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TELEOST WELFARE: BEHAVIOURAL, COGNITIVE AND PHYSIOLOGICAL ASPECTS IN *Oreochromis mossambicus*

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Dissertação de Candidatura ao grau de Doutor em Ciências do Meio Aquático, submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Galhardo, L., Vital, J., Oliveira, R.F. The role of predictability in the stress response of a cichlid fish. Submitted to *Physiology and Behaviour*.

CONTENTS

Abstract

Fish welfare has been mainly associated with a balanced physiological functioning, as assessed through measures of stress. However, what the animals feel about their own circumstances is at the core of the welfare concept, which relies on the assumption that animals are sentient and thus posses some degree of consciousness. Recent neuroanatomic, cognitive and behavioural evidence, reviewed in chapter 1, strongly suggest that the concept of sentience can be extended to fish. Therefore, the study of fish welfare should no longer be restricted to stress, rather requiring the development of methods to assess their mental states and a better understanding of psychological aspects as part of coping mechanisms.

In response to environmental changes, fish process information mentally and develop coping mechanisms with the purpose of maintaining allostasis. The aims of the present thesis are to identify behavioural and physiological indicators that indirectly inform about the internal states of fish under particular circumstances, and to identify psychological modulators of the stress response, namely the role of social support and predictability of salient events in this process. The model used was the Mozambique tilapia (*Oreochromis mossambicus*), as its biology is well known, it is very adaptable to artificial conditions, and it has a growing economic importance. This species has an elaborate social system in which territorial males dig spawning pits in the substrate, to which they attract females for breeding.

In chapter 2, groups of fish living with and without substrate were compared. The absence of substrate weakened male dominance establishment, decreased male sexual and territorial behaviours (pit digging and hovering over the pit), promoted possible abnormal behaviours (e.g. vacuum pit digging), decreased levels of general activity, decreased behavioural diversity and had no influence in levels of aggression. At the physiological level, there were no differences in cortisol or in glucose levels, but the haematocrit was significantly lower in males without access to substrate. In chapter 3, the assessment of preferences for substrate has shown that territorial males choose to spend more time in a substrate compartment, and this preference was further strengthened in a breeding context. Both territorial and non-territorial animals preferred to forage in the substrate compartment. Chapter 4 aimed to adapt a ‘push-door’ paradigm to study fish motivation to have access to food, to social partner and to substrate only (control). Latency to open the door, work efficiency (as a measure of attention) and maximum price paid were the measures adopted. Results suggest that males value food and social partners in a similar way, and more than a substrate-only compartment. It was apparent that territorial males tended to value the social partner more than non-territorial males. Chapter 5, aimed to validate the use of cortisol as a measure of stress in Mozambique tilapia. Cortisol daily variation showed a gradual increase during the dark period and a peak in the early morning. Social isolation has caused an increase of cortisol levels in non-territorial males. An *in vivo* ACTH challenge showed a threshold response, with cortisol levels varying from an identified baseline to an upper plateau with increasing dosages of ACTH stimulation. In chapter 6, fish were subjected to neophobia (novel object) and confinement stress tests under different social contexts. The novel object promoted exploratory behaviour in males that were left undisturbed in visual contact with a familiar female, and did not affect males' patterns of inactivity nor interactions with females in any social contexts. The stress response to confinement was not affected by social context. Chapter 7 analysed how predictability can affect the stress response to stimuli with different valences. Increased levels of cortisol were the response to unpredictable confinement. Higher levels of anticipatory behaviour and a tendency for increased cortisol suggest that predictable feeding events also trigger a stress response.

Mental states are a key component of the welfare assessment but they are not directly accessible to human scrutiny. When interpreted independently of each other, physiological or behavioural measures are not sufficient to provide information on internal states. It is concluded that a combination of deprivation, preference and motivational studies, with the measurement of physiological parameters such as cortisol, is likely to be a relevant approach to infer indirect subjective experiences in fish. Fish processing of external information embraces a psychological component. This fact has to be taken into consideration when interpreting stress responses and managing fish welfare in artificial conditions.

Resumo

O bem-estar de peixes tem sido predominantemente associado a um funcionamento fisiológico equilibrado, tal como avaliado através de medidas de stress. No entanto, aquilo que os animais sentem acerca das suas próprias circunstâncias constitui o cerne do conceito de bem-estar, o qual depende pois da assunção de que os animais são sencientes e detêm algum grau de consciência. Dados recentes de neuro-anatomia, cognição e comportamento, revistos no Capítulo 1, sugerem fortemente que o conceito de senciência pode ser alargado aos peixes. Assim, o estudo do bem-estar dos peixes não se deve continuar a restringir ao stress, antes exigindo o desenvolvimento de métodos para avaliar estados mentais, bem como uma melhor compreensão dos aspectos psicológicos enquanto partes de mecanismos de ajuste.

Em resposta a alterações do ambiente, os peixes processam a informação mentalmente e desenvolvem mecanismos de ajuste com o objectivo de manterem a alostase. Os objectivos da presente tese consistem na identificação de indicadores comportamentais e fisiológicos que informem indirectamente sobre os estados mentais dos peixes em circunstâncias particulares, e na identificação de moduladores psicológicos da resposta ao stress, nomeadamente o papel da envolvente social e da previsibilidade de eventos relevantes neste processo. O modelo usado foi a tilápia moçambicana (*Oreochromis mossambicus*), tendo em conta que a sua biologia é bem conhecida, que é muito adaptável a condições artificiais, e que tem uma importância económica crescente. Esta espécie possui um sistema social elaborado, no qual os machos territoriais escavam depressões no substrato, para os quais atraem as fêmeas para a reprodução.

No Capítulo 2, foram comparados grupos de peixes vivendo com e sem substrato. A ausência de substrato enfraqueceu o estabelecimento de dominância pelos machos, diminuiu os comportamentos sexuais e territoriais (escavação do ninho e pairar sobre o ninho), promoveu possíveis comportamentos anormais (e.g. escavação de ninho no vácuo), diminuiu os níveis gerais de actividade e a diversidade de comportamentos, e não teve influência nos níveis de agressão. Ao nível fisiológico, não houve diferenças nos níveis de cortisol e de glucose, mas o hematócrito foi significativamente inferior nos machos sem acesso ao substrato. No Capítulo 3, a avaliação de preferência pelo substrato mostrou que os machos territoriais preferem passar mais tempo num compartimento com substrato, tendo esta preferência sido ainda maior num contexto de reprodução. Tantos os machos territoriais como os não territoriais preferiram alimentar-se no compartimento com substrato. No Capítulo 4 procurou-se adaptar um paradigma de “porta de empurrar” (‘push-door’) ao estudo da motivação dos peixes para acesso a alimento, parceiro social ou apenas substrato (controlo). As medidas adoptadas foram a latência da abertura da porta, a eficiência do trabalho (enquanto medida da atenção) e o custo máximo pago. Os resultados sugeriram que os machos valorizam o alimento e o parceiro social de uma forma similar, e mais que um compartimento apenas com substrato. Foi aparente que os machos territoriais tendem a valorizar o parceiro social mais que os machos não territoriais. No Capítulo 5 procurou-se validar o uso de cortisol como medida de stress na tilápia moçambicana. A variação diária de cortisol mostrou um aumento gradual durante o período nocturno e um pico no início da manhã. O isolamento social causou um aumento nos níveis de cortisol dos machos não territoriais. Foi feito um desafio *in vivo* que mostrou uma resposta por patamares, com os níveis de cortisol variando desde uma nível de base até um patamar superior sob níveis crescentes da dosagem de estimulação por ACTH. No Capítulo 6, os peixes foram submetidos a um teste de neofobia (objecto novo) e de confinamento, em diferentes contextos sociais. O objecto novo promoveu o comportamento exploratório dos machos quando não perturbados e em contacto visual com uma fêmea familiar, mas não afectou os padrões de inactividade nem as interacções com as fêmeas, em qualquer contexto social. A resposta de stress ao confinamento não foi afectada pelo contexto social. O Capítulo 7 analisou como a previsibilidade pode afectar a resposta ao stress por estímulos com diferentes valências. Um aumento dos níveis de cortisol foi a resposta ao confinamento não previsível. Níveis mais elevados de comportamento antecipatório e uma tendência para aumento do cortisol sugerem que eventos previsíveis de alimentação também podem despoletar uma resposta ao stress.

Os estados mentais são uma componente fundamental da avaliação do bem-estar, mas não são acessíveis ao escrutínio humano directo. Quando interpretados independentemente umas das outras, as medidas fisiológicas e comportamentais não são suficientes para informar sobre os estados internos. Desta tese pode-se concluir que uma combinação de estudos de privação, preferência e motivação, junto com a medida de parâmetros fisiológicos como o cortisol, é provavelmente uma abordagem relevante para inferir indirectamente sobre as experiências subjectivas dos peixes. O processamento da informação externa pelos peixes envolve uma componente psicológica. Este facto tem de ser tido em conta na interpretação das resposta ao stress e na gestão do bem-estar de peixes em condições artificiais.

Résumé

Le bien-être des poissons est essentiellement associé à un fonctionnement physiologique équilibré, évalué par des mesures de stress. Pourtant, ce que les animaux perçoivent sur leur propre situation est au cœur de la définition même de bien-être animal, laquelle repose sur la notion qu'ils sont sensibles et possèdent donc un certain degré de conscience. De récentes données neuro-anatomiques, cognitives et comportementales, examinées dans le chapitre 1, suggèrent fortement que la notion de sentience peut être aussi appliquée aux poissons. Par conséquent, l'étude du bien-être des poissons ne doit plus se limiter aux stress. Elle exige un développement des méthodes d'évaluation de leur état mental, en vue d'une meilleure compréhension des aspects psychologiques, dans le cadre des mécanismes d'adaptation.

Face aux changements environnementaux, les poissons traitent l'information et développent des mécanismes d'adaptation, dans le but de maintenir leur allostase. Les objectifs de cette thèse consistent à identifier des indicateurs comportementaux et physiologiques qui puissent informer indirectement sur les états internes de poissons en des circonstances particulières. Ils consistent également à identifier des modulateurs psychologiques de leur réponse au stress, notamment le rôle du soutien social, tout comme la prévisibilité des événements marquants dans ce processus. La tilapia du Mozambique (*Oreochromis mossambicus*), une espèce très adaptable à des conditions artificielles et dont la biologie est bien connue, a été le modèle utilisé. Elle possède un système social élaboré, dans lequel les mâles territoriaux creusent des fosses dans le substrat, vers lesquelles ils attirent les femelles pour la reproduction.

Dans le chapitre 2, des groupes de poissons vivant avec et sans substrat ont été comparés. L'absence de substrat affaiblit l’affirmation de la domination masculine et diminue les comportements sexuels et territoriaux des mâles (creuser des fosses et planer sur elles), favorise des comportements anormaux (creuser des fosses sur le vide) et diminue les niveaux d'activité générale, tout comme la diversité des comportements. Par contre, elle n’a pas d’influence dans l’agressivité. Au niveau physiologique, on n'observe pas de différence dans les niveaux de cortisol, ni de glucose, chez les mâles sans substrat, alors que leurs niveaux d'hématocrite étaient significativement réduits. Dans le chapitre 3, l'observation comparée des mâles territoriaux a montré qu'ils choisissent de passer plus de temps auprès du substrat et que cette préférence est renforcée dans un contexte de reproduction. Tous les animaux préfèrent se nourrir dans le compartiment ayant du substrat. Le chapitre 4 a

cherché à adapter le paradigme «push-door» à l'étude de la motivation des poissons, concernant l'accès à la nourriture, à des partenaires sociaux et au substrat (comme témoin). Le temps de latence, l'efficacité du travail (en tant que mesure de l'attention) et le prix maximal payé ont été les mesures employées. Les résultats suggèrent que les mâles valorisent la nourriture et les partenaires sociaux de façon similaire, mais plus que le substrat. Il a été évident que les mâles territoriaux ont tendance à valoriser le partenaire social plus que les mâles non-territoriaux. Le Chapitre 5 a eu le but de valider l'utilisation du cortisol comme mesure de stress chez la tilapia du Mozambique. La variation quotidienne de cortisol a montré une augmentation progressive au cours de la période sombre et un pic en début de matinée. L'isolement social a provoqué une augmentation des niveaux de cortisol chez les mâles non-territoriaux. Une expérience avec de l'ACTH *in vivo*, avec des dosages croissants de stimulation, a montré une réponse ouverte, avec des variations de cortisol entre la référence de base et un niveau supérieur. Au chapitre 6, les poissons ont été soumis à un test de néophobie (nouvel objet) et à un test de stress par confinement dans des contextes sociaux différents. Le nouvel objet a donné lieu à un comportement d'exploration chez des mâles laissés intacts et en contact visuel avec une femelle connue. Le cadre d'inactivité ou d'interactions avec les femelles n'a pas changé dans tous les contextes sociaux. La réaction de stress au confinement n'a pas été affectée par le contexte social. Le Chapitre 7 a analysé, avec des stimuli différents, la manière dont la prévisibilité peut affecter la réponse au stress. L'augmentation des niveaux de cortisol a été la réponse à un confinement imprévisible. Des niveaux plus élevés de comportements d'anticipation et une tendance à l'augmentation du cortisol suggèrent que les événements d'alimentation prévisibles déclenchent aussi une réaction au stress.

Les états mentaux sont une composante clé de l'évaluation du bien-être, mais ils ne sont pas directement accessibles à l'examen humain. Quand les mesures physiologiques et comportementales sont interprétées de façon indépendante les unes des autres, elles ne s'avèrent pas suffisantes pour fournir des informations sur les états internes. On peut en conclure que l'articulation d'études de privation, de préférence et de motivation, associée à la mesure de paramètres physiologiques comme le cortisol, peut être une procédure pertinente pour déduire indirectement les expériences subjectives chez les poissons. Le traitement de l'information externe par les poissons englobe une composante psychologique. Ce fait doit être pris en considération dans l'interprétation des réactions de stress et dans la gestion du bien-être des poissons dans des conditions artificielles.

CHAPTER I

General Introduction

1. Animal Welfare

1.1. Origin of the concept

The concept of animal welfare had its origin in public concerns about how animals were treated. The book “Animal Machines”, published by Ruth Harrison (1964) in the United Kingdom, was a landmark in tracing the history of the animal welfare concept (1999). Harrison’s descriptions of how animals were being kept and raised in the intensive agricultural production systems elicited a public outcry with relevant social, political and scientific consequences. In 1965, the British Government set up a technical committee chaired by Professor F.W.R. Brambell to *Inquire into the Welfare of Animals Kept Under Intensive Livestock Husbandry Systems*. The result of this, the ‘Brambell report’, as it came to be known, was published. In this report original ideas like the importance of behavioural assessment and the relevance of respecting animal’s needs were defended, and a number of important recommendations were made. The Brambell report highlighted the need for animal welfare research, and promoted the emergence of a social ethics towards farm animals (Rollin, 1990). This was the formal beginning of animal welfare as a scientific area.

Since then, animal welfare science developed as a multidisciplinary area, with the contribution of many different fields of research, such as applied ethology (e.g. Stolba and Wood-Gush, 1989), stress physiology (e.g. Mormède et al., 2007), evolutionary ecology (Dawkins, 1998a), cognitive psychology (e.g. Mendl and Paul, 2004), neuroscience (e.g. Würbel, 2001), veterinary medicine (e.g. Broom, 1987), and genetics (e.g. Muir and Craig, 1998), among many others. Animal welfare research started within farming systems, but soon extended its action to animals used in research and toxicological tests. Nowadays, it targets all animals kept in captivity, and also, albeit to a lesser extent - in natural habitats.

1.2. Approaches and definitions

Animal welfare has to do with the animals’ quality of life (Appleby, 1999). In general, the approaches of animal welfare scientists portray different ethical concerns and can fall in three interrelated domains: feelings-based (mind), functioning-based (body) and nature-based (nature) (Fraser et al., 1997). For the first of these, the *feelings-based approach*, the subjective experiences of animals count more than anything else. This approach is based on the assumption that the majority of species kept for human use are capable of experiencing negative and positive mental experiences (emotions). For this reason, they deserve moral standing and thus their suffering and pleasure matters and should be object of scientific scrutiny (e.g. Dawkins, 1990; Duncan, 1996). A growing number of scientists have been valuing this approach, tending to rely on behavioural and, more recently, on cognitive methods and neurophysiological techniques for studying welfare (a more detailed description of these methods can be found in section 2.2.). The *functioning-based approach* highlights the bodily functioning and relates well-being to the absence of disease, injury and mal-nutrition. In general, scientists relying on this approach tend to prefer health measurements like levels of growth, reproduction, longevity, and normal physiological and behavioural parameters as welfare indicators (Broom and Johnson, 1993). A third approach, the *nature-based approach*, is based upon the idea that animals have their own nature ('telos') (Rollin, 1990). Therefore, they should be able to live natural lives, meaning that animals should be able to perform their natural repertoire of behaviour (Duncan and Fraser, 1997). Scientists adopting this approach tend to study animals in their natural habitats and compare behaviour with artificial conditions (e.g. Stolba and Wood-Gush, 1989; Veasey et al., 1996). Different scientists give different weights to each of these approaches, but there has been a tendency to integrate them all as, in fact, altogether they offer a more holistic picture of animal welfare (Fraser et al., 1997).

Many definitions of animal welfare have been proposed over the last forty years. For example, Hughes (1976) defined animal welfare as a "*state of complete mental and physical health, where the animal is in harmony with its environment.*" This definition is comparable to the World Health Organisation's definition of human health, in which "*health is not only the absence of infirmity and disease but also a state of physical, mental and social well-being*". The wider focus including also the mental and social well-being is what essentially differentiates the concept of animal welfare from that of animal health. Welfare, from the animal's point of view, is not only how well its body is doing but also and mainly its perception of its own life (Dawkins, 1980; Duncan and Petherick, 1991). Hughes's definition of welfare lacks in operationality, and thus it has not been much used (Fraser, 1995). But a very frequent definition in the literature is the one by Broom (1991), in which welfare is the "*animal's state as regards its attempts to cope with its environment*". While it was traditionally associated with the functioning-approach, Broom (1998; 2008) claims that feelings, assumed as part of coping mechanisms, should be considered as an important part of coping mechanisms.

1.3. Ethical views

Animal welfare has always been inextricably linked to animal ethics, since it is a function of how humans decide to treat animals. Humans are moral agents and, for many, animals are assumed to have moral standing (Dawkins, 1998b). The welfare questions addressed, why they matter and even how they are tackled are embedded in ethical values (Fraser, 1999). However, animal welfare scientists follow a scientific methodology, in relation to data collection and analysis, aiming to reach objective and unbiased results from their research. Animal welfare science covers a wide range of relevant issues, like levels of stress and associated mental experiences, animals' needs and preferences, among many others. It is not up to science to take moral decisions about what we do to animals or what is the acceptable degree of suffering in a given situation (Sandøe and Simonsen, 1992; Broom, 2008). While supported by scientific knowledge, these decisions are or shall be grounded on ethical approaches.

In fact, what is considered right or wrong acts towards animals varies considerably with different ethical principles. For example, to kill an animal may be very differently judged by the utilitarian and the most influential deontological approach, the animal rights view, just to mention two of the most prominent theories of ethics. Utilitarianism confers automatic moral standing to those animals for which sentience is assumed based on their capability to feel. In his book "Animal Liberation", first published in 1975, Singer (1991) highlights the fact that a certain act is morally right if it has the best possible consequences, i.e. if it enhances the overall happiness and decreases the overall suffering. In doing so, there must be equal consideration for suffering and interests, regardless of the species concerned and whether the animal in question has further mental capacities. According to this view, certain contexts where a painless killing and a replacement of the killed animal by a new one that goes on pursuing its interests in life, may be morally acceptable. The deontological view applied to animals by Tom Regan (1984) confers moral rights to animals that are "subjects-of-a-life" referring to animals that are not only sentient but also have certain cognitive features such as intentionality, self-awareness, believes, wishes and some sense of the future. Under this view, the intrinsic value of those animals overrides human interests, and therefore killing is by all means simply unjustifiable.

Outside the realm of formal moral philosophy, our ethical decisions regarding how to treat animals are frequently based on hybrid views which combine elements of various ethical approaches (Sandøe et al., 1997). Legislation and codes of good practices also address animal protection in the same way. To some extent they adopt an utilitarist approach but, over a certain point, a deontological view may prevail. Based on this hybrid approach, Oliveira and Galhardo (2007) suggested a theoretical decision-making model to evaluate whether a human activity is acceptable in view of animal welfare criteria (Figure 1). The model defines two areas: one of acceptable animals use (human benefit > animal suffering) and another one of unacceptable animals' use (human benefit < animal suffering). Above a certain threshold no further suffering is acceptable, no matter how much is the human benefit. Conceptually this reflects a common ethical approach to animal protection, which seems to be operational although limited by the fact that straightforward comparisons between variables into play (animal suffering vs. human benefits) are frequently not possible.

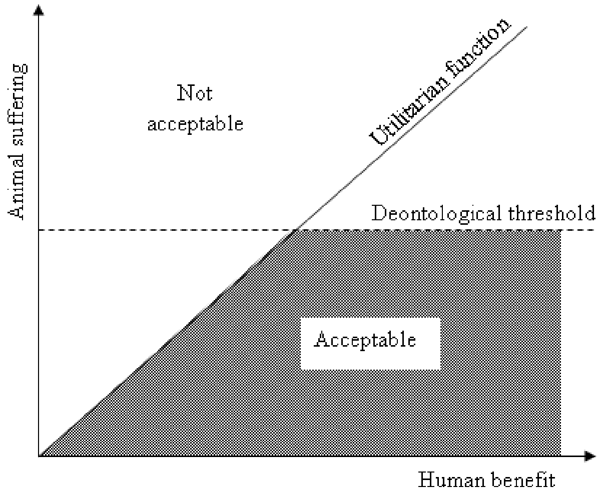

Figure 1. Decision-making model for the evaluation of humane activities' acceptability. From Oliveira and Galhardo (2007).

2. Fish Welfare

A functioning-based approach has clearly prevailed in fish welfare, with numerous studies focusing on fish stress mechanisms and responses to a wide variety of conditions and procedures (Huntingford et al., 2006). Measures of productivity and health have also been a major part of the fish welfare research (section 2.3.). In many of these studies, suffering is not directly addressed but some implicitly assume it as a possibility based on behavioural and physiological criteria. For example, Lambooiij et al (2002) claim that it is not recommended "*placing conscious eels in cold brine water, because it takes more than 27 seconds before unconsciousness may be induced*". However, as it will be discussed below, the question of whether fish suffer or not is not consensual, and has been the object of scientific debate over the last decade (Rose, 2002; Sneddon, 2003a; Chandroo et al., 2004b; Arlinghaus et al., 2007; Rose, 2007).

2.1. Fish stress[1]

2.1.1. Physiological response to stress

The three stages of the stress response

The stress response in fish is very similar to that of other vertebrates and can be described in three stages (Barton, 2002; Iwama, 2007). The primary response involves the activation of two neuroendocrine axes (Figure 2). The hypothalamus-sympathetic-chromaffin cell axis produces cathecolamines (adrenaline and noradrenaline) from the chromaffin cells, the equivalent of adrenal medulla in tetrapods. The second axis is the hypothalamic-pituitary-interrenal tissue (HPI) axis, with the production of corticosteroids (mainly cortisol, in teleosts) from the interrenal tissue, the equivalent of adrenal cortex in tetrapods. The interrenal tissue surrounds the dorsal posterior cardinal (DPC) veins of the anterior kidney, with the chromaffin cells scattered within it and in the walls of the DPC veins (Janz and Weber, 2000). The sympatho-adrenergic response activates cardiovascular and respiratory functions and assists in the mobilisation of energy

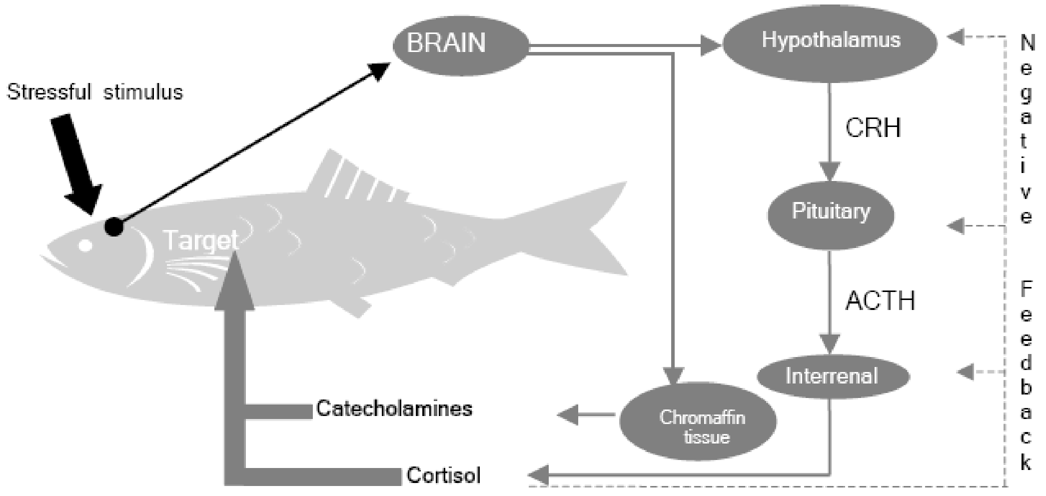

Figure 2. The main hormonal components of the stress response in fish. From FSBI (2002).

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[1] This section is an adaptation of part of the published article: Galhardo, L., Oliveira, R.F., 2009. Psychological stress and welfare in fish. Annual Review of Biomedical Sciences 11, 1-20.

reserves for the increased metabolic requirements (Sumpter, 1997). As in the other vertebrates, the HPI response is related to the energy metabolism and hydromineral balance (Mommsen *et al.*, 1999).

The secondary response is a physiological and behavioural adjustment to the stress conditions. It includes the activation of a number of metabolic pathways which induce a wide range of changes in blood chemistry and haematology, respiration, acid-base disturbances with ion losses at the gills, hydrothermal balance, cellular response and immune function (McDonald and Milligan, 1997; Barton, 2002; Iwama, 2007). It has been demonstrated that there is a close relationship between the immune system and the stress response, with glucocorticoids having a strong anti-inflammatory effect and inducing relevant changes in immune cells, as well as cytokines having the power to stimulate the cortisol production (Fulford and Harbuz, 2005). In fish these interactions are also present, with the anterior kidney being the common site for haematopoiesis, antibody and stress hormones production (Weyts *et al.*, 1999).

The tertiary response has to do with prolonged stressor exposure, and refers to changes in the whole organism, with an eventual impact oin the population (Iwama, 2007). Stress has inhibitory effects on fish reproduction and inhibits growth due to metabolic effects and by affecting growth endocrine pathways (Pankhurst and Van Der Kraak, 1997). Overall resistance to disease can also suffer profound changes and lead to immune suppression, dramatically increasing disease incidence and mortality rates (Broom and Johnson, 1993; McDonald and Milligan, 1997).

The sympatho-adrenergic response

In the presence of a stressor the sympathetic nervous system is activated by means of autonomic neurons coming from the paraventricular nucleus (PVN) of the hypothalamus to the locus coeruleus in the brainstem and spinal cord (Peter, 1990). The chromaffin cells are stimulated by the sympathetic preganglionic fibers via cholinergic receptors to release stored catecholamines (Sumpter, 1997). In teleost fish, adrenaline has the most important role in the stress response (Barton, 2002). Within seconds, the combined action of the sympathetic nerves and adrenaline prepares the body tissues with β-adrenoreceptors for 'fight or flight'; accelerating heart and respiration rate, increasing blood pressure and flow at the muscles, and stimulating glucose release (Peter, 1990). The activity of the parasympathetic nervous system becomes suppressed in these emergency times - energy storage, digestion or growing are automatically 'judged' as luxuries in a body with more urgent priorities (Sapolsky, 2004).

During acute stress, catecholamine concentration decreases in a few minutes, but if the stressor is prolonged it can remain high for longer periods of up to some days. Chronic stress can induce desensitisation of the system or impairment in catecholamine storage (Sumpter, 1997).

The HPI axis response

The first level of the HPI cascade is the hypothalamus. The hypothalamic preoptic nucleus (PON) secretes the peptide corticotrophin-releasing hormone (CRH), which belongs to a very relevant family of hormones, known as the CRH system, and is present in all vertebrates (Janz and Weber, 2000; Chen and Fernald, 2008). This includes a number of related peptides also involved in the HPI regulation and expressed in a number of important central and peripheral tissues. Even though CRH's mechanisms of action are still not entirely understood, its control upon adrenocorticotrophic hormone (ACTH) release is well established (Huising and Flik, 2005; Flik et al., 2006). In turn, a variety of neurotransmitters and cytokines are able to stimulate the synthesis of CRH (Johnson *et al.*, 1992). Arginine vasotocin (AVT) is also produced by the hypothalamus, with likely differences in the subsets of neurons involved and in the way they modulate the HPI response (Balment *et al.*, 2006). Other neuropeptides can interact with CRH neurons and in this way influence subsequent hormone production.

The second level of the HPI axis is the pituitary gland. The pituitary cells are innervated directly by the hypothalamic neurosecretory fibres (Peter, 1990). CRH and AVT act synergistically to enhance ACTH production at the anterior pituitary (corticotrophs). The presence of AVT allows an increased flexibility over ACTH control as CRH and AVT gene expression are regulated independently (Balment *et al.*, 2006). ACTH is secreted very rapidly in response to a stressor and it may fall back to the basal levels during prolonged exposure showing that habituation occurs above the level of the pituitary gland (Sumpter, 1997). CRH also controls the release of α-melanocyte stimulating hormone (α -MSH) and β-endorphin from the melanotrophs at the intermediate lobe of the pituitary (Sumpter, 1997; Huising *et al.*, 2005). β-endorphin is an agonist of opiate receptors in the nervous system, producing analgesia and regulating mood (Lovallo, 2005). It has been suggested that α -MSH and β-endorphin can act in synergy with ACTH for the cortisol synthesis (Sumpter, 1997).

The third level of the HPI axis is the interrenal tissue, which produces cortisol upon stimulation by ACTH (and other hormones) levels (Barton, 2002). It is generally agreed that cortisol rises after the exposure to a stressor within the first 4-10 minutes and lasts for a few hours (Foo and Lam, 1993; Sumpter, 1997). When fish are subjected to chronic stress, plasma cortisol can be elevated for many days or even weeks or may return to basal levels due to habituation mechanisms or impairment of the HPI axis (Sumpter, 1997; Hontela, 1998; Barton, 2002).

Cortisol is a fundamental hormone for the maintenance of allostasis as it has both a permissive role (it permits other hormones to accomplish their function in basal conditions) and a stress-induced regulatory role (Johnson *et al.*, 1992). There are two types of known cortisol receptors: the mineralocorticoid receptors (MR) and the glucocorticoid receptors (GR). In mammals, MR receptors are sensitive to low levels of cortisol, being responsible for body fluid balance, for cortisol negative feedback over the diurnal cycle and to the normal metabolic glucose demands (de Kloet et al., 2005; Lovallo, 2005). The GR receptors have much less affinity to cortisol, only detecting it if in very high concentrations, and thus they are more related to the stress response and its recovery (Johnson *et al.*, 1992). Until recently, teleosts were thought to have only GR receptors with both osmotic and stress-related functions (Mommsen *et al.*, 1999). However, MR receptors were recently described in common carp (Prunet *et al.*, 2006; Stolte *et al.*, 2008). Transactivation studies show that both receptors have similar affinities to cortisol, although the main MR ligand is still not entirely defined (Stolte *et al.*, 2008). Expression of GRs and MRs mRNA was found in the forebrain (especially in the lateral pallium) and in all relevant neuroendocrine components of the HPI axis, in a very similar pattern to that described in other vertebrates (de Kloet et al., 2005; Stolte et al., 2008). The HPI is regulated by a series of negative feedback loops, with cortisol mainly acting back at the hypothalamus, pituitary gland and interrenal tissue to inhibit CRH, ACTH and its own synthesis (Sumpter, 1997). However, other hormones, neurotransmitters and cytokines also contribute to the HPI regulation (Johnson *et al.*, 1992).

The coordination between the sympatho-adrenergic and the HPI axes

The sympatho-adrenergic response and the HPI axis interact with each other, in order to avoid excessive activation of each system and concomitant negative consequences (Johnson *et al.*, 1992). Hypothalamic neuronal projections activate sympathetic outputs leading to the adrenaline release. This hormone is indirectly regulated by autonomic reflexes and by cortisol feedback to the hypothalamus, which reduces activation of brainstem sympathetic brain centres (Lovallo, 2005). CRH and ACTH synthesis may be in turn activated by catecholamines (Johnson *et al.*, 1992).

2.1.2. Behavioural response to stress

Behavioural responses to acute stress

The first alarm response is an essential aspect of the behavioural response (Ursin and Eriksen, 2004). The arousal response affects sensory, perceptual and cognitive mechanisms, serving the purpose of narrowing the attention to a given source of threat (Steckler, 2005). This attentive state favours the retrieval of acquired memories and the acquisition of new ones. This is fundamental to produce the most appropriate response to a given stressor. During arousal, the sympathetic system is turned on, while all behaviours commanded by the parasympathetic are switched off. The animal interrupts what it is doing, whether swimming around, feeding or exploring and directs its attention to the stressor.

In response to the stressor, the inhibitory behavioural system is one of the possible defensive systems to be activated. It occurs in conflict contexts and contributes to increased vigilance and arousal, frequently leading to enhanced exposure to the stressor (Steckler, 2005). In fish, as described by a number of authors, the most paradigmatic response of this type is the freezing behaviour during which the animal remains motionless on the bottom or hovering in the water column, with no fin movement (e.g. Vilhunen and Hirvonen, 2003). This behaviour may be elicited by fear and act as a defensive mechanism (e.g. Yue *et al.*, 2004), or as an anti-predator defence (e.g. Vilhunen and Hirvonen, 2003). However, as it will be discussed later, this coping strategy is not exclusively context-dependent; personality types or other specificities can promote this or other defensive systems.

Fully opposed to freezing-like behaviours is the fight-or-flight defensive mechanism with the active purpose of removing the threat imposed by the stressor, whether by acting on it or by moving away (Schreck *et al.*, 1997; Steckler, 2005). A number of different fish behavioural patterns have been related to this defensive system (Huntingford et al., 2006; Ashley and Sneddon, 2008). In response to a threatening event, they may include protective motor reactions like fleeing, hiding or shoaling. Aggressive responses are also extensively described and include chases and attacks of variable intensities (e.g. Oliveira and Almada, 1998a). Body rocking and rubbing against surfaces were described as nociceptive and pain responses (Sneddon et al., 2003). Locomotory patterns are also sensitive to many stressors, and much used in behavioural analysis (Huntingford *et al.*, 2006). A number of environmental factors, like exposure to noxious chemicals, water temperature, etc., may affect the effectiveness of the fight-or-flight response (Schreck *et al.*, 1997).

Behavioural responses during chronic stress

When behavioural defence mechanisms are not enough to remove the animal from the source of threat, changes of behaviour in a larger scope may occur, reflecting the imposed allostatic load over the animal. Schreck and colleagues (1997) reviewed the range of fish behaviours that may be affected during exposure to chronic stress. Beyond significant changes in the swimming patterns, sustained levels of stress can decrease or change the anti-predator behaviour, disrupt feeding behaviour, increase shelter seeking (sometimes in an inappropriate manner), change territorial behaviour and interfere with learning processes (Schreck *et al.*, 1997).

2.1.3. Factors influencing the stress response

Depending on a number of factors, the neuroendocrine system responds differently in speed, magnitude and patterning of hormones and neurotransmitters activity, as well as in behavioural patterns (Sapolsky, 2004; Lovallo, 2005). Differences in individual and stressor related aspects will be discussed below.

Individual-related

Barton (2002) reviewed a number of environmental, genetic and developmental factors that may account for different endocrine responses. The state of the internal and external environment may alter how an individual is affected by and respond to the stressors. The magnitude of the cortisol response also varies with species, even if closely related, between wild and domesticated fish, and among strains or stocks. Sex differences were found between females and immature males of rainbow trout, with females recovering from stress quicker than males (Øverli *et al.*, 2006). Developmental factors also account for relevant differences in stress vulnerability. Stress responses develop early in life and early experiences may have long-lasting effects (Barton, 2002; Flik *et al.*, 2006). Auperin and Geslin (2008) just demonstrated that early exposure to a brief stressor reduced the cortisol response later in life in rainbow trout.

The definition of coping styles in fish greatly contributed to the understanding of individual differences in the stress responses (Koolhaas *et al.*, 1999; Øverli *et al.*, 2002). Two coping styles have been defined based on their consistent neuroendocrine and behavioural characteristics: the reactive (shy) and the pro-active (bold) coping response. The first is characterised by higher levels of cortisol release and by inhibitory behaviours such as freezing. The latter is typical of the fight-and-flight response with lower levels of cortisol production. Shy individuals tend to be less aggressive and eventually subordinate, while the bold are predictably dominants and more aggressive (Pottinger and Carrick, 2001; Øverli et al., 2004a). However, these clear-cut distinctions may be flexible or even disappear in certain contexts or as a result of experience (Schjolden *et al.*, 2005; Brown *et al.*, 2007; Frost *et al.*, 2007).

Stressor-related

The stressors that may affect the life of a fish both in natural conditions and in captivity have been reviewed by a number of authors (e.g. Schreck *et al.*, 1997; Conte, 2004; Huntingford *et al.*, 2006). The relevance of a stressor to an individual has not only to do with its nature, but also with the pattern of exposure (Broom and Johnson, 1993). Intensity and duration are two main attributes to take into consideration, but also the effect of repeated stressors or the length of time left between discrete stressors (Barton, 2002).

There is no clear-cut distinction between physical and psychological stressors as most of them involve both components, though in variable proportions (Steckler, 2005). Lovallo (2005) distinguishes between those starting in the body (physical) and those starting in the mind (psychological), with the cortisol response being particularly sensitive to the psychological stressors in mammals. In fish, one major source of psychological stress is the intra-specific social environment due to the formation of hierarchies and the competition for territories and social partners. Many studies have been evaluating the effect of social status and social stressors on the HPI axis and other related systems (e.g. Munro and Pitcher, 1985; Fox et al., 1997; Pottinger and Carrick, 2001; Clement et al., 2005; Gilmour et al., 2005; Earley et al., 2006; Fernandes-De-Castilho et al., 2008). Predation is another important source of psychological stress for fish (e.g. Griffiths, 1997; Kelley and Magurran, 2003; Mesquita and Young, 2007; Vilhunen et al., 2008). In captivity, many husbandry procedures have a number of similarities with predation, and are also relevant sources of potentially strong psychological components. Intra-specific and inter-specific social stressors as well as certain husbandry procedures may primarily elicit fear, danger recognition and anticipation of threatening events with concomitant behavioural and neuroendocrine responses (Chandroo et al., 2004a).

However, even more important than the physical or psychological nature of the stressors *per se*, are the individual appraisals (Ursin and Eriksen, 2004). This purely psychological mediator between any stressor and the stress response is a major source of variation to stress vulnerability. This aspect will be discussed in section 3.

2.1.4. Stress and welfare: the concept of allostasis

Cannon's concept of *homeostasis* as "*a relatively stable condition by which the body maintains its internal balance*" gave a major contribution to the understanding of stress mechanisms (Cooper and Dewe, 2004). His concept was more recently changed into what Sapolsky (2004) called the 'homeostasis plus': the notion of allostasis. Meaning "stability through change", allostasis introduces a more dynamic and flexible view of the internal balance (Mc Ewen, 1998). According to different life cycle stages (breeding, migrating, molting, etc.), the internal balance is reshaped to accommodate different requirements and adapt to them. The organism is able to anticipate predictable changes through feedforward mechanisms, during which physiological and behavioural adjustments are undertaken in anticipation of a changing demand (Korte et al., 2005). For example, bears' preparation for winter includes eating large quantities of food and put on body fat to act as an energy source during hibernation (Mc Ewen, 2006). But the organism also responds to unpredictable changes, such as a fight with an intruder, through feedback mechanisms, in an attempt to restore the former balance. The means through which the animal copes with unpredictable and predictable environmental inputs require a set of physiological and behavioural processes coordinated by the brain. These are usually the indicators used to measure stress. Whether they measure welfare, in the sense of subjective experiences, is presently one of the most discussed questions (for further details see section 2.2.2.) (e.g. Dawkins, 1998b). The fact is that these responses may be activated differentially in accordance to the nature of the imposed changes and have a biological cost of different levels (allostatic load) (Mc Ewen and Wingfield, 2003). This cost may also be reflected in transient or more prolonged negative mental states. However, to a certain extent this is essentially an adaptive mechanism that leads the animals to overcome the stressors (to cope). However, when the physiological and behavioural responses become misadjusted (due to individual or environmental-related factors), the animals are said to be in a state of allostatic overload. This is then considered to be within a pathological dominion (or pre-pathological for some authors), which is when stress becomes truly maladaptive and a real concern in terms of welfare (Moberg, 1985; Mc Ewen, 1998; Broom 2008).

2.2. Fish sentience

Sentience is the capacity to feel emotions, and involves being conscious of internal and external stimuli, requiring for that some cognitive competences (Chandroo et al., 2004a). Sentience shapes motivational states and therefore modulates behavioural and physiological responses of animals. As mentioned before, sentience is a pre-requisite for conferring welfare status to animals. The following sections define the scope of relevant terms commonly used in sentience and welfare research.

2.2.1. Consciousness, cognition, emotions and motivation: the scope of these terms

Consciousness

"Consciousness is the difference between sleeping and waking, seeing and blindness, understanding and forgetting. To ignore the great looming mountain of consciousness is to do bad science. Physics cannot be done by evading gravity, and biology cannot succeed by turning a blind eye to the origin of species. Likewise, the mind–brain sciences cannot be pursued without consciousness." (Baars, 2003)

Philosophers, psychologists, biologists and neuroscientists tend to emphasize different properties of this mental phenomenon. In its simplest form, consciousness implies the perception of sensations or feelings (phenomenal consciousness or sentience) (Panksepp, 2003). This requires the capability for mental representation and relates to the basic experiences of seeing, hearing, feeling pain or joy (Baars et al., 2003). In a somewhat more elaborated form (access or higher-order consciousness), according to Edelman et al. (2005), consciousness implies higher level thoughts such as possibly the perception of own thoughts (metacognition), the perception of past and future events (episodic memory) and even the ability for language. It refers to more complex experiences of being able to think about or report on a mental state either in the present or the past.

Although some argue that consciousness is an epiphenomenon (Rolls, 1999; LeDoux, 2000), it seems to be largely accepted that it has an adaptive value on its own. Cabanac (1996) describes consciousness as a multi-dimensional phenomenon, in which affectivity, i.e. the psychological quality of pleasantness or unpleasantness is what confers it the best adaptive advantage: "*it saves the animals to store an astronomical number of potentially useful or noxious stimulus-response reflexes*" (Cabanac, 1999). In other words, consciousness provides behavioural flexibility, efficiency and faster reactions. In evolutionary terms, it offered the organisms an added tool to deal with more complex environments (especially social environments) and longer time scales (Dawkins, 1998b).

In attempting to localise the emergence of consciousness in the phylogenetic tree, the different approaches to consciousness are reflected in the divergent conclusions reached by a number of authors. For example, Dennett (1996) excludes from the conscious universe human babies and all non-human animals on the basis of their lack of language. For Rose (2002), consciousness is an attribute of humans and possibly some primates due to the existence of a developed neocortex. Rosenthal (1993) only attributes consciousness to those capable of metacognition, i.e. capable of thinking about their own thoughts (humans and some primates). Defending a progressive emergence of consciousness among species, Cabanac (1999) locates the emergence of consciousness in amniotes, as he clearly denies the existence of fear and other emotions in fish and amphibians. Baars (2001) based on cognitive and learning arguments, believes that phenomenal consciousness must be present in all vertebrates and possibly also in some invertebrates. Merker (2007) seems to corroborate this position when he claims that "*an adequate account of neural mechanisms of conscious function cannot be confined to the thalamocortical complex alone*" but shall be extended to the brainstem, which mechanisms are "*integral to the constitution of the conscious state*".

The perception of the self is embedded in the concept of consciousness (Damasio, 2001). It has its roots on the ability of the brain to constantly map the state of the organism through a complex number of brain structures and neural circuits (e.g. amygdala, thalamus, ventral tegmental area, ventral pallidum, nucleus accumbens, peri-aqueductal grey, hippocampus) (Baars et al., 2003; Damasio, 2003). According to Damasio (2003), cortical and subcortical structures of the brain allow many species to have a continuous representation of the body capable of supporting feelings of emotion and a sense of self. According to this author, only primates might, through the addition of cortical structures that allow high-level convergence of neural information (insular, anterior cingulated and prefrontal cortices), have a more encompassing sense of self.

Cognition

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

The boundaries between cognition and consciousness/emotions are not clear and opinions diverge. Some authors (Lazarus, 1999; Damasio, 2003) argue that they are inextricably connected (see below). Despite assuming a close relation between them, others regard the two as independent aspects: cognition is more cortical and evolutionary recent, emotions are subcortical and more primitive (Panksepp, 2003). Shettleworth (2001) makes a clear distinction between cognitive studies and inferences about consciousness, on the basis of the incapability of animals to verbally report their experiences. In the welfare field, many researchers cautiously claim that a better knowledge in these areas can provide new tools to understand sentience and its manifestation in animals (e.g. Duncan and Petherick, 1991; Duncan, 2006). While recognising the importance of cognitive studies, some draw the attention to potential pitfalls of a mixed approach, namely the risk of considering that only the cognitively complex organisms are conscious and thus only these are capable of suffering (Dawkins, 2001).

The study of memory in cognitive ethology is frequently used to assert evidence of sentience in animals. The short-term or working memory implies the temporary storage and manipulation of information required for specific tasks. Duration and number of items stored in this type of memory are limited and decay over time (Baddeley, 2000). The long-term memory systems include implicit memory (procedural representations), which affects behaviour unconsciously (habituation, simple forms of conditioning), and explicit memory which involves declarative or conscious mental representations (Hampton and Schwartz, 2004). It includes the semantic memory (general knowledge about the world) and episodic memory (personal knowledge about one's own past).

Emotions

Some authors (e.g. Damasio, 2001) define emotions as a patterned collection of chemical and neural responses produced by the brain when it detects the presence of an internal or external stimulus with affective relevance. This process may be conscious or not and the main target of emotions is the body (viscera and musculoskeletal system), but also some areas of the brain. For Damasio (2001) feelings are the mental representations of these physiological changes. In other words, feelings are the mental perception of emotions. While the latter can be assessed directly by means of behavioural and physiological measurements, feelings are indeed private and, thus, very subjective, being impossible any direct assessment of them.

Other authors (e.g. Cabanac, 2002) do not make such a clear-cut distinction between emotions and feelings and describe emotions as mental experiences with behavioural, physiological, cognitive and subjective components. The cognitive definition of emotions by Lazarus (1999) describes emotions in close interconnection with cognition and as involving judgement, evaluation and thoughts. Emotions are related to how individuals appraise the stimuli (a development of this idea can be found in section 3.2.). Despite this cognitive mediation, Lazarus still accepts that some emotions are unconscious.

Motivation

Motivation refers to an internal state caused by an intricate net of internal and external factors which result in short-term, reversible changes in behaviour (Manning and Dawkins, 1998). Internal stimuli accounting for motivational states are individual-related and some examples of these are endogenous rhythms and specific physiological states like those related to hunger or the phase of the reproductive cycle. How external stimuli affect motivations depends on the type, intensity, context and other attributes of environmental factors. As motivation arises from the internal processing of the different stimuli, motivational states are also closely influenced by cognitive factors related to early and later learning in life as well as to memory (Colgan, 1989).

Different approaches have been attempted to explain motivation. A behavioural approach describes motivation through the analysis of decision-making mechanisms (Manning and Dawkins, 1998) and more or less complex homeostatic models (Hughes and Duncan, 1988; Hogan, 1997). A more hardware approach is physiologically orientated and covers the study of neuro-ethological and hormonal mechanisms (Toates, 2002). Motivation strongly influences the control of behaviour, and depends upon three brain processing levels of growing complexity: a stimulus-response system, a dopamine-mediated pathway (responsible for ‘wanting’ a pleasant reward) and a cognitive system, requiring mental representations and flexible ways to process information (Toates, 2004). Hedonistic theories establish a close link between emotions and motivational states, assuming that pleasure and other hedonic states underlie the reinforcement processes (Colgan, 1989).

The interdependent relationship between cognition, motivation, emotions and behaviour (Toates, 2004) is the reason why measuring motivation, i.e. how much an animal wants to have access to a given resource, is assumed to be an indirect way of measuring emotions, and thus of measuring subjective states relevant to welfare (Dawkins, 1990; Kirkden and Pajor, 2006). Methods of measuring motivation and their relationship with mental experiences will be further developed in chapter 8.

Affect or ‘motivational affective state’ is a frequently used term in the scope of emotions and motivation research (e.g. Colgan, 1989; Cabanac, 1996). It refers to the valence (positive or pleasant/negative or unpleasant) of the subjective mental states. Under the scope of the present thesis, emotions, feelings, affect or ‘motivational affective states’ are regarded as synonymous terms.

2.2.2. The study of sentience

Brief historical background

As defined above, sentience is the most basic form of consciousness, the ability of experiencing positive (pleasure) and negative (suffering) affective states (Duncan, 2006). Although Darwin defended the existence of emotions in animals, this idea was completely abandoned in the first half of the 20th century, when Europe and America were dominated by the positivism movement (see Rollin, 1990). Any reference to emotions, consciousness and other subjective terms was discarded from science as a plain non-existence or at least as not available to scientific study. It was under this philosophical atmosphere that Behaviorism flourished in North America: animals were seen as an exclusive function of their present or past environment and treated as black boxes in terms of mental processes (McFarland, 1989; Duncan, 2006). With the view that all vertebrate behaviour could be explained through basic principles, animal experiments in the laboratory focused on unravelling these principles, in particular of learning. In Europe, ethologists followed a very different route for the study of animal behaviour, viewing behaviour primarily in its biological and evolutionary context. Despite re-introducing terms such as ‘hunger’, ‘pain’, ‘fear’ or ‘frustration’, they also rejected to scientifically address subjective questions (McFarland, 1989; Rollin, 1990). In a classical study by Baerends and Baerends-Van Roon (1950) on the ethology of cichlid fish, subjective terms were used to describe behaviour, albeit no proper research was carried out to properly address the question of mental experiences. They state the following while describing aspects of the fish social behaviour:

> *"That they can experience the same emotional reaction simultaneously is illustrated by the following observation in* Tilapia natalensis*. These fish when immature and living in schools show either a pattern of vertical bars or two longitudinal bands. The latter pattern they assume when they are not disturbed, the former when they feel anxiety. Now, as soon as we brought a small* Hemichromis *into a tank containing a school of* Tilapia*'s showing the longitudinal bands, all at once assumed the cross bands. From this we conclude that they became anxious irrespective of whether they individually had been attacked or not." (Pg. 79)*

The animals' sentience folder was finally re-opened in the 70's with the ideas of Donald Griffin (1981) on the mental lives of animals and of Jane Goodall (1992) with her account of the social complexities in a chimpanzee's group at Gombe. The question of animal suffering made its definitive progress within animal welfare science with the pioneering work of Marian Dawkins (1980). For her, one essential animal welfare question was "*what the animals feel about their own lives?*". For other authors, this is indeed the only question that matters (Duncan and Petherick, 1991; Duncan, 2006). In recent years, fast developments in areas like cognitive ethology, physiology and neuroscience brought new arguments and points of view to the very difficult task of understanding sentience (e.g. Baars, 2001; Damasio, 2003; Panksepp, 2003; Merker, 2007; Cabanac et al., 2009).

Assessment of affective states

In tackling animal welfare through the study of sentience, scientists do not only search for means of identifying and reducing suffering. Recently, there is a clear trend to value ways of identifying and promoting positive affective states, like comfort and contentment (Boissy et al., 2007b; Yeates and Main, 2008; Balcombe, 2009; Fraser, 2009). The fact that pleasure also plays a role on decision-making in sentient animals has increasingly been recognised (Balcombe, 2009). In fish, for example, it is apparent that tactile stimulation by cleaners (e.g. cleaner wrasses *Labroides dimidiatus*) can promote pleasure in client reef fish, if interpreting behaviour from a proximate, rather than from an exclusive evolutionary perspective. Cleaners use their pelvic and pectoral fins to massage their visiting clientele, which enable them to freely inspect each client during longer episodes of time and also, to reconcile after a cheating episode and during interactions with predatory clients (pre-conflict management) (Bshary and Würth, 2001; Grutter, 2004; Bshary, 2006). For clients, the benefits are not as clear but reduced levels of cortisol associated to cleaning episodes suggest that receiving tactile stimulation might ultimately reduce stress (Bshary et al., 2007; M.C. Soares, unpublished data). It is therefore conceivable that clients unable to receive tactile reward are more easily stressed which ultimately can result in a reduced fitness (M.C. Soares, personal communication).

Methods to infer affective states in animals are necessarily indirect and more complicated than in humans, since animals cannot report through language how they feel. In this context, behaviour and physiological indicators are frequently combined with arguments by analogy with humans (Sandøe and Simonsen, 1992; Würbel, 2009).

In the assessment of subjective mental states, behaviour has been an essential tool, but not the exclusive one (Dawkins, 2004). Facial expressions, body postures, vocalisations or play are among usual indicators of positive affective states. Tests of preference between resources and tests of motivation, where animals are made to pay a cost for accessing a wanted resource have been developed since 30 years ago with the purpose of identifying particular needs and inferring positive welfare (Dawkins, 1980; 1988). Observations of abnormal behaviours have also been used to uncover states of possible poor welfare (Duncan, 2006). However, this approach has its difficulties, as can be illustrated by the discussion on the relationship between stereotypies and suffering (Mason and Latham, 2004). A number of behavioural paradigms aiming to assess animal models of affective disorders, such as anxiety and depression, have also been frequent, e.g. open field, elevated plus maze, T and radial-maze (Ennaceur et al., 2006).

Combined with behaviour, a number of physiological markers are, despite complex and context-dependent, inevitably used in the scope of sentience research (Broom and Johnson, 1993; Yeates and Main, 2008). One of their main limitations is that they only measure arousal and not the actual valence of stimuli. For example, cortisol may rise in response to a positive context of sexual activity, a neutral context of physical exercise or a negative context of fear (Lovallo, 2005; Paul et al., 2005). In any case, some of the measures undertaken include heart rate, stress hormones (especially corticosteroids) and other hormones (e.g. oxytocin), neurotransmitters (such as endorphins) and monoamines (such as dopamine and serotonin).

As already mentioned, the contribution of cognition for the assessment of emotions is very relevant. A number of complex cognitive attributes have been found in animals, being an invaluable contribution to the understanding of ways and contexts under which animals are more likely to suffer or feel pleasure. Cognition allows inferences about consciousness on animals with sophisticated cognitive abilities (Mendl and Paul, 2004). One example of this is a number of studies developed on the declarative memory of European scrub jays (*Aphlemocoma coerulescens*), a food storing bird species. In the lack of language, it was assumed that if these animals could prove to know what, where and when a past event occurred, then they would have the basis for episodic memory (Clayton et al., 2003). These birds have shown capacity for conscious forms of complex memory of the type what/where/when, revealing ability for selective attention to stimuli, anticipation and flexible and integrated behavioural responses (Clayton et al., 2003). These capacities may have a profound effect on the range of situations in which scrub jays might suffer, which is valuable information in terms of welfare management (Mendl and Paul, 2004).

Another important contribution of cognition to the assessment of emotions is that it provides information not only on the emotional arousal but also on the actual valence of affective states (i.e. whether they are positive or negative). In this context, a promising approach is the cognitive bias experiments, so far undertaken in rats, in which moods (pessimism/optimism) are assessed through the negative or positive interpretation of ambiguous stimuli (Harding et al., 2004). Another line of research is the use of anticipatory behaviour and dopaminergic activity to assess potential positive emotional states between the sign and the actual arrival of a positive stimulus (Spruijt et al., 2001). Reaction to reward loss was also suggested as an indicator of animal emotion and welfare (Burman et al., 2008), on the basis that animals in negative affective states are particularly sensitive to reward losses. However, this is arguable, as it may be highly dependent on animals' previous expectations (Crespi, 1942), which can be lower in case of poor affective states (e.g. depression). Animals' appraisal of events is also an emergent area of cognitive research applied to welfare (Désiré et al., 2002), as will be described in section 3.2.2.. Studies of aversive learning have been used to identify negative emotions of pain and fear (e.g. Yue et al., 2004; Dunlop et al., 2006). Yue et al. (2004) showed evidence that fish exhibit a conscious voluntary response to a conditioned stimulus rather than a reflexive, stereotypic response. Rainbow trout learnt to avoid a neutral stimulus (light) associated to a plunging net. The authors observed that the latency of responding to the light was double than to the net in a demonstration that avoiding the light was more deliberate in nature than escaping to the net only. In an experiment run by Dunlop et al. (2006), the presence of a conspecific significantly changed the avoidance response to an electrical shock. Rainbow trout were willing to remain close to the conspecific during low intensity shock stimuli previously shown to elicit avoidance. Goldfish tended to leave the area closer to the conspecific, but remained in the adjacent area, rather than at the end of the tank. These results suggest that shock avoidance in fish is not purely a reflex action.

Research on pain and fear in humans and animal models has also strongly contributed to understanding sentience in animals. Some studies involve the analysis of subjects' behavioural and physiological response to analgesic and anxiolytic drugs, brain lesions, electrical stimulation and comparison of brain activity in humans and non-humans by means of various brain imaging techniques (Schnitzler and Ploner, 2000; Paul et al., 2005; Dawkins, 2006).

2.2.3. Evidence of sentience in fish

As discussed above, the emergence of consciousness in the phylogenetic tree is a matter of scientific disagreement. One of the authors that have been most prominent in defending that fish are not conscious is Rose (2002; 2007) on the basis of their lack of neocortex. For him, fish behaviour is simple and relatively stereotyped, implying simple forms of learning (habituation, simple classical conditioning) only, all regulated exclusively at the brainstem level (Rose, 2002). However, recent neuroanatomic, physiological, cognitive and behavioural studies offer compelling evidence suggesting the contrary (reviewed by Chandroo et al., 2004a). Fish exhibit complex and flexible patterns of behaviour, highly developed cognitive abilities and perception of emotions. The indirect evidence that fish are sentient beings is the object of the following sections.

Brain neuroanatomic evidence

During embryonic development, the teleost telencephalon suffers an eversion and bending outward, when the dorsal part of the neural tube starts to thicken while the ventral part remains unchanged. The result of this is a pair of telencephalic hemispheres disposed laterally to a single ventricular cavity. This process contrasts with the development of the terrestrial vertebrates' telencephalon, which consists of an evagination, producing paired telencephalic hemispheres with a number of internal ventricles (Figure 3). As a consequence of this divergent development it is difficult to establish topographic homologies between the pallial areas of teleosts and those of land vertebrates (Portavella and Vargas, 2005). However, recent research on spatial memory of goldfish has shown homologies between the lateral and medial pallia of fish and the hippocampus and amygdala of terrestrial vertebrates, respectively (Rodriguez et al., 2006).

Fishes are the largest group of vertebrates, having evolved in the most diverse environments which determined a high variety of anatomical, physiological and behavioural adaptations (Helfman et al., 1997). The fish brain's organisation reflects this radiation, with structures – in particular those that process sensory information – that are more or less developed in accordance to particular specialisations to certain environments (Kotrschal et al., 1998). In any case, all fish have a basic brain organisation which also coincides with a common vertebrate model (Butler and Hodos, 2005). This organisation consists of the following regions (Figure 4): the hindbrain or rhombencephalon (subdivided in metencephalon, which is composed by the pons and the cerebellum, and myelencephalon, composed by the medulla), the midbrain with the mesencephalon (composed by the optic tectum and tegmentum) and the diencephalon (with the thalamus and hypothalamus) and the forebrain or telencephalon (composed by a pair of cerebral hemispheres and the associated olfactory lobes). The forebrain is divided in pallial (dorsal area) and subpallial (underneath) areas. The pallial areas can be further subdivided into central and peripheral regions, where the distinctions between these areas are attributed to the central region having large scattered neurons. The peripheral area is composed of three regions: the medial, dorsal and lateral pallial nuclei.

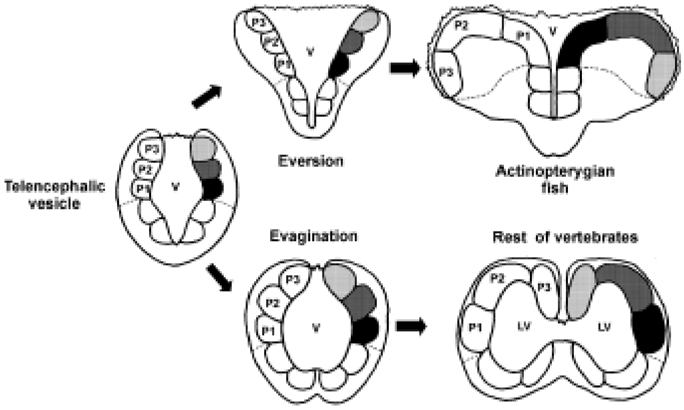

Figure 3. Schematic representation of the two different processes of embryonic development of the telencephalic vesicle in vertebrates. From Portavela and Vargas (2005).

Sensory information comes from many possible sources: visual cues, olfactory cues and lateral line information captured by mechanoreceptors and electroreceptors. The lateral line organization in fish seems very similar to the auditory systems found in terrestrial vertebrates, both heavily relying on mechanoreceptors. The somatosensory information is received by the brain through the cranial nerves (trigeminus, facialis and vagus) and three lateral line nerves (Braithwaite, 2006). Like in terrestrial vertebrates, there is considerable interconnectivity between different areas of the teleost brain (Braithwaite, 2006). The brainstem (parts of the rhombencephalon: pons, medulla and midbrain) and the diencephalon are continuous with each other. Various sensory inputs are integrated in the hypothalamus which, as already mentioned above, can influence behaviour through the secretion of hormones. Large projection neurons connect the telencephalon and the diencephalon via the olfactory tracts.

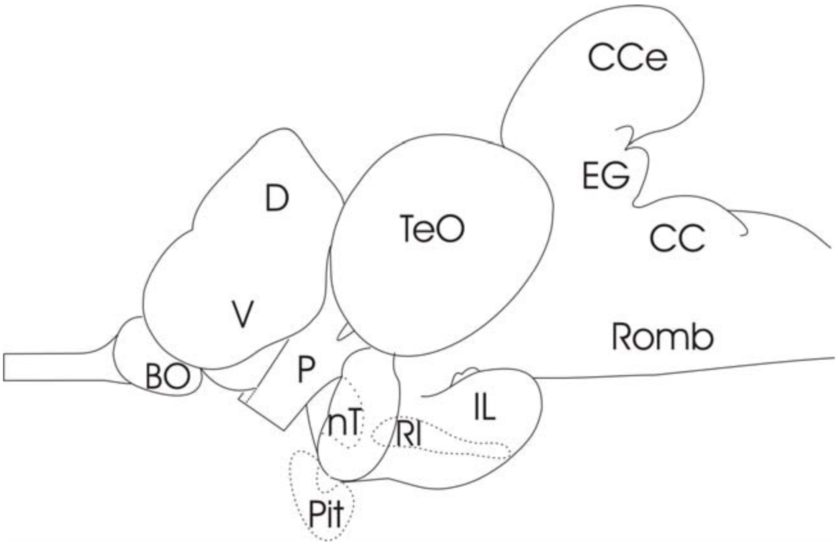

Figure 4. Schematic representation of a brain of the cichlid fish *Oreochromis mossambicus*. From Magda Teles (unpublished data). Rhombencephalon (Romb), includes CCe - corpus cerebelli; EG - eminentia granularis; CC- crista cerebellaris; Mesencephalon represented by the TeO - optic tectum; Diencephalon includes P - preoptic region, nT - nucleus tuberis ; Pit - pituitary; IL - inferior lobe of the hypothalamus ; Rl - recessus lateralis; and Telencephalon with the BO - olfactory bulb; D - dorsal telencephalon; V - ventral telencephalon.

As in land vertebrates, the fish forebrain plays a key role in processing and coordinating sensory and motor information. It seems to be extremely important in influencing emotional, social and reproductive behaviour, as well as learning and memory (Braithwaite, 2006; Rodriguez et al., 2006). In fact, there appears to be sufficient capacity for a number of high-level processes to occur within this structure. For example, there is evidence that the fish lateral pallium is essential for processing and encoding complex spatial information to form cognitive maps (Broglio et al., 2003). Areas such as the optic tectum, the cerebellum and the medial pallium seem to be responsible for body-centred orientation strategies and emotional learning (Broglio et al., 2003). Similarities with other vertebrates also occur at the level of the cerebellum, a structure that has been found to be involved in emotional learning in mammals, as well as in fish (Rodriguez et al., 2005).

In face of what was said above, the case for attributing consciousness to fish can be made on the basis of the various brain homologies (Butler and Hodos, 2005; Rodriguez et al., 2006) and possibly also some analogies (Bshary *et al.*, 2002; Chandroo *et al.*, 2004b) with higher vertebrates. From the neuroanatomical point of view, consciousness does not arise in one precise site in the mammalian brain, rather it is postulated to be the result of an intricate system of neuronal connections with specific characteristics that work together in producing conscious thoughts and in keeping the allostasis of the central nervous system (Taylor, 2001; Baars et al., 2003; Damasio, 2003; Lovallo, 2005). As discussed above, these interconnections also occur in fish involving homologous neuronal areas of the brain, and similar neurotransmitter mechanisms (e.g. dopaminergic and serotonergic brain systems) (Chandroo *et al.*, 2004a).Furthermore, cases of convergence can also occur. For example, between some primates and cetaceans there is highly convergent social behaviour and cognitive competences, despite the evolutionary divergence, the adaptation to very different environments and the different brain organization (Marino, 2002).

Cognitive evidence

Recent research on cognitive capabilities of fish has shown behaviours indicative of declarative mental representations, complex and flexible memory and learning abilities (reviewed by Bshary et al., 2002; Chandroo et al., 2004a; Braithwaite, 2006), which are assumed to form the basis of consciousness (Duncan and Petherick, 1991; Baars, 2001). Table 1 describes some examples of these studies performed in a variety of contexts such as intra and inter-specific social interactions, spatial orientation, feeding and predators' avoidance.

Emotional evidence

In fish, the study of emotions has been especially focused on pain and fear. Lynne Sneddon and colleagues (Sneddon, 2002; 2003a; 2003b) have described not only the

Table 1. Examples of studies showing evidence of declarative mental representations in fish

Cognitive abilities	**Species**	**Authors**
Individual recognition in the social network and subsequent behavioural adjustment (e.g. fighting strategy, body colour)	- Rainbow trout (*Oncorhynchus mykiss*) - Atlantic salmon (*Salmo salar*) - Artic charr (*Salvenius alpinus*)	(Johnsson, 1997) (O'Connor et al., 1999; 2000) (Hoglund et al., 2000)
Observational learning and posterior use of information during social interactions ("eavesdrop")	- Guppy (*Poecilia reticulata*) - Fighting fish (*Betta spendens*) - Rainbow trout (*Oncorhynchus mykiss*) - Peacock blenny (*Salaria pavo*)	(Dugatkin and Godin, 1992) (Oliveira et al., 1998; McGregor et al., 2001) (Johnson and Åkerman, 1998) (Gonçalves et al., 2003)
Transitive inference (in a fighting context)	- Burton's mouthbrooder *(Astatotilapia burtoni)*	(Grosenick et al., 2007)
Sophisticated cooperative hunting through strategies that include prior solicitation for hunting together and different roles during hunt	- Moray eel (*Gymnothorax javanicus*) with red sea coral grouper (*Plectropomus pessuliferus*) and lunartail grouper (*Variola louti*)	(Diamant and Shpigel, 1985; Bshary et al., 2006)
Cooperative predator inspection	- Guppy (*Poecilia reticulata*) - Stickleback (*Gasterosteus aculeatus*)	(Pitcher et al., 1986)
Inter-specific cleaning symbioses involving numerous complex mechanisms (Machiavellian intelligence) of manipulation, punishment and reconciliation. Some other complex social aspects include inter and intra-specific recognition, altruism, social prestige and 'bookkeeping'.	- Cleaner wrasses (*Labroides dimidiatus*)	(Bshary and Würth, 2001; Bshary et al., 2002)
Avoidance of dangerous sites (showing anticipatory abilities)	- Stickleback spp. - Various species	(Huntingford and Wright, 1989) (Kelley and Magurran, 2003)
Complex and flexible spatial memory, use of different adaptive strategies (within species) such as formation of internal map-like representations	- Gobiid (*Bathygobius soporator*) - Goldfish (*Carassius auratus*) - Atlantic salmon (*Salmo salar*) - Stickleback (*Gasterosteus aculeatus*)	(Aronson, 1951; 1971) (Salas et al., 1996; López et al., 1999; López et al., 2000; Rodriguez et al., 2002) (Braithwaite et al., 1996) (Odling-Smee and Braithwaite, 2003)
Special foraging skills, cases of tool use (e.g. instrumental use of leaves or substrate), and choice of mating sites sometimes associated to stable cultural traditions	- Various species	(reviewed by Bshary et al., 2002; Braithwaite, 2006) (Warner, 1988; 1990) (Helfman and Schultz, 1984)
Association of unpaired, distinct stimuli showing anticipatory and predictive behaviour as a consequence of a combination of that association and memories of previous experiences	- Paradise fish (*Macropodus opercularis)*	(Topál and Csányi, 1999)
Memory of time-place associations (one of the pre-requisites for episodic memory)	- Paradise fish *(Notemigonus crysoleucas)* - Inanga *(Galaxias maculatus)* - Angelfish *(Pterophyllum scalare)* - Nile tilapia *(Oreochromis niloticus)*	(Reebs, 1996) (Reebs, 1999) (Barreto et al., 2006a) (Delicio and Barreto, 2008)

nociceptive system, but also pain perception in rainbow trout. The nociceptive system is part of the peripheral and central nervous system responsible for detecting noxious stimuli and transmitting them to the brain. Nociception triggers several autonomic responses and, when reaching higher brain areas, may also involve pain, which is the mental experience of nociception in sentient animals (Zimmermann, 1986). According to Ashley and Sneddon (2008), the requirements for the existence of pain include the possession of nociceptors, pathways leading to higher brain structures, brain structures involved in pain processing, presence of opioid receptors, endogenous opioids and enkephalins, effect of analgesics in reducing nociceptive responses, avoidance learning and non-reflex responses to noxious stimuli.

Nociceptors are free nerve endings in the periphery or internal organs. In land vertebrates, they are usually of two fibre or nerve types: small myelinated A-delta fibres with high conduction velocities and smaller unmyelinated C fibres with slower conduction velocities (Ashley and Sneddon, 2008). The trigeminal nerve (the fifth cranial nerve that innervates the majority of sensory information from the head of vertebrates) also exists in fish, conveying information from oral and facial areas. Both fibre types are present in this nerve and their size ranges are similar in all vertebrates (Sneddon, 2002). The nociceptor properties are similar in fish and tetrapods, namely in relation to diameter of receptive fields, type of action potentials and conduction velocity. The nociceptive system requires the existence of brain areas (e.g. pons, medulla, thalamus) where the nociceptive processing occurs. As in humans, the fish telencephalon receives neural projections from the thalamus and regions of the pallium processes somatosensory information, like possibly nociception via the trigeminal nerve (Ashley and Sneddon, 2008). Recent electrophysiological recordings in rainbow trout and goldfish have shown that the spine, cerebellum, tectum and telencephalon are all structures involved during nociceptive stimulation (Dunlop et al., 2006). Preliminary studies of gene expression also suggest that these brain areas are likely to play different roles in the processing of nociceptive signals. An additional requirement to prove the existence of a nociceptive system is the possession of opioid receptors, endogenous opioids and enkephalins. These structures and substances were found in the brain of a number of different fish species following a similar pattern as that known for land vertebrates (Ashley and Sneddon, 2008).

Bateson (1991) suggests that, although subjective, the behaviour is the best way to assess the existence of pain in animals. The observation of non-reflexive reactions to a noxious stimulus has been assumed as indicative of pain perception. Sneddon (2003a) have found prolonged changes in behaviour of rainbow trout (enhanced respiratory rate, body rocking and rubbing on surfaces) over the long period of time during which the noxious stimulus was activated. These responses were much reduced under the effect of the pain-killer, morphine. Avoidance learning as a response to painful stimuli was also proved in goldfish and other fish species (e.g. Dunlop et al., 2006).

Fear enhances a common stress response, generally involving the activation of the defensive behavioural system (mentioned above in section 2.1.2). As it is the case for other emotions, it has the likely effect of protecting organisms against potential danger. In higher vertebrates, the neural basis of fear mainly involves the amygdala and hippocampus which, as mentioned above, have homologous pallial structures in fish (Rodriguez et al., 2006). Avoidance learning tasks are also common paradigm in fear research, revealing a consistent pattern of avoidance behaviours (e.g. Portavella et al., 2003; Yue et al., 2004). Yue and colleagues (2004) have shown how the behavioural responses of rainbow trout to fearful stimuli are of a non-reflexive nature and how they can make conscious choices to avoid future frightening stimuli. Ashley & Sneddon (2008) review studies where the administration of benzodiazepines (anti-anxiety drugs) to fish can reduce the typical fear response.

2.3. Fish welfare assessment

Measurement of fish welfare largely depends on the adopted welfare concept, approach and objective. Welfare measures of fish in an aquaculture (e.g. growth rates) are likely to have different components from those in an exhibition aquarium (e.g. natural behaviour). As mentioned in the first section of this review, preferential welfare measures are taken according to function, feelings or nature-based approaches. Since there is no easy way to measure animal welfare, there has been a search for some integration of the various approaches (Mason and Mendl, 1993; Volpato et al., 2007). This integration is indeed necessary because many measures (e.g. hormones) can vary with a wide variety of factors and thus, when taken alone, their value as a welfare indicator is limited. However, taking too many measures on-board frequently result in inconsistent picture in terms of welfare.

In an effort to grasp the essential approach to animal welfare, Dawkins (2004) claimed that knowing ‘whether animals are healthy’ and asking them ‘whether they have what they want’ is all that is necessary. In contrast, more complex approaches have been explored by other authors, which list enormous amounts of potential welfare indicators

(Broom and Johnson, 1993). More recently, the concept of ‘quality of life’ (QoL) – defined by Scott et al. (2007) as the subjective and dynamic evaluation by the individual of its circumstances and the extent to which these meet its expectations, which results in affective responses to those circumstances – led to the development of multi-dimensional evaluation indices with special application on farms and companion animals. These are quantifiable measurements related to different dimensions of welfare (e.g. pain, disease, stress) and use behavioural, physiological and biochemical markers (Scott et al., 2003). They differ from on-farm evaluation schemes which rely on environmental-based (e.g. housing system, management routines, etc.) instead of animal-based parameters (Bracke, 2007). The standard method for measuring QoL in people is the self report. For animals, this approach was adapted to questionnaires to be completed by the owner, caretaker or veterinary (Scott et al., 2007).

2.3.1. Physiological indicators

To a large extent fish welfare measures coincide with short or long term measures of stress. Pottinger (2008) reviews the stress measures in fish, showing that almost all elements of the primary stress response in fish, as described in section 2.1.1., can presently be measured by a diversity of methods (e.g. CRF, ACTH). However, some of these have associated problems. For example, the quickness of the adrenergic response makes the measurement of catecholamines without an effect of the associated sampling procedures difficult. Only with a previously implanted catheter this problem may be overcome, which makes this measure less practical.

Cortisol can be measured from blood plasma, urine, bile or body homogenates but, as Pottinger (2008) highlights, plasma cortisol reflects better the secretory activity and the levels to which the target organs are exposed at a given moment in time. It requires anaesthetising the animals and taking the sample within a short period of time (4 minutes, Foo and Lam, 1993) in order for the measure to reflect baseline levels rather than the response to handling and anaesthesia. A non-invasive method to sample cortisol is its measurement from holding water (Scott et al., 2001). This reflects a temporal integration of the cortisol levels that were in circulation and were then excreted (by urine and faeces) or diffused through the gills. It is for this reason considered more conservative and less vulnerable to time fluctuations of cortisol levels than plasma assays (Bshary et al., 2007).

Measures of the secondary response include changes in blood metabolites (e.g. glucose, lactate, hepatic glycogen, heatshock proteins and metabolically active enzymes) and in immune system function (e.g. number of circulating lymphocytes). Some degree of inconsistency can however occur among all the possible indices described (Broom and Johnson, 1993; Mommsen et al., 1999; Iwama, 2007). Less accessible measurements include modifications in target-issue corticosteroid receptor abundance and gene expression.

2.3.2. Health and productivity indicators

The tertiary stress response relates to changes in the whole organism, thus it is measured at a different level than the primary and secondary responses. So the level of its measurement is different from previous measurements. In accordance with what was described in section 2.1.1., it includes measures of growth, physical condition, incidence of disease and reproductive status (Pankhurst and Van Der Kraak, 1997; Pottinger, 2008).

Stress can affect reproductive fitness at various levels: reproductive hormones, gamete development and quality, egg and larval survival and development. Growth is also affected by stress through unbalance of complex endocrine pathways and due to metabolic effects (Pankhurst and Van Der Kraak, 1997). Spinal deformities are among common problems in aquaculture attributed both to embryonic abnormalities and growth alterations of vertebral and intervertebral tissues (Branson and Turnbull, 2008).

A common indicator of physical condition in fish is fin erosion, which is rather frequent in aquaculture systems. It is a multicausal condition which relates to the physical abrasion of fin tissue and probably reflects the production techniques at various levels (physical, chemical, bacteriological, nutritional, social and behavioural environment) (Ellis et al., 2008). Despite the unknown relationship between this condition and suffering, it is believed that it is potentially associated to nociception, osmotic disturbance and infection and permanently altered fin function (Ellis et al., 2008).

Elevated levels of corticosteroids are immunosuppressive which result in higher pathogen vulnerability and the consequent higher incidence of diseases (Fletcher, 1997). Generally, diseases bring with them higher mortality, and both are common indicators in commercial and non-commercial settings.

In aquaculture contexts, health, physical condition, growth and reproductive rates are frequently considered as measures of productivity since they are necessary for good performance which is the ultimate goal of this activity. However, as indicators of welfare, the tertiary stress responses represent a late stage of stress/poor welfare, often with consequences that are difficult and expensive to revert. Therefore, earlier and sensitive indicators of stress have been increasingly preferred because interventions in minimising stress can then be put into practice at an earlier stage. In this context, simple measures involving behaviour have increasingly been used.

2.3.3. Behavioural indicators

Behaviour has many advantages as a welfare indicator since its measurement does not necessarily imply to disturb the animal's current activities, it is the result of the animal's decision-making processes and, ultimately, it is the expression of its emotions or 'motivational affective states' (Dawkins, 2004). Evaluating behaviour is easily accessible to skilled and attentive caretakers and thus it really represents the earliest potential signal of disturbed welfare. Some examples of these are the detection of changes in body or eye colour, ventilation rate, swimming patterns or food intake (FSBI, 2002). In aquaculture tanks, methods of monitoring behaviour have been developed and these include the installation of sub aquatic cameras and remotely operated vehicles (Frost et al., 1996). Nevertheless, some authors refer to limitations in using behaviour as a welfare indicator due to problems in its observation and interpretation (e.g. Pottinger, 2008).

The fish behavioural responses to acute and chronic stress have been described under section 2.1.2., and these coincide with the most basic and prompt fish behavioural indicators of welfare. Fish preferences are increasingly being used as indicators of welfare of fish (FSBI, 2002). Some authors even rely on this measurement almost exclusive based on the argument that it is not possible to portray the fish internal states in terms of welfare (i.e. sentience) by using physiological measures (Volpato et al 2008). In fish welfare, behaviour has not only been useful to indicate signs of poor welfare but it has also been an obvious indirect tool in the study of fish sentience and cognition. As described in the above sections, cognitive studies heavily rely on behaviour and studies in pain and fear also have a strong behavioural component.

3. Fish psychological stress[2]

Despite the lack of knowledge on the precise mechanisms underlying the integration of psychological aspects in the stress physiology of fish, a growing body of evidence indicate mental processes in these animals (e.g. Barreto and Volpato, 2006; Barreto et al., 2006b). Although the topic is controversial, results from stress studies also strongly suggest the existence of a subjective element in how the organism processes the input from stressors which result in the behavioural and psychological stress response. Therefore, forcefully based on indirect evidence, a case is made here for the existence of that psychological mediator in the fish stress response.

3.1. Evolution of the psychological stress concept

The basic idea of psychological stress is as old as the Greek classical philosophers. Epicurus suggested that coping with emotional factors was a way to improve the 'quality' of life (Johnson *et al.*, 1992). But it was much later that these factors became fully integrated in the concept of stress.

In 1859, Claude Bernard first introduced the idea of 'milieu interieur', which had to be kept harmonious in response to the external changes (Johnson *et al.*, 1992). Cannon's ideas, put forward in the first half of the XX century and never using the word 'stress', gave a major contribution to the understanding of homeostatic and stress mechanisms. In 1956, Hans Selye described his concept of stress and the 'General Adaptation Syndrome', giving great relevance to the adrenal cortex response (Levine, 2005). While being the first acknowledging psychological elements of the stress concept, Cannon and Selye highlighted the non-specificity of the stress response, regardless of the nature of the stressor (Cooper and Dewe, 2004).

In the 1970s', John W. Mason was the first questioning the generality of the stress response and the lack of relevance given to psychological factors in explaining it (Cooper and Dewe, 2004). He mentioned the likelihood of a higher involvement of the central nervous system in what he called the "emotional arousal to a threat", which could possibly modulate the stress response (Cooper and Dewe, 2004; Veissier and Boissy, 2007). The contribution of Richard Lazarus, in the second half of the twentieth century, was a milestone to the modern concept of stress. Based on his work for the

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[2] This section is an adaptation of part of the published article: Galhardo, L., Oliveira, R.F., 2009. Psychological stress and welfare in fish. Annual Review of Biomedical Sciences 11, 1-20.

military in the Second World War, he claimed that individual differences in stress responses were based on the respective 'personal meaning' attached to particular events. The concept of 'appraisal' became a central piece of his work: a somewhat 'enriched' perception also involving a personal evaluation, which is dependant on individual goals and beliefs (Lazarus, 1999). The appraisal process detects the stimuli and prepares the body to react, consisting in two stages: the primary appraisal or the process of evaluating the personal relevance of a given stimuli, and the secondary appraisal or the process of assessing the available coping options. His contribution is highly founded in interconnected cognitive and emotional mechanisms, but he also acknowledges that some appraisals can be extremely rapid and unconscious, leading to fast coping responses (Lazarus, 1999). Other appraisal-related theories were developed (e.g. Scherer, 1984; Frijda, 1987; Ellsworth, 1991), mainly differing on the types of criteria that contribute to the evaluation process. These criteria were reviewed by Desiré (2002) and can be summarised in four main components: relevance (stimuli intrinsic characteristics like novelty and pleasantness), implications (stimuli importance to individual's needs and goals), coping potential (individual resources to deal with the stimuli like control), and normative significance (degree to which the stimuli is compatible with individual and social standards).

More recently, Ursin & Eriksen (2004) presented their 'Cognitive Activation Theory of Stress' (CATS), formally defining some concepts frequently used in confounding terms (e.g. coping, hopelessness, helplessness). The stress response involves neurophysiological activation and arousal and it is regarded as a healthy process, if not sustained over time. A major aspect of this theory is the assumption that the stress response depends on learned expectancies related to the stimuli and to the result of the available coping resources. These expectancies and the way they influence appraisal are the major source of individual differences in the stress response (Eriksen *et al.*, 2005).

3.2. Appraisal approach applied to fish

Psychological stress depends primarily on the ability to appraise the stimuli in two steps. Firstly, they are perceived and evaluated in relation to their properties and specific importance, and secondly coping mechanisms will be mentally assessed in preparation to the effective bodily reaction (Lazarus, 1999; Ursin and Eriksen, 2004). In humans, this mechanism has been well described (e.g. Lovallo, 2005), and is summarised below. As mentioned before, some authors already applied this framework to farm animals in their studies of emotions (e.g. Désiré et al., 2002; Boissy et al., 2007a). In fish, the regulation of stress involving psychological components is not well studied. However, considering all the above described neuroanatomical, cognitive and emotional evidence described above, the suggestion that fish also appraise stimuli is discussed in the following sections.

3.2.1. Physiological integration of psychological components of stress

The stimuli appraisal neural mechanism

In humans, sensory information mediated by the peripheral nervous system arrives at primary sensory cortical areas. It is then relayed to frontal areas of the cortex, where a cognitive evaluation of the stimulus takes place, and to the limbic system (hippocampus and amygdala), where it is emotionally coloured (Lovallo, 2005). The frontal areas of the cortex are specialised in working memory, required for the cognitive assessment of the inputs. The hippocampus is known to be important in the formation of non-emotional memory (e.g. spatial memory) and in the recall of familiar events (declarative memory), while the amygdala invokes contextual aspects of these events through its particularly important role in classical conditioning (emotional memory) (Prickaerts and Steckler, 2005). By attributing an emotional context, the amygdala modulates the consolidation of long-term memories at the hippocampus (Akirav and Richter-Levin, 2005) being activated whenever the affective value of a stressor, signalled by a conditioned stimulus, has to be assessed. Its emotional output is then sent to the frontal cortex, through basal ganglia pathways, where it is integrated with the cognitive evaluation of the stimulus in the cingulate cortex (Lovallo, 2005). This cortical structure then elaborates on the choices to make under a given motivational context sending projections back to the amygdala (Steckler, 2005).

The appraised information is then sent to the hypothalamus and brainstem by either the central nuclei of the amygdala or the pre-frontal cortex (Lovallo, 2005). The outputs of the amygdala have an extensive indirect effect on a number of peripheral responses, namely on behaviours involved in the stress response (e.g. arousal, attention, freezing, fear, conflict, etc.) (Akirav and Richter-Levin, 2005). Therefore, the subsequent signals of the lateral nuclei and paraventricular nuclei of hypothalamus and the signals of the different structures of the brainstem will express the emotional valence of the received stimulus. This expression occurs through the activation of the autonomic, neuroendocrine and behavioural responses. In the brainstem, a complex feedback system, composed by descending and ascending actions of the reticular formation and aminergic nuclei and the CRF system of neurons, coordinates the behavioural arousal, emotional profile and general state of the brain, having the CRF a particularly important role in the integration of the acute fight-or-flight response (Lovallo, 2005).

Despite not knowing the exact mechanisms, fish possess all the neural, cognitive and emotional requirements necessary for appraisal, i.e. the detection of sensory inputs, generation of mental representations involving both working and declarative memory and associated emotional states. As described above, the limbic system is a fundamental brain area for appraisal and homologies between fish and other vertebrates were found for the hippocampus (lateral pallium) and the amygdala (medial pallium) (Rodriguez *et al.*, 2006). While the neocortex plays an important role for human appraisal, it is not necessarily a condition for the processing of sensory information, decision-making and motor output coordination in other species (Braithwaite & Boulcott 2008). Other homologous and analogous areas can also take over this responsibility in the simpler brain of a fish (Butler & Hodos 2005, Taylor 2001, Chandroo et al 2004a,b, Merker 2007).

The neuroendocrine regulation at the higher level of the brain

In mammals, there are MR and GR receptors in a number of cortical (prefrontal cortex) and limbic areas (amygdala and hippocampus), located especially in CRH neurons. This fact suggests that the regulation of the stress response does not always start at the level of the hypothalamus and brainstem. Rather, whenever there are sensory inputs and the need for their interpretation as described above, the stress response starts at the frontal cortex, amygdala and hippocampus (de Kloet et al., 2005). Noradrenergic pathways are also important for this high level regulation, as they send inputs to the amygdala, influencing arousal and various memory mechanisms (Berridge, 2005).

Normally, cortisol exerts an inhibitory effect at the hypothalamic paraventricular nucleus, via GABAergic neurons which form a net surrounding it. At the amygdala, cortisol has an opposite effect, since it inhibits GABAergic neurons at the hypothalamus, in this way activating the HPA axis and generating a positive feedback in the cortisol production (de Kloet et al., 2005). It is apparent that prolonged effects of corticosteroids switches cognitive improvement into impairment (Akirav and Richter-Levin, 2005). Therefore, the influence of their action at the higher levels of the brain during acute stress is different from those under chronic stress. Acute elevations of glucocorticoids enhance memory consolidation and retrieval, and possibly attention, at the level of GR receptors mainly in the hippocampus but also in the amygdala (Prickaerts and Steckler, 2005). During chronic stress, sustained high levels of corticoids reduce the hippocampus volume, which impairs learning and memory and seem to disrupt attentional mechanisms (Lovallo, 2005). Prolonged cortisol exposure has the additional effect of disrupting the hippocampus inhibitory influence over the hypothalamus, in this way stimulating the HPA axis and the further production of cortisol (de Kloet et al., 2005).

Corticosteroid receptors were recently found in fish forebrain areas, namely in the lateral pallium. This fact suggests that these brain areas – like in mammals - are the primary targets during stress control, suggesting a potential effect of stress on cognitive processes (Stolte *et al.*, 2008). There is also experimental evidence supporting such an effect. Schreck and colleagues (1997) reviewed a number of studies showing how stress can affect the memory and learning processes in fish and concluded that very similar processes are also described in higher vertebrates. Examples of these include how pathogens, toxicants, seasonal rhythms or procedures, such as transport, may affect the learning process of species like the rainbow trout, goldfish or coho salmon. The memory induced by a stress context was demonstrated more recently by Moreira & Volpato (2004) in Nile tilapia with a conditioned endocrine response to confinement (unconditioned stimulus) signalled by light (conditioned stimulus). A similar investigation was undertaken with rainbow trout, selectively bred for divergent plasma cortisol responses. Retention of a conditioned response was longer in the low than in the high responsive fish, showing that different cortisol responses correspond to different cognitive profiles (Moreira *et al.*, 2004). A subsequent study using partial emersion as stimulus, which most certainly involves fear (Brydges et al., 2009), confirmed that exogenous cortisol accelerated the extinction of a conditioned response in unselected rainbow trout (Barreto et al., 2006b). These findings were in line with the above mentioned detection of cortisol receptors in areas of the forebrain responsible for memory.

3.2.2. Psychological modulators of the stress response

As already mentioned, one of the most, if not the most, relevant reasons accounting for the differential stress responses is the way the stressor is appraised (Sapolsky, 2004; Ursin and Eriksen, 2004; Steckler, 2005). This fact has implications not only for the perception of the stimuli but also for the evaluation of the available coping responses (Lazarus, 1999; Ursin and Eriksen, 2004). According to the Cognitive Activation Theory of Stress, these two appraisal stages are associated with specific internal expectancies (set values) which are compared with the reality (actual value). The degree of the resulted discrepancy dictates the level of arousal and the related stress response (Ursin and Eriksen, 2004). However, the expectancies can be cognitively manipulated by modifying the psychological context in which the stressor is applied. This manipulation can be linked to the stimulus appraisal or to the coping responses appraisal. Some examples of this are given below. References to fish research are limited by the studies available.

Stimuli-related modulators: predictability and social support

The predictability of a stressor affects the stress response and this fact has been extensively described for humans and animals (Sapolsky, 2004; Lovallo, 2005). Despite the inconsistent physiological responses of various studies, it is apparent that animals prefer predictable over unpredictable negative events (Bassett and Buchanan-Smith, 2007). For example, in rats different predictable stressors alleviate stress responses and promote place preferences and spatial learning when compared to the same stressors presented in an unpredictable schedule (Orsini *et al.*, 2002; Prior, 2002). However, the relieving effect of predictability depends a great deal on a number of the stressor's properties (nature, intensity and frequency) as well as on the reliability of the signalling system and on the time elapsed between the sign and the onset of the aversive event (Sapolsky, 2004). For example, it is highly unlikely that the anticipation of a very damaging or a very frequent event will alleviate the stress response.

Social support creates a psychological context where the stress response is much reduced and where animals show better welfare (DeVries et al., 2003; Sapolsky, 2004). It is well identified that both isolation and group formation with unfamiliar conspecifics enhance behavioural and physiological responses to stress in social mammals (e.g. Gust et al., 1996; Jensen, 2001; Ruis et al., 2001; DeVries et al., 2003; Takeda et al., 2003; Désiré et al., 2006). In fish, the same effect has been shown: social isolation increases cortisol levels and modifies the feeding and agonistic patterns of interaction (Earley *et al.*, 2006; Øverli *et al.*, 2006) and encounters with unfamiliar fish also produces a cortisol response (Yue *et al.*, 2006). A number of studies on the effect of social support on the stress response have been undertaken in human and non-human primates (Johnson *et al.*, 1996; Sapolsky, 2004), but not in fish.

Response-related modulators: control and outlets for frustration

Control is understood as the capability to successfully decrease the exposure to a stressor. It has been regarded as an important factor for the subject's judgment of its coping possibilities (Steckler, 2005), and thus it is a relevant modulator of the stress responses. However, providing an animal with an actual degree of control may not be enough for it to appraise coping as likely to be successful. The self evaluation of possible coping responses is limited by prior experiences (Ursin and Eriksen, 2004). Thus, if prior responses were successful in removing the source of stress, the animal acquires a sense of control that will lower its level of arousal and keep it prepared to cope with following challenges (Eriksen *et al.*, 2005). This is probably one of the reasons why dominant fish tend to have lower cortisol levels than subordinates (Earley *et al.*, 2006) and may also be one of the cognitive contributes to the 'winner and loser effect' (Eriksen *et al.*, 2005), where winners have a higher probability of winning a subsequent encounter (Rutte *et al.*, 2006). Frost and colleagues (2007), for example, have shown how bold rainbow trout reduced their boldness as a consequence of negative prior experiences (loosing fights). On the other hand, if the animal learned from past experiences that there was no relationship between its attempts to cope and the outcome result of the stress experience (total lack of control), it may develop learned helplessness, a condition that has been associated with depression (Ursin and Eriksen, 2004; Lovallo, 2005). Under this state, the animals may be unable to realise that objective control is eventually available and that they could make use of it; learning and avoidance responses becomes impaired (Steckler, 2005). Ursin and Eriksen (2004) distinguish helplessness from hopelessness, in which the latter means some form of negative control, where the response may even contribute to worsen the stressful situation, and consider it a better depression model. Both conditions are known to be powerful in cortisol activation (Ursin and Eriksen, 2004; Lovallo, 2005) and it is possible that subordinate fish may frequently find themselves in these circumstances (Earley *et al.*, 2006).

Outlets for frustration distract the subject from the stressor (Sapolsky, 2004). Typically, the behavioural responses do not help to remove the source of stress, but they do probably give the animal a perception that it is doing something about it. In this way, they contribute to alleviate the adverse effect of the stressor, as shown by the decreased physiological signs of stress. Examples of possible outlets for frustration are the displacement activities (maybe including self-directed behaviours) and eventually stereotypies. Sapolsky (2004) gives the example of exercise and displaced aggression as powerful outlets for human frustration. Displaced aggression is understood as aggression that, in the impossibility of being directed towards the original aggressor (dominant), it is targeted towards weaker conspecifics (e.g. smaller animals, females) or even towards elements of the environment (e.g. substrate). This type of aggression is also common in non-humane primates. Studies with baboons, conducted by Sapolsky and his team, found subsets of subordinates, which displaced aggression and showed low levels of cortisol (DeVries et al., 2003). In fish, displaced aggression as an outlet for stressful social encounters was also observed. Øverli and his team (2004b) have shown how rainbow trout facing larger animals reacts with increased aggression against smaller fish, with patterns of brain serotonin and plasma cortisol compatible with decreased stress. Clement and colleagues (2005) also found, with *Astatotilapia burtoni*, that territorial males engaged in directed aggression towards a potential threat (a dominant male in a video display) while non-territorial males directed their aggression towards the tank mates. The physiological correlate of this study is though less clear, as two subsets of cortisol levels were found for the non-territorial males. Outlets for aggression in the form of interactions with substrate have also been suggested by other authors for different cichlid species (Munro and Pitcher, 1985). Performing stereotypies is an additional outlet for frustration. Indeed, in their review of stereotypies and animal welfare, Mason and Latham (2004) suggested that one of the potential explanations for these repetitive and invariable behaviours could be their function as alternative coping strategies to deal with non-controllable stressors, since in a number of situations stereotyping individuals were found to show a reduced response to stress. In fish, Ashley (2007) mentions some studies where the locomotory pattern of Atlantic salmon and Atlantic halibut resembles stereotypic pacing in mammals. However, these mechanisms are still not well studied in fishes.

4. *Oreochromis mossambicus* as an experimental model

The Mozambique tilapia, *Oreochromis mossambicus* (Peters 1852), is an African mouthbrooder cichlid. The family cichlidae is a very large and diverse family of perciform fish distributed all over the planet (Fryer and Iles, 1972). In the large lakes of Africa, cichlids have evolved rapidly into a large number of closely related but morphologically diverse species, which makes them very important models for the study of speciation, evolution and genetics (Seehausen et al., 2008). Cichlid diversity is reflected in the wide range of body sizes, shapes and colours, natural habitats, diet compositions and feeding behaviours. Reproduction is also characterised by a variety of mating behaviours and parental care strategies.

Oreochromis mossambicus belongs to the large group of fishes of the tilapiine cichlid tribe, endemic to Africa and Israel. It comprises three main genera which differ in their mating and parental behaviour. *Tilapia* (40 species) are monogamous, substrate incubators and with biparental care. *Sarotherodon* (30 species) are monogamous, with biparental or paternal mouthbrooding. *Oreochromis* (10 species) are polygamous and maternal mouthbooders. These behavioural differences between genera have also been confirmed by evolutive analysis of mtDNA (Nagl et al., 2001).

4.1. Biology and natural behaviour

Mozambique tilapia is endemic from the lakes and rivers of the East Coast of Africa (Trewavas, 1983). Since the 1930s, this and other species of tilapias have been intentionally dispersed worldwide for biological control of aquatic weeds and insects, as bait for certain captures, for aquaria, and as a food source. As an exotic species, it became the most widely distributed group worldwide, virtually in all countries where it is cultured (Canonico et al., 2005). Its natural occurrence depends on a number of environmental factors, namely the strength of the current and the temperature. This species avoids strong streams, protecting themselves in vegetation near the banks (Trewavas, 1983). Temperatures below 10ºC appear to limit their dispersion (Bruton and Boltt, 1975). However, the Mozambique tilapia tolerates a wide range of temperatures that can reach 29ºC. This species also tolerates considerably high levels but not rapid changes of salinity. Therefore, it can be found in habitats such as brackish and coastal waters (Trewavas, 1983).

The juveniles of *O. mossambicus* are omnivorous and adults mainly rely on a detritivorus diet. As opportunistic feeders, they profit from whatever food items are available, from plankton to algae, or even small fish, such as their own young, as reported in aquaria (Neil, 1966).

This species is sexually dimorphic. Males have upper jaws and body sizes larger than the females, and they also develop a very dark body colour during breeding contexts (Trewavas, 1983). But what definitely distinguishes males from females at any phase of their life cycle is the shape and openings number of the genital papilla: males have a tubular papilla with one single opening (urogenital), while females have a heart-shaped papilla with two openings (urinary pore and oviduct) (Oliveira and Almada, 1995).

Very few studies have described the behaviour of *Oreochromis mossambicus* in natural or semi-natural conditions. Two examples of these studies were carried out by Brutton and Boltt (1975) on a natural population living in the Lake Sibaya (South Africa) and by Neil (1966) on a stocked population inhabiting Hawaiian ponds. Both have reached very convergent descriptions of the natural behaviour of this species, and Neil (1966) also compared it with behaviour of animals raised in aquaria.

The natural behaviour of the Mozambique Tilapia is characterized by two main life stages which seem to depend essentially on the temperature level: the non-breeding phase (temperatures below 18ºC) and the breeding phase (temperatures above 18-20ºC) (Neil, 1966). In tropical waters, where temperatures remain constant and high, they breed throughout the year. The non-breeding behaviour is related to schooling, generally in deeper waters where the temperature is more stable (Bruton and Boltt, 1975). Very little is know about other type of behaviours performed during this phase, but animals occasionally leave the shoals to feed in shallow waters (Trewavas, 1983).

The breeding behaviour is characterized by the territoriality of the lek-breeding males. At the onset of the warm season, groups of mature males leave the schools and assemble in densely aggregated groups in shallow waters with sparse or moderate vegetation (arenas) (Bruton and Boltt, 1975). In these areas, mature males, compete for the acquisition and defense of their own territories. Neil (1966) reported that fighting among the introduced males in Hawaii was very violent, with serious injury and even death occurring. But although males are highly aggressive during the breeding season, under natural conditions most interactions appear to have evolved to ritualized displays, normally without serious injury resulting (Fryer and Iles, 1972; Bruton and Boltt, 1975). Male territoriality is expressed by aggression, pit digging and hovering over the territory (Neil, 1966; Fryer and Iles, 1972; Bruton and Boltt, 1975).

Male territories are spawning pits which consist of circular depressions on the substrate with variable dimensions (Fryer and Iles, 1972). In the Hawaiian ponds, the spawning pits had an average width of 51 cm on the shallower areas (terrace) (Neil, 1966). They are built by the breeding males through mouthdigging and expulsion of sand portions to adjacent areas, involving a number of behavioural patterns to which males devote a considerable part of their time (Neil, 1966). Ripe females, which leave the school and enter the arenas, are then attracted to these sites through a sequence of male courtship behavioural patterns (Neil, 1966). Nelson (1995) suggested that the sexual selection in this species include not only the inter-male competition but also the females' preference for spawning pits and interacting males of larger size. Once male and female are in the spawning pit, the oviposition starts, without major external interferences, while the male ejects sperm over the eggs (Neil, 1966). Within a matter of seconds, the female takes the eggs and sperm into the mouth, where fertilization and mouthbrooding takes place (Bruton and Boltt, 1975; Trewavas, 1983). The female then leaves the spawning pit and form schools of mouthbrooding females in shallow and protected areas (Neil, 1966; Bruton and Boltt, 1975). Hatching occurs 3-5 days after fertilization (Trewavas, 1983). The fry can leave the mother's mouth temporarily after the first days of incubation. A 'call', consisting of a mother's visual signal (slight snapping movements of the head) and water disturbance, induces the fry to re-enter the mother's mouth for protection (Neil, 1966; Fryer and Iles, 1972). After a period of 20-22 days, the fry are definitely released and left in very shallow and densely vegetated areas, where temperatures can reach 32ºC (Bruton and Boltt, 1975). Then, they form large schools in water 1-15 cm deep during the day, and move to deeper waters during the night. As they grow, they start to move to deeper waters (Bruton and Boltt, 1975).

4.2. Behaviour observed in aquaria

Aquarium-bred and raised *O. mossambicus* have been the target of many behavioural studies. A complete description of their behaviour was done by a number of authors among which Baerends and Baerends-Van Roon (1950) and Neil (1964) are classic examples.

Neil (1966) described behavioural patterns and respective time distribution in aquaria as being essentially the same to that observed in more natural settings. According to him, the non-reproductive behaviour is dominated by shoaling – *'swimming to and fro in midwater'*. Other observed activities in this phase were feeding (including catching prey), nipping at the surface or substrate, yawning and chafing (which are both functionally considered to be comfort behaviours).

In aquaria, during the process of group formation Oliveira & Almada (1998a) defined a regular temporal sequence of activities which consisted of mutual assessment and hierarchy formation, territorial establishment and pit digging and courtship. These authors suggest that this same sequence may be present in the natural lek formation at the onset of the breeding season.

Breeding behaviour involves the building and defense of spawning pits just like in the natural conditions. Territorial aggression includes what Oliveira & Almada (1998a) defined to be symmetrical and asymmetrical interactions, the later involving high or low intensity patterns of aggression. Symmetrical interactions involved retaliation of aggressive acts (e.g. mouthfighting, mutual displays). Asymmetrical interactions are those where the receiver of aggression does not respond. High-intensity asymmetrical interactions involve attack-flee and bite-flee, whereas low-intensity interactions can involve approach-withdrawal. Some behaviour is more typical of territorial intrusions, such as agonistic displays and chasings. In aquaria, the occurrence of more than one territory (spawning pits) built by other males is possible (Trewavas, 1983). Fighting patterns like ‘pendelling’ (head-to-head ritualized fight, where both fish rush against each other without ever colliding) are typical of interterritorial threats arising from the co-existence of more than one territory in the aquarium (Neil, 1966).

In aquaria, depending on its dimensions, it is frequent that the shoal remains swimming over the territories of the breeding males. In this context, males ascend to the shoal and initiate their courtship towards a female. Courtship implies a sequence of behaviours already very well described and including “tilting” (body held downwards at an angle of 10-30° oriented laterally to her), “leading” the female to the nest, “rolling” over (lies on its side) once in the pit and circling around it as soon as the female also enters. As described by Neil (1966), this sequence ‘tilting-leading-roll’ did not occur in the Hawaiian ponds, where the whole courtship sequence was much more rapid than the one reported in aquaria. Furthermore, this author describes constant interferences of other males and the female displacement as a consequence of fights during the courtship and spawning processes in aquaria. When oviposition is completed, after four to seven batches of eggs, the female takes the eggs and sperm into the mouth and reenter the school swimming over the spawning pit, usually chased by the male (Neil, 1966). When there is a cover available, the female leaves the school seven days later and seeks shelter, where fry can temporarily be released before the terminus of mouthbrooding (Baerends and Baerends-Van Roon, 1950).

The Mozambique tilapia is a very visual species like many other cichlid species (Fernald, 1984), using this perceptive channel to communicate within the social group. This is well reflected not only in their behavioural repertoire (e.g. agonistic displays, patterns of courtship) but also in relation to the different colour patterns the body and eyes can assume. For example, beyond the very dark colour of the breeding male, other colour patterns of Mozambique tilapia have been associated to certain internal states and patterns of behaviour. For example the “anxiety pattern” consists of a general darkening of the dorsal half of the body. The arousal or mild aggression patterns is characterised by a darkening of the caudal, anal and pelvic fins and also the ventral side of the fish. The spawning and brooding females also assume specific patterns, with the first having a darker body pattern and the second a striped pattern with black eyes, lower jaw and operculum. Aggressive males also have their body and fins considerably darkened (Lanzing and Bower, 1974).

But this species also uses non-visual communication pathways. Aquarium-bred adult and juveniles can produce sounds of varying frequency (between 1-16 kHz), duration and intensity. These sounds vary with different contexts like feeding, breeding, territoriality or fry school formation (Fryer and Iles, 1972; Lanzing, 1974). For example, Amorim et al (2003) found that only territorial males produced sounds during courtship and pit-digging, which were probably related to the advertising of the spawning readiness and synchronisation of gamete release. Amorim & Almada (2005) also suggest that differences in courtship between winners and losers of recent agonistic interactions can modulate subsequent courtship behaviour including sound production, eventually due to transient physiological changes induced by the encounter outcome (e.g. in circulating monoamines, cortisol and androgens). Lanzing (1974) notes that minor environmental disturbances may cause the fish to fall silent for long periods. Olfaction may also play an important role on the social communication of Mozambique tilapia as some studies have been demonstrating. For example, Barata et al (2007) suggested that males actively advertise their dominant status through urinary odorants which may act as a 'dominance' pheromone to modulate aggression in rivals, thereby contributing to social stability within the lek. Chemical communication also decreases aggression and increases individual recognition in the closely related species, *Oreochromis niloticus* (Giaquinto and Volpato, 1997).

4.3. Relevant features as a behavioural and welfare research model

O. mossambicus is the model species used in the present thesis. There are substantial reasons to consider it as a good model for behavioural and welfare studies. Its biology and behaviour are already very well described by classical and more recent research. As mentioned before, a detailed ethogram of this species is available and can be used as the basis of any behavioural research. Recent studies of its social behaviour offered a very consistent picture of important behavioural and neuroendocrine elements related to patterns of social hierarchies (e.g. Oliveira and Almada, 1996), aggressive behaviour (e.g. Oliveira et al., 2001), mating systems (e.g. Borges et al., 1998), mechanisms of mating choice (e.g. Oliveira et al 2003) and maternal behaviour (e.g. Oliveira and Almada, 1998b). Knowledge on this species' patterns of chemical communication (e.g. Barata et al., 2007) and sound production (e.g. Amorim and Almada, 2005) has also been growing. These and other studies have provided important contributions to the understanding of mental capabilities of *O. mossambicus* (see Table 1, section 2.2.3.). Several physiological and cognitive aspects of stress studies have also been developed in this species and the closely related Nile tilapia (Foo and Lam, 1993; Barreto et al., 2006b).

In welfare studies, it is very challenging and useful to identify particular needs, sources of disturbance and associated coping mechanisms in a species as resilient as the Mozambique tilapia is. Its natural behaviour is not essentially different from that in artificial conditions. Schooling, various aspects of breeding behaviour and other important behaviour patterns are qualitatively similar in both conditions (Neil, 1966). Its sexual dimorphism and variation of body/eyes' colour with contexts and internal states are relevant tools in behavioural observations. Patterns of behaviour and time devoted to specific activities (e.g. courtship, spawning pit building) also provide the sampling of significant behaviour patterns within a reasonable amount of time. This species is very robust and ecologically tolerant. As mentioned above, it is highly tolerant to different salinities, temperature variation and high temperatures. It also shows good resilience to low dissolved oxygen and high ammonia concentrations. Furthermore, Mozambique tilapia is not prone to diseases, having high resistance to most viral, bacterial and parasitic infections (Kohler, 2000). All these features make this species easy to keep in aquaria, to breed and to manipulate under experimental conditions.

Studying the behaviour and welfare of *O. mossambicus* is scientifically relevant as this species is frequently used as a teleost model in various fields of research, such as genetics, neuroendocrinology, stress, toxicology, ecology, and others. This species has also a very relevant economic value as a cultured species, a fact which has led to a relevant body of related research. Actually, this and other tilapias are the third most common species in aquaculture systems around the world, after carps and salmonids (Kohler, 2000; El-Sayed, 2006). They play a specifically important role as a source of animal protein in developing countries. Asian countries like China, Philippines and Indonesia represent 79% of the total production, but tilapia culture is also fast developing in African (mainly Egypt), and South-American countries. The Mozambique tilapia is the second most important farmed tilapia species in the world (3,6%), after the

Nile tilapia, which accounts for 80% of the total production (El-Sayed, 2006). In some countries, like the United States of America, most aquaculture systems involve hybrid forms of tilapia (e.g. red tilapia: *O. mossambicus* x *O. niloticus*). Tilapias are presently considered the “new white fish” and viewed as the natural replacement of the depleted ocean stocks of cod, among other species (Kohler, 2000). For this and other reasons, its importance as a food source is increasing with consequent increases in production (El-Sayed, 2006). Tilapias are grown in a variety of systems, ranging from extensive to very intensive ones. Different husbandry procedures, feeding regimes and social groups are provided in the variety of the existent culturing systems (El-Sayed, 2006). These may pose welfare problems that are still largely unknown. For example, producing exclusively all-male groups is attractive from the production perspective as it avoids the losses of slower-growing females, but under uncontrolled conditions, it has potential associated welfare problems which so far were not addressed.

Therefore, given the good scientific knowledge of Mozambique tilapia, its resilience in artificial conditions and its growing importance as a cultured fish, using it as a research model in welfare studies is not only scientifically appropriate but also potentially very useful for future applications in aquaculture.

5. Aims of this thesis

5.1. Identifying behavioural and physiological welfare indicators

In response to an unfavourable environment, animals activate coping mechanisms in an attempt to keep their allostasis. Behavioural and physiological responses are accessible aspects of these mechanisms and can provide indirect information of internal states. With this objective I aimed to identify behavioural and physiological indicators that can reflect particular needs of *Oreochromis mossambicus* and give information about this species welfare under specific conditions.

5.2. Identifying psychological modulators of the stress response

The perception and cognitive evaluation of a stressor can be more relevant for the modulation of the stress response than the stressor’s nature itself. In fish this is to a large extent an unexplored field of research, despite the existence of indirect evidence that fish can appraise stimuli. With this objective I aimed to identify how social support and predictability change the appraisal of stressors in *Oreochromis mossambicus*.

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CHAPTER II

The effect of substrate availability on behavioural and physiological indicators of welfare in a cichlid fish

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

Abstract

Male African cichlids (*Oreochromis mossambicus*) establish territories on the substrate upon which spawning pits are dug, thus attracting females. The substrate, therefore, plays a very significant role in its lifecycle. The effects of substrate access on behaviour and physiology in captivity were assessed. Mixed-sex, all-male and all-female groups were observed for five days, with and without substrate. Social patterns, behaviour directed towards the substrate, locomotor activity and spatial behaviour were recorded, and haematocrit, plasma cortisol and glucose levels were measured. Substrate inclusion saw a significant increase in behavioural diversity, sexual behaviour of dominant males in mixed groups, pit digging and territoriality whereas a lack of substrate was characterised by increased chafing and inactivity. Vacuum-pit digging was also observed. Frequency of aggression did not differ significantly and female behaviour was not affected by the presence of substrate. For both sexes, no differences in cortisol and glucose levels were found between the two treatments, but haematocrit increased with substrate. The key role played by substrate in territorial males is consistent with the behavioural and physiological data reported. In the absence of substrate, decreased territorial behaviour is contrasted with similar levels of aggression, cortisol and glucose; all of which are suggestive of a stress-related context. Moreover, the exhibition of vacuum activities is a signal that behavioural needs are not being met and may be some form of coping mechanism. These findings, taken in conjunction with the variations in behavioural diversity and inactivity, suggest that the welfare of male cichlids may be adversely affected by the absence of substrate.

1. Introduction

The concept of animal welfare remains hugely relevant to all human activities involving live animals, and shapes the manner in which animals are kept and treated in captivity. Housing and husbandry standards as well as legislative requirements have been established in a number of different areas. Although all vertebrates are included, a significant portion of the acquired knowledge is concerned primarily with studies focused on mammals and birds. However, since fishes constitute a very important resource - not only in aquaculture, but also in public aquaria, in research and as pets - fish welfare has emerged as an area of growing interest.

There have been three basic approaches addressing the concept of welfare and its subsequent measurement. One of these is the comparison between natural behaviour and the behaviour expressed under artificial settings (Duncan and Fraser, 1997). Excluding fear responses, it assumes that natural behaviour is positively motivated and promotes biological functioning, as it is the best way of portraying the preferences and evolutionary capabilities of a species to adapt to a given environment (Bracke and Hopster, 2006). However, caution must be exercised in interpreting the value of natural behaviour in terms of the individual's welfare. Certain context-dependent behavioural patterns may disappear in artificial environments without negative consequences for welfare, but the restriction or prevention of those which exclusively derive from an internal motivation (behavioural needs) may impact negatively on welfare (Dawkins, 1990). Furthermore, as Barnard and Hurst (1996) have pointed out, some behavioural patterns that may be interpreted at first sight as an indication of poor welfare may be linked inextricably to vital aspects of the species' evolutionary history and should, thus, not be considered to impairing welfare. A second approach to the concept of welfare relates to body functioning, where health indicators (e.g. mortality, reproductive success) and physiological parameters (e.g. corticosteroids, blood chemistry) assume great significance as welfare indicators (Broom and Johnson, 1993). Although extremely useful in providing information regarding the manner in which the body functions in certain circumstances, this approach should not be used to the detriment of behavioural observations and a detailed evaluation of the animal's context (Mason and Mendl, 1993). For example, glucocorticoids are produced in order to prepare the body to react to a great variety of stressors. However, their generalised use as stress indicators may be limited by a number of factors such as circadian rhythms, age, physiological status, hierarchical position in social groups, etc (Lane, 2006). A third approach to a definition and evaluation of welfare is the existence of subjective mental experiences, where attention is paid to how the animal perceives the situation it finds itself in (Dawkins, 1980). To some authors this is encapsulates what welfare is all about (Dawkins, 1990; Duncan and Fraser, 1997). Unavoidably indirect, the indicators of such mental experiences rely greatly on behaviour. Measurement of preferences and motivation has been the most popular approach in the recent years. The exhibition of certain behaviours indicating frustration or conflict have also been used as possible indicators of impaired welfare (Mench and Mason, 1997). At present, sophisticated strategies which expand upon the cognitive abilities of animals have been used to address the issue of subjective experiences on animals (Mendl and Paul, 2004).

In previous decades, the vast majority of studies conducted on fish relied on health and productivity indicators in aquaculture systems and on studies of stress. Although the existence of subjective experiences in fishes as been a matter of deep controversy (Rose, 2002; Sneddon, 2003; Sneddon et al., 2003), there is a considerable body of evidence regarding conscious subjective experiences (Braithwaite and Huntingford, 2004; Chandroo et al., 2004). Fish welfare remains a relatively recent field of research and a number of blindspots in current understanding exist (for a review, see Huntingford *et al* (2006)). Among the areas considered to warrant more attention are a better understanding of fishes' behavioural needs and an improved array of welfare indicators.

In the present study, the African cichlid Mozambique tilapia (*Oreochromis mossambicus*) was used as a model as it is widely used in both aquaculture and research, it is robust, easy-to-keep and easy to breed in captivity and its biology and behaviour are well known (Baerends and Baerends-Van Roon, 1950; Neil, 1966; Fryer and Iles, 1972; Trewavas, 1983).

In this species, during the breeding season, males aggregate in shallow waters forming arenas. Here, individual territories are defined through the building of spawning pits (nests) on the substrate, to which ripe females will be attracted for spawning (Neil, 1966; Nelson, 1995). The attainment of specific territory and dominance position are achieved through aggression, and short bouts of combats are reported (Turner, 1994; Oliveira and Almada, 1996). However, according to Fryer and Iles (1972), agonistic encounters during maintenance of territories have evolved into more threatening displays, which are less deleterious. It is apparent, therefore, that the substrate plays a key role in reproduction and in the regulation of social interaction.

It generally felt that in captivity the presence of a substrate is also a highly relevant environmental feature in modulating social interaction, as the behavioural repertoire is essentially the same (Baerends and Baerends-Van Roon, 1950; Pinheiro, 1980). A number of authors only report some qualitative differences in behaviour. Aggression appears more prevalent because the school spends more time swimming on the dominant animals nesting areas and individuals remain in permanent contact with each other (Barlow, 1974; Munro and Pitcher, 1985). The period of time between the onset of courtship and effective spawning may be longer, with males exhibiting a particular sequence of courtship behaviours (tilt-lead-roll) more frequently in captivity than in the wild (Neil, 1966).

In some artificial systems, this species, like many other cichlids for which the substrate plays a similar role, is kept in tanks without substrate. The lack of consideration for species-specific needs has adversely affected the welfare of many animals in captivity. It is this concern which as led to the development of environmental enrichment strategies with the primary objective of increasing physical and mental well-being through the promotion of more opportunities for species-typical behaviour (Kreger et al., 1998).

The aim of the present study, therefore, is to discuss the importance of substrate to the Mozambique tilapia through measurement of behavioural and physiological parameters, recorded in relation to substrate availability and, in doing so, contribute to the current paucity of information on this subject.

2. Materials and Methods

2.1. Animals and Housing

Ninety-two adult Mozambique tilapias were used in this study (46 females and 46 males). The experimental fish were part of a stock held at ISPA, in Lisbon (Portugal). The stock was maintained in glass aquaria (120 x 40 x 50 cm, 240 l), at a temperature of 26ºC±2ºC and on a 12L:12D photoperiod. Each tank had a layer of fine gravel substrate, a double-filtering system (sand and external biofilter, Eheim) and a constant air flow into the water. The water quality was analysed weekly for nitrites (0.2-0.5 ppm), ammonia (<0.5 ppm) (Pallintest kit®) and pH (6.0-6.2). All individuals are identified by means of a transponder (Trovan ID 100 – 2,2x11,5 mm) and/or a combination of three colour beads attached underneath of the dorsal fin, by a nylon line. Fish were fed daily *ad libitum* with commercial cichlid sticks (ASTRA). The experimental conditions in testing aquaria (100x40x50 cm, 200 l) and isolation aquaria (50x25x31 cm, 40 l) matched those described for stock conditions.

2.2. Experimental Procedures

Animals were grouped into three different sex ratio groups composed by four individuals: all males (MM), all females (FF) and half males/half females (MF). Each replicate involved one of each of these three groups, in a total of eight replicates. One MF group was removed from the experiment due to the death of one male during the course of the experiment. Variation in body size within each group was kept as uniform as possible. (standard length coefficients of variation: 0.06±0.02 % for FF, 0.06±0.03 % for females of MF, 0.05±0.02 % for males of MF, 0.07±0.02 % for MM).

For each replicate, the three described groups were subjected to two conditions in testing aquaria: (a) without substrate and (b) with a seven cm layer of sand as a substrate. Prior to experimentation, individuals spent seven days in social isolation, to minimise possible effects of previous social experience and each condition lasted for a period of five days. (Oliveira and Almada, 1998a). Each replicate took 24 days to be finalised and to protect against any bias, the presentation of the two conditions was balanced among the eight groups (Figure 1).

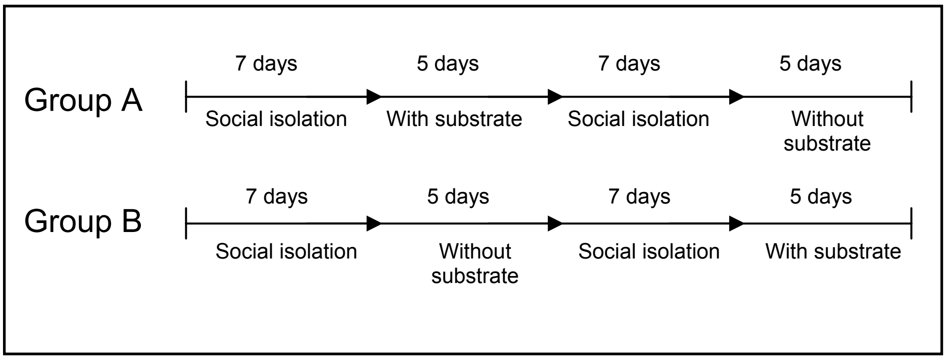

Figure 1. Schematic representation of the experimental design. Groups were run in pairs (A and B) in order to balance potential order effects of the access to the substrate.

2.3. Behavioural Sampling

Behavioural sampling was carried out, twice daily (11.30h and 15.30h) during five days of each condition, in accordance with the following protocol (Martin and Bateson, 1993):

a) Behaviour Continuous Sampling – a sampling period of 10 minutes for social interaction and behaviour associated to the substrate;
b) Scanning Instantaneous Sampling – at intervals of 30 seconds, the position of the four individuals on a grid (with six squares marked on the aquaria glass) was recorded for a total period of 10 minutes.
c) Behaviour Continuous Sampling – a period of five minutes only for behaviours associated to the substrate.
d) Focal Continuous Sampling – a period of 30 seconds per individual for locomotor activity (number of times the fish head crossed the lines of the mentioned grid).

Therefore, the daily total sampling effort was 58 minutes per aquaria. The behaviour patterns identified and recorded in this study have already been used in a number of studies (Oliveira and Almada, 1998a) and were originally described by Baerends and Baerends van Roon (1950) and Neil (1966). For analysis the behavioural patterns were grouped into behavioural categories (Table 1).

2.4. Social Status

Since territoriality and courtship behaviour are shaped by dominance relationship in this species (Fryer and Iles, 1972; Oliveira and Almada, 1998a), the determination of social status was judged the best way to identify where possible differences between conditions (with and without substrate) exist. In order to estimate the social status of each individual the method validated by Almada and Oliveira (1997) was followed and adapted to the present data. Using a simulation programme [ACTUS (Estabrook and Estabrook, 1989)], the analysis of 4x2 contingency tables (individuals x number of performed agonistic behaviours/number of received agonistic behaviours) allowed individuals to be classified into three different social status: (a) dominants, when the number of aggressive acts was larger than that expected by chance ($P<0.05$); (b) subordinates, when the number of received aggressive acts was larger than that expected by chance ($P<0.05$); (c) intermediates, when the number of performed/received aggressive acts did not differ significantly from the simulated one ($P > 0.05$). In order to be able to be included in the statistical analysis, an Overall Dominance score (OD) was defined being: (a) OD=0, when in both conditions males were never dominant; (b) OD=1, when males were dominant only in one condition, (c) OD=2, when males were dominant in both conditions. According to this, in the all-male groups (MM) there were seven totally dominant males (2), two partially dominant males (1) and 23 males that were never dominant (0). In the mixed groups (MF), there were

Table 1. Brief description of the behaviour patterns and respective categories.

Categories of behaviour[1]	Behavioural pattern[2]	Description
Asymmetric low-intensity agonistic interactions (ALI)	Frontal display	Facing opponent, fish erects gill covers and branchiostegal membrane, usually with mouth opened.
	Lateral display	In parallel/antiparallel position to opponent, fish fully erects dorsal and anal fins, with spread caudal and pelvics, and eventually branchiostegal membrane erected.
	Tail beating	In lateral display and with vigorous body undulations, fish beats tail sideways.
	Approaching	Fish swims towards the opponent to a distance inferior to its body length, eventually in frontal display; it can or cannot touch and/or attempt to bite.
	Biting attempt	Fish approaches opponent with mouth opened and, just before touching its body, swims backwards.
Asymmetric high-intensity agonistic interactions (AHI)	Attacking	Fish swims rapid and suddenly towards the opponent. It can or cannot be followed by touching or biting.
	Chasing	Usually after approaching or attacking, fish pursues opponent in a very rapid swimming
	Circling	Fish swims around opponent describing circles; it may be followed by more escalated forms of aggression.
	Touching	Fish induces physical contact with opponents' body (head, flanks, fins).
	Biting	Fish approaches or attacks opponent with mouth opened and rams it in any part of the body, but especially in the flanks and near the head.
Symmetrical agonistic interactions (SIM)	Carouseling	In lateral display, opponents circle each other.
	Mouth fighting	Mutual attempt to grip the opponents' jaws; once firmly seized, opponents pull and push with tail beats.
	Pendelling	Two nest holder males, facing each other's heads, rush at each other with dorsal and anal fin closed against the body, suddenly breaking just before contact.
Sexual interactions (SEX)	Tilting	The fish holds body at an angle of about 30º with the horizontal, with the unpaired fins against the body.
	Leading	While tilting, the male swims in front of the female towards the spawning pit.
	Circling	With the female close to the pit, the male circles around it; when the female joins the male, they circle the pit with the male behind.
	Quivering	The male vibrates the body and presumably ejects sperm.
Behaviours associated to the substrate	Pit Digging (DIG)	In vertical position or at an angle of 45º, with mouth opened male digs a depression on the substrate. Two main movements are included: with head downwards, mouth is pushed against substrate, filled with particles, which are ejected in the pit periphery and, in deeper pits, fish may swim up the slope pushing the substrate to the periphery.
	Territoriality (TER)	Male hovers near the bottom or remains immobile in touch with the substrate above the spawning pit.
	Inactivity (INA)	Fish remains immobile in touch with the substrate or/and hovering in non-territorial areas.
	Nipping substrate (NIP)	With head downwards, body at an angle of 45º, and fins half-erected, fish thoroughly nips out substrate; sand and particles may be carried to some distance before being expelled, while fish describes an apparent wandering route.
	Chafing (CHA)	Fish shoots downwards to the bottom, lays itself on the flank, and chafes over the substrate, and then it rises again and resumes the previous position.
	Moving in the bottom (MOV)	Fish swims just above the substrate, very low in the water column.

[1] ALI includes all non-reciprocal display patterns of aggression; AHI includes all non-reciprocal overt patterns of aggression; SIM includes all reciprocal patterns of aggression.

[2] Adapted from Baerands & Baerands-Van Roon 1950; Oliveira & Almada 1998a.

five totally dominant males (2), two partially dominant males (1) and seven males that were never dominant (0).

Female social status was not considered, as hierarchic. Although there is a reference to a nip-order hierarchy in females of the blue acara cichlid *Aequidens pulcher* (Munro and Pitcher, 1985), and the occurrence of complex agonistic behaviour (e.g. displays) among females in aquaria, in nature they tend to spend their time shoaling with the exception of when they depart the schools to visit the males' arenas and incubate.

The aggressive encounters that occurred during this study did not lead to any injuries or fatalities in the males involved. Furthermore, the subordinates' behaviour did not appear to demonstrate any signs of major behavioural restriction (e.g. movements' restriction, excessive submissive postures, feeding inhibition, etc.), in which case the procedure for the prospective group would have been discontinued.

2.5. Blood sampling and assays of physiological parameters

In order to avoid circadian effects, sampling always took place at the same time (13.00-14.00h). Only one time point was chosen in order to avoid cumulative anaesthesia and handling stress which would interfere with the results. At the end of each treatment phase (day five), fish were removed individually from the aquarium and lightly anaesthetised [Stage two (Ross, 2001)] in a solution of MS-222 (tricaine methane sulphonate, Sigma; 200 ppm). Samples of 100-200 µl of blood were taken from the caudal vein (1 ml syringes; 25G/16 mm needles) and body length (total and standard) and weight were measured. The fish were then placed in aerated water, and took between 30 seconds to one minute to recover from the anaesthesia. Induction from the anaesthesia and blood sampling took no longer than minutes, which is the latency for cortisol release into the systemic circulation in response to handling stress (Foo and Lam, 1993). The sequence in which fish were removed from the tanks, within each group, did not affect the cortisol level (2 Way-ANOVA, F3,82 = 0.71, *P*>0.05).

For each blood sample, two heparinized capillary tubes were used for the haematocrit. These tubes were then centrifuged (3.5 minutes, 16000 rpm) and the haematocrit read in accordance with Morgan and Iwama (1997). Remaining blood was centrifuged for 10 minutes at 3500 rpm to isolate the plasma, which was then stored at – 20ºC until assayed for cortisol and glucose.

The free cortisol fraction was extracted from the plasma through addition of diethyl ether as the steroid solvent. The samples were then centrifuged (5 minutes, 1000 rpm, 4ºC) and frozen (10 minutes, -80ºC) to separate the ether fraction, which remained liquid. The steroids were then isolated through evaporation of the ether. This process was repeated twice. Levels of free cortisol fraction were then determined via radioimmunoassay, using the commercial antibody 'Anti-rabbit, Cortisol-3' [ref: 20-CR50, Brand Interchim (Fitzgerald), Montluçon, França, cross-reactivity: cortisol 100%, Prednisolone 36%, 11-Desoxycortisol 5.7%, Corticosterone 3.3%, Cortisone < 0.7%]. Intra- and inter-assay variability is 6.4% and 4.2%, respectively. Plasma glucose was measured by the enzymatic method with glucose oxidase in accordance with the Randox glucose assay kit protocol (Randox GL 2623, Randox Laboratories, Antrim, UK).

2.6. Data Analysis

Statistical analysis was conducted in order to assess the effect of substrate on each behavioural and physiological parameters, using multiple repeated measures analyses of variance for males (repeated factor: substrate; independent factors: type of group, overall social status) and two-way repeated measures analysis of variance for females (repeated factor: substrate; independent factor: type of group). When significant differences in the variances between the two conditions were found (Levene's Test), data were normalised using the transformations proposed by Zar (1984), namely logarithmic transformation for continuous variables (cortisol), Poisson transformation for frequencies (behavioural patterns) and *arcsin* transformation for percentages (haematocrit, position in the water column). In the event of data not meeting the parametric assumptions, ANOVAs were still undertaken due to the lack of equivalent non-parametric tests and because the *F*-statistic is remarkably robust to deviations of normality and heterogeneity of variances (Lindman, 1974). Following the ANOVAs, planned comparisons of least squares means were performed between the two conditions (with and without substrate).

An index of behavioural diversity was computed through the adaptation of the ecological Shannon diversity index (Zar, 1984; Galhardo et al., 1996; Wemelsfelder et al., 2000). Absolute diversity (H) provides a measure of diversity within a given sequence of behaviour. It is represented by the equation $H = -\sum_{i=1}^{S} Pi * \ln Pi$, where S is the number of behaviours in the sequence and Pi, the proportion of each behaviour. Relative diversity (R) is given by dividing H per H_{max} (maximum absolute diversity possible within a given condition, i.e. all possible behaviours each of them occurring in equal proportions). Therefore, relative diversity means the diversity of behaviour in a given environment (H) as a function of the behavioural options available in that environment (H_{max}) (Wemelsfelder et al., 2000). To compare the differences between the diversity of behaviour in each of the two conditions the Wilcoxon Matched Pairs Test was used. Pearson correlations were used to compare social status in both conditions presented (with and without substrate) and also to establish the degree of association between the variation of glucose and cortisol in the tested fish.

A value of $P<0.05$ was taken for significance in all statistical tests. The statistical package used for analysis was Statistica V.7.1® (StatSoft Inc, USA, 2205).

3. Results

3.1. Social Status

Variation of male social status was independent of the order of substrate presentation (ACTUS: $\chi^2 = 3.36$, df = 2, $P>0.05$). However, a tendency towards a higher social status consistency was noted when substrate was available as the first condition. When an absence of substrate was the first condition tested (half of the groups), 58% of males (13 out of 22) maintained the same social status (four of which were dominant), four gained it (two became dominant) and five lost social status (two lost dominance). Social status was correlated between both conditions ($r_s = 0.6$, n = 22, $P<0.01$). However, when presence of a substrate was the first tested condition, more males maintained social status (83%, 20 out of 24) and only four changed it (two gained and two lost). Eight dominant males remained without any change between conditions and, here, social status showed greater correlation between both conditions ($r_s = 0.7$, n = 24, $P<0.001$).

3.2. Social Behaviour

Having access to substrate did not influence the frequency of agonistic interactions in male groups, with exception of the intermediate social status (n=2) in the MM group (Figure 2; Table 2). However, an effect was noted on the asymmetric aggression (low and high intensity) in relation to the social status, since these patterns of behaviour were more frequent in the overall dominant males (Figure 2; Table 2). Agonistic behaviour did not change with substrate in females, but it was more expressive in the FF groups than in the MF ones (Figure 3; Table 3).

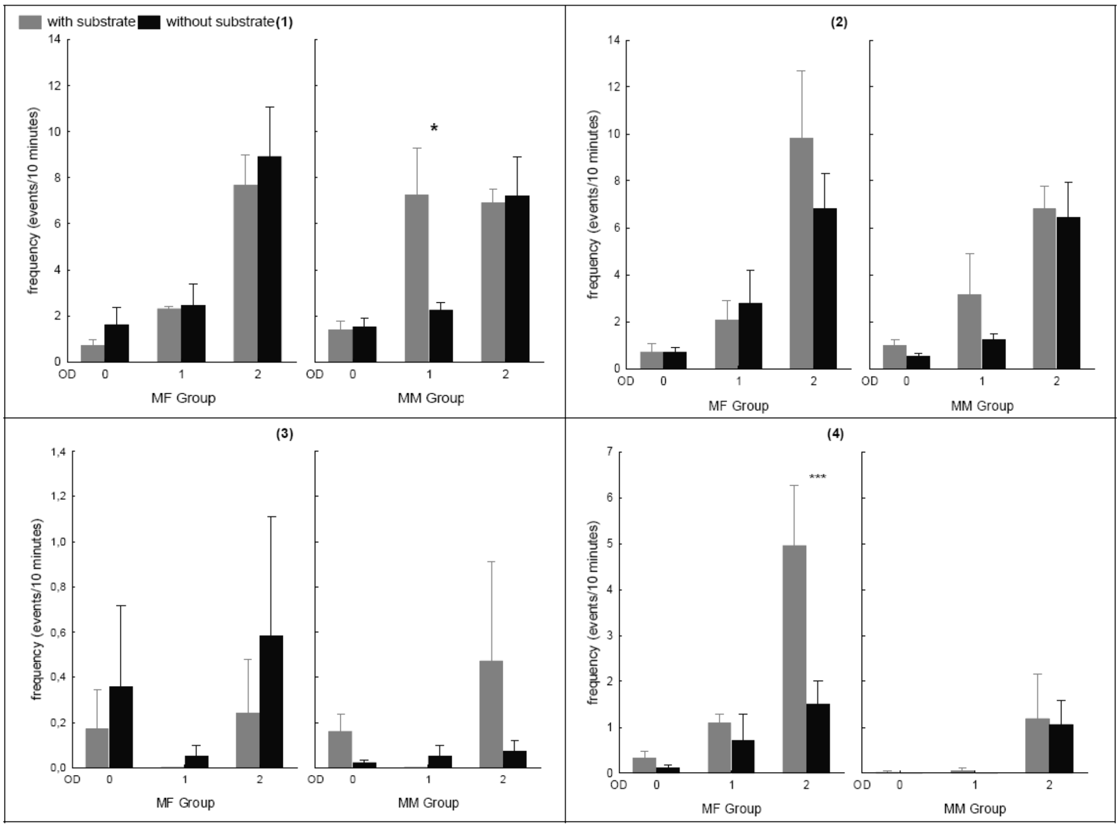

Figure 2. Males social behaviour (mean±SE). (1) Asymmetrical low-intensity agonistic interactions (2) Asymmetrical high-intensity agonistic interactions (3) Symmetrical agonistic interactions (4) Sexual behaviour. OD – Overall Dominance; 0 – Never dominant 1 – Dominant in one condition 2 – Always dominant. Levels of significance: *P*<0.05 (*); *P*<0.01 (**); *P*<0.001 (***).

Regarding sexual behaviour, a clear effect of the substrate was noted as was the influence of type of group and social status among males. Frequency of sexual behaviour was higher in the dominant males of MF groups in the presence of substrate (Figure 2; Table 2).

As Figure 2 shows, sexual behaviour was also observed among males in the male groups. In an FF group, *ad libitum* observations showed two females building a nest, courting each other and spawning and, one of them, incubating unfertilised eggs which where eaten later. In mixed groups, no intra-sex courtship was observed.

Table 2. MANOVA repeated measures results for male behavioural and physiological parameters.

Variables	G $F_{1,40}$	OD $F_{2,40}$	S $F_{1,40}$	GxOD $F_{2,40}$	SxG $F_{1,40}$	SxOD $F_{2,40}$	SxGxOD $F_{2,40}$
ALI	0.40, ns	46.45, $P < 0.001$	0.37, ns	1.60, ns	3.39, ns	1.83, ns	0.92, ns
AHI	0.59, ns	80.89, $P < 0.001$	2.28, ns	0.54, ns	0.76, ns	0.61, ns	1.91, ns
SIM	0.30, ns	0.77, ns	0.01, ns	0.05, ns	1.83, ns	0.05, ns	0.56, ns
SEX	13.24, $P < 0.001$	27.87, $P < 0.001$	9.33, $P < 0.01$	5.16, $P < 0.05$	8.44, $P < 0.01$	6.53, $P < 0.01$	7.08, $P < 0.01$
DIG	0.20, ns	16.94, $P < 0.001$	71.99, $P < 0.001$	1.39, ns	0.11, ns	22.70, $P < 0.001$	7.14, $P < 0.01$
TER	4.56, $P < 0.05$	21.69, $P < 0.001$	32.39, $P < 0.001$	0.58, ns	5.89, $P < 0.05$	5.87, $P < 0.01$	1.68, ns
CHA	2.53, ns	5.22, $P < 0.05$	9.11, $P < 0.01$	0.84, ns	0.95, ns	1.69, ns	0.21, ns
INA	2.00, ns	0.88, ns	7.34, $P < 0.01$	0.01, ns	1.90, ns	1.51, ns	0.15, ns
COR	0.85, ns	0.71, ns	0.01, ns	0.07, ns	0.03, ns	0.12, ns	0.16, ns
GLU	0.00, ns	0.97, ns	0.67, ns	0.19, ns	0.17, ns	0.05, ns	0.63, ns
HMT	0.00, ns	0.27, ns	27.82, $P < 0.001$	0.04, ns	0.04, ns	0.06, ns	0.07, ns

S – Substrate (with; without); G – Group (MM; MF); OD – Overall Dominance (0; 1; 2). ALI - asymmetric low-intensity aggression; AHI – asymmetric high-intensity aggression; SIM – symmetrical aggression; SEX – sexual interactions; DIG – Pit digging; TER – Territoriality;; CHA – Chafing; INA – Inactivity; COR - Cortisol; GLU – Glucose; HMT – Haematocrit.

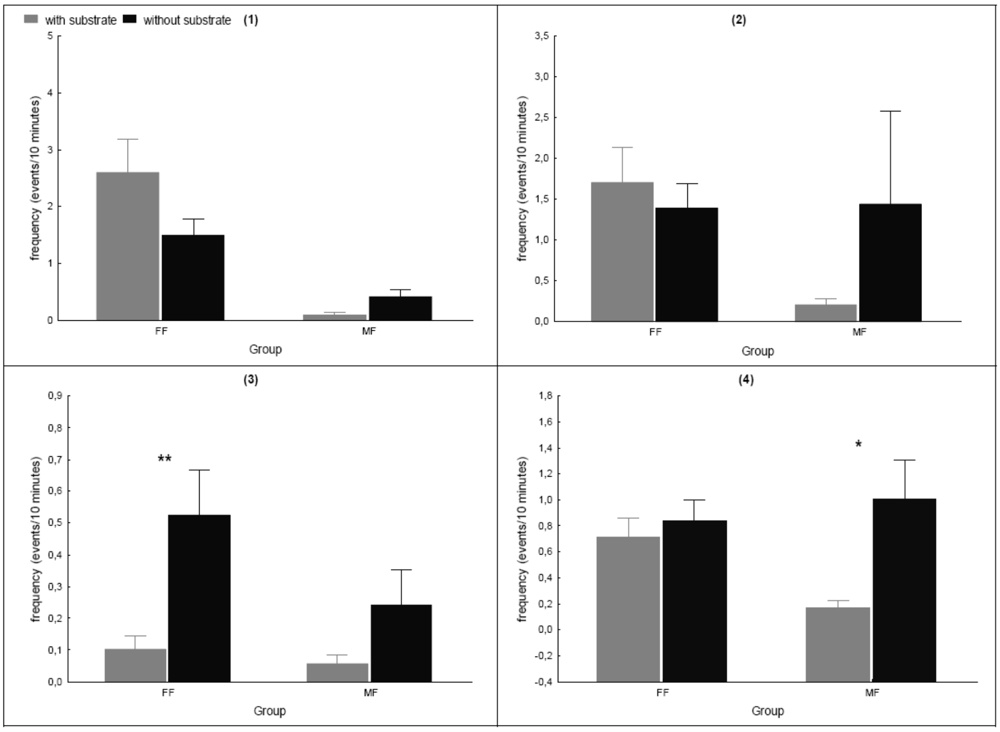

Figure 3. Females Behaviour (mean ± SE). (1) Asymmetrical low-intensity agonistic interactions (2) Asymmetrical high-intensity agonistic interactions (3) Chafing (4) Inactivity. Levels of significance: $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***).

Table 3. 2-Way ANOVA repeated measures results for female behavioural and physiological parameters.

Variables	G $F_{1,44}$	S $F_{1,44}$	SxG $F_{1,44}$
ALI	13.24, $P < 0.001$	0.08, ns	3.25, ns
AHI	4.80, $P < 0.05$	0.17, ns	1.73, ns
CHA	1.61, ns	6.30, $P < 0.05$	0.59, ns
INA	1.09, ns	7.54, $P < 0.01$	3.92, ns
COR	0.97, ns	3.53, ns	1.76, ns
GLU	0.00, ns	1.13, ns	0.03, ns
HMT	0.02, ns	56.04, $P < 0.001$	0.36, ns

3.3. Behaviours associated to the substrate

Pit-digging behaviour was more frequent in males with elevated social status (dominants once or twice in the two conditions), with access to the substrate. This behaviour was performed equally in MM and MF groups. Territorial behaviour varied in a similar manner, apart from the fact that a more marked difference was seen in the MF groups (Figure 4; Table 2). Nesting and territorial behaviour was also observed in the absence of substrate (see Figure 5, for pit digging). For this reason, the frequency of territorial behaviour without substrate by the dominant males of MM groups did not differ significantly from the “with substrate” condition. Both patterns of behaviour were conducted by five dominant males (total number of dominant males = 12) and their frequency is also represented in Figure 4.

For both sexes, chafing was significantly higher in the absence of substrate. Among males, this difference was particularly relevant in the dominants of MM groups (Figure 4; Table 2). Females exhibited more chafing in the FF groups (Table 3).

An increased frequency in patterns of inactivity was observed in the absence of substrate, in both sexes of mixed groups (males: Figure 4, Table 2; females: Figure 3, Table 3).

No relevant substrate-dependent differences were found in locomotory patterns in either males or females.

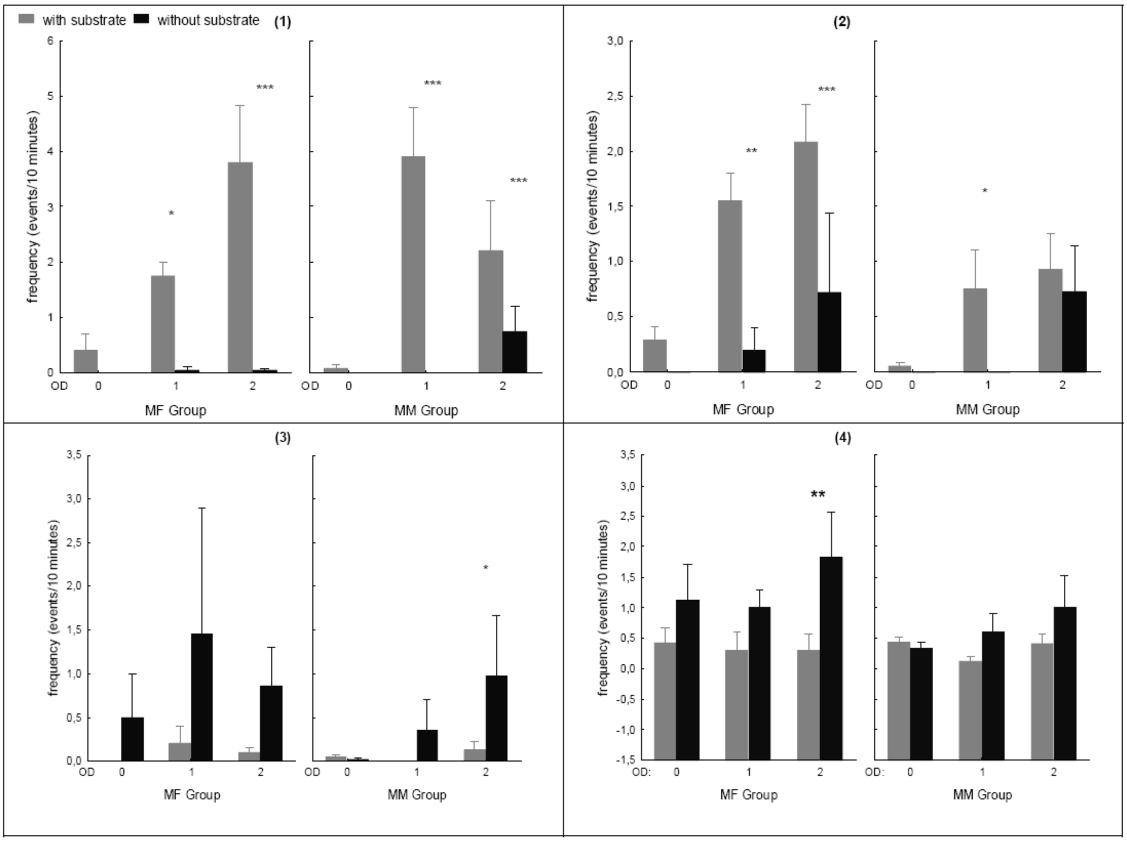

Figure 4. Males Behaviour Associated to the Substrate (mean ± SE). (1) Pit digging (2) Territorial behaviour (3) Chaffing (4) Inactivity OD – Overall Dominance; 0 – Never dominant 1 – Dominant in one condition 2 – Always dominant. Levels of significance: *P* < 0.05 (*); *P* < 0.01 (**); *P* < 0.001 (***).

3.4. Behavioural Diversity

Substrate availability appears to increase behavioural diversity in males, but this difference is significant only in the MM groups (mixed groups: Wilcoxon Matched Pairs Test, Z = 1.66, *P* = 0.09; MM groups, Z = 2.25, *P*=0.02) (Figure 5). Among the females, substrate does not appear to influence behavioural diversity, but in the FF group there is a tendency for greater diversity without substrate (Wilcoxon Matched Pairs Test, FF: Z = 1.78, *P*=0.08) (Figure 5).

3.5. Cortisol and glucose levels and haematocrit

Access to substrate did not influence cortisol or glucose levels of either males or females (Figure 6; Tables 2, 3). However, the latter, had a tendency to show higher cortisol levels in MF groups that had substrate (Figure 6). Cortisol and glucose levels were correlated with each other in males (r = 0.23, n = 88, P<0.05), but not in females (r = 0.14, n = 82, *P*>0.05). The haematocrit values were higher with substrate for both males and females.

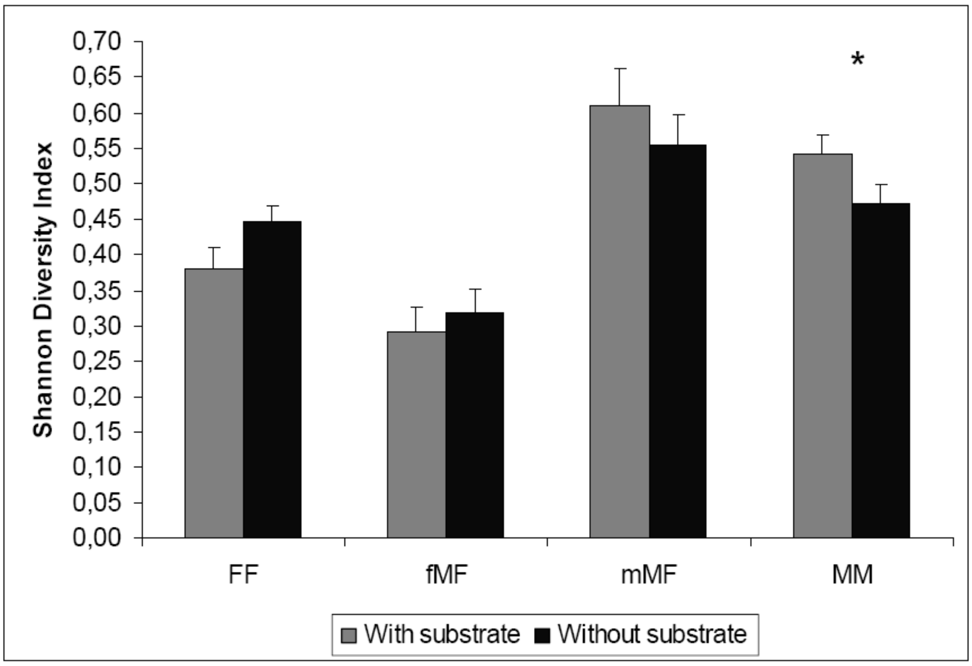

Figure 5. Behavioural Diversity (mean+SE). Level of significance: *P*<0.05 (*), Wilcoxon matched pairs test.

4. Discussion

Male social status was correlated in both conditions, especially when presence of substrate was the first condition presented. Substrate availability did not influence the agonistic interactions of both sexes but increased sexual behaviour of dominant males in MF groups. Similarly, pit digging and territorial behaviour were more frequent with substrate, although this also occurred without this resource. Chafing and inactivity were more frequent in the absence of substrate in both sexes. Behavioural diversity decreased in males, but not in females. Physiological data show no differences in cortisol and glucose plasma concentration between the two conditions, but haematocrit clearly increased with substrate.

4.1. Behavioural parameters

The majority of the males assumed the same social status regardless of substrate availability. However, this relationship was clearer in the groups where substrate was presented first. In these instances, 83% of males retained its previous social status,

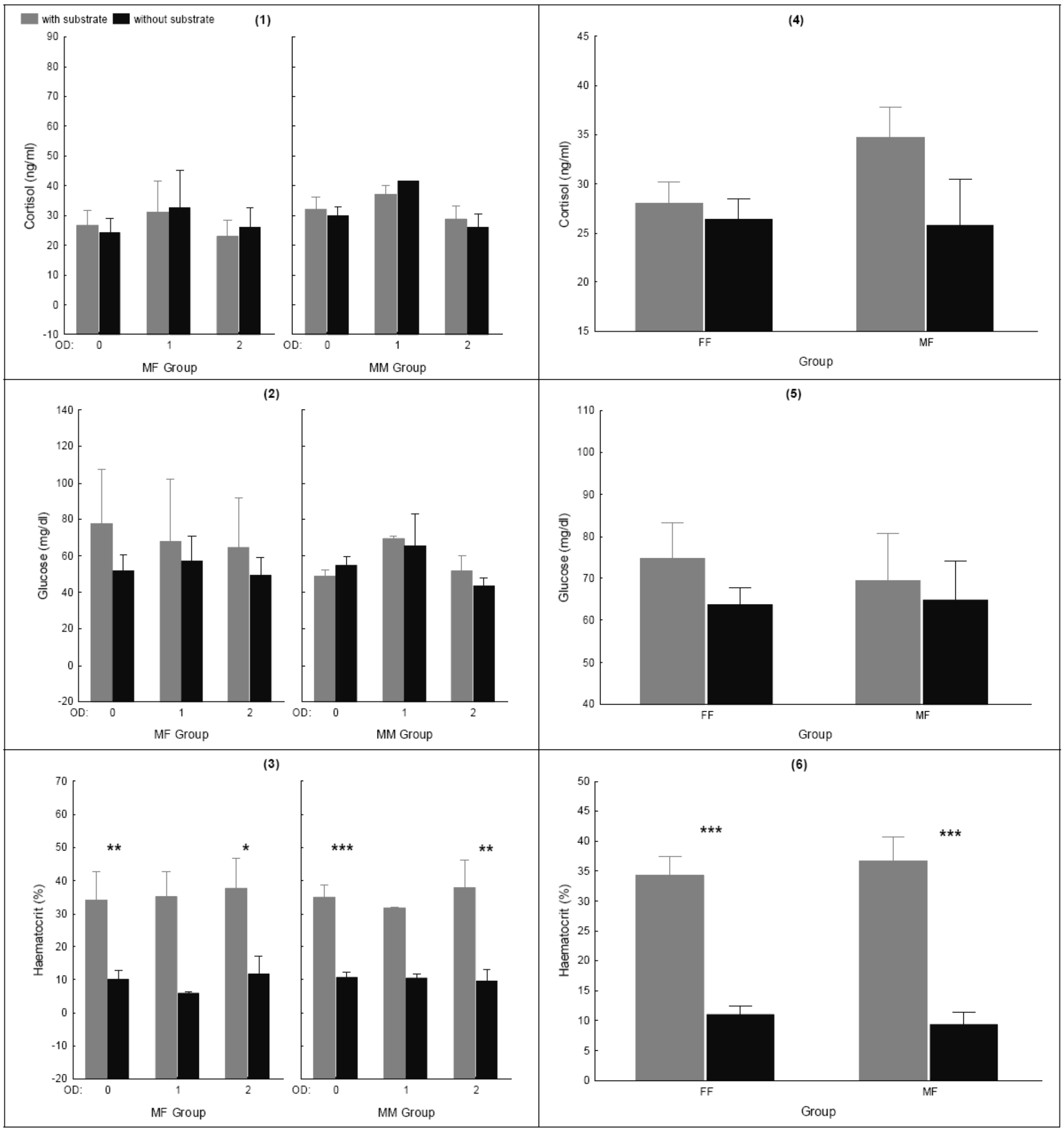

Figure 7. Physiological Parameters (mean ± SE). (1) Males cortisol (2) Females cortisol (3) Males glucose (4) Females glucose (5) Males haematocrit (6) Females haematocrit. OD – Overall Dominance; 0 – Never dominant 1 – Dominant in one condition 2 – Always dominant. Levels of significance: *P*<0.05 (*); *P*<0.01 (**); *P*<0.001 (***).

and all dominants kept their status, even after a period of seven days of social isolation. Since defining social status is important in the Mozambique tilapia in order to gain access to and defend territories in the substrate (Fryer and Iles, 1972), this result suggests this resource has the potential to strengthen dominance-subordinant relationships.

Sexual behaviour increased in dominant males of MF groups in the presence of a substrate, which suggests that the substrate may be an environmental facilitator for the exhibition of such behaviour. Dominant males in MM groups also exhibited sexual behaviour but this was not dependent on substrate. Oliveira and Almada (1998c) suggested that courtship among males may be associated with a low partner selectiveness in the early stages of the courtship process and that, for the courted males, this may reduce aggression.

Symmetric aggressive interactions (fights) have been associated with the initial group formation and the establishment of social status (Oliveira and Almada, 1998a) and substrate availability did not affect their frequency. Asymmetric aggression of low intensity (displays) was higher in the intermediate social status of males in the MM groups. This could suggest that, for individuals such as these, tank minus substrate would be more beneficial. However, as the attainment of a social position is very important for reproductive success, aggressive encounters (especially displays, such as these) are part of their full species-specific repertoire and have evolutionary value. In captive conditions, however, care must be taken to avoid escalated fights from which the subordinants may struggle to escape. Among dominant males, asymmetric aggression was invariant with and without substrate. Overt aggression appears to have a function in establishing and defending the nest site (Oliveira and Almada, 1998a), but no variation occurred with substrate. This suggests it may have been stimulated by factors other than the competition for the nest/territory. In the Mozambique tilapia, agonistic and sexual behaviour are part of the same behavioural axis with the androgen 11-ketotestosterone being implied in both behaviours (Borges et al., 1998). The high frequency of overt aggression without substrate may be related to this hormonal association and to a high motivation to breed, in an environmental where he lack of substrate does not facilitate it. Additionally, some authors (Heiligenberg, 1965; Barlow, 1974; Oliveira and Almada, 1998a; Mendonça, 2006) have suggested that substrate may have a regulatory role in aggression, and that digging behaviour could play a role as a re-directed pattern of aggression. Displaced aggression towards tank mates (Clement et al., 2005) and in the form of foraging (Munro and Pitcher, 1985) have also been reported for other cichlid species.

Among females, also, no differences in aggression were observed with the availability of substrate, but increased aggression was noted among the FF groups. *Ad libitum* observations of FF groups also showed occasional nest building, courtship and incubation of unfertilised eggs by ovulated females. It is possible that aggression is the result of a high breeding motivation that does not find the appropriate social context in which to be expressed. Aggression among females has been reported exclusively in the breeding context, particularly during pre-spawning or brooding (Neil, 1966; Oliveira and Almada, 1998b); also corresponding to two peaks of testosterone during the females reproductive cycle (Smith and Haley, 1988).

As expected, male pit digging and territorial behaviour followed a similar pattern and were generally more frequent with substrate. As mentioned above, establishment and maintenance of territories plays a very important role in the sexual and agonistic behaviour of this species. It is probably due to this that certain dominant males performed these behaviours even in the absence of substrate. Vacuum activities, such as these, have been used as possible indicators of behavioural needs, i.e., behaviours that are internally motivated and that can cause frustration and disturbance when prevented (Manning and Dawkins, 1992). For this reason, they have also been interpreted in the context of animal welfare, despite lingering doubts surrounding them and the link with the occurrence of actual suffering (Dawkins, 1990).

Chafing increased significantly without substrate, mainly among males of higher social status but also among females of FF groups (where more aggression was also noted). Chafing may be performed to release parasites or particles from the body surface and it may be associated with fighting animals which leave clouds of particles in suspension (Pinheiro, 1980). However, the possibility that this behaviour is undertaken as a displacement activity in response to an adverse context (e.g. lack of substrate in which to dig the nest in the context of male social interactions) should also be considered and evaluated in the scope of specific work. The above conclusion was reached in light of the administration of two thyroid hormones, thyrotropin-releasing hormone and 3-Me-His2-TRH, in jewel cichlid (*Hemichromis bimaculatus*) (Christ, 1984). Behavioural patterns exhibited in displaced contexts may serve as coping mechanisms, revealing conflict, frustration or a disturbance of some description (Mench and Mason, 1997). When prolonged or intense, observation of such patterns may be an indication of poor welfare (Dawkins, 1980).

Inactivity in non-territorial areas without substrate was greater for both sexes, especially in MF groups. It is possible that some of the behaviours originally directed towards the substrate were replaced by an increased immobility in its absence. Fish can rest when they are satiated or there are no predators in the surrounding area and sleep has been reported for the Mozambique tilapia (Shapiro and Hepburn, 1976).

Activity and inactivity patterns can be very plastic in fishes, even at the individual level, and are modulated by a number of factors such as light intensity, temperature, shoal size, predation risk or intraspecific competition (Reebs, 2002). For this reason, resting behaviours in fish, as in other animals, are not necessarily indicators of poor welfare. However, when prolonged or performed in unusual contexts, they can be a signal of barren environments (Broom and Johnson, 1993). In this study, as no other condition changed but the substrate availability, it is suggested that this increased immobility may be a response to a poorer environment.

Males showed a tendency for greater behavioural diversity with the presence of substrate. The fact that behavioural diversity did not reach levels of significance in MF groups is probably attributable to the smaller sample number which served to amplify the strong differences in behavioural diversity between dominants and subordinates, diluting the final result. It would appear that the presence of substrate stimulates a number of behaviours that, by definition, disappear from the non-substrate environment. This would lead ultimately to a reduction of motivational drive to perform substrate-linked behaviours. However, the exhibition of vacuum activities seems to suggest that this motivation is still present, even without substrate. Behavioural diversity has been used often in the assessment of welfare, on the basis that the greater diversity of behaviour, the greater the likelihood animals have of making use of their species-specific repertoire (Wemelsfelder et al., 2000). However, caution must be exercised regarding its interpretation because certain functional behaviours (e.g. courtship, pit digging and other territorial patterns) may be replaced by non-related behaviours (e.g. hovering and immobility in non-territorial areas) or even by possible coping mechanisms (e.g. vacuum digging and territorial behaviours, chafing), despite demonstrating behavioural diversity. Therefore, it is the actual nature of the behaviours in question, and not merely their general diversity, that is crucial in interpreting possible indicators of welfare.

4.2. Physiological parameters

Cortisol was not found to vary between both conditions. Specific variations in cortisol related to group composition or social status were also not found. Glucocorticoids, such as cortisol, have been used as primary indicators of stress, and are affected by a multitude of factors (Lane, 2006). Cortisol may be elevated during mating as a result of aggression, conflict and other endocrine changes that occur in the sexual context. Social status may also have an effect on both dominants (Correa et al., 2003) and subordinates (Gilmour et al., 2005). However, Earley *et al* (2006) point out that this relationship is not always clear since cortisol may vary with a number of subtle characteristics related to the social dynamics or housing system. Furthermore, individual characteristics may lead to different behavioural and physiological coping strategies within the same social status (Clement et al., 2005). In light of this, it is probably the case that the social dynamics intrinsic to each group may have a combined effect on the lack of a cortisol pattern found in this study; the presence or absence of substrate being an indirect factor affecting the social interactions. Despite the lack of significant differences, there was a tendency towards increased cortisol in females of MF groups with substrate which may be the result of increased sexual context in these aquaria.

Elevated glucose is a secondary response to stress and has been used in a variety of fish species as an indicator of stress (Cnaani et al., 2004). In this study, levels of glucose were equivalent to those expressed in stress-induced studies [air exposure, 45±13.2 mg/dl (Cnaani et al., 2004); maintenance in high densities, 30-69 mg/dl (Silveira-Coffigny et al., 2004)]. Glucose and cortisol levels were correlated in males but not in females. They did not vary significantly between both substrate conditions. The actual relationship between cortisol and glucose is still not fully understood in fish (Mommsen et al., 1999). It is generally accepted that cortisol stimulates the release of glucose in the blood stream, which may explain the correlation found in this study for these two physiological variables in males. This relationship, though, is not clear as plasma glucose concentration does not always increase with higher cortisol levels and can also vary with a number of factors, including physiological state, external disturbances to the animal or even the nature of the stressors involved (Mommsen et al., 1999; Barreto and Volpato, 2006). These aspects or the simple fact that males and females perceive the same stimuli (e.g. substrate) differently may be linked to the lack of a correlation in the females.

Haematocrit was clearly higher in fish with access to substrate compared to those without. It is apparent that haematocrit levels are often elevated during short term stress as a way of increasing oxygen supply in response to a higher metabolic demand (Cnaani et al., 2004), but it still remains unclear whether it can be used as a welfare indicator on a longer term basis (Broom and Johnson, 1993). In this instance, the observed increase may be linked to reduced inactivity in both sexes and an increase in energy demand associated with nest digging in males.

4.3. Animal welfare implications

Aggression related to territoriality plays a crucial role in the survival and reproductive success of male Mozambique tilapia (Fryer and Iles, 1972). Therefore, any assessment of welfare needs to take this into account; analysing aggressive behaviour in its appropriate functional and evolutionary context (Barnard and Hurst, 1996). A naturalistic approach to welfare allows suggesting, perhaps, that for males the lack of substrate does impact on their natural behaviour. This study allowed identification of a number of behavioural changes that occurred in the Mozambique tilapia when in the presence or absence of substrate, which can be summarised as follows: natural territorial behaviour, possible coping behaviours, immobility and behavioural diversity. For males lacking substrate, territorial behaviour and subsequent breeding were affected; as well as sexual behaviour, pit digging and hovering over the nest (territoriality). The nature of aggression also changed as it was not based on competition for territories. This change may have negative implications for welfare as the aggression lacks its natural outcome (territory acquisition and defence) and subordinates may find it difficult to avoid agonistic encounters due to the nature of aquaria. Without substrate, some behavioural patterns were exhibited with a putative coping function. These included vacuum pit digging and territoriality, and eventually chafing in the absence of substrate. The relevance of pit digging as a behavioural need should be further assessed through the development of preference and motivational studies. Immobility also increased as did behavioural diversity in males, suggesting a change in the usual behavioural time budget due probably to an impoverished environment. The effect of substrate availability is not apparent for females but a more specific study should be carried out in ovulating females for which the substrate could be more significant.

When physiological indicators are analysed together with behaviour in the presence of substrate, they seem to be consistent with the natural function of sexual and territorial behaviour. However, without substrate, this relationship is less clear for cortisol and glucose.

In conclusion, a lack of substrate affects males' behaviour, reducing certain patterns that are crucial to their fitness in the natural environment, and increasing others that may be linked to a high motivation to perform substrate-dependent behaviours. These facts suggest a negative effect on welfare and a requirement for further investigation.

5. Acknowledgments

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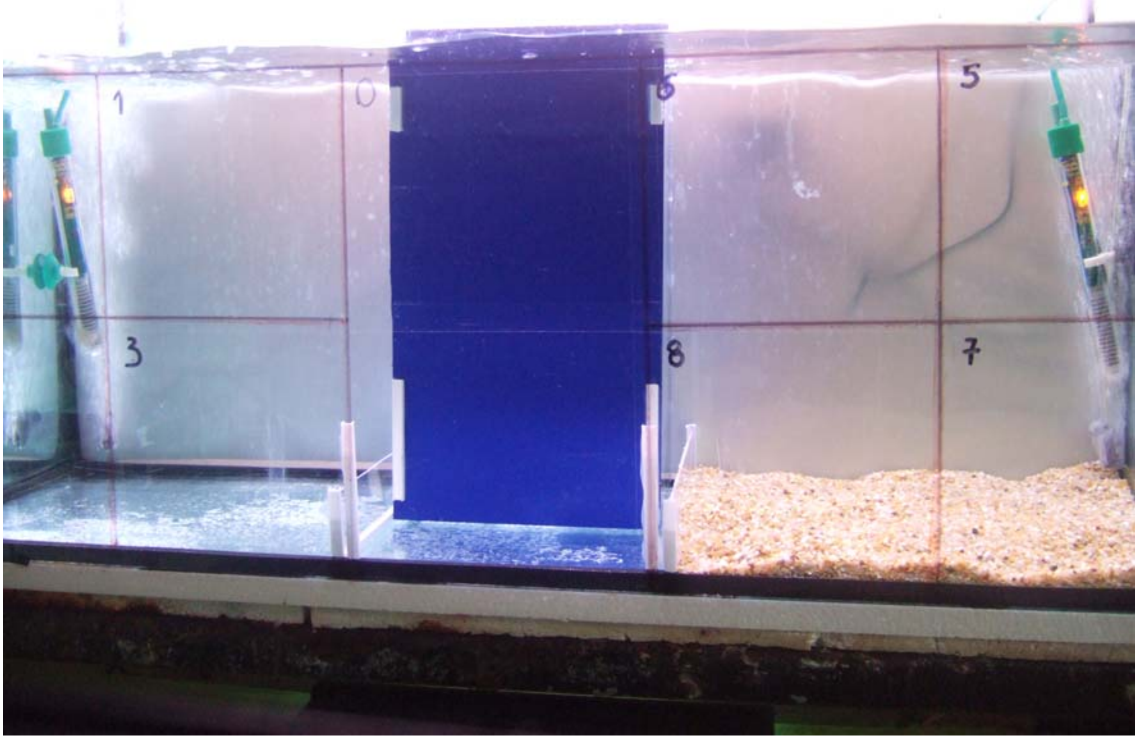
CHAPTER III

Preference for the presence of substrate in male cichlid fish: effects of social dominance and context



Galhardo, L., Almeida, O., Oliveira, R.F., 2009. Preference for the presence of substrate in male cichlid fish: effects of social dominance and context. Applied Animal Behaviour Science 120, 224-230.

Abstract

Many cichlid species dig spawning pits or nests in soft bottoms and exhibit many substrate-oriented activities. Despite this fact being of general knowledge, captive cichlids in laboratory and aquaculture set-ups are often kept in the absence of a soft substrate that they can manipulate. This raises a potential welfare issue, depending on how the substrate is valued by cichlid fish. The aim of this study is to assess the importance of substrate for male Mozambique tilapia (*Oreochromis mossambicus*) in social and non-social contexts. Preferences were established as a measure of time spent in two choice compartments, before and after the presence of a female. Locomotory activity, social interactions and substrate-related behaviours were recorded. Results show that dominant males prefer the area with substrate regardless of social context, and that female's presence strengthens this preference. The same preference is not apparent in the subordinate males, except for foraging. To draw conclusions on the importance of substrate to subordinates, preferences should also be assessed in agonistic contexts, during which substrate may serve to displace aggression. These results, together with related previous studies, show that the lack of substrate is particularly deleterious in a reproductive context, and thus it is likely to decrease the welfare state of breeding males of Mozambique tilapia.

1. Introduction

Preference tests started to be developed in the scope of welfare studies in the 70's by Dawkins and others on floor preferences in hens (reviewed by Dawkins, 1980). The aim of preference tests is to assess the choice of an animal faced with a number of available resources. Although these tests have been widely used in fish to investigate aggression, mate choice, parental care, schooling, etc. (e.g. Schluter et al., 1998; Landmann et al., 1999; Schlupp et al., 1999; e.g. Gonçalves and Oliveira, 2003; Webster and Hart, 2004), their application to fish welfare is not frequent (Huntingford et al., 2006).

Fish welfare has been measured using several physiological and behavioural responses to stress as well as other indicators of organic functioning, such as incidence of diseases, growth and mortality rates (Iwama et al., 1997). However, the interpretation of these approaches in the light of the welfare concept may be very complex, even contradictory. Instead, the preference tests are probably among the most well described approaches to obtain an indirect expression of the animal's mental states (Mason and Mendl, 1993; Dawkins, 2004; Duncan, 2006; Volpato et al., 2007).

Substrate may serve different functional purposes according to the fish species, or even within the same species. Many African cichlids use substrate during the breeding season for nest building (Fryer and Iles, 1972). As some cichlid species have substrate-related feeding habits (Fryer and Iles, 1972), it is also possible that different substrate types have different impacts on foraging. For example, Webster and Hart (2004) show that three-sticklebacks prefer complex over simple substrate for foraging purposes. The feeding context also allowed Horstkotte and Plath (2008) to relate different diets with substrate preference in pupfish. Hoglund and others (2005), in their study of monoamines and avoidance behaviour, have demonstrated the role of substrate in crucian carp as a relevant anti-predator feature of the environment.

In the present study, the relevance of substrate for males of Mozambique tilapia (*Oreochromis mossambicus*) is assessed. This species is an African mouthbrooder cichlid for which substrate is likely to have an important role, at least for territorial males during the breeding season. Males aggregate in leks in shallow waters during this season, where they establish social hierarchies (Neil, 1966; Nelson, 1995). Territorial males are usually bigger and stronger, with larger mouths and therefore more likely to win territorial contests (Oliveira and Almada, 1995). Disputes are mainly related to the acquisition of a territory, depressions built in the substrate, to which ripped females are attracted for spawning (Fryer and Iles, 1972; Oliveira and Almada, 1996). Mouthbrooding takes place in areas outside the lek (Baerends and Baerends-Van Roon, 1950).

The lack of substrate can potentially disrupt the stability of the social hierarchy and decreases the sexual behaviour of territorial males of Mozambique tilapia (Chapter II). Males try to build nests, even in the absence of substrate and become more inactive when this resource is not available (Chapter II). Therefore, the lack of substrate may affect the welfare of, at least, territorial males during breeding contexts. Yet, these animals are commonly maintained in aquaria without substrate in experimental and aquaculture settings.

The aim of this study is to evaluate the preference for substrate in males of Mozambique tilapia. Due to the mentioned social differences in territories' acquisition, territorial and non-territorial males will be tested. The effect of a female's arrival to the set up where males were placed alone is also assessed. Our main hypothesis is that territorial males prefer substrate, at least when a breeding context is created.

2. Material and Methods

2.1. Fish

The experimental fish were 29 males (weight: 99 ± 3.1g) of the species *O. mossambicus*, belonging to a stock held at ISPA. They were maintained in glass aquaria (120 x 40 x 50 cm, 240 l), at a temperature of 26ºC ± 2ºC on a 12L:12D photoperiod. All experimental fish were part of eight mixed sex social groups (3-5 males/5-6 females), forming stable social hierarchies. Their social status was identified prior to the experiment. Territorial males adopt a specific nuptial black coloration and a frequent territorial behaviour which includes the nest building and its defence. Each tank had a layer of fine gravel substrate of the same kind as that used in the experimental aquaria. Tanks were supplied with a double filtering system (sand and external biofilter, Eheim) and constant aeration. Water quality was weekly analysed for nitrites (0.2-0.5 ppm), ammonia (<0.5 ppm) (Pallintest kit®) and pH (6.0-6.2). All individuals were marked by means of a combination of three colour beads attached underneath the dorsal fin, by a nylon line. This tagging method was adopted in order to differentiate animals in the stock with minimum manipulation. The experience in this lab is that it does not affect individual or social behaviour, namely the social ranks, and it does not cause local problems. Fish were fed daily *ad libitum* with commercial cichlid sticks (ASTRA).

2.2. Experimental aquaria

The experimental aquarium (100 x 40 x 50 cm, 200 l) was divided in three distinctive compartments (Figure 1). The lateral or choice compartments have similar dimensions (40 x 39 cm) and the bottom of only one of them was covered with a 7 cm layer of fine gravel. To control for side effects, substrate was placed in each lateral compartment for half of the trials. The central or neutral compartment (20 x 19 cm) was the start box, where the male was placed to start the experiment. Behind it, there was a glass box (20 x 19 x 40 cm) physically isolated from the main tank, where a female is placed. Visual contact with the female compartment was only possible from the lateral compartments, since there was an opaque Plexiglas partition between it and the central compartment.

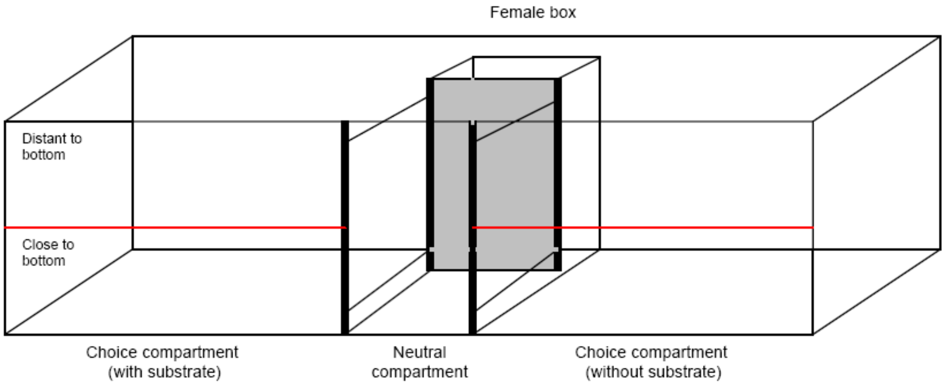


Figure 1. Representation of the experimental set up.

2.3. Choice Test

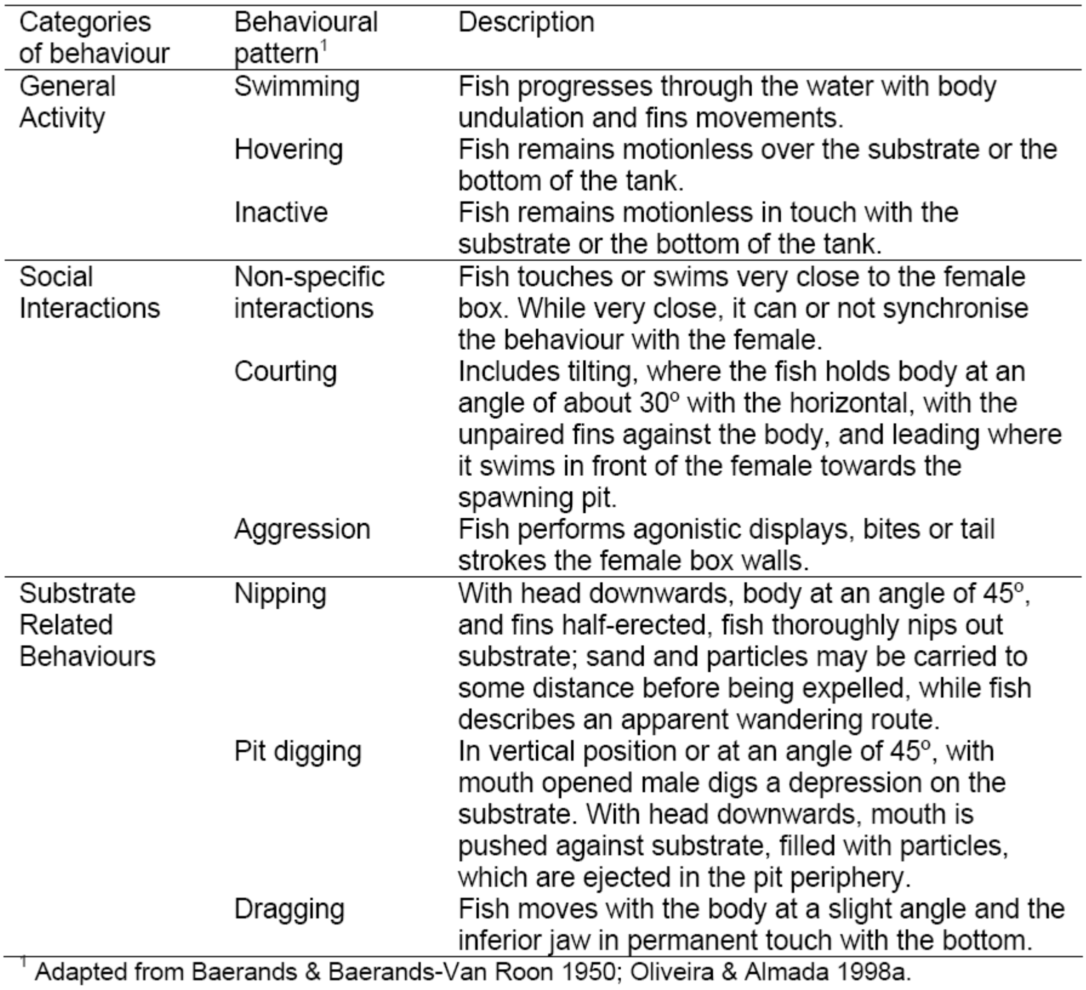
The choice test was divided in two parts during which six observation periods of 10 min each were conducted. During the first part no female was placed in the female box, with the objective of testing the compartment choice in a non-social context. A male was removed from the stock tank and placed in the start box, separated from the lateral compartments by two removable transparent Plexiglas partitions. Two minutes later, when it reduced the ventilatory rate, assumed a non-stressed colour pattern (no dark stripes) and started to swim around, the partitions were removed and the male was allowed to swim freely in the experimental set up. The first observation period was conducted immediately after the start box opening with the objective of following the behaviour during initial acclimation (observation 1). Two behavioural observations without female were respectively conducted one and two hours later (observations 2 and 3). In the second part of the choice test, a female was placed in the experimental set up, with the objective of testing the compartment choice in a social/breeding context. A female belonging to the same stock tank (familiar) was placed in the female box, while the male was confined again in the start box. A total number of 29 females were used (one per male). After two minutes, the start box was opened and the behaviour of the male was immediately sampled (observation 4). Two trials on a social context were then conducted one and two hours after the female has been placed in the set up (observations 5 and 6).

2.4. Behavioural sampling, exclusion and preference criteria

All observation periods involved a focal and continuous behavioural sampling (Martin and Bateson, 2007). Behaviours sampled are described in Table 1. In each lateral compartment two sub-areas were assigned in relation to their distance to the bottom (close and distant) for constant position sampling (Figure 1). In this way, 4 areas were defined: substrate/close, substrate/distant, no substrate/close, no substrate/distant. Time spent in each of these four areas was used as a measure of preference. Latency to enter the first choice compartment was not considered because when the partitions of the start box were lifted, the animals simply entered the compartment to which they were randomly faced to.

Six males (4 territorial and 2 non-territorial) were excluded from trials since they have shown high indicators of stress, namely a characteristic colour pattern (body and eye dark stripes), high ventilatory rates and very high immobility. The excluded fish spent more than 85% of the time in the start box or in the first compartment to which they moved, and remained immobile during the two first sampling periods. The remaining sample was constituted by 23 males, of which 11 were dominants and 12 subordinates. All animals visited both compartments during the acclimation period and thus their choices were fully informed. Preference was defined as a fish spending more than 50% of time in one of the choice compartments, whether close or distant to the bottom (Schluter et al., 1998). These criteria were not applied to observations 1 and 4, which were both periods of acclimation to a new event.

Table 1. Brief description of the behaviour patterns and respective categories



Categories of behaviour	Behavioural pattern[1]	Description
General Activity	Swimming	Fish progresses through the water with body undulation and fins movements.
	Hovering	Fish remains motionless over the substrate or the bottom of the tank.
	Inactive	Fish remains motionless in touch with the substrate or the bottom of the tank.
Social Interactions	Non-specific interactions	Fish touches or swims very close to the female box. While very close, it can or not synchronise the behaviour with the female.
	Courting	Includes tilting, where the fish holds body at an angle of about 30° with the horizontal, with the unpaired fins against the body, and leading where it swims in front of the female towards the spawning pit.
	Aggression	Fish performs agonistic displays, bites or tail strokes the female box walls.
Substrate Related Behaviours	Nipping	With head downwards, body at an angle of 45°, and fins half-erected, fish thoroughly nips out substrate; sand and particles may be carried to some distance before being expelled, while fish describes an apparent wandering route.
	Pit digging	In vertical position or at an angle of 45°, with mouth opened male digs a depression on the substrate. With head downwards, mouth is pushed against substrate, filled with particles, which are ejected in the pit periphery.
	Dragging	Fish moves with the body at a slight angle and the inferior jaw in permanent touch with the bottom.

[1] Adapted from Baerands & Baerands-Van Roon 1950; Oliveira & Almada 1998a.

2.5. Data Analysis

For analysis, observations 2 and 3 and observations 5 and 6 were pooled together since preliminary analysis have shown no differences between them (Wilcoxon Matched Pairs Tests were run for observations 2/3 and 5/6 for behavioural patterns and use of area, P>0.05). Discrimination of sub-areas (close or distant to the bottom) was analysed using the Wilcoxon Matched Pairs Test. Analysis of the frequencies of pit digging among the different observation periods was carried out with Friedman ANOVA. Time spent in the different areas of the experimental set up as well as time spent performing specific behaviours in the two social contexts were analysed using a repeated measures analyses of variance (two repeated factors: social context, choice areas; categorical factor: social status). When there were significant differences in the variances between the two conditions (Levene's Test), data were normalised using the transformations proposed by Zar (1984), namely Poisson transformation for frequencies (total number of behavioural patterns) and *arcsin* transformation for percentages (duration of behavioural patterns). Even, if the data did not meet the parametric assumptions, ANOVAs were still undertaken due to the lack of equivalent non-parametric tests and also because the *F*-statistic has been shown to be remarkably robust to deviations of normality and heterogeneity of variances (Lindman, 1974). Following the ANOVAs, planned comparisons of least squares means were performed where relevant.

A value of $P<0.05$ was taken for significance in all statistical tests. All analysis was performed using the statistical package Statistica V.8.0® (StatSoft Inc, USA, 2205).

3. RESULTS

3.1. General behaviour and preferences

Before the female's placement in the experimental set up, males spent the majority of the time engaged in locomotory activities. This pattern changes with the female's arrival, due to the increase of social interactions and behaviours directed to the substrate (mainly pit digging). In the last observation period, locomotory and substrate-related activities increase, while levels of interactions tend to decrease.

The use of space seems to be different in the two choice compartments in terms of being close or distant to the bottom (Figure 2). Without substrate, the males do not seem to prefer being close or distant to the bottom both without (Wilcoxon Matched Pairs: Z=1.07, P=ns) and with female (Wilcoxon Matched Pairs: Z=1.03, P=ns). On the contrary, in the substrate compartment, males spend more time close to the substrate in both social contexts (Wilcoxon Matched Pairs, no female: Z=3.50, P<0.001; female: Z=4.08, P<0.001).

When analysing the effect of social status on the use of the four areas, there is a clear preference of the territorial males for being in the compartment with substrate, and close to it, regardless of the social context (Figure 2; Table 2). This difference is accentuated after the female's arrival. The non-territorial males do not show this preference in any of the social contexts under analysis (Figure 2; Table 2).

3.2. Locomotor activity

Territorial males prefer to swim in the substrate compartment, close to the gravel, regardless of social context (Table 2). In these males, the female arrival tends to decrease the time spent swimming in areas distant to the bottom. Non-territorial males do not express any preference.

Time spent hovering is not influenced by the female and there are no marked differences between social status. However, in general hovering tended to occur in the substrate compartment (Table 2).

Inactivity was a rare behavioural pattern in the males that were not excluded from the trials.

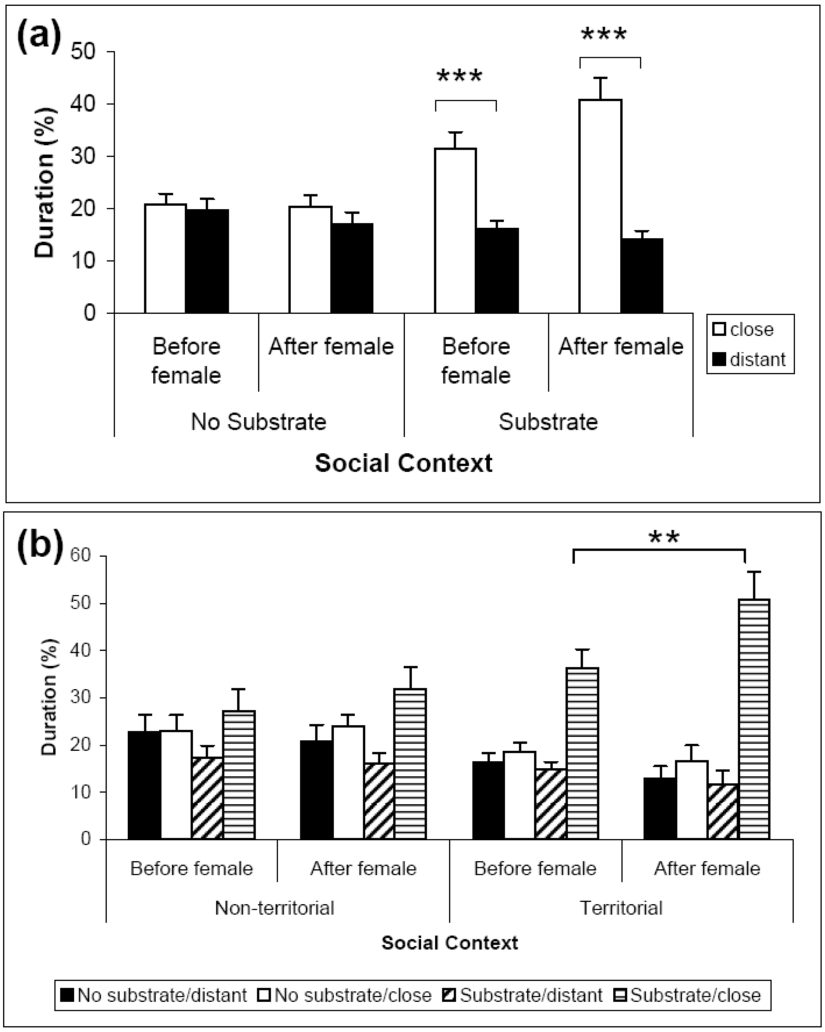
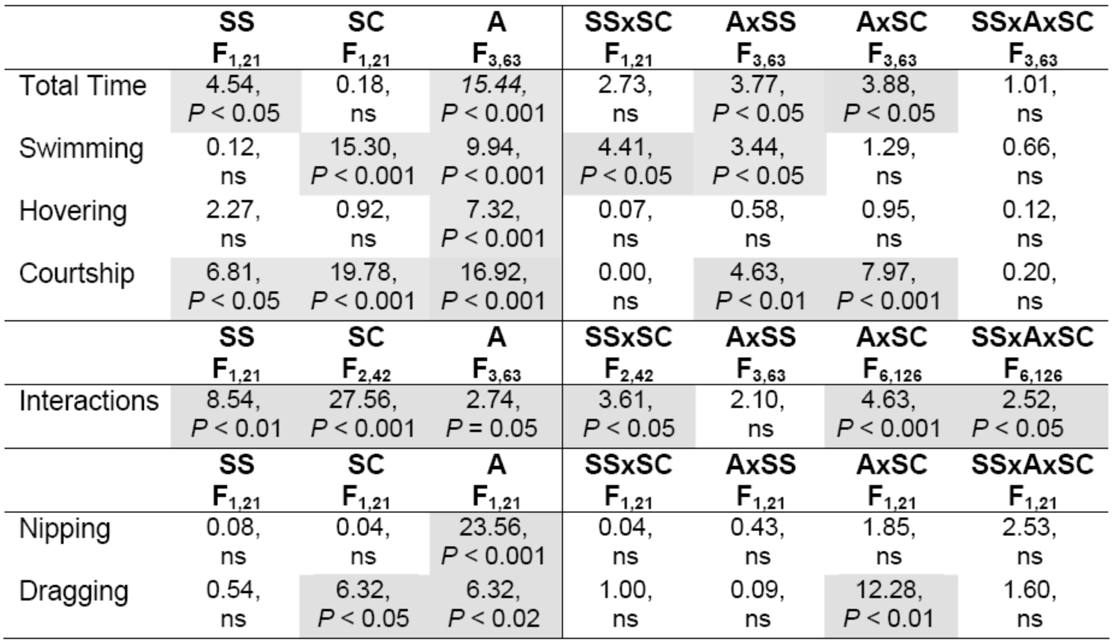


Figure 2. Total time spent (a) close and distant to the bottom in both choice compartments 1 hour before and after the female's arrival (b) in different areas by territorial and non-territorial males in the two social contexts. **P< 0.01; ***P< 0.001.

Table 2. ANOVA repeated measures results for time spent in specific behaviours. SS – Social Status (territorial/non-territorial); SC – Social Context (courtship: female arrival/after female; interactions: before female/female arrival/after female; other variables: before/after female); A – Area (with substrate, close/distant; without substrate, close/distant; nipping and dragging: with, without substrate).



	SS **$F_{1,21}$**	**SC** **$F_{1,21}$**	**A** **$F_{3,63}$**	**SSxSC** **$F_{1,21}$**	**AxSS** **$F_{3,63}$**	**AxSC** **$F_{3,63}$**	**SSxAxSC** **$F_{3,63}$**
Total Time	4.54, $P < 0.05$	0.18, ns	*15.44,* $P < 0.001$	2.73, ns	3.77, $P < 0.05$	3.88, $P < 0.05$	1.01, ns
Swimming	0.12, ns	15.30, $P < 0.001$	9.94, $P < 0.001$	4.41, $P < 0.05$	3.44, $P < 0.05$	1.29, ns	0.66, ns
Hovering	2.27, ns	0.92, ns	7.32, $P < 0.001$	0.07, ns	0.58, ns	0.95, ns	0.12, ns
Courtship	6.81, $P < 0.05$	19.78, $P < 0.001$	16.92, $P < 0.001$	0.00, ns	4.63, $P < 0.01$	7.97, $P < 0.001$	0.20, ns
	SS **$F_{1,21}$**	**SC** **$F_{2,42}$**	**A** **$F_{3,63}$**	**SSxSC** **$F_{2,42}$**	**AxSS** **$F_{3,63}$**	**AxSC** **$F_{6,126}$**	**SSxAxSC** **$F_{6,126}$**
Interactions	8.54, $P < 0.01$	27.56, $P < 0.001$	2.74, $P = 0.05$	3.61, $P < 0.05$	2.10, ns	4.63, $P < 0.001$	2.52, $P < 0.05$
	SS **$F_{1,21}$**	**SC** **$F_{1,21}$**	**A** **$F_{1,21}$**	**SSxSC** **$F_{1,21}$**	**AxSS** **$F_{1,21}$**	**AxSC** **$F_{1,21}$**	**SSxAxSC** **$F_{1,21}$**
Nipping	0.08, ns	0.04, ns	23.56, $P < 0.001$	0.04, ns	0.43, ns	1.85, ns	2.53, ns
Dragging	0.54, ns	6.32, $P < 0.05$	6.32, $P < 0.02$	1.00, ns	0.09, ns	12.28, $P < 0.01$	1.60, ns

When analysing the effect of social status on the use of the four areas, there is a clear preference of the territorial males for being in the compartment with substrate, and close to it, regardless of the social context (Figure 2; Table 2). This difference is accentuated after the female's arrival. The non-territorial males do not show this preference in any of the social contexts under analysis (Figure 2; Table 2).

3.2. Locomotor activity

Territorial males prefer to swim in the substrate compartment, close to the gravel, regardless of social context (Table 2). In these males, the female arrival tends to decrease the time spent swimming in areas distant to the bottom. Non-territorial males do not express any preference.

Time spent hovering is not influenced by the female and there are no marked differences between social status. However, in general hovering tended to occur in the substrate compartment (Table 2).

Inactivity was a rare behavioural pattern in the males that were not excluded from the trials.

3.3. Social Interactions

Courtship was intense immediately after the female's placement in the experimental set up. Therefore, the males' behaviour was analysed immediately at female's arrival and one hour after it.

On female's arrival, males of both social status exhibited the highest levels of courtship behaviour (Figure 3). In this observation period, territorial males used both compartments, because they swam constantly from one to the other, while subordinates exhibited more courtship in the non-substrate compartment. In the subsequent observation, subordinates no longer courted the females, while the dominants' courtship became mainly performed in the substrate compartment (Figure 3, Table 2).

'Non-specific' social interactions were also intense immediately after the female's arrival. Therefore, the males' behaviour was analysed in three social contexts: before the female's placement (for a baseline comparison), on its arrival and some time after it. Non-territorial males spent the highest proportion of time in 'non-specific' interactions immediately after the female's arrival. These interactions occurred especially in both sub-areas of the non-substrate compartment (Table 2). Territorial males did not tend to engage in this kind of interactions and when doing so, they did not choose a specific area or compartment (Table 2).

3.4. Behaviours related to the substrate

Pit digging occurred exclusively on the substrate compartment and mainly by the territorial males when after the female's arrival (Friedman ANOVA, $\chi^2 = 7.29$, df = 2, $P < 0.05$; Mann-Whitney, Female: $Z=3.00$, $P<0.01$) (Figure 3).

Nipping on the bottom was particularly carried out in the substrate compartment by males of both social status and irrespective of social context (Table 2). On the other hand, dragging on the bottom was almost exclusively exhibited in the non-substrate compartment by all males when the female is not present (Table 2).

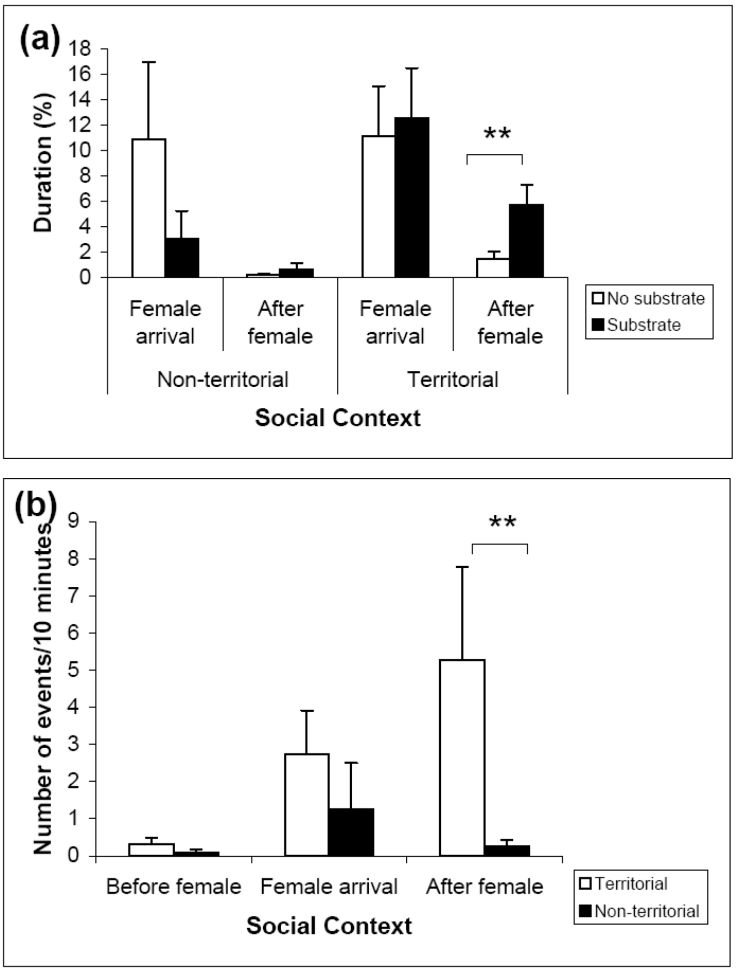


Figure 3. Activities of territorial and non-territorial males (a) Time spent courting in the two compartments immediately after female arrival (Female arrival) and 1 hour later with the female in the set up (After female). (b) Pattern of pit digging in the different social contexts. **P<0.01.

4. DISCUSSION

4.1. General preferences

There was a clear preference for substrate by territorial males regardless of the social context created by the presence of a female in the set up. Substrate preference by territorial males was identified when analysing a number of behaviours, namely: patterns of activity (swimming and hovering), social interactions (courtship) and substrate-directed behaviours (pit digging, nipping). This preference was even more expressive when they engage in reproductive behaviours (courtship and pit digging). Non-territorial males did not express a substrate preference, except for nipping on the substrate.

The locomotory activity of territorial males occurred especially over the substrate independently of the social context, while courtship and pit digging were behaviours incremented by the female's arrival. This fact is in line with the natural behaviour of Mozambique tilapia where, prior to courtship, a male is tuned to find and defend a territory, establishing a social hierarchy with the remaining males in the lek (Fryer and Iles, 1972; Oliveira and Almada, 1998a). In this case, as the male had already acquired the dominant status in the stock, it is likely that it was highly motivated for territorial and sexual behaviours, which are both substrate-related, even before the female's arrival. Males spent most time courting the female immediately after it had been placed in the aquarium. In this period, no substrate preference was shown. However, in subsequent observations, dominants maintained their motivation for courtship, together with an increased time digging the spawning pit. Then, their preference for substrate while courting became clear and more meaningful in the context of this species reproductive behaviour. Courtship has a number of distinctive behavioural patterns, an important part of which are related to the spawning site, such as leading the female to the nest or circling it (Oliveira and Almada, 1998b). Furthermore, males were seen to intersperse courtship with dig pitting.

Despite the fact that in natural conditions, subordinates may also establish territories, though in periphery of the lek and less successfully (Fryer and Iles, 1972), the constraints created by artificial conditions, may totally inhibit territorial behaviours. In this study, subordinates did not show a preference for substrate neither through their general locomotor activity nor during social interactions. Despite these males have been tested without dominant males, their reproductive subordinate status brought from the stock may have decreased the reproductive motivation. In fact, while territorial males remained motivated to court the female for hours, non-territorial males have shown a completely different pattern of social interactions. Immediately after the female's arrival, they have attempted to court and exhibited the highest level of 'non-specific' social interactions. However, their motivation to interact soon decreased substantially, as judged by the low levels of interactions and no courtship in subsequent observations. It is interesting to note that during this period subordinates clearly spent their time interacting in the non-substrate compartment instead of sharing the time between compartments (as was the case for dominants). This fact suggests that subordinates may have a perception (simply conditioned or more cognitively elaborated) that substrate is a resource that "belongs" to territorial males, in this way actively avoiding it when interacting with females.

Despite the results related to non-territorial males, it is inappropriate to conclude that substrate is not relevant to them. In the present study, the social environment was created by a female, thus promoting reproductive behaviour in the motivated males. In fact, in this context, substrate was not particularly relevant to subordinates. But it is well known how preferences may change with different contexts, physiological states, life cycle stages, etc. as reviewed by Bateson (2004). Therefore, the importance of substrate for subordinates may well change in a different social context. For example, in the actual presence of dominant males, it is possible that substrate would become a target for displaced aggression for subordinates, which in that case might be regarded as an important resource to decrease social stress in the aquarium (Chapter II). The use of substrate, in the form of digging or foraging, or as a moderator of aggression and of levels of social stress has been suggested by some authors for different cichlid species (Heiligenberg, 1965; Barlow, 1974; Munro and Pitcher, 1985; Oliveira and Almada, 1998a).

In a non-social context, all males have shown similar patterns of nipping and dragging. Nipping occurred mainly in the substrate compartment and regardless of the female's presence, which shows that gravel may offer an additional opportunity for foraging and exploration in any context. Dragging occurred mainly when the female was not yet in the aquarium and almost exclusively in the non-substrate compartment. This behavioural pattern, and its context of occurrence, is suggestive of some type of inspecting-like behaviour. If this is the case, it denotes that a bottom without substrate is somewhat an unfamiliar environment deserving inspection.

This study has two methodological aspects that deserve to be addressed. The first is related to the non-balanced order of female's presentation to the focal male. In fact, the aim of the present study was to detect changes in the substrate's use in a fish before and after a female's arrival. To balance the presentation order would not be adequate because the duration of the male's internal effects induced by the female's presence would not be controllable. In any case, the different results between territorial and non-territorial animals in this study show that there were no biases created by order effects. It would be interesting to undertake a complementary study where males would be separately tested for substrate's preference in isolation and in a social context, to confirm the preference for substrate of territorial males in a more permanent social context. A second methodological aspect is related to the use of familiar-looking gravel as the substrate option in this study. The choice for familiar environments is one of the identified problems in interpreting animals' preferences (Fraser and Matthews, 1997).

For the animals under study, the ‘familiar-looking’ floor likely includes a number of different properties: the existence of removable particles, the texture, the colour, reflexes or shadows created by the particles. To simply change the size of the particles would not necessarily eliminate the ‘familiar-looking’ aspect and, furthermore, this lab’s experience is that different gravel sizes, as long as they are still removable, have no impact on fish behaviour towards the substrate. Therefore, an attempt to remove the familiarity components of the substrate from the experimental design, would risk the adoption of a solution with no biological meaning for these animals. In any case, in this particular study, ‘familiarity’ did not become a confounding factor as the results show that only the territorial males had a clear preference for gravel.

4.2. Conclusion and implications for welfare

Substrate is a multiple-function resource for Mozambique tilapia. It is important for territoriality and reproduction, and it seems to provide additional opportunities for foraging and exploration. As discussed, territoriality and reproductive behaviour are natural behavioural features of this species and fundamental for their ecological success. Recently vacuum nest-building has been observed in this species in the absence of substrate (Chapter II), suggesting that nest-building should be viewed as a behavioural need in cichlid fish (Dawkins, 1988; 1990; Jensen and Toates, 1993). These aspects, together with the expressed preference for substrate by territorial males, allow the conclusion that substrate is a relevant resource for these males because it is the most appropriate environmental outlet for pit digging behaviour. Therefore, its lack of availability is likely to decrease the welfare of dominant males. In the reproductive context, substrate does not seem to be a valuable resource for subordinates, but this fact should be further investigated in agonistic contexts where substrate may be important in the aggression displacement as previously discussed. In non-social contexts, substrate increases environmental complexity, offering new opportunities for foraging and exploration. It seems to promote behavioural diversity and reduce inactivity, which are both signs of increased welfare (Chapter II).

In conclusion, substrate seems to be important for the welfare of *Oreochromis mossambicus* males, based on previous knowledge of cichlids natural behaviour (e.g. Fryer and Iles, 1972) and comparative laboratory studies with and without this resource (Chapter II). The present preference study clearly confirmed the substrate relevance for territorial males.

5. Acknowledgments

Thanks are due to David Gonçalves for his help with the statistical analysis. This study was funded by the Pluriannual Program of Fundação para a Ciência e a Tecnologia (FCT, UI&D 331/2001) and L.G. was supported by a PhD fellowship from FCT (SFRH/BD/16162/2004).

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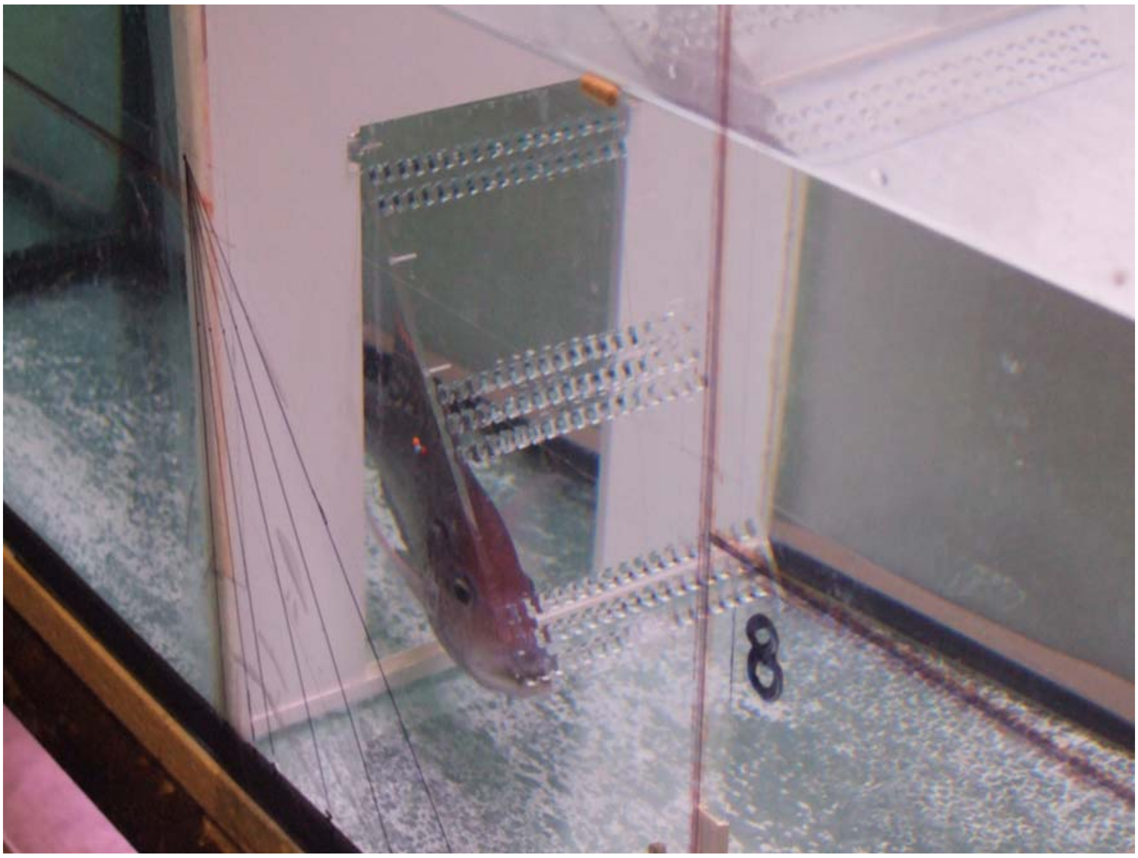
CHAPTER IV

Measuring motivation in a cichlid fish:

an adaptation of the push-door paradigm



Galhardo, L., Almeida, O., Oliveira, R.F. Measuring motivation in a cichlid fish: an adaptation of the push-door paradigm. Submitted to *Applied Animal Behaviour Science*.

Abstract

Recent behavioural, cognitive and neurophysiological studies strongly suggest that fish are capable of mental experiences. Therefore, identifying needs from the animals' point of view is likely to be one of the best approaches to understand their welfare. Motivational tests, as a measure of what animals want, have been developed and refined for some decades. Despite many earlier studies on fish motivational systems, none attempted to quantify their motivation using this approach. Motivation studies often imply operant tasks for which various devices are used. The aim of this study was to adapt for the first time a push-door to quantify motivation in a cichlid fish, the Mozambique tilapia (*Oreochromis mossambicus*). Males of this species have strong snouts which they use for a number of activities and thus they are suited to push. Twelve males of different social status were tested for three kinds of reinforcers: food, social partner and a control (additional space with substrate only). The animals were required to work at the door (push/touch) at an ascending cost in order to have access to the resources. Measures of motivation included latency to open the door, work efficiency and maximum price paid. Latency to open the door increased with increasing cost for all resources, with the highest latency for the control reinforcer. Work efficiency was constant with increasing cost for social partner and food, and higher than the control. Work efficiency decreased for the control as cost increased. Maximum price paid was consistent with these results, being higher for social partner and food than for the control. The results of the three measures were consistent with each other and showed that the push-door can be used to measure motivation in this species. Further refinement of the present experimental set up will allow the use of this paradigm in the future, in order to improve knowledge on how this species values and ranks their needs.

1. Introduction

Fish welfare has been an area of growing interest and concern (Huntingford et al., 2006). Recent evidence on fish behavioural, neuroendocrine and cognitive processes suggests that they are capable of subjective feelings (reviewed in Galhardo and Oliveira, 2009). Since animal welfare has to do with what animals feel (Dawkins, 1990), to know what they want has been regarded one of the most fruitful approaches to understand animals' perception of their own needs (Duncan, 2006; Kirkden and Pajor, 2006) and this idea was recently extended to fish (Volpato et al., 2007). Based on arguments from analogy, a clear link was established between animals' needs, motivation and feelings: rewards they want to achieve (or avoid) may contribute to either reduce suffering or increase pleasure (Dawkins, 1990; Widowski and Duncan, 2000; Kirkden and Pajor, 2006). Therefore, attempts to measure motivation are an indirect approach to understanding the subjective states of animals.

During the last thirty decades, methods from experimental psychology and application of economic concepts have been used to measure motivation (Lea, 1978; Dawkins, 1983; Kirkden, 2003). Fish were never included in these studies, despite the fact that they also reveal motivation for certain resources. For example, goldfish exhibit trade-offs between the cost of a discrete shock and social contact (Dunlop et al., 2006) and feeding (Millsopp and Laming, 2008). Motivation tests are experimental procedures aiming to measure how much an animal is prepared to pay to have access to (or avoid) a given reinforcer (Kirkden and Pajor, 2006). Positive reinforcers used in these studies may be food, water, shelter, or many other species-specific enrichment items (Sherwin and Nicol, 1996; Mason et al., 2001; Hovland et al., 2006). Quantification of motivation requires the definition of an ascending reinforcement schedule per session, with costs being imposed on access to a given resource (e.g. Olsson and Keeling, 2002) or on its consumption (e.g. Dawkins, 1983). Costs may either involve price increase (e.g. weight of pushing doors, Petherick and Rutter, 1990) or income decrease (e.g. time available to spend with the resource, Dawkins, 1983). As cost increases, the animals' response may be measured as a function of work for the resource's access (e.g. Olsson et al., 2002; Hovland et al., 2006) or based on consumption measures (e.g. Matthews and Ladewig, 1994; Hansen and Jensen, 2006). A quantifiable comparison among resources must involve an additional resource of known value (usually food) (Kirkden and Pajor, 2006).

Motivation measurements are frequently based on a demand curve aiming to characterise consumption (demand or expenditure) as cost increases (Matthews and Ladewig, 1994). The consumption elasticity measures how much animals consider a resource a ‘necessity’ (inelastic demand) or a ‘luxury’ (elastic demand) (Dawkins, 1983). However, demand curves are not always considered to be the most valid measure of motivation: they require that animals have exclusive access to the resource under the test environment (closed economies), that the cost and resource-use vary proportionally and that the price per unit of resource is kept constant (Mason et al., 1998b; Kirkden and Pajor, 2006). Under different circumstances, instead of using elasticity of demand, it has been proposed to use the maximum price paid for a single access to the resource (Olsson and Keeling, 2002; Kirkden and Pajor, 2006).

Push-doors, as operant devices to study motivation, were first used in hens by Duncan and Kite (1987), where increased cost was imposed by added weights attached to the door. This paradigm was further developed by Petherick and Rutter (1990) and used by a number of researchers (e.g. Olsson et al., 2002; Cooper and Appleby, 2003). Examples of operant studies in fish, although outside the context of motivational studies, include pendulum-pressing in rainbow trout (Yue et al., 2008), switch-pulling in Nile tilapia (Endo et al., 2002) and rod-pushing in tench (Herrero et al., 2005) for food delivery. Earlier examples include operant tasks for access to food and mirrors for agonistic displays in Siamese fighting fish (Hogan et al., 1970).

The aim of the present study is to adapt the push-door paradigm for quantification of motivation in the cichlid fish Mozambique tilapia. Males of this species have very strong snouts, which they use to build pits in the substrate and during mouth-to-mouth fights (Oliveira and Almada, 1998), and they easily learn conditioning paradigms (Antunes and Oliveira, 2009). The hypothesis is that they could learn an operant task related to a push-door placed between them and a resource. The primary aim is to test the usefulness of push-doors for studying motivation in this species, rather than to establish a comprehensive identification of resources’ value.

2. Materials and Methods

2.1. Animals and housing

This study involved the use of 12 males (weight 115±9.6 g) of the species *O. mossambicus*. Their social status was identified prior to the experiment, being six territorial and six non-territorials. Territorial males adopt a specific nuptial black coloration and a frequent territorial behaviour which includes the nest building and its defence (Oliveira and Almada, 1996). They were part of a stock held at ISPA and were maintained in glass tanks (120 x 40 x 50 cm, 240 l) with a fine gravel substrate, in stable social groups of 3-5 males and 5-6 females. The stock temperature was held at 26ºC ± 2ºC, with a 12L:12D photoperiod. Tanks were supplied with a double filtering system (sand and external biofilter, Eheim) and constant aeration. Water quality was weekly analysed for nitrites (0.2-0.5 ppm), ammonia (<0.5 ppm) (Pallintest kit®) and pH (6.0-6.2). Fish were fed twice a day, 1-2% of their body weight with commercial cichlid floating and sinking sticks (ASTRA). The latter started to be given to the animals one month before the beginning of the experiment.

2.2. Apparatus and push-door

The experimental aquarium (100x40x50 cm, 200 l; Figure 1a) was divided in two distinctive compartments: the start compartment ([SC], 40x40x50 cm), and the resource compartment ([RC], 60x40x50 cm). The SC was a fully barren compartment. The RC had a 5 cm layer of substrate (fine gravel) in area RC_1 as a baseline environmental feature. Substrate was not placed in area RC_0 to avoid interference with the door functioning. The female used for the social partner testing was placed in a smaller perforated aquarium (12x20x50 cm) in front of the door (20 cm of distance) (Figure 1a). A fixed and opaque partition with a transparent swing-door (17x15.5 cm) hinged in the centre separated the two compartments (Figure 1b). The swing-door was made of Plexiglas and perforated to facilitate its movement. The fish were able to see the resources in the RC and to touch and push the access door for variable periods of time. Occasionally, some fish pushed the door wide enough to pass under it. For practical reasons, the door of this experimental design was operated manually (as in Olsson and Keeling, 2002; Hovland et al., 2006), by a transparent nylon string attached to the door's bottom, which could be pulled from outside the visual field of the animals. Therefore, when the animals reached a given number of touches or pushes (see below reinforcement schedule), the string would be pulled and the door opened. After the animal passed through (Figure 1b), the door would remain opened until the end of the trial. In order to increase the opening cost, different plumb weights were attached (adapted from Duncan and Kite, 1987) to the external and bottom area of the door (Figure 1c). The aquarium was laterally covered with opaque partitions in order to avoid incoming stimuli from outside the experimental set up. Aeration was removed during the tests, and reintroduced in between tests and overnight.

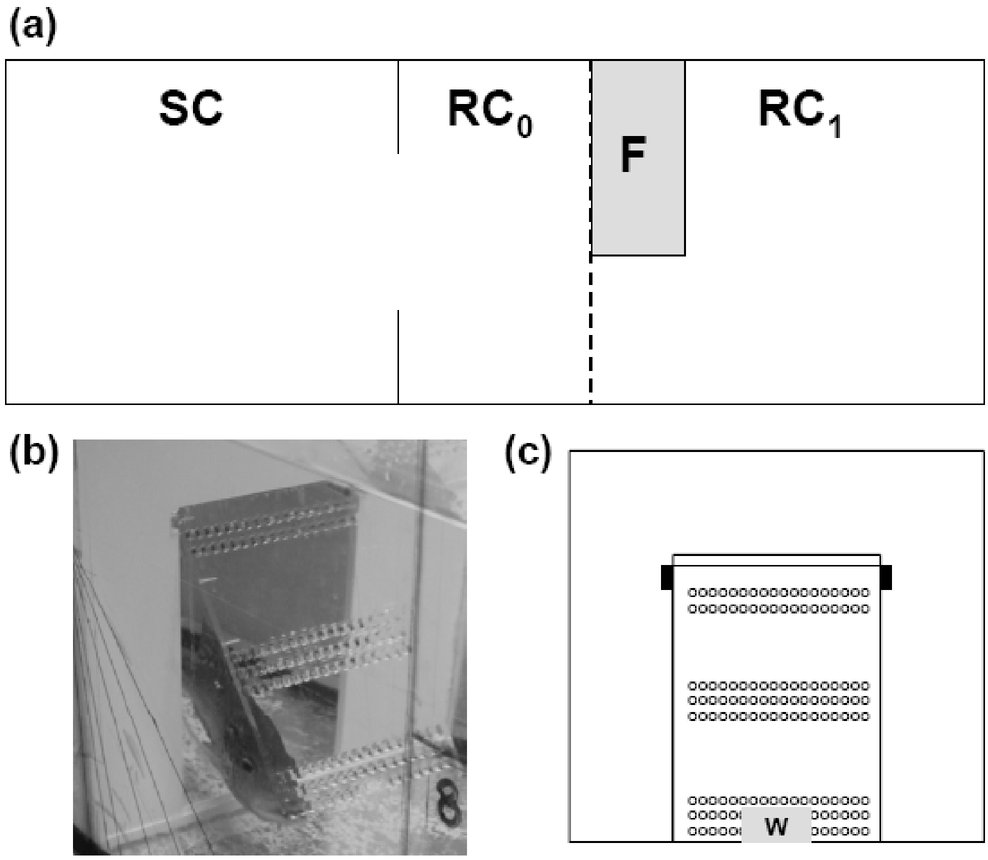


Figure 1. (a) Schematic representation of the experimental set up (SC) Start compartment (RC0) Resource compartment, area without sand (RC1) Resource compartment, area with sand. F – Female aquarium used in social partner trials (b) Schematic representation of the push-door. W – area where weigths were attached to. (c) Photo of a Mozambique tilapia passing the push-door.

2.3. Experimental procedure

2.3.1. Reinforcers

This experiment involved three kinds of reinforcers, tested one at a time in a balanced order: food, social partner (female) and additional space/substrate (as a control). The food reward was kept constant throughout the study and consisted of three pellets of commercial food for cichlids per trial (two trials per day were run, which completed the individual daily portion). Two different kinds of pellets were used to amplify the food attractiveness: one out of the group of three pellets – divided in two halves - immediately sank and remained in front of the door (visual stimulus), while the other two remained floating. As the latter were the pellets usually given to the fish, their use aimed to ensure the olfactory and rewarding properties of more familiar food. The pellets were put in the set up after the animals' acclimation. Fish were induced to anticipating this event by exposure to the sight of a yellow container previously associated with food. Pellets were placed in the RC immediately after that. A previous study has shown that Mozambique tilapia is able to anticipate the delivery of food when it is predictably signed (Chapter VII). In fact, the presentation of a visual cue five minutes before the food delivery has determined an increase of swimming activity in the front glass of aquaria, interspersed with frequent surfacing in the area where food was usually placed. Despite the fact that the individual daily amount of food was delivered during the trials, the animals were considered to be tested in an open economy, i.e. with access to the reward outside the experimental set up because they could still eat food particles in the home tanks.

The social partner was also tested in an open economy as test males were in full contact with their social group until arrival to the set up (where they spent only 30 minutes in isolation prior to trials). After having passed the door for access to the social partner, fish were allowed to remain in the RC for 30 minutes with free access to the SC (away from social partner). This time spent with the reward (reward size) was a long enough period for animals not to devalue their partner due to artificial interruptions of social bouts (Mason et al., 1998a). The third reinforcer (additional space/substrate) was intended as a control. Although it is known that males value substrate (Chapters II and III), here the RC with substrate was simply used as a baseline comparison to the remaining resources. Due to laboratory restrictions, only five males (two dominants, three subordinates) were tested for the control.

2.3.2. Reinforcement schedule

Animals expressed their interest in the RC by frequency of touching and/or strength of pushes at the door. Touching the door was defined in the scope of this work as the physical contact established between the tip of the fish's snout and the door surface, without moving the door. Pushing the door was essentially a similar behaviour (probably belonging to the same continuum) but performed with variable strengths which caused the door's movement to different extents. Occasionally, the strength of the door's push was sufficiently strong to open the door and allow the fish to pass through without external intervention. When this was not the case the door was operated manually in accordance to the reinforcement schedule described below. Touch and push were two spontaneous behaviours which were incorporated as measures of fish's work in this experimental approach in order to keep the behavioural response to the task as natural and spontaneous as possible, and avoiding complex training protocols which could interfere with motivation (Würbel, 2009).

A fixed ratio (fixed number of responses required to obtain one reinforcer) was imposed which consisted of five work levels (from easiest, level 1 to most difficult, level 5) of an ascending cost to have access to the test resource, as described in Table 1. The costs imposed during the course of this experiment are related to the two exhibited behaviours - touches and pushes – and were defined as described in Table 1a. Therefore, ascending costs included an increased frequency of touches required for the door to be opened (from 20 touches, at the easiest level to 70 touches, at the most difficult level) or increased weights attached to the door (from 0g, at the easiest level to 200g, which is the double of fish's body weight, at the most difficult level). As described in Table 1b, the animals were required to perform a given number of pushes depending on their amplitudes. Range of difficulty, whether in relation to the frequency of touches or weights attached to the door and number of pushes, were decided during a preliminary pilot study.

Table 1. (a) Reinforcement schedules for touches (frequency) and pushes (door's weight) (b) Number *versus* amplitude of pushes required to open the door at any cost.

(a)

Price	**Touches (N.º)**	**Door weight (g)**
Level 1	20	0
Level 2	40	25
Level 3	50	50
Level 4	60	100
Level 5	70	200

(b)

Pushes to open the door	**Amplitude**	**Number**
	<15º	12
	≥15º <30º	3
	≥30º <45º	2
	≥45º	1

The animals had a total of 15 minutes to complete each trial, i.e. to have access to the RC, but their responses (touches/pushes) were only considered if performed within five consecutive minutes. This fixed amount of time was chosen on the basis of a previous pilot study where animals tended to respond in behavioural bouts of approximately five minutes. After that they seemed to loose attention in relation to the operant task, swimming around and performing behaviours such as surfacing, nipping or dragging at the bottom (moving with inferior jaw in permanent touch with the bottom). To avoid overestimating the meaning of touches at the push-door, all those also performed in other surfaces (walls) were discounted from the total amount of touches at the door.

In summary, for each imposed cost, the weighted door would be opened as soon as the established number of touches or ‘number *versus* amplitude’ pushes was reached.

2.4. Training and Trials

As mentioned, the spontaneous behavioural response to the door was touching and pushing, occasionally causing it to open. Thus, training simply aimed to reinforce both behaviours in such a way that levels of their performance could become a measure of fish attention and effort (work) at the door and inherently a measure of the motivation to access the resource on the other side. Training was done through a combination of operant conditioning and shaping techniques (Prescott and Buchanan-Smith, 2003). The reinforcer used was food. An initial training period lasted for approximately five days (approx. 1h per day). This period aimed to acclimatise animals to the set up and door (wide and semi-opened). At this stage, fish hesitantly approached the semi-opened door, took some time to touch it and eventually passed through the lateral opening. The training method at this point was shaped according to each individual with the objective of leading all the animals to contact and pass the door. After this time, during which they generally became less fearful, six training sessions (in three days) were undertaken immediately prior to the trials. In the first training session the door was semi-opened and all animals passed to the RC without difficulties. The following training sessions always started with the door closed, with no weights attached (the same as the first cost level). During the three next training sessions, different animals still passed the door at different number of touches or pushes. If they would not touch or push the door an acceptable number of times, it was anyway semi-opened in order to avoid frustration and subsequent disconnection from the task (Olsson et al., 2002). However, animals that at the 4th training session would still not work enough to open the door within 15 minutes were removed from the study. At sessions 5-6 the decision that animals were already trained was taken on the basis of a consistent decrease in latency to open the door in relation to the first sessions. On the basis of these criteria four animals were removed from the experiment due to their inconsistent and stressful behaviour. Stressed behaviour was characterised by excessive inactivity, stressed body colour pattern (dark stripes) and elevated ventilatory rate.

Trials started the day immediately after training. Each male was individually transferred from its home tank directly to the experimental set up just before the trial started for the food trials, and 30 minutes before for the social partner and control trials. This short period of isolation in the set up intended to create a change of context, improving attractiveness for social partners, without generating stress (see discussion, for further details). The acclimation period for the food trials never exceeded three minutes, the time enough for the animal to recover from being moved (as indicated by reduced ventilatory rate, non-stressed colour pattern and swimming around). After the acclimation period, the food was placed in the RC, or an opaque partition (placed next to the door) was lifted to establish visual contact with the female in the RC. Costs increased progressively from minimum (level 1) to maximum work (level 5) in five consecutive trials as described above. Animals were tested twice a day for food, following the usual feeding routine, and once a day for social partner and control/substrate. The experiment was run in two separate blocks of five and seven males (all individually tested) with a balanced presentation of the reinforcers (the second group was not tested for the control). After the fish having achieved the established price to open the door and pass to the resource compartment, the door remained open for free access to both compartments. After each trial, the fish was removed from the set up back to its home tank. The animal was always captured in the SC, to where it swam freely or was gently conducted, in order to avoid the establishment of an aversive relationship with the RC.

2.5. Behavioural sampling

The behavioural sampling was focal and continuous (Martin and Bateson, 2007). It was undertaken by two observers with different roles: one observed the fish’s behaviour and opened the door at the required moments (and left it opened), while the other registered the number of touches/pushes and the remaining behavioural patterns.

2.5.1. Motivation measures

The use of consumption measures to infer motivation (and hence demand curves) was not considered valid in this study for three reasons: it was an open economy; there was a prolonged access to the social partner (and control) without associated costs; non-substitute resources were being compared. Therefore, measures of motivation were related to the resource access and included latency to door opening, work efficiency and maximum price paid. Latency to door opening was the period of time between the trial start and the door opening and reflected the work performed in a given amount of time: the faster the work performance, the lower the latency. Work efficiency (%) was the time spent working at the door (period of time between the first touch/push and the door's opening, discounting the periods not investing at the door) divided by the latency to door's opening. It expresses the proportion of time spent working at the door before it opened. This measure intended to evaluate the degree of attention paid to the door/RC, regardless of the behavioural strategy adopted to open it (touches, pushes or both). Maximum price paid is the maximum cost at which the door was opened.

2.5.2. Behaviour in relation to reinforcers

Despite the fact that no measures of consumption were used to assess motivation, an exploratory analysis of behaviour in relation to reinforcers was undertaken. After the animals have passed the door, the latency to start eating was measured. In relation to the social partner reinforcer, 'time close to female' (within 2 cm from the female's tank), 'time interacting with female' (mutual touching of the female's tank walls) and 'time interacting with substrate' (nipping and pit digging) were the sampled behaviours. The amount of time spent in the three different areas of the experimental set up was recorded for both social partner and control. These observations were undertaken during the first 10 minutes out of 30 minutes in contact with the reinforcer.

2.6. Data analysis

Latency and work efficiency were both analysed using a repeated measures analyses of variance (two repeated factors: cost [levels 1-5] and reinforcers). Differences between trials involving the social partner and food reinforcers were analysed with the total sample of 12 animals. The categorical factor 'social status' (territorial/non-territorial) was added to this ANOVA model in order to assess its possible influence in the variation. Planned comparisons of least squares means were carried out when appropriate. All the analyses involving the control reinforcer included only five animals (the first block of tested animals). When there were significant differences in the variances between conditions (Levene's Test), data were normalised using the transformations proposed by Zar (1984), namely log transformation for latencies (seconds) and *arcsin* transformation for work efficiency (percentages). Differences in maximum price paid between social partner and food reinforcers were analysed using the Wilcoxon Matched Pairs Test. Differences in maximum price paid among the three reinforcers (including control) were analysed with a Friedman ANOVA (n=5). All analyses after access to the RC also involved repeated measures ANOVAs (repeated factor: cost [levels1-5]; categorical predictor: social status or area). A value of $P<0.05$ was taken for significance in all statistical tests. All analysis was performed using the statistical package (StatSoft Inc, USA, 1984-2008).

2.7. Ethical Note

The experiments described were conducted in accordance to national legal standards on protection of animals used for experimental purposes and are part of a project approved by the national authorities (Ref. 30489, 29/11/2007).

3. RESULTS

3.1. Behavioural strategy for reinforcers access

In general, there was a mixed exhibition of pushes and touches, but each animal tended to increase the performance of one of them and in this way reached the amount of work pre-established to open the door. When the reinforcer was food, 51% of the animals which passed the door, opened it through pushes and 49% through touches. For the social partner, touches prevailed with 81% of the animals, against 18% which opened the door by pushes. The control resource was never accessed through pushes since 100% of the animals which accessed the resource compartment did it by reaching the scheduled number of touches. Occasionally, some animals achieved the cost imposed simultaneously through the required number of touches and pushes.

3.2. Measures of motivation

Latency to open the door increased with cost for social partner and food (repeated measures ANOVA, $F_{(4,44)} = 67.6$, $P<0.001$; Figure 2a), with no differences between these reinforcers (repeated measures ANOVA, $F_{(1,11)} = 1.8$, NS; Figure 2a). The latency to access the control reinforcer (additional space/substrate) also increased with cost (repeated measures ANOVA, $F_{(4,16)} = 19.8$, $P<0.001$; Figure 2a) and it was the resource to which the access latency was the highest (repeated measures ANOVA, Reinforcer*Price (n=5): $F_{(8,32)} = 3.3$, $P=0.007$; Figure 2a). There was no overall significant influence of social status in the latency to get access to social partner or to food (repeated measures ANOVA, $F_{(4,40)} = 0.3$, NS; Figure 2b). However, at the highest prices (level 4 and 5), latency of non-territorial males increased significantly in contrast with the behaviour of territorial males (planned comparisons of LS means, $P<0.001$; Figure 2b).

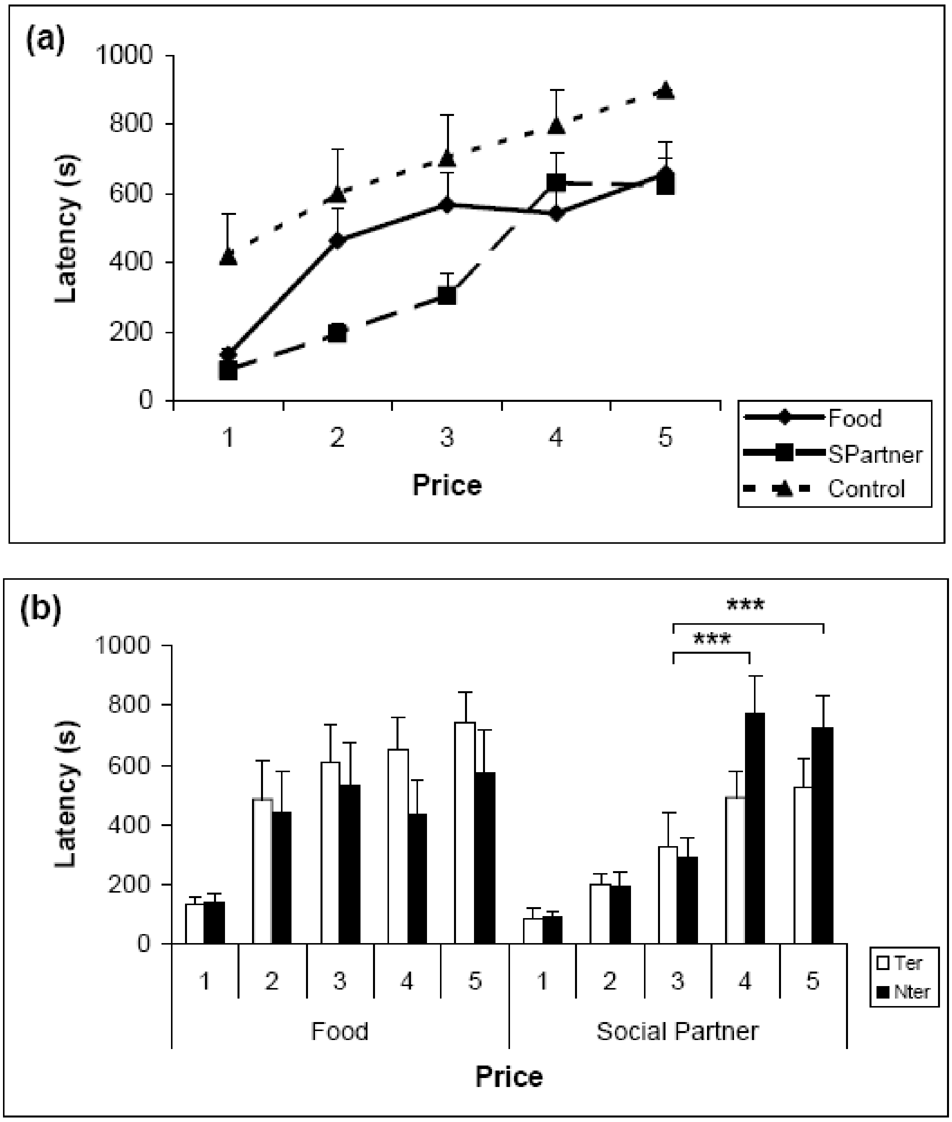


Figure 2. Latency (s) for opening the door as price increases (a) with different reinforcers: food (n=12), social partner (n=12) and control (n=5) (b) in territorial and non-territorial males with food and social partner (n=12). ***P<0.001

Work efficiency (percentage of time spent working at the door in relation to the latency to door's opening) to access a social partner or food was similar at any cost (average value of 77.5% for food and 78.2% for social partner; repeated measures ANOVA, Reinforcer*Price: $F_{(4,44)}$ = 1.8, NS; Figure 3a). Work efficiency to get access to the control reinforcer (additional space/substrate) was lower than for social partner and food (average value of 51.9%; repeated measures ANOVA, (n=5): $F_{(2,8)}$ = 8.4, P=0.01, Figure 3a) and tended to decrease with cost (from 66.5% at FR1 to 32.4 at FR%; repeated measures ANOVA, (n=5): $F_{(4,16)}$ = 1.6, P=0.08, Figure 3a). Social status tended to influence work efficiency to access a social partner or food (repeated measures ANOVA, $F_{(4,40)}$ = 2.5, P=0.06; Figure 3b). Similarly to latency, this is particularly clear at high access prices (in this case, level 4), where work efficiency of non-territorial males decreased significantly in contrast with behaviour of territorial males (planned comparisons of LS means, P<0.05, Figure 3b).

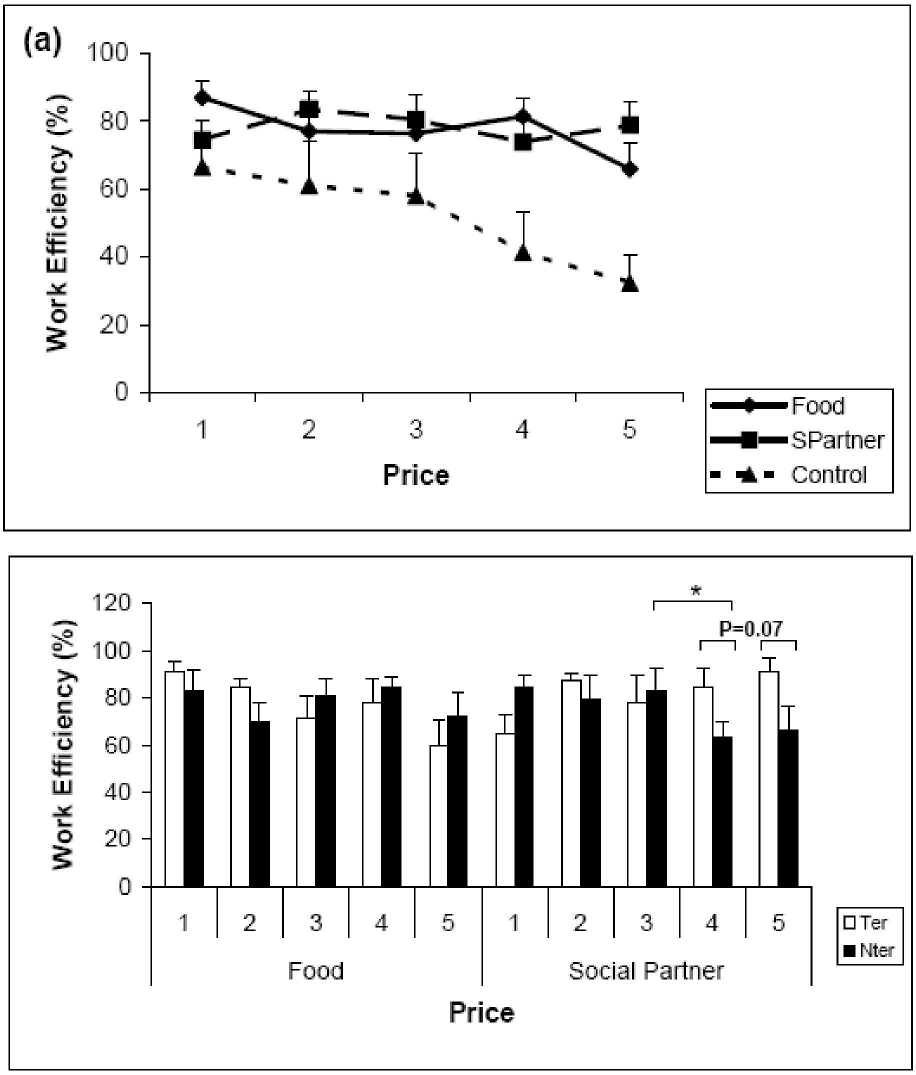


Figure 3. Work efficiency (%) for opening the door as price increases (a) with different reinforcers: food (n=12), social partner (n=12) and control (n=5) (b) in territorial and non-territorial males with food and social partner (n=12). *P<0.05.

There was no difference between maximum price paid to have access to social partner or food (Wilcoxon matched pairs test, Z=0.6, NS [n=12]; Table 2). Differences between territorial and non-territorial males were not statistically significant. Maximum price paid for access to the control reinforcer (additional space/substrate) is lower than to social partner/food (Friedman ANOVA, χ^2 [n=5; df = 2] = 7.7, *P*<0.05; Table 2).

Table 2. Maximum price paid (average ± standard error) by territorial and non-territorial males for food (n=12), social partner (n=12) and control (n=5) as expressed by the weight of the door.

	Food	Social Partner	Control
Territorial*	100±37g	170±29g	25±25g
Non-territorial**	121±38g	100±32g	50±25g

*N=6 except for control (N=2)
**N=6 except for control (N=3)

3.3. Behaviour with reinforcers

All animals ate the food immediately after entering the RC. There was no difference in latency to start eating the food with different access costs or between social status (repeated measures ANOVA: $F_{(4,40)} = 0.8$, NS). All animals ate the available pellets without interruptions.

Use of area after having access to the reinforcer was compared for social partner and the control (additional space/substrate). In both cases there were clear differences in the use of area, as time spent in the area with substrate (RC_1) was higher than in the area without substrate (RC_0), with or without female (repeated measures ANOVA, social partner [n=12]: $F_{(2,33)} = 213.3$, $P<0.001$; substrate [n=5]: $F_{(2,12)} = 70.6$, $P<0.001$, Figure 4). When the social partner reinforcer was tested, males hardly visited the start compartment (SC), while when there was no social partner (control reinforcer) areas SC and RC_0 were indistinctively used.

After having passed the door, time spent close to the female was the same regardless of the cost paid and social status (average of time spent close to female along the five different costs: 32.8±2.9%; repeated measures ANOVA: $F_{(4,40)} = 1.2$, NS). However, territorial males spent more time interacting with the female than non-territorial males (average of time interacting with the female along the five different costs: territorial, 29.0±4.2%; non-territorial, 22.9±3.4%; repeated measures ANOVA: $F_{(1,10)} = 6.8$, P=0.03). There was no difference in male ‘time interacting with substrate’ along the different access costs (repeated measures ANOVA: $F_{(4,40)} = 0.55$, NS) and between social status when the reinforcer was the social partner (repeated measures ANOVA: $F_{(1,10)} = 1.71$, NS) and when it was only substrate/control (repeated measures ANOVA: $F_{(4,12)} = 0.62$, NS).

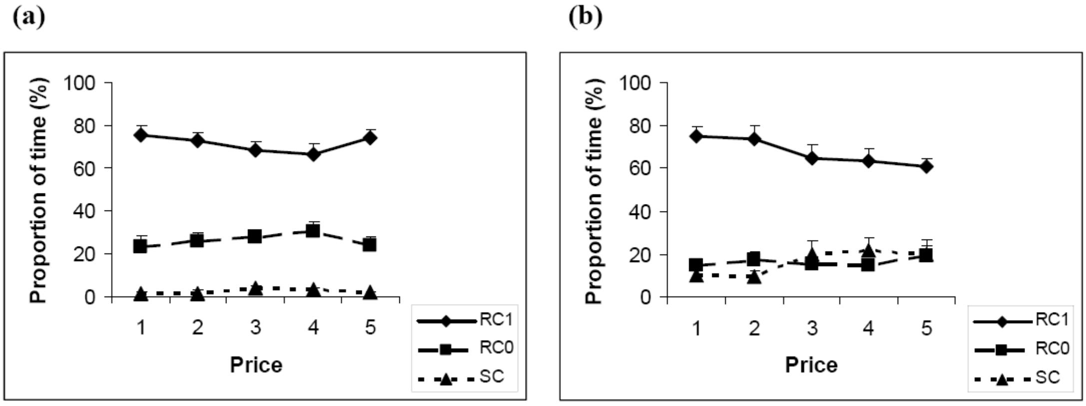


Figure 4. Proportion of time (%) spent in area 1 of resource compartment (RC1), area 0 of resource compartment (RC0) and in the start compartment (SC) during the period of access to the reinforcer (a) social partner (n=12) (b) control (n=5).

4. DISCUSSION

Latency to open the door increased with access cost for all resources, with the highest latency linked to the control reinforcer. Work efficiency was higher and constant for the social partner and food, and lower and decreasing with increasing price for the control reward. Maximum price paid was consistent with these results, being higher for social partner and food than for the control reinforcer. Non-territorial males seemed less willing to work at higher costs for access to the social partner.

4.1. The push-door, behavioural strategies to open it and reinforcement schedule

This study is the first that used a push-door to quantify motivation in fish. The results suggest that the adapted push-door is an operant paradigm potentially successful for Mozambique tilapia. Animals easily became familiar with the door and quickly adjusted to the task. They developed two spontaneous behavioural responses that may correspond in fact to a behavioural continuum (touches and pushes at the door). One of the criticisms of motivational tests is related to the very extensive and complex training protocols, which may jeopardise the original objective of these tests, the measurement of spontaneous motivation (Würbel, 2009). For this reason, training was kept to the minimum necessary to ensure that animals did understand the fact that their own behaviour towards the door could open it. Therefore touches and pushes were both accepted as work developed at the door. Despite the decision to manually open the door at certain fixed costs, some males did push it strongly enough to open and pass (amplitude of push ≥45°, Table 1).

When the reinforcer was food, more animals opened the door through pushes than touches. The social partner caused a visible decrease in number of animals opening the door through pushes, and the control resource was never accessed by pushes. It is possible that the access for food triggered a more intense physical reaction in the fish. This does not mean that they have worked faster or that they paid more attention to the task of accessing food, as latency and work efficiency have respectively shown. In other words, they were not necessarily more motivated for access to food. This preference for pushing for food may reflect the fact that they were trained exclusively with food. Thus, they may have had an additional opportunity to learn a more intense physical response related to the acquisition of this particular reinforcer.

The reinforcement schedules imposed whether for number of touches or door’s weight, and the five minute bouts, were decided on the basis of a previous pilot study and confirmed to be well adjusted to the aim. The adopted measures of motivation showed that animals progressively and consistently adjusted their behaviour to increased costs. It is possible that some males could have opened doors at even higher costs than those imposed. However, the inclusion of more trials in the experiment would be too time-consuming for the objectives of the present study.

4.2. Open *versus* closed economy, training and rewards

The three reinforcers used (female, food and additional space with substrate only) are all potential resources for the ecology and welfare of this species (Chapters II and III). The present study was developed in an open economy set up, despite the acknowledged criticisms in the validity of open economies to measure motivation (Mason et al., 1998b; Kirkden and Pajor, 2006). The aim was to test the usefulness of the push-door for this species and not to establish definitive comparisons between resources. Therefore, the main priority was to design a relatively simple apparatus and to keep the animals non-stressed, enabling them to handle the task as well as possible. A closed economy would have required maintaining the animals in social isolation during the study or to design an apparatus in which the animals could live as in Mason’s studies (e.g. Mason et al., 2001; Hovland et al., 2006), which was not feasible without first validating the use of the adapted push-doors. A previous pilot study showed how fish did not pay attention to the push-door after a period of social deprivation (some hours to 2 days). Some authors have already shown how fish learning can be affected by stress and high levels of cortisol (Moreira et al., 2004; Moreira and Volpato, 2004; Barreto et al., 2006).This is in line with recent studies where males of this species have shown the highest cortisol levels in result of social deprivation (Chapter V). The potential detrimental effects of social isolation for operant tasks were also discussed for pigs (Pedersen et al., 2002). An open economy was also adopted for the control reinforcer (substrate only) to avoid changing the stability of the home tanks by removing the substrate (Chapter II). The food reinforcer was tested in a somewhat less open economy as the daily amount of food was delivered exclusively during the trials. However, as mentioned before, the animals could still nip particles of food in the substrate when back to the home tanks. The constraints of the present experimental design were taken into consideration when interpreting the behavioural results to have access to the three reinforcers. It is clear that fish were able to rank reinforcers, as social partner and food seemed more attractive than substrate only, but conclusions about absolute value of these resources can only be drawn with further refinement.

The adopted training method was very successful in controlling fear and in getting animals familiar with the operant task. However, the decision of using food as the training reinforcer had two main disadvantages. First, some fish less motivated to eat could have presented unnecessary difficulties during training. Second, by using food as a training reinforcer and testing it later with other rewards, this resource may have been favoured over others. Training sessions involving the simultaneous use of all rewards under test (if compatible) could be a strategy to take maximum advantage of individual motivations without favouring any reward in particular. At the same time, such a strategy could function as a particularly attractive stimulus which is welcome in training sessions.

A caution note should be added in relation to food as a yardstick, beyond the aspects already discussed by Hovland et al. (2007) on how food is presented. In studies involving mammals and birds, this resource is usually assumed as the most inelastic, against which motivation for access to other resources is compared (Dawkins, 1990; Kirkden and Pajor, 2006). However, this is not necessarily the case for fish due to a very different metabolism, where food is frequently not the most limiting need (Monaghan, 1990). A high priority to eat is also not only a species-dependant issue, but can vary with different life stages. For example, during breeding contexts in natural conditions, Mozambique tilapia spends periods of less or no eating (Neil, 1966). Therefore, it is expected that motivation to breed may decrease motivation to feed in this species.

Reward size in this experiment was simply related to the maintenance of its attractiveness throughout the trials. This was because the objective was to measure access to reward and not reward consumption (demand measures). In relation to food, the balance achieved was to keep the usual meal size per trial session. This approach did not satiate the animals, but given the usual routine, it was also unlike to frustrate them. Instead of investing in quantity, an effort was made to manipulate the fish positive perception about food (see material & methods above for details, Galhardo and Oliveira, 2009). In relation to access to the social partner, 30 minutes of free contact with the female was judged to be enough time to avoid aversive interruption of social interactions, as mentioned before. Probably, as a consequence of this unconstrained interaction, consumption in relation to the female did not change with access cost. However, it is interesting to note that once with the female, males no longer left the resource compartment (swimming back to the start compartment). In contrast, when tested for control (additional space/substrate), males turned back to the start compartment a number of times, probably because they valued this extra space (SC). In any case, all males preferred to stay in the area with substrate. For a more complete understanding of behaviour with the reward, it would have been useful making behavioural samples for the complete period of 30 minutes, and not only for the first 10 minutes, as was the case.

4.3. Measures of motivation

The three measures of motivation adopted (latency, work efficiency and maximum price paid) were chosen as the most appropriate indices to measure access to a reward in face of the experimental design. All of them distinguished between social partner/food rewards and substrate (control) in a consistent way. The increased latencies for the control (additional space with substrate only) suggested that animals worked harder for the social partner and food access, thus preferring these resources. In order to avoid inappropriate analysis of motivation based in work effort when two different behaviours were involved (pushes and touches), ‘work efficiency’ was adopted as a measure of motivation. This parameter measured degree of attention and investment at the door regardless the type of behaviour the animals engaged in. Results have shown that work efficiency was similar to social partners and food and lower to a substrate-only compartment. That is, social partner and food attracted more of fish attention and made them to develop a more prolonged behavioural response than substrate-only. Maximum price paid has also pointed in the same direction. It is to be noted that once animals stopped opening the door, they would not open it again in subsequent trials (except for two animals, out of 12), which made this index quite consistent.

Social status influenced the motivation indices to have access to the social partner: the non-territorial males showed higher latencies and lower work efficiency at higher costs. On the other hand, territorial males interacted more with the females at any costs. Therefore, the results consistently suggest that territorial males were more motivated to have access to females at higher costs than non-territorial males.

4.4. Conclusion

The existence of a paradigm to quantify the value fish attach to certain resources is potentially very useful in a number of behavioural, physiological, cognitive and welfare studies. Information derived from these studies has also relevant implications for the management of their welfare in captivity, namely in the identification of relevant needs.

The present study has shown that the push-door can be used to measure motivation for access to resources in this species. In future studies the experimental set up can be refined in order to increase validity of motivation measures in relation to the present and other resources. The adaptation to an automated, computer controlled push-door is one of the aspects to improve, as it can reduce sampling effort. In this way, sampling sizes can increase, which is important for better interpretation of individual differences. To test the animals in closed economy, to deal with all the resources in the same way and, eventually, to refine the reward sizes are other important steps towards a more precise motivation measuring. It is also relevant to keep the animals healthy, not stressed and appropriately trained. Provided these aspects are taken into consideration, the push-door can successfully measure this species motivation.

5. Acknowledgments

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CHAPTER V

Measuring cortisol in Mozambique tilapia: the effects of diurnal variation, social isolation and ACTH challenge

Galhardo, L. Almeida, O., Oliveira, R.F. Measuring cortisol in Mozambique tilapia: the effects of diurnal variation, social isolation and ACTH challenge. Submitted to *Journal of Fish Biology*.

Abstract

Cortisol is widely used as an indicator of stress in teleosts but the interpretation of its variation is complicated due to a wide range of internal and external factors. This study was designed to characterize several factors that may modulate cortisol levels in a cichlid fish commonly used in fundamental and applied research, the Mozambique tilapia *Oreochromis mossambicus*, namely diurnal variation, social status, social isolation and response to an ACTH challenge. Androgen (testosterone and 11-ketotestosterone) levels in social isolation were also analysed for better understanding of eventual relationships between cortisol and these hormones.

Diurnal variation showed a gradual increase of cortisol levels in the dark period, with a peak in early morning just before the lights were switched on, and no significant variation during the light period. These cortisol levels were found comparable to those described for other diurnal animals, including some fish species. Social isolation caused an increase of cortisol in non-territorial males and a decrease of 11-ketotestosterone (11-KT) in territorial males. No differences were found in levels of 11-KT and testosterone between these two male types in isolation. The activation of the hypothalamus-pituitary-interrenal tissue axis induced by social isolation should be taken into consideration when using this experimental procedure as a way to obtain baseline hormonal levels in stress studies. The *in vivo* ACTH challenge showed a threshold mechanism. Cortisol response varies from an identifiable baseline (approximately 25 ng/ml, for ACTH doses below 10^{-3} µg/g), which was not elevated by handling stress, to an upper plateau (100-120 ng/ml for ACTH doses above 10^{-2} µg/g), against which other stress levels under similar conditions can be compared.

These findings can contribute to better understand the cortisol response of *O. mossambicus* and allow comparisons with stress studies undertaken under similar conditions. This kind of knowledge is central for a better stress management of fish species in captivity.

1. Introduction

Cortisol is widely measured as an indicator for stress in teleosts. This cholesterol-derived steroid is synthesized in the interrenal cells (homologous of adrenal cortex in mammals), which are distributed around the walls of the posterior cardinal veins of the head-kidney (Janz and Weber, 2000). Cortisol is produced upon the stimulation of a number of hormones, but especially under the control of the pituitary adrenocorticotrophic hormone (ACTH) (Flik et al., 2006). ACTH is synthesised in the corticotroph cells, located at the anterior pituitary and its release is triggered by the synergistically action of corticotrophin-releasing hormone (CRH) and arginine vasotocin (AVT) (Balment et al., 2006). Both hormones are secreted in the hypothalamus: CRH in preoptic nucleus (Chen and Fernald, 2008) and AVT in different subsets of neurons (Balment et al., 2006). Hypothalamic nuclei receive numerous inputs related to metabolic, circadian, sensorial and emotional processes, which makes the hypothalamic-pituitary-interrenal tissue axis very sensitive to all these internal and external inputs (Mormède et al., 2007). Cortisol plays an important role in maintaining the osmotic homeostasis and in modulating the allocation of metabolic resources (Mommsen et al., 1999; Mormède et al., 2007). Under stress conditions, it is responsible for increasing blood glucose levels which helps animals to cope with the stressors (Mommsen et al., 1999). Therefore, under acute stress conditions cortisol rise is an adaptive and desired response (Sapolsky, 2004). However, in conditions of prolonged exposure to stressors, where more complex metabolic adaptations are required, cortisol can impair growth, depress the immune system, reduce the reproduction success and significantly alter cognitive function (Mc Ewen, 1998).

Cortisol turned into a very common indicator of stress due to a number of reasons. Its levels increase in response to a great number of putative stressors. It can be easily measured in blood plasma (e.g. Foo and Lam, 1993), urine (e.g. Oliveira and Almada, 1996) and water (e.g. Scott et al., 2008; e.g. Leong et al., 2009) through, among others, commercially available radio or enzyme immunoassay (RIA or LIA) or enzyme-linked immunosorbent assay kits (ELISA). It is possible to measure experimental baseline levels with appropriate sampling and anaesthesia (Mommsen et al., 1999). In contexts of prolonged stressors' exposure, it is also possible to adopt more dynamic approaches through cortisol measurement in response to HPI stimulation (through CRH or ACTH challenges) or inhibition (with dexamethasone) (Hontela, 1998; Mormède et al., 2007).

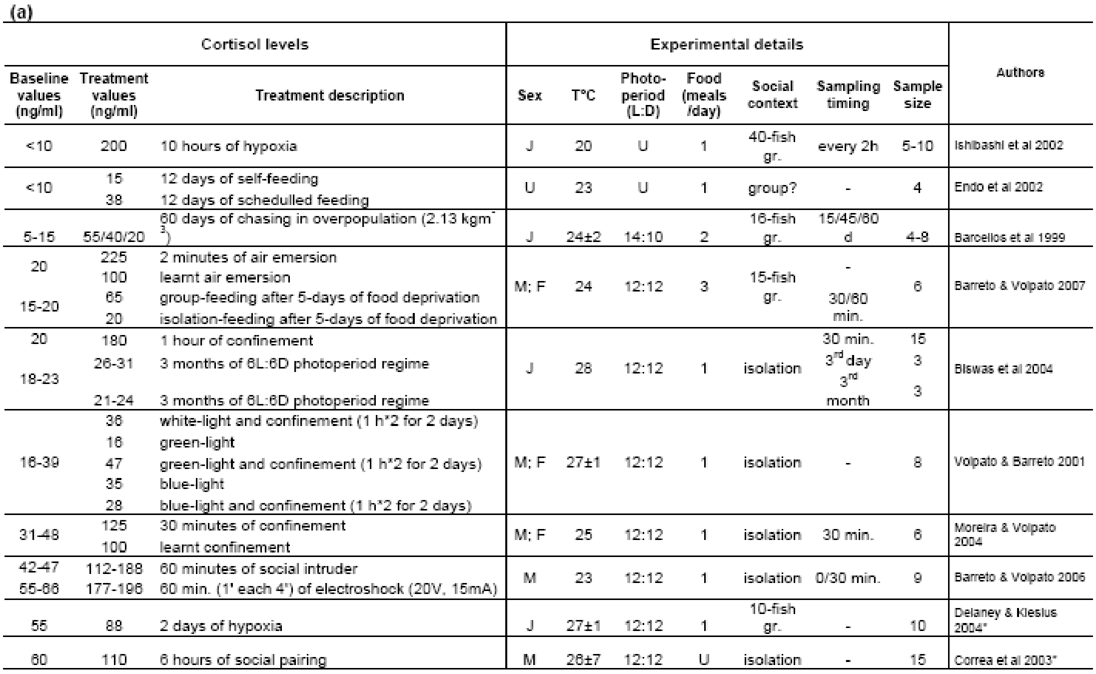
The HPI axis functioning is well studied in a number of fish species (Sumpter, 1997; Barton, 2002), but the interpretation of its activation is less clear (Lane, 2006; Mormède et al., 2007). Very different stress levels of cortisol are described in literature. For example, a number of studies restricted to the closely related tilapia species, Nile tilapia *Oreochromis niloticus* (Linnaeus) and Mozambique tilapia *Oreochromis mossambicus* (Peter), have produced very different levels of baseline and stress cortisol levels (Table 1a and 1b, respectively). Wide variation in teleost cortisol levels may be due to an extensive range of factors.

Internal factors are related to differences among species and breeds (Barton, 2002), sex (Øverli et al., 2006), individual coping styles (Koolhaas, 1994) and sexual maturity (Goymann and Wingfield, 2004). Seasonal and diel rhythms are responsible for non-behavioural related fluctuations of cortisol in a number of fish species (Sánchez-Vázquez et al., 1998; Lankford et al., 2003). Exposure to stressors early in life may have long-lasting consequences for cortisol responses, as recently demonstrated by Auperin and Geslin (2008), with a cortisol reduction in rainbow trout. A further internal confounding factor is that a return to basal levels during chronic exposure to stressors might not indicate adaptation to the external stimuli, but rather an exhaustion of the cortisol production by overstimulation of the HPI axis (Sumpter, 1997; Barton, 2002).

Important external factors also induce variations of cortisol levels. The type of stressor and its intensity and duration are well known modulators of the stress response (Barton, 2002). Factors such as photoperiod, environmental temperature and feeding time not only can affect the stress response but in particular may interfere with the cortisol circadian rhythm in different fish species (Cerdá-Reverter et al., 1998; Lankford et al., 2003). Experimental procedures can also be a major source of variation in cortisol levels. For example, the timing and duration of handling, anaesthesia and blood sampling procedures can activate the HPI axis *per se* (Foo and Lam, 1993). Handling stress can produce variable levels of stress, which some authors try to minimise through habituation procedures (Quabius et al., 1997; Nolan et al., 1999) or conditioning techniques (Schreck et al., 1995). Social dynamics can be responsible for variable cortisol responses. Some studies found elevated cortisol levels in individuals with a non-territorial/subordinate position in the social rank (e.g. Munro and Pitcher, 1985; Gilmour et al., 2005). Others reveal cortisol increases in both subordinate and dominant animals under unstable social contexts (e.g. Correa et al., 2003). Clement and colleagues (2005) proposed that the influence of social dynamics on cortisol levels

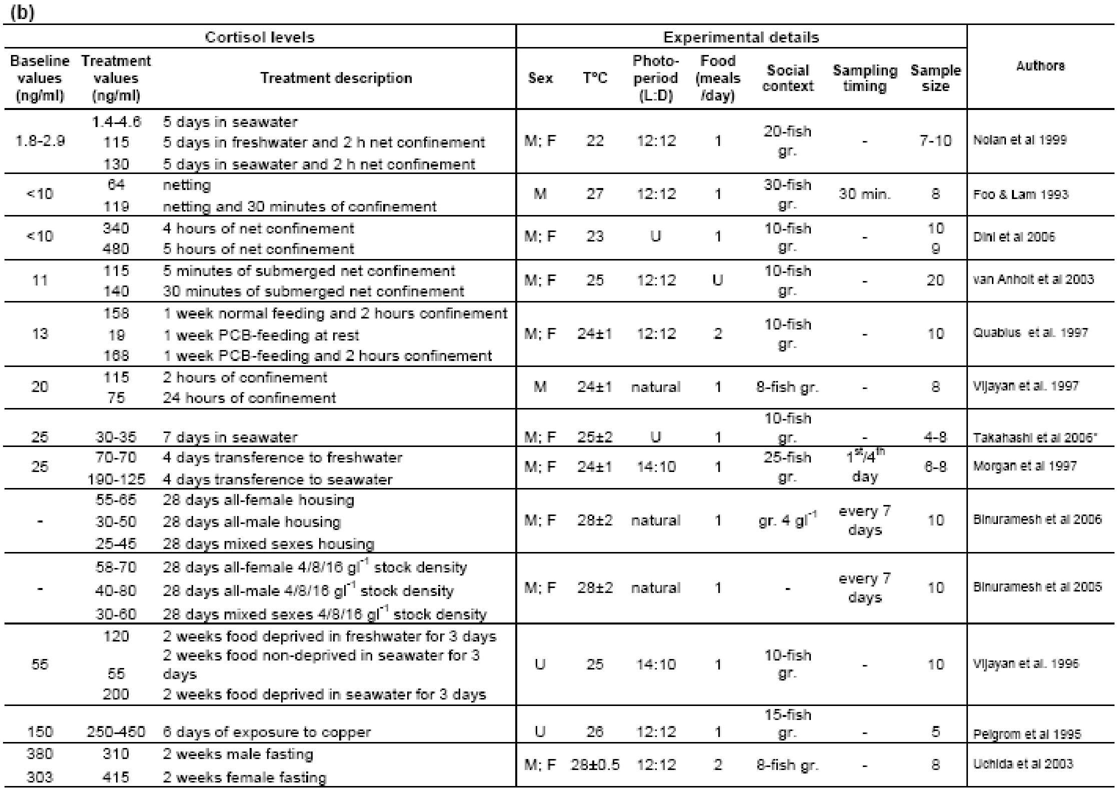
Table 1. Baseline and treatment cortisol levels of (a) *Oreochromis niloticus* and (b) *Oreochromis mossambicus* in different studies. Baseline levels were sampled under resting/undisturbed or pre-treatment conditions. Treatment description excludes periods of acclimation. Sex: M, males; F, females; J, juveniles; U, undetermined. Photoperiod: L, light; D, dark periods. All cortisol levels were measured from blood, through radioimmunoassays with exception of studies marked with* in the 'authors' column, which have used enzyme immunoassays. Samplings' time of day were rarely mentioned.

(a)



Cortisol levels			Experimental details							Authors
Baseline values (ng/ml)	Treatment values (ng/ml)	Treatment description	Sex	T°C	Photo-period (L:D)	Food (meals /day)	Social context	Sampling timing	Sample size	
<10	200	10 hours of hypoxia	J	20	U	1	40-fish gr.	every 2h	5-10	Ishibashi et al 2002
<10	15 38	12 days of self-feeding 12 days of schedulled feeding	U	23	U	1	group?	-	4	Endo et al 2002
5-15	55/40/20	60 days of chasing in overpopulation (2.13 kgm^{-3})	J	24±2	14:10	2	16-fish gr.	15/45/60 d	4-8	Barcellos et al 1999
20 15-20	225 100 65 20	2 minutes of air emersion learnt air emersion group-feeding after 5-days of food deprivation isolation-feeding after 5-days of food deprivation	M; F	24	12:12	3	15-fish gr.	- 30/60 min.	6	Barreto & Volpato 2007
20 18-23	180 26-31 21-24	1 hour of confinement 3 months of 6L:6D photoperiod regime 3 months of 6L:6D photoperiod regime	J	28	12:12	1	isolation	30 min. 3rd day 3rd month	15 3 3	Biswas et al 2004
16-39	36 16 47 35 28	white-light and confinement (1 h*2 for 2 days) green-light green-light and confinement (1 h*2 for 2 days) blue-light blue-light and confinement (1 h*2 for 2 days)	M; F	27±1	12:12	1	isolation	-	8	Volpato & Barreto 2001
31-48	125 100	30 minutes of confinement learnt confinement	M; F	25	12:12	1	isolation	30 min.	6	Moreira & Volpato 2004
42-47 55-66	112-188 177-196	60 minutes of social intruder 60 min. (1' each 4') of electroshock (20V, 15mA)	M	23	12:12	1	isolation	0/30 min.	9	Barreto & Volpato 2006
55	88	2 days of hypoxia	J	27±1	12:12	1	10-fish gr.	-	10	Delaney & Klesius 2004*
60	110	6 hours of social pairing	M	26±7	12:12	U	isolation	-	15	Correa et al 2003*

(b)



Cortisol levels			Experimental details							Authors
Baseline values (ng/ml)	Treatment values (ng/ml)	Treatment description	Sex	T°C	Photo-period (L:D)	Food (meals /day)	Social context	Sampling timing	Sample size	
1.8-2.9	1.4-4.6 115 130	5 days in seawater 5 days in freshwater and 2 h net confinement 5 days in seawater and 2 h net confinement	M; F	22	12:12	1	20-fish gr.	-	7-10	Nolan et al 1999
<10	64 119	netting netting and 30 minutes of confinement	M	27	12:12	1	30-fish gr.	30 min.	8	Foo & Lam 1993
<10	340 480	4 hours of net confinement 5 hours of net confinement	M; F	23	U	1	10-fish gr.	-	10 9	Dini et al 2006
11	115 140	5 minutes of submerged net confinement 30 minutes of submerged net confinement	M; F	25	12:12	U	10-fish gr.	-	20	van Anholt et al 2003
13	158 19 168	1 week normal feeding and 2 hours confinement 1 week PCB-feeding at rest 1 week PCB-feeding and 2 hours confinement	M; F	24±1	12:12	2	10-fish gr.	-	10	Quabius et al. 1997
20	115 75	2 hours of confinement 24 hours of confinement	M	24±1	natural	1	8-fish gr.	-	8	Vijayan et al. 1997
25	30-35	7 days in seawater	M; F	25±2	U	1	10-fish gr.	-	4-8	Takahashi et al 2006*
25	70-70 190-125	4 days transference to freshwater 4 days transference to seawater	M; F	24±1	14:10	1	25-fish gr.	1st/4th day	6-8	Morgan et al 1997
-	55-65 30-50 25-45	28 days all-female housing 28 days all-male housing 28 days mixed sexes housing	M; F	28±2	natural	1	gr. 4 gl^{-1}	every 7 days	10	Binuramesh et al 2006
-	58-70 40-80 30-60	28 days all-female 4/8/16 gl^{-1} stock density 28 days all-male 4/8/16 gl^{-1} stock density 28 days mixed sexes 4/8/16 gl^{-1} stock density	M; F	28±2	natural	1	-	every 7 days	10	Binuramesh et al 2005
55	120 55 200	2 weeks food deprived in freshwater for 3 days 2 weeks food non-deprived in seawater for 3 days 2 weeks food deprived in seawater for 3 days	U	25	14:10	1	10-fish gr.	-	10	Vijayan et al. 1996
150	250-450	6 days of exposure to copper	U	26	12:12	1	15-fish gr.	-	5	Pelgrom et al 1995
380 303	310 415	2 weeks male fasting 2 weeks female fasting	M; F	28±0.5	12:12	2	8-fish gr.	-	8	Uchida et al 2003

may also be dependent on individual coping strategies, such as the performance of displaced aggression.

The use of cortisol levels as an indicator of stress demands to take into account these sources of variation (Mormède et al., 2007). The aim of our study is to contribute to a better interpretation of cortisol response in the cichlid fish *Oreochromis mossambicus* by considering some of the above mentioned factors, such as diurnal variation, social isolation, and social status. *O. mossambicus* is a territorial species, with males forming leks in shallow waters during the breeding season. Individual territories are established and pits are built to attract ripe females for spawning (Neil, 1966; Nelson, 1995). This species is widely cultured in tropical parts of the world (Binuramesh et al., 2006) and is increasingly used for scientific purposes (e.g. Vijayan et al., 1997a; van Anholt et al., 2003; Takahashi et al., 2006), which imply potentially stressful artificial conditions. A better description of its cortisol response is thus a relevant step for stress-management in captivity for this species, and for a clearer interpretation of stress levels measured in scientific studies with captive individuals.

The present study has the following aims: 1. to characterise the diurnal variation of cortisol in territorial and non-territorial males' kept in social groups (experiment 1a); 2. to analyse the effect of social isolation on cortisol levels of territorial and non-territorial males, and to analyse levels of 11-ketotestosterone and testosterone for better results' interpretation (experiment 1b); 3. to identify the cortisol range of response, using an ACTH challenge (experiment 2).

2. Materials and Methods

2.1. Fish and housing conditions

Experimental fish of the species *O. mossambicus* (experiment 1 and 2) belong to a stock held at ISPA (Oliveira et al., 1996). In their home tanks, they were kept in social groups of 2-3 males and 4-5 females. Fish were maintained in 240l aquaria (glass, 120x40x50 cm), at a temperature of 26ºC±1ºC on a 12L: 12D photoperiod. Each tank had a layer of fine gravel substrate. Tanks were supplied with a double filtering system (gravel and external biofilter, Eheim) and constant aeration. Water quality was analysed twice per month for nitrites (0.2-0.5 ppm), ammonia (<0.5 ppm) (Pallintest kit®) and pH (6.0-6.2). Fish were daily fed (in the morning) with 2% of their weight of commercial cichlid sticks (ASTRA).

2.2. Diurnal variation and effects of social isolation

2.2.1. Experimental conditions

Sixteen males (weight: 82±5.3 g) were used in both studies reported here. In the first study (diurnal cortisol variation), social groups were composed by two males (focal animals) and three females per tank (M/F sex ratio=0.67), left under the same environmental conditions as described for their home tanks. These groups were left undisturbed for three weeks, in order to allow the formation of stable hierarchical structures (Oliveira and Almada, 1998). Males' social status was identified prior to the experiment. Territorial males adopt a conspicuous nuptial black coloration and exhibit reproductive behaviour, including territory defence and digging of a spawning pit in the substrate.

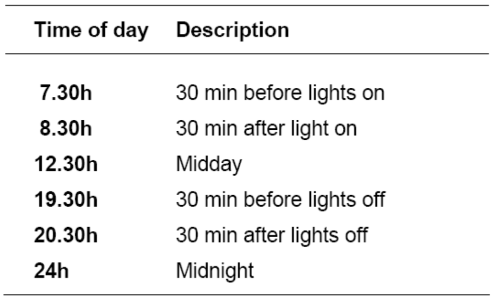
In the second study (effects of social isolation on cortisol and androgens), after having been in stable social groups, the sixteen territorial and non-territorial males were individually housed in aquaria (50×25×31cm, 40 l) of which the walls were made opaque in order to visually isolate fish from each other. For one week, all conditions remained the same as in the home tanks.

2.2.2. Sampling

In the first study (diurnal variation), the behavioural and blood sampling method included six different sampling points at different times of the day (Table 2). Due to technical problems in the laboratory, only 7 out of 16 animals could be sampled for the sampling point at 20.30h. The experiment lasted for six weeks (after three weeks of social acclimation), in which each fish was sampled once a week for the correspondent sampling point. The two males belonging to the same aquarium were sampled in different days to minimise disturbance in the aquarium. Locomotory activity of each fish was measured for two minutes just before capture, by counting the number of times the snout crossed a line in a grid marked on the front part of the glass aquaria (eight squares). For that purpose a focal continuous sampling method was adopted (Martin and Bateson, 2007). Locomotory sampling and manipulations during the lights-off periods were undertaken under an illumination provided by a low intensity background light (red lamp) which is likely to provoke minimum nocturnal disturbance (Oliveira et al., 2007; Seehausen et al., 2008).

In the second study of this first experiment (social isolation), blood sampling for steroids analysis took place at the 7th day of isolation. The procedure was the same for

Table 2. Sampling points for the daily variation of cortisol in *O. mossambicus*.



Time of day	Description
7.30h	30 min before lights on
8.30h	30 min after light on
12.30h	Midday
19.30h	30 min before lights off
20.30h	30 min after lights off
24h	Midnight

both parts of the experiment: fish were rapidly hand net captured, where after they were lightly anaesthetised [Stage two (Ross, 2001)] in a solution of MS-222 (tricaine methane sulphonate, Sigma, St. Louis, MO, 200 ppm). Samples of 100-150 µl of blood were taken from the caudal vasculature (1 ml heparinised syringes; 25G/16 mm needles). The fish were then placed in aerated water 30 seconds to one minute, in order for them to recover from anaesthesia. The anaesthesia induction and blood sampling were performed within a maximum of four minutes, which is the latency for cortisol release into systemic circulation in response to handling stress at 27ºC (Foo and Lam, 1993). The non-conjugated, referred to as free, cortisol fraction was extracted from the plasma by adding to the sample diethyl ether, as the steroid solvent. The samples were then centrifuged (5 minutes, 1000 rpm, 4ºC) and cooled for 10 minutes at -80ºC to separate the ether fraction, which remained liquid, from the frozen aquatic fraction. The steroids were isolated by evaporating the ether. This process was repeated twice.

All hormone levels were quantified by radioimmunoassay. Cortisol assays used the commercial antibody ‘Anti-rabbit, Cortisol-3’ [ref: 20-CR50, Interchim (Fitzgerald), Montluçon, France, cross-reactivity: cortisol 100%, Prednisolone 36%, 11-Desoxycortisol 5.7%, Corticosterone 3.3%, Cortisone < 0.7%] and the radioactive marker [1,2,6,7-3H] Cortisol [ref: TRK407-250mCi, Amersham Biosciences, Piscataway, NJ/USA]. Cortisol intra- and inter-assay variability was 5.8% and 6.5% respectively. Levels of free 11-ketotestosterone fraction were determined using an antibody kindly donated by D. E. Kime, which the corresponding specificity table was published in Kime and Manning (1982). The testosterone antibody (reference: RDITRK2T2) was purchased from Research Diagnostics Inc. (Concord, USA). Testosterone and 11-ketotestosterone intra-assay variability was 2.5% and 1.1% respectively.

2.2.3. Data Analysis

Statistical analysis was conducted to assess the effect of time of day on locomotory activity and cortisol levels, using a two-way repeated measures analyses of variance for males (repeated factor: time of day [diurnal variation], social context [social isolation]; categorical factor: social status). Variances between conditions (Levene's Test) were not significantly different. Following the ANOVAs, planned comparisons of least squares means were performed between conditions (experiment 1a: dark/light periods and territorial/non-territorial; experiment 1b: social group/isolation and territorial/non-territorial). A value of $P < 0.05$ was taken for significance in all statistical tests. The statistical package used for analysis was Statistica V.8® (StatSoft Inc, USA, 2008).

2.3. ACTH challenge

2.3.1. Experimental conditions

Twenty eight males (weight: 59.5±5.9 g) were used in this experiment. Only territorial males were involved in order to minimise eventual cortisol variability. Since prior experiments have shown that cortisol levels are the lowest under stable social conditions, the animals were tested for the ACTH challenge without being moved from their home tanks (M/F sex ration=0.4).

2.3.2. ACTH challenge: experimental set up, treatments and sampling

The ACTH challenge test was designed in accordance to the experimental protocol proposed by Hontela (1998). Six different concentrations of ACTH$^{1\text{-}39}$ (Adrenocorticotrophic hormone porcine pituitary, A-6303, Sigma Chemical Company, St. Louis, MO, USA) were administered to a sample of four animals, for each dose in an unpaired experimental design. The ACTH doses (treatment) were 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10 mg/ml physiological saline solution 0.9%. The treatment was weight-adjusted (100 µl of ACTH solution/100 g body weight). As a control, four additional males were injected with a physiological saline solution only. The twenty-eight animals were sampled around midday. Animals were rapidly hand net captured without chasing, lightly anaesthetised (as above) and intraperitoneal injected with the respective treatment. After recovering (within one minute), they were put back in the home tanks. One hour and forty-five minutes later (Hontela, 1998), fish were blood sampled for cortisol analysis, following the procedure described in experiment 1. Cortisol assays were also carried out as in experiment 1. Cortisol intra- and inter-assay variability was 2.7% and 4.7% respectively.

2.3.3. Data Analysis

Statistical analysis was conducted to assess the effect of ACTH doses on cortisol levels, using one-way analyses of variance. Data was normalised through logarithmic transformation for continuous variables (Zar, 1984). Following the ANOVAs, planned comparisons of least squares means were performed between conditions (ACTH 10^{-3} and 10^{-2} doses). A value of $P < 0.05$ was taken for significance in all statistical tests. The statistical package used for analysis was Statistica V.7.1® (StatSoft Inc, USA, 1984-2008).

2.4. Ethical note

This experiment did not involve mortality of animals and all males were returned to their previous home tanks after the experiments. The ACTH injections did not cause behavioural signs of distress associated to four of the six ACTH doses. However, the two stronger doses induced behavioural signs of stress (inactive behaviour and dark stripes in the body). These signs disappeared within a period of approximately four hours. The experimental procedures involved in these studies were in compliance with the regulations on animal experimentation in Portugal.

3. RESULTS

3.1. Diurnal variation of cortisol

The diurnal variation of cortisol showed significant differences, with a gradual increase during the dark period and a peak in the early morning, before the lights went on (one-way ANOVA, excluding sampling point 20.30h, n=16: $F_{(4,56)}=4.6$, $P<0.01$; including sampling point 20.30h, n=7: $F_{(5,25)}=4.2$, $P<0.01$, planned comparisons between dark and light period: $P=0.08$; Figure 1a). There were no differences in cortisol levels between territorial and non-territorial fish in any of the sampled points (one-way ANOVA, excluding sampling point 20.30h, n=16: $F_{(4,56)}=0.1$, ns; Figure 1a). Daily variation of locomotory activity has shown marked differences related to time of day and to social status (one-way ANOVA: $F_{(4,56)}=6.3$, $P<0.001$; Figure 1b). Activity is higher during the light period (one-way ANOVA: $F_{(4,56)}=2.9$, $P<0.05$, planned comp. $P<0.02$), and territorial fish are more active than the non-territorial (one-way ANOVA: $F_{(4,56)}=5.6$, $P<0.05$; Figure 1b). At midnight all animals were seen resting in the bottom of the tanks, therefore no locomotory activity was registered during the two-minute sample.

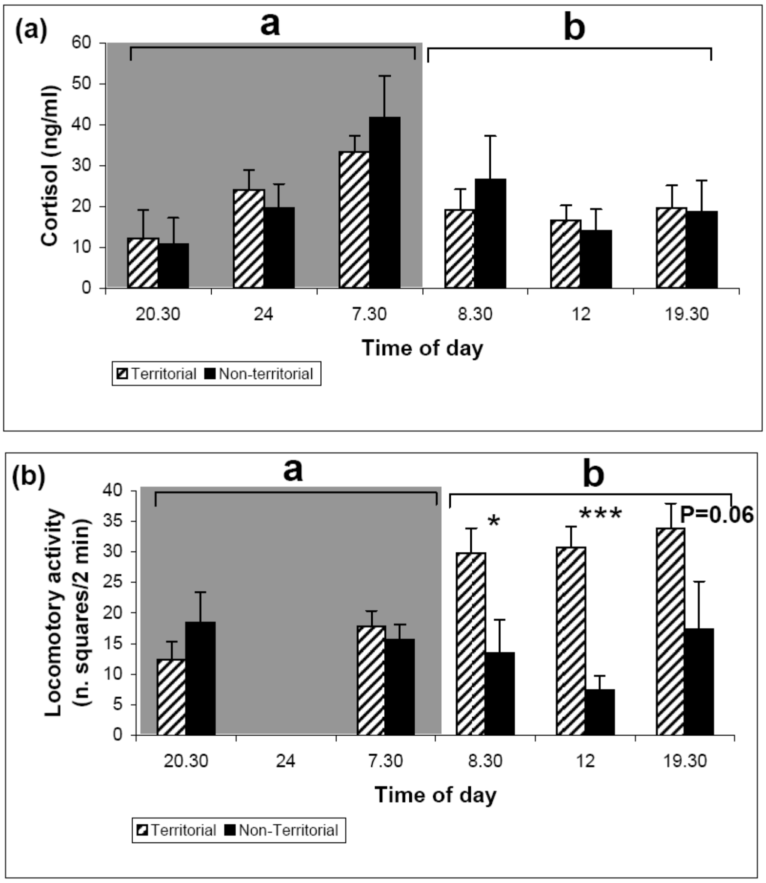


Figure 1. Diurnal variation of (a) cortisol levels (ng/ml) in territorial (n=11, n=4 at 20.30h) and non-territorial (n=5, n=3 at 20.30h) fish and (b) locomotory activity (number of squares crossed in two minutes) in territorial (n=11) and non-territorial (n=5) fish. *P<0.05 ***P<0.001

3.2. Effects of social isolation on cortisol, testosterone and 11-ketosterone

In social conditions there were no differences between territorial and non-territorial levels of cortisol (one-way ANOVA: $F_{(1,14)}$=2.5, ns, plan comp. ns; Figure 2). They increased in social isolation (one-way ANOVA: $F_{(1,14)}$=13.4, P<0.01; Figure 2), with this variation being especially due to the non-territorial males (planned comp., non-territorial: $F_{(1,14)}$=11.2, P<0.01; territorial: ns). Therefore, in social isolation, non-territorial fish tended to show higher levels of cortisol than territorial fish (planned comp. $F_{(1,14)}$=3.9, P=0.07; Figure 2). Levels of 11-ketotestosterone were higher in territorial males in social groups (one-way ANOVA: $F_{(1,14)}$=7.6, P=0.02, planned comp. P<0.001; Figure 2). Once in isolation, levels of this hormone became similar between both social status especially due to its decrease in territorial males (one-way ANOVA: $F_{(1,14)}$=8.5, P=0.01; Figure 2). Changes in testosterone followed a similar pattern but with a weaker difference between social status when males are integrated in social groups (one-way ANOVA: $F_{(1,14)}$=3.5, P=0.08, planned comp. P<0.01; Figure 2). Differences of testosterone levels between territorial and non-territorial males disappeared after isolation (one-way ANOVA, $F_{(1,14)}$=3.5, ns; Figure 2).

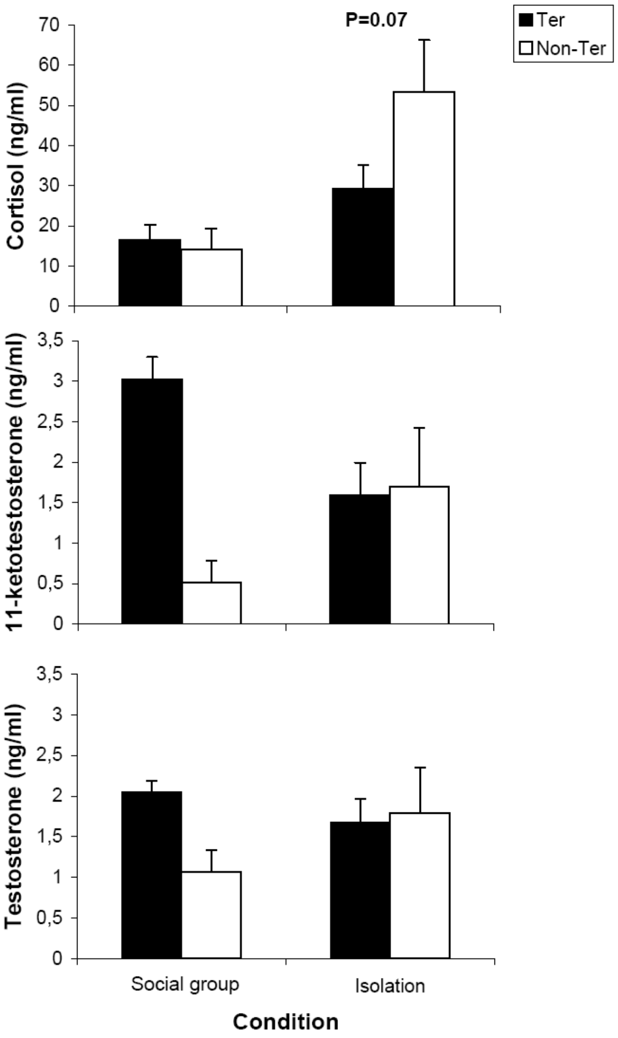


Figure 2. Cortisol, 11-Ketotestosterone and testosterone levels (ng/ml) in stock and isolated territorial (n=11) and non-territorial (n=5) males of *O. mossambicus*. **P<0.01 ***P<0.001

3.3. ACTH challenge

Comparing all sampling points, there were two different levels of cortisol responses to treatments, with a clear difference between the 10^{-3} µg/g and the 10^{-2} µg/g ACTH doses (one-way ANOVA: $F_{(6,21)}$=13.1, P<0.001, planned comp. P<0.0001; Figure 3). ACTH doses below 10^{-3} µg/g produced cortisol responses of approximately 25 µg/ml, which did not differ significantly from the control (15 µg/ml; planned comp., ns). ACTH doses above 10^{-2} µg/g produced higher cortisol responses between approximately 100-120 µg/ml. No significant differences were found among these doses, with cortisol levels forming an upper plateau (planned comp., ns; Figure 3). These levels of cortisol (two higher ACTH doses) corresponded to a very inactive behaviour and species-specific colour patterns of stress (dark stripes in the body).

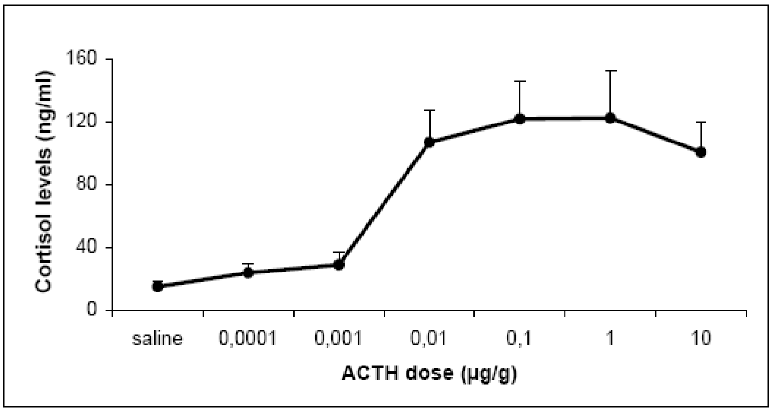


Figure 3. Levels of cortisol (ng/ml) to a *in vivo* weight-adjusted ACTH challenge in *O. mossambicus* (n=4)

4. DISCUSSION

4.1. Diurnal variation of cortisol

Cortisol diurnal changes of male *O. mossambicus* showed a gradual increase over the night (20.30h to 7.30h, corresponding to the dark period) with a peak in the early morning (7.30h, just before the dark period ends) and basal levels during the rest of the light period. No differences between territorial and non-territorial males were found. Lane (2006) describes this pattern for diurnal animals, with a peak early in the morning, to gear the body for diurnal action, a plateau phase during the day and a fall at the beginning of the night, to promote an uninterrupted night sleep. Studies in fish seem to follow the same pattern, that is, animals display a cortisol peak at the beginning of their active period. For example, studies on male sword-tails (Hannes and Franck, 1983) and rainbow trout (Reddy and Leatherland, 1995) have reported early morning peaks of cortisol probably associated to their diurnal feeding habits. Studies in sea bass have described cortisol peaks at both light onset and offset (Cerdá-Reverter et al., 1998). Probably, this pattern is related to the exhibition of both diurnal and nocturnal feeding behaviour in this species, modulated by the photoperiod and water temperature (Sánchez-Vázquez et al., 1998; Azzaydi et al., 2000). Lorenzi et al. (2008) found cortisol peaks in bluebanded goby late in the morning and intermediate levels for the rest of the day, likely related to the highly variable intertidal zone, where this species lives. In fact, tide phases, along with day/night cycles, probably contribute to modulate peaks of activity. This study describes *O. mossambicus* diurnal variation of cortisol but is not conclusive in terms of a true circadian cycle, since each time of day was sampled only once for each individual. In order to demonstrate the occurrence of the same peaks across days, it would have been necessary to carry out at least two days of continuous sampling (Lorenzi et al., 2008). In the same way relevant factors for the synchronisation of circadian rhythms such as photoperiod, feeding time, and water temperature were not manipulated in the present study. Therefore, the diurnal variation reported here is associated to the existent laboratory conditions and shall be interpreted with this fact in mind.

4.2. Effects of social isolation on cortisol, 11-ketosterone and testosterone

Under stable social conditions males of both social status showed identical levels of cortisol, but after isolation cortisol increased significantly only in the non-territorial males. Other studies have also reported similar levels of cortisol between territorial and non-territorial males under social conditions (e.g. Correa et al., 2003; Chapter II) and increase in cortisol responsiveness due to social isolation (Earley et al., 2006). The fact that the non-territorial males have shown an increased cortisol response in relation to the territorial males may be related to two different possible coping styles. Despite the fact that territorial and non-territorial males can be both related to pro-active or reactive coping strategies (Clement et al., 2005), Gómez-Laplaza and Morgan (2003) have shown how non-territorial males, placed in isolation, increased locomotor and decreased feeding behaviour in relation to territorial males in the same context. Therefore, the present cortisol results and the published behavioural data suggest that social isolation is a stressful event, to which the subordinates may be more responsive, and that it does not compensate the social stress created by the constant presence of a territorial male.

In social conditions, 11-ketotestosterone (11-KT) and testosterone (T) were more elevated in territorial males, as it has also been shown in previous studies (Oliveira et al., 1996). This difference between territorial and non-territorial males has disappeared after seven days of social isolation, reason why all males are routinely placed in social isolation prior to certain studies involving social dynamics (Oliveira et al., 1996; Oliveira et al., 2005). However, this procedure may have negative consequences in relation to the stress response.

4.3. ACTH challenge

Results of ACTH challenge showed a threshold effect, i.e., low levels of ACTH do not affect cortisol release but above a certain level, the HPI is activated. A lower plateau, generated by ACTH doses below 10^{-3} µg/g, varied around 25 ng/ml; an upper plateau, produced by ACTH doses above 10^{-2} µg/g, varied around 100-120 ng/ml. The same kind of maximum plateau was also found in white sturgeon for ACTH doses above 5.0 µM (Belanger et al., 2001). It is possible that by refining ACTH doses between 10^{-3} µg/g and 10^{-2} µg/g, a gradual increase in cortisol levels would be found.

The saline control of this experiment produced very similar cortisol values (15 ± 6.3 ng/ml) to those found in the first experiment (16.6 ± 3.6 ng/ml) at midday, and confirm the low levels of cortisol produced by animals in stable social conditions. This fact also shows that handling (for the ACTH injections) did not produce any measurable long lasting (2h) effect. Many studies have obtained similar baseline/undisturbed levels for Mozambique tilapia (e.g. Morgan and Iwama, 1997; Quabius et al., 1997; Vijayan et al., 1997b; van Anholt et al., 2003; Takahashi et al., 2006). However, there are also those which reported lower (below 10 ng/ml: Foo and Lam, 1993; Nolan et al., 1999; Dini et al., 2006) or higher values (150 ng/ml: Pelgrom et al., 1995; 55 ng/ml: Vijayan et al., 1996; 303-380 ng/ml: Uchida et al., 2003). A wide range of factors can affect what is assumed to be a cortisol baseline value (see introduction), which makes difficult or impossible to define a single circumstance that will reflect a baseline level.

In vivo and *in vitro* ACTH challenges have been used to evaluate the HPI range of responses in a number of different fish species (Brodeur et al., 1998; Hontela, 1998; Belanger et al., 2001). As mentioned above, maximum cortisol level in this study was 120 ng/ml, which corresponded to a visible stressed behaviour (inactivity and black stripes in the body). Similar levels were reached in yellow perch two hours after ACTH administration, under the same procedure as in the present study (Hontela, 1998). As no analysis of temporal cortisol dynamics was carried out, it is impossible to be sure that this level corresponds to the cortisol physiological maximum. In fact, various studies in fish have shown a response peak at 30 minutes after the onset of stressors (e.g. Mozambique tilapia: Foo and Lam, 1993; white sturgeon: Belanger et al., 2001; Nile tilapia: Biswas et al., 2004) or the in vitro ACTH stimulation (e.g. juvenile rainbow trout: Brodeur et al., 1998). Therefore if cortisol peaks have a short duration, our maximum levels may reflect a descendant interrenal activity or an ongoing process of cortisol clearance from the plasma. In future studies of this kind, it would be relevant to analyse the dynamics of the cortisol response over a period of time (e.g. 15’, 30’, 60’, 120’, 180’, 240’). It should however be noted that some studies report peaks of cortisol with high duration of permanence. For example, cortisol peaks in white sturgeon are reached within 30 minutes and remain elevated for further 90 minutes (Belanger et al., 2001). If that was the case in Mozambique tilapia, than the maximum levels found in this study would be closer to maximum interrenal activity.

The present characterisation of baseline levels and maximum responses of cortisol to an ACTH challenge can be used to compare and evaluate the relative severity of a range of stressors. For example, a number of stress studies involving stressors such as confinement in low water level and social isolation were already undertaken this laboratory, and the cortisol levels sampled consistently varied between 40 and 60 ng/ml (Chapter VI and VII). In face of the present results, the severity of the mentioned stressors can be classified as mild in comparison with the maximum response to ACTH administration.

4.4. Conclusion

Cortisol levels are highly variable among stress studies due to a wide range of internal and external factors, which makes very difficult to draw comparative analysis. Thus, for each species and respective contexts, it is important to validate cortisol measurements. In *O. mossambicus*, diurnal variation has shown a cortisol peak in the early morning just prior to the light phase. Social isolation induces an increase of cortisol levels which has to be taken into account when using this procedure to infer baseline cortisol levels. The ACTH challenge has shown a threshold mechanism with two distinct groups of dose-responses relationship, which can be compared with other stress responses. These findings contribute to better understand the cortisol response of *O. mossambicus* and allow comparisons with stress studies undertaken under similar conditions. These findings can be directly applied in the improvement of stress management and welfare of *O. mossambicus* in captivity.

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CHAPTER VI

The effect of social context in the stress response and neophobia of a cichlid fish

Galhardo, L., Vitorino, A., Oliveira, R.F. The effect of social context in the stress response and neophobia of a cichlid fish. Submitted to *Hormones and Behaviour*.

Abstract

Social groups have a buffer role against stress in humans and many vertebrate species. Despite the stress created by hierarchical structures, social groups have numerous adaptive advantages. Familiar preferences within groups tend to reduce aggression and increase group efficiency. Therefore, any kind of social disruption can cause stress responses. Despite the extensively studied role of social groups as modulators of the stress response in social mammals, in fish this aspect is less well known. Therefore, the aim of this study was to analyse how the territorial cichlid fish Mozambique tilapia responded to a neophobia test and a confinement stress test in three different social contexts: (a) in the presence of a familiar conspecific; (b) in the presence of an unfamiliar conspecific; and (c) in social isolation. The stability of the social context was also considered. Patterns of activity, social interactions and exploration were sampled as well as blood for cortisol measurement. The novel object (NO) did not affect inactivity and social interactions, but the presence of a familiar conspecific in an undisturbed context promoted more exploratory behaviour than in any other treatments. The behavioural and cortisol responses to confinement do not suggest the influence of social support in the stress response. In conclusion, under particular stable social conditions, social support can reduce neophobia but not the stress response of male Mozambique tilapia as measured by cortisol, but this effect is likely to vary with the nature of the stressors.

1. Introduction

The group living system can be a permanent source of stress for some individuals. Agonistic encounters stimulate an acute increase in cortisol levels in males of both social status, which seems to induce the expression of aggressive behaviour (Øverli et al., 1999). However, it is apparent that the dominant's level rapidly decreases, while the subordinate levels is sustained or increases (reviewed by Oliveira and Gonçalves, 2008). Therefore, dominant animals have been related to lower baseline levels of corticosteroids or to faster corticosteroids recover after agonistic encounters (Øverli et al., 1999). They have preferential access to relevant group resources and they usually have better control over the agonistic bouts (DeVries et al., 2003). In turn, low social status has been associated to chronic high levels of corticosteroids, higher brain serotonergic activity, reduced immune responses and inhibitory behaviours (e.g. reduced aggression) (Øverli et al., 2004; Gilmour et al., 2005). However, these patterns can change with a wide range of individually, socially or environmentally-related factors (Fox et al., 1997; DeVries et al., 2003; Earley et al., 2006). Stability of social groups is one such factor. In an unstable group, social positions are permanently challenged due to the income of new members or to the individual profiles of the residents. The behavioural outcome of unstable groups is a rise in alertness and aggressive encounters among conspecifics. This is accompanied by an increase in cortisol levels in all group members and the typical relationship between cortisol levels and social rank may be altered (DeVries et al., 2003).

Despite the associated costs of competition, life in social groups has well known adaptive advantages for fish and other animal species. Social information seems to travel fast within a fish group, promoting more efficient behaviours relevant to survival (Hoare and Krause, 2003). Shoaling offers benefits in foraging, mating and predator defence contexts (Frommen et al., 2007). Many studies have demonstrated a non-random interactions pattern within a group: animals express individual preferences, based on conspecifics recognition (Hoare and Krause, 2003). For example, choice for familiar animals has been shown in different species of fish (Griffiths, 2003; Griffiths et al., 2004; Bhat and Magurran, 2006). In territorial fish, the 'dear enemy' effect, in which animals are frequently more aggressive towards non-familiar neighbours than towards familiar ones, is a well known behaviour (Frostman and Sherman, 2004). Gómez-Laplaza & Fuente (2007) have also shown how familiarity is combined with social status in angelfish: subordinates choose familiar subordinates over unfamiliar subordinates, and unfamiliar subordinates over familiar dominants. Kin preferences are known in various fish species: unfamiliar kin are preferred to non-kin, and familiar to unfamiliar kin in sticklebacks (Frommen et al., 2007) and in zebrafish (Gerlach et al., 2008).

Familiarity promotes social learning, cooperation, cohesion and less aggression (Swaney et al., 2001; Bhat and Magurran, 2006). Therefore, it is expectable that disruptions of the social group such as isolation, mixture with non-familiar animals or any other form of instability may produce stress responses. Many studies have provided evidence that these social manipulations produce behavioural and physiological social responses in mammals (e.g. Gust et al., 1996; Jensen, 2001; Ruis et al., 2001; DeVries et al., 2003; Takeda et al., 2003; Désiré et al., 2006). In some fish species, social isolation increases cortisol levels and modifies the feeding and agonistic patterns of interaction (Øverli et al., 2002; Earley et al., 2006; Martins et al., 2006). Some forms of learning seem to be disrupted by isolation in goldfish (Laudien et al., 1986). Encounters with unfamiliar fish and unstable social groups also produce a cortisol response (Fox et al., 1997; Yue et al., 2006).

In face of the clear adaptive function of the group, it is also expected that the social context has a function in ameliorating the adverse effects of stressors on its members. This has been thoroughly shown in humans (e.g. Kiecolt-Glaser et al., 2005; DeVries et al., 2007), and also in non-human primates (Johnson et al., 1996; Sapolsky, 2004) and other mammals (e.g. Ruis et al., 1999; Andrade and Guimarães, 2003). Devries et al (2003) suggest that affiliative interactions, also present in groups with social hierarchies, may suppress the production of corticosteroids, despite the relatively unknown underlying mechanism. These authors and others (e.g. Taylor et al., 2000; Heinrichs et al., 2003) further suggest that oxytocin may be the main hormone modulating pro-social behaviours and the HPA axis, thus producing a stress-protective effect. In fish, very little is known about the effect of social support as a psychological buffer to stressors. Thompson & Walton (2004) measured the effects of vasotocin and isotocin in goldfish (the homologous peptides to arginine-vasopressin and oxytocin in mammals) on social approach behaviour. They have shown that isotocin stimulated approach reactions towards the visual stimuli of conspecifics in the absence of aggressive or sexual olfactory contextual cues. In a recent study, undertaken with lake sturgeon on cohesive social behaviour, a shortened stress response to aerial exposure was found in animals with social partners, in opposition to those maintained in isolation (Allen et al., 2009).

In the present study, the effect of social context in the stress and neophobia response of a territorial fish was analysed. The model used was the African mouthbrooder cichlid, *Oreochromis mossambicus*, due to its very well known social dynamics. The males form lek aggregations in the breeding season, where social hierarchies are established. The dominants defend their territories and build pits in the substrate, to where ripe females are attracted for spawning (Fryer and Iles, 1972; Oliveira and Almada, 1998). There is growing evidence that different social contexts produce physiological effects in this species. For example, the simple observation of fights has the power to elevate plasma androgen levels (Oliveira et al., 2001). Higher androgen levels are also produced by males in contact with non-familiar neighbours in contrast with the lower levels of androgens produced by males in the boundary of already established neighbours (R. Aires and R. F. Oliveira, unpublished data). Furthermore, social isolation produces the highest cortisol levels among an array of different stressors (Chapter V). Therefore, the aim of this study was to analyse how the Mozambique tilapia responds to a neophobia test and to a confinement test in different social contexts.

2. Material and Methods

2.1. Animals and Housing

Twelve adult male fish of the species *O. mossambicus* were the focal animals of the present study. Twelve adult females of the same species were used as non-focal animals in order to create the social context of the experiment. These animals were housed in stock aquaria of 240 l in groups of eight animals each (three males: five females). Temperature was held at 26ºC ± 2ºC, with a photoperiod of 12L: 12D. Each tank had a sand filter, an external biofilter (Eheim) and constant aeration. Water quality was weekly monitored for nitrites (0.2-0.5 ppm), ammonia (<0.5 ppm) (Pallintest kit®) and pH (6.0-6.2). Fish were daily fed *ad libitum* with commercial cichlid sticks (ASTRA). Under experimental conditions, the animals were housed individually in testing aquaria of 40 l, with all the remaining conditions the same as described for the stock.

2.2. Experimental Procedures

2.2.1. Social context

The present experiment was conducted in two stages under three different social contexts: ‘familiar’ (male with 4-days of visual contact with a familiar female), ‘unfamiliar’ (male with 1-hour of visual contact with an unfamiliar female) and ‘isolated’ (male with 4 days of total social deprivation). Females were chosen to create a social context more affiliative and less competitive than it could be with males in visual contact. Familiar individuals had been held in the same stock aquaria. Conversely, unfamiliar females originated from different stock aquaria than the focal male. All females were used only once during the experiment.

In the first stage (experiment 1), the three social contexts were compared under stable conditions in accordance to the experimental set up represented in Figure 1. All males remained in acclimation for four days before the behavioural tests. During this period, the males that would be subjected to the ‘familiar’ and ‘unfamiliar’ conditions were in visual contact with familiar females. The “isolated” males spent this period in social isolation. All groups remained untouched until the fifth day, only the unfamiliar males suffered a disturbance with the exchange of the female (familiar to unfamiliar) thirty minutes to one hour prior to the beginning of the experiment. To control for this ‘instability effect’, a second stage took place (experiment 2) consisting of testing the same males in the same three social contexts, but under unstable conditions. Males were removed from the stock to the experimental aquaria, allowing them an acclimation period of three hours (experiment 1 had shown no differences in behaviour after the first three hours and the forth day of acclimation), after which a familiar and an unfamiliar female were introduced to the ‘unstable familiar’ and ‘unstable unfamiliar’ male respectively. The ‘isolated male’ remained as such until the beginning of the behavioural observations, though a ‘sham’ female introduction was performed in their adjacent aquarium. Thirty minutes to one hour after the females’ placement in the set up, the behavioural observations started.

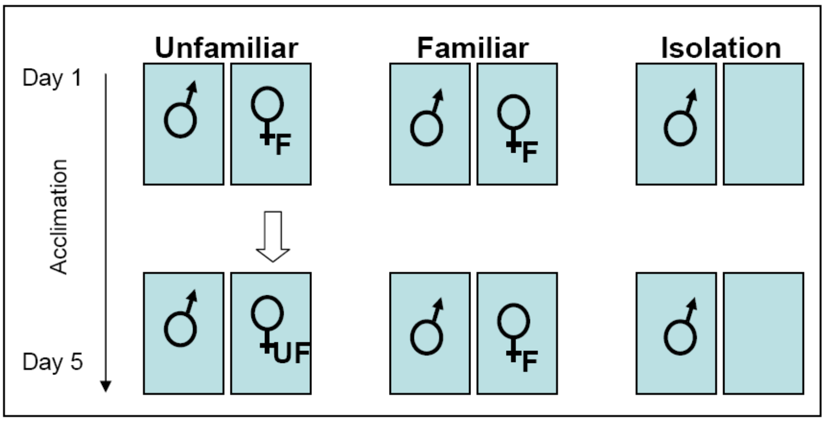


Figure 1. Representation of the experimental set up (experiment 1).

In both experiments, after the respective acclimation period, a sampling for baseline behaviour of the three groups took place, followed by two behavioural tests: a neophobia (novel object) and a confinement stress test. All observations were carried out through a small opening in a black curtain placed in front of the tanks. After the tests, all animals (males and females) were put back in their original stock tanks where social and physical conditions remained the same. Males spent one week in the stock between treatments (different social contexts) and treatment order was balanced between males.

2.2.2. Neophobia test (Novel Object)

This test has been used in a number of studies to assess fear and stress responses in fishes (e.g. Sneddon et al., 2003; Schjolden et al., 2005) and in other vertebrate species (e.g. pigs: Janczak et al., 2003; quails: Miller et al., 2006). It involves a motivational conflict between fear from potential threats and exploration of new relevant stimuli. This conflict generates an approach-avoidance set of behaviours that can vary with properties of the environment (e.g. familiar environments in rats promote approach behaviours) (Powell et al., 2004). A novel object (ping-pong ball filled with sand) was carefully placed in the focal male tank in a fixed point near the front glass, after which behaviour was immediately recorded for 10 minutes. The object was then removed and the animals were left undisturbed for 30-60 minutes before the confinement stress test took place.

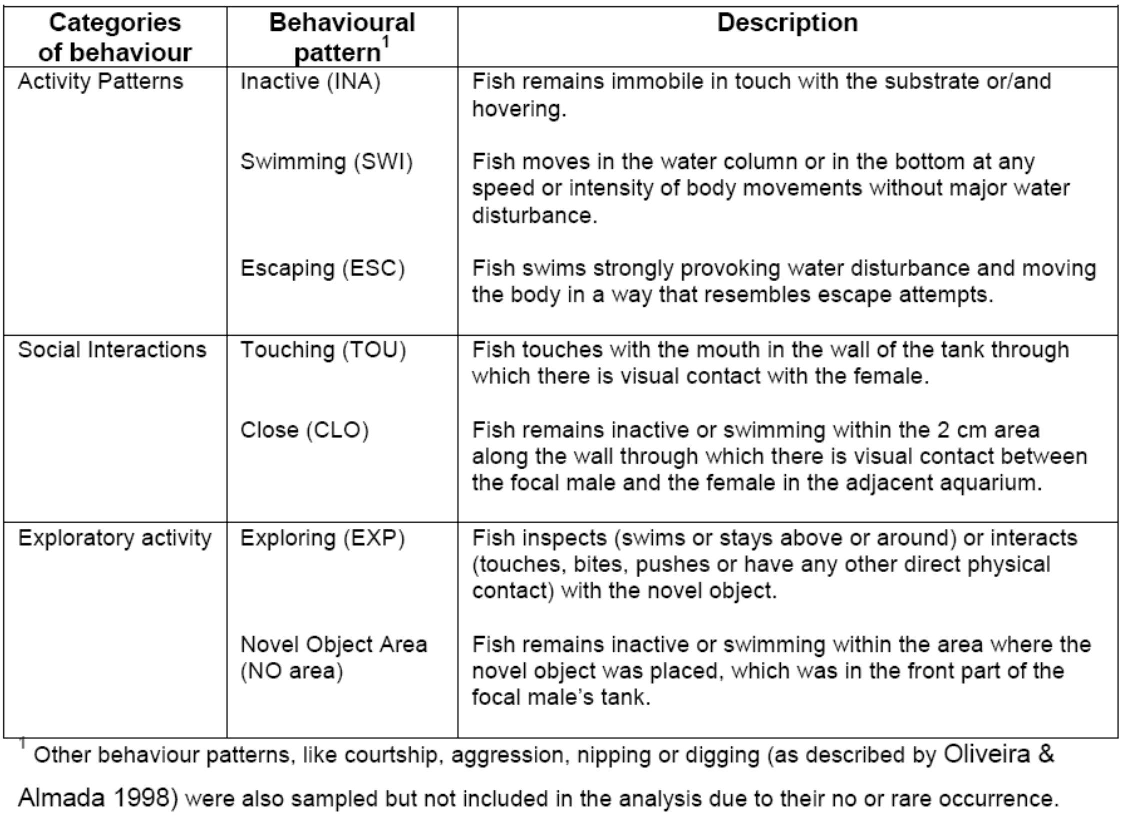
2.2.3. Confinement stress test

The confinement test, with variable methodologies, has been used before to assess stress responses (e.g. behavioural reactivity, cortisol responses) in various fish species (e.g. Øverli et al., 2004; Brelin et al., 2005; Schjolden et al., 2005; Øverli et al., 2006) including *O. mossambicus* (e.g. Vijayan et al., 1997). In this study, the confinement stressor consisted in lowering the water column to approximately 10 cm by means of a manual water pump previously installed in each tank, so that the height of the water column does not allow the fish to swim in its tank. Behavioural response was recorded during the first 10 minutes of confinement. The total duration of the test was 30 minutes, after which blood sampling, for cortisol measurement, took place.

2.3. Behavioural sampling

Behaviour of all focal males and females was recorded before (baseline sampling) and during tests, between 10:30h and 14:30h, according to Table 1. The baseline behaviour and neophobia test were sampled through focal instantaneous sampling during 10 minutes per individual (Martin and Bateson, 2007). The confinement test was sampled through instantaneous scans of three individuals at a time. Referential lines marked in the front glass of the tanks allowed determining the position of the fish in relation to the adjacent tank (Close) or to the novel object (NO) area as described in Table 1.

Table 1. Brief description of the behaviour patterns and use of specific areas of the aquaria adopted for sampling.



Categories of behaviour	Behavioural pattern[1]	Description
Activity Patterns	Inactive (INA)	Fish remains immobile in touch with the substrate or/and hovering.
	Swimming (SWI)	Fish moves in the water column or in the bottom at any speed or intensity of body movements without major water disturbance.
	Escaping (ESC)	Fish swims strongly provoking water disturbance and moving the body in a way that resembles escape attempts.
Social Interactions	Touching (TOU)	Fish touches with the mouth in the wall of the tank through which there is visual contact with the female.
	Close (CLO)	Fish remains inactive or swimming within the 2 cm area along the wall through which there is visual contact between the focal male and the female in the adjacent aquarium.
Exploratory activity	Exploring (EXP)	Fish inspects (swims or stays above or around) or interacts (touches, bites, pushes or have any other direct physical contact) with the novel object.
	Novel Object Area (NO area)	Fish remains inactive or swimming within the area where the novel object was placed, which was in the front part of the focal male's tank.

[1] Other behaviour patterns, like courtship, aggression, nipping or digging (as described by Oliveira & Almada 1998) were also sampled but not included in the analysis due to their no or rare occurrence.

2.4. Blood sampling and cortisol assay

At the end of the confinement test, fish were individually removed from the test aquarium and lightly anaesthetised [Stage two (Ross, 2001)] in a solution of MS-222 (tricaine methane sulphonate, Sigma; 200 ppm). Samples of 100-200 µl of blood were taken from the caudal vein (1 ml syringes; 25G/16 mm needles). The fish were then placed in aerated water, recovering from the anaesthesia within 30 seconds to one minute. The anaesthesia induction and blood sampling were performed within a maximum of four minutes, which is the latency for cortisol release into the systemic circulation in response to handling stress (Foo and Lam, 1993).

Free cortisol fraction was extracted from the plasma by adding to the sample diethyl ether, as the steroid solvent. The samples were then centrifuged (5 minutes, 1000 rpm, 4ºC) and frozen (10 minutes, -80ºC) to separate the ether fraction. The steroids were isolated by evaporating the ether. This process was repeated twice. Levels of free cortisol fraction were then determined by radioimmunoassay, using the commercial antibody 'Anti-rabbit, Cortisol-3' [ref: 20-CR50, Interchim (Fitzgerald), Montluçon, France, cross-reactivity: cortisol 100%, Prednisolone 36%, 11-Desoxycortisol 5.7%, Corticosterone 3.3%, Cortisone < 0.7%] and the radioactive marker [1,2,6,7-3H] Cortisol [ref: TRK407-250mCi, Amersham Biosciences, Piscataway, NJ/USA]. Intra- and inter-assay variability was of 5.8% and 6.5%, respectively for experiment 1 and 2.7% in both cases for experiment 2.

2.5. Data Analysis

To assess the effect of different social contexts on the baseline behavioural parameters, a one-way repeated measures analyses of variance for males was conducted (repeated factor: social context). Data was normalised using the transformations proposed by Zar (1984), namely *arcsin* transformation for proportion of behavioural patterns. Following the ANOVAs, planned comparisons of least squares means were performed where appropriate. The variation between the behaviours performed during the neophobia/confinement tests and the baseline was calculated in order to correct behavioural responses to the respective baseline. Differences in variation of behaviour among social contexts for both tests were then assessed through one-way repeated measures analyses of variance (repeated factor: social context). A value of $P < 0.05$ was taken for significance in all statistical tests. The statistical package used for analysis was Statistica V.8 ® (StatSoft Inc, USA, 1984-2008).

2.6. Ethical Note

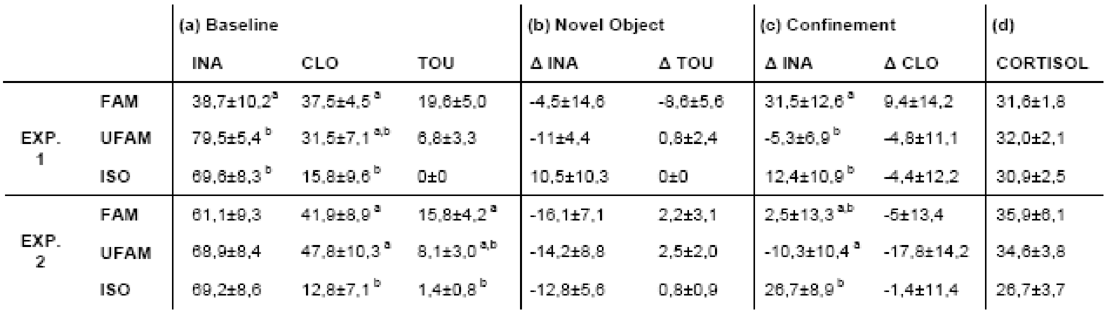
The experiments described were conducted in accordance to national legal standards on protection of animals used for experimental purposes and are part of a project approved by the national authorities (Ref. 30489, 29/11/2007).

3. Results

3.1. Baseline behaviour

In experiment 1, the familiar treatment showed the lower levels of inactivity (repeated measures ANOVA, $F_{2,\ 22}= 5.9$, $P<0.01$; Table 2), while in experiment 2, there were no differences in activity among social contexts (repeated measures ANOVA, $F_{2, 22}$= 0.6, NS; Table 2). In both experiments the patterns of social interactions showed the same trend: both males presented with a familiar and with unfamiliar females stayed closer to the adjacent aquarium (with the stimulus female inside) than isolated males (repeated measures ANOVA, Exp 1: $F_{2, 22}$= 3.3, P=0.05; Exp. 2: $F_{2, 22}$= 11.7, P<0.001; Table 2). In the same way all males in social contact with females touched more in the contact glass than the isolated males, with no significant differences between males from the familiar and unfamiliar treatments (repeated measures ANOVA, Exp 1: $F_{1, 11}$= 3.5, P=0.08; Exp. 2: $F_{2, 22}$= 8.3, P<0.01; Table 2).

Table 2. Means ± standard error of duration (%) of (a) baseline patterns of behaviour (inactivity, touching and time spent close), (b) variation of inactivity and touch during the novel object test, (c) variation of inactivity and time spent close during the confinement test, and (d) cortisol levels (ng/ml) for the three social contexts (familiar, unfamiliar and isolated). a,b Significant differences between groups (planned comparisons, P<0.05).



		(a) Baseline			(b) Novel Object		(c) Confinement		(d)
		INA	CLO	TOU	Δ INA	Δ TOU	Δ INA	Δ CLO	CORTISOL
EXP. 1	FAM	38,7±10,2^{a}	37,5±4,5^{a}	19,6±5,0	-4,5±14,6	-8,6±5,6	31,5±12,6^{a}	9,4±14,2	31,6±1,8
	UFAM	79,5±5,4^{b}	31,5±7,1^{a,b}	6,8±3,3	-11±4,4	0,8±2,4	-5,3±6,9^{b}	-4,8±11,1	32,0±2,1
	ISO	69,6±8,3^{b}	15,8±9,6^{b}	0±0	10,5±10,3	0±0	12,4±10,9^{b}	-4,4±12,2	30,9±2,5
EXP. 2	FAM	61,1±9,3	41,9±8,9^{a}	15,8±4,2^{a}	-16,1±7,1	2,2±3,1	2,5±13,3^{a,b}	-5±13,4	35,9±6,1
	UFAM	68,9±8,4	47,8±10,3^{a}	8,1±3,0^{a,b}	-14,2±8,8	2,5±2,0	-10,3±10,4^{a}	-17,8±14,2	34,6±3,8
	ISO	69,2±8,6	12,8±7,1^{b}	1,4±0,8^{b}	-12,8±5,6	0,8±0,9	26,7±8,9^{b}	-1,4±11,4	26,7±3,7

3.2. Neophobia test

The novel object (NO) test did not produce significant changes in patterns of activity (repeated measures ANOVA, Exp 1: $F_{2, 22}$= 1.4, NS; Exp. 2: $F_{2, 22}$= 0.1, NS; Table 2) and social interactions (Touch: repeated measures ANOVA, Exp 1: $F_{2, 22}$= 2.6, NS; Exp. 2: $F_{2, 22}$= 0.2, NS; Table 2) between the 3 treatments. However, in experiment 1, the exploratory behaviour of the NO was clearly higher in males exposed to familiar females (repeated measures ANOVA, $F_{2, 22}$= 19.1, P<0.001; Figure 2A). Variation of time spent in the NO area in relation to baseline, as an additional measure of exploratory motivation, also showed significant changes: males from the familiar treatment increased their time in this area in relation to baseline while, in contrast, the isolated males actively avoided it (repeated measures ANOVA, $F_{2, 22}$= 19.0, P<0.001; Figure 2B). This pattern was an inversion of the baseline use of the same area (in front of the tank), where the lowest use was seen in males exposed to familiar females and the highest by the isolated males, with an intermediate use by males exposed to unfamiliar females (repeated measures ANOVA, $F_{2,\ 22}$= 3.1, P=0.07; Figure 2C). By contrast, in experiment 2, there was no evidence of differences in the exploratory behaviour (repeated measures ANOVA, $F_{2,\ 22}$= 0.5, NS; Figure 2D) or use of NO area (repeated measures ANOVA, $F_{2,\ 22}$= 0.9, NS; Figure 2 E-F) by males belonging to each of the three social contexts.

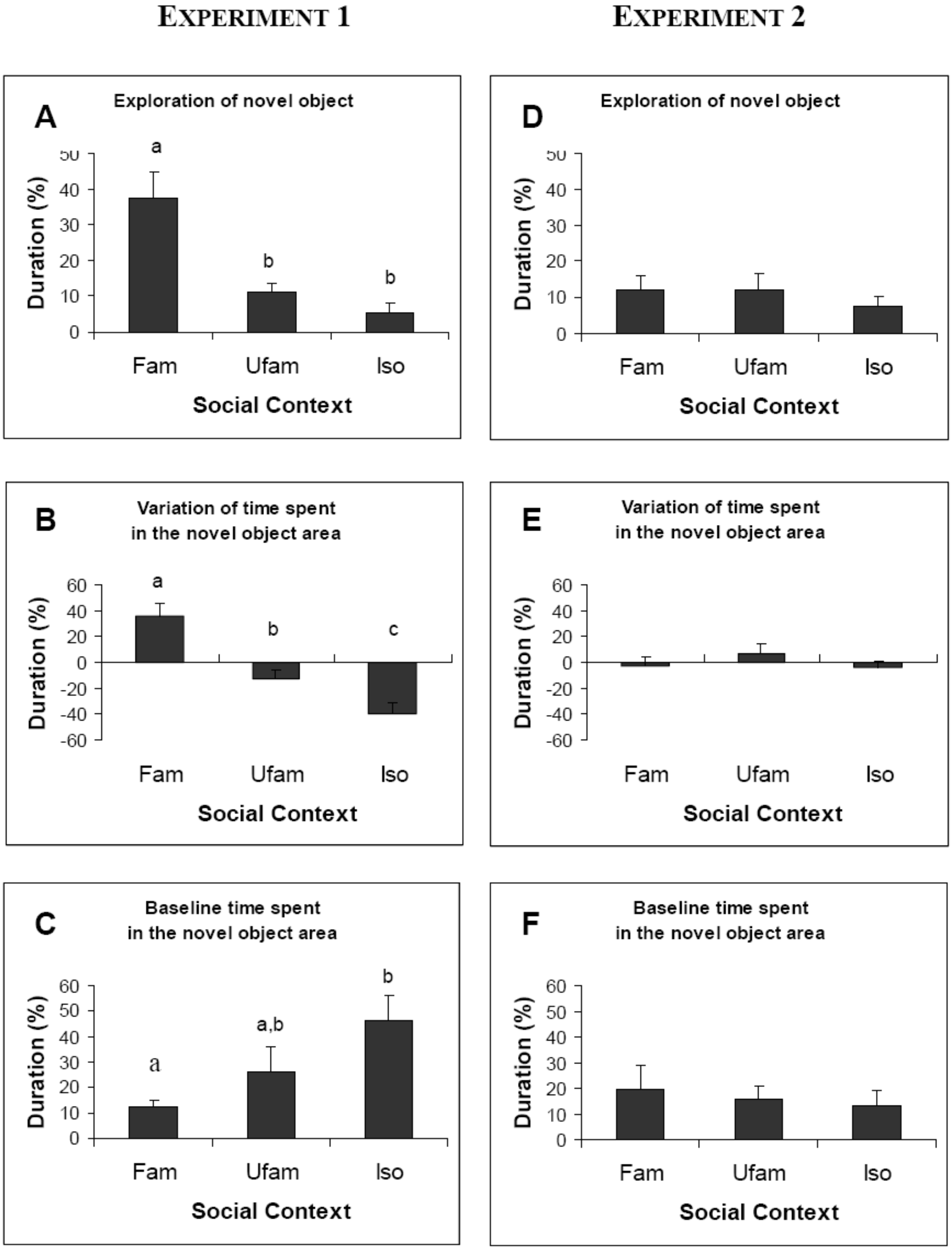


Figure 2. Behavioural patterns related to the exploration of the novel object in the three social contexts (familiar, unfamiliar and isolated). Experiment 1 (A-C): Time spent in novel object'sexploration (A) Variation of time spent in the novel object area (B) Baseline time spent in the novel object area (C). Experiment 2 (D-F): Time spent in novel object's exploration (D) Variation of time spent in the novel object area (E) Baseline time spent in the novel object area (F). a,b Significant differences between groups (planned comparisons, P<0.05).

3.3. Confinement stress test

Confinement increased inactivity of males exposed to familiar females in experiment 1 (repeated measures ANOVA, $F_{2,\ 22}$= 3.2, P=0.06; Table 2) and of isolated males in experiment 2 (repeated measures ANOVA, $F_{2,\ 22}$= 3.8, P<0.05; Table 2). Time spent closer to the adjacent tank did not reveal significant changes in both experiments for all social contexts (repeated measures ANOVA, Exp 1: $F_{2,\ 22}$= 0.5, NS; Exp. 2: $F_{2,\ 22}$= 0.6, NS; Table 2).

The cortisol response to confinement was the same for all males of both experiments, regardless their social context (repeated measures ANOVA, Exp 1: $F_{2,\ 22}$= 0.1, NS; Exp. 2: $F_{2,\ 22}$= 1.9, NS; Table 2).

4. Discussion

4.1. Baseline behaviour

The baseline behaviour recorded in experiment 1 has shown that animals kept in stable conditions and in the presence of familiar conspecifics have a higher activity than animals kept in isolation or in the presence of unfamiliar conspecifics. Patterns of baseline inactivity were the same for all males of experiment 2 (unstable/disturbed males). Social interactions, as measured by touch in the contact glass and by use of the area closer to the adjacent conspecific’s tank, were similar between males of the familiar and unfamiliar treatments regardless of the stability of the context (experiment 1 and 2). The results of the baseline behaviour suggest that stability has a likely influence on behaviour of the tested males, namely in their baseline patterns of activity. As it will be further developed below, it is possible that instability is related to the need for further attention (Griffiths et al., 2004), which promotes higher patterns of inactivity.

4.2. Neophobia Test

Patterns of activity and social interactions of both experiments remained unchanged with the presentation of the novel object. However, in experiment 1, males exposed to familiar females, under stable/undisturbed conditions explored the novel object more than those exposed to unfamiliar females or kept in isolation. This result did not happen in experiment 2, where males exposed to familiar females in the disturbed context behaved like the males of the other two treatments (unfamiliar female and social isolation).

The more relevant difference between experiment 1 and 2 was the stability/disturbance of the social context to which the males were exposed. While in experiment 1 males exposed to familiar females and isolated males remained undisturbed, in experiment 2, they had to face a disturbance in the adjacent aquarium, similarly to what males exposed to unfamiliar females experienced in both experiments. Therefore, when in face of the novel object, the "familiar/disturbed" males (experiment 2) were less available to explore it, as any other disturbed groups, in contrast with the "familiar/undisturbed" male of the experiment 1. In fact, instability of the social environment revealed to be a limiting factor to the exploration of the novel object. Unstable social contexts keep the animals more alert and attentive to the social environment, probably due to its unpredictable and potentially threatening properties (Griffiths et al., 2004). This is why to some extent, this attention cannot be shared and compete with certain non-obviously threatening environmental novelties, as it was the case of the novel object. Therefore, the present results suggest an effect of social support, probably not so related to a familiar social environment, but especially to its stability.

The social support effect is well known in mammals, favouring a reduction of their stress responses (e.g. Andrade and Guimarães, 2003; Sapolsky, 2004). In a study developed by Dunlop et al (2006) on fish pain perception, the presence of a conspecific significantly changed the behavioural response to a noxious stimulus: trout were willing to remain close to the conspecific while being subjected to low intensity shock stimuli previously shown to elicit avoidance. In the present study, the clear avoidance behaviour towards the novel object exhibited by the undisturbed 4-days isolated males (experiment 1) additionally suggests how social support can contribute to reduce neophobia. The same effect was not seen in males isolated for a shorter period (experiment 2), whose behaviour was the same as for other social contexts. It is likely that for these animals, beyond the shortened isolation period (only 4 hours), the disturbance acted as a distraction factor. Social isolation enhances stress responses in social species of higher vertebrates (e.g. Jensen, 2001; Ruis et al., 2001). In fish, it can affect feeding behaviour (Rainbow trout, Øverli et al., 2002; African catfish, Martins et al., 2006) as well as aggressive behaviour and the cortisol response (territorial convict cichlid fish, Earley et al., 2006). Social isolation can induce the highest cortisol responses among a number of moderate stressors in the Mozambique tilapia (Chapter V). Therefore, it is apparent that the lack of a social partner promoted a stress response in isolated males which was also revealed by the avoidance behaviours towards the novel object (neophobia).

4.3. Confinement stress test

Confinement increased inactivity of males exposed to familiar females (experiment 1) and isolated males (experiment 2), but did not affect the use of area closer to the adjacent aquarium. Confinement did not produce significant differences in cortisol levels in both experiments for the three social contexts.

The confinement test raised inactivity of undisturbed males exposed to familiar females (experiment 1) to the level of the other social contexts pattern. In experiment 2, the three-hour isolated males showed an increase in their inactivity, to levels that are higher than usually exhibited in both experiments, which is suggestive of a coping mechanism. The 4-day isolated males of experiment 1 were not so extreme in activating this eventual coping mechanism probably because the confinement may have represented for them a relief/enrichment in comparison to the four days of isolation lacking any environmental stimuli. In fact, the confinement paradigm (as performed in this study) seems to be only a mild stressor for this species. The levels of cortisol induced by it were similar to those evoked by other mild stressors and lower than those reported for socially isolated animals (Chapters V and VII). Trewavas (1983) also reports that *O. mossambicus* is able to survive to very extreme reduction of water level during the dry season in natural conditions, which probably confers a high level of tolerance for confinement. This procedure did not change the previous pattern of use of the area closer to the female (males exposed to familiar and unfamiliar females remained more time in this area than isolated fish). Despite this, the behavioural data available is not enough to suggest an effect of social support in reducing the stress response. The same is also true for cortisol, in relation to which no differences were found among groups. The present results are compatible with the possibility that the value of social support may vary with the nature of the stressors (DeVries et al., 2003). Allen et al (2009) have also studied the effect of the social partners' presence on the stress response to a brief aerial exposure in sturgeons (*Acipenser fulvescens*). They measured cortisol at regular time intervals after the stressor exposure via an implanted *cannula*, and have shown that the social contact did not change the peak cortisol response. However, this study has also demonstrated that the overall cortisol response was shorter in duration in the non-isolated animals. Furthermore, they have measured plasma ions and glucose, which also revealed differences between social and isolated contexts.

4.4. Conclusion

Social support reduces neophobia in the territorial *O. mossambicus* males, under stable social contexts. However, behavioural parameters and the measured levels of cortisol do not indicate any social support influence in response to confinement stress.

5. Acknowledgments

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CHAPTER VII

The role of predictability in the stress response of a cichlid fish

Galhardo, L., Vital, J., Oliveira, R.F. The role of predictability in the stress response of a cichlid fish. Submitted to *Physiology and Behaviour*.

Abstract

In recent years there has been an increasing interest in the cognitive abilities of fish with implications for their welfare management. Although it is known that psychological factors can modulate the stress response in mammals, this aspect has seldom been investigated within fish stress. Whether the perception (appraisal) that fish make of significant environmental events modifies their behavioural and physiological response was investigated in this study. For this purpose, a predictable *vs*. unpredictable paradigm for positive (food) and negative (confinement stress) events was adopted, using the cichlid fish *Oreochromis mossambicus* as a model species.

Results show that there is a differential effect of predictability for the negative and positive events. In the confinement experiment, predictability involved more attention to the visual cue and lower cortisol. The feeding event has triggered higher levels of anticipatory behaviour and a tendency for higher cortisol in the predictable group. Therefore, predictable negative events reduce the stress response, while predictable positive events may induce it, when there is a significant delay between the visual cue and the actual occurrence of the event. These findings demonstrate that fish can appraise relevant aspects of the environment which has welfare implications for housing, husbandry and experimental procedures.

1. Introduction

The relevance of psychological factors in the modulation of the stress response became clear in the second half of last century (Cooper and Dewe, 2004). The same sensory input may produce a completely different output, depending on the way the stressor is appraised by different individuals or by the same individual in different contexts. The appraisal process involves two stages in which cognitive and emotional areas of the brain are involved. The first stage (primary appraisal) involves the perception of the event and evaluation of its properties on the basis of previous memory and learned facts. The second stage (secondary appraisal) involves the assessment of the available coping mechanisms (Lazarus, 1999; Ursin and Eriksen, 2004). In humans, the appraisal mechanism involves the frontal areas of the cortex, where the cognitive evaluation takes place, and areas of the limbic system (hippocampus and amygdala), where the emotional valence is defined (Steckler, 2005). While the appraisal concept was already applied to the study of emotions in mammals (Désiré et al., 2002), in fish the whole concept of psychological stress has been rarely addressed.

The physiological and behavioural stress response in fish is well known and it is very similar to that of other vertebrates (Iwama, 2007). However, very little is known on the effect of psychological components on stress and there is even a current debate on whether fish possess certain mental abilities (Rose, 2002; Iwama, 2007) . Despite this, recent studies show evidence of far more complex fish behavioural and cognitive capacities than previously described (Chandroo et al., 2004; Braithwaite, 2006). Fish receive and process sensory inputs in the forebrain, have homologous areas to the tetrapods' limbic structures and similar neurotransmitter systems (Chandroo et al., 2004; Butler and Hodos, 2005; Rodriguez et al., 2006). Studies of pain and fear show how teleosts can also experience these emotions in a similar way as other vertebrates (Sneddon, 2007; Yue et al., 2008). Furthermore, the recent finding of corticosteroid receptors in the forebrain of carp (Stolte et al., 2008), strongly suggest that the regulation of the stress response starts above the level of hypothalamus, which is in line with recent studies showing how learning and memory of fish can be disrupted by high cortisol levels during stressful contexts (e.g. Barreto et al., 2006). Therefore, despite missing knowledge on exact mechanisms, fish are likely to have the necessary central mechanisms for the psychological assessment of environmental stimuli related to stress (appraisal) (for a review, see Galhardo and Oliveira, 2009).

Predictability of events (i.e. the perceived occurrence of a stimulus (Ursin and Eriksen, 2004)), is one of the most discussed psychological modulators of the stress response (Sapolsky, 2004; Lovallo, 2005), and therefore it is used here to test for the occurrence of appraisal in fish. One of the earliest studies on the effects of predictability was undertaken by Weiss (1970) in rats with electric shocks. He found that the same intensity and duration shocks, when delivered in a predictable way, produced less physiological responses and less gastric ulcers. Since then, several studies on the effects of predictability of aversive and attractive events have been developed. Basset and Buchanan-Smith (2007) have reviewed these studies and their effects on the well being of animals. Predictability of aversive events is more associated to lower stress responses than unpredictability (Orsini et al., 2002; Prior, 2002) despite some inconsistent results attributed to different study lengths and to the inappropriate comparison of different stress measurements (Bassett and Buchanan-Smith, 2007). In fact, unpredictability, even for biologically non-relevant events, is enough to stimulate the amygdala, a limbic structure responsible for contextual emotional learning (Whalen, 2007). Concerning positive events, which are usually food-related, the relationship of predictability with stress signals is less clear. A number of studies, especially based on behavioural parameters, indicate that predictable feeding regimes are less stressful for the animals (Carlstead, 1986; Wiepkema and Koolhaas, 1993; Bloomsmith and Lambeth, 1995; Gilbert-Norton et al., 2009). However, other studies point out in a different direction: predictable schedules induce high levels of anticipatory behaviours, characterised by increased general activity, with eventual stereotypic behaviour (Carlstead, 1996; Johannesson and Ladewig, 2000; Waitt and Buchanan-Smith, 2001). Sánchez and colleagues (Sánchez et al., 2009) have studied the effects of a random and scheduled feeding regime on sea bream levels of cortisol and concluded that the latter regime stimulated anticipatory behaviour and higher cortisol levels in this species. Loss of predictability (predictable followed by unpredictable regimes) is clearly reported as a source of stressful behavioural and physiological responses (Bloomsmith and Lambeth, 1995; Johannesson and Ladewig, 2000; Bassett and Buchanan-Smith, 2007; Gilbert-Norton et al., 2009). Furthermore, the relieving effect of predictability depends on a number of aspects related to the stressor’s properties (nature, intensity, frequency) as well as on the reliability of the signalling system (Sapolsky, 2004; Bassett and Buchanan-Smith, 2007).

How males of a cichlid fish, the Mozambique tilapia (*Oreochromis mossambicus*), cope with the presentation of signalled predictable and unpredictable events that differ in their valence - 1) negative event: confinement stress (experiment 1); 2) positive event:

feeding episode (experiment 2) – are investigated in this study. The working hypothesis is that predictability will produce differential behavioural and physiological stress responses according to the valence of the stimulus.

2. Material and Methods

2.1. Animals and housing

72 *O. mossambicus* males were used in this study (experiment 1, n=29; experiment 2, n=24; isolation control, n=19; average weight, 99.5±3.8g). Females were not used in order to decrease potential variability in the sample. They belong to a stock held at ISPA and were maintained in glass aquaria (120 x 40 x 50 cm, 240 l) with a fine gravel substrate, in stable social groups of 3-5 males and 5-6 females. The temperature was held at 26ºC ± 2ºC, with a 12L:12D photoperiod. Tanks were equipped with a double filtering system (sand and external biofilter, Eheim) and constant aeration. Water quality was weekly analysed for nitrites (0.2-0.5 ppm), ammonia (<0.5 ppm) (Pallintest kit®) and pH (6.0-6.2). Fish were fed daily *ad libitum* with commercial cichlid sticks (ASTRA).

2.2. Experimental aquaria and set up

Under experimental conditions, the animals were housed individually in testing aquaria of approximately 40 l (50 x 25 x 30 cm). The remaining conditions were the same as described for the stock tanks. All aquaria were laterally covered with opaque partitions in order to avoid visual contact between animals. The confinement aquaria (21x19x35 cm) were placed next to the experimental ones, also in visual isolation and filled with 2 litters of water (enough to cover the body height, with the dorsal fin closed) and no substrate.

The experimental set up was the same for the 2 experiments, with the exception of the nature of the stimuli/stressor used: a confinement paradigm, as a negative event (experiment 1) and feeding, as a positive event (experiment 2). A schematic representation of the experimental set up is presented in Figure 1. Both experiments involved a paired design, with predictable and unpredictable balanced treatments. Each experiment lasted for 19 days, during which the animals were maintained isolated in the experimental aquaria to control for variation due to different social ranks (Oliveira et al., 2005). A blood sampling for baseline plasma cortisol took place at day 4, training occurred at days 8-9 (predictable or unpredictable treatment) and days 17-18 (unpredictable or predictable treatment), immediately followed by the test trials at day 10 and 19, respectively. As control groups for cortisol baseline levels, blood samples were obtained from 19 males in three different contexts: in stable groups in stock tanks, and after 4-days and after 10-days of social isolation.

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Procedure	I	I	I	B	I	I	I	PRE	PRE	T_{PRE}	I	I	I	I	I	I	UNP	UNP	T_{UNP}

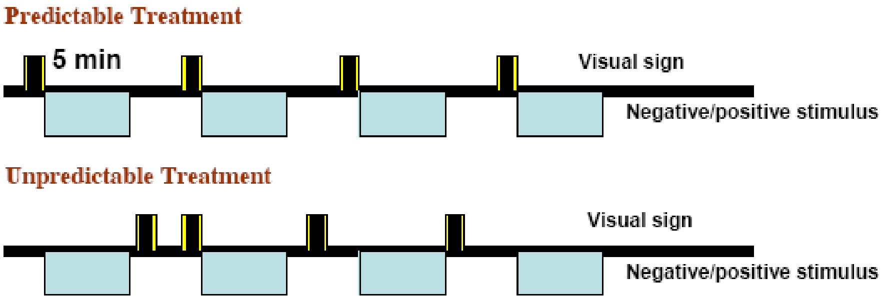


Figure 1. Schematic representation of the experimental set up. I – Social Isolation B – Blood sampling for baseline cortisol; PRE – Training for predictable treatment; TPRE – Trial for predictable treatment; UNP – Training for unpredictable treatment; TUNP – Trial for unpredictable treatment. Order of predictable and unpredictable treatments was balanced and the presentation of the signal in the unpredictable group was randomized.

2.3. Visual cue, valence events, training and trial procedures

Predictability was signalled in both experiments by means of a visual cue consisting of a yellow and black stripped card (20x20 cm). The card was placed in the outside of the aquarium lateral wall, being visually accessible to the fish for five minutes before the occurrence of the signalled event: confinement (experiment 1) or food (experiment 2). The unpredictable treatment involved the presentation of the same sign dissociated from the subsequent event, that is, at least 30 minutes before or after the session in a random way.

The negative valence event (confinement) consisted quickly capturing (preventing chasing) the animal from the experimental aquarium with a hand net and placing it in the confinement aquarium for 30 minutes. This paradigm, with variable methodologies, has been generally adopted as a stress test in fish (e.g. Schjolden et al., 2005). Behavioural sampling was undertaken during the first ten minutes and blood sampling for plasma cortisol measurement at the end of the confinement period.

The positive valence event (feeding) consisted of the delivery of two pellets of the usually used commercial food for cichlids per session (four training sessions per day covered the individual daily portion). These pellets fluctuate for some time before swelling and sinking. Usually, fish take them from the surface. The pellets were delivered in the front part of the aquarium using the opposite corner at which the signal for predictable treatments was given.

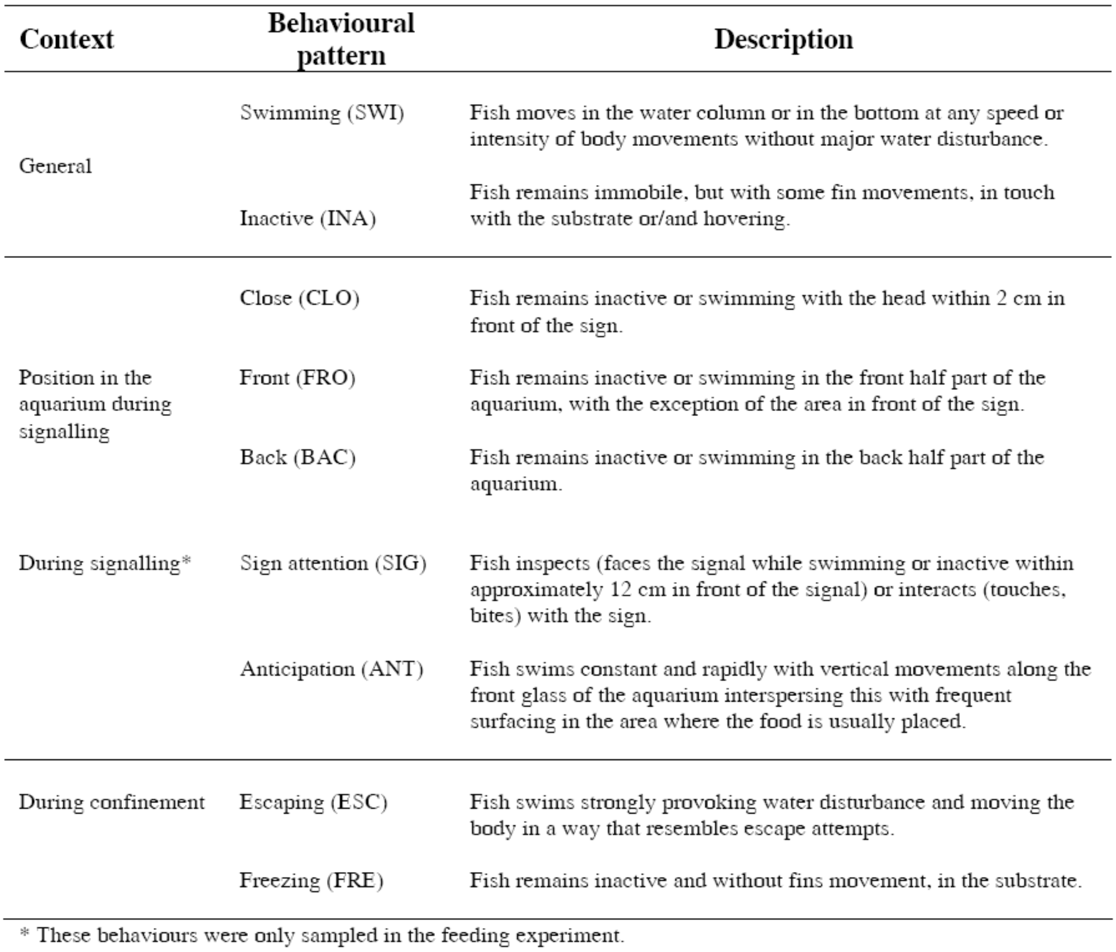
Each experiment involved eight training sessions, developed during 2 days, at 10.00, 12.00, 14.00 and 16.00h. A study developed in our laboratory has shown that males of this species can be conditioned within 6 to 8 training sessions (Antunes and Oliveira, 2009). A schematic representation of the predictable and unpredictable training is presented in Figure 1. The trials took place the day after the 2-day training sessions at 10.00h. In experiment 1, confinement took place as usual. In experiment 2, after the 5' signalling, no fish were fed but immediately anaesthetised to avoid anaesthesia complications.

2.4. Behavioural and blood sampling and cortisol assay

Behaviour during signalling was recorded by means of five minute scan sampling, with a total of 10 sampling points per individual (Martin and Bateson, 2007). In experiment 1 (confinement), only 12 out of the total 29 involved animals were observed during the presentation of the visual cue while the whole set of fish was sampled in experiment 2 (feeding) (n=24). Behaviour during confinement was also sampled through scan sampling, during the first 10 minutes of the test, with a total of 60 sampling points. The behavioural activities and position of fish in the tank were noted as described in Table 1.

At the end of the confinement test (experiment 1), fish were lightly anaesthetised in the confinement aquarium without additional disturbance (Stage two anaesthesia following (Ross, 2001)) by adding a solution of MS-222 (tricaine methane sulphonate, Sigma; 200 ppm) to the water. Samples of 100-200 µl of blood were taken from the caudal vein (1 ml syringes; 25G/16 mm needles). The fish were then placed in aerated water, recovering from the anaesthesia within 30 seconds to one minute. Blood sampling was performed within a maximum of four minutes from the induction of anaesthesia, which is within the latency for cortisol release into the systemic circulation in response to handling stress in this species (Foo and Lam, 1993).

Table 1. Brief description of the behaviour patterns and use of specific areas of the aquaria adopted for sampling.



Context	Behavioural pattern	Description
General	Swimming (SWI)	Fish moves in the water column or in the bottom at any speed or intensity of body movements without major water disturbance.
	Inactive (INA)	Fish remains immobile, but with some fin movements, in touch with the substrate or/and hovering.
Position in the aquarium during signalling	Close (CLO)	Fish remains inactive or swimming with the head within 2 cm in front of the sign.
	Front (FRO)	Fish remains inactive or swimming in the front half part of the aquarium, with the exception of the area in front of the sign.
	Back (BAC)	Fish remains inactive or swimming in the back half part of the aquarium.
During signalling*	Sign attention (SIG)	Fish inspects (faces the signal while swimming or inactive within approximately 12 cm in front of the signal) or interacts (touches, bites) with the sign.
	Anticipation (ANT)	Fish swims constant and rapidly with vertical movements along the front glass of the aquarium interspersing this with frequent surfacing in the area where the food is usually placed.
During confinement	Escaping (ESC)	Fish swims strongly provoking water disturbance and moving the body in a way that resembles escape attempts.
	Freezing (FRE)	Fish remains inactive and without fins movement, in the substrate.

* These behaviours were only sampled in the feeding experiment.

Free cortisol fraction was extracted from the plasma by adding diethyl ether to the sample, as the steroid solvent. The samples were then centrifuged (5 minutes, 1000 rpm, 4ºC) and frozen (10 minutes, -80ºC) to separate the ether fraction. The steroids were isolated by evaporating the ether. This process was repeated twice. Levels of free cortisol fraction were then determined by radioimmunoassay, using the commercial antibody ‘Anti-rabbit, Cortisol-3’ [ref: 20-CR50, Interchim (Fitzgerald), Montluçon, France, cross-reactivity: cortisol 100%, Prednisolone 36%, 11-Desoxycortisol 5.7%, Corticosterone 3.3%, Cortisone < 0.7%] and the radioactive marker [1,2,6,7-3H] Cortisol [ref: TRK407-250mCi, Amersham Biosciences, Piscataway, NJ/USA]. Intra- and inter-assay variability were respectively as follows: experiment 1, 3.8% and 5.9%; experiment 2 and ‘cortisol control’, 5.8% and 6.5%.

2.5. Data Analysis

All analyses were conducted using the statistical package Statistica V.8.0® (StatSoft Inc, USA, 1984-2008). For analysis of cortisol among treatments (baseline/predictable/unpredictable) and for the 'cortisol control', a one-way repeated measures ANOVA was used followed by planned comparisons of least squares means. Behavioural comparisons of predictable vs. unpredictable treatments were carried out using Wilcoxon Matched Pairs Test. For comparisons of cortisol between changes from baseline of predictable and unpredictable treatments, the same test was used after calculating the variation between the baseline in social isolation and each of the treatments. A significance value of $P < 0.05$ was used in all statistical tests.

2.6. Ethical Note

The experiments described were conducted in accordance to national legal standards on protection of animals used for experimental purposes and are part of a project approved by the national authorities (Ref. 30489, 29/11/2007).

3. RESULTS

3.1. Baseline cortisol levels

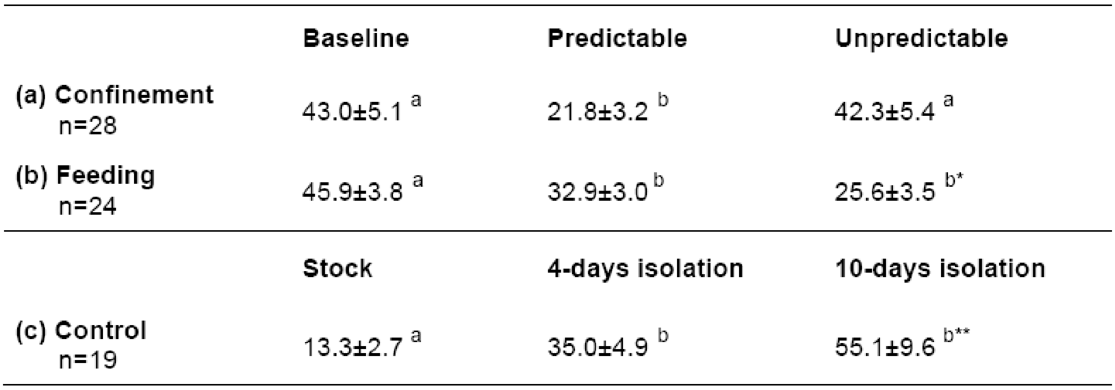
The baseline cortisol levels of experiment 1 (confinement) and 2 (feeding) were measured after 4 days of isolation and are summarised in Table 2. In experiment 1, the baseline level is higher than the predictable treatment level (one-way repeated measures ANOVA, $F_{(2,54)} = 6.9$, $P<0.01$, planned comparisons of LS means $P=0.002$). In experiment 2, the baseline cortisol is also higher than the both treatments level (one-way repeated measures ANOVA, $F_{(2,46)} = 12.3$, $P<0.001$, planned comparisons of LS means: predictable, $P=0.004$; unpredictable, $P<0.001$).

Control levels of cortisol changed considerably with the social context (Table 2). Males exhibited the lowest levels of cortisol when they were housed in groups (stock), and significantly increased when they were placed in social isolation (one-way repeated measures ANOVA, $F_{(2,36)} = 11.3$, $P<0.001$, planned comparisons of LS means $P<0.001$).

3.2. Experiment 1 – Negative valence event: confinement stress

During stimulus signalling, the animals spent more time close to the visual sign in the predictable treatment than in the unpredictable one (Wilcoxon matched pairs test, Z=2.4, P=0.02 [n=12]; Figure 2a). There were no differences in the use of the front or back parts of the aquarium between treatments (Wilcoxon matched pairs test, front and back: Z=0.8, ns [n=12]; Figure 2a).

Table 2. Mean ± standard error of cortisol values (ng/ml) in (a) confinement (b) feeding (c) cortisol control in stock and after 4 and 10 days of isolation. Letters a and b express differences between treatments (one-way repeated measures ANOVA, planned comparisons of least square means, P<0.01). *P=0.09; **P=0.08.



	Baseline	**Predictable**	**Unpredictable**
(a) Confinement n=28	43.0±5.1 ^{a}	21.8±3.2 ^{b}	42.3±5.4 ^{a}
(b) Feeding n=24	45.9±3.8 ^{a}	32.9±3.0 ^{b}	25.6±3.5 ^{b*}
	Stock	**4-days isolation**	**10-days isolation**
(c) Control n=19	13.3±2.7 ^{a}	35.0±4.9 ^{b}	55.1±9.6 ^{b**}

During confinement, no differences between treatments were found in frequency of swimming (Wilcoxon matched pairs test, Z=0.9, ns [n=29]) and inactivity (Wilcoxon matched pairs test, Z=1.4, ns [n=29]). However, freezing behaviour tended to be more frequent in the unpredictable treatment (Wilcoxon matched pairs test, Z=1.7, P=0.09 [n=29]). Escaping was very rare in both treatments.

Cortisol levels of the predictable treatment were significantly lower than those of both the control group (measured in social isolation) and of the unpredictable treatment (Wilcoxon matched pairs test, Z=3.1, P=0.002 [n=28]; Table 2, Figure 2b).

3.3. Experiment 2 – Positive valence event: feeding

During stimulus signalling, the animals spent the same amount of time close to the visual sign in the predictable and in the unpredictable treatment (Wilcoxon matched pairs test, Z=0.5, ns [n=24]; Figure 2c). Also no difference between treatments was found for attention towards visual cue (Wilcoxon matched pairs test, Z=1.1, ns [n=24]; Table 1, Figure 3). However, there were differences in the use of space: animals spent more time in the front areas of the aquarium in the predictable treatment (Wilcoxon matched pairs test, Z=3.3, P=0.001 [n=24]; Figure 2c) and in the back in the unpredictable one (Wilcoxon matched pairs test, Z=3.2, P=0.002 [n=24]; Figure 2c).

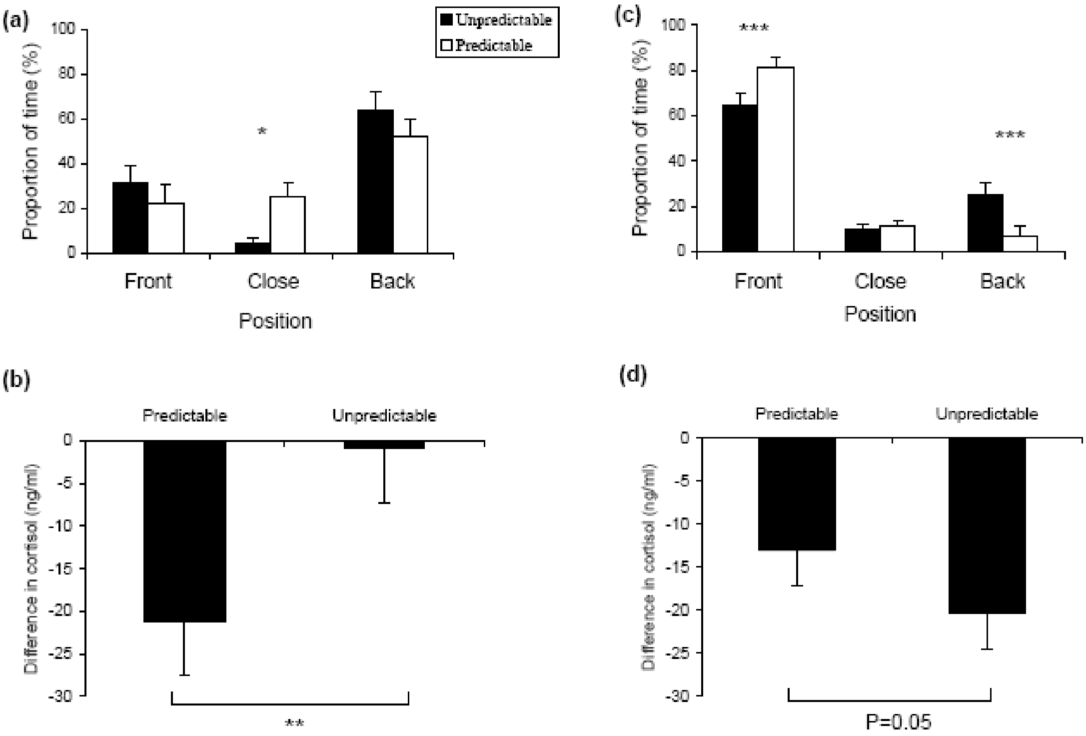


Figure 2. Proportion of time (%) spent in different areas of the aquarium (front, close and back in relation to the visual cue) during signalling of (a) confinement and (c) feeding. Difference in cortisol between baseline values and predictable/unpredictable treatments after (b) confinement (d) feeding *P<0.05 **P<0.01 ***P=0.001

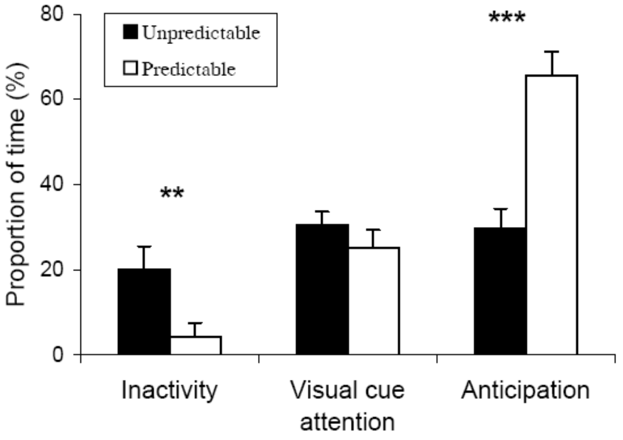


Figure 3. Proportion of time (%) spent in different behavioural patterns during the feeding event signalling **P<0.01 ***P<0.001

Levels of activity were also measured, with a higher inactivity being shown during the unpredictable treatment (Wilcoxon matched pairs test, Z=2.6, P=0.009 [n=24]; Figure 3). During signalling, levels of anticipatory behaviour were significantly higher in animals of the predictable treatment (Wilcoxon matched pairs test, Z=3.9, P<0.001 [n=24]; Table 1, Figure 3).

In relation to baseline levels, cortisol levels were significantly more decreased during the unpredictable treatment than during the predictable treatment (Wilcoxon matched pairs test, Z=2.0, P=0.05 [n=24]; Table 2, Figure 2d).

4. DISCUSSION

The current study suggests that the fish response to stressors and positive events is modified by their appraisal of the situation (i.e. predictable vs. unpredictable). There is a differential effect of predictability depending on the valence of the event in relation to the negative (confinement) and positive (feeding) events. In the confinement experiment (negative valence event), the predictable group paid more attention to the visual sign, expressed less freezing behaviour and a diminished cortisol response in relation to the baseline in social isolation. On the other hand, the signalling of food presentation (positive valence event) has triggered higher levels of anticipatory behaviour and activity in the predictable group. Despite the decreased cortisol levels in relation to the baseline in social isolation in the predictable group, there was a more marked cortisol decrease in the unpredictable group in response to food.

4.1. Baseline cortisol levels

Social isolation, whether for 4 or 10 days, increases the cortisol levels of male tilapia. This fact suggests that isolation is a stressful procedure, which is in accordance with the fact that *O. mossambicus* is a group living species (Oliveira and Almada, 1996). In this experiment males were placed in social isolation prior to the experimental treatment, which has been a routine procedure to minimize behavioural and androgen variation due to different social ranks (Oliveira et al., 2005; Chapter II). However, data presented here shows that this is not the most appropriate procedure to obtain baseline cortisol levels, since social isolation activated the hypothalamus-pituitary-interrenal axis. Therefore, the use of cortisol levels from males kept in stable social groups (stock) as a proxy for a measure of cortisol baseline levels in this species is a better approach. Even acknowledging that cortisol levels in control groups exposed to social isolation do not represent baseline values these were used as reference values in the analyses of cortisol levels of experimental treatments, since they are the matched groups in terms of experimental design. These analyses, suggest that predictable confinement and (predictable or unpredictable) presentation of food were both less stressful procedures than social isolation *per se*.

4.2. Predictability of a negative valence event (experiment 1)

During confinement, the males subjected to the predictable treatment tended to show less freezing behaviour and showed lower cortisol levels. Freezing behaviour is a well known inhibitory response to stress and it has been previously described for fish under stressful contexts (e.g. Vilhunen and Hirvonen, 2003; Yue et al., 2004). Together, the behaviour under confinement and the cortisol response, strongly suggest that the males subjected to the predictable treatment were less stressed than males in the unpredictable treatment. This is in line with the cognitive activation theory of stress, whereby predictability decreases the degree of discrepancy between internal expectancies (set values) and the reality (actual value), with the concomitant decrease in levels of arousal and stress response (Ursin and Eriksen, 2004). These results are also consistent with many published studies on the effect of predictability of aversive events in other species (Orsini et al., 2002; Prior, 2002). The animals seemed to pay more attention to the visual cue in the predictable treatment, as judged by the time they spent close to it, suggesting that the cue was being investigated and, thus, its meaning processed accordingly. The ‘safety signal hypothesis’, discussed by Basset and Buchanan-Smith (2007), suggests that predictability is less stressful because it signals the safety periods in the absence of cue. Additionally, predictability may provide a perception of control, as it allows self-preparation of the incoming event, even if only internally and not through behaviour (Orsini et al., 2002).

4.3. Predictability of a positive valence event (experiment 2)

When the stimulus was food, there was no difference in attentiveness to the visual cue (as measured by time close by or exploration) between the predictable and unpredictable treatments. However, a very conspicuous difference was noted in increased activity and anticipatory behaviour by the predictable group. A strong tendency to an increased cortisol level was also noted in this same group. Together with the behavioural data, the results show that the predictable context is potentially more stressful than the unpredictable one. The occurrence of increased activity and anticipatory behaviour in predictable feeding schedules is in accordance with many other studies where ‘Food Anticipatory Activity’ has been reported (Carlstead, 1996;

Bassett and Buchanan-Smith, 2007). These studies rarely show physiological data related to the expression of anticipatory behaviour. However, in a study of feeding predictability in sea bream, Sánchez et al. (Sánchez et al., 2009) also observed an increased cortisol response associated to anticipatory behaviour. While some authors highlight the role of anticipatory behaviour as an expression of positive emotions in animals (e.g. Spruijt et al., 2001), its subjective meaning has been less discussed (but see also Manteuffel et al., 2009). In our view, the meaning of anticipatory behaviour in relation to well being is inextricably linked to the delay between the triggering signal and the onset of the expected event. If it is short enough, the expression of anticipatory behaviour may mean arousal related to positive emotions, also expressed by means of less aggression and more exploratory behaviour (e.g. de Jonge et al., 2008b). This is the case in a number of studies where predictable schedules for positive events is reported as positive and where time period between the signal and the event was only of some seconds (Dudink et al., 2006; de Jonge et al., 2008a). However, in studies of temporal predictability (fixed times) or when the time span between the signal and the expected event is longer (from minutes to hours), this may induce expectation, frustration and indeed some loss of control (and, thus, unpredictability). In this case, animals may engage in high levels of anticipatory behaviour, even stereotypies, with related aggression and other stress-related behaviours (Carlstead, 1996; Gilbert-Norton et al., 2009). To be also noted that some reported beneficial predictable feeding schedules may be due to experimental designs where the unpredictable treatment represented in fact a loss of predictability (e.g. Ulyan et al., 2006). In the present study, the increased anticipatory behaviour and cortisol suggest that five minutes of food signalling may be an enough delay to potentially develop negative subjective effects in Mozambique tilapia. Therefore, levels of arousal observed in the predictable feeding may be subjectively linked to negative emotions and thus have a negative impact on welfare. Cortisol tended to be higher, whether due to the expectation/loss of control or to the increased activity, or to both. In fish, the relationship between cortisol and affective states has not been established, but in humans it is well known that the hypothalamus-pituitary-adrenal axis is more sensitive to negative emotions than to positive ones (Lovallo, 2005).

In conclusion, Mozambique tilapia males are able to appraise stimuli predictability. They show an increased stress response to unpredictable negative events and to predictable positive ones, in the latter case probably due to the expectation generated by a delay between signalling and actual occurrence of the event. These findings demonstrate the occurrence of appraisal in fish which have important welfare implications for the management of teleosts in captivity. Husbandry and experimental procedures should include the reliable signalling of aversive events and avoid delays of predictable attractive events, such as feeding times.

5. Acknowledgments

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CHAPTER VIII

General Discussion

1. Overview of results

The aims of the present thesis were to identify behavioural and physiological indicators of welfare in *Oreochromis mossambicus* and to investigate the existence of a psychological dimension in the stress response using the same species as a fish model.

In Chapter II, social groups of two males and two females were observed for five days with and without access to substrate. Absence of substrate was found to weaken the establishment of dominance relationships, decrease male sexual and territorial behaviours (pit digging and hovering over the pit), promote possible abnormal behaviours (such as vacuum pit digging and high levels of chafing), decrease levels of general activity and, at least for some of the males, decrease behavioural diversity. There was no difference in levels of aggression between conditions with and without substrate. At the physiological level, there were no differences in cortisol and glucose levels between conditions, but the haematocrit was significantly lower without substrate.

In Chapter III, the objective was to assess preferences for substrate in territorial and non-territorial males before and after the arrival of a female. A clear choice for the substrate compartment by territorial males was observed, where they spent more time swimming, hovering, courting, pit digging and nipping. This preference was expressed before a female's arrival to the tank, but the female's presence has even strengthened the time spent in the substrate compartment. Both territorial and non-territorial animals preferred to forage in the substrate compartment, and both exhibited more dragging in the non-substrate compartment.

Chapter IV aimed to develop a new approach to evaluate fish motivation to gain access to certain resources. The 'push-door' paradigm was adapted to fish, which were required to touch and push a number of times before having access to the resources (social partner, food and substrate only). Mozambique tilapia was able to quickly develop a strategy to respond to the operant task, keeping a high level of attention to the push-door. Latency to open the door increased with access cost for all resources, but it was always higher for the control reinforcer (substrate only). Work efficiency and maximum price paid was higher for social partner and food and lower for the control. Work efficiency decreased with cost for the control resource. Territorial males tended to work more for the social partner at higher costs than non-territorial males. Results show that males value food and social partners in a similar way, and more than a substrate-only compartment. It was apparent that territorial males tended to value social partners more than non-territorial males.

In Chapter V, the objective was to validate the use of cortisol as a measure of stress in Mozambique tilapia. Diurnal variation and social status, social isolation and response to an ACTH challenge were the aspects studied. Results were used as reference figures to compare and interpret levels of cortisol obtained from all experiments. Diurnal cortisol variation was found to be very similar to that of other diurnal vertebrates, showing a gradual increase during the dark period and a peak in the early morning. Social isolation has caused an increase of cortisol levels in non-territorial males. The *in vivo* ACTH challenge showed a threshold mechanism, with cortisol levels varying from an identified baseline (approximately 25 ng/ml, for ACTH doses below 10^{-3} µg/g), to an upper plateau (100-120 ng/ml for ACTH doses above 10^{-2} µg/g).

The physiological stress response can be modulated by psychological factors as reviewed in Chapter I. Two similar stressors can be appraised differently with completely different allostatic consequences, as shown by the measurable stress responses. Despite the existent knowledge on psychological factors affecting the stress response in other vertebrates, in fish very little is known since the emphasis has been the physiological stress processes.

Chapter VI analyses the effect of social support in the stress response of my model species, *O. mossambicus*. With that purpose, fish were subjected to a neophobia and a confinement test under different social contexts (stable familiar, unstable familiar unstable unfamiliar, isolated). The novel object promoted exploratory behaviour in stable familiar males as compared with the unstable familiar, unfamiliar and isolated males, and did not affect males' patterns of inactivity and interactions with females in any social contexts. The stress response to confinement was not affected by social contexts.

Chapter VII studies the effect of predictability to a negative (confinement) and a positive (feeding) stimulus arrival in the stress response. An increased stress response was related to the unpredictable confinement, as measured by higher levels of cortisol and a tendency for increased freezing. On the other hand, higher levels of anticipatory behaviour and a tendency for increased cortisol have suggested that the predictable presentation of food triggered a more conspicuous stress reaction.

2. Behavioural and physiological welfare indicators

2.1. Understanding needs in a cichlid fish

Needs are basic biological requirements to obtain a particular environmental or social resource or to perform a particular behaviour in response to internal or/and external stimuli (Broom, 2008). Animals are highly motivated to fulfil their needs and if they are prevented from doing so, their welfare is likely to be affected (Duncan, 2006). In natural conditions, unsatisfied needs are likely to have a cost for short or long-term survival and fitness. Therefore, animals developed evolutionary strategies to fulfil their needs. In captivity, even if the fitness cost no longer exists, unfulfilled needs may affect them through negative mental states. Below I discuss the existence of some needs in *O. mossambicus* and the respective context of occurrence, based on the experiments of Chapters II-IV.

Global results of experiments from Chapters II-III have shown evidence that substrate is probably a need for males in a breeding stage. The same experiments also suggest that this resource may in fact be multi-functional, and thus relevant in a number of different contexts. In Chapter II, substrate seemed to play a role in the social hierarchy and territory establishment by territorial fish, in the spawning pit building and in frequency of courtship, very much in accordance with descriptions of behaviour in natural conditions (Neil, 1966; Bruton and Boltt, 1975). On the other hand, during breeding dynamics, non-territorial males tend to keep away from substrate (Chapter III), although this resource may also be important for these males. For example, in Chapter II, both type of males preferred substrate for foraging, which is compatible with the detritivorous nature of this species (Fryer and Iles, 1972; Trewavas, 1983). In Chapter II, the occurrence of similar levels of aggressive behaviour in aquaria with and without substrate led to the hypothesis that this environmental element may contribute to the maintenance of lower levels of non-territorial aggression due to displaced nipping by the subordinates. Other authors have suggested that nipping and digging at the substrate may be a re-directed pattern of aggression, thus helping to balance levels of agonism (Heiligenberg, 1965; Barlow, 1974; Munro and Pitcher, 1985; Oliveira and Almada, 1998). A preference test, similar to that of Chapter III, could further investigate the role of substrate for subordinate males during aggressive bouts. Females' need for this resource was not apparent in the study undertaken in Chapter II. However, personal observations of pre-spawning females building nests when no males were available, also reported in earlier studies (Baerends and Baerends-Van Roon, 1950), suggest that substrate may have an indirect relevance for females which could also deserve further research.

In Chapter IV, animals were less motivated for access to a compartment with substrate only, than that with food or a social partner (female). This result further suggests that substrate as a resource might have context-specific relevance, that is, might be relevant under breeding, agonistic, foraging or other particular contexts. The use of substrate in natural conditions is consistent with this possibility: non-breeding animals form schools in deeper waters and substrate assumes particular relevance during the breeding season only, when males move to shallower areas in order to feed or form territories (Bruton and Boltt, 1975). The context-dependence hypothesis would require further research, but it is apparent that the same occurs in other fish species. For example, a study on substrate preferences in sticklebacks concluded that substrate preference is context-dependent (Webster and Hart, 2004). It concluded that fish did prefer complex over simple substrate for foraging, but only if hunger-motivated and when both substrates had the same amounts of prey or when the complex had more prey than simple substrate.

Chapter IV also pointed out other potentially important needs, corroborated by experiments in other chapters. In fact, results of the motivation test showed that fish value social contact as much as they value food. This result not only agrees with a variety of other similar studies in social vertebrates (Mason et al., 2001; Pedersen et al., 2002; Hovland et al., 2006), but also with the fact that social isolation has been shown to be a major stressor in this species (Chapters VI and VII). However, the value attributed to these resources may vary and cannot be easily generalised. For example, in Chapter IV, territorial males tended to work more at higher cost for access to the social partner than non-territorial males. In relation to food, despite being a commodity of primary importance, in fish times of shortage do not have the same impact as in warm-blooded vertebrates (Monaghan, 1990). Furthermore, at least in natural conditions, territorial males suspend their feeding behaviour during the most active breeding phase (Neil, 1966).

From the welfare point of view, it is important to consider that the expression of needs may vary with context (e.g. breeding, foraging, aggression, etc.) and with different life cycle stages (e.g. territorial/non-territorial) (Fraser and Duncan, 1998). The expression of needs may also change with different coping styles, since in reacting to stressors, different coping mechanisms may lead to responses that may vary in a continuum between reactive (higher cortisol levels and inhibitory behaviour) and pro-active (lower cortisol levels and fight-or-flight defensive behaviour) modes (Koolhaas et al., 1999). Therefore, undue generalisations of needs' identification in animals may lead to wrong decisions and subsequent negative impacts on management of species in captivity. For example, different studies on environmental colour effect in *O. niloticus* failed in explaining results in relation to particular contexts (conditions of experiments), thus reaching different conclusions on which colour is more appropriate for fish (Volpato and Barreto, 2001; Merighe et al., 2004; McLean et al., 2008; Luchiari and Freire, 2009).

In conclusion, the results of the presented studies show that substrate, social partners and food are likely to be important to *O. mossambicus*, but the expression of these needs must be appropriately contextualised.

2.2. On the measurement of what a cichlid needs

Assessing how animals react towards relevant environmental or social resources contributes not only to a better understanding of their behavioural responses but also to what might be considered a need. Authors that regard mental experiences as a relevant aspect of the welfare concept, agree that the best way of assessing welfare is through the observation of behavioural reactions in response to the manipulation of eventual needs (Dawkins, 1990; Duncan, 1996; Kirkden and Pajor, 2006; Volpato et al., 2007). Animal's mental states associated to the satisfaction of needs can be measured by two different approaches, which were undertaken in the first part of this thesis. The first, adopted in Chapter II, implies the observation of the animal's responses, in an environment containing or lacking a given resource (substrate). In this approach, animals cannot control their access to the resource and, under deprivation conditions the occurrence of abnormal behaviours (e.g. vacuum or displaced activities) is regarded as an indicator of disturbed welfare (Kirkden and Pajor, 2006). The second approach involves two different kinds of tests in which animals' decisions are observed, after providing them with control over its environment (Dawkins, 1990; Kirkden and Pajor, 2006). The preference test concerns experiments in which animals can choose between alternative resources (e.g. in Chapter III, between having substrate or not). Motivation tests (Chapter IV) quantify the motivation strength for access to a given resource through the payment of a given cost which is often associated to the performance of an operant response (pushing a door).

Preference and motivation tests are more sensitive than most deprivation studies, since poor mental states are not always revealed by behavioural disturbances (Kirkden and Pajor, 2006). Preference tests provide important information on animals' preferences but can imply a few assumptions that are not always valid. Among them is the fact that animals, particularly domestic animals, make the best adaptive choices, which is not always the case (Fraser and Matthews, 1997). A preference test portrays choices that make animals feel better in each moment, which does not necessarily correspond to the best adaptive choices in a longer term. Other limitations relate to the experimental designs and to results' interpretation and their meaning in terms of welfare. All these aspects have been discussed and reviewed by several authors (Dawkins, 1980; Fraser and Matthews, 1997; Bateson, 2004; Duncan, 2006; Volpato et al., 2007). Design and interpretation of motivational tests have been the source of much debate and refinement (see Chapter IV). In any case, these tests represent a relevant advantage of allowing quantification in comparison to deprivation studies. Motivation tests can quantify the relevance of accessing a given resource, while deprivation studies can not quantify the amount of behavioural abnormality or levels of cortisol before it can be said that suffering occurs (Dawkins, 1990). However as Kirkden and Pajor (2006) point out, preference and motivation tests, by their very nature, provide external stimuli that may induce motivation in tested animals. Therefore, exclusively based on these tests, it is not possible to conclude whether a given resource would be needed if 'out of sight'. To overcome this limitation, motivation tests are complemented with deprivation studies during which behavioural and physiological data are collected. One elegant example of this is the study by Mason et al. (2001) where they demonstrated that mink would work for swimming as much as for food, and that when deprived from swimming their cortisol levels were comparable to food deprivation. Taken together, the work developed for accessing a social partner (Chapter IV) and the high levels of cortisol in socially isolated fish (Chapter VI and VII), present good evidence that social contact is a need for *O. mossambicus* males.

Chapter II-III include enough evidence to consider substrate as a need for territorial males in breeding contexts. Chapter II has shown how substrate's deprivation contributed to a less well established social hierarchy. When animals were living without substrate, a number of behavioural changes were observed (to be discussed in the following section). But the most relevant and direct expression of needs was the performance of vacuum activities by dominant males when substrate was not available. Vacuum activities are highly motivated behaviours that can be carried out even if the appropriate stimulus is not present (Manning and Dawkins, 1998). Animals were seen performing the entire sequence of digging behaviour and hovering over the area where the pit would have been built if substrate was available. The expression of vacuum activities is generally indicative of behavioural needs associated to a specific functional goal (e.g. a spawning pit) or be rewarding in itself even if the functional aspect of the behaviour is not attained (Jensen and Toates, 1997). For example, studies in hens have shown that nest building is a behavioural need; pre-laying hens perform it even if a fully built nest is provided (Duncan and Kite, 1987). Vacuum activities have been related to states of frustration, although it is unclear how they relate to suffering (Dawkins, 1990). Since the mere performance of the behaviour may be rewarding in itself, vacuum activities may well be part of a coping mechanism, thus relieving suffering. In any case, the existence of vacuum activities is highly suggestive of an inadequacy of some kind, which is why they have been associated to reduced welfare (Dawkins, 1990). The preference test of Chapter III corroborated the relevance of substrate for dominant males as measured by the increased time spent in the substrate compartment and the increased number of interactions with the resource, in particular after a female's arrival. As mentioned before, Chapter IV has shown preliminary evidence that animals worked less for the substrate-only compartment than to gain access to a social partner or to food, being possible that substrate, without contextual integration, may be devalued.

2.3. Motivational tests as a measure of wants

Wants tend to be associated to a cognitive modulation of needs, thus to a conscious representation of needs (Duncan and Petherick, 1991; Boissy et al., 2007). However, Berridge and Robinson (1998) claim that the core process of 'wants' may exist even in the absence of conscious experiences. These authors establish a difference between 'wanting' and 'liking', in that the first is associated to the incentive motivational value of a stimulus (goal-directed behaviour, attraction and consumption) and is modulated by the mesolimbic dopamine system and the second to the conscious hedonic state of pleasure ('liking'), modulated by opioids. This distinction has been use to explain the addictive effect of drugs, in which too much dopamine produces excessive 'wanting' even without 'liking'. However, as the same authors mention, it is adaptive that 'wanting' and 'liking' are associated processes in order to potentiate the acquisition of biologically relevant resources.

It is common to use motivational tests as a measure of 'wants' associated by analogy with humans to the subjective states (pleasure or aversion) caused by wanting to have access or to avoid a given stimulus (e.g. Dawkins, 1990; Kirkden and Pajor, 2006). Motivational tests are based on the assumption that the stimulus relevance is proportional to a cost the animal pays to get access to it, and thus it can be quantified (reviewed in Chapter IV). The first step for motivation to produce behaviour is the selection of the salient goal (e.g. the resource under test), which involves selective attention (Vimal, 2008). According to Vimal (2008), attention means focalization, concentration and information-selection, for which at least some degree of consciousness is required. The second step involves appraisal mechanisms, thus associated to the subjective states referred above to ‘liking’ mechanisms. Attention increases the sensitivity to reward, by activating the brain reward systems, closely related to emotions. These processes are generally linked to decision-making processes in the organisation of the behavioural responses. Reward systems operate as if they balance benefits of reward against costs (energy spent); the positive balance possibly corresponding to the experience of pleasure (Boissy et al., 2007). The animal then acts accordingly by anticipation. Animals will work for a reward as long as the balance between benefit/cost remains positive. In their simpler format, decision-making processes do not necessarily imply consciousness, involving simpler learning forms and innate mechanisms expressed by means of reflexive and simple behaviour (McFarland, 1989; Manning and Dawkins, 1998; Dawkins, 2006). However, behaviours requiring attention, flexible integration of various elements, complex forms of learning and memory are necessarily conscious (Boissy et al., 2007; Braithwaite and Boulcott, 2007). Behaviours of this type were extensively observed in fish (as reviewed in Chapter I, Table 1).

The push-door paradigm of Chapter IV seemed to be a suitable way to measure wants, and thus some cognitive features and possible associated affective states. It demanded from fish attention and a flexible strategy. Primarily used in hens and later in some mammal species, this paradigm could be adapted to this species because males of *O. mossambicus* have strong snouts that they use for natural ‘pushing’ activities, such as pit digging and mouth-to-mouth fights. Animals were in permanent visual (and chemical) contact with the reward placed in the adjacent compartment, but they had a barrier (the push-door) blocking their way. It seems evident that animals would be physically capable of performing the task. However, pushing a door was not simply a reflexive or innate behaviour because fish did not evolve responding to functionally similar stimuli in nature. Fish had to adapt their physical competences in a new situation and responded to it in a meaningful way. They have kept attention to the door, systematically directing their behaviour towards it. Attention was measured under the designation of ‘work efficiency’ which consisted of a time proportion spent working at the door (touches or pushes) before it opened to give access to the commodity. Through a short training period they have learnt that a given number of touches and pushes would cause the door to open. Therefore, they increased levels of attention and work as access costs increased for the more attractive rewards (social partner and food). Reversely, decreased attention (dispersion), as measured by increases in behaviours like nipping at surfaces other than the door, surfacing and chafing, were observed when the least attractive of the three resources (substrate-only) was under test.

2.4. To fight or not to fight: the importance of species-specific evolutionary history in interpreting welfare

When interpreting behaviour to make inferences about welfare in captivity, expression of aggression is often regarded as a symptom of reduced well-being. In aquaria, territorial fish, such as *O. mossambicus*, tend to keep swimming over territories which induce frequent territorial invasions, often with no hidden places for non-territorial animals to retreat. These circumstances tend to increase aggression levels, with escalated fights, risking integrity and survival of individuals. Therefore, aggression is generally perceived in the welfare context – even for territorial species - as an undesirable behaviour, to be avoided at any cost (e.g. Ashley, 2007).

Nevertheless, aggressive behaviour is common among animals, particularly in territorial species. Early experiments on preference and motivation in a territorial species, the Siamese fighting fish, have shown a very high motivation to fight (Hogan, 1967; Bols, 1977). In *O. mossambicus*, male conflict has an important role in survival and reproduction. In this species, it is frequently associated to breeding contexts, as previously discussed. In natural conditions, overt aggression gave rise to more ritualised fights, with no physical contact, in this way saving fish from serious injuries (Fryer and Iles, 1972). In captivity, bouts of aggression can occur without an obvious relationship with territoriality. That was the case in Chapter I, when animals were observed with and without substrate. In the aquaria containing substrate, territorial fights took place as described for natural conditions. In the absence of substrate, levels of aggression remained the same, but without an obvious goal. Various concurrent aspects may explain the reason for these sustained levels of ‘non-functional’ aggression. It may be determined by persistent endogenous factors, like increased levels of androgens associated with breeding behaviour (Oliveira, 2004). Aggression may then be a behavioural need, and thus highly motivated and performed, regardless the attainment of the functional goal. Aggression may also be promoted by stress factors (Barlow, 1974; Munro and Pitcher, 1985), or by the absence of suitable outlets for frustration (e.g. substrate, as previously discussed).

It can be concluded that the role of aggression in the evaluation of welfare should be rooted in the evolutionary history of the species (Barnard and Hurst, 1996). In interpreting behaviour as a coping response, it is highly relevant to consider the functional contexts in which it occurs. Failure in approaching behaviour in this way can lead to poor understanding of welfare and management of territorial fish in captivity. For example, some authors claim that a blue background increases aggression in Nile tilapia, thus suggesting that this colour is not appropriate (Fanta, 1995; Merighe et al., 2004). Volpato & Barreto (2001) reached a different conclusion, considering that aggression is a desirable behaviour in this species. However, none of these studies took into consideration the physical and social context in which aggression occurred, which is what provides information on possible good or poor welfare. In short, if aggression is performed in a functional context, it should be considered a positive indicator of welfare (Chapter I, aquaria with substrate). If not, it should be interpreted with caution, considering that it can be the expression of reduced welfare (Chapter I, aquaria without substrate).

In any case, consequences of aggression may be dramatic in terms of welfare if subordinate males are not provided with means of withdrawal. Therefore, promoting welfare in territorial species is a sensitive balance between allowing the performance of aggression in a biologically meaningful environment, and providing suitable protective conditions for subordinates (e.g. enough space to flee, refuges, appropriate group size and composition).

2.5. When the behavioural repertoire speaks about welfare

Behavioural patterns are the best possible tool to understand welfare (Dawkins, 2004) and to identify very subtle changes in internal states of animals. The objective of this section is to discuss which behaviours can signal reduced welfare. Brief considerations are also made in relation to behaviours that may indicate not only absence of suffering but rather pleasure (positive welfare).

In coping with a given context, behaviour can contribute to the animals' adaptation or not. In the first case, behaviour is interpreted as a 'normal behaviour' and in the second one as an 'indicator of poor welfare'. In natural conditions, species' evolution tended to adapt behaviour to predictable and unpredictable environmental changes (allostatic process). Therefore, knowledge of species specific behavioural repertoire, respective daily time budgets and stages of life cycles are very important for the understanding of their specific requirements or needs in captivity (Mench and Mason, 1997). This is why a common rule-of-thumb to promote individual's welfare in captivity is to produce artificial environments as similar as possible to natural living conditions.

Normal behaviour in captivity is thus often regarded as natural behaviour. But beyond specific evolutionary adaptations, species' behavioural plasticity may, to some extent, contribute to animals' adaptation to new circumstances. Therefore, some changes in natural behaviour do not always mean reduced welfare or suffering. Mench and Mason (1997) point out three important adjustments of natural behaviour to captive conditions. The first is related to behaviour that animals no longer need to do in captivity. For example, predator avoidance behaviour (e.g. Vilhunen and Hirvonen, 2003). The second refers to behaviour that animals need to do if specific circumstances occur and for which they require a suitable environment. For example, a study with crucian carp exposed to olfactory cues from injured skin of conspecifics has shown how lack of hiding material has altered fish avoidance behaviour and increased brain serotonergic levels (Hoglund et al., 2005). The third category of natural behaviour includes those behaviours that animals are motivated to do whatever the circumstances. An example of this is the spawning pit building in Mozambique tilapia (Chapter II). As discussed in previous sections, these behaviours fall in the category of 'behavioural needs', which are very important to be kept in captivity, as they are the most prone to induce suffering. Therefore, normal behaviour should not be strictly regarded as natural behaviour, but rather the behaviour that successfully responds to specific artificial conditions.

In practice, normal behaviour largely overlaps with natural behaviour. Discrepancies in behaviour in relation to natural circumstances are then used to assess needs and welfare (Stolba and Wood-Gush, 1989). In Chapter II, it was assumed that fish with access to substrate exhibited behaviours similar to those in natural circumstances (Chapter I). The comparison of this condition with substrate deprivation aimed to identify discrepancies in behaviour. In other words, finding deviations from what was considered to be normal behaviour in captivity for this species was the aim of this experiment. Results clearly have shown that normal behaviour persists, but subtle changes can highlight aspects that might be relevant from the welfare point of view. These changes fall in the following categories:

a) Changes in the frequency of normal behaviour: the significant decrease of sexual behaviour, pit digging and territorial behaviour of dominant males has shown that breeding behaviour was less expressed, which can have interfered with welfare, given the ecological importance of this category of behaviours; the inactivity levels increased, which can be a sign of barren environments (for a more detailed discussion of this aspect see Chapter II).

b) Suppression of normal behaviour: suppression of pit digging was determined by lack of substrate. As mentioned before, certain behaviours can be suppressed in captivity without costs for the animals, while others clearly induce suffering. Results of Chapters II and III, e.g. exhibition of vacuum pit digging and clear preference for substrate by territorials, have shown that suppression of pit digging may cause suffering.

c) Normal behaviour out of context: this includes behaviours such as displacement activities (e.g. nipping the substrate under agonistic contexts, chaffing during social interactions; see Chapter II for further details on these behaviours) or vacuum activities (e.g. pit digging when no substrate is available). As already mentioned, these behaviours are typically used as indicators of poor welfare, and they constitute a symptom of a stressful context. However, as Mason and Latham (2004) point out, these behaviours are coping mechanisms, thus probably contributing to ameliorate the negative perception of the situation.

d) Changes in global diversity of behaviour: increased diversity of behaviour is used by some authors as an indicator of welfare, under the assumption that through diversity a relevant extent of the species behavioural repertoire is being expressed (Wemelsfelder et al., 2000). However, the use of this measure requires careful attention to the nature of the behavioural patterns actually concurring to the diversity. For example, in Chapter II, part of the behavioural diversity observed without substrate were in fact behaviours with no obvious function (e.g. aggression) or even interpreted as indicators of reduced welfare (e.g. vacuum activities).

Normal behaviour in captivity can fall outside the scope of natural behaviour. In fact, certain species in artificial conditions can well reshape natural behaviours to serve different functions. For example, Mozambique tilapia’s expression of feeding anticipatory behaviour includes repeated patterns of swimming and surfacing in the area where food is expected (Chapter VII). Repetitive patterns of swimming in the same area are not described in natural conditions, and surfacing in the wild is not likely to serve a feeding purpose in detritivorous species. Depending on cognitive and physical plasticity of species, some may even learn or spontaneously develop completely new patterns of behaviour to adapt to artificial conditions. This is the case in highly complex species such as dolphins that spontaneously perform a number of aerial behaviours in captivity (Galhardo et al., 1996). In Chapter II, I speculated that one observed behavioural pattern could be one such behaviour as it was exclusively seen when animals were substrate-deprived. It was named it 'dragging', and it consisted of a swimming pattern performed on the bottom, with a slight inclination of the body and with the edge of the snout dragging while progressing forward. This behaviour, exhibited by both territorial and non-territorial males (and also females), was systematically observed in experiments of Chapter III, in the non-substrate compartment. In Chapter II, it was also sampled but under the name of 'swimming on the bottom', while in Chapter IV it was observed when animals were acclimating around a substrate-less compartment. While performing this behaviour, animals moved around the aquarium as if they were carefully patrolling the floor. When in group (Chapter II) they would perform it in a school formation, which suggests a protective behaviour. In functional terms I speculate that the animals could have been exploring the substrate, trying to understand what type of 'substrate' that was. If this interpretation is valid, this behaviour *per se*, just like other coping mechanisms, cannot be considered indicative of poor welfare, as it is probably the fish's tool to adjust to novelty.

A final consideration relates to behaviours that can indicate positive welfare. In the scope of this thesis, I suggest that three kinds of behaviours can be used with this purpose: reward-related operant behaviours, exploration and reward's anticipation. As already discussed, Chapter IV has shown that animals were able to increase their attention and spontaneous responses to the push-door (touch and push) in order to access what they regarded as a reward. In this sense, the amount of attention paid to the stimulus and the concomitant behavioural response that gave access to rewards should be considered signs of positive welfare. But, as the majority of behaviours, attention should be interpreted in due context. For example, in Chapter VII, increased levels of attention to a visual cue signalling a predictable stressor (confinement) does not probably show more than that animals were learning the association between the visual cue and confinement. The ability to explore familiar or novel surroundings is ecologically vital and, in captivity, it has been used as a sign of low anxiety (e.g. Sneddon et al., 2003; Frost et al., 2007) or even positive welfare (Wemelsfelder and

Birke, 1997). Chapter VI shows that fish in stable visual contact with others were more prone to explore a novel object than animals in isolation, which showed neophobia, suggesting that the former were less prone to anxiety than the latter. Signs of reward's anticipation of the kind observed in Chapter VII may also be considered positive to some extent. However, if the time elapsed between the event's warning and its onset is excessive, this anticipation may easily become anxiety (for a development of this discussion, see Chapter VII). A better identification of this possible phenomenon requires crossing these behavioural indicators with physiological measurements, such as cortisol levels.

2.6. Physiological approach: what can be learnt from cortisol

Measuring corticosteroids in the scope of animal welfare studies has been a routine procedure. Their frequent use as stress hormones is due to the fact that they easily rise in response to a wide range of stressors, they are easily measured from blood, urine or water with commercial kits and baseline levels can be defined (see Chapter V). Corticosteroids, such as cortisol, are a measure of stress activation but are recognised to be an insufficient indicator of internal states (Volpato et al., 2007). This is so because production of this hormone measures arousal of the HPA axis, but hardly informs on the valence of associated stimuli, that is whether the stimulus is positive or negatively appraised by the animal (Boissy et al., 2007; Yeates and Main, 2008). Furthermore, cortisol can vary with a wide variety of internal and external factors, not always directly associated to a specific stress response. These sources of variation were extensively discussed in Chapter V, in general and in relation to *O. mossambicus*.

In the present discussion, I compare and interpret levels of cortisol found in different procedures throughout the experiments of Chapters II, V, VI and VII (Figure 1). The context in which *O. mossambicus* expressed the lowest levels of cortisol was the social stable condition (i.e. under a period of a few weeks without disturbance) in the stock aquaria within a social group composed by 3-4 males and 5-6 females. This is in full agreement with general literature on fish and other vertebrates covering the effects of social stable environments on baseline cortisol (e.g. DeVries et al 2003, Fox et al 1997, Yue et al 2006). During periods of social stability, dominant males tend to lower their cortisol levels and subordinates develop behavioural strategies to avoid openly competing with dominants (e.g. Gomez-Laplaza and Morgan, 2003). In artificial conditions, social stability may be disrupted by environmental inadequacies (e.g. lack of enough space). However in the present studies, the results of the three experiments (Chapter V and VII) show that animals seem to be well adapted to the environmental stock conditions, in which they have exhibited the lowest activation of the HPI axis, and thus the lowest levels of stress. Therefore, it became suggestive that this is the best context in artificial conditions to obtain a general baseline level of cortisol for *O. mossambicus*.

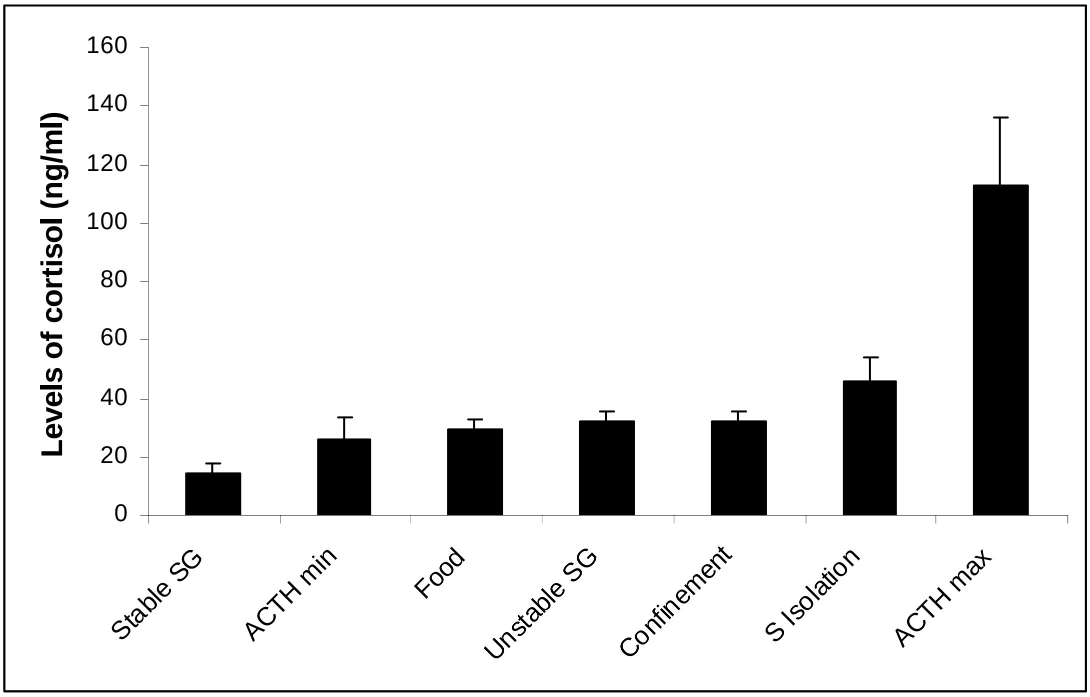

Figure 1. Mean levels of cortisol (ng/ml) expressed in different procedures undertaken under the scope of four separated experiments. Stable SG (stable social group, Chapters V and VII), ACTH min (mean level of lower threshold of ACTH challenge, Chapter V), Food (food anticipation, Chapter VII), Unstable SG (unstable social group with 4-days formation, Chapter II), Confinement (confinement in a restricted water level for 30 minutes, Chapters VI-VII), S Isolation (social isolation for periods between 4 and 10 days, Chapters V and VII), ACTH max (mean level of upper threshold of ACTH challenge, Chapter V).

The ACTH challenge undertaken in Chapter V has shown that there are two distinct thresholds of cortisol response. The lowest was approximately 25 ng/ml and corresponded to ACTH doses below 10^{-3} µg/g. The upper threshold produced cortisol levels of 100-120 ng/ml with ACTH doses above 10^{-2} µg/g. These results were discussed in detail in Chapter V. For the purpose of this discussion, the knowledge of these cortisol levels is very useful in allowing a better understanding on how strongly other stressors were imposed over the HPI axis's activity.

Food is generally regarded as a positive/rewarding stimulus. In Chapter VII, this resource was signalled five minutes before available. This procedure has promoted expectation, as observed by behaviour, and a tendency for higher levels of cortisol in relation to unpredictable food distribution. In fact, food-anticipatory response is a well described phenomenon in fish and other species (e.g. Mistlberger, 1994; Consten et al., 2001). Lovallo (2005) argues that cortisol tends to be less produced in contexts where there are positive appraisals, i.e. where stimuli are regarded as positive events. Therefore, it would be expectable that levels of cortisol would not be raised due to a feeding episode. However, feeding events are frequently associated to subtle contexts, difficult to isolate and to which the HPI is reactive. For example, under group conditions, a meal is closely related to competition, which raises cortisol (Gomez-Laplaza and Morgan, 2003; Øverli et al., 2005; Barreto and Volpato, 2007). Feeding in isolation does not seem to reduce levels of cortisol due to the negative effects of isolation (Gomez-Laplaza and Morgan, 2003). To my present knowledge, it is not known how fish cortisol responds in a situation where the mentioned variables (expectation, group *versus* isolation feeding) have been disentangled from the feeding event. In the experiment of Chapter VII, raised levels of cortisol can be considered moderate and corresponding to a minor ACTH challenge, thus it is possible that even with the mental perception of expectation for five minutes, food expectation does not present a major stressor for the fish.

Unstable social conditions mean that social status in the hierarchical organisation is being established or challenged due to variation of the social group's composition. The unstable social conditions of the present study (Chapter II) corresponded to the first 4 days of group formation, which is a very active period within the group, involving considerable levels of territorial competition and therefore increased levels of cortisol (Oliveira and Almada, 1998). However, when compared with responses to the ACTH challenge, cortisol increase due to group formation does not represent more than a moderate stressor. This result is expectable since the evolutionary history of this species shaped it to develop response mechanisms to successfully cope with recurrent contexts of hierarchical definition and acquisition of territories.

Confinement is a well known stress paradigm commonly used in *O. mossambicus* and producing physiological responses such as increased levels of cortisol and glucose and high opercular rate (Vijayan et al., 1997; Binuramesh et al., 2005). In the studies of Chapters VI and VII, individual confinement lasted for 30 minutes in a column of water just enough to cover the body's height (200 ml). Levels of cortisol produced in response to this stressor were moderate and comparable to presentation of food or formation of the social group. In one of the studies (Chapter VII), confinement even seemed to play a role of environmental enrichment, as levels of cortisol decreased in relation to the higher baseline levels in social isolation (for further details, see Chapter VII). Confinement was pre-assumed to be a more severe stressor. However, the fact that this type of confinement turned out to be only a moderate stressor becomes more understandable with the reports from natural habitats where these fish were found to survive in conditions of extreme reduction of water. Trewavas (1983) describes a situation where *O. mossambicus* individuals survived in a 1 meter layer of moist sand covered by an additional layer of 2 meters of dry sand.

Not surprisingly, social isolation undertaken in experiments of Chapters V and VII were stronger stressors than the previous ones, as far as cortisol levels are regarded. This is again in accordance with the animals' natural history, since they are social species in all stages of their life cycle (Fryer and Iles, 1972; Oliveira and Almada, 1996). Social isolation is known to activate the HPI axis also in other fish species such as convict cichlids (e.g. Earley et al., 2006), angelfish (e.g. Gomez-Laplaza and Morgan, 2003) and rainbow trout (Øverli et al., 2006).

In summary, the best baseline levels of cortisol, in the sense of lowest activation of HPI axis, are those corresponding to stable social groups (ca. 15 ng/ml). HPI's arousal in animals kept under the conditions of our laboratory range from mean levels of 25 to 120 ng/ml. Stressors used in the scope of the present studies could be considered moderate (around 30 ng/ml) in relation to maximum cortisol response. Levels found in social isolation are higher than for other stressors (above 40 ng/ml), which suggest that this stressor was the strongest used in the scope of this project. Provided sources of variation are taken into consideration, I agree with other authors' approaches (e.g. Mason et al., 2001) in using cortisol in conjunction with normal or abnormal behaviour. In this way, cortisol remains very valuable as a measure of stress, and thus of welfare.

3. Psychological modulators of the stress response

3.1. Appraisal, fish stress and welfare

As far as mental processes are concerned, fish have been considerably treated as 'black boxes', to borrow this term from behaviourism. In the research area of fish stress this is particularly true: a wide range of stressors have been identified, as well as the physiological and behavioural responses they invoke. Decades ago, this mechanistic approach to stress proved to be insufficient to explain differential reactions to the same stressors in humans and other terrestrial vertebrates (Sapolsky, 2004). The general stress picture became then more complex with the progressive acceptance that psychological aspects were relevant components of the whole process. Presently, a great deal of psychological variables is known to account for a variety of stress responses in humans and other animals. Only very recently, psychological aspects of fish biology started to be treated in a scientific and systematic way in the context of numerous studies mentioned in Chapter I. Evidence that fish are far more complex and brain-mediated entities than expected came from neuroanatomic, cognitive and behavioural studies (Chapter I, for appropriate references). With this platform of studies, and despite an extensive area of obscurity on the exact mechanisms, it is no longer possible to disregard a mental component in the fish stress response. This fact is reflected in the growing number of studies in the last decade, addressing mental or psychological processes in fish (e.g. Moreira et al., 2004; Moreira and Volpato, 2004; Barreto et al., 2006; Dunlop et al., 2006) Therefore, in fish too, psychological factors are highly likely to interfere with the brain mechanisms to produce appropriate responses. This interference may change the way fish ‘look’ at stimuli, that is, the way they appraise them.

In face of a stressor, the protective role of social support is well known in humans, primates and other animals (e.g. Johnson et al., 1996; Ruis et al., 1999; Andrade and Guimarães, 2003; Kiecolt-Glaser et al., 2005). Chapter VI has shown that *O. mossambicus* males, in a stable social context, have revealed less anxiety signs in face of a novel object (for details, see Chapter VI). This result is in agreement with a recent study on lake sturgeons, for which it was also concluded that social support played a beneficial role in regulating their stress response to brief air exposure (Allen et al., 2009). A different study involving rainbow trout and goldfish concluded that animals would pay a price (low intensity shock) to remain close to a social partner (Dunlop et al., 2006). However, the same study of Chapter VI found that the visual contact with a tank mate did not relieve the stress response to confinement. A number of different reasons may have contributed to it. On one hand, the patterns of cortisol variation may not have been sufficiently portrayed with a single sampling point (for further details, see Chapter VI). On the other hand, the experimental procedure may have not been the most appropriate to study social support since animals, due to confinement, had a limited visual contact to the partner. It is also possible that the result is not linked to intrinsic aspects of the experiment but that the effect of social support depends on the nature of the stressor, a possibility analysed in other studies (DeVries et al., 2003).

Predictability is a further psychological variable which seems to influence fish response to stimuli. Studies conducted in Chapter VII showed a higher stress response when fish were subjected to an unpredictable than to a predictable confinement. It has been suggested that appraisal of a predictable stimulus produces less arousal due to a sense of safety during periods of non-signalisation (Bassett and Buchanan-Smith, 2007). Additionally, predictability provides some sense of control, as it allows self-preparation to the incoming event, even if only internally and not by means of behaviour (Orsini *et al.*, 2002). However, when associated to a positive stimulus (e.g. food), predictability causes arousal as measured by levels of cortisol and anticipatory-like behaviours. Whether this arousal produced negative or positive mental states was not analysed under the scope of my studies. It is possible that associated mental states are dependent on the delay between the onset of the expected event and its previous signalisation. If the period is short enough, simple reward anticipation may produce positive mental states. However, if the delay is extended, animals may feel a loss of predictability in that they fail in anticipating exactly when the positive event will occur. In this case, signs of anxiety are very likely to be exhibited (Gilbert-Norton et al., 2009).

As I have already extensively discussed, pathological stress, allostatic overload or poor welfare occur when coping mechanisms fail to adjust the organism to its environment. Adaptation does not only consist of a homeostatic mechanism always ready to be triggered in response to threats. A more dynamic view of adaptation considers the body preparation in anticipation to predictable changes, as shown in Chapter VII. This allostatic view (Korte et al., 2007) is extremely important to the welfare concept: the animals' ability to change their coping strategies in anticipation and along with incoming challenges. These efficient adjustment mechanisms require the fine coordination of a brain. For that purpose, appraisal processes have a marked role in evaluating the nature and importance of those challenges. Thus, poor welfare means a reduced ability to cope with environmental challenges, and it is associated not only to the actual situation (incoming stressors) the animals are living but also, and most importantly, to how they appraise it.

3.2. Implications of appraisal for management of fish in captivity

As reviewed in Chapter I, the fish appraisal of stimuli and associated memory and learning abilities should be manipulated with the purpose of promoting more positive appraisals of the environment. Schreck and colleagues (1995) did it by conditioning chinook salmon to associate feeding to prior handling and transport. As a consequence, these animals showed a reduced physiological response to stress, an improved fitness during transport and a better coping response when exposed to additional stressors. In fact, by buffering the adverse impact of stressors, positive reinforcement training has good advantages for fish and other vertebrates' welfare (Laule and Desmond, 1998).

Operant conditioning techniques can provide means for the fish to control its environment, which as reviewed in Chapter I, is an important response-related variable modulating appraisal. For example, provision of correctly adjusted self-feeders (operated by the fish) may promote growth and feed conversion ratios in rainbow trout (Alanara, 1996). One of the control components is the ability for choosing between options. Appropriate environmental features which allow such choice may promote a positive perception of individual coping ability (Chapter III and IV). In carp, the provision of hiding material to undertake avoidance behaviour in face of a threatening olfactory cue, reduced the physiological response to stress and allowed avoidance behaviour (Hoglund *et al.*, 2005). Additionally, an appropriately enriched environment can provide animals with outlets for frustration. As discussed above, substrate may regulate levels of aggression in captivity and can provide an enlarged scope for foraging in environments that are poorer than in nature (Chapters II and III).

Keeping animals in appropriate social groups is a well known stabilising element in captivity as well as a powerful mean of providing environmental complexity and enrichment (Carlstead, 1996). As mentioned above, since isolation has a negative impact (Chapters V-VII), and conversely social support seems to reduce fear and anxiety (Chapter VI), the permanent access to adequate social contact should be used to improve welfare.

Considering that predictability modifies the fish coping responses, its manipulation can reduce the negative impact of certain stressful procedures in captivity. To be noted, however, that in line with results of Chapter VII, predictability for positive events may have a different influence on the fish response. In any case, a generalised idea in environmental enrichment research is that excessive predictability may enhance symptoms of boredom, reason why the controlled presentation of novel stimuli is favoured (Bassett and Buchanan-Smith, 2007; Morgan and Tromborg, 2007). Therefore, it seems reasonable to assume that for a good husbandry, predictable or unpredictable events must be managed on a case-by-case basis, taking into consideration the anticipation's factor and in relation to the events' nature, intensity and duration.

It is important to highlight the role of an appropriate level of challenges in the promotion of welfare in captivity (Wemelsfelder and Birke, 1997; Meehan and Mench, 2007). A prolonged deficit of environmental stimulation may impair mental and physical mechanisms due to chronic lack of opportunities to interact with the environment. Animals may show symptoms of apathy and boredom (Wemelsfelder and Birke, 1997). On the other hand, excessive environmental stimulation can sensitise coping mechanisms, enhancing permanent arousal and leading to a generalised state of exhaustion, which is the cause of many anxiety-related pathologies (Meehan and Mench, 2007). Therefore, inappropriate stimulation induces an allostatic overload of different types which is incompatible with good welfare (Mc Ewen and Wingfield, 2003).

I suggest that an adjusted level of environmental complexity, control and predictability, with the concomitant learning opportunities, are the most important factors to keep biological mechanisms within the boundaries of an acceptable allostatic load. In other words, these are key elements to promote a positive appraisal of the environment and hence a good welfare.

4. Conclusion

Animal welfare has to do with how an animal feels about its own situation, thus involving at least some degree of consciousness. Present scientific evidence allows including fish in the sphere of sentient animals. Specific motivational states are determined by a combination of internal and external factors modulated by memories, learning abilities and emotions. In dealing with internal and environmental stimuli (stressors), these motivational states lead to specific appraisals and coping mechanisms. A complex and well coordinated brain receives sensorial inputs, processes these mechanisms and organises responses. If stimuli are within the ecological limits of the species concerned, the animal has a major ability to cope successfully with them. This kind of allostatic load is considered a healthy stress response, and has no negative consequences for overall welfare, even if accompanied by transient and moderate negative mental states. However, if stressors become sustained over time or stronger than the animal's ability to cope, than the animal enters a state of allostatic overload, with pre-pathological or pathological signs of stress and definitely with a reduced welfare.

Welfare measurement implies interpreting the behavioural and physiological expression of coping mechanisms under specific contexts. I identified a number of behavioural patterns that, due to their context of occurrence or frequency patterns, were interpreted as possible indicators of poor (e.g. vacuum pit digging, reduced expression of courtship, aggression out of a territorial context, etc.) or good (e.g. exploration of novel objects) welfare. Important life and welfare requirements (needs) may vary with context, life cycle stages, evolutionary history and individual coping styles. Based on the present studies, I have concluded that substrate is an important environmental resource in the breeding phase of territorial Mozambique tilapia males. Social deprivation as produced the highest cortisol levels of all used stressors, while stable social groups the lowest cortisol levels. Motivation to access a social partner was as strong as motivation to feed. Together, these studies highlight the importance of a social resource to this species. A combination of deprivation, preference and motivational studies with a physiological measurement, such as cortisol, is likely to be the most fruitful approach to have an indirect indication of mental states. Promotion of welfare benefits from the understanding of fish appraisal mechanisms. The results of my studies suggest that social support reduces neophobia and that predictability of negative events decreases the stress response in comparison with unpredictable negative stimuli. Both can be used to reduce stress in a number of husbandry and experimental practices in captivity.

The welfare and sentience of fish is a relatively new field of research and thus presents numerous opportunities to develop relevant and innovative studies in many different scientific areas such as ethology, cognition, physiology, neuroscience, etc.. Under the specific objectives of this thesis, the identification of other possible needs, behaviours expressing them, and related contexts of occurrence deserves further investigation. For example, it would be interesting to investigate in detail patterns of behaviours that act as displacement activities and the possible occurrence of stereotypies. Studies of preference and motivation to have access to a diversity of resources under controlled contexts would also provide important information in terms of needs. With this purpose, the push-door paradigm can be a tool to be further refined. In the field of psychological modulators of the stress response, other factors beyond social support and predictability could also be investigated. Some examples of these may be related to the stimuli presentation, like the effect of stimuli relative intensity, or to the response, like providing the fish means of control over a given event.

In conclusion, identification of specific needs, behavioural and physiological indicators of welfare and appraisal mechanisms are relevant tools to understand and improve welfare of Mozambique tilapia in captivity. Provision of means to satisfy needs through appropriately enriched environments, with some means of control, a degree of predictability and opportunities for learning are likely to be the features that best guarantee the welfare of Mozambique tilapia and, in fact, of any sentient species.

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