Ruiz-Gomez et al., 2011) and have a higher locomotor response to stress (Øverli et al., 2002, 2007). Another interesting fact is that these lines also differ in their predisposition to retain learned information (Moreira et al., 2004) and develop and follow routines (Ruiz-Gomez et al., 2011). In the latter experiment HR fish exhibited a more flexible foraging behaviour than LR fish, with the latter retaineing learned feeding routines after food patches had been experimentally relocated. Due to the possible intrinsic relationship between the retention of acquired threat information and other cues, it could be hypothesised that threat sensitive behaviour may also differ between individuals depending on coping style. Physiologically, HR fish respond to stress with a low sympathetic activity (blood epinephrine) when compared to LR fish (Scholden et al., 2006). In terms of neurobiology a higher monoaminergic activity was shown in HR fish when compared to LR fish when these were exposed to confinement stress (Scholden et al., 2006). The traits found until now for these rainbow trout lines, show similarities to the traits indicating a proactive and reactive stress coping style (Table 2), with HR fish showing reactive patterns and LR fish showing proactive patterns.

Table 2. Resume of behavioural and endocrine profiles of HR and LR rainbow trout models described in the literature.

	HR	**LR**
Aggression	LOW	HIGH
Avoidance	HIGH	LOW
Boldness	LOW	HIGH
Cortisol response (HPI-output)	HIGH	LOW
Flexibility	HIGH	LOW
Locomotor response	HIGH	LOW
Routine formation	LOW	HIGH
Sympathetic activity	LOW	HIGH

In a study by Moreira et al. (2004) HR and LR fish were exposed to a paired conditioned stimulus (CS-water off) and unconditioned stimulus (US-confinement stressor). After 18 pairings of CS-US, most individuals of both lines acquired a conditioned response manifested by the elevation of blood cortisol. Afterwards, both lines were post-conditioned. In post-conditioning fish were exposed to the CS alone on a weekly basis to compare the extinction of the conditioned response. The results showed that HR fish retained the conditioned response for a shorter period than LR fish suggesting differences in cognitive function. These results can be interpreted as a conditioned threat response since fish are presented with a CS followed by an aversive US. Hence it would appear that LR fish retained the conditioned threat response for a longer period than HR fish, suggesting differences in threat extinction. This topic will be addressed in paper III of the present thesis using Nile tilapia. Furthermore in paper IV of the present thesis using the HR/LR model plasticity in learned responses under threat will also be addressed.

Research over the past years has provided evidence that coping behaviours and correlated physiological outputs are subserved by a basic circuitry of emotional limbic areas which include prefrontal cortex, nucleus accumbens, amygdala, BNST (bed nucleous of the stria terminalis), septum, hippocampus, and hypothalamus (Koolhaas et al., 2010). This circuitry is highly dependent on brainstem ascending monoaminergic inputs (Dalgleish, 2004).

Differences in 5-HT function are associated with individual differences in temperament and personality in mammals as well as non-mammalian vertebrates (Schjolden et al., 2006; Koolhaas et al., 2010). For example, the 5-HT system has been indicated to be essential in the control of aggressive behaviour in several animal species including fish (e.g. Silva et al., 2013). Research on the establishment of social dominance during dyadic contests, shows that increased serotonergic activity is commonly seen in subordinate individuals (Winberg et al., 1991; 1992a; 1993; Winberg and Nilsson, 1993a; Øverli et al., 1999; 2001; Höglund et al., 2000; Schjolden et al., 2006). These data suggest that reactive coping strategies are associated with increased serotonergic activity, but also opens up for potential confusion regarding causes and effects of social position (see e.g. commentary by Øverli, 2007b). Aggression in vertebrates is also regulated by DAergic activity. Evidence suggests that elevated DA levels are associated with increased levels of aggression and dominance in fish (Winberg et al., 1991; Winberg and Nilsson, 1992; Vindas et al., 2014b).

Furthermore, Valentino et al. (2010) have proposed and reviewed that different coping strategies can be mediated by the neuropeptide corticotropin-releasing hormone (CRF). These authors support a model in which CRF_1 and CRF_2 receptors promote different coping-styles. CRF_1-mediated inhibition and CRF_2-mediated excitation of the 5-HT system in the dorsal raphé facilitate proactive and reactive coping strategies, accordingly.

2. Outline of the thesis

The aim of this thesis was to study the neural basis of threat-sensitive behaviour in teleost fish, by focusing on brain monoaminergic activity and the magnitude of individual differences. In this context, the role of coping styles in explaining individual differences in threat sensitive behaviour and monoaminergic neurotransmission was particularly addressed.

The thesis is composed of a general introduction, material and methods, summary of results and a discussion where the findings are summarized and discussed in a wider context. This thesis also includes 4 experimental chapters (Papers I-IV).

The first paper of this thesis focuses on individual differences in behaviour in Nile tilapia (*Oreochromis niloticus*), one of the most produced species in aquaculture worldwide. Resumption of feed intake in a novel environment, latency to react to introduced food and feeding anticipation were used to assess different coping styles in Nile tilapia. Crucial to the definition of coping styles, the consistency in behaviour was also studied. Furthermore, this study correlated serotonergic activity in the hypothalamus with a collapsed principal component incorporating all the mentioned feeding parameters (i.e. an overall measure of feeding motivation). The results confirmed that individual variation in brain serotonergic neurotransmission correlates to a complex behavioural syndrome related to feeding motivation.

The second paper of the thesis investigated to what extent Nile tilapia exhibits a physiological, behavioural and monoaminergic response to alarm cues, one of the most used paradigms to study threat-sensitive behaviour in fish. Alarm cues decreased foraging behaviour. However, no changes in monoaminergic activity were detected when comparing to a control group.

In the third paper of this thesis, another paradigm, avoidance conditioning, was used to study if individual variation in personality traits predict threat sensitive responses in Nile tilapia. Fish previously screened for personality traits were given the possibility to escape a signalled aversive stimulus. This study showed that individual personality traits predict how stimuli are appraised and the subsequent degree of threat sensitive behaviour.

In the fourth and last paper, I aimed to utilize an established comparative model of contrasting coping styles, the HR/LR trout lines, to investigate to what degree proactive and reactive individuals react differently to threat. In this case a social avoidance paradigm was used because it has been previously validated in rainbow trout. HR/LR fish were exposed to a larger dominant fish and given the possibility to escape. After seven encounters transparent walls were inserted in the escape route or between both fish. This study showed that LR fish attempted to escape more often and took less time to escape when physically in contact with a bigger fish. When exposed to the visual contact only, the LR fish exhibited a higher locomotor behaviour than HR fish. This experiment also provided insight into the role played by monoamines in social induced threat-sensitive responses.

Figure 3 demonstrates all driving questions and decision points made throughout the development of this thesis.

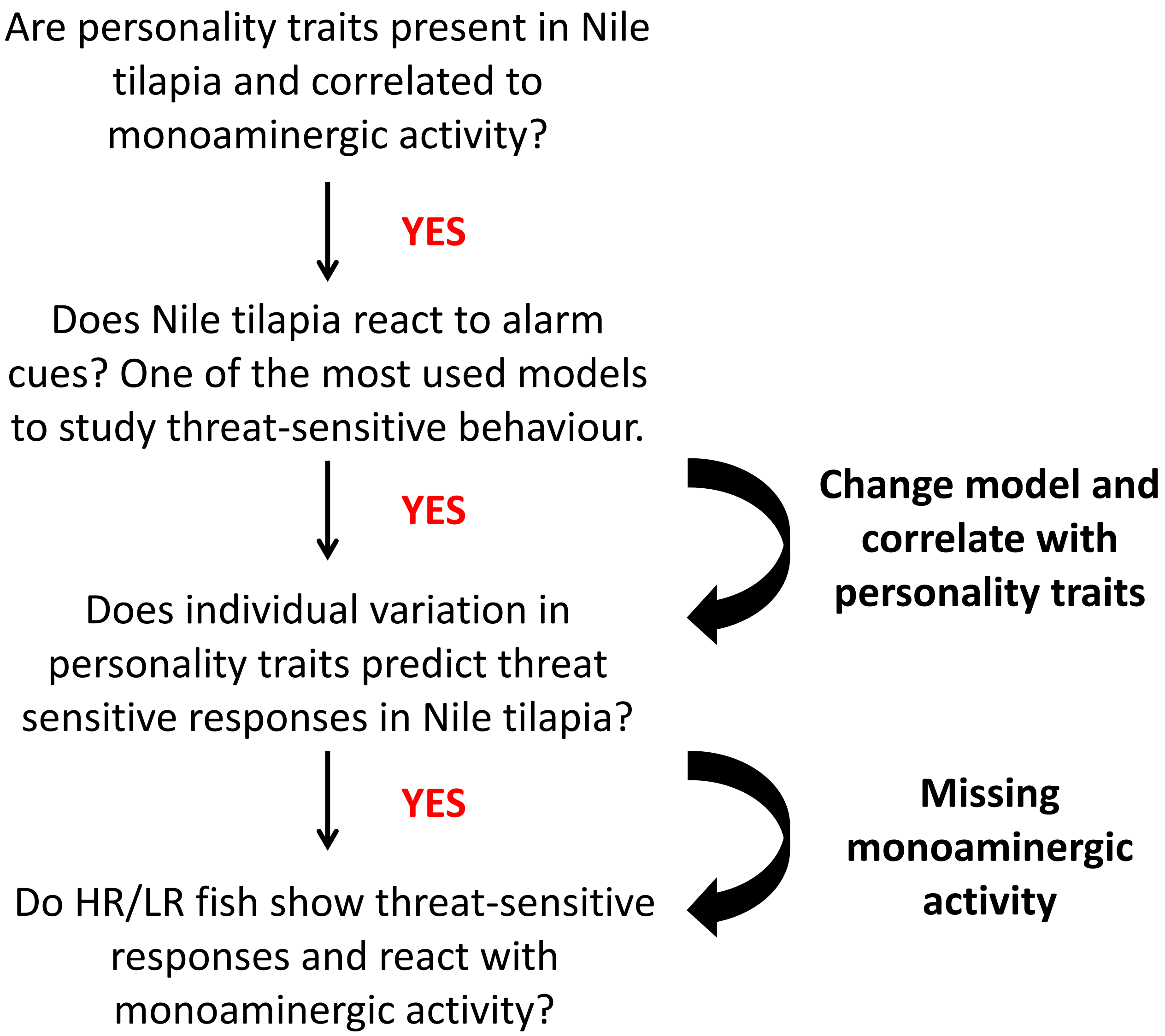

Figure 3. Driving questions and decision points.

3. Material and Methods

3.1. Experimental fish and housing conditions

In the experiments presented in this thesis, Nile tilapia (*Oreochromis niloticus*) and HR/LR rainbow trout (*Oncorhynchus mykiss*) were used as experimental animals. Nile tilapia were used as one of the model fish species in these experiments because it is one of the major fresh water species being commercially cultured worldwide. Furthermore, its production in RAS using lower water exchange rates is expected to increase in the coming years, and thus potential problems with the accumulation of alarm cues in the water. In the last experiment HR/LR rainbow trout lines were chosen due to their well studied characteristics with respect to behaviour and physiology.

In papers I and II thirty sexually immature Nile tilapia (23.53 ± 3.47 g) (mean ± SD) were used. All fish were randomly netted and transferred from the holding tank into isolation. Observation aquaria were divided into four chambers each with size 22 x 25 x 50 cm (width x length x depth) and each fish was kept isolated in one chamber. All fish were originated from the 14th generation of Genomar Supreme tilapia, GST$^{(tm)}$, and kept at the fish-lab facilities at the Norwegian University of Life Sciences. In paper III, forty-two juveniles of Nile tilapia with an initial body weight of 40.8 ± 6.5 g were used. From these, 24 individuals, randomly selected, were used to characterize coping styles and avoidance learning while the remaining fish were used as controls in the avoidance learning test. All fish were housed individually in a 40-L glass aquarium (40 cm length×30 cm width×35 cm height, 30 L water capacity).

In paper IV, 24 HR (321.63 ± 86.02 g) and 21 LR (244.02 ± 51.00 g) rainbow trout were used. All fish were obtained at the Danish Technological University (DTU-Aqua). Each individual was transferred from the holding tank and placed individually and randomly in a chamber with size 50 x 25 x 50 cm. In this experiment, 11 large brood-stock rainbow trout (1018.63 ± 115.98 g), also obtained at DTU-Aqua, were used as large dominant fish. Each of the dominant fish was placed individually in a chamber with size 50 x 50 x 50 cm next to the chamber of the previously described fish.

Fish used in all experiments were kept in recirculation systems under a 12h:12h light:dark photoperiod.

3.2.Feeding in isolation

During isolation, in all experiments fish were hand fed twice a day. In papers I, II and IV, fish were offered pelleted food, one at a time, allowing for registration of the number of consumed pellets and feeding latency. Furthermore, behaviour during each feeding occasion was scored according to a 4-step scale (Table 3). Accumulated scores reflect how quickly fish resume normal feeding behaviour.

Table 3. Feeding scores during feeding tests.

Score	Behaviour
0	Fish does not respond to food.
1	Fish eats only pellets that fall directly in front of the snout, and does not move to take food.
2	Fish moves more than one body length to take food, but returns to the original position in the aquarium between each food item.
3	Fish moves continuously to take each food item.

In paper III fish were hand fed *ad libitum*, for a maximum period of 1 h, after which the remaining pellets were collected and counted. The average feed intake was used as indicative of feed intake recovery.

3.3.Conditioning with alarm cues

In paper II fish were exposed to alarm cues. Each fish conditioned with alarm cues was video recorded for 30 minutes, during which they received two 5 ml injections of skin extracts. The first injection was given after 10 minutes and the second after 20 minutes to guarantee the exposure of the fish to stable levels of alarm substance. The videos were analyzed for locomotory activity and bottom-grazing behaviour. For the preparation of the used skin extract, Nile tilapias were killed by decapitation and skin was taken from the sides of the fish. Approximately 2 g of skin were homogenized in 100 ml of distilled water. The homogenate was centrifuged at 2400 rpm for 5 min at 4 ºC. The supernatant was frozen and a concentration of 1:5 in distilled water was used in the aquariums.

3.4.Classical conditioning

In paper III a classical conditioning was applied. Four different experimental groups of fish were established in this experiment: A treatment group (*T- learning*) underwent the full avoidance learning test utilising a signalled aversive stimulus (unconditioned stimulus, US). The conditioned stimulus (CS) consisted of stopping the water inflow for 30 sec. The US consisted of lowering an iron frame into the tank until it was touching the dorsal fin of the fish. It remained there for a period of 15 min. Additionally, 3 different control groups were established (*C1- water off*, *C2- confinement* and *C3- water off/confinement*). Controls were used to test the influence of the CS only (*C1*: fish were exposed to *water off* once daily during 8 days), US only (*C2*: fish were exposed during 8 days to the confinement frame only, without previous signaling) and the CS–US pairing (*C3*: fish were exposed to CS–US pairing for 8 days). *C3* and *T* were exposed to the same procedures during 7 days of training, but on day 8, *T* was exposed to CS only while *C3* to CS followed by US. Each tank was divided in 2 partitions using a PVC divider containing an escape door (half circle, 8 cm diameter) that was opened upon CS presentation. Fish were trained to associate US with CS for 7 days (1 training session per day). The latency to escape (i.e. to swim to the side with no confinement frame) was determined daily. In addition to the latency to escape, the time taken between the first escape and the first return, the total number of returns and the total time spent in the (previous) confinement area were registered. These behaviours were used as a measure of the degree of responsiveness to a frightening stimulus.

3.5. Agonistic behaviour

Salmonids establish social hierarchies by displaying aggressive behaviour. In paper IV agonistic behaviour was used as a social stressor and was quantified from video recordings. Each encounter between test fish and large brood-stock fish lasted for 15 minutes. Agonistic behaviour was quantified as the following: latency to first attack, total number of attacks, submissive behaviour and escape time. Attacks were characterized as one fish biting another and was is usually the culmination of a charge. In this experiment each test fish was given the opportunity to escape from the large dominant fish and the time needed to escape was recorded.

3.6. Cortisol Analysis

Body cortisol was measured in Paper II and III. In paper II 4g of white muscle of each fish were separated for quantification of cortisol. Each sample was divided in two subsamples (2g each) and one was used for monitoring procedural losses which, was spiked with 450ng of Hydrocortisone (HO888 – 1G Sigma). Each of the subsamples was homogenized in 2ml of PBS and resuspended in 20ml of ethyl acetate for extraction. Ethyl acetate was separated from the tissue by centrifugation and evaporated by vacuum centrifugation. Dry residue was then resuspended twice (2×3ml) in 30% v/v methanol - mili-Q water, filtered on a 0.2µm filter and loaded on a 500mg Amprep C_{18} microcolumn. Impurities were washed out with 10ml of mili - Q water and cortisol was eluted in 2.5ml of 90% v/v methanol - mili-Q water. The subsamples were dried for a second time by vacuum centrifugation, resuspended in 400µl of HPLC buffer, filtered through a 0.2µm filter. After extraction each subsample was run through HPLC for cortisol separation and later quantified by ELISA (Neogen, #402710). For specific assay procedures please refer to paper II.

In paper III plasma cortisol levels were measured with a commercially available competitive binding Coat-A-Count® Cortisol kit (SIEMENS Medical Solutions Diagnostics, Los Angeles, CA, USA). Briefly, 50 µl of blood plasma was transferred into an Ab-Coated tube and 1 ml of ^{125}I Cortisol added. The tubes were then incubated for 45 min at 37°C in a water bath. The contents of all tubes were decanted, and allowed to drain for 5 min before being readonagammacounter (2470 WIZARD^{2TM}, PerkinElmer^{TM}, Inc., Zaventem, Belgium) for 1 min. A calibration curve was constructed on logit-log graph paper and used to convert results from percent binding cortisol to concentration (ng ml^{-1}).

3.7.Analysis of brain monoaminergic activity

High pressure liquid chromatography (HPLC) with electrochemical detection was conducted in papers I, II and IV in order to quantify brain monoamine neurochemistry. In short, dissected brains were placed in tissue-tek, frozen on dry-ice and then sliced in serial 300-µm sections. Afterwards, brain slices were quickly thaw mounted on glass slides, and immediately refrozen at -80ºC for microdissection using a brain punch technique. Brain punch samples (including an internal standard) were then run through an HPLC system by a mobile phase in order to separate its components through a filter column. The monoamines in the samples were analyzed in contrast with standards of known concentrations and actual sample concentrations were quantified. For specific procedures please refer to papers I, II and IV.

4. Synopsis of Results

4.1. Paper I

The primary goal of paper I was to assess the level of consistency in different measures of feeding behaviour, to scrutiny which measures of feeding could be best used to evaluate consistent personality traits, and further to determine if serotonergic activity in the hypothalamus was correlated with these measures. Using Nile tilapia as a model, a strong degree of individual consistency in different measures of feeding behaviour (feeding latency and feeding score) was observed. Furthermore, low serotonergic activity in the hypothalamus was highly correlated with a personality characterized by high feeding motivation. Feeding motivation was represented as an overall measure, incorporating several behavioural parameters; feeding latency, days to achieve latency zero, grazing behaviour, and locomotory activity. The data was examined using a Principle Component Analyses (PCA) (Figure 4). This study thus confirms individual variation in brain serotonergic neurotransmission as a correlate to complex behavioural syndromes related to feeding motivation.

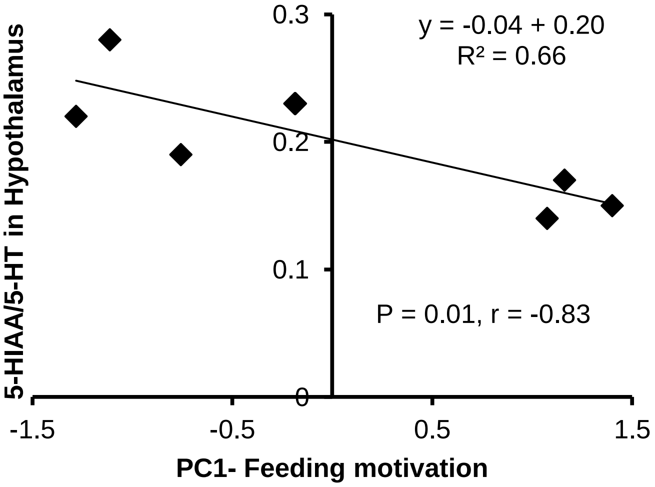

Figure 4. Relationship between serotonergic activity in the hypothalamus (5-HIAA/5-HT) and feeding motivation (N=8). Pearson correlation r and P values are given on graph.

4.2.Paper II

In paper II the involvement of dorsolateral (Dl) and dorsomedial telencephalon (Dm) in the response to stress and predatory cues was investigated by exposing Nile tilapia to a standardized confinement stress and to skin extracts from conspecifics. Also, the effects of skin extract on feeding anticipatory activity and locomotor activity was investigated to characterize threat sensitive behaviour in this species. Confinement stress resulted in an elevation of cortisol, and serotonin metabolism in Dl and Dm. Even if the result was not significant, the same general pattern was seen after exposure to alarm cues. Nile tilapia responded behaviourally to conspecific skin extract by reducing feeding anticipatory behaviour. This may reflect a general elevation of awareness, and further studies combining skin extract with other challenges are needed to reveal neuroendocrine effects associated with this predatory cue.

4.3.Paper III

The primary goal of paper III was to investigate if different coping styles can predict fear responses in Nile tilapia using the principle of avoidance learning (combination of classical and operant conditioning). In this study fish previously screened for coping style were given the possibility to escape a signalled aversive stimulus. Proactive individuals were characterized by having a faster feed intake recovery after transfer into a novel environment and a less neophobic behaviour when exposed to a novel object, as compared to reactive individuals. Fish avoiding the area of previous confinement (aversive stimulus) were the fish exhibiting characteristics usually ascribed to reactive individuals. Also, cortisol concentrations were strongly linked to behaviours indicating fearfulness. In summary, fearful individuals showed a range of typically reactive traits; such as slow recovery of feed intake in a novel environment, neophobia, and high post-stress cortisol levels.

4.4.Paper IV

The primary goal of paper IV was to investigate if physical (i.e. neurogenic) and psychological (psychogenic) stressors (c.f. Anisman et al., 1997) affected individuals with contrasting stress coping styles differently, and if this is reflected in monoaminergic neurotransmission in areas with functional homologies to the mammalian hippocampus and amygdala. In order to achieve this, the HR/LR trout model and a social learning avoidance paradigm were used. In the group subjected to physical stress (physical interaction with a large dominant fish) LR fish attempted to escape more frequently than HR fish when in contact with a larger sized dominant fish, even though the strains suffered the same number of attacks. Also, latency to attempt to escape differed between HR and LR individuals, in that LR fish started to show escape behaviour sooner than HR fish. In the group subjected to psychological stress (visual exposure to a large dominant fish), differences in locomotor activity between HR and LR were observed during the first 5 minutes after insertion of transparent walls. Differences in monoaminergic activity between strains (HR/LR) and treatments are reported in detail in paper 4.

5. Discussion

In this thesis, I investigated the neural basis of threat-sensitive behaviour in teleost fish, by focusing on brain monoaminergic activity and the magnitude of individual differences in the behavioural response to threatening and/or stressful stimuli. The concept of stress coping styles (Koolhaas et al., 1999) was employed in explaining individual differences in threat sensitive behaviour and monoaminergic neurotransmission. The link between coping styles and emotions such as fear has previously been investigated in mammals and birds. It is generally understood that individual variation in the threshold for when a stimulus becomes inhibiting rather than stimulatory is likely correlated to the individuals' subjective experience of that stimulus in a given situation. Understanding the link between coping styles and threat sensitive behaviour in fish may thus unravel whether emotions are an essential component of coping styles in species distributed throughout the vertebrate subphylum.

To develop this thesis a series of questions were asked. The first question was whether a set of correlated behavioural traits could be explained by variation in brain monoaminergic activity. In paper I, using Nile tilapia it was demonstrated that individual variation in hypothalamic 5-HT neurotransmission correlated to complex behavioural syndromes related to feeding motivation. In paper II it was shown that Nile tilapia responded to conspecific alarm cues by reducing bottom-grazing behaviour. Furthermore, acute stress in the same species resulted in cortisol elevation, as well as increased serotonergic activity in the dorsolateral and dorsomedial area of the telencephalon. The same general neuroendocrine pattern was seen after exposure to alarm cues. However, this response was less pronounced than in acutely stressed fish, and not statistically separate from non-stressed controls. In paper III it was demonstrated that individual variation in coping styles predicts fear responses in Nile tilapia, using the principle of avoidance learning. Fish previously screened for coping style were given the chance to escape a signalled aversive stimulus. Fearful individuals showed a range of typically reactive traits such as slow recovery of feed intake in a novel environment, neophobia, and high post-stress cortisol levels. Finally in paper IV it was further demonstrated that proactive LR individuals have a higher tendency to establish and maintain routines, even under stressful conditions. It is assumed that the dorsolateral (Dl) and dorsomedial (Dm) telencephalon in fish correspond in function to the mammalian hippocampus and amygdala, respectively, and the results pinpoint elevated dopamine (DA) signalling in these limbic structures as underlying generally more fearful behaviour in HR fish. In fact, LR fish responded with increased DA metabolism only to physical interaction with a larger conspecific, while simultaneously showing a reduced ability to change previously established avoidance routines.

5.1. Are coping styles present in Nile tilapia and correlated to monoaminergic activity?

Different aspects of feeding behaviour were used in paper I to characterize coping styles since feeding behaviour in novel environments have been used previously as personality measures, due to their correlation with both physiological traits such as stress induced cortisol production (Øverli et al., 2002; 2007) and behavioural characteristics such as locomotor response to acute stress (Øverli et al., 2006a) and the ability to win fights for social dominance (Øverli et al., 2004a). In this first study we demonstrated that both feeding latency and feeding score (measured during nine days after transfer to social isolation) were consistent over time in Nile tilapia. Furthermore, feeding latency was correlated with bottom grazing behaviour and locomotory activity, measurements taken when fish were fully acclimatized. Consequently, feeding latency taken during acclimation to a new environment also predicts feeding anticipatory behaviour in fully acclimatized fish, indicating the presence of personality traits and coping styles. To this point, our results are in agreement with previous studies indicating the presence of coping styles in Nile tilapia (Barreto and Volpato, 2011), as well as an established role for 5-HT in feeding behaviour (de Pedro et al., 1998; Øverli et al. 1998, Ortega et al., 2013).

From a proximate viewpoint, it is reasonable to believe that behaviours may be correlated because they share the same neurobiological, neuroendocrine and/or genetic mechanisms (Coppens et al., 2010). In paper I it was indicated (although by a correlative approach only) that serotonergic activity in the hypothalamus mediates feeding motivation. Fish with lower serotonergic activity in the hypothalamus took shorter periods of time to achieve an immediate reaction to distributed food (zero latency) and exhibited higher bottom-grazing behaviour and locomotory activity prior to feeding. Hence, it would appear that fish with lower serotonergic activity in the hypothalamus are generally more motivated towards appetitive stimuli such as food. Notably, serotonergic activity in the hypothalamus was correlated to a complex behavioural syndrome, components of which were recorded a considerable time prior to the neurochemical measurement. Clearly, the 5-HT system of both fish and mammals is also very responsive to environmental stimuli, particularly stressful stimuli (Winberg et al., 1992b; 1993a; Bethea et al., 2005; Sloman et al., 2005; Beekman et al., 2005; Hegazi and Hasanein, 2010; Weber et al., 2012) as well as corticosteroid hormones (Medeiros and MacDonald, 2013), but in a stable environment the individual variation may well be generated endogenously. This, in turn, indicates the presence of chronic individual differences in 5-HT transmission, caused either by genetic differences (Lesch and Merschdorf, 2000; Donaldson et al., 2013; Klucken et al., 2013) or early experiences (Lesch and Merschdorf, 2000; Lukkes et al., 2009). After demonstrating that feeding motivation (measurement used to characterize coping styles) was correlated with monoaminergic activity, I wanted to determine how Nile tilapia reacted to alarm cues, one of the most used models to study threat sensitive behaviour.

5.2.What are the behavioural and physiological responses of Nile tilapia in the presence of alarm cues?

In order investigate the responses to alarm-cues in Nile tilapia, changes in locomotor activity and feeding anticipatory behaviour in response to skin extracts were investigated. Specificity of the neuroendocrine response was also adressed by comparing muscle cortisol and brain monoaminergic responses in Dl and Dm between undisturbed controls, fish exposed to skin extracts, and fish exposed to acute confinement stress.

Confinement stress resulted in significantly elevated cortisol values and increased serotonergic activity in Dl and Dm. Several studies indicate that stress increases serotonergic activity in the telencephalon in fish (e.g. Winberg et al., 1992b; Øverli et al., 2004b; Schjolden et al., 2006; Gesto et al., 2013), and serotonin in turn regulates both physiological and behavioural stress responses (Medeiros et al., 2010; Nowicki et al., 2014). However, to my knowledge there are relatively few studies focusing on the involvement of regional brain monoamine signalling in Dl and Dm in response to stress (Øverli et al., 2004b; Basic et al., 2013; Vindas et al., 2014). My results (paper II) clearly show that confinement stress resulted in a distinct rise in serotonergic activity in Dl and Dm, manifesting the involvement of these brain structures in the stress response. The same general pattern was seen after exposure to alarm cues, i.e., increased cortisol concentration levels and serotonergic activity in Dl and Dm. However, this response was not statistically different from controls and contradictory to studies carried out in rodents. For instance, rats exposed to fox odour displayed a significant elevation in corticosterone (Morrow et al., 2000). Furthermore, Hayley et al. (2001) demonstrated that mice exposed to a predator odour presented an increase in 5-HIAA and MHPG concentrations within the hippocampus and amygdala which are areas regarded as homologous to Dl and Dm regions in fish, respectively. In addition, Morrow et al. (2000) also found that rats exposed to predator odour exhibited an increase in dopaminergic activity in the amygdala. Moreover, it has been demonstrated previously that brain dopaminergic activity in the telencephalon of crucian carp is affected by skin extracts, suggesting that this neurotransmitter is affected by skin extracts (Höglund et al., 2005a). The fact that in our study no effects of skin extracts were found on dopaminergic signalling in limbic regions (Dl and Dm) of the brain in Nile tilapia suggests that skin extract exposure by itself was not an intense enough stimuli to significantly affect brain monoaminergic signalling. The fact that a significant effect of confinement stress was observed, but not of alarm cues, may indicate that the Dl and Dm regions are activated during confinement stress, while alarm cues produce a weaker activation of subsets of neurons in these areas. Alarm cues may also elicit a more regionalised effect within the structures studied, as both Dl and Dm are subdivided (Simões et al., 2012). Imaging methods may offer the possibility of studying the effects of alarm cues at a finer resolution further on.

Behaviourally, we could not detect any effects of skin extract on locomotor activity. Höglund et al. (2005a), on the other hand, described a decrease in locomotor activity in crucian carp after a repeated administration of skin extract. In Nile tilapia skin extract in stead reduced feeding anticipatory behaviour (paper II). The shift of behaviour from foraging to predator awareness may indicate a general anti-predator response by increased apprehension. In this context, apprehension is defined as a reduction or suppression of other activities such as foraging or mate seeking as a result of increased attention to detecting and/or responding to potential predators (Kavaliers and Choleris, 2001).

In summary, at this point of the thesis it had been demonstrated that Nile tilapia exhibit coping-styles which can be explained by different neurobiological responses and also exhibit a threat-sensitive response to alarm-cues. However, in the limbic brain regions investigated here the monoaminergic response to alarm-cues was not statistically distinguishable from undisturbed fish. Therefore, for the next step I opted for a new model to test threat-sensitive behaviour while testing whether variation in coping styles could predict different threat sensitive responses in Nile tilapia.

5.3. Does individual variation in coping styles predict threat sensitive responses in Nile tilapia?

Paper III confirmed that Nile tilapia exhibit divergent coping styles, with proactive individuals exhibiting a faster feed intake recovery after transfer into a novel environment and reduced neophobic tendency when exposed to a novel object, as compared to reactive individuals. In classical conditioning, repeated CS–US (conditioned-stimulus – unconditioned-stimulus) pairing resulted in the acquisition of a behavioural conditioned response (CR). In this study, behavioural conditioned response was observed after fish were exposed to the avoidance learning test and the escape behaviour differed significantly between groups (see summary of experimental groups of paper III in Table 4).

Table 4. Summary of experimental groups in paper III. One treatment group and 3 different control groups which were used to test the influence of CS only, US only and CS–US pairing. *C3* and *T* were exposed to the same procedures during 7 days of training, but on day 8, *T* was exposed to CS only while *C3* to CS followed by US. An escape door was opened upon CS presentation. Fish were trained to associate US with CS for 7 days (1 training per day).

Experimental Groups	
T-learning	Treatment group underwent the full avoidance learning test using a signalled aversive stimulus (US). The CS consisted of stopping the water inflow for 30 sec. The US consisted of an iron frame lowered into the tank until touching the dorsal fin of the fish, and then remaining there for 15 min.
C1- water off	Fish were exposed to *water off* - CS only.
C2-confinement	Fish were exposed to the confinement frame, without previous signalling - US only.
C3- water off/confinement	Fish were exposed to CS–US pairing.

In C1-water off, the use of the escape door was presumably more related to exploration than to escape behaviour. Fish exposed to the US alone or in combination with the CS escaped to the undisturbed side of the tank, and did not return during the observation period (15 min). Fish exposed to T-learning took longer to return to the initial position as compared to fish exposed to water off only. Despite fish in C1-water off and T-learning being exposed to the same stimuli (water off), their behaviour differed significantly suggesting that the way the stimuli was interpreted or appraised also differed. This indicates that Nile tilapia can learn how to avoid aversive stimuli by conditioning.

Presumably, the fish returned to the area of the tank where they had been confined as this area was used for feeding. Therefore, one possibility is that the motivation to feed played a role in returning to a potentially dangerous area. Avoidance learning has been a method used to investigate fear in different animal species (e.g. in fish: Yue et al., 2004; 2008). In our study, the observed differences in escape behaviour between fish exposed to C1-water off and T-learning suggest that these responses are not merely reflexive in nature but are associated with a subjective interpretation of the stimuli. The way individual fish behaved on T-learning group was correlated with traits indicative of coping styles. This suggests that the individual variation in how negative the CS was interpreted (negative appraisal) depends of an individuals' coping style. Even though several studies indicate the presence of both coping styles and emotions in fish, the link between both had never been investigated in fish. In paper III it is demonstrated that "risk-aversive" fish (i.e. avoiding the area of previous confinement) also previously had shown reactive patterns such as slower feed intake recovery in a novel environment, more neophobic behaviour, and higher HPI responsiveness after net restraining (Table 5).

Table 5. Correlation between variables indicating coping styles and fearfulness in Nile tilapia.

Coping styles/Fearfulness	Latency to escape (sec)	Time between 1st escape and return (sec)	# returns	Time spent in confinement area (sec)
Plasma cortisol after net restraining (ng/ml)	Ns	$r_s = 0.60$ $p = 0.009$	Ns	$r_s = -0.48$ $p = 0.025$
#escape attempts during net restraining	Ns	Ns	$r_s = 0.58$ $p = 0.005$	Ns
FI recovery novel environment (%BWd^{-1})	Ns	Ns	$r_s = 0.44$ $p = 0.04$	Ns
#times entering 10cm radius from novel object	Ns	Ns	$r_s = 0.54$ $p = 0.01$	Ns

(n = 22 when considering # of returns and time spent in confinement area 2 - 2 out of the 24 fish did not escape on day 8 - and n = 19 when considering the time between escape and return 2 - 2 out of the 24 fish did not escape on day 8 and 3 fish escaped but never returned to the confinement area).

The most plausible explanation is that proactive individuals were less fearful when presented with a signal previously associated with an aversive stimulus, when compared to reactive individuals. Fear is an important component of personality in humans (McCrae and Costa, 1997; Pineles et al., 2009), other mammals (e.g., in dogs: Svartberg and Forkman, 2002; in rats: Steimer et al., 1997; Steimer and Driscoll, 2003) and in birds (Garamszegi et al., 2008). In an evolutionary perspective fearfulness may be adaptive as it allows individuals to avoid potential threat or danger; from this view, it follows that individual variation in the threshold for when a stimuli becomes inhibitory or stimulatory, i.e. coping style, is likely to be linked with the subjective experience of that stimulus in a particular situation. As unpredictable situations provide reactive coping with more benefits, predictable situations favour proactive responses (Wingfield, 2003). Therefore, emotional distress is likely an essential component of reactive coping.

In paper I, II and III of this thesis I demonstrated that Nile tilapia exhibit coping-styles which can in part be explained by different neurobiological responses and also exhibit a threat-sensitive behavioral response both to alarm-cues (chemical) and after avoidance learning (physical cue, water inlet turned off). Furthermore, it was demonstrated that individual variation in coping styles predicts threat-sensitive responses after conditioning in Nile tilapia. However, until now statistically significant neurobiological responses to threat-sensitive behaviour had not yet been demonstrated (c.f. paper II). Therefore, in paper IV using the HR/LR trout lines demonstrated to express distinct stress coping styles, I aimed to describe possible differences in monoaminergic activity in a social learning avoidance paradigm (developed by Carpenter and Summers, 2009). After learning an escape route when confronted with a larger, dominant conspecific (used as the aversive stimulus), fish were either exposed to the dominant individual (physical stress - stressors caused by physical interaction) or just exposed to the sight of the opponent (psychological stress).

5.4. Do HR/LR fish show different neurobiological responses when exposed to a threat?

In paper IV, I start by demonstrating that fish displaying a proactive coping style present behavioural inflexibility. When confronted with a known aggressive and dominant conspecific LR fish attempted to escape more through a previously used, but now blocked exit than HR fish. Furthermore, the sight of the larger conspecific through a transparent wall was enough to significantly increase locomotor activity in LR fish, as compared to HR. Both of these behavioural responses indicate that LR fish base their behaviour on expectations, impeding behavioural adjustment in new situations. Hr fish on the other hand show a more apprehensive behaviour, limiting their behavioural reaction during visual exposure (i.e. when aggressive attacks would be expected to occur, but did not). This is in agreement with previous findings by Ruiz-Gomez et al. (2011), who report that fish from the LR strain took longer time to find a re-located feeding source. Even earlier results indicate that LR fish retained a conditioned response longer than HR fish (Moreira et al., 2004). From the mammalian literature, it is known that proactive individuals generally have a higher predisposition to develop and maintain routines, which suggests that their behavioural responses are based on internally organized predictions of the present environment. Reactive animals adjust their behaviour according to the environment (Coppens et al., 2010). In this context, there appears to fundamental cognitive differences between proactive and reactive animals, which appear to be crucial in the way they perceive stimuli valence such as a threat.

The monoaminergic control of behavioural outputs during stress and social interactions has been well documented also in comparative vertebrate models in mammals (Winberg and Nilsson, 1993a; Summers et al., 2005; Summers and Winberg, 2006). In mammals there is evidence of the cathecolaminergic (CA) systems (both DA and NE) being fundamental in the variation of behavioural flexibility through salience modification, attention, perception and impulse control (Coppens et al., 2010; Schultz, 2010; Arnsten and Pliszka, 2011; Economidou et al., 2012). In many situations, animals encounter alternative course of actions and specific outputs may have to be inhibited, to allow the emergence of the most appropriate goal-directed behaviour. In other words, in certain situations it is necessary to override a planned or already initiated action. In such cases a deficient inhibitory process may affect individuals by allowing impulsive, potentially detrimental, conduct (Bari and Robbins, 2013). In our experiment, after exposure to physical stress, LR individuals showed a region-specific significant increase in DA activity in the Dm. This response however brought the LR fish to the level shown by HR fish in all experimental situations (including the undisturbed control situation). In mammals, the regulation of perception, attention, inhibition and impulsivity, takes place in the forebrain, under CA regulation (Arnsten and Pliszka, 2011; Economidou et al., 2012). Specifically, high levels of NE and DA have been associated with increased arousal, as for example during stressful situations (Aston-Jones et al., 1999; Matsumoto and Hikosaka, 2009). In fish species, it has been now established, that there is a high level of conserved brain function in monoaminergic systems (Parker et al., 2013; O'connell and Hofmann, 2011; Winberg and Nilsson, 1993a). Therefore, increased DA activity in the Dm of LR fish may be regulating the increase in impulsive behaviour (i.e. lack of inhibition) towards trying to escape through a previously learned escape route, which was no longer available. Importantly, when CA levels are excessive, then neuron firing may be suppressed and animals are not able to react optimally. In other words, specific CA amounts are important in order to elicit the right behavioural outputs (Arnsten and Pliszka, 2011). In HR fish, DA activity levels in the Dm appear to remain the same after physical stress as compared to basal levels. Although speculative, levels in these individuals may be optimal in order to maintain high arousal and attention levels towards environmental change, and allow them to inhibit behavioural outputs when situations change (*i.e.*the locked escape route). Meanwhile, the change in DA activity in LR fish elicited by physical stress appears to greatly increase their impulsive behaviour. Interestingly, NE activity in the Dm was downregulated after physical stress in both HR and LR individuals. Even though both DA and NE may regulate the same processes in similar manners, they show region-specific effects and have been reported to affect subregions of the same areas in different manners. For example, while increased NE release in the nucleus accumbens (NAcc) shell of rats, decreases impulsive behaviour, a similar increase in both NE and DA in the NAcc core, increases impulsivity (Economidou et al., 2012). In light of these results, it would be interesting to investigate CAergic regulation in other forebrain areas, particularly those functionally homologous to the NAcc and the striatum, in order to elucidate further region-specific effects. The decrease in NE activity found in the Dm of HR individuals could be associated with the inhibition of the escape behaviour (*i.e.* decrease in impulsivity), while in LR individuals the subsequent increased in DA but decrease in NE in the Dm may favour impulsive behaviour. This could exemplify how possessing a balance in monoaminergic release affects behavioural outputs (Arnsten and Pliszka, 2011).

It has been reported that 5-HT mediates fear-like behaviours in the amygdala (e.g. Amat et al., 1998). Hayley et al. (2001) reported an increase in 5-HT and NE activity in the amygdala of two strains of mice when presented with psychological stressors. However, in paper IV, 5-HT effects were only observed with physical stress. Although physical stress response generally resulted in a more pronounced response when compared to psychological stress, NE concentrations in response to psychological stress were more pronounced than with physical stress. HR individuals showed a significant decrease in NE turnover after psychological stress. This appears to be a recurrent response to stress in these individuals and might be associated with their reactive coping style to these situations, in this specific brain area.

In fish species, there is ample evidence linking both the 5-HT and DA systems with stress and social behaviour regulation (for reviews see Winberg and Nilsson, 1993a; Sørensen et al., 2013). It has been reported in mammals that, even though fear stress may result in increased 5-HT and DA metabolism in limbic areas, this response is dependent on stimuli intensity (Inoue et al., 1994). In other words, physical and psychological stressors represent different stimuli intensities and therefore activate monoaminergic systems differently. In the present study, both physical and psychological stress affected brain monoamine signalling in Dm and Dl regions. However, the response to physical stress only elicited an increase in both DA and 5-HT turnover in both the Dm and the Dl. This is in accordance with the results from paper II, showing that confinement stress elicited similar, but a stronger, activation of 5-HT than olfactory cues of predation in Dm and Dl of Nile tilapia.

5.5.Concluding remarks

In this thesis, I have demonstrated that individual variation in brain 5-HT neurotransmission correlates to complex behavioural syndromes related to feeding motivation in Nile tilapia. The understanding that differences in feeding behaviour are likely to be part of behavioural syndromes with underlying brain mechanisms may have practical implications. One example is the possibility that selection programs aimed at improved feeding motivation may result in co-selection of other variables that are part of the behavioural syndrome. Understanding the proximate and ultimate mechanisms behind the development of individual behavioural and physiological profiles is of importance for population management (Conrad et al., 2011), biomedicine (Koolhaas et al., 2010), and aquaculture (e.g. Huntingford and Adams, 2005; Øverli et al., 2006b; Martins et al., 2011b; 2012). Furthermore, increased understanding of the proximate basis for individual behavioural profiles provides support for using fish as models in biomedical research for depression and affective states such as frustration (Vindas et al., 2014) and eating disorders (Eggert et al., 2007; Hancock and Olmstead, 2011).

In paper II, I demonstrated that Nile tilapia responded to conspecific alarm cues (a model used to study threat-sensitive behaviour) by reducing bottom-grazing behaviour, although the neuroendocrine response to alarm-cues was not statistically different from undisturbed individuals. Understanding the mechanism underlying threat-sensitive behaviour in fish is of major importance not only in fundamental research but also in more applied sciences such as aquaculture. By exhibiting threat-sensitive behaviour fish spend energy on activities others than growth which could ultimately lead to economical losses. It is worth highlighting the possible increase of threat-sensitive behaviour in recirculation aquaculture systems as this type of production system is foreseen to increase in the coming years due to its environmental advantages. In these systems the water is re-used to produce fish and over time alarm substances released from fish due to handling/grading may accumulate in the systems and potentiate the perception of threat. Furthermore, if the neural basis for threat-sensitive behaviour in fish is homologous to fear and anxiety behaviours found in humans then the use of fish in drug screening could be considered. It is possible that the suppression of feeding anticipatory behaviour observed in paper II reflects a general elevation of awareness, and combination skin extract with other challenges may reveal neorendocrine effects associated with this predatory cue.

In paper III, it is demonstrated that individual variation in coping styles predicts fear responses in Nile tilapia where fearful individuals showed a range of typically reactive traits. This study suggests that the link between coping styles and the expression of emotional or affective states such as fear is an evolutionary widespread phenomenon throughout the vertebrate subphylum, including fish. In this study we showed for the first time that cortisol is strongly linked to behaviours indicating fearfulness. However one question remains: does the fear reaction potentiate cortisol response, or does elevated cortisol exposure over time alter limbic structures in the brain that mediate fear responses? Further studies are needed to unravel the time course and coordination of psychological and biological stress responses.

Paper IV shows further evidence that LR individuals have a higher tendency to establish and maintain routines, even under stressful conditions. Furthermore, this response appears to be under DA/NE regulation in an area homologous to the mammalian amygdala. Therefore, these results suggest that cathecolaminergic activity in the Dm may be important regulating behavioural flexibility and consequently, stress coping styles.

In conclusion, in this thesis it is demonstrated that fish with contrasting stress coping styles present clear differences in their behavioural output when exposed to a fearful situation where, reactive individuals display a more fearful behaviour. Proactive individuals appear to be less reactive to changes in the environment suggesting that proactive individuals are less observant or simply appraise the surrounding environment differently. Nonetheless, individual behavioural traits appear to be predictive of how a stimuli is perceived. When exposed to a fearful stimulus fish present regionally discrete brain responses in brain regions homologues to the hippocampus and amygdala in mammals, and these reactions appear to be conserved through the vertebrate lineage.

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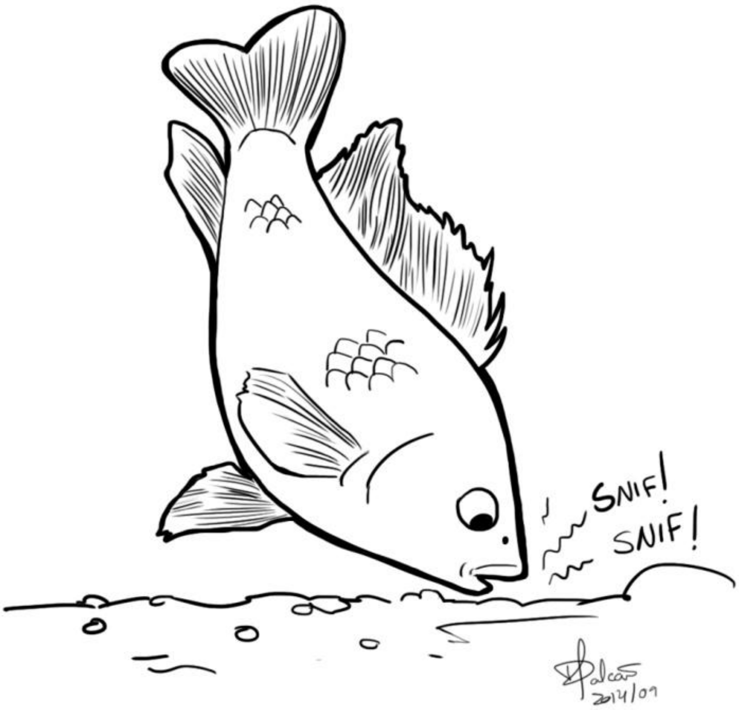
Paper I

Feeding motivation as a personality trait in Nile tilapia (Oreochromis niloticus): Role of serotonergic neurotransmission



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Feeding motivation as a personality trait in Nile tilapia (*Oreochromis niloticus*): role of serotonergic neurotransmission

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Abstract Consistent individual variation in behaviour and physiology (i.e. animal personality or coping style) has emerged as a central topic in many biological disciplines. Yet, underlying mechanisms of crucial personality traits like feeding behaviour in novel environments remain unclear. Comparative studies, however, reveal a strong degree of evolutionary conservation of neural mechanisms controlling such behaviours throughout the vertebrate lineage. Previous studies have indicated duration of stress-induced anorexia as a consistent individual characteristic in teleost fishes. This study aims to determine to what degree brain 5-hydroxytryptamine (5-HT, serotonin) activity pertains to this aspect of animal personality, as a correlate to feed anticipatory behaviour and recovery of feed intake after transfer to a novel environment. Crucial to the definition of animal personality, a strong degree of individual consistency in different measures of feeding behaviour (feeding latency and feeding score), was demonstrated. Furthermore, low serotonergic activity in the hypothalamus was highly correlated with a personality characterized by high feeding motivation, with feeding motivation represented as an overall measure incorporating several behavioural parameters in a Principle Component Analyses (PCA). This study thus confirms individual variation in brain 5-HT neurotransmission as a correlate to complex behavioural syndromes related to feeding motivation.

Keywords Behavioural syndromes · Temperament · Locomotory activity · Brain punch · Monoamines · Cichlids

Introduction

The study of consistent, individually specific behavioural, physiological and cognitive/emotional patterns has lately become a central subject in a wide range of different biological disciplines ranging from evolutionary ecology to health sciences (Koolhaas et al. 1999, 2010; Koolhaas 2008; Gosling 2001; Korte et al. 2005; Réale et al. 2007; Sih et al. 2004) and has even been used to explain political and social phenomena (Anderson and Summers 2007). When consistent over time and across situations, individual behavioural differences are referred to as personality (Gosling

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2001), temperament (Réale et al. 2007) or shyness/boldness (Wilson et al. 1994). Some researchers go further in the conceptualization of personality to include not only consistency in single behavioural traits, but also correlations between multiple traits. This approach has yielded definitions such as behavioural syndromes (Sih et al. 2004) and coping styles (Koolhaas et al. 1999). Behavioural syndromes are defined as sets of correlated behaviours which are consistent over time and across situations (Sih et al. 2004), while coping styles are defined as sets of behavioural and physiological stress responses which are consistent over time and characteristic to a certain group of individuals (Koolhaas et al. 1999).

Despite the diversity of terminology and underlying definitions, there seems to be a consensus that consistency over time should always be considered when referring to individual behavioural tendencies. From a behavioural neuroscience angle, it is reasonable to propose that such consistency reflects underlying causal mechanisms of a persistent nature (Øverli et al. 2007; Coppens et al. 2010). To better understand animal personalities, it would thus be valuable to determine the mechanistic basis for individual variation in behavioural types.

In view of the compelling evolutionary implications of consistent trait correlations, integrative research using comparative models such as teleost fishes highlights a range of organizing principles. Research on fish provided early documentation on individual consistency in behaviour (Huntingford 1976; Wilson et al. 1993) followed by research into underlying proximate physiological mechanisms (Øverli et al. 2005; Schjolden et al. 2005). Coping styles are characterized by the discrepancy between two primarily different types of behavioural and physiological stress responses (proactive and reactive individuals), and a series of common patterns have been identified throughout the vertebrate subphylum (Koolhaas et al. 1999; Groothuis and Carere 2005; Øverli et al. 2004a, 2007; Schjolden and Winberg 2007; Coppens et al. 2010). Behaviourally, proactive individuals are typically identified by high levels of aggression, territorial control, active avoidance and other behavioural responses that suggest active efforts to offset a negative stimulus (Koolhaas et al. 1999, 2010). Reactive individuals on the other hand are identified by low levels of aggression and passive avoidance of a negative stimulus (Koolhaas et al. 1999, 2010). In addition, proactive individuals typically show low levels of behavioural flexibility and appear to be best adapted to stable environments (Benus et al. 1991; Verbeek et al. 1994; Bolhuis et al. 2004; Ruiz-Gomez et al. 2011). Physiologically, the proactive coping strategy is characterized by low hypothalamus–pituitary–adrenal (HPA axis) reactivity and low parasympathetic reactivity, whereas sympathetic reactivity is high. In contrast, animals with a reactive coping style show high HPA reactivity, high parasympathetic reactivity and low sympathetic reactivity (Koolhaas et al. 1999).

Resumption of feed intake in novel environments or after stress has become one of the most used parameters to assess different personalities in fish (Øverli et al. 2006a, 2007; Kittilsen et al. 2009a, b; Silva et al. 2010; Martins et al. 2011a; Basic et al. 2012). Latency to react to the presence of food has also previously been used to assess personalities in Senegalese sole (*Solea senegalensis*) (Silva et al. 2010). Another alternative parameter is feeding anticipation. Feeding anticipation is usually seen in animals that have restricted but predictable feeding schedules and not in animals that are randomly fed (Sánchez et al. 2009; Mistlberger 2011). Feed anticipation is characterized by an increase in locomotor activity, beginning several hours prior to meal time, and rising to a peak at mealtime in rats (Mistlberger 2011) as well as in fish (Sánchez et al. 2009). Such anticipatory behaviour has been recently used as an indicator of stress response and recovery in Atlantic salmon (Folkedal et al. 2012); however, the neural mechanisms underlying feed anticipatory activity remain unclear (Mistlberger 1994; Stephan 2002; Hsu et al. 2010) and so does the uncertainty as to whether feeding anticipation could be used as a personality measure.

The monoamine neurotransmitter/neuromodulator serotonin (5-hydroxytryptamine; 5-HT) has been shown to be important in the control of several aspects of personality such as aggression and impulsivity (Ho et al. 1998; Øverli et al. 1999; Lesch and Merschdorf 2000; Miczek et al. 2002; Kravitz and Huber 2003; Summers et al. 2005; Koolhaas et al. 2007; Coppens et al. 2010). In addition, 5-HT is centrally involved in feed intake in both fish and mammals (De Pedro et al. 1998). 5-HT is reported to have anorectic affects in several species from nematodes (Luedtke et al. 2010), to fish (Ortega et al. 2013), to humans (Sargent and Henderson 2011). One of the first studies concerning the role of serotonin in feeding in fish was done by De Pedro et al. (1998) where they indicate a highly conserved anorectic function of 5-HT throughout phylogeny. Similar conclusions were drawn by Øverli et al. (1998).

There are a number of studies on the neural control of feeding in fishes (Lin et al. 2000; Bernier and Peter 2001; Bernier 2006; Matsuda 2009; Kulczykowska and Vazquez 2010; Matsuda et al. 2012), but as far as, we are aware only a few address individual differences in feeding behaviour as a consistent personality trait (Di-Poï et al. 2007; Castanheira et al. 2013). Therefore, the primary goal of this study was to assess the level of consistency in different measures of feeding behaviour to scrutiny which measures of feeding could be best used to assess consistent personality traits, and further to determine whether 5-HT activity in the hypothalamus is correlated with these measures. Our model species was Nile tilapia (*Oreochromis niloticus*) due to its well characterized behaviour (Barreto and Volpato 2011; Martins et al. 2011a). Furthermore, Nile tilapia is one of the major fresh water species being produced worldwide (FAO 2005–2013).

Materials and methods

Fish, housing and experimental procedures

The experiment was carried out at the fish laboratory facility at the Norwegian University of Life Sciences, Aas, Norway, using freshwater system (temperature: 27.42 ± 1.12 °C) (mean ± SD) and a photoperiod of 12 h light:12 h dark. The behavioural experiment lasted for 10 days. Eight separate observation aquaria were divided into four chambers each with size 22 × 25 × 50 cm (width × length × depth) (following Winberg and Lepage 1998). The fish used in this experiment were 2.5 months old. Before the experiment, they were kept in a common holding tank and fed 5% of body weight a day using belt feeders.

Thirty sexually immature Nile tilapia (23.53 ± 3.47 g) were transferred from the holding tank into isolation (one fish per chamber—note that two fish were removed from the experimental set up since they did not consume any given pellets). During the following 9 days, each fish was hand fed 2.5% of body weight twice a day, at 9 a.m. and 5 p.m. This amount corresponds to the ration given daily in the stock holding tanks. During each feeding period (with a duration of 3 min), feeding latency was measured and a feeding score was attributed to each fish. Uneaten food was removed from the tanks after the feeding period. Feeding latency was measured as the time (seconds) from food entered the water until the first pellet was consumed, and feeding score was attributed from a range of 0–3 (c.f. Øverli et al. 2006a) according to the following scale: 0—the fish does not eat or swim towards the feed; 1—the fish only eats pellets that are immediately in front of its' mouth; 2—the fish moves to get the feed but always returns to its' initial position between each pellet; 3—the fish moves freely to find feed and eats most or all of it. Accumulated feeding score and sum of feeding latencies (over 9 days) were used as a personality measure (Kittilsen et al. 2009a). After the initial 9 days, a subset of nine fish were chosen randomly and video recorded (Sony, Handycam, DCR-HC32 NTSC) on day 10 for 30 min for analyses of locomotory activity and bottom-grazing behaviour (i.e. time that each fish spent grazing the bottom of the tanks in search for food) beginning at 8 a.m. (1 h prior to feeding). These measurements were done using a stopwatch. Locomotory activity was measured as the percentage of time that fish spent swimming and bottom-grazing behaviour was measured in seconds. Locomotory activity and bottom-grazing behaviour at this time were taken as a measure of feed anticipatory activity, presuming that fish at this time had learned to associate the human presence with food distribution. After 30 min, all nine fish were over anaesthetized with MS222 (0.5 g l^{-1}) and brains collected for monoamine analyses. One brain sample was lost due to a technical error.

Serotonergic neurochemistry

Whole brains of sampled fish were dissected out in <2 min, placed immediately in tissue-tek (Sakura Finetek) and frozen on dry ice. Brains were then sliced in serial 300-µm sections (MNT cryostat at −19 °C), quickly thaw mounted on glass slides, and immediately refrozen at −80 °C for microdissection using a brain punch technique (Øverli et al. 2004b; Shaw et al. 2009; adapted from Korzan et al. 2000). Hypothalamus was identified using a stereotaxic atlas of *Oreochromis mossambicus* brain (Sakharkar et al. 2005) and microdissected with a 300-µm-diameter needle at −14 °C using a BFS-MP freezing stage for microtomes (the microdissected areas included preoptic area (PO) and nucleous preopticus (NPO)). After microdissection, brain tissue punches were ejected into 100 μl sodium acetate buffer (pH 5), to which an internal standard (3,4 dihydroxybenzylamine) was added. Samples were frozen at −80 °C to facilitate cell lysis, thawed on ice and centrifuged at 17,000 rpm for 5 min. The supernatant was removed, and 5-HT and its principle catabolite 5-hydroxyindoleacetic acid (5-HIAA) were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection. The HPLC system consisted of a solvent-delivery system (Shimadzu, LC-10AD), an auto injector (Famos, Spark), a reverse phase column (4.6 mm × 100 mm, Hichrom, C18, 3.5 mm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at −40 and +320 mV. A conditioning electrode with a potential of +40 mV was employed before the analytical electrodes to oxidise any contaminants. The mobile phase consisted of 86.25 mmol l^{-1} sodium phosphate, 1.4 mmol l^{-1} sodium octyl sulphate and 12.26 μmol l^{-1} EDTA in deionized (resistance 18.2 MW) water containing 7 % acetonitril brought to pH 3.1 with phosphoric acid. Samples were quantified by comparison with standard solutions of known concentrations and corrected for recovery of the internal standard using HPLC software (CSW, DataApex Ltd, Czech Republic).

Data analysis

For each individual and observation day, an average of the two daily behavioural recordings of feeding latency and feeding score was calculated. Based on the resulting measure, individual consistency in feeding behaviour between days was analysed by non-parametric correlation (Spearman), due to non-normal distribution of data.

To test for the correlation between feeding motivation and serotonergic activity in the hypothalamus, individual traits that represented feeding behaviour (feeding latency, days to achieve latency zero, grazing behaviour and locomotory activity) were collapsed into first principal component scores using Principal Component Analyses (PCA). A correlation matrix was used to check multicollinearity, i.e. to identify variables that did not correlate with any other variable (this was the case for feeding score which was only correlated with feeding latency so it was not considered for the PCA), or correlated very highly ($r = 0.9$) with one or more other variables. KMO test was greater than 0.7, and the Bartlett's test of sphericity was significant. Correlation between the principal component score (representing feeding motivation) and serotonergic activity was then analysed using a Pearson correlation, as both these variables passed the normality test.

All statistical analyses were performed using SPSS (version17.0) for windows. Statistical significance was accepted at $P < 0.05$.

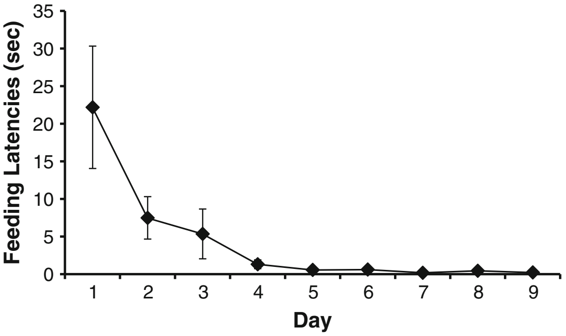

Fig. 1 Decreasing of feeding latencies averages ($n = 30$) in Nile tilapia housed individually over a period of 9 days

Results

Behavioural consistency

Feeding latencies decreased rapidly during the experiment (Fig. 1), and all fish achieved a feeding score of 3 during the experimental period of nine days.

When transferred into isolation, Nile tilapia showed strong individual consistency in feeding behaviour. All present relationships were positive indicating that a fish that exhibit high feeding motivation on any given day also exhibited such behaviour on succeeding days. Regarding latency to take distributed food, average latency between two feeding occasions on day 1 correlated to this measure on day 2 to 5, and then again at day 8 and 9 (Table 1), while comparing latencies from one day to the next always yielded a statistically significant correlation. Similar consistency was observed in feeding score. Individual average feeding score on day 1 correlated with the same measure on day 2 to 4 and 9 (Table 1). Similar to

Table 1 Spearman correlations [correlation coefficients (up row) and *P* values (down row)] between feeding latencies (FL) and feeding scores (FS) on different experimental days and day 1 ($N = 30$)

	Day 2	Day3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
FL Day 1	0.542	0.531	0.496	0.361	0.338	0.183	0.429	0.368
	0.002*	0.003*	0.005*	0.050*	0.068	0.334	0.018*	0.046*
FS Day 1	0.593	0.637	0.439	0.293	0.316	0.351	0.262	0.428
	0.001*	<0.001*	0.015*	0.116	0.089	0.057	0.162	0.018*

* Significant correlations

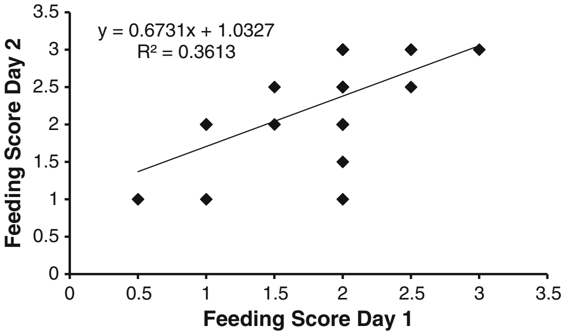

Fig. 2 Day to day consistency in a personality measure in Nile tilapia. Relationship between feeding score on day 1 and feeding score on day 2 ($n = 30$). *P* value = 0.001. Note the existence of several overlapping data points. Each day a feeding score was attributed at 9 a.m. and 5 p.m

feeding latency, correlating individual scores between two successive days revealed a statistically significant relationship in every case (Fig. 2).

Correlations between different accumulated measures of feeding behaviour are shown in Table 2. During the initial 9 days after transfer into isolation, accumulated feeding score over 9 days was significantly correlated with both feeding latency (sum of 9 days) and days to achieve latency zero. Both feeding latency and days to achieve latency zero were correlated with parameters of feeding anticipatory activity (locomotory activity (33.02 ± 8.69 % of time) (mean ± SE) and bottom-grazing behaviour (16.44 ± 7.31 s)) (Fig. 3), but no such correlation was found between accumulated feeding score and any measure of feeding anticipatory activity on day 10. Time spent in bottom-grazing behaviour and locomotory activity on day 10 were highly correlated ($P = 0.001$).

Feeding behaviour and serotonergic activity in the hypothalamus

Individual traits that represented feeding behaviour were collapsed into first principal component scores using PCA. Table 3 shows the PCA loadings of the feeding behaviour variables used to generate a principal component scores (PC1) to assess feeding

Table 2 Pearson correlations [correlation coefficients (up row) and *P* values (down row)] between the different behaviours measured throughout the experiment ($N = 9$)

	Feeding latency (s)	No. of days to reach latency zero	Time spent in bottom-grazing behaviour (s)	Locomotory activity (% time)
Feeding score	−0.757	−0.710	0.629	0.497
	0.018*	0.032*	0.070	0.173
Feeding latency (s)		0.569	−0.809	−0.749
		0.110	0.008*	0.020*
No. of days to reach latency zero			−0.875	−0.683
			0.002*	0.043*
Time spent in bottom-grazing behaviour (s)				0.896
				0.001*

* Significant correlations

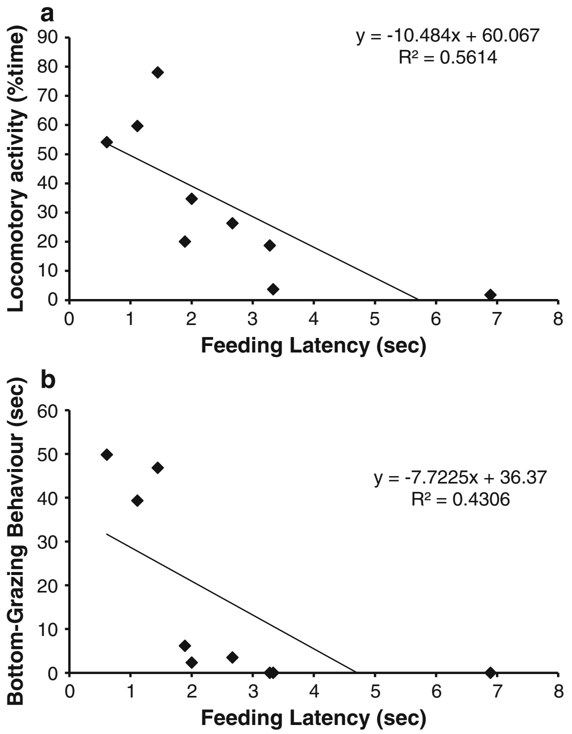

Fig. 3 Correlation between different personality measures taken at different time points. **a** Correlation between the average of feeding latency and the average locomotory activity ($n = 9$). *P* value = 0.020. **b** Correlation between the average of feeding latency and the average bottom-grazing behaviour ($n = 9$). *P* value = 0.008

Table 3 PCA loadings of the feeding behaviour variables used to generate a principal component scores (PC1) to assess feeding motivation after transfer into a novel environment in juveniles of Nile tilapia

Behaviours	Loadings for PC1 (component matrix)	% Variation explained
Feeding latency (s)	0.689	79.243
No. of days to reach latency zero	0.713	
Time spent in bottom-grazing behaviour (s)	0.886	
Locomotory activity (%time)	0.881	

motivation after transfer into a novel environment in juveniles of Nile tilapia. The variable with the highest loading is the one contributing more to the PCA which in this case was bottom-grazing behaviour.

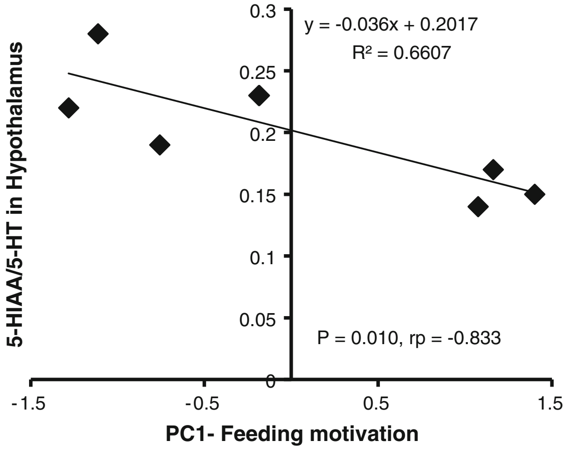

Fig. 4 Relationship between serotonergic activity in the hypothalamus (5-HIAA/5-HT) and feeding motivation ($N = 8$). *P* value (*P*) and Pearson correlation (rp). Note that there is an overlapping of two data points (the closest points to the *y* axis)

Serotonergic activity in the hypothalamus was negatively correlated with feeding motivation (PC1) ($P = 0.010$, Pearson correlation $= -0.833$, Fig. 4) which indicate that individuals with higher 5-HIAA/5-HT ratios in the hypothalamus seem to be consistently less motivated towards food. However, no correlation was found between feeding motivation and concentrations of 5-HT or 5-HIAA (data not shown).

Discussion

Feeding behaviour in novel environments has been used previously by several authors as a personality measure due to its correlation with both physiological traits such as stress-induced corticosteroid production (Øverli et al. 2002, 2007) and behavioural characteristics such as locomotor response to acute stress (Øverli et al. 2006a), ability to win fights for social dominance (Øverli et al. 2004a), neophobia (Martins et al. 2011b), aggression and reaction time to mating opportunities (Korzan et al. 2006). In the present study, both feeding latency and feeding score measured during the initial 9 days after transfer to social isolation were indicated as consistent personality traits. Moreover, individual summated feeding latency was correlated with bottom-grazing behaviour and locomotory activity, measurements taken when fish were fully acclimatized. Consequently, feeding latency taken during acclimation to a new environment also predicts feeding anticipatory behaviour in fully acclimatized fish, indicating a feeding behaviour syndrome. In this respect, our results comply with previous studies indicating the presence of coping styles in Nile tilapia (Martins et al. 2011b; Barreto and Volpato 2011).

From a proximate viewpoint, behaviours may be correlated because they share the same neurobiological, neuroendocrine and/or genetic mechanisms (Coppens et al. 2010). In the present study, it would appear that serotonergic activity in the hypothalamus, measured in fully acclimatized fish, mediates feeding motivation. Fish with lower serotonergic activity in the hypothalamus took shorter periods of time to achieve latency zero and exhibited higher bottom-grazing and locomotory activity prior to feeding. Thus, fish with lower serotonergic activity in this brain region seem more motivated towards food, and our study goes in agreement with previous studies that have identified sites in the hypothalamus which may potentially regulate feeding anticipatory activity (Hsu et al. 2010). It is, however, a novel observation that serotonergic activity in hypothalamus correlates with a complex behavioural syndrome, components of which were recorded a considerable time prior to the actual neurochemical measurement. PCA loading for summated feeding latency was, for instance, 0.689 (c.f. Table 3), and feeding latencies decreased dramatically during the experiment (c.f. Fig. 1), so how fish performed initially had the largest impact on the accumulated measure. Considering the well documented link between hypothalamic 5-HT and feeding behaviour (Leibowitz et al. 1990; De Pedro et al. 1998; Takada et al. 1998; Lin et al. 2000), it follows that this neurobiological trait is a relatively constant individual feature, at least for fish in isolation. Clearly, the 5-HT system of both fish and mammals is also very responsive to environmental stimuli, particularly stressful stimuli (Winberg et al. 1992; Winberg and Nilsson 1993; Bethea et al. 2005; Sloman et al. 2005; Beekman et al. 2005; Hegazi and Hasanein 2010; Weber et al. 2012), but in a stable environment, the individual variation appears to be generated endogenously.

Furthermore, the relationship between feeding motivation and serotonergic activity seen with the current experimental design suggest differences in feeding behaviour unrelated to variability in stress-induced elevation of 5-HT. This, in turn, indicates the presence of chronic individual differences in 5-HT transmission, caused either by genetic differences (Lesch and Merschdorf 2000) or early experiences (Lesch and Merschdorf 2000; Lukkes et al. 2009).

Serotonin has previously been associated with individual differences in temperament and personality (Koolhaas et al. 2010). Koolhaas et al. (2010) reviewed several studies indicating the correlation of 5-HT in aggression and how 5-HT may be related to the coping-style axis rather than the emotional axis as it has been related until now. The present data indeed shows that 5-HIAA/5-HT ratio in the hypothalamus relate to different coping styles since this ratio is related to different aspects of feeding behaviour during and after transfer to a novel environment. Differences in nutritional background (i.e. amount of tryptophan, the 5-HT precursor, intake) between individuals could be a possible mechanism linking serotonergic activity and foraging behaviour. However, in the present study, all fish were fully adapted and fed the same amount of food and therefore it is unlikely that the nutritional state of the fish affected the individual serotonergic activity in the hypothalamus since all individual had access to the same amount of 5-HT precursor, L-tryptophan. An alternative mechanism could be individual differences related to polymorphism genes influencing 5-HT transmission. In humans, personality differences appear to be related to a polymorphism of genes that influence the central 5-HT transmission, by affecting 5-HT production rate, synaptic release and degradation (Pavlov et al. 2012). Also, functional polymorphisms in the monoamine oxidase A and the 5-HT transporter have been linked with individual variation in personality in both humans and in rhesus monkeys (Lesch and Gutknecht 2005; Suomi 2006). To which extend such polymorphisms are present in fish and contribute to explain differences in feeding motivation still need to be investigated.

In conclusion, the results of the present study showed that in Nile tilapia feeding latency and feeding score are behavioural traits consistent over time and fish with lower serotonergic activity in the hypothalamus exhibited a higher feeding motivation over time. These results underline the concept that consistent differences in behaviour are due to consistent differences in physiology and neurobiology. Future studies should aim to cover both the behavioural syndromes and the underlying mechanisms, giving perhaps particularly focus on the conditions under which such normally occurring relationships become dissociated (c.f. Ruiz-Gomez et al. 2008). The results obtained in this study indicate that individual differences in feeding behaviour are consistent over time. The understanding that differences in feeding behaviour are likely to be part of behavioural syndromes with underlying brain mechanisms may have practical implications. One example is the possibility that selection programs aimed at improved feeding motivation may result in co-selection of other variables that are part of the behaviour syndrome. Understanding the proximate and ultimate mechanisms behind the development of individual behavioural and physiological profiles is of importance for population management (Conrad et al. 2011), biomedicine (Koolhaas et al. 2010) and aquaculture (e.g. Huntingford and Adams 2005; Øverli et al. 2006b; Martins et al. 2011c, 2012). Our findings are in agreement with other studies exploring non-invasive manipulation of 5-HT signalling to reduce stress and aggression in aquaculture (Winberg et al. 2001; Lepage et al. 2002, 2005; Höglund et al. 2007). Furthermore, increased understanding of the proximate basis for individual behavioural profiles provides support for using fish as models in biomedical research for depression and affective states such as frustration (Vindas et al. 2014) and eating disorders (Eggert et al. 2007; Hancock and Olmstead 2011).

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Conflict of interests The authors declare that there are no conflicts of interests.

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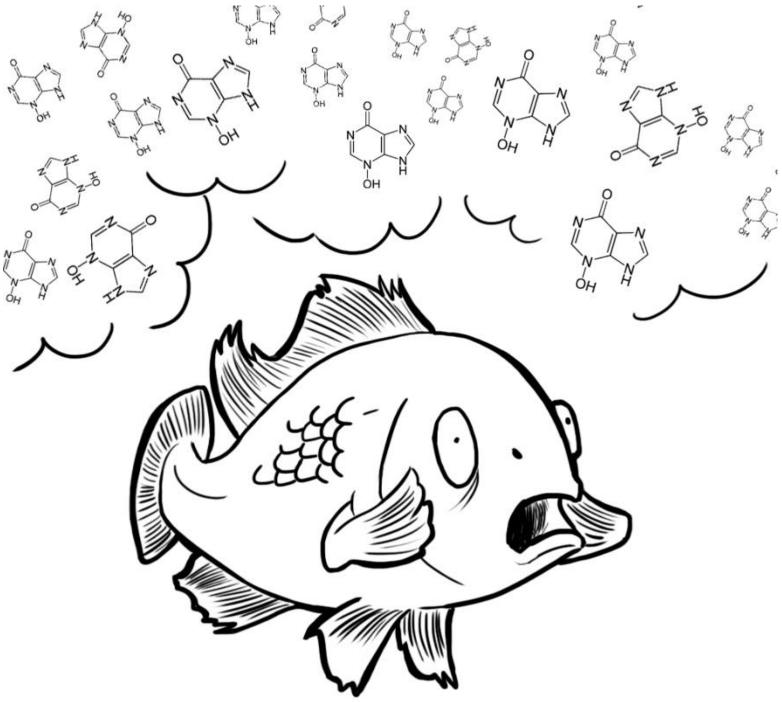
Paper II

Stress and fear responses in the teleost pallium



Title:

Stress and fear responses in the teleost pallium

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Abstract:

Evolution has resulted in behavioural responses to threat which show extensive similarities between different animal species. The reaction to predator cues is one example of such prevailing responses, and functional homologies to mammalian limbic regions involved in threat-sensitive behaviour have been found in the teleost telencephalon. The dorsolateral (Dl) and dorsomedial (Dm) regions of the pallium are thought to perform hippocampus and amygdala-like functions respectively. To what degree these regions are involved in the neuroendocrine responses to stress and predatory cues however remains largely unknown. In the present study the involvement of Dl and Dm in the response to stress and predatory cues was investigated by exposing Nile tilapia (*Oreochromis niloticus*) to a standardized confinement stress and to skin extracts from conspecifics. Nile tilapia develops a characteristic anticipatory behaviour to hand feeding, and effects of skin extract on this behaviour and locomotor activity were studied to characterise threat sensitive behaviour. Nile tilapia responded behaviourally to conspecific alarm cues by reducing feeding anticipatory behaviour. This may reflect a general elevation of alertness, and further studies combining skin extract with other challenges are needed to reveal neuroendocrine effects associated with this predatory cue. Confinement stress resulted in an elevation of cortisol, and serotonin (5-hydroxytryptamine, 5-HT) metabolism in both Dl and Dm. A similar tendency was observed in fish exposed to chemical alarm cues, but this effect did not reach the level of statistical significance. Hence, limbic responses to stress and fear, akin to those seen in extant mammals, are also present in the teleost lineage.

Key words:

Alarm cues, skin extracts, brain punch, cichlids, fish.

1. Introduction

The ability to detect and avoid threats such as predators has a very clear fitness effect. Essential components of threat-sensitive behaviour show great similarity between different animal groups from very simple organisms (e.g. protozoan) to more complex organisms (e.g. fish and mammals) [1], [2]. These homologies have promoted the use of reactions to predatory cues in rodents as models for studying psychopathologies associated with anxiety and fear in humans [3], and anxiety models in fish are also becoming more common in ethopharmacological studies [4] and [5]. Also, animal welfare research has been pointing at common underlying physiological mechanisms within vertebrates as an argument that fish experience suffering and fear [6]. Teleost fishes have emerged as an alternative to small mammals in both neurobiology and behavioural research [7], [8] and [9], and the neural substrates involved in fear-related responses such as panic/erratic swimming and freezing are being revealed in zebrafish [10] and [11]. The neuroendocrine control of more subtle behaviours (e.g feeding) which are not easily captured by computerised observation systems remain largely unknown.

The brain monoamines, serotonin (5-HT), dopamine (DA) and norepinephrine (NE) play a regulatory role in the limbic system, which controls several responses related to social, emotional and motivational stimuli, including defence and fear reactions [12]. Specially, 5-HT- and DA-ergic signalling in the amygdala within the limbic system have been shown to take part in responses to fear, including responses to olfactory predator cues [13] and [14]. Moreover, several studies have demonstrated that the amygdala together with the hippocampus, another brain region located in the limbic system, regulate the activity of the hypothalamic-pituitary-adrenal (HPA)-axis [15] and [16]. A different developmental pattern of the forebrain in fish has been constraining comparative studies of these brain regions [17]. However, brain lesion studies indicate functional homologies between the mammalian hippocampus and amygdala and the dorsolateral (Dl) and dorsomedial (Dm) telencephalon respectively in teleosts [18], [19], [20] and [21]. However, the response of these brain parts to stressful paradigms is strongly dose- and context-dependent [22], [23] and [24], and possible responses to predatory cues have not been investigated. Studies investigating if changes in monoamine signalling in Dl and Dm are affected by stress and olfactory predatory cues in fish are needed to further establish these areas as functional homologues of hippocampus and amygdala. However, previous studies focusing on the effects of predatory cues on brain monaminergic signalling have been performed in whole brains or in crude divisions of the brain, such as telencephalon, hypothalamus, olfactory bulbs and brain stem. In fish, Bell et al. [25] showed that risk-taking behaviour under predator presence was positively correlated with changes in 5-HT signalling. Also, higher 5-HT turnover has been shown in fish exposed to alarm cues without having hiding substrate [26].

In cichlid fish avoidance reactions to alarm cues have been demonstrated in Mozambique tilapia (*Oreochromis mossambicus*) [27] and convict cichlids (*Archocentrus nigrofasciatus*) [28], which indicates that this type of reaction is present in this family of fish. Moreover, Nile tilapia (*Oreochromis niloticus*) easily develops a distinct feeding anticipatory behaviour during hand feeding [29] which makes this species a suitable model for testing whether or not olfactory cues of predation elicit acute avoidance and/or suppress feeding motivation. The aim of this study was thus to characterize the behavioural response to injured conspecifics in Nile tilapia, and to compare the neurochemical activation of hippocampal (Dl) and amygdalar (Dm) like structures between this stimuli and a standardized stressor. In order to do this, changes in locomotor activity and feeding anticipatory behaviour in response to skin extracts were investigated. The specificity of the neuroendocrine response was investigated by comparing muscle cortisol and brain monoaminergic responses in Dl and Dm between fish exposed to skin extracts or confinement stress.

2. Material and Methods

2.1. Fish, housing and experimental procedures

The experiment was carried out at the Norwegian University of Life Sciences, Aas, Norway, using a closed recirculating freshwater system (temperature: 27.42 ± 1.12 ºC) (mean ± SD) with a photoperiod of 12h light: 12h dark. This experiment lasted for 10 days. Observation aquaria were divided into four chambers with size 22 x 25 x 50 cm (width x length x depth).

Thirty sexually immature Nile tilapia (23.53 ± 3.47 g - 8 weeks old) were transferred from a stock holding tank into visual isolation (one fish per chamber). During the following nine days of acclimation each fish was fed 2.5% of body weight twice a day with commercial pelleted feed, at 9am and 5pm. After the acclimation period, on day 10, all fish were divided into three groups: control (n = 9), olfactory alarm cues (n = 10) and, acute confinement stress (n=11). Each individual fish in control and alarm cues groups was video recorded (Sony, Handycam, DCR-HC32 NTSC) for 30 minutes, during which two 5 ml doses of either distilled water or skin extracts were injected into their tanks, respectively. The protocol for injection of skin extract followed Höglund et al. [26]. In short, the first injection was given after 10 minutes and the second after 20 minutes (as described in [26]) after the start of the video recording. The videos were analysed for locomotor activity and bottom-grazing behaviour, a behaviour used to measure feeding anticipatory activity. Bottom-grazing behaviour was measured as the time (seconds) fish spend inspecting the bottom of the aquarium in the search for food. Locomotor activity was measured as the % of time fish spent in active locomotion; this is, when fish moved more than 10% of its body length. Both behaviours were analysed using a stop-watch. Analysis of the video recording revealed that not all fish within control and alarm cues groups exhibited bottom-grazing behaviour. As no significant differences were found in fish performing and not performing bottom-grazing behaviour within groups in either cortisol (control group $p = 0.410$; alarm cues group $p = 1.000$) or serotonergic activity in Dl (control group $p = 0.876$; alarm cues group $p = 0.858$) and in Dm (control group $p = 0.527$; alarm cues group $p = 0.947$) (t-test was used to study significant differences) both grazers and non-grazers were pooled together. Each fish of the acute confinement stress group was inserted inside a transparent plastic box (20 x 12 x 7 cm) (length, width, deep) for 20 minutes. After video recording all fish were euthanized with MS222, brains were collected for monoamine analyses (all samples were collected and frozen in less than 2 minutes at -80ºC) and bodies collected for cortisol measurements. One brain sample in control group was lost due to technical reasons.

2.2. Preparation of skin extract

For the preparation of the skin extract used in the experiment, Nile tilapias from the same batch as experimental fish were killed by decapitation and skin was taken from the sides of the fish. Approximately 2 g of skin were homogenized in 100 ml of distilled water. The homogenate was centrifuged at 2400 rpm for 5 min at 4 ºC [26]. The supernatant was frozen and a concentration of 1:5 in distilled water was used in the aquariums.

2.3. Cortisol Analysis

The muscle cortisol analysis followed the protocol detailed by Åberg Andersson et al. [30]. Four g of white muscle of each fish were separated for quantification of cortisol. Each sample was divided in two subsamples (2g each) and one was used for monitoring procedural losses which, was spiked with 450ng of Hydrocortisone (HO888 – 1G Sigma). Each of the subsamples was homogenized in 2ml of PBS and re-suspended in 20ml of ethyl acetate for extraction. Ethyl acetate was separated from the tissue by centrifugation and evaporated by vacuum centrifugation. Dry residue was then resuspended twice (2×3ml) in 30% v/v methanol - mili-Q water, filtered on a 0.2μm filter and loaded on a 500mg Amprep C_{18} microcolumn. Impurities were washed out with 10ml of mili - Q water and cortisol was eluted in 2.5ml of 90% v/v methanol - mili-Q water. The subsamples were dried for a second time by vacuum centrifugation, resuspended in 400μl of HPLC buffer, filtered through a 0.2μm filter and stored at -80ºC for further analyses.

After extraction each subsample was run through high-performance liquid chromatography (HPLC) for cortisol separation and later quantified by ELISA. Each subsample was injected on a 250×4.6mm column packed with C_{18}-silica gel (5μm particle size) and the chromatogram was developed using a four-step gradient eluent (1 - 45% v/v methanol in 0.01 M sodium dihydrogen phosphate buffer (pH=5.3) between 0 and 25 minutes; 2 – 51% v/v between 26 and 65 minutes; 3 - 64% v/v between 66 and 80 minutes; 4 - 45% v/v between 80 and 90 minutes). Chromatography was performed at 4ºC with 1 ml/min flow rate. The elution of the steroid was monitored by U.V. absorbance at 239 nm. The elute was dried under vacuum centrifugation, resuspended in the same volume of ELISA buffer and cortisol was quantified with ELISA kits (Neogen, #402710).

2.4. 5-Hydroxytryptamine neurochemistry

Brains were cut frozen (MNT cryostat at -19ºC) in serial 300-µm sections, quickly thaw mounted on glass slides, and immediately refrozen at -80ºC for microdissection of Dm and Dl [22], [31] and [32]. Brain regions were identified using a stereotaxic atlas of *Oreochromis mossambicus* brain [33] and microdissected with a modified syringe needle with an inner diameter of 300-µm-diameter at -14ºC (BFS-MP freezing stage to microtomes). After microdissection, brain tissue punches were ejected into 100 µl sodium acetate buffer (pH=5), to which an internal standard (3,4 Dihydroxybenzilamine Hydrobromide) was added. Samples were frozen at -80˚ C to facilitate cell lysis, thawed on ice and centrifuged at 17,000 rpm for 5 minutes. The supernatant was removed and analysed by HPLC. 5-HT, 5-HIAA, DA and Dopac were quantified using HPLC with electrochemical detection. The HPLC system consisted of a solvent-delivery system (Shimadzu, LC-10AD), an autoinjector (Famos, Spark), a reversephase column (4.6mm´100 mm, Hichrom, C18, 3.5 mm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at -40 mV and +320 mV. A conditioning electrode with a potential of +40 mV was employed before the analytical electrodes to oxidise any contaminants. The mobile phase consisted of 86.25 mmol l^{-1} sodium phosphate, 1.4 mmol l^{-1} sodium octyl sulphate and 12.26 µmol l^{-1} EDTA in deionized (resistance 18.2MW) water containing 7 % acetonitril brought to pH 3.1 with phosphoric acid. Samples were quantified by comparison with standard solutions of known concentrations and corrected for recovery of the internal standard using HPLC software (CSW, DataApex Ltd, the Czech Republic). Monoaminergic activities were calculated dividing the concentration of the monoamine metabolite by the concentration of the main monoamine (e.g. 5-HIAA/5-HT). Due to low concentration levels of DA and interacting peaks we were not able to analyze DA concentrations in Dm and as a consequence dopaminergic activity in Dm.

2.5. Data Analysis

The normality of data was tested by Lilliefors tests. Monoaminergic activities were arcsin transformed and monoamine values log transformed to achieve normality. Comparisons of cortisol concentrations, monoaminergic activities and monoamine concentrations between the different test groups were done using one-way Anova and Unequal N HSD Post-hoc. Behavioural data was analysed with non-parametric tests (Friedman tests), to evaluate differences in locomotory activity and bottom grazing behaviour over time in control and alarm cues groups.

All statistical analyses were performed using Statistica (version 11) for windows. Statistical significance was taken at $P < 0.05$.

3. Results

3.1. Whole body cortisol and neurochemistry in Dl and Dm

Confinement stress resulted in an elevation of cortisol, when compared to fish exposed to alarm cues ($p = 0.005$) and controls ($p < 0.001$), but no differences were observed between alarm cues group and controls ($p = 0.66$) (Anova: $F = 11.902$; $p < 0.001$; $df = 26$) (Fig. 1).

The same general pattern was observed in serotonergic activity. Confinement stress resulted in an elevation of serotonin metabolism in the Dl, when compared fish exposed to alarm cues ($p = 0.03$) and controls ($p = 0.01$), but no differences were observed between alarm cues group and controls ($p = 0.75$) (Anova: $F = 6.774$; $p = 0.004$; $df = 26$) (Fig. 2A). The same pattern was observed in Dm, where confinement stress resulted in an elevation of serotonin metabolism, when compared to fish exposed to alarm cues ($p = 0.04$) and controls ($p = 0.02$), but again no differences were observed between alarm cues group and controls ($p = 0.89$) (Anova: $F = 5.728$; $p = 0.009$; $df = 26$) (Fig. 2B). Nonetheless, a similar general pattern was observed in cortisol and monoaminergic activation between alarm cues and control group. However, as stated before this response was less pronounced than in confined stressed fish, and not statistically significant. No differences where found between groups in 5-HT and 5-HIAA concentrations or in dopaminergic activity, DA and Dopac concentrations in both Dl and Dm ($p > 0.05$). Monoamine concentrations and activities are shown in Table 1.

3.2. Behaviour

Behaviourally the injection of alarm cues did not resulted in differences of locomotory activity over time ($p > 0.05$). Friedman analysis revealed that fish that presented bottom-grazing behaviour initially reduced this behaviour over time when presented with skin extracts ($p = 0.049$) (Fig. 3A). In control group no behavioural differences were observed over time ($p = 0.85$) (Fig. 3B).

4. Discussion

The study of monoaminergic systems in fish over the past years has drawn growing interest, especially since zebrafish models appear to be suitable to study complex behaviours and the neuroendocrine responses behind such behaviours [34]. In the present study confinement stress resulted elevated cortisol values and increased 5-HTergic activity in Dl and Dm. There are a number of studies showing that stress increases 5-HTergic activity in the telencephalon in fish (e.g. [22], [35], [36] and [37]). To our knowledge there are relatively few studies focusing on the involvement of regional brain monoamine signalling in Dl and Dm in response to stress. Vindas et al. [24] reported that omission of expected reward results in elevated 5-HT levels in Dm. Furthermore, Øverli et al. [22] demonstrated that social defeat leads to increased 5-HTergic turnover in Dl. However, Basic et al. [23] demonstrated that confinement stress did not affect serotonergic activity in Dl, which is in contrast to the latter study. Our results clearly show that confinement stress resulted in a distinct rise in 5-HT activity in Dl and Dm, manifesting the involvement of these brain structures in the stress response.

Although, exposure to skin extracts did not significantly differ from controls, the same general pattern seen with confinement stress was seen after exposure to alarm cues, i.e., increased cortisol concentration levels and serotonergic activity in Dl and Dm. However, this response was less pronounced than that seen in confined stressed fish. The necessity of studying stress responses at the level of specific brain structures as it was performed in the present study is of growing importance as previous studies using hole-brain monoaminergic concentrations did not reveal monoaminergic differences in acutely stressed fish [38]. The present data is slightly in contrast with studies in rodents. For example, rats exposed to fox odour presented a significant elevation in corticosterone [14]. Moreover, mice exposed to predator odour showed an increase in 5-HIAA and MHPG (norepinephrine metabolite) concentrations within the hippocampus and amygdala [13] areas homologous to Dl and Dm regions in fish, respectively. Furthermore, rats exposed to predator odour exhibited an increase in dopaminergic activity in the amygdala but not an increase in dopamine concentration [14]. Moreover, Höglund et al. [26] demonstrated that skin extracts affected brain dopaminergic activity in the telencephalon of crucian carp, suggesting that this neurotransmitter should be affected by skin extract in Dl and Dm. However, in the present study we could not detect any effect of skin extract on dopaminergic signalling in these parts of the brain.

Taken together, the present results suggest that skin extract exposure by itself was not an intense enough stressor to significantly affect brain monoaminergic signalling in Dl and Dm. Still, it is possible that other stimulus, such as visual predator cues can (or could) elicit specific changes in the monoaminergic activity of telencephalic regions in Nile tilapia.

In the present study we could not detect any effects of skin extract on locomotor activity. The present results are opposed to the results obtained by Höglund et al. [26] who described a decrease in locomotor activity after a second injection of skin extract in the water. Their study however, was performed in crucian carp (*Carassius carassius*) so the possibility remains that differences in locomotor activity in the presence of skin extracts may be species dependent.

Exposure of Nile tilapia to skin extracts reduced feeding anticipatory behaviour in the present study, indicating a shift of behaviour from foraging to predator awareness. This may reflect a general anti-predator apprehension. Anti-predator apprehension is defined as a reduction or suppression of other activities such as foraging or mate seeking as a result of increased attention to detecting and/or responding to potential predators [3]. The fact that threat sensitive behaviour shift in the present study was not accompanied with a direct effect on brain activation pattern, HPI-axis or increased locomotor activity is in accordance with a recent study performed by Brown and co-workers [39] who demonstrated that fish pre-exposed to high-predation risk display higher levels of neophobia towards novel predator cues. This suggests indirect effects of predatory cues, where alertness to changes in the environment is elevated. Future studies, combining acute challenges with skin extracts are needed to verify if such changes in alertness underlie the suppression of feeding anticipatory behaviour in the present study.

Furthermore, understanding the mechanism underlying threat-sensitive behaviour in fish is of major importance not only in fundamental research but also in more applied sciences such as aquaculture. By exhibiting threat-sensitive behaviour fish spend energy in activities others than growth which necessarily leads to economical losses. Worthy of mention is the possible increase of threat-sensitive behaviour in recirculation aquaculture systems, a type of production system that is foreseen to increase in the coming years due to its environmental advantages [40]. In these systems the water is re-used to produce fish and over time alarm substances released from fish due to handling/grading may accumulate in the systems and potentiate the perception of threat. Furthermore, if the neural basis for threat-sensitive behaviour in fish is homologous to fear and anxiety behaviours found in humans then the use of fish in drug screening could be considered.

In conclusion, increased 5-HTergic activity in Dl and Dm in response to a standardized confinement stress manifests the involvement of these brain structures in the neuroendocrine stress response in fish. Exposure to skin extracts suppressed feeding anticipatory behaviour, indicating a threat sensitive behaviour as response to a predatory cue. Even if this threat sensitive behaviour was not accompanied with a significant effect on pallial activation pattern, it showed the same general pattern as observed in confinement stressed individuals. It is possible that the suppression of feeding anticipatory behaviour reflects a general elevation of awareness, and combining skin extract with other challenges may reveal neuroendocrine effects associated with this predatory cue. Taken together, the present results suggest that limbic responses to stress and fear are similar in mammals and teleosts, supporting the use of teleost models in the study of affective states.

Acknowledgements

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Figures and Table

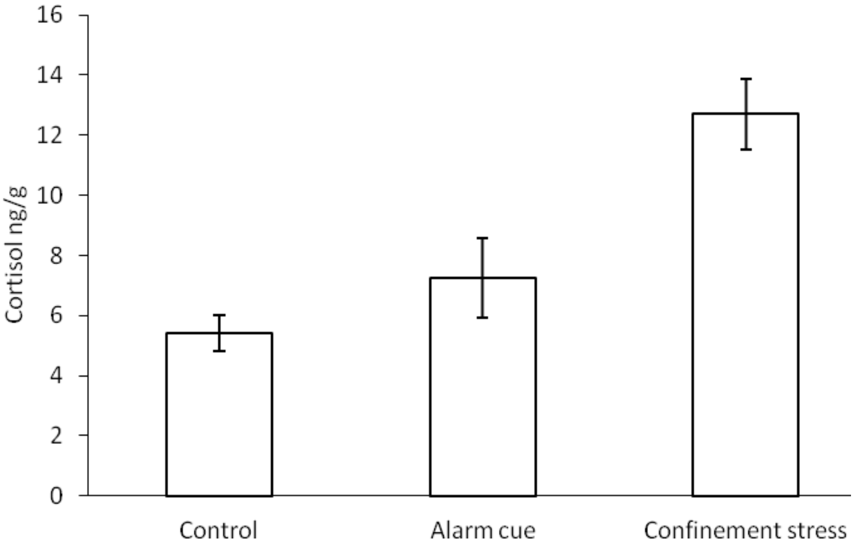


Figure 1. Cortisol concentrations. Differences in muscle cortisol concentrations between control (N = 9), alarm cues (N = 10) and confinement stress (N = 11) groups. Different letters indicate statistical differences (P-value ≤ 0.005).

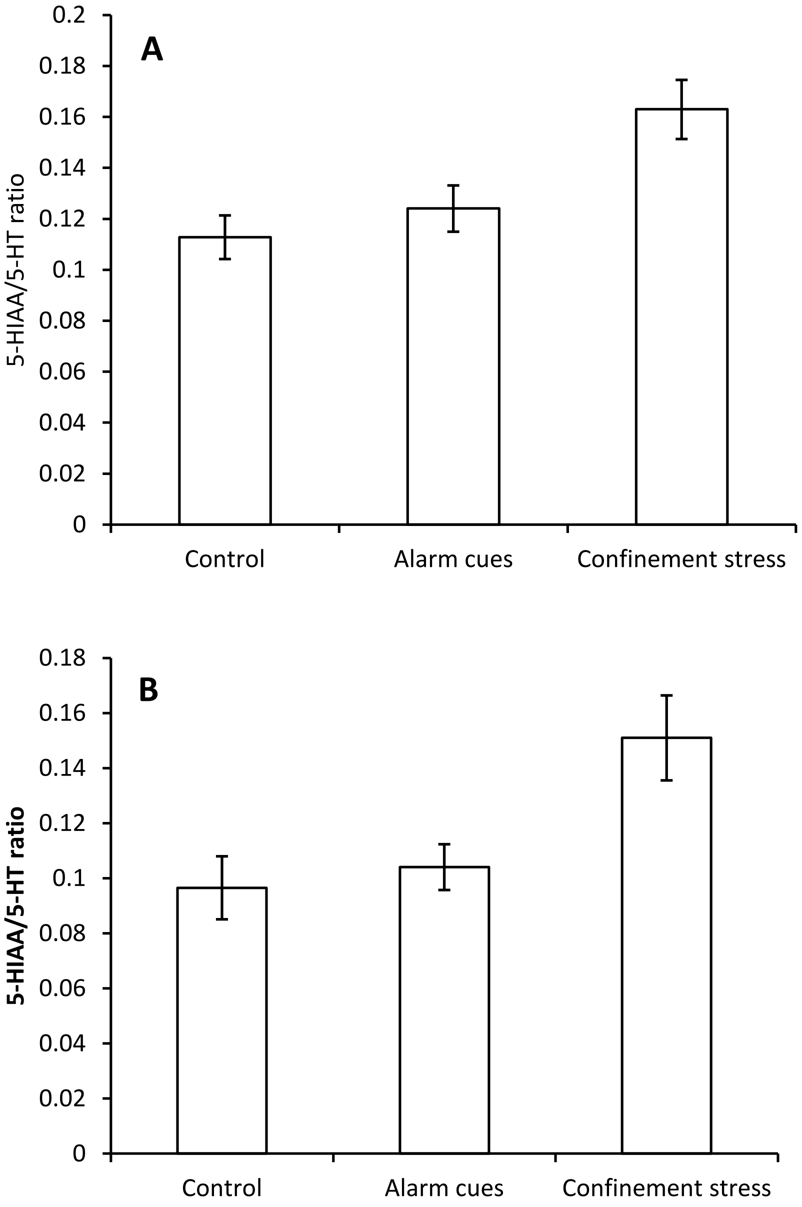


Figure 2. Serotonergic activity in A - dorsolateral (Dl) and in B - dorsomedial (Dm) telencephalon of Nile tilapia exposed to either control conditions (N = 9), conspecific alarm cues (N = 10) or confinement stress (N =11). Error bars designate standard error of mean. Different letters indicate statistical differences (P-value ≤ 0.05). For detailed ANOVA statistics see table 1.

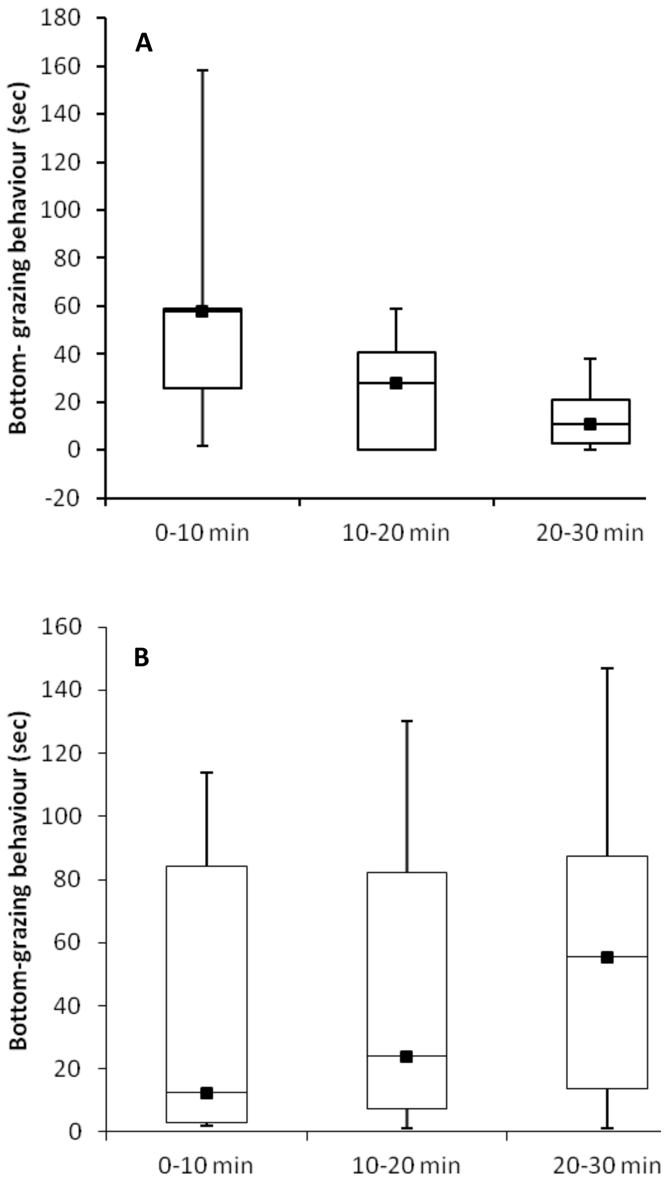


Figure 3. Bottom-grazing behaviour. Median (solid squares), interquartile range (box) and minimum and maximum of the time spent bottom-grazing during 3 periods. Each figure compares bottom-grazing behaviour before injection and after two injections within groups. **A** – alarm cues group – (0-10 minutes: time before alarm cues injection; 10-20 minutes: time after first alarm cues injection; 20-30 minutes: time after second alarm cues injection) – Friedman ANOVA p = 0.049; **B** – control group – (0-10 minutes: time before distilled water injection; 10-20 minutes: time after first distilled water injection; 20-30 minutes: time after second distilled water injection) – Friedman ANOVA p = 0.846.

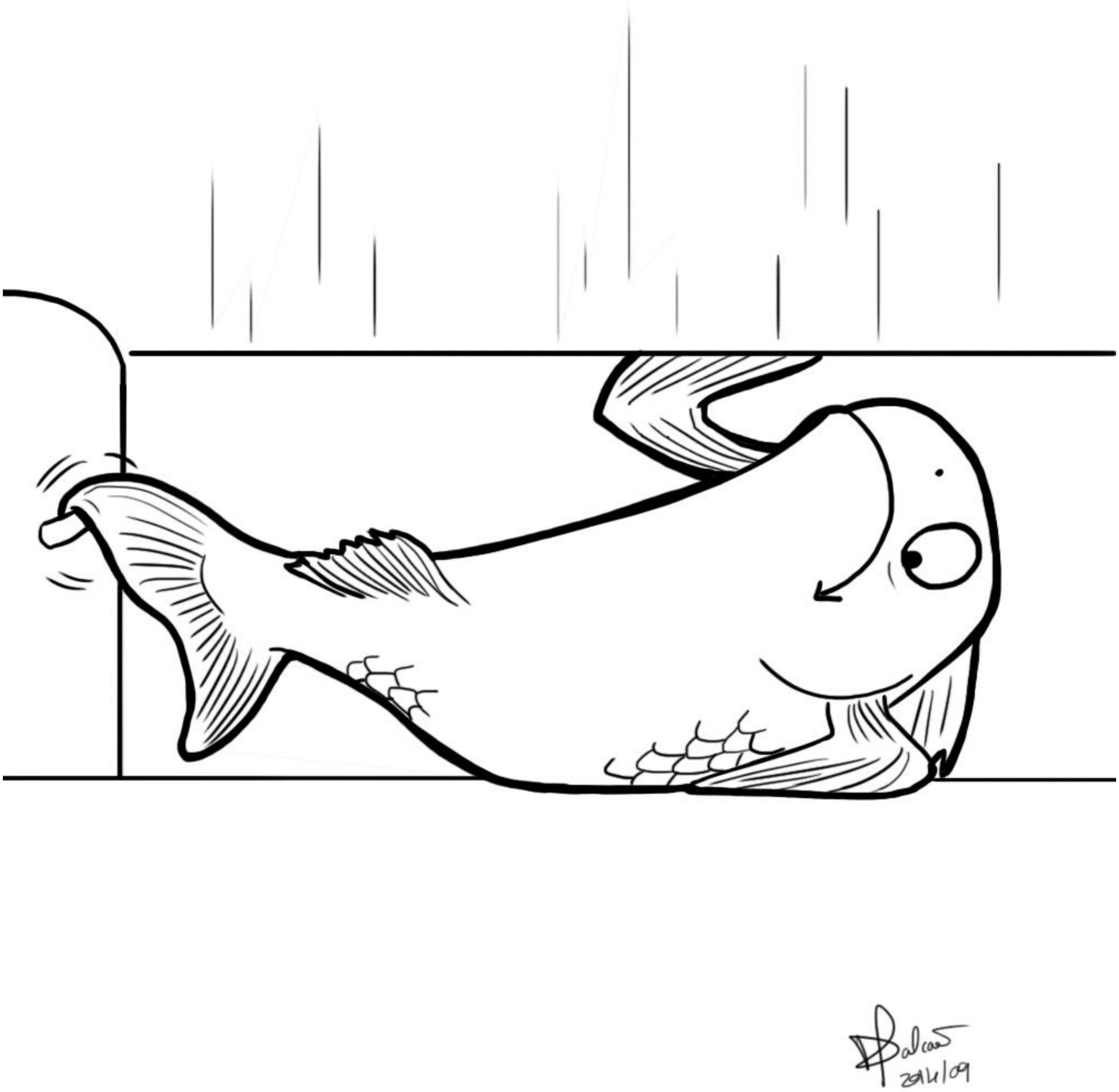
Table 1. Monoaminergic concentrations and activities in Dl and Dm. Values of concentrations of 5-HT, 5-HIAA, DA and Dopac (µg/g of protein) (mean ± SE) in Dl and Dm and serotonergic and dopaminergic activity in Dl and Dm. n values are shown within ( ).

	Control	**Alarm cues**	**Confinement stress**	**Interaction**	**Direction**
Dl					
5-HT	43±6 (8)	38±4 (10)	47±9 (11)	*F*= 0.18 $p = 0.83$	
5-HIAA	4.9±0.8 (8)	4.8±0.7 (10)	7.7±1.5 (11)	*F*= 2.0 $p = 0.16$	
5-HT/5-HIAA	0.11±0.01 (8)	0.12±0.01 (10)	0.16±0.01 (11)	*F*= 6.8 $p < 0.01$	↑ in confinement stress
DA	7.3±1.2 (8)	5.1±1.3 (9)	6.3±1.0 (11)	*F*= 1.9 $p = 0.18$	
DOPAC	1.7±0.5 (8)	1.4±0.3 (9)	2.3±0.4 (11)	*F*= 1.3 $p = 0.30$	
DOPAC/DA	0.23±0.05 (8)	0.31±0.07 (8)	0.44±0.10 (11)	*F*= 2.0 $p = 0.16$	
Dm					
5-HT	17±2 (8)	19±2 (10)	16±3 (11)	*F*= 0.65 $p = 0.53$	
5-HIAA	1.7±0.3 (8)	1.9±0.2 (10)	2.2±0.3 (11)	*F*= 1.0 $p = 0.40$	
5-HT/5-HIAA	0.10±0.01 (8)	0.10±0.01 (10)	0.15±0.02 (11)	*F*= 5.7 $p = 0.01$	↑ in confinement stress
DA	N.A.	N.A.	N.A.	N.A.	
DOPAC	0.97±0.22 (8)	0.69±0.05 (10)	1.0±0.2 (11)	*F*= 1.1 $p = 0.36$	
DOPAC/DA	N.A.	N.A.	N.A.	N.A.	

*N.A.-not applicable

Paper III

Linking Fearfulness and Coping Styles in Fish



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Linking Fearfulness and Coping Styles in Fish

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Abstract

Consistent individual differences in cognitive appraisal and emotional reactivity, including fearfulness, are important personality traits in humans, non-human mammals, and birds. Comparative studies on teleost fishes support the existence of coping styles and behavioral syndromes also in poikilothermic animals. The functionalist approach to emotions hold that emotions have evolved to ensure appropriate behavioral responses to dangerous or rewarding stimuli. Little information is however available on how evolutionary widespread these putative links between personality and the expression of emotional or affective states such as fear are. Here we disclose that individual variation in coping style predicts fear responses in Nile tilapia *Oreochromis niloticus,* using the principle of avoidance learning. Fish previously screened for coping style were given the possibility to escape a signalled aversive stimulus. Fearful individuals showed a range of typically reactive traits such as slow recovery of feed intake in a novel environment, neophobia, and high post-stress cortisol levels. Hence, emotional reactivity and appraisal would appear to be an essential component of animal personality in species distributed throughout the vertebrate subphylum.

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Introduction

Individual variation in the physiological and behavioural responses to aversive stimuli is increasingly viewed as adaptive responses that are crucial for survival in a continuously changing environment [1]. In contrast to the presumed advantages of flexible responses, when faced with changing environmental conditions, individuals of the same species or population show consistent responses in stressful and dangerous situations [2,3,4]. This phenomenon is referred to as animal personality [5], behavioural syndrome [6], temperament [7], or coping style [2]. In general, some individuals show a proactive behavioural pattern, consistently being more aggressive, more explorative, more neophilic, and more actively avoiding danger than their reactive counterparts. In addition to consistent differences in behavioural traits that correlate among each other, proactive and reactive individuals also differ in neuro-endocrine traits. Proactive individuals have a low hypothalamus-pituitary adrenal/ interrenal (HPA, HPI in fish) axis responsiveness, but high sympathetic reactivity, while the opposite is true for reactive individuals [2,3,8]. There is evidence that the physiological traits correlated to animal personality are heritable (e.g. [9,10]), and contrasting personalities are associated with different fitness consequences [5], which suggests that personality is subjected to evolutionary processes. Likewise, emotions are thought to confer survival advantages by giving animals the ability to avoid harm/punishments and seek valuable resources/reward (e.g. [11,12]). Under an evolutionary point of view, therefore, emotions - by being functional and adaptive - are unlikely to have evolved spontaneously in the recent human lineage. In addition, the capacity for emotions is likely to differ substantially between species as a consequence of both evolutionary lineage and selective pressures associated with life history [13]. Fear, for example, as a negative emotion increases precautionary behaviour, allowing individuals to avoid potential threat or danger and, therefore has an adaptive value [14].

There are indications that certain stimuli are appraised as fearful in a wide variety of animal groups. This has been demonstrated by behavioural responses to direct exposure to novelty and/or predators (e.g. [15–19]). Such responses in fish have been used to describe differences in boldness, and have been interpreted in different ways, such as neophobia [19], reduced exploration or hesitancy [17] or emotional reactivity [18] including fearfulness [15,16]. However, to which extent responses to direct exposure to aversive stimuli involves common phylogenic roots of cognitive processes involved in fear, such as appraisal, is largely unknown.

The link between personality or coping styles and emotions, including fear, has been addressed in humans, non-human mammals and birds. The individual variation in the threshold for when a stimulus becomes inhibiting rather than stimulatory, i.e. coping style (sensu [2]) is likely correlated to the individual's subjective experience of that stimulus in a given situation. Different personality types have been shown to differ in emotional reactivity [20], the reactivity to negative appraisals [21] and susceptibility to psychological illness [22]. Fear reactivity, for example, has been shown to be a dimension of temperament in humans [23,24] influencing the susceptibility to depression and anxiety [25]. However, how evolutionary widespread these putative links between personality and the expression of fear are remains to be studied.

Utilizing a teleost fish as a comparative vertebrate model allows investigation of the link between emotions and endocrinal and behavioural dimensions of coping styles in this animal group. Further, this will add to our understanding of the evolutionary relevance and adaptive value of personality, and unravel whether emotions are an essential component of coping styles in species distributed throughout the vertebrate subphylum.

We investigated whether coping styles can predict fear responses in fish using the principle of avoidance learning (combination of classical and operant conditioning). Fish previously screened along the proactive-reactive styles continuum (using 3 subsequent tests: feed recovery after transfer itno a novel environemnt, novel object and net restraining) were given the possibility to escape an aversive stimulation that was associated with a cue signalling the onset of the aversive stimuli. In this study, individuals of Nile tilapia were subjected to a signaled aversive stimulus for 7 days (conditioned stimulus, CS: stopping water inflow for 30 sec; unconditioned stimulus, US: confinement stress by lowering a frame into the tank until touching the dorsal fin). Afterwards fish were exposed to the CS only and were allowed to escape from the previous confinement area by using an escape door. The individual variation in escape behavior in this fish was registered and related with the behavior and neuro-endocrine profiling of the same fish screened for coping styles.

Nile tilapia, *Oreochromis niloticus* was used as a model species due to its well characterized behaviour, endocrine and physiological profiles in different behavioural paradigms, including conditioning [26,27].

Results

Coping styles in Nile tilapia

Feed intake recovery after transfer into a novel environment was shown to predict neophobia ($r_s = 0.45$, $p = 0.027$, Fig. 1). This suggests that fish recovering their feed intake faster after transfer to a novel environment show lower neophobic response when exposed to a novel object, i.e. traits typically ascribed to bold individuals.

No correlation was however found between cortisol after the net restraining stress, feed intake recovery and the behaviour during the novel object test ($p > 0.05$).

Avoidance learning

Latency to escape from the conditioned stimulus (CS, stopping the water inflow, from now on *water off*) decreased significantly over the 7 days of training (one-way repeated measures ANOVA, $F_{3.10,71.3} = 14.6$, $p < 0.001$). On training day 1 fish took, on average, 513 sec to escape, and by day 7 fish were escaping in less than 30 sec ($p = 0.001$, Bonferroni comparison, Fig. 2). During avoidance learning, 22 fish (out of 24) learned to associate the CS (*water off*) with the unconditioned stimulus (US, exposure to a confinement stress); i.e. escaped even in the absence of the confinement frame on day 8. The 2 fish that did not learn were excluded from the analysis concerning the link between coping styles and avoidance learning. It should be noted, however, that these fish did not represent outlier values in regard to previously measured variables.

Control and treatment fish did not differ significantly in the latency to escape (Fig. 3, $p > 0.05$, Kruskall Wallis test). However, when the time between first escape and return is considered (Figure 3C) significant differences were detected ($p < 0.001$). Fish exposed to the confinement stressor only (*C2- confinement*) and in combination with *water off* (*C3-water off/confinement*), escaped through the partition door and did not return to the side where the confinement frame was inserted. Fish exposed to *water off* only during the 7 days of training exhibited the lowest time between escaping and returning (25.2±12.09 sec) while fish exposed to water off only on day 8 after 7 days of pairing between *water off* and confinement showed a significantly higher time between escaping and returning (343.9±71.44 sec, $p = 0.003$, Dunn's comparison). The number of returns and time spent in the confinement area was also higher in *C1-water off* (# returns: 6.4±1.3; time spent in confinement area: 488.4±76.6 sec) as compared with *T-learning* (# returns: 4.9±0.9; time spent in confinement area: 378.2±61.8 sec) but not significantly different ($p > 0.05$).

The relationship between coping styles and avoidance learning

Fish exposed to *T-learning* showed a pronounced individual variation in escape responses. Individuals that took less time to escape were also the individuals that took longer to return to the side of previous confinement ($r_s = -0.60$, $p = 0.009$) and spent less time in the confinement area on day 8 ($r_s = 0.44$, $p = 0.039$) while in addition showing the highest cortisol levels in the end of the avoidance learning test ($r_s = -0.44$, $p = 0.045$), suggesting that fish escaping faster, taking longer to return and spending less time in the confinement area were more stressed even in the absence of the confinement frame.

Time to return after escaping was shown to be correlated positively to cortisol level after the net restraining stress applied on day 35 ($r_s = 0.60$, $p = 0.009$, Table 1). On the contrary, individuals returning more often to the area of previous confinement (number of returns) and spending more time in that area, exhibited typical characteristics of bold individuals such as lower cortisol response after net restraining ($r_s = -0.48$, $p = 0.025$,), higher feed intake after transfer to a novel environment ($r = 0.44$, $p = 0.041$), less neophobia when exposed to a novel object ($r = 0.54$, $p = 0.01$ with number of times entering 10 cm radius and $r = 0.47$, $p = 0.029$ with number of times entering 5 cm radius) and more actively trying to escape when restrained ($r_s = 0.58$, $p = 0.005$).

Discussion

It is now generally accepted that in fish, individual variation in behaviour and physiology when exposed to environmental challenges, reflect the existence of coping styles [3,28]. This study showed, for the first time, that Nile tilapia *Oreochromis niloticus,* also exhibits divergent coping styles with proactive individuals being characterized by a faster feed intake recovery after transfer into a novel environment and less neophobic when exposed to a novel object, as compared to reactive individuals. Such behavioural responses to challenges have also been described in other fish species [29–35].

In classical conditioning, repeated CS–US pairing results in the acquisition of a behavioural conditioned response (CR). In this study, behavioural conditioned response was observed after fish were exposed to the avoidance learning test. The escape behaviour differed significantly between *C1-water off* and the other controls and *T-learning,* as these fish, despite using the escape door returned very quickly to the side where the inflow water was interrupted. In *C1-watter off,* the use of the escape door is probably more related to exploration than to escape behaviour. Fish exposed to the US both alone or in combination with the CS, escaped to the other side of

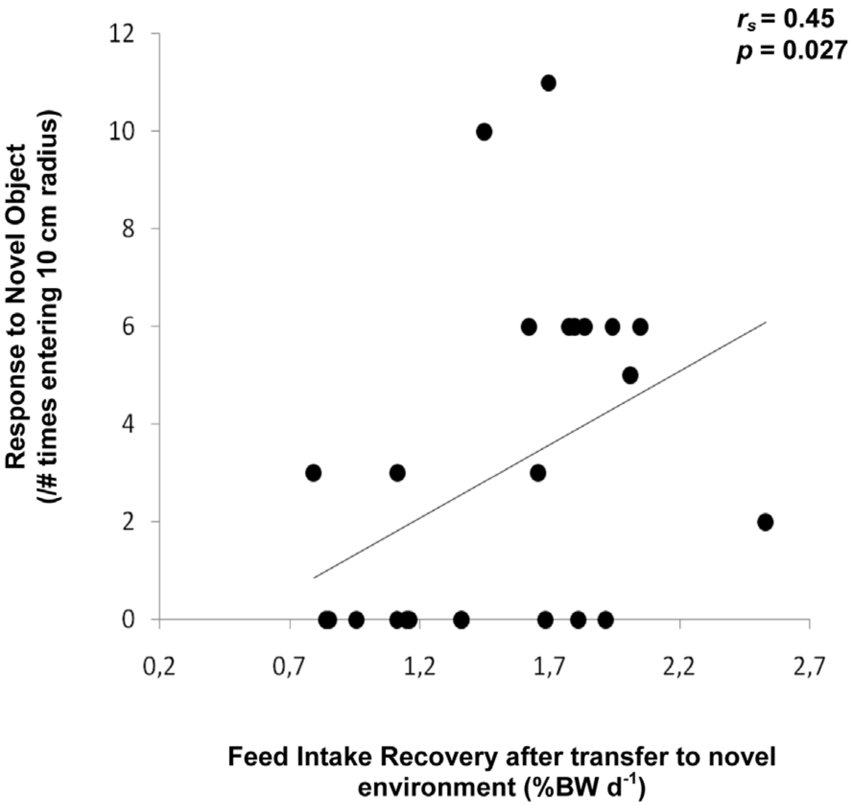

Figure 1. Relationship between feed intake recovery after transfer to a novel environment and neophobia (n = 24).
doi:10.1371/journal.pone.0028084.g001

the tank and never returned during the 15 minutes of observation. Fish exposed to *T-learning* (pairing CS–US for 7 days followed by exposure to CS only on day 8) took longer to return to the area where the confinement frame was previously used as compared to fish exposed to *water off* only. Despite fish in *C1-water off* and *T-learning* were exposed to the same stimuli (*water off*), their behaviour differed significantly suggesting that the way the stimuli was interpreted or appraised also differed. This indicates that Nile tilapia can learn how to avoid aversive stimuli by conditioning. A previous study by [26] showed that Nile tilapia can be conditioned

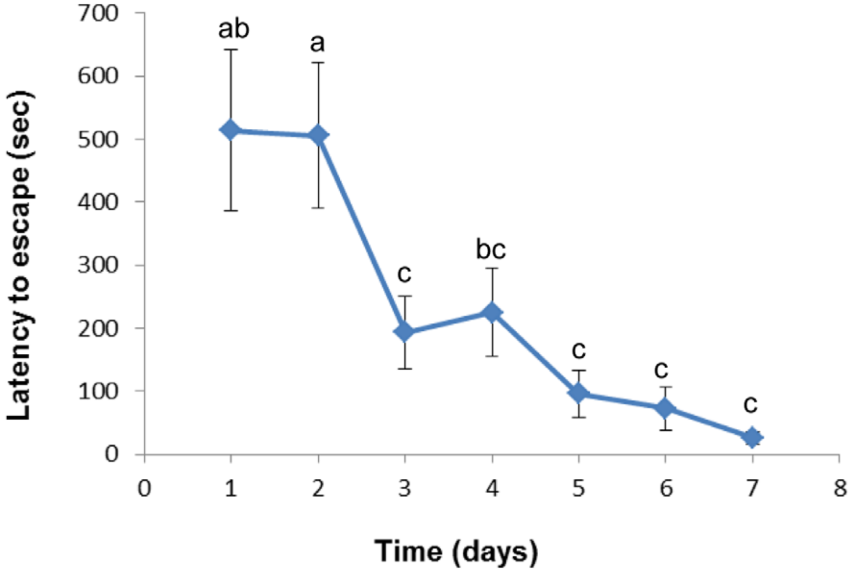

Figure 2. Reduction in latency to escape of *T* fish over the 7 days of CS-US pairing. Each point represents the mean ± SE of 24 individuals. Different letters denote statistical significance at a significant level of $p<0.05$ after repeated ANOVA and Bonferroni comparisons.
doi:10.1371/journal.pone.0028084.g002

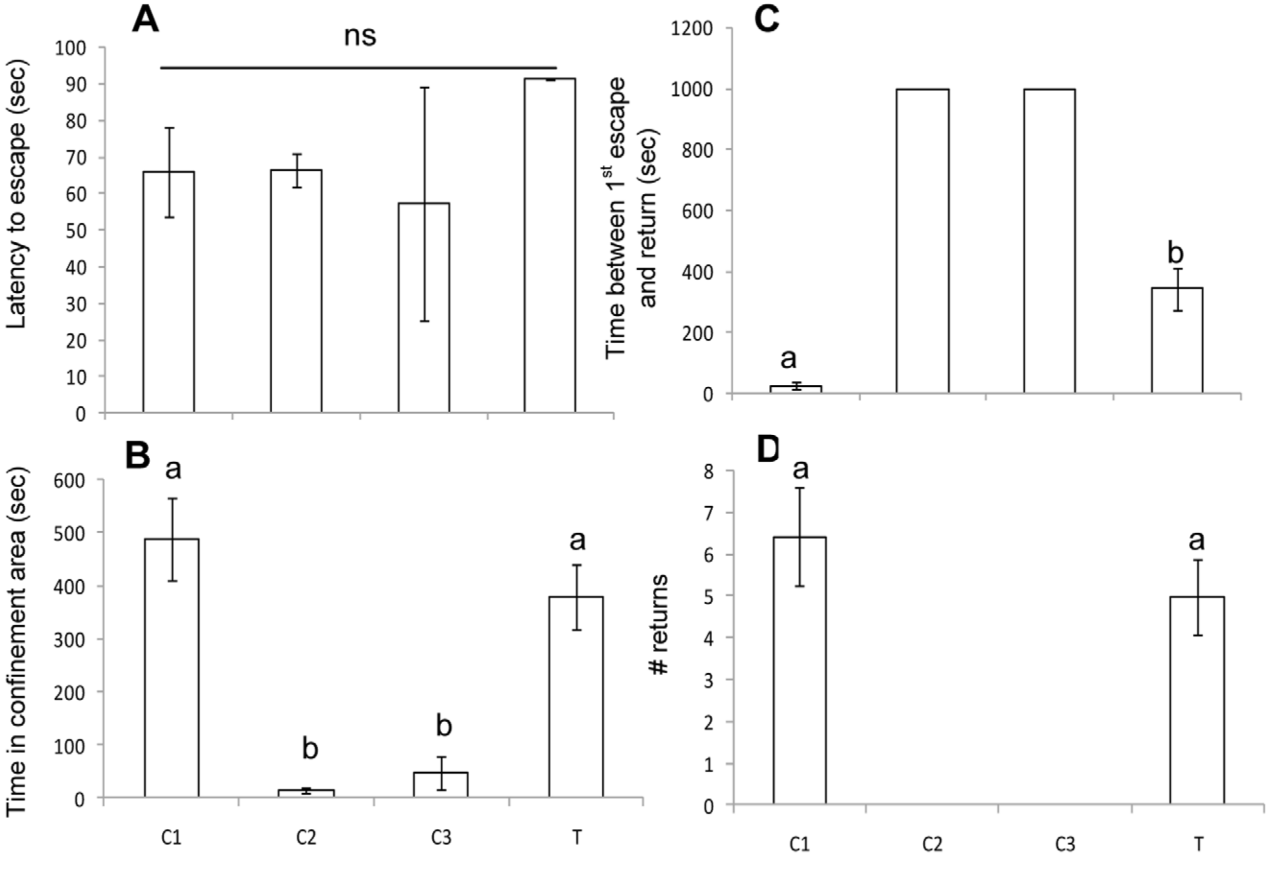

Figure 3. Comparison of escape behavior between *T* and *C1-C3* fish. Latency to escape (A), time spent in confinement area (B), time between 1st escape and 1st return to confinement area (C) and total number of returns to confinement area (D) in *C1–C3* (n = 6 in *C1* and *C2* and n = 5 in *C3*) and *T* on day 8, after 7 days of training (n = 22, 2 fish did not escape on day 8 and were not included).

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to display a stress response in response to conditioned stimuli. In the present study, in addition to classical conditioning, we allowed fish to escape from the aversive stimuli and the results suggest that Nile tilapia is capable of conditioned avoidance learning.

The reason why fish returned to the area of the tank where the confinement frame has been previously used is not clear. It should be noted that the area used for confinement was also the area used for feeding, therefore, one possibility is that the motivation to feed played a role in returning to a potentially dangerous area.

The concept of avoidance learning has been used to investigate fear in different animal species (e.g. in fish [36,37]). The emergence of consciousness and feelings in fish has been a matter of intense scientific debate (e.g. [38–41]). Some authors [39–41] argue that this is not possible because their behaviour is simple and

Table 1. Correlation between variables indicating coping styles and fearfulness.

Coping styles\Fearfulness	Latency to escape (sec)	Time between 1st escape and return (sec)	# returns	Time spent in confinement area (sec)
Plasma cortisol after Net Restraining (ng/ml)	ns	$r_s = 0.60$ $p = 0.009$	ns	$r_s = -0.48$ $p = 0.025$
# escape attempts during Net Restraining	ns	ns	$r_s = 0.58$ $p = 0.005$	ns
FI recovery Novel Environment (%BW d^{-1})	ns	ns	$r_s = 0.44$ $p = 0.04$	ns
# times entering 10 cm radius from Novel Object	ns	ns	$r_s = 0.54$ $p = 0.01$	ns

(n = 22 when considering # of returns and time spent in confinement area − 2 out of the 24 fish did not escape on day 8 - and n = 19 when considering the time between escape and return − 2 out of the 24 fish did not escape on day 8 and 3 fish escaped but never returned to the confinement area).

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reflexive and they lack a neocortex. Yet, a growing body of evidence related to cognitive [42], neuroanatomic [43,44] and emotional [36,37,45] aspects of fish behaviour provides strong support for the ability to feel in fish. In the present study, the observed differences in escape behaviour between fish exposed to *C1-water off* and *T-learning* suggest that these responses are not merely reflexive in nature but are associated with a subjective interpretation of the stimuli. If a reflexive response would be present one would have expected a similar behavioural response between fish exposed to the same stimulus (in our case, *C1-water off* and *T-learning*), which was not the case.

The way individual fish behaved when exposed to *water off* on day 8 (after 7 days of CS–US pairing) was shown to be correlated with traits indicative of coping styles. This suggests that the individual variation in how negative the CS was interpreted (negative appraisal) depends of an individuals' coping style. The link between coping styles and the subjective experience of stimuli and emotional responses has never been investigated in fish, despite studies showing that both (i.e. coping styles and emotions) are possible in fish. This study showed that fish avoiding the area of previous confinement were the fish exhibiting characteristics usually ascribed to reactive or shy individuals, such as lower feed intake recovery after transfer into a novel environment, more neophobic and higher HPI responsiveness after net restraining as compared to proactive or bold individuals. One possible explanation could be a difference in behaviour flexibility between reactive and proactive individuals, in what proactive individuals would be more flexible and therefore prone to modify learned behaviours (in this case the association between *water off* and the onset of confinement resulting in escaping behaviour). This explanation seems, however, unlikely as proactive individuals were shown to be less flexible in modifying learned behaviour than reactive individuals [46]. An alternative explanation is that individuals of the proactive type were less fearful when presented with a signal previously associated with an aversive stimulus, as compared to individuals of the reactive type. Fear is an important component of personality in humans [24,47], other mammals (e.g., in dogs [48]; in rats [20,49]) and in birds [50]. The argument for the link between coping styles and fearfulness in fish is evolutionary: fearfulness may be adaptive as it allows individuals to avoid potential threat or danger; from this view, it follows that individual variation in the threshold for when a stimuli becomes inhibitory or stimulatory, i.e. coping style, is likely to be linked with the subjective experience of that stimulus in a particular situation. Severe, chronic and/or unpredictable conditions are likely to provide reactive coping more benefits while mild, intermittent stress and/or predictable conditions are likely to favor proactive responses [51]. Therefore, emotional distress is likely an essential component of reactive coping. This study suggests that the link between coping styles/personality and the expression of emotional or affective states such as fear is an evolutionary widespread phenomenon throughout the vertebrate subphylum, including fish.

This study showed for the first time that cortisol is strongly linked to behaviours indicating fearfulness. A key question that remains to be investigated is whether the link between cortisol responsiveness and fear responses is based on a cause or effect connection. Does the fear reaction potentiate cortisol response, or does elevated cortisol exposure over time alter limbic structures in the brain that mediate fear responses [52]? Further studies are needed to unravel the time course and coordination of psychological and biological stress responses. Extensions of this study could be the investigation of the underlying brain activity in (e.g. through monoamine activity) in differential brain parts, particularly in the medial pallium, an area that is believed to be homologous of the amygdala of land vertebrates [53] and to play an important role in fear responses [54].

This study provides the first evidence that in fish, similarly to what has been found in other vertebrates, individual's coping style is predictive of how stimuli are appraised and the subsequent degree of avoidance behaviour. These results support the inclusion of emotional reactivity and appraisal as essential component of animal personality in species distributed throughout the vertebrate subphylum.

Materials and Methods

This experiment was approved by the Ethical Committee judging Animal Experiments (DEC no 2009049) of the Wageningen University, The Netherlands.

Experimental animals, housing and feeding

Forty-two juveniles of Nile tilapia *Oreochromis niloticus* with an initial body weight of 40.8±0.8 g (means±SE) were used as experimental animals. From these, 24 individuals, randomly selected, were used to characterize coping styles and avoidance learning while the remaining fish were used as controls in the avoidance learning test. All animals were obtained from a local tilapia producer (all-male, TilAqua, The Netherlands) where they had experienced common housing and feeding conditions. Upon arrival at Wageningen University, fish were group-housed in a stock tank for 15 days until the start of the experimental procedures. During this period fish were fed *ad libitum* with a commercial diet (2 mm floating pellets; 44% crude protein, 10% fat, 25% carbohydrates, 11.5% ash; Skretting, France) twice a day (08:00 and 16:00) by hand. The same feed was used during the experimental procedures.

During the screening for coping styles (35 days) and avoidance learning (8 days), fish were housed individually in a 40-L glass aquarium (40 cm length×30 cm width×35 cm height, 30 L water capacity, water flow rate was 4 L min^{-1}). Tanks were part of a recirculation system operated at a water refreshment rate of 1500 L kg feed^{-1} d^{-1} [55].

Water temperature (26.5±0.1°C), pH (range between 8.6 and 8.7), conductivity (1.96±0.01 mS cm^{-1}), TAN (0.05±0.03 mg L^{-1}), NO_2-N (0.00±0.00 mg L^{-1}) and NO_3-N (46.0±2.7 mg L^{-1}) were checked daily. A 12 h: 12 h light: dark photoperiod was maintained with daybreak set at 7:00 h.

Coping styles

Screening for coping styles consisted of subjecting each fish to 3 subsequent tests: 1) novel environment (based on [29,56]), 2) novel object test (based on [57]) and 3) net restraining test (based on [55]).

The novel environment test consisted of transferring individual fish to a 40-L glass aquarium and following daily feed intake recovery for 14 days. Fish (n = 24) were fed *ad libitum*, by hand, twice per day (08:00 and 16:00) using the same commercial feed as used during the previous 15 days. Feeding continued for a maximum of 1 h, after which the remaining pellets were collected and counted. The average feed intake of the 1st week after transfer to the novel environment was used as indicative of feed intake recovery.

Individually housed fish were kept visually isolated from one another by black plastic around tanks, except for the front side which allowed daily visual observations of the fish.

The novel object test (day 30, after onset of isolation) consisted of a sudden drop of a weighted red LEGO brick (3×3×2 cm, length×width×height) in the middle of the tank, using transparent fishing line attached to the brick to avoid visual contact between the fish and researcher. A mesh screen with squared holes (1 cm) was used on top of the aquarium to allow the determination of the number of times fish entered a 5 and 10 cm radius around the novel object. The latency to enter the 5 cm radius area was also determined using a stopwatch. Fish was considered within the 10 or 5 cm cut-offs when the head was inside that area. The observation period lasted 15 minutes after which the novel object was gently removed.

The net restraining test was conducted on day 35 and consisted of keeping each fish in an emerged net for 60 sec followed by 1 h in the respective tanks (based on [55]). While in the net, the escape behaviour of each fish was determined by counting the number of escape attempts (i.e. body displacements). Blood samples were collected 1 h after the start of net restraining. Fish were rapidly netted and placed in 0.3 g L^{-1} of tricaine methanesulfonate (TMS, Crescent Research Chemicals, Phoenix, Arizona, USA using 0.6 g L^{-1} of sodium bicarbonate as buffer). One mL of blood was collected from all fish by hypodermic syringe (containing 3 mg of Na_2EDTA) from the caudal blood vessels. This procedure was finalized within 3 min after fish were caught and anaesthetized. The collected blood was placed in cooled 1.5 mL plastic tubes, mixed and centrifuged at 6000×*g* for 5 min at 4°C. After centrifugation plasma was collected and stored at −20°C until cortisol analysis (see below).

Avoidance learning

After being screened for coping styles each fish was exposed to an avoidance learning paradigm for 8 days (Fig. 4). Four different experimental groups of fish were established: A treatment group (*T- learning*, n = 24) underwent the full avoidance learning test utilising a signalled aversive stimulus (unconditioned stimulus, US). The conditioned stimulus (CS) consisted of stopping the water inflow for 30 sec (from now on *water off*). The US consisted of an iron frame (14 cm×35 cm) lowered into the tank until touching the dorsal fin of the fish, and then remaining there for 15 min. Additionally, 3 different control groups were established (*C1- water off*, *C2-confinement* and *C3- water off/confinement*). Controls were used to test the influence of CS only (*C1*: n = 6 fish were exposed to *water off* once daily during 8 days), US only (*C2*: n = 6 fish were exposed during 8 days to the confinement frame only, without previous signaling) and CS–US pairing (*C3*, n = 5, fish were exposed to CS–

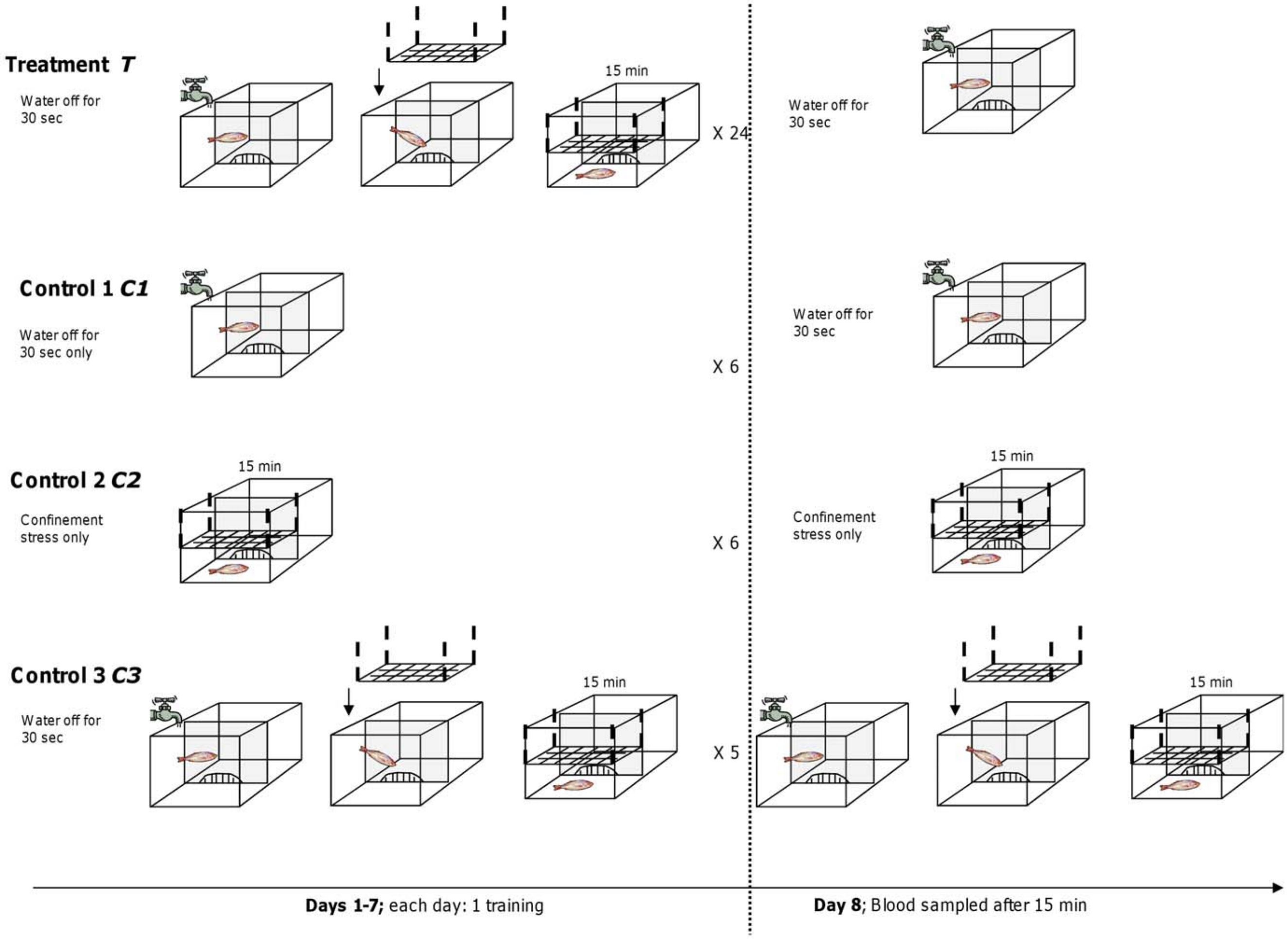

Figure 4. Schematic representation of the experimental set-up used during the avoidance learning test. Fish exposed to avoidance learning (*T-learning*, n = 24) were trained for 7 days to associate *water off* (CS) with the onset of a confinement stress (US) followed by exposure to CS only on day 8. Fish in *C1-water off* (n = 6) were exposed to the CS only, i.e. *water off* during 8 days; Fish in *C2- confinement* (n = 6) were exposed to the US only, i.e., confinement during 8 days without previous signaling by stopping the water inflow; Fish in *C3-water off/confinement* (n = 5) were exposed to CS–US pairing for 8 days. During the 7 days of training the latency to escape was determined. On day 8 in addition to the escape behaviour measures also blood was collected (15 minutes after the start of the US or CS) for cortisol measurements.
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US pairing for 8 days, see Figure 1). *C3* and *T* were exposed to the same procedures during 7 days of training, but on day 8, *T* was exposed to CS only while *C3* to CS followed by US.

Each tank was divided in 2 partitions using a PVC divider containing an escape door (half circle, 8 cm diameter) that was opened upon CS presentation. Fish were trained to associate US with CS for 7 days (1 training per day). The latency to escape (i.e. to swim to the side with no confinement frame) was determined daily. In addition to the latency to escape, at this step also the time taken between the first escape and the first return, the total number of returns and the total time spent in the (previous) confinement area, were registered. These behaviours were used as a measure of the degree of responsiveness to a frightening stimulus (based on [36]). After 15 min of observation on day 8 (during this time fish could choose whether and when to return to the previous confinement area), fish were netted and rapidly killed by severing the spinal cord just behind the head. Afterwards, blood (for cortisol analysis) were immediately collected. Blood was processed as described earlier.

Control fish were sampled (for blood), 15 minutes after the start of the US or CS. Fish used in *C1–C3* and *T* were all exposed to the experimental conditions prior to the start of the avoidance learning test (however in C1–C3 no coping styles data were collected).

Analysis of cortisol

Plasma cortisol levels were measured with a commercially available competitive binding Coat-A-Count® Cortisol kit (SIEMENS Medical Solutions Diagnostics, Los Angeles, CA, USA) adapted from [58]. Briefly, 50 µl of each sample to be assayed was transferred into an Ab-Coated tube and 1 ml of ^{125}I Cortisol added. The tubes were then incubated for 45 min at 37°C in a water bath. The contents of all tubes were decanted, and allowed to drain for 5 min before being readonagammacounter (2470 WIZARD^{2TM}, PerkinElmer^{TM}, Inc., Zaventem, Belgium) for 1 min. A calibration curve was constructed on logit-log graph paper and used to convert results from percent binding cortisol to concentration (ng ml^{-1}). The Coat-A-Count cortisol antiserum cross-reacts 100% with cortisol, 11.4% with 11-deoxycortisol, 0.98% with cortisone, 0.94% ith corticosterone and 0.02% with progesterone.

Data analysis

Statistical analyses were performed using SPSS 16.0 for windows. Relationships between variables were investigated using Spearman correlation. To determine whether latency to escape changed over the learning period, a repeated ANOVA (n = 24) was used followed by Bonferroni comparisons. The value of 1000 sec was used when fish did not escape during the 15 minutes observation period. Kruskal Wallis test and Dunn's post-hoc comparison were used to compare the escape behaviour (homogeneity of variances could not be obtained even after data transformation) between controls and treatments. Statistical significance was taken at $p<0.05$.

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Author Contributions

Conceived and designed the experiments: CM PS LC EH OO JS. Performed the experiments: CM. Analyzed the data: CM EH OO JS. Contributed reagents/materials/analysis tools: CM BC. Wrote the paper: CM EH OO JS.

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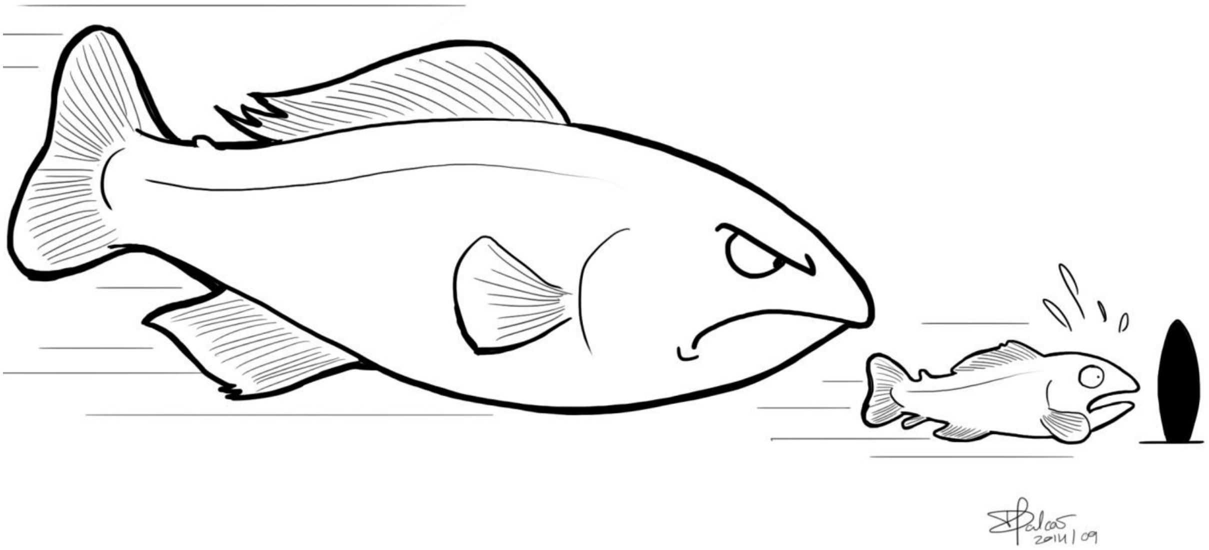
Paper IV

Stimuli gradation and threat sensitive behaviour in fish: effect of heritable coping styles



Title:

Stimuli gradation and threat sensitive behaviour in fish: effect of heritable coping styles

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Abstract

Activation of defensive behavioural systems protecting against potential threats, i.e. threat sensitive behaviour, or fear responses, include both innate and learned responses, and are closely associated with neuroendocrine stress reactions. Animals exhibit consistent individual variation in the stress response, thus it is likely that perception of threat will also differ between individuals. Such traits are critical for survival, and the fact that variability in coping style has been maintained through evolution suggest context dependent fitness effects. The phylogenic roots of fear and threat sensitive behaviour may thus shield light on vulnerability to anxiety and other disorders, also in man. Here we observe that selection for low (LR) vs. high (HR) post-stress cortisol levels in rainbow trout (*Oncorhynchus mykiss*), yielded strains showing proactive and reactive behaviour in threatening situations, with stimulus graded from visual to physical exposure to a larger, aggressive conspecific. A previously available escape route was closed during a final exposure, prior to sampling of plasma (circulating cortisol) and brain tissue (monoamine neurochemistry). It is assumed that the dorsolateral (Dl) and dorsomedial (Dm) telencephalon in fish correspond in function to the mammalian hippocampus and amygdala, respectively, and our results pinpoints elevated dopamine (DA) signalling in these limbic structures as underlying generally more fearful behaviour in HR fish. Interestingly, LR fish responded with increased DA metabolism only to physical interaction with a larger conspecific, while simultaneously showing a reduced ability to change their previous, now futile, avoidance routines.

Key words: threat, fear, anxiety, personality, monoamines, limbic system, teleosts.

Introduction

Comparative models are indispensable with regards to providing fundamental principles of nervous system organization in vertebrates (Striedter, et al. 2014), and teleost fishes have emerged as an alternative to small mammals in both neurobiology and behavioural research (e.g. Clark et al., 2011; Sørensen et al., 2013; Kalueff et al, 2014). Particular attention has been directed to the evolution of cognitive and emotional processes, questioning from both fundamental and applied perspectives to what degree aversive vs. rewarding situations are accompanied by conscious experience in fish (Chandroo et al., 2004; Braithwaite, and Boulcott, 2007; Cottee, 2012; Millot et al, 2014a). Whilst unable to ascertain whether fish possess capacities for suffering and fear, comparative studies have clearly showed that neuroendocrine mechanisms involved in stress coping and affective states are strongly conserved by evolution (Winberg and Nilsson, 1993; Höglund et al., 2000; Carpenter et al., 2007; Steenbergen et al., 2011; Sørensen et al., 2013; Vindas et al., 2014).

In this context, stable individual differences in intraspecific phenotype (animal personalities or stress coping styles) have frequently been identified and utilised to reveal both proximate mechanisms and evolutionary principles (Øverli et al., 2007, Ruiz-Gomez et al, 2011; Martins et al., 2011; Rey et al., 2013; Tudorache et al., 2013; Millot et al. 2014b). This variation often takes the form of suites of behavioural and physiological traits, where sympathetic reactivity, the propensity to express a fight or flight response to stress, and the tendency to develop and follow behavioural routines are positively correlated (Coppens et al., 2010). Furthermore, these traits show a negative relationship with reactivity of the hypothalamic pituitary adrenal axis (HPA-axis) or its homologue in fish, the hypothalamic pituitary axis (HPI-axis) (Ruiz-Gomez et al., 2011). The extremes of this continuum are often referred to as pro- and reactive stress coping styles (Koolhaas et al., 1999), contrasting phenotypes which have now been recognised and further characterized throughout the vertebrate lineage (Øverli et al., 2007; Koolhaas et al., 2010; Lendvai et al., 2011; Baugh et al, 2013).

In general, proactive coping is characterized by low flexibility expressed as rigid, routine-like behaviour tendencies and reduced impulse control (e.g lack of behavioural inhibition during stress). Whilst considerable effort is made to develop comparative models for anxiety and stress research (e.g. Clark et al., 2011; Stewart et al., 2012, Rihel and Schier et al., 2012, Dahlbom et al., 2012, Maximino et al., 2014), the neurobiological background for contrasting plasticity in the response to sudden changes in the environment are rarely addressed in fish (see e.g. Johansen et al, 2012).

Threat sensitive behaviour or fear can be defined as the activation of a defensive behavioural system that protects animals against potential threats (Fendt and Fanselow, 1999). Threat reactions include innate and learned responses to both predators and conspecifics (Kelley and Magurran, 2003) and are associated with neuroendocrine and physiological stress responses (e.g.: Watt et al., 2007; Hegab and Wei, 2014). Furthermore, since animals exhibit a considerable and consistent individual variation in their stress responses, it is likely that the individual perception of threat also will differ between individuals. For example, a higher propensity for routine formation in proactive animals, contributing to a fast execution of their anticipatory responses, may result in that proactive individuals experience environmental changes disrupting learned behavioural patterns, such as fear avoidance, as more anxiogenic. Such individual differences in stimuli interpretation is further elucidated by recent behavioural studies, suggesting that coping styles appear to predict stimuli appraisal and the subsequent behavioural responses both in mammals and fish (Martins et al. 2011). Still, the underlying central mechanisms for such individual differences in cognition need to be investigated to disclose the phylogenic roots of fear and threat sensitive behavior.

In mammals, it has been shown that interpretations of challenges, such as direct confrontations (neurogenic stressors) and threats (psychogenic stressors), are reflected in the reactivity of the limbic system (Sokolowski and Corbin, 2012). For example, psychogenic stressors, such as predatory cues, evoke several neurochemical alterations, which include increased turnover in the monoamines, norepinephrine (NE), dopamine (DA), and serotonin (5-hydroxytryptamine; 5-HT). Furthermore, that these changes seem to be related individually in the stress response (Hayley et al., 2001), suggests a link between stress coping style and limbic monoaminergic signalling.

Recent studies in fish demonstrates that the dorsolateral (Dl) and dorsomedial (Dm) telencephalon in fish correspond to the mammalian hippocampus and amygdala, respectively (Portavella and Vargas, 2005; Vargas et al., 2009; O'connell & Hofmann 2011, Goodson & Kingsbury, 2013). Furthermore, unpublished results from our lab indicate a more pronounced monoaminergic activation in Dm and Dl on response to netting and confinement compared to a predator cue (Silva et al., submitted), resembling the mammalian neurogenic and psychogenic stressors, respectively. Still, studies of the link between cognitive differences in fish with contrasting stress coping style and monoaminergic signalling in forebrain areas with limbic function is needed to elucidate fundamental mechanisms underlying individuality in responses to different types of stressors.

Thus, the aims of the current study were to investigate how psycho- and neurogenic stressors affected neurochemistry in Dm and Dl, respectively, in individuals with contrasting stress coping styles. In order to achieve this, we utilized the HR/LR trout model; two strains of rainbow trout selected for high (HR) or low (LR) post stress plasma cortisol, resembling the proactive and reactive stress coping style (Øverli et al. 2007; Schjolden et al., 2005; Ruiz-Gomez et al., 2011). Furthermore, we applied a social learning avoidance paradigm, developed by Carpenter and Summers (2009). After learning an escape route when confronted to a bigger conspecific, fish were either re-confronted to the bigger conspecific (neurogenic stressor) or just exposed to the sight of the bigger conspecific (psychogenic stressor) while the escape route was blocked with a transparent wall. After confrontation/exposure, neurochemical changes in Dl and Dm were compared between proactive and reactive fish.

Material and Methods

Housing and experimental fish

The experiment was carried out at The Danish Institute for fisheries Research Station (DTU Aqua), Hirtshals, Denmark, on rainbow trout (*Oncorhyncus mykkiss*) from the 6th generation of HR/LR strains, which had been selected on post-stress cortisol levels to confinement stress; i.e. HR are high- and LR low responders (Pottinger and Carrick, 1999). Furthermore, these strains have been reported to display a behavioural and physiological profile in agreement with the proactive (*i.e.* LR) and reactive (*i.e.* HR) coping styles described for mammals (Øverli et al. 2007; Schjolden et al., 2005; Ruiz-Gomez et al., 2011). LR/HR fish were reared in indoor tanks (100 x 100 x 60 cm, 600L) in a closed recirculating freshwater system on a 12:12 light/dark photoperiod and at an ambient temperature (mean temperature at time of experiment: 13.13 ± 0.63 ºC). Fish were fed 3mm dry pellets (BioMar, Denmark) corresponding to an equivalent of 1.5% of their body weight by use of belt feeders (running for 12 hours).

Experimental design

Experimental aquaria (50 x 100 x 50 cm, 250 l) were divided by PVC walls into one 125 l (“*A*” compartment) and two adjacent 62.5 l chambers (“*B*” and “*C*”). The wall separating the two small chambers (*B* and *C*) contained an escape route (a 8.3 cm ⌀ hole positioned as shown in Figure 1), while the wall separating the *A* and *B* chamber was intact but removable. Initiating the experiment, HR (321 ± 86 g) and LR (244 ± 51 g) trout were individually transferred to the *B* chamber of each aquarium. The escape route was left open and the fish were able to move between the *B* and *C* compartments and familiarize themselves with the escape route. Experimental fish were considered to be acclimated when they had moved through the escape route at least 2 times and had displayed active feeding behaviour over at least 2 consecutive days (hand feeding 0.7 % of body mass and scoring feeding behaviour following Øverli et al., 2006).

Social learning avoidance paradigm

The social learning avoidance paradigm used for this experiment was modified from Carpenter and Summers (2009), in which a larger conspecific is used as both a visual (psychogenic) and physical (neurogenic) stressor. Large brood stock fish from an aquaculture population (not selected for HR vs. LR) (1019 ± 116 g) were individually placed in each *A* chamber one day before the start of avoidance learning. Small fish were maintained in the *B* chambers, and the escape route was closed by transparent PVC. Experimental fish were divided into one of three treatments: control (HR: n=8, LR: n=8), neurogenic (HR: n=7, LR: n=6) or psychogenic stress (HR: n=9, LR: n=7). Control fish never interacted with their larger conspecifics, were fed daily, and otherwise kept non-disturbed. Other fish interacted with their larger conspecific neighbours 15 min twice daily, and were fed after (9am and 5pm) for four days. During each social interaction, the solid wall separating *A* and *B* chambers were removed and the *B* to *C* escape route was left open. All interactions were video recorded (Sony, Handycam, DCR-HC32 NTSC) in order to quantify agonistic interactions (latency to first attack, total number of attacks and total time to contest resolution following, and latency to escape (set at 900 s, if no escape attempt). The number of interactions required before an escape was performed was also recorded. After each social interaction, fish were separated and left isolated until the next interaction. After seven interactions a psychogenic/neurogenic stress paradigm was conducted as follows:

- Neurogenic (*i.e.* physical exposure) stress: fish were allowed to interact as in previous sessions, but a transparent wall was inserted at the escape route. Video recordings were used in order to quantify agonistic interactions and unsuccessful escape attempts during 15 min.
- Psychogenic (*i.e.* visual exposure) stress: A transparent wall was inserted between *A* and *B* chambers in order to present the larger fish as a visual threat only. In this case, video recordings were used to quantify escape attempts (if any) and locomotor activity, for 30 min (15 min before the stressor and 15 min during stress).

Sampling procedure

All fish were anaesthetized with a high dose of ethylene glycol monophenyl ether (2 ml l^{-1}) until no opercular movement was observed. Fish were then weighed and brains were excised within 2 min. Brains were placed in a container with Tissue-Tek O.C.T compound (Sakura Finetek) and immediately frozen in dry ice and stored at -80 °C for later brain punch micro-dissection and monoamine neurochemistry analysis.

Monoamine analysis

Whole brains were sliced with a SLEE Cryostat MNT machine (SLEE Mainz, Germany) at -19ºC in serial 300-µm sections quickly thaw mounted on glass slides, and immediately refrozen at -80ºC. Micro-dissections were conducted on a BF-30 MP freezing stage for microtomes (Physitemp Instruments, USA), set at -14ºC using a 337µm ⌀ punch needle. The forebrain dorsolateral (Dl) and dorsomedial (Dm) pallium areas were identified using a stereotaxic atlas for rainbow trout (Navas et al., 1995). Punched tissue samples were ejected into 100 µl of sodium acetate buffer (pH=5) containing 3,4 Dihydroxybenzylamine as an internal standard. Samples were frozen at -80˚C to facilitate cell lysis. Prior to analysis, samples were thawed on ice and centrifuged at 17,000 rpm for 5 minutes. The supernatant was then removed and the monoamines 5-HT, DA, NE and their principal catabolites 5-Hydroxyindoleacetic acid (5-HIAA), 3,4-Dihydroxyphenylacetic acid (DOPAC) and 3-Methoxy-4-hydroxyphenylglycol (MHPG), respectively, were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection. The HPLC system consisted of a solvent-delivery system (Shimadzu, LC-10AD), an auto injector (Famos, Spark), a reverse phase column (4.6mm´100 mm, Hichrom, C18, 3.5 mm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at -40 and +320 mV. A conditioning electrode with a potential of +40 mV was employed before the analytical electrodes, in order to oxidise possible contaminants. The mobile phase consisted of 86.25 mM l^{-1} sodium phosphate, 1.4 mM l^{-1} sodium octyl sulphate and 12.26 µM l^{-1} EDTA in deionized (resistance 18.2MW) water containing 7 % acetonitril brought to a pH of 3.1 with phosphoric acid. Samples were quantified by comparison with standard solutions of known concentrations and corrected for recovery of the internal standard using HPLC software (CSW, DataApex Ltd, Czech Republic).

Data analysis

Differences between the HR and LR fish in attacks received, latency to the attempt escape (via closed escape route) and numbers of failed escape attempts were analysed by Mann-Whitney U tests. Locomotor activity before presentation of the bigger conspecific (basal locomotor activity) and after visual exposure was analysed by Mann-Whitney U test followed by Bonferroni correction for multiple comparisons. After being tested for normal distribution with a Lillefors test, MHPG/NE, 5HIAA/5-HT and DOPAC/DA ratios in micro-dissected Dm and Dl were analysed by a two-way analysis of variance (ANOVAs), with treatment (social confinement vs. visual threat vs. control) and strain (HR vs. LR) as independent variables, followed by a Tukey–HSD test post-hoc test when required.

Results

Behaviour

Prior to closure of the escape route, there were no difference between HR and LR fish in either received aggression (HR median: 52 total, LR median: 85 total, P=0.19) or in the number of training sessions needed to escape for the first time (HR median: 2.0, LR median: 2.5, P=0.98). During the final physical encounter, with a closed escape route, LR fish showed a shorter latency to attempt escape (P=0.01; Figure 2A) and made a higher number of failed escape attempts at the blocked escape route (P=0.02; Figure 2B). Neither LR nor HR fish attempted any escape attempts during visual exposure. However, LR fish increased their locomotor activity significantly compared to HR fish during the first 5 minutes of visual exposure (P=0.048; Figure 3), but this strain effect disappeared thereafter. Baseline locomotor activity was not different between the strains (P=0.68, Figure 3).

Monoamine turnover

Detailed statistics for monoamine neurochemistry are summarized in Figure 4 and 5. Strain specific effects, which were independent of treatment, were observed in both investigated brain parts. DOPAC/DA ratio in Dl was higher in HR compared to LR trout (P<0.001). 5-HIAA/5-HT (P=0.54) and MHPG/NE (P=0.32) did not differ significantly between the strains in this brain part. The same pattern was observed in Dm. In this brain part the DOPAC/DA ratios were significantly higher in the HR strain compared to the LR strain (P<0.002), while there were no significant differences between strains in 5-HIAA/5-HT (P<0.15) or in MHPG/NE ratios (P<0.90). Treatment effects, which were independent of strain origin, were observed in Dl and Dm. Generally, physical interaction had higher impact on 5-HIAA/5-HT ratios compared to being visually exposed to a large, previously known aggressor in the Dl. This was reflected in a significant elevation of 5-HIAA/5-HT ratios in individuals that interacted physically with the bigger conspecific, compared to undisturbed controls (P=0.04). In fish visually exposed to the bigger conspecific, 5-HIAA/5-HT ratio showed a weak tendency to be elevated compared to undisturbed controls (P=0.1). In the Dl, DOPAC/DA ratios tended to be elevated in the group that interacted physically with a bigger conspecific (compared to visual exposure, P=0.08), but visual exposure yielded no response (e.g. compared to controls, P=0.7). Dm 5-HIAA/5-HT ratios showed a similar pattern as in Dl. In this brain part, 5-HIAA/5-HT was significantly higher in fish that interacted physically with a bigger conspecific, compared to undisturbed controls (P=0.03), while again visual exposure yielded no effect. Dm DOPAC/DA ratios were on the other hand significantly higher in physically interacting fish compared to controls (P=0.02) and to visually exposed fish (0.04). Dl MHPG/NE ratios showed significant lower values in fish that were visually exposed to a bigger fish (P<0.001) and in fish that interacted physically with a bigger fish (P<0.001) compared to controls.

Interaction effects between treatment and strain were observed in DOPAC/DA and MHGP/NE ratios in Dm. Physical interaction with a bigger individual resulted in elevated DOPAC/DA ratios in LR fish compared to LR fish visually exposed to a bigger conspecific (P<0.001) and undisturbed LR controls. As a result, there was no significant difference between LR and HR fish when interacting physically with a bigger conspecific (P=0.99), despite the overall strain effect. Regarding MHPG/NE ratio in Dm, physical interaction with bigger individual resulted in lower values compared to controls (HR; P<0.006 and LR; P<0.001), and there were no strain effects. Visually exposure to a bigger conspecific also resulted in a lower MHPG/NE ratio compared to control fish in the HR strain (P<0.001). However, this effect was not seen in LR trout, where fish visually exposed to a bigger conspecific did not differ from undisturbed LR controls (P<0.22). There were no significant differences between strain in undisturbed controls or visually exposed fish.

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Discussion

The consistency between LR and HR trout, in rate of learning the escape route when being confronted with a bigger conspecific in the present study, is in accordance with previous studies demonstrating no difference in learning-skills between these strains (Moreira et al., 2004; Ruiz.Gomez et al., 2011). However, when the fish was reintroduced to a bigger conspecific, LR trout performed higher numbers of escape attempts towards the transparent wall blocking the learned escape route, demonstrating a reduced ability to adjust to this change in the environment. Moreover, fish from this strain responded with higher locomotor activity to visual exposure of the bigger fish compared to HR trout. Both of these behavioural responses indicate that LR fish base their behaviour on expectations, impeding behavioural adjustment in new situations. This is in line with generally stronger tendency to develop and follow routines in proactive animals (Bolhuis et al., 2004; Verbeek et al., 1994; Ruiz-Gomez et al., 2011). Reduced behavioural activity in HR fish may also indicate a more apprehensive or fearful behaviour in this strain. In mammals, the amygdala is of paramount importance for the modulation of fear and anxiety, a function that seems to be partly mediated by DA (for references see de la Mora et al., 2010). Furthermore, DA release in this brain part is likely related to higher levels of arousal during stressful situations (Inglis and Moghaddam, 1999). In the present study, DA turnover in Dm was generally lower in LR compared to HR trout, and only elevated in LR trout during physical interaction with a bigger conspecific while, additionally, a previously available escape route was blocked. Taking the higher propensity for anticipatory responses in proactive animals in consideration (Benus et al., 1991), this neurochemical change indicates that disrupting a learned escape response has a larger impact on LR trout limbic functions. Furthermore, the elevated DA turnover in Dm might indicate a fear like state in these animals, specifically released by the unexpected environmental change (i.e. blocking the known escape route). However, in addition to being involved in fear responses, DA has the general function of facilitating neural processes involved in goal-directed behaviour, and it cannot be excluded that the elevated DA turnover in Dm just reflects more pronounced behaviour response in LR individuals when interacting with a bigger conspecific.

In the present study, a general strain specific effect, independent of seeing or interacting with a bigger conspecific, was detected in Dl. In this brain part, having hippocampal like functions, DA turnover was higher in the HR compared to LR trout strain. There is growing evidence that, in addition to its well-known role in memory formation, the hippocampus may act as a novelty detector; identifying the salience of a stimulus by comparing incoming and stored information (Jenkins et al., 2004). Moreover, DA seems to play an important role in the process of detection and storage of unpredicted events in this brain part (for references see Lemon and Manahan-Vaughan, 2006). This is also reflected in the role of this neurotransmitter in attention and perception (Coppens et al., 2010, Schultz 2010, Arnsten and Pliszka, 2011, Economidou et al. 2012). A general response pattern in the HR strain seems to be that they have lower threshold for reacting to challenges (Øverli et al., 2002) compared to the LR strain, and it is tempting to suggest that the higher DA dopaminergic turnover in Dl reflects a generally higher capability to detect and react to novelty in this strain. Moreover, in the present study, DA turnover showed the same general pattern in Dm as in Dl, with elevated values in HR compared to LR trout. In mammals, the amygdala has been shown to act together with hippocampus in detection of environmental novelty (Blackford et al., 2010). Further studies is needed to investigate if this strain specificity in DA turnover, with generally higher levels in structures with limbic like functions in the teleostean brain, is related to a higher awareness and if this state makes them more sensible to fearful situations.

General treatment effects, independent of strain, were also observed in the present study. Physical interaction with a bigger conspecific resulted in more pronounced 5-HT and DA turnover in Dm and Dl compared to fish that was visually exposed to the bigger conspecific. Both DA and 5-HT have been shown to be involved in responses to stress in fishes and other vertebrates. Moreover, in mammals, it has been reported that both physiological stress and cues of stressful events may result in similar increased 5-HT and DA turnover and metabolism in limbic areas, and that these neurochemical changes are related to intensity of the stressor (Inoue et al. 1994). This is in accordance with unpublished results from our laboratory, showing that confinement stress elicited similar, but a stronger, activation of 5-HT compared olfactory cues of predation in Dm and Dl of Nile tilapia. Taken together, this suggests that fear like states related to threats, such as exposure to cues of predation or socially dominant individuals, give rise to similar regional (but less pronounced) changes as stressors of a more physic nature. NE showed an opposite pattern than DA and 5-HT turnover in the present study, showing higher levels in undisturbed controls. This is in somewhat contrast to previous studies of NE and stress in fish, showing a positive relationship between the endocrinal stress response and central NE levels (Øverli et al., 1999; Höglund et al., 2002). Still, it is important to keep in mind that we here report regional activity monoamine neurochemistry, and previous studies have only reported data on overall forebrain effects. This suggested region specificity in NE turnover is however not supported by mammalian studies; showing a positive relationship between stimuli averseness and limbic NE turnover and release (Tanaka et al., 1983; Tanaka et al., 1990; Thomas et al., 1992).

In conclusion, the present study has demonstrated a generally higher Dl DA turnover in HR compared to LR individuals in forebrain limbic regions, which may be related to a higher ability to adjust to environmental changes in the HR strain. Moreover, attempts to escape during physical interaction with a large and aggressive conspecific were seen more quickly in LR fish, and this was also the only condition during which DA metabolism of LR approached that of HR fish. Furthermore, in the present study, effects which are independent of strain origin was observed, suggesting that fear like states related to threats give rise to similar regional, but less pronounced, changes in 5-HT and DA, in response to a more physical stressor, in teleostean brain areas having limbic like functions.

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Tables and Figures

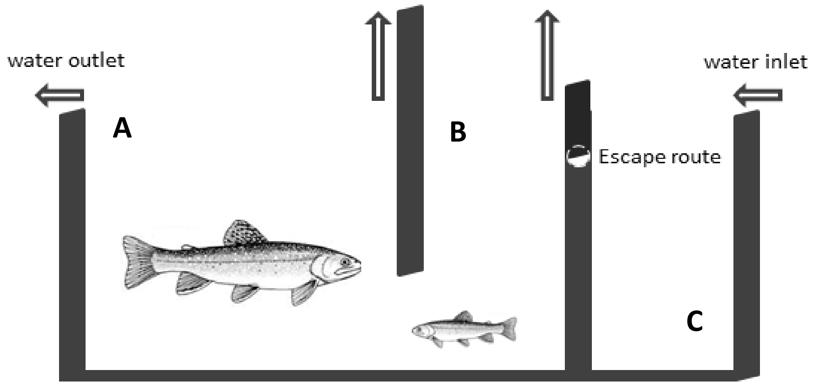


Figure 1. Experimental aquaria. Each aquarium consisted of one big chamber and two small chambers. The wall between the two smallest chambers had a hole (*i.e.* escape route) which could be opened or closed. The wall separating the small test fish from the large aggressive conspecific was removable and could be replaced (from opaque to transparent).

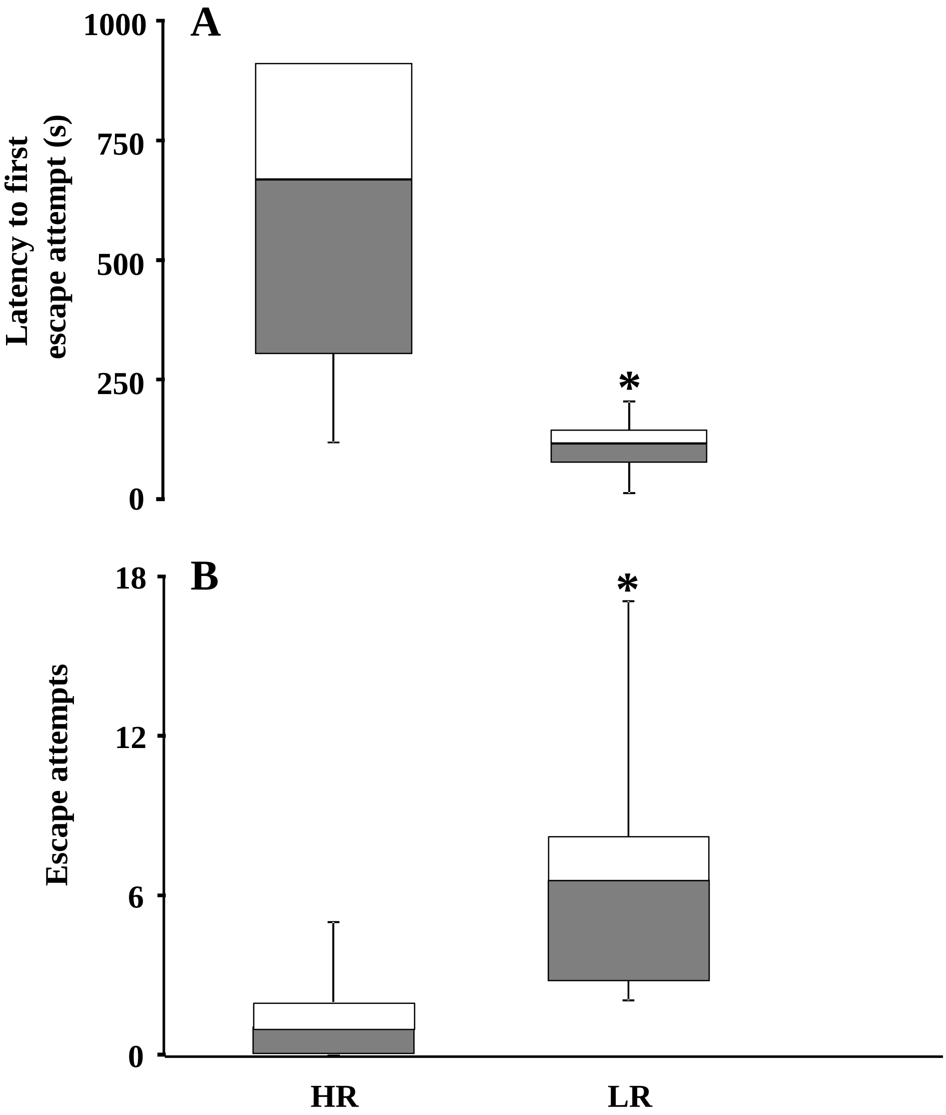


Figure 2. A. Latency to first escape attempt by HR and LR fish when subjected to neurogenic stress. Statistics: Mann-Whitney U test, Z = 2.500, *p* = 0.012). **B.** Number of escape attempts (mean ± S.E.M) conducted during neurogenic stress (*i.e.* physical stress) by HR and LR fish. . Statistics: Mann-Whitney U test, *p* = 0.022).

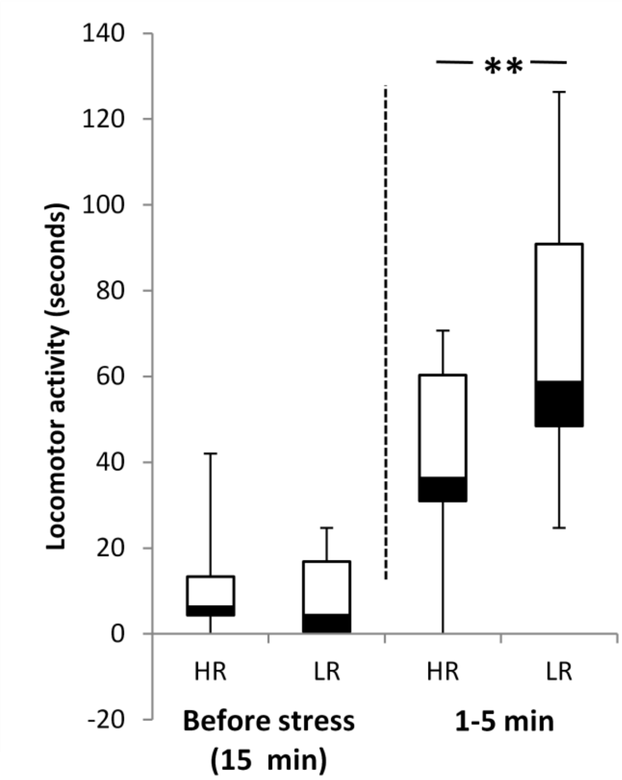
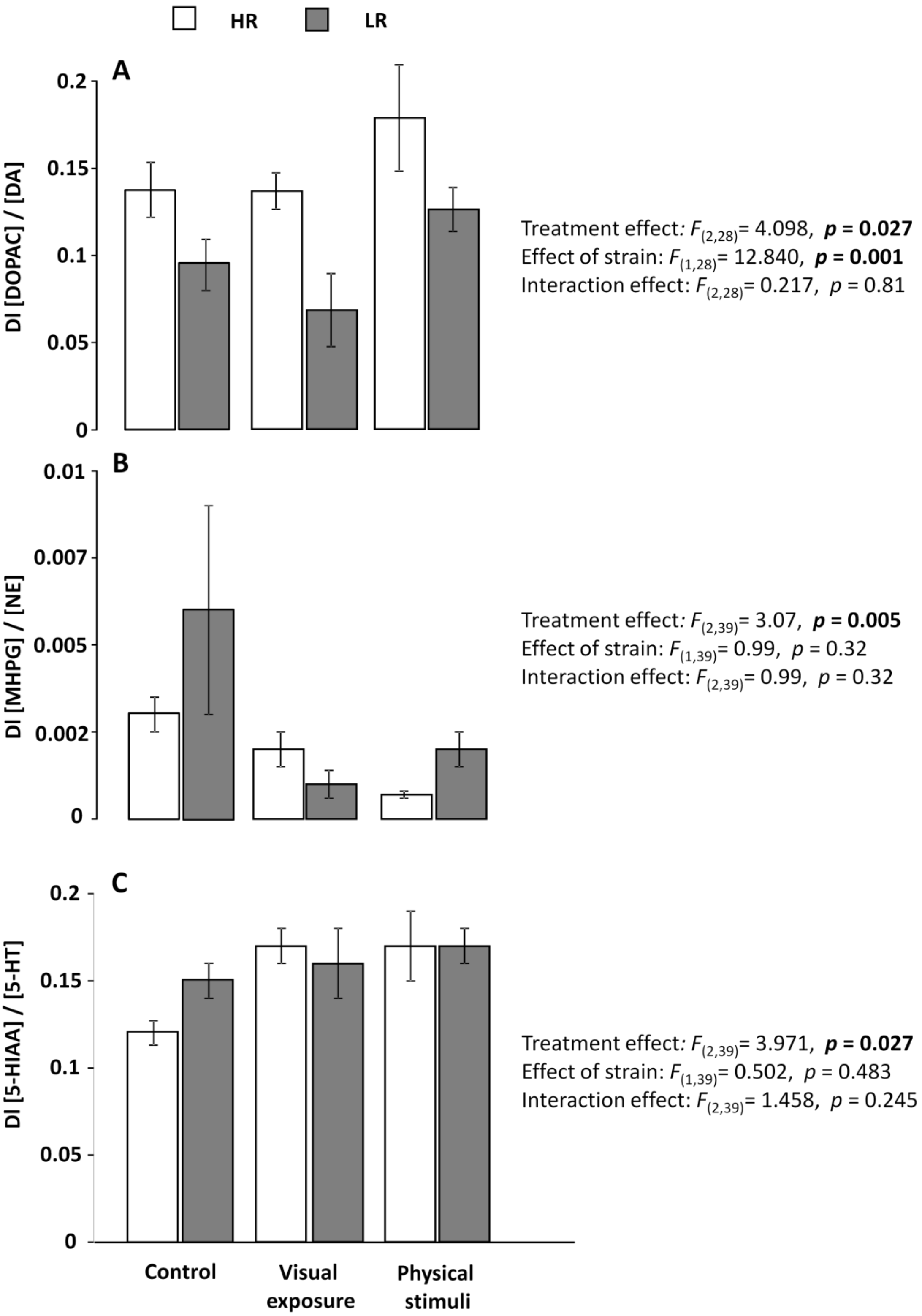


Figure 3. Locomotor activity at basal levels and 5 minutes after physical interaction for HR and LR fish. Values are seconds moved in each 5 min interval.



Treatment effect: $F_{(2,28)}$= 4.098, ***p* = 0.027**
Effect of strain: $F_{(1,28)}$= 12.840, ***p* = 0.001**
Interaction effect: $F_{(2,28)}$= 0.217, *p* = 0.81

Treatment effect: $F_{(2,39)}$= 3.07, ***p* = 0.005**
Effect of strain: $F_{(1,39)}$= 0.99, *p* = 0.32
Interaction effect: $F_{(2,39)}$= 0.99, *p* = 0.32

Treatment effect: $F_{(2,39)}$= 3.971, ***p* = 0.027**
Effect of strain: $F_{(1,39)}$= 0.502, *p* = 0.483
Interaction effect: $F_{(2,39)}$= 1.458, *p* = 0.245

Figure 4. Treatment and strain effects on A: DOPAC/DA ratios, B: The MHPG/NE ratios, and C: 5-HIAA/5-HT ratios in the dorsolateral pallium (Dl) of HR and LR fish. Two-way ANOVA statistics results are presented in figure for each panel. Small letters indicate Tukey-Kramer HSD post-hoc differences.

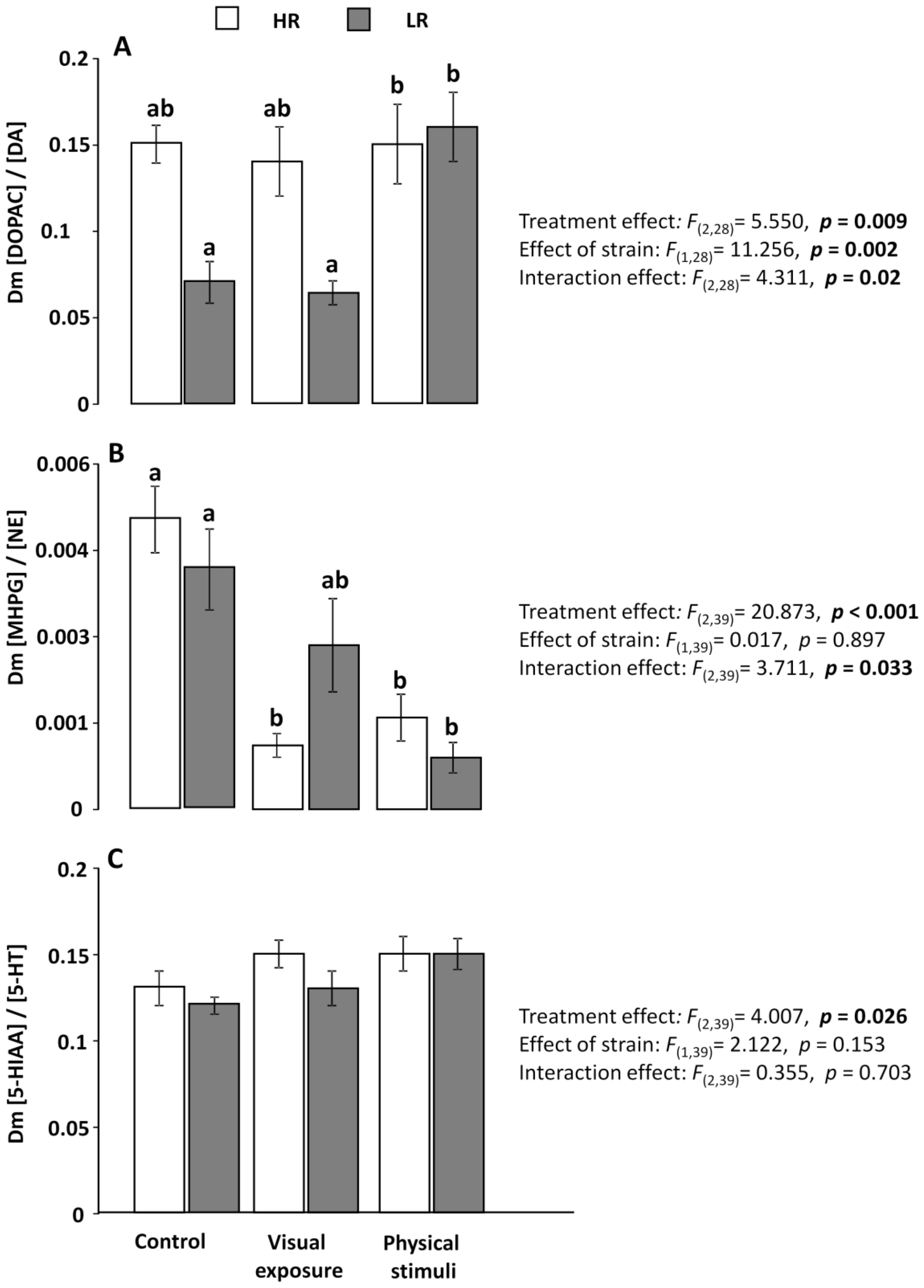


Figure 5. Treatment and strain effects on A: DOPAC/DA ratios, B: MHPG/NE ratios, and C: 5-HIAA/5-HT ratios in the dorsolateral pallium (Dl) of HR and LR fish. Two-way ANOVA statistics results are presented in figure for each panel. Small letters indicate Tukey-Kramer HSD post-hoc differences.