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NEUROENDOCRINE REGULATION OF SOCIAL INTERACTIONS IN A CICHLID FISH

Ana Sofia Mendes da Silva Santos Félix

Tese submetida como requisito parcial para obtenção do grau de

Doutoramento em Biologia do Comportamento

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Dissertação orientada por Professor Doutor Rui Filipe Pais de Oliveira
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Palavras-Chave

Comportamento social, Cérebro, Hormonas, Tilápia de Moçambique

Keywords

Social behavior, Brain, Hormones, Mozambique tilapia

PsycINFO Classification Categories and Codes

2400 Animal Experimental & Comparative Psychology

2440 Social & Instinctive Behaviour

2500 Physiological Psychology & Neuroscience

2540 Physiological Processes

RESUMO

O estudo do comportamento animal e em particular do comportamento social tem atraído investigadores desde há muito tempo. Todos os animais interagem com os outros, característica fundamental para a sua sobrevivência e reprodução. No entanto, para obter uma total compreensão do comportamento social, é necessária a integração de seus vários componentes. Com esta tese, pretendemos clarificar este tópico, estudando como o cérebro controla o comportamento através da ação conjunta de seus circuitos neurais, genes e moléculas, e também como o ambiente social de forma recíproca influencia o cérebro. Baseado neste objetivo e usando a tilápia de Moçambique (*Oreochromis mossambicus*) como espécie modelo, num primeiro estudo investigámos como o comportamento social é controlado por uma rede dinâmica de regiões cerebrais, a *Social Decision Making Network* (SDMN). Aqui, tentámos entender quais são as pistas específicas que desencadeiam mudanças no padrão de ativação dessa rede neural, usando lutas entre machos. Os nossos resultados sugerem que é a avaliação mútua do comportamento de combate que impulsiona mudanças temporárias no estado do SDMN, e não a avaliação do resultado da luta ou apenas a expressão de comportamento agressivo. Em seguida, explorámos a modulação hormonal do comportamento social, em particular pelo neuropeptídeo vasotocina. Para isso, manipulámos o sistema da vasotocina injetando vasotocina e um antagonista específico dos receptores de vasotocina V1A em machos. Para distinguir se a vasotocina afeta o comportamento isoladamente ou em combinação com andrógenios, conduzimos esta experiência em peixes castrados e peixes controlo. Curiosamente, descobrimos que a vasotocina afetou o comportamento dos machos em relação às fêmeas, mas não em relação aos machos, e que os andrógenios e a vasotocina modularam a agressividade dos machos em relação às fêmeas. Em seguida, procurámos compreender como as interações sociais afetam os sistemas neuroendócrinos. Nesse sentido, utilizámos um paradigma de intrusões territoriais para avaliar os padrões temporais dos níveis de andrógenios e tentámos relacioná-los ao fenótipo comportamental de cada indivíduo. Obtivemos padrões distintos de resposta androgénica às interações sociais devido a diferenças individuais subjacentes em sua extensão de resposta. Este estudo oferece uma importante contribuição para a área de investigação, fornecendo possíveis razões para as discrepâncias associadas à hipótese de desafio, o principal modelo em endocrinologia comportamental que descreve a relação entre andrógenios e interações sociais. Finalmente, pensa-se que os andrógenios respondem às interações sociais como forma de preparar os indivíduos para outras interações. Assim, tentámos descobrir como um aumento de andrógenios no sangue afeta o cérebro. Para esse efeito, injetámos peixes com andrógenios e estudámos as mudanças transcriptómicas que ocorrem no cérebro usando a técnica de RNAseq, permitindo uma compreensão mais detalhada do efeito dos andrógenios no cérebro. Em suma, o comportamento social é complexo e depende de vários fatores internos e externos. Os resultados desta tese fornecem um contributo significativo para pesquisas futuras.

ABSTRACT

The study of animal behavior and in specific of social behavior has attracted researchers for a long time. All animals interact with others, a feature which is fundamental to their survival and reproduction. However, to get a complete understanding of social behavior, the integration of its various components is required. In this thesis, we aimed to shed light on this topic, studying how the brain controls behavior through the concerted action of its neural circuits, genes and molecules, and also how the social environment feedbacks and impacts the brain. Grounded upon this objective and using the Mozambique tilapia (*Oreochromis mossambicus*) as a model species, in a first study we investigated how social behavior is controlled by a dynamic network of brain regions, the Social Decision Making Network (SDMN). Here, we tried to understand what are the specific cues that trigger changes in the pattern of activation of this neural network, by using staged fights between males. Our results suggest that it is the mutual assessment of relative fighting behavior that drives acute changes in the state of the SDMN, and not the assessment of fight outcome or just the expression of aggressive behavior. Then, we explored the hormonal modulation of social behavior, in particular of the neuropeptide vasopressin. For this purpose, we manipulated the vasotocin system by injecting vasotocin and a specific antagonist of vasotocin receptors V1A in males. To distinguish if vasotocin affected behavior alone or in combination with androgens, we conducted this experiment in both castrated and control fish. Interestingly, we found that vasotocin affected the behavior of males towards females but not towards males and that both androgens and vasotocin modulated aggressiveness towards females. Next, we sought to comprehend how social interactions affect neuroendocrine systems. In that sense, we used a paradigm of territorial intrusions to assess temporal patterns of androgen levels and tried to relate them to the behavioral phenotype of each individual. We obtained distinct patterns of androgen response to social interactions due to underlying individual differences in their scope for response. This study makes an important contribution to the field by providing possible reasons for discrepancies associated with the Challenge Hypothesis, the major framework in behavioral endocrinology which describes the relationship between androgens and social interactions. Finally, it is believed that androgens respond to social interactions as a way to prepare individuals for further interactions. Thus, we tried to uncover how an androgen surge in the blood affects the brain. To accomplish this, we injected fish with androgens and studied brain transcriptomic changes with the RNAseq technique, allowing the achievement of a thorough understanding of the effect of androgens on the brain. In sum, social behavior is complex and dependent on several internal and external factors. The findings from this thesis provide significant insights for future research.

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CHAPTER 1

General Introduction

This chapter includes material published in:

Gonçalves, D., Félix, A. S., & Oliveira, R. F. (2017). Neuroendocrinology of Social Behavior in Teleost Fish. In D. W. Pfaff & M. Joëls (Eds.), *Hormones, Brain and Behavior* (3rd ed., Vol. 2, pp. 3–18). Oxford: Academic Press. (Appendix A)

and

Félix A.S., & Oliveira R.F. Integrative Neurobiology of Social Behavior in Cichlid Fish. In M. E. Abate & D. L. G. Noakes (Eds.), *The Behavior, Ecology and Evolution of Cichlid Fishes: A Contemporary Modern Synthesis*. Springer Academic. (Appendix B) *in press*

1. Introduction

Social behavior encompasses the interactions between individuals of the same species, fundamental to their survival and reproduction. The study of this kind of behavioral patterns and the unraveling of its underpinnings is a fascinating research area. However, to fully understand social behavior it is essential to integrate the various components underlying social interactions. From a mechanistic point of view, we ought to grasp specifically how the brain controls behavior, through the concerted action of its neural circuits, cells, genes and molecules, and also how the social environment feedbacks and impacts the brain.

This chapter begins by introducing the neural basis of social behavior, in particular the Social Decision Making Network, a set of core brain areas, interconnected with each other, that together control social behavior and is modulated by hormones and neuromodulators. Next, I explore how hormones, specifically androgens and the neuropeptide vasotocin, exert their action on social behavior and conversely how social behavior affects these hormones in teleost fish. In this section, I also present a brief overview of the Challenge Hypothesis, a model that tries to explain the reciprocal relationship between androgens and social behavior. The third section of this chapter addresses the importance to account for individual variability in neuroendocrinology studies. Finally, the model species, *Oreochromis mossambicus*, used in this thesis as a model organism, is described, including why it is a well-suited model to address questions in the scope of behavioural neuroendocrinology.

2. The Social Decision Making Network

In 1999, Newman challenged the neuroscientific community by proposing the existence of a core set of brain areas that collectively regulate social behavior in mammals. Each one of these areas is reciprocally connected with the others, contains sex steroid hormone receptors and is involved in the regulation of multiple social behaviors. It was designated as Social Behavior Network (SBN) and it is composed of six limbic areas: the lateral septum, the medial extended amygdala, the medial preoptic area, the anterior hypothalamus, the ventromedial and ventrolateral hypothalamus, all localized in the forebrain, and the midbrain periaqueductal gray and tegmentum. Her model was based on a considerable amount of behavioral, neuroanatomical and neuroendocrine evidence from diverse studies in rodents and other mammals, which used electrical stimulation, neuropharmacological manipulations, specific brain lesions and detection of immediate early gene expression (IEG). Together, these data show that common areas jointly influence sexual, parental or even aggressive behavior,

counteracting the idea of one area (or even a separate mini-circuit) determining a specific behavior. Instead, this specific set of areas represent the nodes of a neuroanatomical network, whose dynamic activation patterns are responsible for multiple social behaviors. For instance, male sexual behavior would be the result of successive behavioral responses such as sniffing, mounting, ejaculation or grooming, which altogether are activated by this integrated circuit and modulated by environmental stimuli and sex steroids.

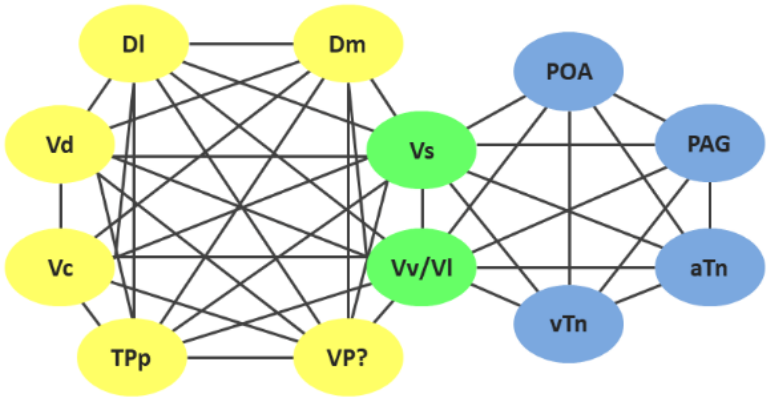
Later, this model was expanded to a wider framework, the Social Decision Making Network (SDMN), in order to include the Mesolimbic Reward System (O'Connell & Hofmann, 2011), which is proposed to be responsible for the regulation of the evaluation of the salience of a stimulus (via dopaminergic signaling), an inherent feature of an individuals' adaptive response to the environment, and consequently a relevant building block of social behavior (O'Connell & Hofmann, 2011).

In teleosts, the SDMN is presumably constituted by homologue areas to those described in mammals, which have been identified based on hodology, neurochemical profiles, development and gene expression studies or behavioral and functional surveys (reviewed in O'Connell & Hofmann, 2011). The teleost SBN includes the supracommisural part of the ventral pallium (Vs), the ventral (Vv) and lateral (Vl) parts of the ventral telencephalon, the Medial Preoptic Area (POA), the ventral tuberal nucleus (vTn), the anterior tuberal nucleus (aTn), all localized in the forebrain, and the Periaqueductal Gray (PAG) (O'Connell & Hofmann, 2011). The teleost Mesolimbic Reward System is composed of the central (Vc) and dorsal (Vd) parts of the ventral telencephalon, the medial part of the dorsal telencephalon (Dm), the lateral part of the dorsal telencephalon (Dl), the posterior tuberculum (TPp) in the midbrain, and also the Vv/Vl and the Vs, concurring nodes of the SBN (O'Connell & Hofmann, 2011); Figure 1A, see Figure 1B for the mammalian putative homologues).

The nodes of the teleost SDMN have extensive expression of steroid and neuropeptide (e.g. vasopressin, oxytocin) receptors (Goodson, 2005). For instance, in teleosts, estrogen (plainfin midshipman, *Porichthys notatus*, Forlano et al., 2005; Atlantic croaker, *Micropogonias undulatus*, Hawkins et al., 2005; zebrafish, *Danio rerio*, Menuet et al., 2002; Burton's mouthbrooder, *Astatotilapia burtoni*, Munchrath & Hofmann, 2010; european seabass, *Dicentrarchus labrax*, Muriach et al., 2008), progesterone (*A. burtoni*, Munchrath & Hofmann, 2010), androgen (*P. notatus*, Forlano et al., 2010; goldfish, *Carassius auratus*, Gelinas & Callard, 1997; *A. burtoni*, Harbott et al., 2007; Munchrath & Hofmann, 2010), glucocorticoids (Japanese medaka, *Oryzias latipes*, Kikuchi, Hosono, Yamashita, Kawabata, &

Okubo, 2015; rainbow trout, *Oncorhynchus mykiss*, Teitsma et al., 1997, 1998), arginine vasotocin (AVT, the homologue of mammalian arginine vasopressin; *A. burtoni*, Huffman et al., 2012; (Loveland & Fernald, 2017); rock hind, *Epinephelus adscensionis*, Kline et al., 2011), and isotocin (the homologue of mammalian oxytocin; *A. burtoni*, Huffman et al., 2012) receptors have all been found across all areas of the SDMN. Thus, the SDMN is open to modulation by these hormones, probably by altering the weight of its nodes or the strength of their connectivity (Oliveira, 2012).

A)



B)

Abbrev.	Teleosts SDMN Brain Nuclei	Putative Mammalian Correspondence	
POA	Medial preoptic area	Medial preoptic area	Social Behavior Network
vTn	Ventral tuberal nucleus	Anterior hypothalamus	
aTn	Anterior tuberal nucleus	Ventromedial and ventrolateral hypothalamus	
PAG	Periaqueductal gray	Periaqueductal gray and tegmentum	
Vs	Supracommisural part of the ventral pallium	Medial extended amygdala (bed nucleus of the stria terminalis and medial amygdala)	
Vv/Vl	Ventral (Vv) and lateral (Vl) parts of ventral telencephalon	Lateral septum	
Vc	Central part of the ventral telencephalon	Striatum	Mesolimbic Reward System
Vd	Dorsal part of the ventral telencephalon	Nucleus accumbens	
Dm	Medial part of the dorsal telencephalon	Basolateral amygdala	
Dl	Lateral part of the dorsal telencephalon	Hippocampus	
TPp	Posterior tuberculum	Ventral tegmental area	

Figure 1. Representation of the Social Decision Making Network (SDMN) in teleosts (O'Connell & Hofmann, 2011). A) Putative nodes of the Mesolimbic Reward System are in

yellow and the nodes of the Social Behavior Network are in blue. Overlapping nodes of the SBN and the Mesolimbic Reward System are in green. A homologous for the mammalian Ventral Pallidum (VP) node has not yet been identified. B) Putative mammalian correspondence for each teleost brain nuclei.

While the SDMN has been well established in mammals, for other taxa, it has been questioned due to a lack of consistency on some of the proposed homologies and scarce of functional studies (Goodson & Kingsbury, 2013). Thus, a reasonable approach is to use it as a framework to understand how the brain regulates social behavior in non-mammalian species.

A considerable number of studies, centered on the behavioral responses of teleost fishes, have documented the activation of specific sets of SDMN nodes in association to the expression of specific social tests, hence establishing their involvement in the regulation of social behavior. O'Connell et al (2013) presented the Burton's mouthbrooder cichlid, *A. burtoni* males with different social stimuli and discovered that visual information (seeing a female or a male) is sufficient to elicit *c-fos* transcription in dopaminergic neurons of Vc, and this transcription is significantly correlated to aggressive behavior in the case of exposure to an intruder male. These data suggest that Vc seems to be involved in assessing stimulus visual valence. Another interesting survey was also carried out in this species. Since *A. burtoni* males can reversibly switch between dominant and subordinate status and rapidly present distinct phenotypes, investigators examined immediate early gene (IEG) levels in several brain areas of males ascending or descending in social status, as compared with control individuals (Maruska, Becker, Neboori, & Fernald, 2013; Maruska, Zhang, Neboori, & Fernald, 2013). In socially ascending males, both *c-fos* and *egr-1* levels were higher than in control males in the SDMN nuclei (Vv, Vs, POA, vTn, aTn, Dm and Dl) (Maruska, Zhang, et al., 2013). Descending males presented different activation patterns for *c-fos* and *egr-1* across the same areas. *c-fos* expression levels were increased in the Vs, POA and aTn by comparison with controls while *egr-1* mRNA levels were higher in the Vv, Vs, vTn, Dm and Dl (Maruska, Becker, et al., 2013). Finally, a very interesting study with the plainfin midshipman, *P. notatus*, where reproductive behavior is intimately associated with social acoustic signals, measured *c-fos* activation in several brain nuclei including the vTn, aTn and TPp (Petersen et al., 2013). The authors report a significant increase of IEG expression in the aTn and TPp of males exposed to acoustic signals of other males compared to control males, showing the importance of these nuclei in social communication in this species.

Noteworthy, the occurrence of a SDMN has already been tested functionally in teleosts (Roleira, Oliveira, Lopes, & Oliveira, 2017; Teles, Almeida, Lopes, & Oliveira, 2015). A study carried in zebrafish tested the the SDMN hypothesis by analyzing, using qPCR, the patterns of brain activation of males after participating in agonistic interactions (Teles et al., 2015). Zebrafish, *D. rerio*, is a model species commonly used today in neuroscience and behavioral studies (Oliveira, 2013). Even though it is a shoaling fish, individuals are very territorial and present well-characterized agonistic behaviors, such as chases, strikes, bites as well as freezing or fleeing (Oliveira, Silva, & Simões, 2011; Paull et al., 2010). Thus, researchers examined the expression patterns of two IEGs (*c-fos* and *egr-1*), as markers of neuronal activity, in several SDMN nuclei of winners and losers that participated in fights and also of fish that were presented to a mirror. Since fish cannot recognize their own image on a mirror, they attack it and express very intense aggressive behaviors as if it is an intruder (Teles, Dahlbom, Winberg, & Oliveira, 2013). This experimental group was included in this study to allow to distinguish both perceptual and motor features involved on brain activation, inasmuch no fight outcome (winning or losing) is perceived by individuals who interact with a mirror (Teles et al., 2013). By using non-interacting fish as a control, they verified that all treatments originated different behavioral states represented by distinct patterns of functional connectivity across the SDMN nodes. In particular, no localized activity (i.e. IEG expression) of any of these nodes was attributed to neither social phenotype but instead different clusters of brain areas and corresponded densities of connections, supporting the SDMN model (Teles et al., 2015).

3. Neuroendocrinology of Social Behavior

3.1. The Reciprocity between Hormones and Behavior

The relationship between hormones and behavior has been a matter of interest for several centuries. The initial paradigm established hormones as directly responsible for behaviors, grounded in classical experiments of castration and androgen replacement studies (see Oliveira, 2004, for historical background). However, experiments showing that hormones rather increase the probability of the individuals to express behaviors instead of switching on and off behaviors altered this simplistic concept (e.g., Albert, Jonik, & Walsh, 1993). Currently, it is well recognized that hormones act as modulators of the neural mechanisms underlying behavior (Oliveira, 2009). On the other hand, intensive studies in the last decades have focused on the

influence of social interactions on hormones. Actually, the social environment feedbacks on neuroendocrine mechanisms changing hormone levels which, in turn, modulate neural mechanisms and ultimately subsequent social behaviors (Oliveira, 2004). The concept of reciprocity is thus central in the study of social neuroendocrinology: hormones influence behavior but also respond to it (Figure 2).

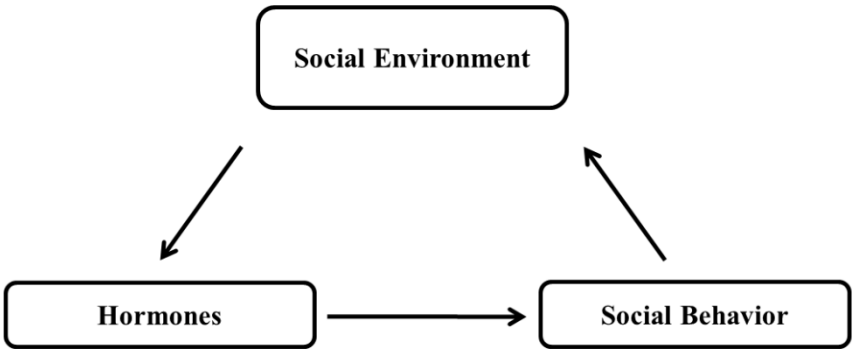


Figure 2. The interaction between hormones and social behavior. Hormones modulate the behavior of an individual while the social environment feedback on its hormonal levels and, in turn, modulate subsequent social behaviors.

Although several hormones have been implicated in this reciprocal interaction, including sex-steroids, glucocorticoids or neuropeptides (e.g. oxytocin) (Gonçalves, Félix, & Oliveira, 2017), in this review, I will only focus on androgens and the neuropeptide vasotocin.

3.1.1. The Challenge Hypothesis

One major model has been proposed to explain the two-way relationship between androgens and behavior. The "challenge hypothesis", proposed by Wingfield and colleagues (1990), postulated that androgen levels would rise above reproductive levels as a function of the social environment. According to this model three androgen levels can be recognized: constitutive circulating levels occur during the non-breeding phase (constitutive baseline, *a*), which, in seasonal breeders, increase in the breeding season up to the concentration needed for the full development of the gonads, the development of secondary sex characteristics (e.g. long-colored tails on peacocks or bright coloration in many birds) and for the expression of reproductive behaviors (breeding baseline, *b*) (Figure 3A, B); then, androgens can rise above the breeding baseline and reach a physiological maximum (*c*) in response to social interactions, either with

males or with sexually receptive females (Figure 3A). Thus, the regime of social interactions an individual participates will determine its hormone levels. While aggressive behavior is absent or very low between levels *a* and *b*, within the breeding season the level of social competition/instability rise and testosterone levels increase from *b* to *c* (Figure 3C). Within this model, one can quantify the androgen responsiveness - given by the ratio (*c*-*a*)/(*b*-*a*) - which allows comparisons between individuals and between species, independently of baseline levels.

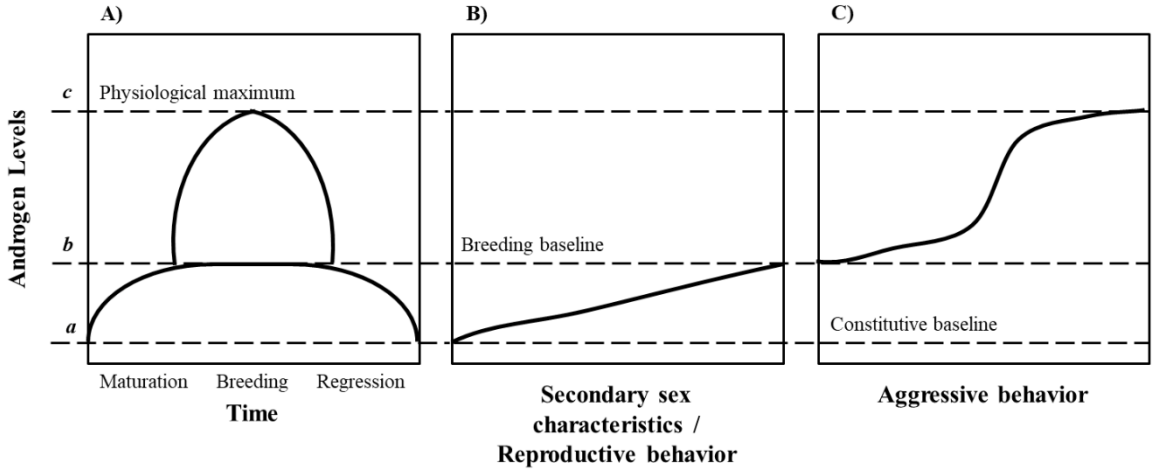


Figure 3. The challenge hypothesis framework (Wingfield et al., 1990)**.** A) The model defines a constitutive baseline, *a*, corresponding to a minimum of androgen levels in the non-breeding phase, which increases in the breeding season to the breeding baseline, *b*, and can reach its physiological maximum, *c*. B) The development of secondary sex characteristics and the expression of reproductive behavior are proportional to androgen levels in the non-breeding phase. C) Aggressive behavior is proportional to androgen levels in the breeding phase.

Even though the challenge hypothesis had its origin in comparative data from bird species, it has been extensively tested across all vertebrate taxa including teleost fish (Hirschenhauser & Oliveira, 2006; Hirschenhauser, Taborsky, Oliveira, Canário, & Oliveira, 2004; Moore, 2007; and see Oliveira, 2004 for a review on this topic).

This model generates a number of predictions regarding the patterns of androgen responsiveness depending on the features of each species' social behavior and social environment, such as its mating system, kind of parental care or breeding density (Wingfield et al., 1990). For example, males from polygynous species should have higher androgen levels than those from monogamous species' and conversely lower androgen responsiveness due to a more pronounced male-male competition (Wingfield et al., 1990; Figure 4A, B). Moreover,

since androgens interfere with paternal care, males from species that provide parental care should have low androgen levels during parental phase that rise in response to male or female interactions (high androgen responsiveness) while species in which males invest less in parental care are expected to have higher androgen levels but lower androgen responsiveness to social interactions (Wingfield et al., 1990; Figure 4C, D).

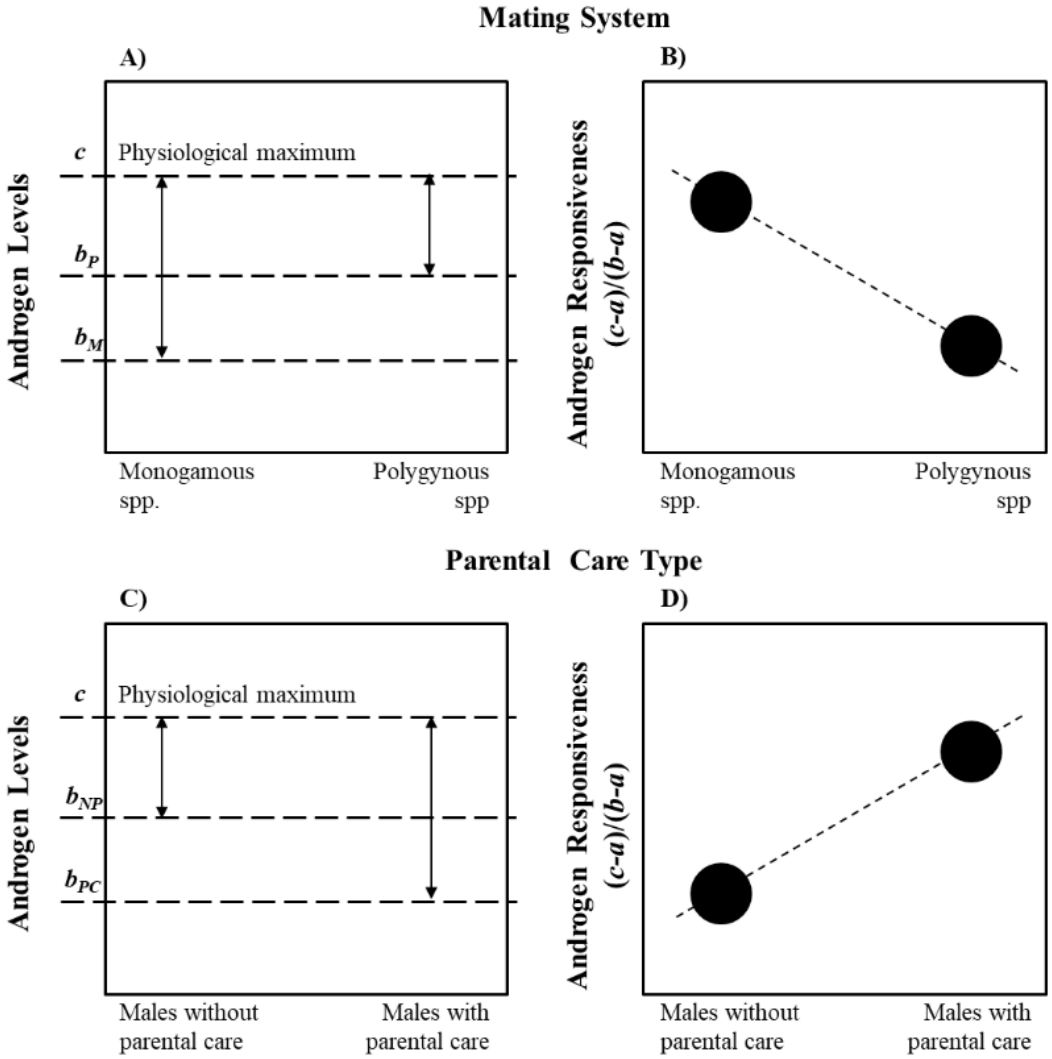


Figure 4. The effect of the mating system and parental care type on androgen levels and androgen responsiveness according to the challenge hypothesis (Wingfield et al., 1990)**.** A) Androgen levels of the breeding baseline are lower for monogamous species than for polygynous species. B) Androgen responsiveness is higher for monogamous species than for polygynous species. C) Androgen levels are lower for males that provide parental care than for males without parental care. D) Androgen responsiveness is higher for males with parental care than for males without parental care.

In sum, the challenge hypothesis has been presented to explain the adaptive nature of the androgen response to social interactions and should be seen as a starting point to achieve a better understanding of the reciprocity between the social environment and neuroendocrine responses.

3.2. Hormones Action on Behavior

Next section will focus on the mechanistic bases of social behavior by exploring the bidirectional relationship between hormones and social behavior at the proximate level (Mayr, 1961). Here, I present the current state of knowledge of the field in teleost fish, using several examples of how hormones are involved in a vast array of social events by acting upon an integrated neural network.

3.2.1. Androgens

Reproductive behavior seems to be intimately associated with sex steroids since castration studies in males abolishes spawning pit digging, nuptial coloration and courtship (Egyptian mouthbrooder, *Pseudocrenilabrus multicolor*, Reinboth & Rixner, 1970; blackchin tilapia**,** *Sarotherodon melanotheron*, Levy & Aronson, 1955; *A. burtoni*, Francis, Jacobson, Wingfield, & Fernald, 1992; *O. mossambicus*, Almeida, Canário, & Oliveira, 2014). Exogenous administration of androgen receptor agonists also supports this association by promoting nest building behavior or courtship (*A. burtoni*, O'Connell & Hofmann, 2012; convict cichlid, *Amatitlania nigrofasciata*, Sessa, Harris, & Hofmann, 2013) while androgen receptor antagonists decrease courtship (*A. burtoni*, O'Connell & Hofmann, 2012; *A. nigrofasciata*, van Breukelen, 2013). Other researchers claim, however, that gonadectomized males maintain reproductive behavior repertoire (jewelfish, *Hemichromis bimaculatus*, Noble & Kumpf, 1936; platinum acara, *Andinoacara latifrons*, Aronson, Scharf, & Silverman, 1960, *S. melanotheron and Oreochromis upembae*, Heinrich, 1967), which suggests that sex steroids influence on behavior is species-specific.

Previous research has established a positive connection between sex steroids and aggression in vertebrates, including teleost fish (see Oliveira, 2004 and Gonçalves, Félix, & Oliveira, 2017 for comprehensive reviews and Hirschenhauser & Oliveira, 2006 for a meta-analysis). For example, in the classical study carried by Fernald (1976) with the Burton's mouthbrooder *A. burtoni*, androgen treatment significantly increased approaching and attacks towards other males while non-aggressive behaviors such as digging or swimming were

unaltered. In this study, rates of approaches and attacks increased 3- to 4- fold in response to androgens (Fernald, 1976). In a study with the sheepshead minnow (*Cyprinodon variegatus*), fish injected with testosterone showed an increase of the frequency of aggressive behaviors, like chasing or bumping conspecifics, and an elevated number of victories in paired contests, compared to controls (Higby, Dwyer, & Beulig, 1991). In line with these results, castration of *A. burtoni* males decreases not only circulating androgens but also aggressive behavior (Francis, Jacobson, Wingfield, & Fernald, 1992). Additionally, blocking androgen receptors with the antagonist cyproterone acetate decreases aggression, either when fish defend their nest from a brood predator (smallmouth bass, *Micropterus dolomieu*, Dey, O'Connor, Gilmour, Van Der Kraak, & Cooke, 2010), or exposed to conspecific intruder males (*A. nigrofasciata*, Sessa, Harris, & Hofmann, 2013).

In species with alternative reproductive tactics, the role of sex steroids on the aggressive behavior males has also been tested. In the Azorean rock-pool blenny, *Parablennius parvicornis*, bourgeois males develop conspicuous secondary sexual characters, like well-developed anal-glands and head humps, defend nests and compete for access to females (Oliveira, Canário, & Grober, 2001). In the breeding season, bourgeois males court females that spawn in their nests, fertilize the eggs and defend them until hatching. Since this is a promiscuous mating system, each male can receive eggs from several females. In contrast, smaller and younger males act as satellites and help defend territories, but also try to fertilize eggs with parasitic fertilizations (Oliveira, Canário, et al., 2001). Ros et al. (2004) implanted nest-holder males with 11-ketotestosterone, the primary androgen in teleost fish, and confirmed a significant increase on aggressive behavior against conspecifics in the field (Ros, Bruintjes, Santos, Canário, & Oliveira, 2004). Interestingly, nest-holder males treated with 11-ketotestosterone also expanded their territory, evidenced by the attacks to other males, chases and exploration behavior observed a longer distance from their nest (Ros et al., 2004). On the other hand, treating satellite males with androgens promoted the development of secondary sex traits but failed to increase aggression in mirror tests (Oliveira, Carneiro, Canário, & Grober, 2001). Apparent inconsistencies are evident as well in the observation that, in some species, castration impairs reproductive behavior but it is not successful in abolishing aggressive behavior (e.g., Mozambique tilapia, *O. mossambicus*, Almeida, Canário, & Oliveira, 2014; three spot gourami, *Trichopodus trichopterus*, Johns & Liley, 1970). Also, Van Breukelen (2013) used flutamide silastic implants to block androgen receptors in the convict cichlid, *A. nigrofasciata*, and observed a decrease in courtship and no effect in aggression. Moreover, O'Connell and Hofmann (2012) pharmacologically manipulated *A. burtoni* males by injecting

androgens or the antagonist cyproterone acetate and observed a clear influence in courtship behavior (androgens treatment increased courtship whereas antagonist decreased courtship behavioral patterns) while aggressive behavior was not affected. However, an interesting finding and possible explanation to these conflicting results is that estrogens enhanced aggression and estrogen receptor antagonist decreased it, pointing to estrogens as major players in aggressive behavior (O'Connell & Hofmann, 2012). As seen in other species (for example, in the Japanese quail, *Coturnix coturnix japonica*, Schlinger & Callard, 1990) androgens can moderate aggressive behavior via aromatization to estrogen by the enzyme aromatase. By way of illustration, Huffman et al. (2013) showed that, in *A. burtoni*, treating fish with an aromatase inhibitor decreased aggression. Altogether, these cases support evidence for a decoupling between the neuroendocrine mechanisms responsible for reproductive and aggressive behaviors. It has been suggested that, at least in teleosts, androgens may have a moderator role on aggression (instead of a mediator one) since they clearly influence it; yet are not strictly necessary (Almeida, Canário, et al., 2014).

3.2.2. Arginine vasotocin (AVT)

Several attempts have been made to examine the association between the nonapeptide arginine vasotocin (AVT) and social behavior. In teleosts, AVT is primarily expressed by neurons located in the POA in the anterior hypothalamus, that project either to the neurohypophysis, where it is released to the bloodstream to act peripherally (reviewed in Godwin & Thompson, 2012), and also to the ventral telencephalon, ventral thalamus and mesencephalum (Huffman et al., 2012; Saito, Komatsuda, & Urano, 2004). There are different populations (parvo-, magno-, and giganto- cellular) of AVT neurons whose anatomy seems highly conserved among taxa (Goodson, 2008). The occurrence of AVT cells in the anterior tuberal nucleus has also been reported (reviewed in Godwin & Thompson, 2012). Several AVT receptors have been described in teleost fish (viz. V1Aa, V1Ab, V2A1, V2A2 since they lack V1B and V2B types and V2C was only found in 3 teleost species, namely, zebrafish, *D. rerio*, three-spined stickleback, *Gasterosteus aculeatus*, and the Southern platyfish, *Xyphophorus maculatus*) (Lagman et al., 2013).

In mammals it is known that gonadal steroids regulate the mammalian homologue of AVT, vasopressin (reviewed in Albers, 2012). For instance, male rats have much more vasopressin cells in the bed nucleus of the stria terminalis and a denser vasopressin innervation of the lateral septum compared with females (van Leeuwen, Caffe, & Vries, 1985). Moreover,

castration of adult male rats leads to a reduction of the number of AVT cell bodies and fiber density in several brain areas which is reversed when rats are treated with testosterone (DeVries, Buijs, van Leeuwen, Caffé, & Swaab, 1985). In teleosts, for long it has been considered that there was no AVT expression in the mammalian homologue of the extended amygdala and septal areas (e.g. Godwin & Thompson, 2012). However, a recent study in *A. burtoni* has found AVT pre-prohormone expression in these regions and also in hippocampus and striatum (Rodriguez-Santiago, Nguyen, Winton, Weitekamp, & Hofmann, 2017), demonstrating that future studies are need to clarify this issue. On the other hand, and at least in some mammals, androgens modulate sensitivity to vasopressin by affecting the number of *V1A* receptors in the medial preoptic nucleus (Syrian hamsters, Young, Wang, Cooper, & Albers, 2000).

In turn, Ramallo and colleagues (2012) provided a detailed characterization of the vasotocinergic system in *Cichlasoma dimerus* and showed that AVT neuron projections are found mostly in the forebrain and the hindbrain while AVT stimulates production of gonadotropins (LH and FSH) on pituitary extracts *in vitro* and androgens on testis culture. They also detected AVT mRNA and peptide in the testis thus showing the influence of AVT in the HPG axis as a neuromodulator in central nervous system and playing a role as a neurohormone at a peripheral level.

Available results from AVT pharmacological studies in teleosts are however inconsistent and a coherent pattern is still missing (Godwin & Thompson, 2012). For instance, intraperitoneal (ip) injections of AVT induced male electric signals used as sexual displays in the weekly electric fish, *Apteronotus leptorhynchus* (Bastian, Schniederjan, & Nguyenkim, 2001). In the bluehead wrasse, *Thalassoma bifasciatum*, AVT injections increased courting in territorial and non-territorial males (Semsar, Kandel, & Godwin, 2001) while in a species with alternative reproductive tactics, the peacock blenny, *Salaria pavo*, AVT administration induced female-typical courtship behavior in females and sneaker males and had no effect in nest-holder males (Carneiro, Oliveira, Canário, & Grober, 2003). However, in the white perch, *Morone americana*, ip injections of AVT had no effect on behavior but intracerebroventricularly (icv) administration increased courting behavior (Salek, Sullivan, & Godwin, 2002). In males of the damselfish beaugregory, *Stegastes leucostictus*, intramuscular treatment of AVT increased aggression while the AVT receptor V1A antagonist, the Manning compound, decreased it, in comparison with saline-treated males (Santangelo & Bass, 2006). In *O. mykiss*, researchers compared the effect of 2 doses of AVT, icv administered, in the agonistic behavior of dominant males (Backström & Winberg, 2009). The higher dosage induced dominant males to descend in status while the Manning compound, had no effect on the fight outcome (Backström &

Winberg, 2009). In *A. burtoni*, ip injection of AVT caused a stress response in animals, originating a decrease of aggression and the loss of status of dominant males, whereas the Manning compound had no effect compared with saline controls (Huffman, Hinz, Wojcik, Aubin-Horth, & Hofmann, 2015).

In the cichlids *Neolamprologus pulcher* and *Telmatocromis temporalis*, aggression seems to be related to higher expression levels of whole brain AVT (O'Connor, Marsh-Rollo, Aubin-Horth, & Balshine, 2016). Actually, AVT (and its receptors) expression in whole brain seem species-specific (O'Connor, Marsh-Rollo, Ghio, Balshine, & Aubin-Horth, 2015). Recent investigations performed a comparative study of the AVT modulation on aggressive behavior, by using different species of gymnotiform electric fish, and performing staged-fights. *Brachyhypopomus gauderio* is a gregarious species which displays aggressive electric pulses in the context of reproduction, specifically in the breeding season, while *Gymnotus omarorum*, a solitary species, is aggressive all year round independent of their breeding season. In this species, it has been established that (aggressive) electric organ discharges (EOD) are under the control of a medullary pacemaker nucleus (Perrone, Batista, Lorenzo, Macadar, & Silva, 2010; Pouso, Quintana, Bolatto, & Silva, 2010). Thus, injection of AVT of both species showed a significant increase only on the non-breeding territorial aggression of *G. omarorum* (to be specific, higher motivation to attack manifested by decreased latency to attack) and no effect on the social species (reviewed in Silva, Perrone, Zubizarreta, Batista, & Stoddard, 2013). Furthermore, injection of AVT on the predicted subordinate in *G. omarorum* blocked the submissive electric signal in the end of the social interaction whilst the AVT receptor V1A antagonist, Manning compound, administered to the presumed dominant male of *B. gauderio* inhibited the electric dominance display (reviewed in Silva et al., 2013). These authors suggested that AVT exerts its influence on the activity of SBN nodes and descending motor output pathways modulating aggressive (electric) behavior in these species (Silva et al., 2013).

The data reported here appear to support the assumption that the observed differential role of AVT depends on each species' social system and even on their distinct social phenotypes. In accordance with this, some researchers indicate that AVT injections reduce aggression in dominant males (*A. burtoni*, Huffman, Hinz, Wojcik, Aubin-Horth, & Hofmann, 2015; *D. rerio*, Filby, Paull, Hickmore, & Tyler, 2010), but have no effect in subordinates (Huffman et al., 2015). Semsar (2001) showed that exogenous AVT either inhibited aggression in territorial males or increased territorial behavior in non-territorial males of the bluehead wrasse *T. bifasciatum*. On the other hand, the AVT antagonist produced no effect (Filby et al.,

2010; Huffman et al., 2015) or reduced aggression (*A. nigrofasciata*, Oldfield & Hofmann, 2011).

Furthermore, the above mentioned lack of consistency has been attributed to differential roles of AVT subpopulations. Based on neuroanatomical and histochemical surveys, Greenwood et al. (2008) suggested that giganto-cellular AVT neurons release AVT in circuits that modulate courtship and aggression, whereas parvo-cellular AVT neurons affect circuits responsible for inducing subordinate behavior in parallel with an activation of the stress axis. More recently, another study has highlighted the key role of magnocellular AVT neurons in aggression contrary to giganto-cellular neurons (Loveland & Fernald, 2017). Still, aggressive behavior seems to be modulated by AVT through a complex regulatory mechanism dependent on the concerted action of two different sub-systems. Moreover, if there are indeed different circuits regulating aggression and courtship through AVT modulation, peripheral administration of AVT fails to stimulate these different central circuits in an independent manner, so targeting specific populations is needed to clarify the role of AVT in this subject.

A noteworthy example has been carried out in the midshipman fish *P. notatus*, a well-studied fish model in the scope of vocal communication (see Bass, 2008; Forlano, Sisneros, Rohmann, & Bass, 2015) for comprehensive reviews). This species is characterized by male dimorphism, namely non-territorial/ 'sneaker' males and territorial larger males which defend nests and attract females by using acoustic signals, agonistic ('grunts') and courtship sounds (long 'hums'), respectively. Interestingly, the AVT delivery either in the forebrain or in the midbrain modulates different vocal circuits as shown by inducing distinct effects. AVT treatment on the preoptic area–anterior hypothalamus decreases burst duration, whereas at the midbrain level (specifically in the paralemniscal midbrain tegmentum), AVT hampers call initiation by decreasing number of vocal bursts and increasing response latency (Goodson & Bass, 2000a, 2000b).

3.3. Social Modulation of Neuroendocrine Mechanisms

In social species, individuals should be socially competent, that is, they should optimize their behavior according to a constantly changing and challenging social environment. To do so, individuals must integrate information about the social environment they live in with internal cues and optimize their responses (Oliveira, 2009). Hormones play a central role in this adaptive and embodied mechanism since social interactions elicit quick responses in circulating

hormones that modulate neural mechanisms through widely distributed hormone receptors (Oliveira & Oliveira, 2014).

3.3.1. Androgens

Males' exposure to social stimuli, either a female or a conspecific male, induce a plasma androgen increase (*O. mossambicus*, Borges, Oliveira, Almada, & Canário, 1998; *N. pulcher*, *Lamprologus callipterus*, blunthead cichlid, *Tropheus moorii*, *Pseudosimochromis curvifrons*, *O. mossambicus*; Hirschenhauser, Ros, Taborsky, Oliveira, & Canário, 2008; Hirschenhauser et al., 2004; *A. nigrofasciata*, Sessa et al., 2013), in accordance with the Challenge Hypothesis. A study on female mate choice revealed that males change their reproductive and aggressive behavior, as well as androgen levels, according to female physiology (hormone release) and/or behavior and in turn females choose mates that release more androgens into water (*A. burtoni*, Kidd et al., 2013). Interestingly, visual information is sufficient to influence hormone systems since in *A. burtoni* seeing a dominant and larger male suppresses dominant behavior of a smaller male and is responsible for a decrease in 11-ketotestosterone levels and an increase in the gene expression of gonadotropin-releasing hormone (GnRH) (Chen & Fernald, 2011).

The HPG (Hypothalamus - Pituitary - Gonads) axis is also affected when individuals participate in agonistic interactions. Reports account for a decrease of the gonadotropins luteinizing hormone *(*LH*)* and follicle-stimulating hormone (FSH) both in winners and losers and a dramatic decrease of androgens in the case of losers (Leshner, 1983). These socially driven changes in circulating steroid levels have been hypothesized to influence subsequent behaviors. For instance, after a fight, losers display less aggressive and more submissive behaviors (Leshner, 1983), and winner and loser effects have been described in many species (i.e. animals which experience victory have higher probability of winning other matches and defeated animals are more likely to lose other fights, respectively) (Hsu, Earley, & Wolf, 2006), including in teleost fish (Hsu et al., 2006). The winner effect seems to be mediated by socially driven changes in androgens. Consecutive wins increase androgen levels in the California mouse, *Peromyscus californicus* (Oyegbile & Marler, 2005) and the administration of an androgen antagonist blocks the winner effect in the Mozambique tilapia, *O. mossambicus* (i.e., cyproterone acetate-treated winners do not win subsequent fights, Oliveira, Silva, & Canário, 2009). In contrast, the administration of androgens in defeated Mozambique tilapia males failed to revert the loser effect (i.e., androgen-treated losers do not win further fights, Oliveira et al., 2009), suggesting that the observed decrease of androgens in losers is not the only mechanism responsible for the loser effect and that other neuromodulators, namely serotonin, could play

an important role (Oliveira et al., 2009). The presumed existence of two different mechanisms is in accordance with the observed differences in the time span and pervasiveness of these two phenomena, with the loser effect lasting longer and being more pronounced than the winner effect (reviewed in Hsu et al., 2006). In summary, prior fighting experience is crucial for subsequent fight outcomes and a positive feedback loop between winning and androgens seems to reinforce dominance status.

Other important evidence on social environment influencing hormones is that plasma androgen levels vary with social status. Dominant males typically have higher levels of androgens than subordinate males (*A. burtoni*, Parikh, Clement, & Fernald, 2006; *N. pulcher*, Desjardins et al., 2008; *O. niloticus*, Pfennig et al., 2012; *C. dimerus*; Morandini, Honji, Ramallo, Moreira, & Pandolfi, 2014). In *N. pulcher*, non-territorial aggregation males have higher testosterone and lower 11-ketotestosterone and helpers have higher cortisol (Bender, Heg-Bachar, Oliveira, Canario, & Taborsky, 2008). Another study in the same species has shown that females' breeders have higher levels of testosterone than helper females or even males (Desjardins et al., 2008), suggesting that androgens may promote parental care. Looking at brain gene expression patterns dominant/breeder females are very similar to dominant males, evidence for a masculinization at the molecular and hormonal level of these females (Aubin-Horth, Desjardins, Martei, Balshine, & Hofmann, 2007). The keynote here is that steroid levels are a consequence of social status. Oliveira et al (1996) demonstrated that urinary sex steroids levels after group formation reflect social establishment; 11-ketotestosterone increased in territorial males and decreased in non-territorial males and no changes were reported in testosterone levels when compared to levels prior to hierarchical establishment (see also Almeida, Gonçalves-de-Freitas, Lopes, & Oliveira, 2014). On the other hand, social challenges induce differential hormonal responses according to individuals' social status. In *N. pulcher*, agonistic interactions elicit higher plasma levels of testosterone and similar 11-ketotestosterone levels in dominant females than subordinate females, and in contrast higher levels of 11-ketotestosterone and equivalent levels of testosterone in dominant males compared to subordinate males (Taves, Desjardins, Mishra, & Balshine, 2009). Likewise, androgen levels of males socially isolated differ in their response according to their previous social status; dominant males decrease 11-ketotestosterone and subordinates show a tendency to increase 11-ketotestosterone whereas cortisol varies depending on prior social context (*O. mossambicus*, Galhardo & Oliveira, 2014).

In turn, androgens modulated by social status determine for instance expression of secondary behavioral (e.g. nuptial coloration, spawning pit volume) and morphological traits

(e.g. mandible width, dorsal fin height) specifically in territorial males (*O. mossambicus,* Oliveira & Almada, 1998a). Dominant males typically have larger GSI (gonadosomatic index) than non-territorial males (*O. mossambicus,* Oliveira & Almada, 1999; *O. niloticus,* Pfennig et al., 2012; *C. dimerus*; Alonso, Honji, Moreira, & Pandolfi, 2012; *A. nigrofasciata,* Chee et al., 2013) however subordinate males are still reproductive active despite differences in testis structure (Pfennig et al., 2012). Androgens likewise modulate color patterns in *A. burtoni* territorial males since 11-ketotestosterone levels are higher in yellow territorial males (as well as aggression) than in blue territorial males (Korzan, Robison, Zhao, & Fernald, 2008). A flexible behavioral strategy seems to underlie this color changing ability. Another very interesting illustration is what is observed in *A. burtoni* females. Sometimes they adopt a male-typical appearance and behavior, namely courtship behavior and aggressive territorial defense mostly towards other females (Renn, Fraser, Aubin-Horth, Trainor, & Hofmann, 2012). This intriguing behavior is associated with higher testosterone levels and a non-significant trend to higher estradiol comparatively to subordinate females (Renn et al., 2012). Data on the mentioned study cannot infer on the ultimate function of this apparently hormonal modulated behavior but one can speculate that this observed behaviorally plasticity could confer them adaptive advantages in the competition for males.

A remarkable example on the reciprocity between hormones and behavior is the social regulation of reproductive plasticity in the Burton's mouthbrooder cichlid, *Astatotilapia burtoni*. A considerable number of studies in the last years provided a very detailed picture of how the social environment impacts dramatically an individual. *A. burtoni* is a maternal mouthbrooding species with a lek-breeding system, where males present two distinct and reversible phenotypes: (1) dominant (territorial) males which are brightly colored, present a black eye-bar and have access to females; and (2) subordinate (non-territorial) males, vanished colored, which are similar to females and usually do not reproduce (Maruska & Fernald, 2013). By giving subordinate males an opportunity to ascend in social status, researchers were able to show that social ascent drives rapid changes in morphology, physiology and behavior (reviewed in Maruska & Fernald, 2011a; Maruska, Levavi-Sivan, Biran, & Fernald, 2011; Maruska, 2015). Within minutes, ascending males display bright body colors and a marked eye-bar and present clear territorial, aggressive and reproductive behaviors (Maruska, 2015). Increased circulating levels of gonadotropins, androgens, estradiol, cortisol and progestins are reported within 30 min (Maruska, 2015; Maruska et al., 2011), and higher expression levels of gonadotropin-releasing hormone gene *(GnRH1*) in the POA, as well as of the immediate-early

genes *c-fos* and *egr-1* and androgen and estrogen receptors in several SDMN brain areas have also been described (reviewed in Maruska & Fernald, 2013; Maruska, 2015). Similarly, the pituitary gland and the testes experience substantial changes (*viz.* LH and FSH mRNA, gonadotropins and steroid receptors, sperm quality) within minutes to days (revised in Maruska & Fernald, 2011a, 2011b), confirming the existence of a complex mechanism of social regulation of the HPG axis at multiple levels.

3.3.2. Arginine Vasotocin

In the case of the AVT system, social status has an impact in a species-specific fashion. For example, in the cooperative breeding cichlid *N. pulcher* subordinate individuals have higher AVT brain levels than dominants (Reddon et al., 2015), whereas in the Mozambique tilapia *O. mossambicus*, there is less AVT in the pituitary and more isotocin in the hindbrain of dominant individuals when compared to subordinates (Almeida, Gozdowska, Kulczykowska, & Oliveira, 2012). Additionally, transcriptomic studies in *N. pulcher* and *A. burtoni* which compared dominant and subordinate animals have found AVT as one of the differentially expressed genes in the brain (Aubin-Horth et al., 2007; Renn, Aubin-Horth, & Hofmann, 2008).

In *A. burtoni*, whole brain AVT expression is higher in territorial compared to non-territorial males. However in the posterior POA (gigantocellular nucleus), territorial males have more AVT mRNA than non-territorial males, in opposition to the anterior POA where the reversed pattern is observed (Greenwood et al., 2008). In the South American cichlid *C. dimerus* subordinates have larger AVT parvo-cellular neurons in the POA than dominant males, pointing to a putative role of these neurons in submissive behavior (Ramallo, Grober, Cánepa, Morandini, & Pandolfi, 2012). In contrast, in *O. mossambicus*, subordinate males have larger cell body areas of AVT neurons in magnocellular POA and gigantocellular POA and submissive behavior correlates with soma size of AVT cells in all three nuclei (parvo-, magno- and gigantocellular) and AVT cell number in the magnocellular POA (Almeida & Oliveira, 2015). Moreover, in a comparative study of two butterfly fish species (Chaetodon spp.) with different social systems, it was shown that individuals from the territorial species have AVT neurons with larger soma size in the POA and a higher density of AVT fibers in several brain areas, than those from a shoaling species (Dewan, Maruska, & Tricas, 2008). Additionally, aggressive behavior in this territorial butterfly fish species is positively correlated with the number of giganto-cellular AVT cells and negatively with the size and number of parvo-cellular AVT cells in the POA (Dewan & Tricas, 2011).

Another clear example of the effect of social environment in the AVT system is exemplified in the clown anemonefish (*Amphiprion ocellaris*). In this species, immature fish sexually differentiate when achieve higher social ranks, so researchers analyzed the number of AVT neurons after hierarchical establishment, during an observational period of one month (Iwata, Nagai, & Sasaki, 2010). Interestingly, subordinate individuals significantly increased the number of AVT magnocellular neurons while higher-ranked individuals suffered a reduction of these neurons (Iwata et al., 2010).

Finally, a more recent study carried in zebrafish demonstrated that acute social interactions elicit quick changes in AVT levels (Teles, Gozdowska, Kalamarz-Kubiak, Kulczykowska, & Oliveira, 2016). Immediately after a fight, winners respond with increased levels of AVT in the forebrain and a decrease of IT in the olfactory bulbs, in contrast to losers that present increased AVT in the forebrain, optic tectum and brainstem, and an increase of isotocin in the diencephalon and a decrease of IT in the cerebellum (Teles et al., 2016).

4. Individual Variation on Androgen Levels

As mentioned above, The Challenge Hypothesis has been tested widely (Hirschenhauser & Oliveira, 2006; Hirschenhauser, Taborsky, Oliveira, Canário, & Oliveira, 2004; Oliveira 2004, Oliveira and Oliveira 2014b, Salvador 2005). However, many published studies do not show the expected androgen response in social interactions (e.g., rodents: Fuxjager et al., 2010; dwarf mongooses: Creel, Wildt, & Monfort, 1993; amphibians: de Assis, Navas, Mendonça, & Gomes, 2012; fish: Ros, Vullioud, Bruintjes, Vallat, & Bshary, 2014; reptiles: Baird, Lovern, & Shine, 2014; birds: Moore et al., 2004; humans: Oliveira and Oliveira 2014b), providing limited support for this model.

On the other hand, few studies account for the individual variability in hormonal responses. By 1987, Bennett already emphasized the need to focus on biological differences among individuals and to shift our attention from the ‘tyranny of the Golden Mean’, particularly in physiological studies (Bennett, 1987). Actually, focusing on the mean of a population or species, one misses the real-life landscape, characterized by dispersion and variability. For example, Figure 5 in (Kempenaers, Peters, & Foerster, 2008) shows a 200-fold variation in testosterone levels in a blue tit (*Cyanistes caeruleus*) population, which cannot be attributed to sampling or measurement errors (Williams, 2008).

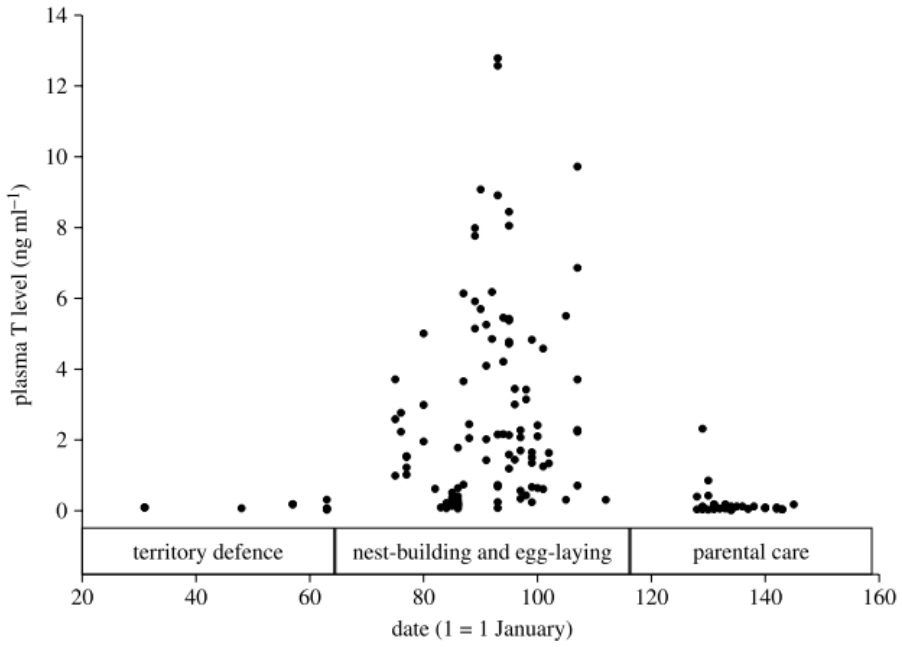


Figure 5. Plasma testosterone levels of blue tits (*Cyanistes caeruleus*). Data is from a wild population sampled in different periods of the year in Vienna, Austria (sample size: 132 individuals; one to four samples per individual (from Kempenaers, Peters, & Foerster, 2008).

Thus, variation may exist among baseline (e.g., eastern bluebirds, *Sialia sialis*: black redstart, *Phoenicurus ochruros*: Wolfgang Goymann, Villavicencio, & Apfelbeck, 2015) and physiological maximum of androgen levels (e.g., Ambardar & Grindstaff, 2017; house sparrow, *Passer domesticus*: Needham, Dochtermann, & Greives, 2017) or in the magnitude of the androgen response (Figure 6, Kempenaers et al., 2008). Several are also the studies that report inter-individual variability in androgen levels which is consistent in time (e.g., Ambardar & Grindstaff, 2017; Bergeon Burns, Rosvall, Hahn, Demas, & Ketterson, 2014; Needham et al., 2017). Consequently, studies with a repeated measures design would be more informative and meaningful than studies which collect discrete samples of individuals, analyze the central tendency and consider variability as "noise" (Bennett, 1987; Kempenaers et al., 2008).

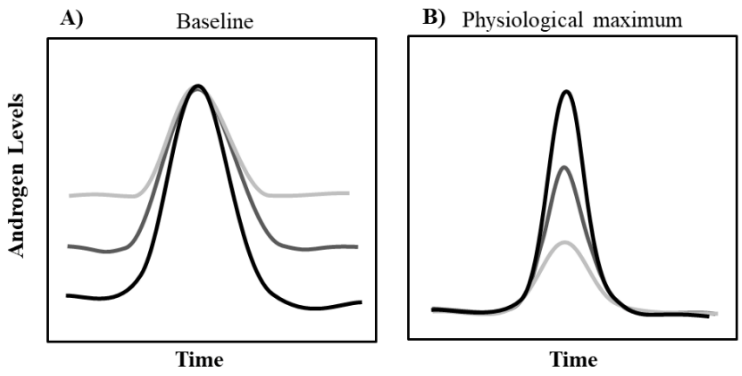


Figure 6. Inter-individual variation in androgen levels. A) Variation in the baseline of androgen levels. B) Variation in the physiological maximum of androgen levels. In both A) and B) the magnitude of the androgen response is different for each individual.

Moreover, another aspect to address is whether this variation is relevant, in other words, if it relates to behavioral phenotype and fitness (Hau & Goymann, 2015). Inter-individual variability of the androgen response could be related to intrinsic psychological features. In recent years, a considerable amount of literature has been published on inter-individual (behavioral) variation. Contrasting patterns are observed whenever individuals behave and interact with their environment. Several definitions with somewhat similar meanings have been proposed for consistent differences between individuals. The term 'temperament' (or personality) is generally understood as the consistency of behavioral differences between individuals over time and across situations (Caramachi, Carere, Sgoifo, & Koolhaas, 2013; Réale, Reader, Sol, McDougall, & Dingemanse, 2007). Distinct behavioral traits (or axes) have been proposed: shyness-boldness (in risky situations, e.g. predator), exploration-avoidance (in new situations), activity (in non-risky and non-novel situations), aggressiveness (towards conspecifics) and sociability (Réale et al., 2007; Sih, Bell, & Johnson, 2004). It is worth noting that, in a population, individuals should be distributed along these axes in a continuum distribution fashion, rather than in a binomial distribution (Réale et al., 2007). Thus, a 'behavioral type' is related to the specific combination of behaviors each individual expresses (Figure 7, Bell, 2007). If a set of behavioral traits correlate between each other, one may define it as a 'behavioral syndrome' (Figure 7, Bell, 2007; Sih et al., 2004), and could mean that the traits are regulated by a common neuroendocrine, genetic or neurobiological mechanism (Coppens, De Boer, & Koolhaas, 2010; Sih et al., 2004). For instance, the best known behavioral syndrome is the proactive-reactive syndrome, studied in the context of stress research to distinguish animals with opposing stress-coping styles (see, for example, Coppens

et al., 2010; Koolhaas et al., 1999; Øverli et al., 2007; Young, Gobrogge, Liu, & Wang, 2011). Proactive individuals are simultaneously bolder, more aggressive and active in response to challenges, show higher exploration rates and, in general, a low HPA (Hypothalamus-Pituitary-Adrenals) axis activity and high sympathetic reactivity. In contrast, reactive individuals seem consistently shy, less aggressive and active, usually freeze in stressful situations and have higher HPA axis and lower sympathetic responses (Koolhaas et al., 1999).

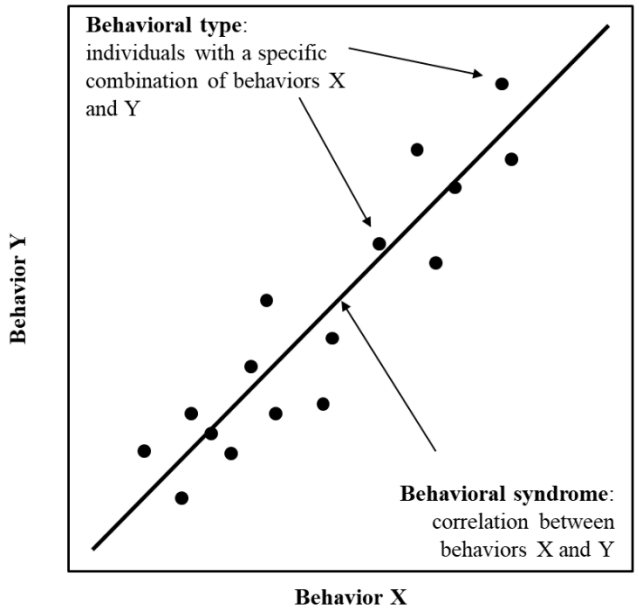


Figure 7. Behavioral type and behavioral syndrome. Each point represents a different individual of the same population.

The literature offers contradictory findings from several authors who attempted to explore the relationship between androgens and behavioral phenotype. For instance, in the mangrove rivulus, *Kryptolebias marmoratus*, there is a positive relation of exploration, boldness and aggression with testosterone baseline levels (Chang, Li, Earley, & Hsu, 2012) while in male great tits, *Parus major*, studies account for a negative relation of exploration and boldness with testosterone baseline levels (van Oers, Buchanan, Thomas, & Drent, 2011). On the other hand, the association between animal personality and fitness has been established (e.g., Dingemanse, Both, Drent, & Tinbergen, 2004; Hau & Goymann, 2015; Smith & Blumstein, 2008). For instance, in bighorn sheep, bold ewes are less docile during handling, whereas shy ewes are more docile (reviewed in Dingemanse & Réale, 2005). Moreover, bold ewes reproduce earlier than shy ewes and in years with a higher risk of predation, bolder and

non-docile ewes have greater survival rates (reviewed in Dingemanse & Réale, 2005). Also, in great tits, *P. major*, there is a negative relationship between exploration and baseline corticosterone levels, while, in turn, reproductive success is linked with corticosterone levels (Hau & Goymann, 2015). In the case of androgens, even though higher levels promote sexual behavior, they also interfere with paternal care and pair bonding, are energy-consuming and have been associated to immunosuppression and oncogenic effects (Oliveira, 2004; Wingfield, Lynn, & Soma, 2001). Interestingly, a study with dark-eyed juncos, *Junco hyemalis*, focused on individual variability in short-term androgen responses and fitness and showed that animals that had a response to a GnRH challenge slight higher than average were the ones with higher survival and offspring rates in opposition to the ones with much higher or much lower responses than average (McGlothlin et al., 2010). In this species, testosterone response to the GnRH challenge consistently varied between individuals but baseline levels did not differ between individuals (Jawor et al., 2006), showing that the magnitude of response was the underlying discriminating factor.

In sum, even though the function and mechanisms that underpin individual variation in androgens are not fully understood, several authors have highlighted the importance of this issue in the context of endocrinology (Hau & Goymann, 2015; Kempenaers et al., 2008; Williams, 2008). As Bennett states: "Real individuals are unique combinations of traits, some above and some below average. It is time to recognize the uniqueness of the individual and to turn it to our advantage as biologists." (Bennett, 1987, p.161).

5. The Mozambique Tilapia as a Model Species in Social Behavior

The Mozambique tilapia, *Oreochromis mossambicus* (Peters, 1852), is a freshwater fish belonging to the Cichlidae family. Cichlids are the most species-rich family of vertebrates, with more than 3,000 species distributed widely along American, African and Asian continents (Kocher, 2004). These fish are subject of particular interest on their explosive and diverse speciation since it is believed that around 2,000 species evolved in a short period of time (Kocher, 2004; Seehausen, 2006). However, besides great phenotypic diversity such as color patterns, body shapes or head morphology, cichlids are characterized by diverse social systems. Behavioral diversity comprises a variety of mating systems (Egger, Obermüller, Sturmbauer, Phiri, & Sefc, 2006; Limberger, 1983; Sato, 1994; Kohda et al., 2009; McKaye, 1983), reproductive (Taborsky, 2001) and fertilization (Mrowka, 1987; Wickler 1962) strategies or even parental care systems (Langen, Thünken, & Bakker, 2013; Balshine-Earn, 1997; Mrowka

1987). The recent development of powerful tools applicable in cichlid species, such as high-throughput sequencing (e.g. RNA-seq, Kasper, Hebert, Aubin-Horth, & Taborsky, 2018), transgenics (Golan & Levavi-Sivan, 2013) with particular emphasis on CRISPR/Cas9 mutagenesis technique (Juntti et al., 2016) and sequencing of several genomes and transcriptomes (namely Nile tilapia, *Oreochromis niloticus, Neolamprologus brichardi/pulcher*, zebra mbuna, *Maylandia zebra*, *Haplochromis nyererei*, and *A. burtoni;* Brawand et al., 2014), bring cichlids forward as prime models for the study of social behavior.

5.1. Biology and Behavior

The Mozambique tilapia is endemic of Southeastern Africa rivers and lagoons (reviewed in Webster & Lim, 2006). This species, which prefers quiet waters, is also seen in some estuaries showing to be resistant to higher salinities environments (reviewed in Webster & Lim, 2006). Although adult fish follow an omnivorous diet they mainly eat vegetation and algae while juveniles feed zooplankton (reviewed in Webster & Lim, 2006). Reproduction is temperature dependent. In temperate areas, fish reproduce seasonally, while in tropical areas, Mozambique tilapia breeds all year (reviewed in Webster & Lim, 2006).

The Mozambique tilapia is a highly social cichlid fish with a lek-breeding system (Fryer & Iles, 1972). Fish aggregate densely in mating territories, where males dig and defend spawning pits and compete for females (Oliveira & Almada, 1998b). Parental care is restricted to females since they lay their eggs into the spits where males fertilize them and then females incubate eggs and fry orally (mouthbrooders), (reviewed in Webster & Lim, 2006). During the brooding period, females suppress feeding and increase aggression towards other individuals to protect the young (Oliveira and Almada 1998a). Fry is usually released from the mouth 20-22 days post-fertilization (Fryer & Iles, 1972). Males present two distinct phenotypes, which can rapidly reverse due to changes in the social environment (Oliveira & Almada, 1998b). Dominant males are larger and darker, establish territories and attract females (Oliveira & Almada, 1998b). In contrast, subordinate males are smaller, silver colored like females, do not establish territories but school with females (Oliveira & Almada, 1998b). Apart from the nuptial coloration, the structure of the jaw and height of dorsal and anal fins are useful morphological traits to discriminate males' social status (Oliveira & Almada, 1995). Also, dominant males have higher androgen levels and higher investment in gonadal tissue than subordinates, but the latter still have mature gonads and may reproduce through sneaking fertilizations (Oliveira & Almada, 1998b, 1998a).

Interestingly, in captivity, males seem to synchronize reproductive behavior, since males in the same tank jointly alternate periods (several days) of territory maintenance and breeding with periods with no breeding activities (Oliveira & Almada, 1998). Also, male alternative mating tactics, such as behaving as floaters or sneakers, are described (Oliveira & Almada, 1998). The former tactic is adopted by intermediate rank (grey-colored) males which occupy others' territories temporarily to court females, while the latter consists in the intrusion of the nests during spawning by males similar to females (Oliveira & Almada, 1998). Male to male courtship is another intriguing phenomenon in this species (Oliveira & Almada, 1998). Dominant males court males that resemble females probably due to a strong sexual motivation and a lack of discriminating ability in primary stages of courtship (Oliveira & Almada, 1998). On the other hand, the reason why courted males perform typical female sexual behaviors is not clear but others hypothesize self-defense mechanisms or indirect sperm competition (Oliveira & Almada, 1998).

5.2. Mozambique Tilapia as a Model to Study Social Neuroendocrinology

The Mozambique tilapia is very robust; tolerating a broad range of salinities and temperatures (Fiess et al., 2007) and proving to be more resistant to diseases and to adverse water quality than other fish species (reviewed in Webster & Lim, 2006). On account of these factors, it is easy to breed and grow in captivity.

Combining these features with their robustness to experimental handling (e.g., repetitive blood or urine collection, pharmacological injections, surgery) and their broad repertoire of social behavior (Baerends & van Roon, 1950), diverse have been the contributions from *O. mossambicus* in the subject area of neuroendocrinology and social behavior, as mentioned already in section 3 of this chapter.

Furthermore, over the years and particularly in this species, existing research has recognized the critical role played by androgens in a variety of social complex phenomena (Oliveira, 2009). For instance, the presence of an audience affects behavior and androgens (audience effect, Roleira et al., 2017) and the androgen response elicited by familiar intruders is less intense than to unfamiliar ones (dear enemy effect, Aires, Oliveira, Oliveira, Ros, & Oliveira, 2015). Mozambique tilapia is able to mount an androgen response in anticipation to territorial intrusions due to associative learning mechanisms (conditioning of the androgen response, Antunes & Oliveira, 2009) while agonistic interactions elicit an androgen increase in

fish spectators (bystander effect, Oliveira, Lopes, Carneiro, & Canario, 2001). Also, in *O. mossambicus*, AVT seems to be rather influential, regulating, as mentioned above, social status.

On the other hand, tools such as a three-dimensional digital map of tilapia brain (Simões et al., 2015) and the sequencing of the *O. niloticus* genome (Brawand et al., 2014), a closed related species, are available, prompting the Mozambique tilapia as a key figure to unravel the underpinnings of social behavior.

Thesis Aims and Structure

This thesis aims to further deepen the knowledge on the neural and endocrine regulation of social behavior, i.e., how the brain controls social behavior, how hormones influence the brain and consequently behavior but also how hormones respond to the social environment. Thus, the empirical part of this thesis is composed of four parts.

First, we focused on the SDMN and how a set of particular core brain areas together control social behavior (CHAPTER 2). In specific, we investigated what are the key aspects of social interactions that originate the neuromolecular restructuring of the brain network. By using agonistic interactions, we tested the hypothesis that it is the assessment that individuals make of the outcome of the fights, rather than the expression of aggressive behavior *per se*, that triggers changes in the pattern of activation of the SDMN.

Next, we examined the effect of the neuropeptide AVT on the breeding behavior of our model species (CHAPTER 3). For this purpose, we manipulated the AVT system in males, with AVT and a *V1A* receptor antagonist, and analyzed their reproductive and aggressive behavior. Also, we carried this experiment in castrated and sham males to investigate the interaction between androgens and the AVT system.

Then, we explored the mechanistic basis of the androgen response to social interactions (CHAPTER 4). Here, we characterized the temporal pattern of the androgen response to social interactions and explored its relationship with inter-individual variation. With this experiment we aimed to address possible reasons for the inconsistencies associated to the Challenge Hypothesis framework.

Finally, in CHAPTER 5, our goal was to unravel which is the specific effect of the androgen response to social interactions on the brain. For this purpose, we studied brain transcriptomic changes associated with a short-term increase of circulating androgens.

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CHAPTER 2

Fighting assessment triggers rapid changes in activity of the brain social decision-making network of cichlid fish

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Abstract

Social living animals have to adjust their behavior to rapid changes in the social environment. It has been hypothesized that the expression of social behavior is better explained by the activity pattern of a diffuse social decision-making network (SDMN) in the brain than by the activity of a single brain region. In this study, we tested the hypothesis that it is the assessment that individuals make of the outcome of the fights, rather than the expression of aggressive behavior per se, that triggers changes in the pattern of activation of the SDMN which are reflected in socially driven behavioral profiles (e.g. dominant vs. subordinate specific behaviors). For this purpose, we manipulated the perception of the outcome of an agonistic interaction in an African cichlid fish (*Oreochromis mossambicus*) and assessed if either the perception of outcome or fighting by itself was sufficient to trigger rapid changes in the activity of the SDMN. We have used the expression of immediate early genes (*c-fos* and *egr-1*) as a proxy to measure the neuronal activity in the brain. Fish fought their own image on a mirror for 15 minutes after which they were allocated to one of three conditions for the two last minutes of the trial: (1) they remained fighting the mirror image (no outcome treatment); (2) the mirror was lifted and a dominant male that had just won a fight was presented behind a transparent partition (perception of defeat treatment); (3) the mirror was lifted and a subordinate male that had just lost a fight was presented behind a transparent partition (perception of victory treatment). Results show that these short-term social interactions elicit distinct patterns in the SDMN and that the perception of the outcome was not a necessary condition to trigger a SDMN response as evidenced in the second treatment (perception of defeat treatment). We suggest that the mutual assessment of relative fighting behavior drives these acute changes in the state of the SDMN.

Keywords: Social Decision Making Network, social competence, immediate early genes, androgens, Challenge Hypothesis.

1. Introduction

Individuals from social species need to combine information about the social environment they live in with information about their internal state, such as previous social experience and organismal condition, in order to adaptively optimize their responses to changes in the social environment (Taborsky and Oliveira, 2012). This ability to rapidly and adaptively adjust behavior to daily social demands is known as social competence and is thought to be accomplished through rapid changes in the state of the neural network underlying social behavior (Oliveira, 2012). Accordingly, consistent changes in social behavior, such as adopting a dominant or subordinate behavioral profile, are associated with distinct behavioral states (that express different behavioral patterns) that are paralleled by specific states of the Social Decision Making Network (SDMN) in the brain (Cardoso et al., 2015). The SDMN consists of an evolutionary conserved set of core brain nuclei that together regulate the expression social behavior, such that the state of the network better explains the behavioral output rather than the activity of a single node *per se* (Goodson, 2005; Newman, 1999; O'Connell and Hofmann, 2011, 2012). All of these brain nuclei are reciprocally interconnected with each other, such that differential activation of the nodes creates dynamic patterns responsible for multiple behaviors. Moreover, the nodes of the SDMN have an extensive expression of steroid, neuropeptide and aminergic receptors, which allows this network to be modulated by these hormones, probably by altering the weight of its nodes or the strength of their connectivity (Goodson, 2005; Oliveira, 2012). Thus, different behavioral states should result from divergent transcriptomes of the SDMN, and changes between states, such as acquiring or losing social status should be associated with rapid changes in patterns of gene expression in the SDMN. Given their fast and transient response to changes in extra- and intra-cellular environment and their effect as transcription factors, immediate early genes (e.g. *c-fos, egr-1*) play a key role in orchestrating transcriptomic responses to environmental changes. Thus, it has been hypothesized that immediate early genes can be the molecular first responders to perceived changes in the social environment that trigger subsequent changes in the neurogenomic state of the SDMN that allows the animal to adjust its behavioral state accordingly (Cardoso et al., 2015). Several studies have documented changes in immediate early gene expression across the SDMN associated with changes in social behavior across different vertebrate taxa (e.g. Faykoo-Martinez et al., 2018; Kabelik et al., 2018; O'Connell and Hofmann, 2012), including teleost fish and also tilapia (e.g., Field and Maruska, 2017; Roleira et al., 2017; Teles et al., 2015). In particular, changes in social status (i.e. ascending or descending in a social hierarchy) have been

associated with rapid changes in immediate early gene expression in the SDMN paralleled by changes in social behavior (Maruska et al., 2013b, 2013a; Teles et al., 2015; Williamson et al., 2018).

In this study, we sought to understand what are the key aspects of an agonistic interaction that trigger an immediate early gene response across the SDMN and concomitantly a socially driven neuromolecular restructuring of this network. We reasoned that in order to be adaptive such network restructuring should match the post-fight social scenario anticipated by the individual in face of the information collected during the interaction. Therefore, the perception of the fight outcome rather than the expression of aggressive behavior per se should play a key role in triggering the SDMN immediate early gene response to an aggressive interaction. Here, we have tested if the perception of the outcome of a single agonistic interaction in an African cichlid fish (Mozambique tilapia, *Oreochromis mossambicus*) is necessary to trigger an immediate early gene (IEG) response across the SDMN or if fighting itself is sufficient to trigger the response.

In order to manipulate the perception of fight outcome, we took advantage of the fact that male Tilapia do not recognize their own image in a mirror and fight aggressively towards it (e.g. Oliveira et al., 2005; Teles et al., 2013). Because in mirror fights the opponent's behavior (i.e. mirror image) always matches the behavior of the focal fish, there is no information available to the participant regarding the fight outcome. That is the males express aggressive behavior without experiencing either a win or a defeat. Thus, an IEG response triggered by a mirror fight would be driven by the experience of fighting and not by the perception of the interaction outcome (i.e. winning vs. losing). In this study, we have used three fighting treatments. After a mirror fighting phase that lasted 15 minutes focal males were allocated to one of three conditions for the last two minutes of the trial: (1) they remained fighting their mirror image (no outcome treatment, where the mirror image remained in both steps of the experiment; MM); (2) the mirror was lifted and a dominant male that had just won a fight was presented behind a transparent partition (opponent becoming dominant treatment, where the mirror image became dominant male; MD); (3) the mirror was lifted and a subordinate male that had just lost a fight was presented behind a transparent partition (opponent becoming subordinate treatment, where the mirror image became a subordinate male; MS). Our prediction was that if the immediate early gene response is challenge dependent, then all three treatments would trigger a similar immediate early gene response; in contrast, if immediate early gene responsiveness is dependent on perceiving a win or a defeat, divergent immediate early gene responses across the SDMN are expected in the MD and MS treatments in relation to the mirror

fights treatment (MM) where no information on outcome is available. Given that socially-driven changes in the SDMN are expected to produce integrated phenotypic responses, at the behavioral and physiological (hormonal) levels, to the social environment and that androgens have been described to respond to social challenges (Challenge hypothesis, Hirschenhauser and Oliveira, 2006; Wingfield et al., 1990), we have also characterized the response of the hypothalamic-pituitary-gonadal axis to our experimental treatments by measuring the expression of gonadotrophin-releasing hormone (*gnrh1*) in the preoptic area and circulating androgen levels (testosterone, T, and 11-ketotestosterone, KT).

2. Materials and methods

2.1. Animals and housing

The Mozambique tilapia is a freshwater fish with a lek-breeding system (Fryer and Iles, 1972). Males aggregate densely in mating territories, where they dig and defend spawning pits and compete for females (Oliveira and Almada, 1998). Males present two distinct phenotypes, which can rapidly reverse due to changes in the social environment (Oliveira and Almada, 1998). Dominant males are usually larger, dark colored, establish territories and attract females. In contrast, subordinate males have a silver color pattern similar to that of females, and fail to establish territories.

O. mossambicus fish from a stock held at ISPA were used in this study. Fish were maintained in stable social groups of 4 males and 5 females per group, in glass tanks (120 x 40 x 50 cm, 240 L) with a fine gravel substrate. Tanks were supplied with a double filtering system (sand and external biofilter; Eheim) and constant aeration. Water quality was monitored on a weekly basis for nitrite (0.2-0.5 ppm), ammonia (<0.5 ppm; Pallintest kit) and pH (6.0-6.2). Fish were kept at a temperature of 26 ± 2 ºC, a 12L:12D photoperiod, and fed with commercial cichlid sticks. The social status of the males was monitored daily and territorial males were identified by dark body coloration and digging of a spawning pit on the substrate (Oliveira and Almada, 1996).

2.2. Experimental procedure

The experimental setup consisted of 2 adjacent tanks (test and demo tank) with an opaque partition between them. Twenty territorial focal males (mean body mass ± SEM: 81.63 g ± 7.06

g) were used in this experiment. Each focal male was isolated for 7 days in the test tank (30 × 50 × 25 cm). On day 6, plasma was collected from the focal male to determine steroids baseline levels. On the same day, a male fish was introduced in the demo tank (30 ×70 × 40 cm), to allow it to adopt this tank as its territory. On the day of the experiment (day 7), an intruder male was introduced in the demo tank and both males were allowed to interact for 30 min. This agonistic interaction was accompanied by the experimenter and fight outcome was assessed by live observation. Accordingly, after fight resolution, winners continue to be aggressive and present a dark coloration while losers only display submissive behavior and present a light coloration. Thus, winners can be seen as clear/explicit dominant males (recently gaining social status) and losers as clear subordinate males (recently losing social status). Fifteen minutes after the beginning of the social interaction in the demo tank, a mirror was placed in the external wall of the test tank, adjacent to the demo tank. The interaction between the mirror and the focal male in the test tank was recorded for 15 min. At the end of the mirror interaction, males in the demo tank were separated by an opaque partition and the focal male in the test tank was allowed to see for 2 min one of the following stimuli: i) its own image in the mirror (MM treatment, N=8), or a real (opponent) male, either ii) the dominant male of the demo tank (Mirror becomes Dominant – MD treatment, N=6) or iii) the subordinate male of the demo tank (Mirror becomes Subordinate – MS treatment, N=6) (Figure 1). Fight outcome was manipulated by controlling the order of introduction of each fish in the demo tank and their size, so the male introduced first (in day 6) was always bigger than the intruder and won all staged fights. Using this procedure, we had no unsolved fights. Focal and opponent males were sized matched and were selected from different family tanks to control for familiarity effects. At the end of the experiment, an opaque partition was placed between the tanks to prevent the males from seeing each other and 20 min later a blood sample was collected from the caudal vein under anaesthesia (MS-222, Pharmaq; 300-400 ppm). Blood sampling always took less than 4 min from the induction of anaesthesia to prevent possible effects of handling stress on steroids levels (Foo and Lam, 1993). Blood samples were centrifuged (10 min, 600 *g*) and plasma was stored at –20ºC until further processing. After blood sampling, the fish were returned to the anaesthesia solution until muscular and opercular movements stopped completely and were then sacrificed by decapitation. The cranial fraction (brain and part of the cranial bones) was embedded in mounting media (OCT Compound, Tissue-Tek, Sakura) and frozen at -80ºC during 15-30 minutes. Coronal sections were obtained at 150 µm thickness using a cryostat (Microm HM 500 M) and collected on previously cleaned slides (70% ethanol). Regions of interest were

microdissected under a steromicroscope (VWR SZB350OH) and collected in 50 µl of Qiazol lysis buffer (RNeasy Lipid Tissue Mini Kit, Qiagen) with a modified 25G needle. Samples were stored at -80 ºC until RNA extraction. The following representative nodes of the SDMN (O'Connell and Hofmann, 2011b) were identified according to Teles et al.(2012): medial part of the ventral subdivision of the ventral telencephalon (VVm; putative homologue of the mammalian lateral septum), supracommissural part of the ventral telencephalon (Vs; putative homologue of the mammalian medial extended amygdala), anterior part of the periventricular preoptic nucleus (PPa), nucleus anterior tuberis (TA; putative homologue of the ventromedial hypothalamus) and central gray (GC).

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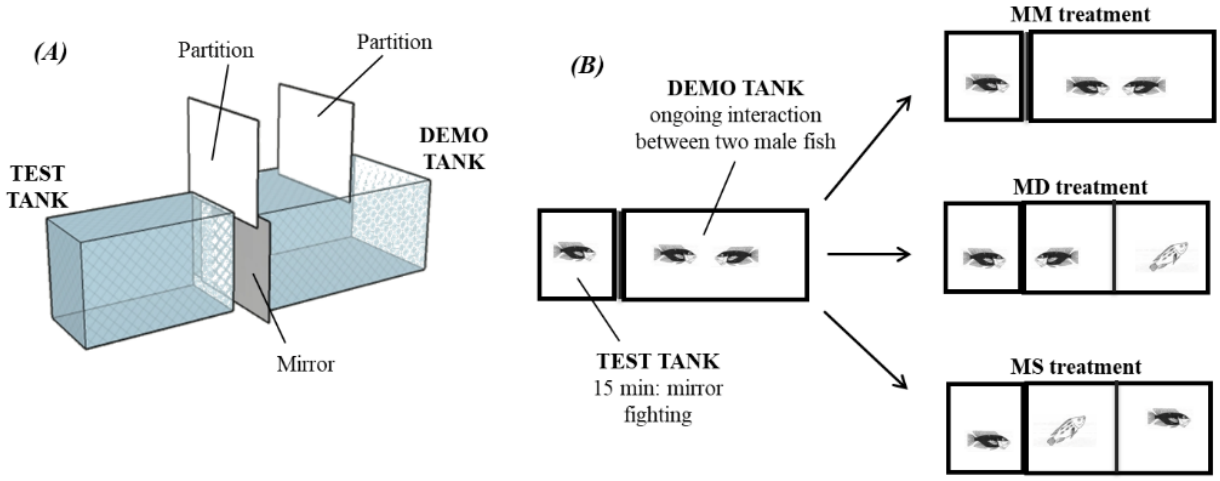

Figure 1. Behavioral paradigm. (A) 3D diagram of the experimental setup. Test tank and demo tank were side-by-side and physically separated. (B) Schematic of the experimental treatments. Focal fish interacted with a mirror for 15 min while two males were fighting in the adjacent compartment. Then, focal fish were allowed to see for 2 min its own image in the mirror (MM treatment), a dominant male (Mirror becomes Dominant – MD treatment) or a subordinate male (Mirror becomes Subordinate – MS treatment).

2.3. Behavioral observations

The behavior of the focal male, either towards the mirror or interacting with the opponent male, was analysed using a computerized multi-event recorder software (Observer, Noldus technology, version 5). The behavior of the opponent male was also analysed with the same software (see Figure S1 for the descriptive statistics of focal and opponent behavioral measures). The analysis was based on the ethogram repertoire provided by Baerends and

Baerends-van Roon (1950). Relevant behavioral patterns were identified to measure male aggressive behavior (i.e. bites, displays, attacks).

2.4. Gene expression analysis

Primers were designed using National Center for Biotechnology Information (NCBI) sequences for *c-fos* (accession #GR607679.1), *egr-1* (accession #AY493348.1), *gnrh1* (accession #AB101665.1) and the housekeeping gene *eef1A* (accession #AB075952.1). Primer3 software (Koressaar and Remm, 2007; Untergasser et al., 2012) was used to design the primers, which were commercially synthesized (Sigma-Aldrich, Hamburg, German). Primers were tested with a cDNA pool in a qRT-PCR, and PCR products were confirmed by sequencing. Amplification products were 106 pb for *c-fos*, 135 pb for *egr-1*, 127 pb for *gnrh1* and 85 pb for *eef1A*. Primer dimer formation was controlled with FastPCR v5.4 software (Kalendar et al., 2009) and optimal annealing temperature was assessed for maximal fluorescence (Table S1). qRT- PCR was performed using the Quantitative PCR System Stratagene MX3000P. The reaction mix included Sybr Green (Fermentas, #K0221), 400 nM of each primer and 1 µl of cDNA in a 25 µl reaction volume. Cycling parameters were: i) denaturation: 5 min at 95 ºC; ii) amplification and quantification: 40 cycles (30 s at 95 °C, 30 s at primer-specific annealing temperature, 30 s at 72 °C); iii) dissociation curve assessment (30 s at 95°C, 30 s at 55°C, 30 s at 95°C). The dissociation curve was performed to confirm a single melting curve proving the inexistence of primer-dimer formation and/or plate contamination. All samples were run in triplicate and controls with water instead of DNA templates showed no amplification. PCR Miner (Zhao and Fernald, 2005) was used to calculate reaction efficiencies (E) and cycle thresholds (CT), based on the kinetics of individual PCR reactions. *c-fos*, *egr-1* and *gnrh1* mRNA levels normalized for housekeeping (HK) gene *eef1A* were determined from the equation: $(1 + E_{HK})^{CT_{HK}} / (1 + E_{gene})^{CT_{gene}}$. Mean values for *eef1A* did not differ between treatments, thus confirming its suitability to be used as a reference gene in this study.

2.5. Quantification of steroids levels

Free steroids (testosterone, T; and 11-ketotestosterone, KT) were extracted from plasma samples by adding diethyl-ether to the samples, centrifuging the mix (800 *g*, 5 min, 4ºC) and freezing it (15 min, -80ºC) to separate the ether fraction (containing the free steroid). This process was repeated twice. The ether fraction was evaporated and the steroids were re-

suspended in phosphate buffer. Steroid concentrations were measured by radioimmunoassay. The testosterone antibody was from Research Diagnostics Inc (#WLI-T3003, rabbit anti-testosterone) and the 11-ketotestosterone antibody was kindly donated by D. E. Kime (the specificity table was published in Kime and Manning 1982). We used a testosterone reactive marker from Amersham Biosciences ([1,2,6,7-3H] testosterone, #TRK402-250 µCi) and a titrated 11-ketotestosterone produced in-house from marked cortisol (Kime and Manning, 1982). Inter-assay variabilities were 4.1 % and 8.9 % for T and KT, respectively. Intra-assay variation coefficients were 2.4 % and 2.0 % for T and 4.1% and 4.0 % for KT.

2.6. Data analysis

Outlier observations were identified and replaced by missing values using a generalized extreme studentized deviate procedure (e.g. Jain, 2010) with a p-value of .05 and a maximum number of outliers set at 20% of the sample size. Behavioral variables and gene expression levels were logarithmically transformed [log10 (x+1)] to meet parametric test assumptions. The behavioral variables (for frequency and latency) were reduced with Principal Component Analysis (PCA) using the variable principle normalization method. Two principal components were obtained that explain 86.3 % of the variance and that seem to represent different aspects of aggressive behavior: “overt aggression” and “aggressive motivation” (see results). The component scores of each case on each of these principal components were analyzed using separate Linear Mixed Models (LMM) with Treatment (MM, MD, MS) as a fixed effect and focal fish as a random effect. Post-hoc tests were used to test for differences between experimental treatments, with p-values adjusted for the number of multiple comparisons (Benjamini and Hochberg, 1995).

Separate LMM were also used to check for differences between treatments in immediate early gene (*c-fos*, *egr-1*) expression in each sampled brain area (GC, TA, Vs, VVm, PPa).Post-hoc tests were used to test for differences between experimental treatments, with p-values adjusted for the number of multiple comparisons (Benjamini and Hochberg, 1995).

Pearson correlations between IEG expression of each brain area and between the behavioral principal component score were used to examine the association between aggressive behavior and gene expression. Pearson correlation matrices between each pair of brain nuclei for each IEG were used as a measure of functional connectivity and tested using a Quadratic Assignment Procedure (QAP) with 5000 permutations. Since the null-hypothesis for QAP states that there is a non-random association between the tested matrices, a QAP with a non-

significant p-value indicates that there is no association between the treatment's IEG activational pattern. The p-values of the Pearson correlation matrices were adjusted (Benjamini and Hochberg, 1995). The brain patterns of IEG expression obtained for each experimental treatment were tested on a network perspective, by measuring density and centrality parameters (Makagon et al., 2012). Density was used as a measure of the network cohesion, given by the proportion of all possible connections that are present in the network (Makagon et al., 2012). Differences in network density between treatments were tested using a t-test (bootstrap set to 5000 sub-samples). As a measure of node centrality we assessed eigenvector centrality, that takes into account not only how well a node is connected to other nodes in the network but also how well connected its relations are (Makagon et al., 2012).

Variation in hormone levels (KT, T) was computed as (Post-treatment levels) - (Baseline levels) for each individual. To test for differences between the treatments we performed unpaired t-tests. Pearson correlation analysis was used to examine the relationship between *gnrh1* gene expression and IEG expression in the PPa. Pearson correlation analysis was also used to examine the relationship between *gnrh1* gene expression in the PPa and androgen circulating levels. A LMM was used to test for differences between treatments in *gnrh1* in the PPa area. Post-hoc tests were used to test for differences between experimental treatments, with p-values adjusted for the number of multiple comparisons (Benjamini and Hochberg, 1995).

Effect sizes were computed for post-hoc tests (Cohen's d).

Statistical analysis was performed using IBM SPSS® statistics v.21, and R (R Core Team, 2015) with the following packages: nlme (LMM), dplyr (t-tests), multcomp (post-hoc comparisons), Hmisc (correlations), ggplots (heatmaps),. Characterization of the SDMN network was obtained with UCINET version 6.653 (Borgatti et al., 2002). Brain nuclei representations of the SDMN network were produced using a custom-made python script. Degrees of freedom may vary between the analyses due to missing values.

2.7. Ethics Statement

In this study, we have staged real opponent agonistic interactions to obtain winner and loser animals, since the use of video-playbacks in this species is inadequate (R. Oliveira, personal observation). However, we have kept sample sizes to a minimum, and limited contests to a short duration. No signs of physical injuries were observed during any of the trials. Animal experimentation procedures were conducted in accordance with the European Communities

Council Directive of 24 November 1986(86/609/EEC) and were approved by the Portuguese Veterinary Authority (Direcção Geral de Alimentação e Veterinária, Portugal; permit # 0421/000/000/2013).

3. Results

3.1. Behavior

A Principal Component Analysis (PCA) of the behavioral variables resulted in two principal components (PC) that together explained 86,3 % of the variance in aggressive behavior (Table 1). PC1 had a high loading (>0.9) of frequency of bites and frequency of attacks, and hence it was interpreted as "overt aggression". The highest loading in PC2 was the latency to display, and hence its symmetric was interpreted as "aggressive motivation".

Table 1. Principal component analysis of behavioral variables.

Behavioral variables	Component loading	
	PC1	PC2
Frequency of displays	.793	-.443
Frequency of bites	.915	-.161
Frequency of attacks	.923	.122
Latency to display	-.595	.717
Latency to bite	-.887	-.293
Latency to attack	-.896	-.287
Eigenvalue	4.262	.919
% of variance explained	71.03	15.32

There was an effect of the experimental treatment in "overt aggression" (i.e. PC1 loadings; $F_{2,17}$= 4.87, p =0.02), with focal fish assigned to the MS condition showing significantly less overt aggression than those in the MM and MD conditions (Figure 2A). In contrast there was no effect of experimental treatment on "aggressive motivation" (PC2 loadings; $F_{2,17}$= 0.50, p =0.62; Figure 2B).

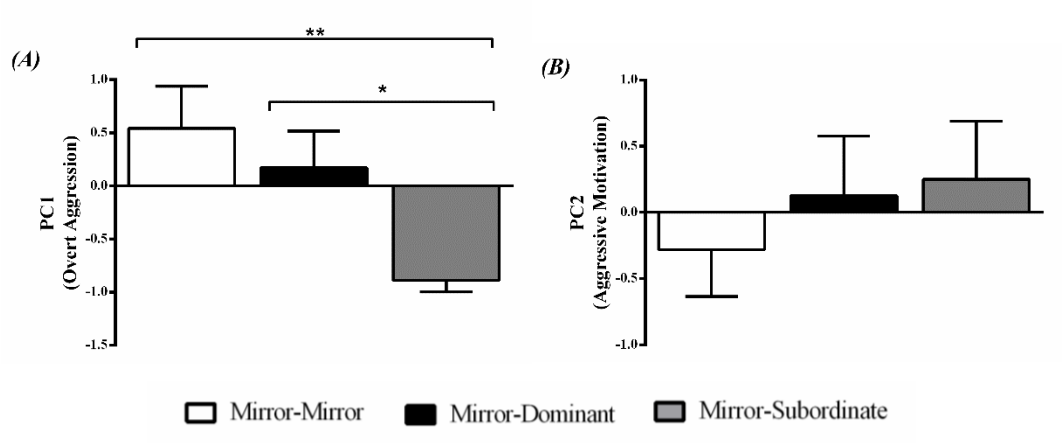

Figure 2. Variation in the behavioral component scores obtained with the Principal Component Analysis (PCA) for each experimental treatment. (A) PC1 interpreted as "overt aggression"; and (B) PC2 interpreted as "aggressive motivation". *Significant difference for $p < 0.05$; **significant difference for $p < 0.01$. Results are expressed as mean ± standard error of the mean (SEM).

3.2. Immediate early gene expression in the Social Decision-Making Network (SDMN)

Significant differences between treatments were only detected for *c-fos* in the TA area, specifically between the MM and the MS treatments (Figure 3; Table 2). No other significant main effect or post-hoc comparison was detected for *c-fos* or *egr-1*.

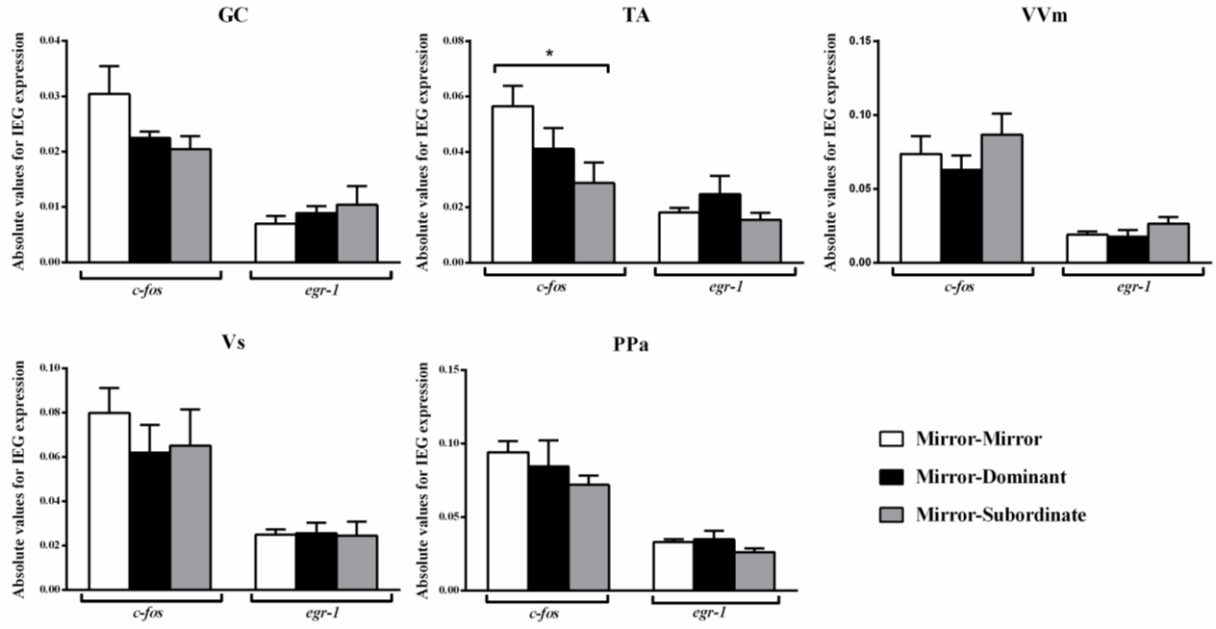

Figure 3. Expression of the immediate early genes *c-fos* and *egr-1* in several brain areas of the SDMN. GC, central gray; PPa, anterior part of the periventricular preoptic nucleus; TA, nucleus

anterior tuberis; VVm, medial part of the ventral subdivision of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon. *Significant difference for $p < 0.05$. Results are expressed as mean ± standard error of the mean (SEM).

Table 2. Effect of treatment on immediate early genes expression in SDMN areas. Main effects and post-hoc comparisons between treatments.

	Main effects		**MM vs MD**			**MM vs MS**			**MD vs MS**		
Areas	**F**	**p**	**t**	**p**	**d**	**t**	**p**	**d**	**t**	**p**	**d**
c-fos											
VVm	.816	.462	.550	.583	.031	.820	.583	.050	1.268	.583	.086
Vs	.821	.458	1.004	.473	.072	1.170	.473	.061	.160	.873	.008
TA	3.839	**.042**	1.250	.211	.081	2.770	**.017**	.140	1.421	.211	.069
GC	.426	.663	.910	.363	.091	.319	.750	.017	.591	.555	.036
PPa	.970	.400	1.027	.457	.047	1.277	.457	.119	.286	.775	.016
egr-1											
VVm	1.528	.247	.675	.500	.038	1.119	.395	.070	1.729	.252	.087
Vs	.156	.857	.166	.868	.010	.552	.868	.030	.362	.868	.018
TA	1.176	.333	.808	.419	.040	.831	.419	.057	.1.533	.376	.074
GC	.918	.419	1.094	.411	.066	1.174	.411	.059	.130	.897	.008
PPa	1.705	.213	.078	.938	.004	1.600	.164	.109	1.618	.164	.081

d: effect size estimate (Cohen's d); Treatments: MM, Mirror-Mirror; MD, Mirror-Dominant; MS, Mirror-Subordinate; GC, central gray; PPa, anterior part of the periventricular preoptic nucleus; TA, nucleus anterior tuberis; VVm, medial part of the ventral subdivision of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon; c-fos degrees of freedom for F-test: GC: (2, 12); PPa: (2, 16); TA: (2, 17); VVm: (2, 14); Vs: (2, 16); egr-1 degrees of freedom for F-test: GC: (2, 16); PPa: (2, 16); TA: (2, 17); VVm: (2, 16); Vs: (2, 17); statistically significant values are in bold.

No significant association between the correlation matrices for *c-fos* and *egr-1* expression in the brain areas of the SDMN was detected using QAP, suggesting that all treatments showed a distinct co-activation pattern for *c-fos* and *egr-1* (Table 3, Figure S2). Thus, the pattern of functional connectivity across the SDMN is specific for each treatment. The density of the *egr-1* network was significantly higher for fish assigned to the MS treatment when compared to the MM and MD treatments (MM vs MS: t=2.815, p=.005; MD vs MS: t=2.061, p=.037) (Table 4). The *egr-1* network density for MM and MD treatments was not significantly different (MM vs MD: t=1.488, p=.137). We have not detected significant differences between treatments for *c-fos* network density (MM vs MD: t=1.861, p=.065; MM vs MS: t=.461, p=.607; MD vs MS: t=1.588, p=.125). The eigenvector centrality measures

suggest that GC is a central node in the *c-fos* and *egr-1* networks for fish in the MM and MS treatments, but that it is a poorly connected node in the MD treatment. (Table 4). The eigenvector centrality measures show that the MD and MS treatment networks are characterized by a high centrality of the PPa node for *egr-1* (Table 4). Centrality measures of the *egr-1* network for fish in the MM treatment show a high centrality for TA and a low centrality for PPa, (Table 4).

Table 3. Association between the correlation matrices for IEG expression in the brain areas of the SDMN. Quadratic assignment procedure (QAP) for *c-fos* and *egr-1* co-activation matrices.

		MM		**MD**	
		r	**p**	**r**	**p**
c-fos	**MS**	-.202	.291	-.119	.409
	MD	.148	.367		
egr-1	**MS**	-.222	.259	-.134	.501
	MD	-.489	.189		

Treatments: MM, Mirror-Mirror; MD, Mirror-Dominant; MS, Mirror-Subordinate

Table 4. Characterization of the SDMN for each experimental treatment using *c-fos* and *egr-1* as reporters of neuronal activity. Values reported correspond to network cohesion (density) and centrality (eigenvector) of each node of the network.

		c-fos			***egr-1***		
		MM	**MD**	**MS**	**MM**	**MD**	**MS**
density		.559	.360	.535	.243	.391	.553
eigenvector	**GC**	.550	.175	.565	.532	.459	.542
	PPa	.408	.579	.382	.127	.576	.518
	TA	.455	.264	.398	.644	.374	.380
	VVm	.456	.523	.375	.454	.188	.444
	Vs	.342	.538	.486	.282	.532	.310

Treatments: MM, Mirror-Mirror; MD, Mirror-Dominant; MS, Mirror-Subordinate; GC, central gray; PPa, anterior part of the periventricular preoptic nucleus; TA, nucleus anterior tuberis; VVm, medial part of the ventral subdivision of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon.

There were no significant correlations between *c-fos* or *egr-1* expression in brain areas of the SDMN and aggressive behavior (Figure 4).

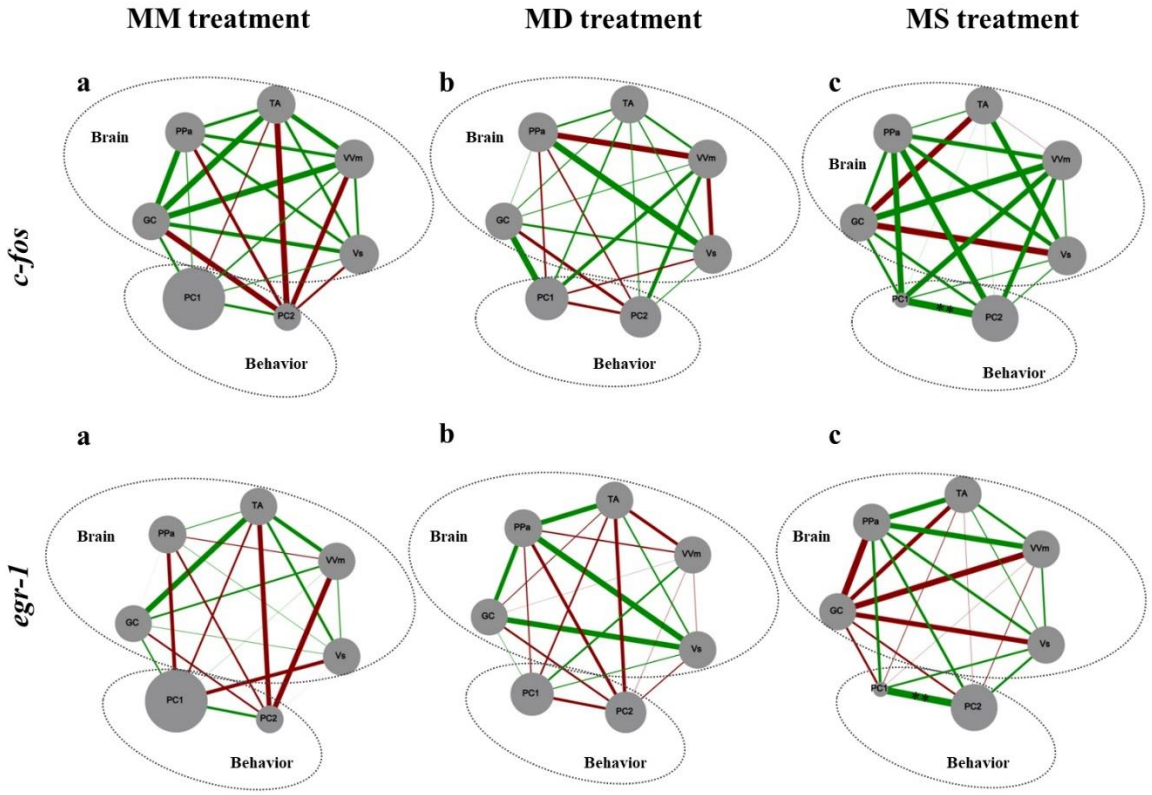

Figure 4. Representation of the state of the SDMN and the behavior for all the experimental treatments. Node size of each brain area indicates the activity level at each network node using *c-fos* and *egr-1* as reporters of neural activity. PC1 and PC2, component loadings obtained with the Principal Component Analysis of aggressive behavior were used as behavioral network nodes, where the node size corresponds to the average of principal component scores within each treatment. Line thickness indicates the strength of the connection between nodes (measured with Pearson correlation coefficients, r-value); green lines represent positive correlations; red lines represent negative correlations. GC, central gray; PPa, anterior part of the periventricular preoptic nucleus; TA, nucleus anterior tuberis; VVm, medial part of the ventral subdivision of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon. PC1, first component loading interpreted as "overt aggression"; PC2, second component loading interpreted as "aggressive motivation". **Significant correlations after p-value adjustment for $p < 0.01$.

3.3. Activity of the hypothalamic-pituitary-gonadal (HPG) axis

There were no significant correlations between the neuronal activation of the PPa as measured by either *c-fos* or *egr-1* and the expression of *gnrh1* in the PPa or circulating androgen levels (*cfos*: r = .170, p = .499, n = 18; *egr-1*: r = .107, p = .673, n = 18). There were also no significant correlations between the expression of *gnrh1* in the PPa and circulating androgen levels (KT: r = .276, p = .283, n = 17; T: r = .371, p = .143, n = 17).

Furthermore, there were no differences between treatments either in *gnrh1* expression in the PPa ($F_{2, 16}$=.407, p=.672; MM vs MD: t_{16}=.380, p=.704, d=.020; MM vs MS: t_{16}=.903, p=.704, d=.053; MD vs MS: t_{16}=.447, p=.704, d=.024), or in the androgen response to the behavioral treatment (KT: MM vs MD: t_{12}=-.644, p=.532, d=.041; MM vs MS: t_{12}=-.905, p=.383, d=.034; MD vs MS: t_{10}=-.441, p=.669, d=.006; T: MM vs MD: t_{10}=-.984, p=.348, d=.306; MM vs MS: t_{11}=-.377, p=.714, d=.034; MD vs MS: t_{9}=.978, p=.353, d=.006), Figure 5.

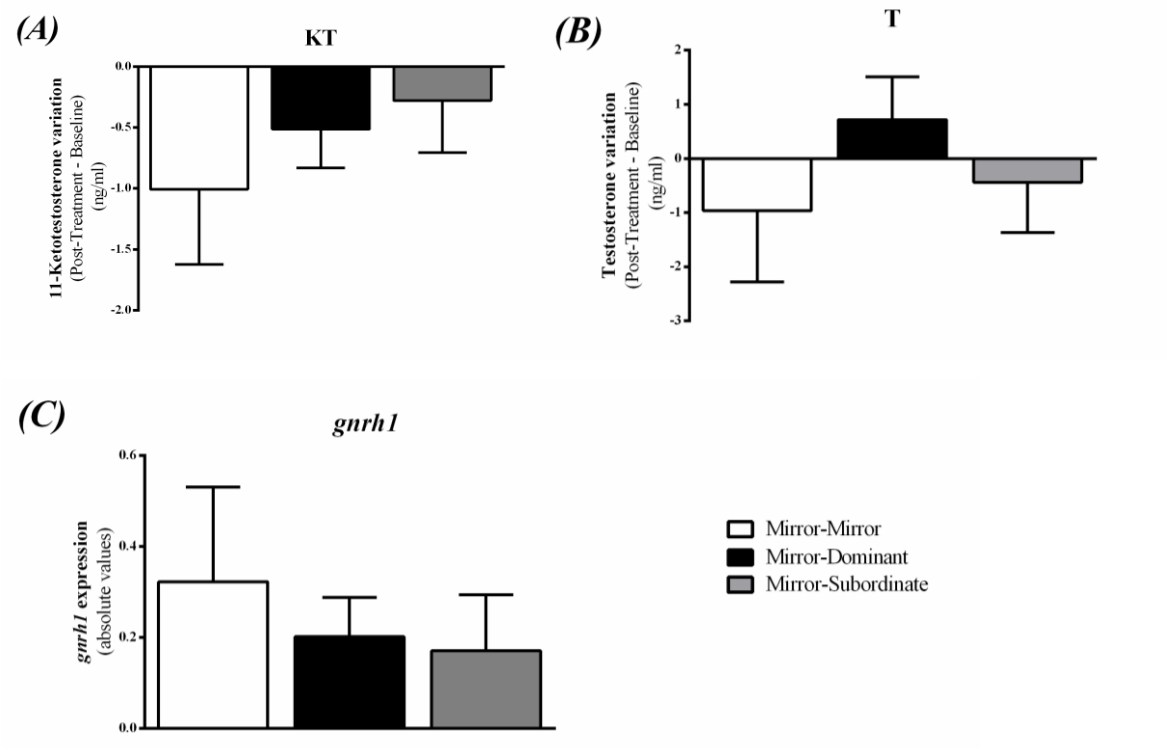

Figure 5. Variation in androgen levels and expression of *gnrh1* in the Ppa of the focal fish for each experimental condition. *(A)* 11-Ketotestosterone (KT) levels; *(B)* Testosterone (T) levels; *(C) gnrh1* expression. Results are expressed as mean ± standard error of the mean (SEM).

4. Discussion

Contrary to our predictions, fish assigned to the MM and the MD treatments showed similar behavioral patterns, that is, they equally fought aggressively their opponents, suggesting that the focal fish of the MD condition did not interpret a recently winning male as having a higher social status than itself, i.e., fish did not perceived the MD interaction as a defeat. In this context, it seems plausible that the visual signal presented was insufficient *per se* to communicate higher status, originating an agonistic interaction that, like the MM, was also unsolved, either because of the short interaction time allowed (only 2 minutes) or because of the symmetry of the fight. A study in another cichlid fish has shown that males previously interacting with a mirror have a higher probability to win a fight than non-mirror stimulated control individuals, probably because of an enhanced aggressive motivation (Dijkstra et al., 2012). On the other hand, the opponent fish had just won a fight, which is known to induce motivational changes that lead to the winner effect (Oliveira et al., 2009). Thus, it seems plausible that the behavior of the MD opponent was paralleled by that of the focal fish due to the heightened motivation of both contestants. In the case of the MS treatment, the losing experience of the opponent leads to a decrease in the willingness to engage in another contest (Hsu et al., 2006). So, it is plausible that the focal fish interpreted the interaction outcome as a win since they performed aggressive displays towards the subordinate opponent male first, which replied much later. Thus, due to a lack of an aggressive motivation by the opponent the focal fish did not further escalate its aggressive behavior (no attacks or bites), hence avoiding extra energetic costs (Hsu et al., 2011). Thus, at least for the MD condition, the experimental treatment may not have effectively altered the focal fish's perception of the outcome, yet fish seem to constantly monitor the social interaction and adjust their behavior according to their internal state and to the behavior of their opponent. The ability of fish to compare their behavior with the one of the opponent and assess their competitive ability (mutual assessment) has few support in the literature (Hsu et al., 2011) but our data suggest its involvement. Of course, future experiments are necessary to fully uncover the underlying cognitive mechanisms.

In the present study, we showed that the pattern of expression of immediate early genes across the SDMN responds to acute changes in social interactions. Only 2 minutes of exposure to different fight outcomes (i.e. MD vs. MS) of an interaction that was already going on for 15 min was sufficient to trigger different patterns of *c-fos* and *egr-1* expression. Given the pivotal role of these immediate early genes in orchestrating integrated transcriptome changes (Clayton, 2000), these short-term responses of *c-fos* and *egr-1* to acute changes in the perceived dynamics

of the interaction suggest that the neurogenomic state of the SDMN can change rapidly in response to perceived social interactions.

Our results also confirm the hypothesis, that the expression of social behavior is better explained by the overall pattern of activation of the SDMN rather than by the activity of a specific region in the brain (e.g. a specific node of the network)(Teles et al., 2015). Indeed, there were no significant correlations between the expression of any of the immediate early genes tested and the expression of aggressive behavior. In contrast, the correlation matrices for the expression of each immediate early gene across the nodes of the SDMN, which capture the co-activation or reciprocal inhibition between brain regions, were specific for each experimental treatment. Moreover, only the expression of *c-fos* in the TA was significantly different between experimental treatments (i.e. MM and MS treatments). The TA is the putative homologue of the ventromedial hypothalamus in mammals, and its ventrolateral subdivision has been strongly associated with aggression. For instance, pharmacogenetic inactivation of this area in mice stops inter-male aggressive behavior while optogenetic activation induces attacks towards females or inanimate objects (Lin et al., 2011). Other study analysed the *c-fos* expression in the brain of subordinate hamsters after a fight and detected elevated activation in several areas including the lateral part of the ventromedial hypothalamus in comparison with dominant males (Kollack-Walker et al., 1997). In a recent review, Hashikawa et al (2017) proposed the involvement of this particular sub-nucleus in the following aspects of aggression: aggressive motivation, specifically that the activation of this area heightens aggressive state (Falkner and Lin, 2014); detection of aggressive signals, such as for example olfactory cues (Falkner and Lin, 2014; Lin et al., 2011); and in the start and execution of aggressive behavioral patterns (Falkner and Lin, 2014). Our results only partially agree with this research in mammals since we report an accentuated expression of *c-fos* only in one of the two treatments (i.e. in MM but not in MD) in which fish express high levels of aggression and a decreased expression of this immediate early gene when fish see a subordinate male after interacting with a mirror (MS) and consequently stop performing attacks and bites. In another cichlid fish (the Burton's mouthbrooder, *Astatotilapia burtoni*) it has been demonstrated that males that were given an opportunity to rise in social rank have higher expression of *c-fos* and *egr-1* in all the areas of the SDMN, including the TA, when compared to stable males, either of a dominant or a subordinate social status (Maruska et al., 2013b). On the other hand, a social descending male has an increase of *c-fos*, and not *egr-1*, expression in this area (Maruska et al., 2013a), corroborating its involvement also in social status transitions, as observed in the current study.

Moreover, a very interesting finding was that fish that saw a subordinate male after fighting with a mirror (MS) showed an increase in the density of the structure of the SDMN, namely on the density of the *egr-1* network, when compared to the other treatments. This evidence suggests that the perception of the fight outcome (which only unequivocally occurred in this treatment) originated a denser brain network, which is characterized by redundant connections and hence a higher robustness to changes in its nodes (i.e. it is less likely affected by the removal of nodes at random (Makagon et al., 2012). Looking into centrality measures obtained with the network analysis it is possible to ascertain that the TA is a more central area while the PPa is a less important node of the *egr-1* network in the MM condition while in the MD and MS conditions the reversed pattern is observed. These results strengthen the idea of the main role of TA in status changes and of the PPa as a link to the bodily changes (e.g. androgen response) that should accompany the changes in brain state.

Androgens are known to respond to social interactions and this response has been hypothesized to play an adaptive role in the adjustment of aggressive behavior to the competitive demands of the social environment (challenge hypothesis, Hirschenhauser and Oliveira, 2006; Wingfield et al., 1990). Therefore, in this study, we have also investigated how androgens responded to the fighting assessment and how the changes in activation of the PPa, where GnRH1 neurons that control the HPG axis are located, were linked to a putative androgen response. Surprisingly, we found no significant changes in androgen levels in any of the treatments with social challenges (MD, MS). Concomitantly, we also did not find a change in the expression of *gnrh1* in the PPa in response to the MD or MS treatments, and there were no correlations between *gnrh1* expression and circulating androgen levels. Moreover, there were no correlations between the expression of any of the immediate early genes and that of *gnrh1*, indicating that the observed activation of the PPa in response to the experimental treatments does not correspond to an activation of the HPG axis. These negative results may result from the short time span of the staged fights with the real opponents, and/or from the failure to induce a perception of fight outcome in the case of the MD treatment.

In summary, our results support the view that it is the assessment that animals make of ongoing fights, and not the perception of the outcome, which trigger rapid changes in gene expression across the SDMN and that the TA is a key node in this network.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

OA and RO designed the experiments. OA performed behavioral experiments. AF processed samples. GO, JL and AF analyzed the data. AF, GO and RO wrote the paper.

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CHAPTER 3

Interaction between arginine vasotocin and gonadal hormones in the regulation of reproductive behavior in a cichlid fish

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Abstract

Nonapeptides of the vasopressin/oxytocin family have been widely associate with the regulation of different aspects of social behavior across different vertebrate species. In particular, arginine vasotocin (AVT), the teleost homologue of vasopressin, has been associated with different aspects of reproductive behavior (i.e. mating and aggression in the context of competition for mates) in fish. Given the fact that androgens are also known to regulate the same aspects of reproductive behavior, we hypothesized that AVT and androgens could be interacting, rather than acting independently, in the regulation of reproductive behavior. In the present study, we aimed to understand the effect of AVT and its interaction with gonadal hormones (putatively androgens) on different aspects of reproductive behavior of a polygynous and territorial cichlid fish, the Mozambique tilapia (*Oreochromis mossambicus*). Using a within-subject design, we treated territorial males, that were previously castrated or sham-operated, with different dosages of AVT as well as with a *V1A* receptor antagonist (Manning compound) and subsequently analyzed their behavior towards females and towards an intruder male. Our results showed that AVT affected the behavior of territorial males towards females but not towards males. Specifically, AVT-treated males interacted less with females than saline-treated males while both gonadectomized and sham-operated males injected with AVT were less aggressive towards females. Moreover, blocking *V1A* receptors increased the frequency of bites towards females in comparison to saline-treated males, in sham-operated males but not in castrated males. This result suggests that AVT down-regulates aggressiveness towards females through the action of *V1A* receptors in the gonads, and that androgens up-regulate this behaviour. In summary, our results provide evidence of the important role of AVT in the modulation of social behavior, through an interaction with gonadal hormones.

Keywords: Reproductive behavior, Aggressive behavior, vasopressin, arginine vasotocin, Manning compound, Mozambique tilapia.

1. Introduction

Both gonadal steroids and neuropeptides have been implicated in the regulation of a wide range of social behaviors (revised recently in Gonçalves et al., 2017 for teleost fish). The canonical explanation for this multiplicity of regulators of social behaviors have relied on the existence of a shared brain network for different social behaviors (aka social behavior network, Goodson, 2005; Newman, 1999; O'Connell and Hofmann, 2011) whose constitutive nodes (i.e. individual brain regions) express receptors for steroid hormones and neuropeptides (e.g., estrogen: plainfin midshipman, *Porichthys notatus*, Forlano et al., 2005; Atlantic croaker, *Micropogonias undulatus*, Hawkins et al., 2005; zebrafish, *Danio rerio*, Menuet et al., 2002; Burton's mouthbrooder, *Astatotilapia burtoni*, Munchrath & Hofmann, 2010; european seabass, *Dicentrarchus labrax*, Muriach et al., 2008; androgen: *P. notatus*, Forlano et al., 2010; goldfish, *Carassius auratus*, Gelinas & Callard, 1997; *A. burtoni*, Harbott et al., 2007; Munchrath & Hofmann, 2010, arginine vasotocin: *A. burtoni*, Huffman et al., 2012; Loveland & Fernald, 2017; rock hind, *Epinephelus adscensionis*, Kline et al., 2011; and isotocin: *A. burtoni*, Huffman et al., 2012), hence allowing the state of this network to be modulated by them. However, a less explored alternative is that some of the effects of these modulators of social behavior can result from a direct interaction between these systems.

Regarding the specific effect of AVT on social behavior, several investigations manipulating the AVT system in teleosts have obtained contrasting results (Godwin and Thompson, 2012). In the case of reproductive behavior, intraperitoneal (ip) injections of AVT induce male electric signals used as sexual displays in the weekly electric fish, *Apteronotus leptorhynchus* (Bastian et al., 2001), and in the bluehead wrasse, *Thalassoma bifasciatum*, AVT injections increase courting in territorial and non-territorial males (Semsar et al., 2001). However, in the white perch, *Morone americana*, ip injections of AVT had no effect on behavior but intracerebroventricular (icv) administration increased courtship behavior (Salek et al., 2002). Several AVT receptors have been described in teleost fish, namely, *V1Aa*, *V1Ab*, *V2A1* and *V2A2* while *V2C* was only found in 3 teleost species (Lagman et al., 2013), but *V1A* receptors are the most distributed receptors in the brain of vertebrates (reviewed in Albers, 2015). In males of the damselfish *Stegastes leucosticus*, intramuscular treatment of AVT increased aggression while the potent AVT receptor *V1A* antagonist, the Manning compound (Manning et al., 2012), decreased it, in comparison with saline-treated males (Santangelo and Bass, 2006). In the rainbow trout, *Onchorhynchus mykiss*, an high dosages of AVT, icv administered, induced dominant males to descend in status while the Manning compound, had

no effect on the outcome of staged fights (Backström and Winberg, 2009). In *A. burtoni*, ip injection of AVT caused a stress response in animals, originating a decrease of aggression and the loss of status of dominant males, whereas the Manning compound had no effect compared with saline controls (Huffman et al., 2015). Moreover, in other studies, AVT injections seem to reduce aggression in dominant males (*D. rerio*, Filby et al., 2010) but increase aggression (*T. bifasciatum*, Semsar et al., 2001) or have no effect (Huffman et al., 2015) in subordinates.

Similarly, the effect of androgens on reproductive and aggressive behaviors is not straightforward. For instance, castration impairs courtship, spawning pit digging and nuptial coloration in some species (e.g. Egyptian mouthbrooder, *Pseudocrenilabrus multicolor*, Reinboth & Rixner, 1970; blackchin tilapia, *Sarotherodon melanotheron*, Levy & Aronson, 1955; *A. burtoni*, Francis, Jacobson, Wingfield, & Fernald, 1992; *c* Almeida, Canário, & Oliveira, 2014) but not in others (jewelfish, *Hemichromis bimaculatus*, Noble & Kumpf, 1936; platinum acara, *Andinoacara latifrons*, Aronson, Scharf, & Silverman, 1960, *S. melanotheron and Oreochromis upembae*, Heinrich, 1967). While in the case of aggressive behavior, the exogenous administration of androgens increases aggression (*A. burtoni*, Fernald, 1976; sheepshead minnow, *Cyprinodon variegatus*, Higby et al., 1991), however androgen receptor antagonists or castration can either inhibit (*Amatitlania nigrofasciata*, Sessa et al. 2013*; A. burtoni*, Francis et al., 1992) or have no effect in aggression (*O. mossambicus*, Almeida et al., 2014; *A. nigrofasciata*, van Breukelen, 2013).

On the other hand, and even though traditionally AVT and androgens have been studied separately in the context of social behavior, some studies account for a crosstalk between these systems. In mammals, it has been shown that androgens modulate the vasopressin neural system, the mammalian homologue of AVT (reviewed in Albers, 2012). For example, castrated male rats present less vasopressin cell bodies and fiber density in several brain areas than control males; a difference which is restored with androgen replacement treatments (DeVries et al., 1985). Moreover, vasopressin seems to regulate gonadal steroidogenesis since *in vitro* studies in rodents report the existence of vasopressin receptors, including *V1A* type, in the testis and that vasopressin influences the production of androgens by Leydig cells (Bathgate and Sernia, 1994; Meidan and Hsueh, 1985; Tahri-Joutei and Pointis, 1989). In teleosts, AVT receptors have also been found in testis (Lema, 2010; Lema et al., 2012) while a study in the Central American cichlid, *Cichlasoma dimerus*, found that AVT stimulated the production of androgens on testis incubation cultures (Ramallo et al., 2012).

In the present study, we aim to understand the interaction of AVT and gonadal hormones on the regulation of the reproductive behavior of a polygynous species, *Oreochromis*

mossambicus. For this purpose, we treated castrated and sham-operated territorial males with different dosages of AVT and a *V1A* receptor antagonist (Manning compound), using a within-subject design, and subsequently analyzed their behavior towards females and males.

2. Materials and methods

2.1. Animals and housing

The Mozambique tilapia is a freshwater fish with a lek-breeding system (Fryer and Iles, 1972). Males aggregate densely in mating territories, where they dig and defend spawning pits and compete for females (Oliveira and Almada, 1998). Males present two distinct phenotypes, which can rapidly reverse due to changes in the social environment (Oliveira and Almada, 1998). Dominant males are usually larger, dark colored, establish territories and court females. These males aggressively defend their territories, while, in contrast, subordinate males have a silver color pattern similar to that of females and fail to establish territories.

Fish used in this study came from a stock held at ISPA. Fish were maintained in stable social groups of 4 males and 5 females per group, in glass tanks (120 x 40 x 50 cm, 240 L) with a fine gravel substrate. Tanks were supplied with a double filtering system (sand and external biofilter; Eheim) and constant aeration. Water quality was monitored on a weekly basis for nitrite (0.2-0.5 ppm), ammonia (<0.5 ppm; Pallintest kit®) and pH (6.0-6.2). Fish were kept at a temperature of 26 ± 2 ºC, a 12L:12D photoperiod and fed with commercial cichlid sticks. The social status of the males was monitored daily. Dominance status of the males was assessed based on the dark body coloration and the possession of a spawning pit on the substrate (Oliveira and Almada, 1996).

2.2. Experimental procedure

Twenty-two territorial males (mean body mass ± SEM: 31.92 g ± 2.25 g; mean standard length ± SEM: 10.20 cm ± 0.27 cm) were isolated in test tanks (47 cm × 24 cm × 30 cm). On one side of the test tank, it was placed an adjacent demonstration tank (70 cm × 37 cm × 30 cm; demo tank 1) containing 4 females, while on the opposite side of the test tank there was another demonstration tank (18 cm × 30 cm × 15 cm; demo tank 2) with an opaque partition between them. Focal fish had visual access to the females of demo tank 1. Two days after isolation (day 2), focal males were submitted to surgery, either a sham operation (SHAM group, n = 11) or

castration (CAST group, n = 11) and returned to the test tank. On day 5, a demonstrator male, of similar sized of the focal male, was placed in demo tank 2. On day 6, focal males received an intraperitoneal injection (ip) with one of the following compounds: vehicle solution, AVT acetate salt (4 different dosages: 0.125, 0.25, 0.5, or 1 μg/g; Sigma V0130) or the specific AVT receptor *V1A* antagonist, Manning compound, ([β-Mercapto-β,β-cyclopentamethylenepropionyl[1], O-Me-Tyr[2], Arg [8]]-Vasopressin (Kruszynski et al., 1980); Sigma V2255). Chemicals were dissolved in saline vehicle solution (0.9% sodium chloride). After the injection, the behavior of the focal fish towards the females of the demo tank 1 was observed for 15 min. Then, an opaque partition was placed between the focal fish and the female's demo tank to avoid visual contact between them and the opaque partition separating the focal male tank and the demo tank 2 was lifted, and the focal fish was given visual access to the male in the demo tank 2 during 15 min, and the behavior of the focal fish was noted. Then, every 2 days, the focal fish were ip injected again with another of the treatments described above and the experimental procedure repeat so that their behavior towards females and the same demonstrator male was noted for each treatment. The experiment was run until all fish were subjected to all treatments and behavioral sampling obtained. The order of exposure of each focal fish to the different treatments was randomized.

2.3. Behavioral observations

Behavior of the focal male, either towards the females or interacting with the demonstrator male, was analysed in real-time using a computerized multi-event recorder software (Observer, Noldus technology, version 5). The analysis was based on the ethogram repertoire provided by Baerends and Baerends-van Roon (1950). Relevant behavioral patterns were quantified during female (i.e. touching the transparent partition, courtship, digging a spawning pit, bites at the transparent partition) and male (i.e. bites at the transparent partition, displays, attacks) interactions. Since only one focal male courted females when injected with the saline treatment and only two males courted females when injected with Manning compound, this variable was excluded from further analyses.

2.4. Data analysis

Behavioral variables were logarithmically transformed [log10 (x+1)] to meet parametric assumptions. However, two variables, the frequency of bites towards females and the frequency

of digging did not follow the assumptions of normality. Outlier observations were identified and replaced by missing values using a generalized extreme studentized deviate procedure (Jain, 2010) with a p-value of .05 and a maximum number of outliers set at 20% of the sample size. For non-parametric variables, the latter test is not possible to apply. Thus, in these cases, extreme values were identified using the SPSS software (SPSS identify values more than 3 box lengths/interquartile range from either hinge) and removed from further analyses.

Behavioral variables were analyzed using Linear Mixed Models (LMM) with castration (sham-operated or castrated) and AVT treatment (saline, AVT 0.125 μg/g, AVT 0.25 μg/g, AVT 0.5 μg/g, AVT 1 μg/g, Manning) as fixed effects and focal fish as a random effect. Homoscedasticity was confirmed with Levene's test. Plots of residuals, fitted values and estimated random effects were used to confirm assumptions of LMM. Planned comparisons were used to test for specific differences between saline and the other treatments and between SHAM and CAST fish within each treatment. P-values were adjusted for multiple testing using the Benjamini & Hochberg (1995) procedure.

Regarding the frequency of bites towards females and the frequency of digging, even though the lack of normality and homoscedasticity of these variables we still used a LMM analysis due to the lack of an equivalent nonparametric test.

Effect sizes were computed for planned comparisons (Cohen's d). Statistical analysis was performed using IBM SPSS® statistics v.21, and R (R Core Team, 2015) with the following packages: nlme (LMM), multcomp (planned comparisons). Degrees of freedom may vary between the analyses due to missing values.

2.5. Ethics Statement

Animal experimentation procedures were conducted in accordance with the European Communities Council Directive of 24 November 1986(86/609/EEC) and were approved by the Portuguese Veterinary Authority (Direcção Geral de Alimentação e Veterinária, Portugal; permit # 0421/000/000/2013).

3. Results

Behavior towards females

The time spend by the focal fish interacting with females changed significantly with AVT treatment ($F_{(5,89)}$ = 19.464, p < 0.001) but did not differ significantly between sham and castrated males ($F_{(1,20)}$ = 0.025, p = 0.875). The interaction between AVT treatment and castration was also not significant ($F_{(5,89)}$ = 1.621, p =0.163). After AVT injection, independently of dosage and castration, males significantly decreased the time spent interacting with females in comparison with the saline injected treatment (Table 1, Figure 1A). Castrated fish injected with Manning decreased the time of interaction with females compared with saline-injected castrated fish (Table 1, Figure 1A).

The frequency of bites towards females decreased significantly with AVT treatment ($F_{(5,90)}$ = 16.253, p <.001) and with castration ($F_{(1,20)}$ = 8.609, p = .008). The interaction between AVT treatment and castration was also significant ($F_{(5,90)}$ = 3.386, p = .008). Baseline (i.e. saline injected fish) frequency of bites towards females was higher in sham-operated than in castrated males. Sham-operated males injected with AVT significantly decreased their bites towards females in comparison with the saline injected treatment and for all dosages (Table 1, Figure 1B). Castrated fish showed no differences in the frequency of bites between the saline and AVT injection treatments (Table 1, Figure 1B). After Manning injection, sham-operated fish significantly increased the frequency of bites in comparison with the saline treatment (Table 1, Figure 1B), and there was a significant difference between the sham-operated and castrated fish in the Manning treatment (Table 1, Figure 1B).

The frequency of spawning pit digging in the presence of females changed significantly with AVT treatment ($F_{(5,91)}$ = 7.440, p < .001) but there was no effect of castration ($F_{(1,20)}$ = 3.727, p = .068). The interaction between AVT treatment and castration was not significant ($F_{(5,91)}$ = 1.629, p = .160). In saline injected males spawning pit digging wass significantly higher in castrated males than in sham-operated (Table 1, Figure 1B). After AVT injection, castrated males significantly decreased digging frequency in comparison with saline injected males, for all dosages, except the AVT 0.5 μg/g dosage (Table 1, Figure 1C). In Sham-operated males there were no differences between the saline and AVT injected treatments (Table 1, Figure 1C). After Manning injection, sham-operated males significantly increased digging in comparison with the saline treatment (Table 1, Figure 1C).

Table 1. Effect of castration and chemical treatment on the behavior of the focal male towards females: effect sizes and planned comparisons.

Planned Comparisons	SHAM			CAST			SHAM vs CAST		
	z	**p**	**d**	**z**	**p**	**d**	**z**	**p**	**d**
TIME SPENT IN INTERACTION									
AVT 0.125 µg/g vs Saline	**-4.635**	**<.001**	**1.231**	**-5.772**	**<.001**	**1.879**			
AVT 0.25 µg/g vs Saline	**-4.699**	**<.001**	**1.463**	**-5.193**	**<.001**	**1.920**			
AVT 0.5 µg/g vs Saline	**-3.708**	**<.001**	**.970**	**-4.021**	**<.001**	**1.186**			
AVT 1 µg/g vs Saline	**-6.478**	**<.001**	**3.341**	**-4.907**	**<.001**	**1.887**			
Manning vs Saline	-.160	.931	.066	**-2.275**	**.041**	**1.405**			
Saline							.495	.828	.496
AVT 0.125 µg/g							-.335	.908	.115
AVT 0.25 µg/g							.087	.931	.036
AVT 0.5 µg/g							.225	.931	.075
AVT 1 µg/g							1.632	.164	1.225
Manning							-.960	.490	1.022
BITES									
AVT 0.125 µg/g vs Saline	**-3.924**	**<.001**	**1.468**	-1.615	.170	nd			
AVT 0.25 µg/g vs Saline	**-3.368**	**.002**	**.966**	-1.615	.170	nd			
AVT 0.5 µg/g vs Saline	**-2.302**	**.049**	**.508**	-1.172	.297	.701			
AVT 1 µg/g vs Saline	**-4.089**	**<.001**	**1.391**	-1.615	.170	nd			
Manning vs Saline	**3.960**	**<.001**	**1.052**	1.225	.294	.541			
Saline							**-2.411**	**.042**	**.877**
AVT 0.125 µg/g							-.337	.736	nd
AVT 0.25 µg/g							-1.056	.332	nd
AVT 0.5 µg/g							-1.497	.195	.510
AVT 1 µg/g							-.417	.722	nd
Manning							**-4.604**	**<.001**	**1.555**
DIGGING SPAWNING PIT									
AVT 0.125 µg/g vs Saline	-.058	1	.064	**-3.342**	**.007**	**nd**			
AVT 0.25 µg/g vs Saline	.203	1	.145	**-3.066**	**.012**	**.997**			
AVT 0.5 µg/g vs Saline	-.058	1	.064	-1.607	.288	.395			
AVT 1 µg/g vs Saline	-.319	1	nd	**-3.342**	**.007**	**nd**			
Manning vs Saline	**2.618**	**.028**	**1.215**	1.353	.352	.345			
Saline							**2.851**	**.017**	**.972**
AVT 0.125 µg/g							-.275	1	nd
AVT 0.25 µg/g							-.275	1	.191
AVT 0.5 µg/g							1.460	.330	.516
AVT 1 µg/g							0	1	nd
Manning							1.282	.355	.385

Groups: SHAM, sham fish; CAST, castrated fish; z: z-test estimate; d: effect size estimate (Cohen's d); p: p-value after multiple comparison adjustment; statistically significant values are in bold.

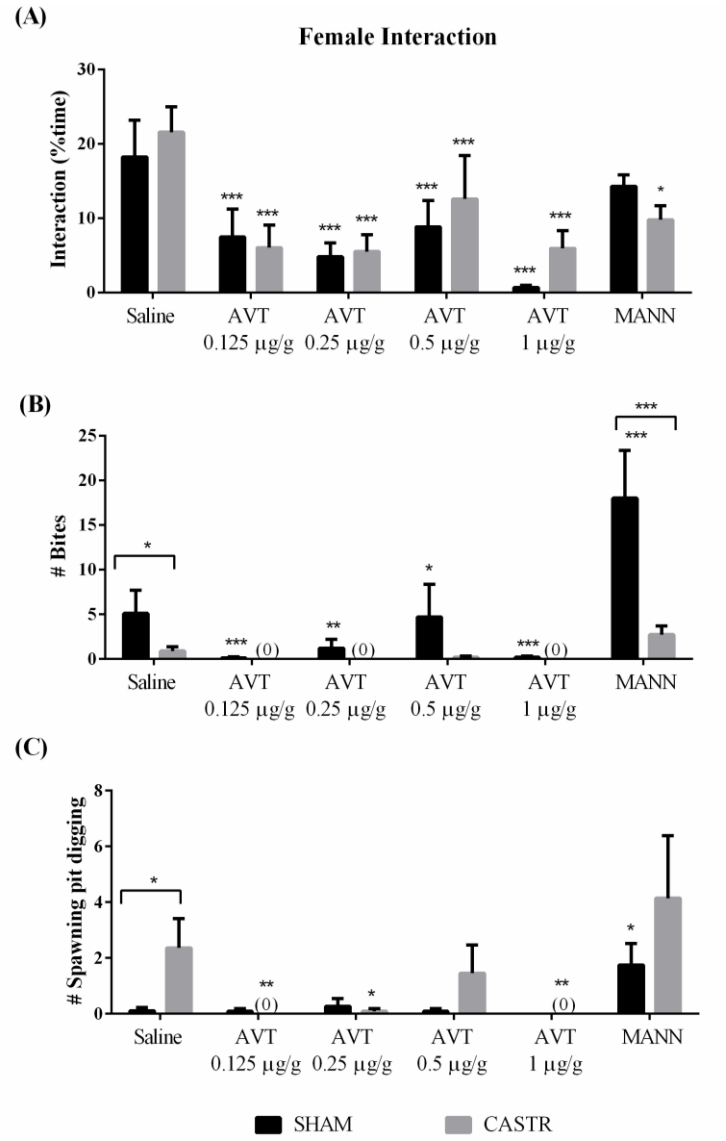

Figure 1. Behavioral measurements of the focal fish during females' interaction after each experimental treatment. (A) time spent interacting with females; (B) number of bites; (C) number of times building the spawning pit. Groups: SHAM, sham fish; CAST, castrated fish. MANN: Manning compound. *significant difference for $p < 0.05$; **significant difference for $p < 0.01$; ***Significant difference for $p < 0.001$; Results are expressed as mean ± standard error of the mean (SEM).

Behavior towards an intruder male

There were no effects of either AVT treatment ($F_{(5,94)} = 1.947$, $p = 0.094$) or castration ($F_{(1,20)} = 1.656$, $p = 0.213$) in the frequency of bites towards the intruder male (Figure 2A). The interaction between AVT treatment and castration was also not significant ($F_{(5,94)} = 0.421$, $p = 0.833$).

There was a significant effect of the AVT treatment ($F_{(5,90)} = 3.013$, $p = 0.015$) but not of castration ($F_{(1,20)} = 0.725$, $p = 0.405$) in the frequency of displays towards the intruder male (Figure 2B). The interaction between AVT treatment and castration was also not significant ($F_{(5,90)} = 0.486$, $p = 0.786$). Visual inspection of Figure 2B suggests the occurrence of an effect for fish injected with AVT (dose 1 μg/g). However, after correcting p-values for multiple comparisons, no significant differences between treatments were observed, despite the high effect sizes (Table 2).

There was a significant effect of AVT treatment ($F_{(5,93)} = 3.526$, $p = 0.006$), but not of castration ($F_{(1,20)} = 1.191$, $p = 0.288$), in the time the focal fish spent displaying towards the intruder male (Figure 2C). The interaction between AVT treatment and castration was not significant ($F_{(5,93)} = 0.645$, $p = 0.666$). Again, after correcting p-values for multiple comparisons, there were no significant differences between treatments despite high effect sizes (Table 2).

Table 2. Effect of castration and chemical treatment on the behavior of the focal male towards the demonstrator male: effect sizes and planned comparisons.

Planned Comparisons	SHAM			CAST			SHAM vs CAST		
	z	p	d	z	p	d	z	p	d
BITES									
AVT 0.125 μg/g vs Saline	.708	.802	.155	.176	.918	.049			
AVT 0.25 μg/g vs Saline	-.182	.918	.041	.673	.802	.173			
AVT 0.5 μg/g vs Saline	.509	.818	.115	.305	.918	.079			
AVT 1 μg/g vs Saline	.101	.920	.022	-1.017	.645	.294			
Manning vs Saline	1.805	.574	.255	1.609	.574	.538			
Saline							-.989	.645	.424
AVT 0.125 μg/g							-1.291	.645	.550
AVT 0.25 μg/g							-.505	.818	.217
AVT 0.5 μg/g							-1.105	.645	.465
AVT 1 μg/g							-1.623	.574	.714
Manning							-1.040	.645	.249
DISPLAYS									
AVT 0.125 μg/g vs Saline	.574	.937	.123	-.066	.947	.017			
AVT 0.25 μg/g vs Saline	-.933	.936	.214	.098	.947	.023			
AVT 0.5 μg/g vs Saline	-.206	.947	.045	.381	.937	.086			
AVT 1 μg/g vs Saline	-1.973	.575	1.035	-1.800	.575	.729			
Manning vs Saline	1.218	.850	.120	.383	.937	.194			
Saline							-.761	.937	.312
AVT 0.125 μg/g							-1.113	.850	.491
AVT 0.25 μg/g							-.195	.947	.086
AVT 0.5 μg/g							-.438	.937	.176
AVT 1 μg/g							-.556	.937	.029
Manning							-1.192	.850	.223

TIME SPENT IN DISPLAYS									
AVT 0.125 µg/g vs Saline	1.212	.588	.241	.039	.969	.010			
AVT 0.25 µg/g vs Saline	.452	.802	.101	.746	.608	.179			
AVT 0.5 µg/g vs Saline	-.272	.838	.055	.931	.588	.205			
AVT 1 µg/g vs Saline	-1.382	.588	.273	-1.942	.588	.611			
Manning vs Saline	1.135	.588	.163	.901	.588	.439			
Saline							-.912	.588	.378
AVT 0.125 µg/g							-1.535	.588	.664
AVT 0.25 µg/g							-.756	.608	.348
AVT 0.5 µg/g							-.274	.838	.105
AVT 1 µg/g							-1.209	.588	.586
Manning							-.952	.588	.144

Groups: SHAM, sham fish; CAST, castrated fish; z: z-test estimate; d: effect size estimate (Cohen's d); p: p-value after multiple comparison adjustment; statistically significant values are in bold.

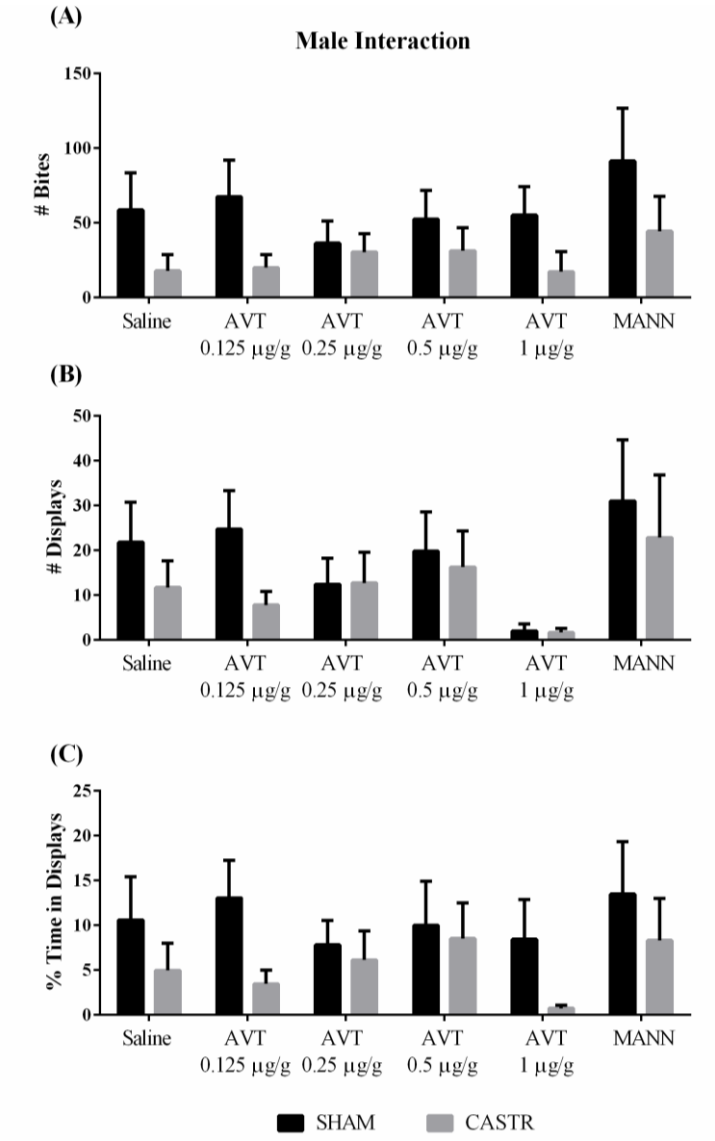

Figure 2. Behavioral measurements of the focal fish during male interaction after each experimental treatment (A) number of bites; (B) number of displays; (C) time spent in displays.

Groups: SHAM, sham fish; CAST, castrated fish. MANN: Manning compound. Results are expressed as mean ± standard error of the mean (SEM).

4. Discussion

In this paper we have investigated the putative effects of gonadal hormones, through castration, AVT, and the interaction between gonadal hormones and AVT on the reproductive behaviour of the cichlid fish *O. mossambicus*.

Castration had no effect on the aggressive behaviour of the focal male towards the intruder male. On the other hand, castration affected the behaviour of breeding males towards females, reducing aggression towards females and increasing the digging behaviour involved in the construction of a spawning pit. Thus, our findings agree to a large extent with a previous study in the Mozambique tilapia, which has shown that gonadectomy impairs the expression of reproductive behaviour, which can be rescued by androgen administration to castrated males, but has no effect on aggressiveness (Almeida et al., 2014). The present study confirms that gonadal hormones (putatively androgens, given the results of Almeida et al., 2014) are pivotal to the expression of reproductive behavior in this species and suggests the existence of independent neural circuits regulating aggressive behaviour directed towards females vs. males (Almeida et al., 2014).

The present study also showed that pharmacological AVT manipulations affected the behavior of focal males towards females but not towards males. Besides gonadectomy, treatment with AVT of gonad-intact males (i.e. sham-operated) also reduced their aggressiveness towards females. Given that all AVT-injected males (i.e. either castrated or sham-operated) interacted less with females, the observed reduction in aggressiveness could be interpreted as a consequence of a reduced interest in females in these males. However, there is a specific effect of the treatment with Manning compound in the frequency of bites in sham-operated but not in castrated males treated that goes in the opposite direction (i.e. an increase in frequency of bites towards females). This specific results, suggests that the observed AVT effect on the reduction of aggressiveness towards females is mediated by V1a receptors located in the gonads, that are involved in the regulation of gonadal hormones (putatively androgens) production or release. This hypothesis, is also supported by the concurrent effects of castration and AVT treatment on the reduction of aggressiveness towards females in this study. In addition, the *V1A* receptor has been detected in fish testis (Lema, 2010; Lema et al., 2012) and a study in the rainbow trout, *O. mykiss*, reported that AVT induced the production of androgens

in immature cultured testes but not in mature testes (Rodríguez and Specker, 1991). However, in the Central American cichlid, *C. dimerus*, AVT stimulates the production of gonadotropins on pituitary extracts *in vitro* and androgens on testis culture of dominant fish (Ramallo et al., 2012). It was also detected AVT mRNA and peptide in the testis thus showing the presence and influence of AVT in the HPG axis at a peripheral level.

In this study we also report an increase of spawning pit building behavior in castrated males compared to sham-operated males after saline injection, and no courtship behavior in sham-operated fish in the saline treatment. A possible explanation for these results may be the stress that fish were subjected to due to handling and injection. Yet, castrated fish decreased the frequency of digging their spawning pit when treated with AVT, while sham-operated fish increased their digging when injected with the Manning compound, implying the activity of *V1A* receptors in the regulation of this behaviour. Also, while in Almeida et al. (2014) there was no difference in the aggressiveness towards females or males between sham and castrated fish, herein we report a significant decrease of aggressiveness towards females as a result of gonadectomy in the saline treatment.

In teleosts, AVT is mainly expressed in neurons located in the POA in the anterior hypothalamus, that project to the neurohypophysis, where it is released to the bloodstream to act peripherally (reviewed in Godwin and Thompson, 2012). These neurons also project to the ventral telencephalon, ventral thalamus and mesencephalon (Huffman et al., 2012; Saito et al., 2004). There are different populations (parvo-, magno-, and giganto- cellular) of AVT neurons that have been proposed to have different modulatory roles in social behavior (Greenwood et al., 2008). In the Mozambique tilapia, subordinates have magno- and giganto-cellular AVT neurons with larger cell body area than dominant males (Almeida and Oliveira, 2015), and there is less AVT in the pituitary of dominant individuals than in subordinates (Almeida et al., 2012), suggesting its involvement in social stress and subordinate status. It is known that AVT influences the stress axis by inducing secretion of adrenocorticotropic hormone (Baker et al., 1996). Thus, in this species, treating dominant males with AVT may elicit a stressful condition, yet it only influences male-female behavior. Goodson (2008) already suggested that social stimuli with distinct valence would evoke contrasting vasopressin neuronal responses ("positive" vs. "negative" conspecifics elicit affiliative vs. aggressive/aversive interactions). However, vasopressin neurons involved in these contrasting effects in mammals are located in the bed nucleus of the stria terminalis, which teleosts seem to lack (Godwin and Thompson, 2012). Alternatively, the absence of effect on aggressive behavior in male-male interactions supports the existence of a complex regulatory mechanism dependent on the concerted action

of different subsystems composed of distinct AVT populations (Greenwood et al., 2008), probably because the peripheral administration of AVT fails to stimulate these contrasting circuits in an independent manner. For instance, in the midshipman fish *P. notatus*, a well-studied fish model in the scope of vocal communication (see Bass, 2008; Forlano et al., 2015; for comprehensive reviews), territorial males defend nests and attract females by using acoustic signals, agonistic ('grunts') and courtship sounds (long 'hums'), respectively. Interestingly, the AVT delivery either in the forebrain or in the midbrain modulates different vocal circuits as shown by inducing distinct effects. AVT treatment on the preoptic area–anterior hypothalamus decreases burst duration, whereas, at the midbrain level (specifically in the paralemniscal midbrain tegmentum), AVT hampers call initiation by decreasing the number of vocal bursts and increasing response latency (Goodson and Bass, 2000a, 2000b).

Finally, AVT neurons can also be modulated by gonadal steroids. Castration of adult male rats leads to a reduction of the number of vasopressin cell bodies and fiber density in the bed nucleus of the stria terminalis (DeVries et al., 1985). As mentioned above, in teleosts, for long it has considered that there was no AVT expression in the teleost homologue of the mammalian extended amygdala and septal areas (e.g. Godwin & Thompson, 2012). However, a recent study in *A. burtoni* has found AVT preprohormone expression in these regions (Rodriguez-Santiago et al., 2017), suggesting that future studies on this subject will need to be undertaken. On the other hand, castration of Syrian hamsters reduces dramatically the expression of V1A receptors and ligand binding in the preoptic nucleus showing that androgens modulate sensitivity to vasopressin by affecting the number of *V1A* receptors (Young et al., 2000). Our study suggests that androgens favor aggressiveness towards females while AVT has an inhibitory action on this behavior via *V1A* receptors. Thus, the presence of androgens and the blocking of *V1A* receptors in the brain have a concerted action on a central neural control mechanism eliciting a substantial increase of aggressive behavior in the presence of females, which is not possible in castrated animals.

In sum, contrary to the literature, in the Mozambique tilapia, AVT did not increase courting or affect aggressive behavior towards males but inhibited interaction and aggressiveness towards females, confirming that the action of this nanopeptide in behavior is species-specific. Moreover, we highlight the need to target specific populations of AVT neurons, in order to clarify the role of AVT in the modulation of social behavior through different putative regulatory circuits and also due to the structural similarity between vasopressin and oxytocin and their receptors (Albers, 2015; Donaldson and Young, 2008) which may lead to relevant crosstalk (reviewed in Kelly and Goodson, 2014; Stoop, 2012).

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

OA and RO designed the experiments. OA performed behavioral experiments. AF analyzed the data. AF and RO wrote the paper.

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CHAPTER 4

Rising to the challenge? Inter-individual variation of the androgen response to social interactions in cichlid fish

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Abstract

The Challenge Hypothesis (Wingfield et al. Am. Nat. 136, 829-846) aims to explain the complex relationship between androgens and social interactions. Despite its well acceptance in the behavioral endocrinology literature, several studies have failed to found an androgen response to staged social interactions. Possible reasons for these inconsistencies are the use of single sampling points that may miss the response peak, and the occurrence of inter-individual variability in the androgen response to social interactions. In this study we addressed this two possible confounding factors by characterizing the temporal pattern of the androgen response to social interactions in the African cichlid, *Oreochromis mossambicus* and relating it to inter-individual variation in terms of the individual scope for androgen response (i.e. the difference between baseline and maximum physiological levels for each fish) and behavioral types. We found that the androgen response to territorial intrusions varies between individuals and is related to their scope for response. Individuals that have a lower scope for androgen response did not increase androgens after a territorial intrusion but were more aggressive and exploratory. In contrast males with a higher scope for response had fewer aggressive and exploratory behaviors and exhibited two peaks of KT, an early response 2 -15 min after the interaction and a late response at 60-90 min post-interaction. Given that the pharmacological challenge of the Hypothalamic-Pituitary-Gonad axis only elicits the late response, we suggest that these two peaks may be regulated by different physiological mechanisms, with the early response being mediated by direct brain-gonad neural pathways. In summary, we suggest that determining the temporal pattern of the androgen response to social interactions and considering inter-individual variation may be the key to understanding the contradictory results of the Challenge Hypothesis.

Highlights

- The time course of the androgen response to social interaction varies between individuals and is related to the scope for response of each fish.
- Individuals with a lower scope for androgen response did not increase androgens after a territorial intrusion.
- Individuals with a higher scope for androgen response exhibit two waves of KT response.
- Responders and non-responders consistently differ in their fewer aggressive and exploratory behaviors.

- The two androgen peaks present in responders seem to be regulated by different physiological mechanisms.

Keywords
Mozambique tilapia, Challenge Hypothesis, androgens, temporal pattern, inter-individual variation, personality

1. Introduction

Almost 30 years ago, Wingfield and colleagues (1990) introduced the Challenge Hypothesis as a framework to study the androgen response to social interactions. Since then, their essay has been a landmark for behavioral endocrinologists that aim to understand the complex relationship between androgens and the social environment. According to this model, constitutive androgen circulating levels (constitutive baseline, *a*) occur during the non-breeding phase, while at the onset of the breeding season they increase up to the concentration needed for the full development of the gonads, the development of secondary sex characteristics and for the expression of reproductive behaviors (breeding baseline, *b*) (Wingfield et al., 1990). Then, androgens can further rise above the breeding baseline and reach a physiological maximum (*c*) in response to social interactions, either with males or with sexually receptive females (Wingfield et al., 1990). Thus, the social interactions of an individual will determine its androgen levels. Moreover, the Challenge Hypothesis generates a number of predictions regarding the seasonal patterns of androgen social responsiveness [quantified by the ratio (*c*-*a*)/(*b*-*a*)] in seasonal breeders according to the mating system and parental care type of the species. For example, since androgens interfere with paternal care, males from species that provide parental care should have low androgen levels during parental phase that rise in response to male or female interactions (high androgen responsiveness), whereas species in which males invest less in parental care are expected to have higher androgen levels but lower androgen responsiveness to social interactions (Wingfield et al., 1990).

Although the Challenge Hypothesis was initially proposed based on comparative data from bird species, it has been extensively tested across all vertebrate taxa, including teleost fish (Hirschenhauser & Oliveira, 2006; Hirschenhauser, Taborsky, Oliveira, Canário, & Oliveira, 2004; Oliveira 2004). Overall, the predictions regarding the seasonal variations in androgen levels have been confirmed (Goymann et al., 2007), but many of the published studies, even in

birds, failed to observe the predicted androgen response to simulated social challenges (e.g., rodents: Fuxjager et al., 2010; dwarf mongooses: Creel et al., 1993; amphibians: de Assis et al., 2012; fish: Ros et al., 2014; reptiles: Baird et al., 2014; birds: Moore et al., 2004). Recently, it has been argued that one possible explanation for these contradictions is the fact that most studies so far have focused on androgen responses to male-male interactions (i.e. territorial intrusions or staged fights), and that male-female interactions would contribute more to the observed seasonal patterns (Goyman et al., 2019). However, there are other possible explanations for the failure in detecting the androgen response to social interactions, namely using inappropriate sampling points that miss the peak of response due to lack of knowledge on the time courses of the response for each studied species and the occurrence of inter-individual variability in androgen response associated to behavioral variation (e.g. personality types).

Indeed, the temporal dynamics of the androgen response to social interactions has only been studied in few species and there is significant variation in the observed patterns. For instance, the response of testosterone in males to the presence of a receptive females peaks between 30 min and 60 min after exposure in male Sprague-Dawley rats (Kamel and Frankel, 1978), whereas in male mice (CBA strain) it peaks at 20 min in Winter and at 40 min in Summer (Amstislavskaya and Popova, 2004). Similarly, aggressive encounters elicit a testosterone peak in winners 45 min after a fight in California mice (*Peromyscus californicus*) (Marler et al., 2005) and at 60 min post-fight in swordtail fish (*Xiphophorus helleri*) (Hannes et al., 1984). Thus, without knowing the temporal dynamics of the androgen response to social interactions for their studied species and using reported sampling points for other species, some studies with negative results may have simply missed the androgen peak they were aiming to characterize, highlighting the need for the characterization of the time course of the response for each species.

On the other hand, few studies account for inter-individual variation in hormonal responses. Usually, comparisons of androgen responsiveness are made between species averaging all sampled individuals (e.g., Goymann, 2009; Hirschenhauser et al., 2004, 2003; Oliveira et al., 2002; Wingfield et al., 1990), ignoring the possible occurrence of alternative phenotypes that may cancel each other in the sample. By 1987, (Bennett, 1987) already emphasized the need to focus on biological differences among individuals and to shift our attention from the ‘tyranny of the Golden Mean’, particularly in physiological studies. Indeed, although the function and mechanisms that underpin individual variation are not fully understood, several authors have highlighted the importance of this issue in the context of

endocrinology (Hau and Goymann, 2015; Kempenaers et al., 2008; Williams, 2008). Actually, for the same population, variation in hormone levels among individuals is quite impressive (e.g. up to two orders of magnitude, (Kempenaers et al., 2008; see also Williams, 2008) and may be related to fitness (Hau and Goymann, 2015). Such differences between individuals in terms of baseline and/or maximum levels could influence the scope for androgen responsiveness leading to inter-individual differences within the same species.

Moreover, inter-individual variation of the androgen response can be related to intrinsic psychological features, such as observed in the stress response (Koolhas et al., 1999). In recent years, a considerable amount of literature has been published on inter-individual variation in behavior profiles. Contrasting patterns are observed whenever individuals behave and interact with their environment. Several definitions with somewhat similar meanings have been proposed for consistent differences between individuals. The term 'temperament' (or personality) is generally understood as the consistency of behavioral differences between individuals over time and across situations (Caramachi et al., 2013; Réale et al., 2007). Distinct behavioral traits (or axes) have been proposed: shyness-boldness (in risky situations, e.g. predator), exploration-avoidance (in new situations), activity (in non-risky and non-novel situations), aggressiveness (towards conspecifics) and sociability (Réale et al., 2007; Sih et al., 2004). It is worth noting that, in a population, the distribution of individuals along these axes is expected to follow a continuum, rather than a bimodal distribution (Réale et al., 2007). If a set of behavioral traits correlate between each other, one may define it as a 'behavioral syndrome' (Bell, 2007; Sih et al., 2004), which could mean that the traits are regulated by a common neuroendocrine, genetic or neurobiological mechanism (Coppens et al., 2010; Sih et al., 2004). For instance, the best known behavioral syndrome is the proactive-reactive syndrome, studied in the context of stress research to distinguish animals with opposing stress-coping styles (see, for example, Coppens et al., 2010; Koolhaas et al., 1999; Øverli et al., 2007; Young et al., 2011). Proactive individuals are simultaneously bolder, more aggressive and active in response to challenges, show higher exploration rates and, in general, a low Hypothalamus-Pituitary-Adrenals (HPA) axis activity and high sympathetic reactivity. In contrast, reactive individuals seem consistently shy, less aggressive and active, usually freeze in stressful situations and have higher HPA axis and lower sympathetic responses (Koolhaas et al., 1999).

The aim of our study is to characterize the temporal pattern of the androgen response to social (male-male) interactions, taking into account the scope for response of each individual and to relate it to inter-individual variation in behavior profiles. For this purpose, we studied

Mozambique tilapia, *Oreochromis mossambicus*, a freshwater fish with a lek-mating system where breeding males, which do not show parental care (Fryer and Iles, 1972), aggregate densely in mating territories, where they dig and defend spawning pits (Oliveira and Almada, 1998). Males present two distinct phenotypes: dominants are usually larger, dark colored, establish territories and attract females; while subordinates have a silver color pattern similar to females and fail to establish territories (Oliveira and Almada, 1998). Our experimental setup allowed focal males to interact with other males and females in a simulated semi-natural environment. In a first longitudinal study (Social challenge experiment), dominant male fish were placed in experimental tanks with a group of females. Every week an acute territorial intrusion was performed and plasma androgen levels were measured at different time points in order to characterize a time course curve for each animal. At the end, each fish was injected with a high dosage of a GnRH analog to assess its physiological maximum. Experimental fish were subjected twice to several behavioral tests to establish individuals' consistent temperament traits. A second study (Physiological challenge experiment) was conducted to characterize the time course of the androgen response to a physiological challenge. Dominant males maintained in the same way as in the previous experiment were injected with a GnRH analog once a week. After injection, plasma was collected at several sampling times as previously and androgen levels were assessed. Our goal was to compare temporal circulating androgen levels obtained in response to social interactions and those elicited by Hypothalamus-Pituitary-Gonads (HPG) axis stimulation to assess the involvement of the HPG in the observed androgen response to social interactions.

2. Materials and methods

2.1. Animals and housing

O. mossambicus adult fish from a stock held at ISPA were used in this experiment. Fish were maintained in glass tanks (120 x 40 x 50 cm, 240 l) with a fine gravel substrate. Each tank was supplied with a double filtering system (gravel and external biofilter) and continuous aeration. Water quality was analized twice per month for nitrites (0.2–0.5 ppm), ammonia (< 0.5 ppm, Pallintest kit) and pH (6.0 – 6.2). Fish were kept at a temperature of 26 ± 2 ºC, a 12L:12D photoperiod, and fed with commercial cichlid floating sticks. Thirty-six focal dominant males (2.5 - 3 years old) were used for the experiments described below. Males' social status was monitored several times per week and territorial males were identified by nuptial black

coloration and exhibition for at least 1 week of reproductive behavior, including territorial defense and digging of a spawning pit in the substrate (Oliveira and Almada, 1996).

2.2. Social challenge experiment

2.2.1. Experimental setup

Twenty focal dominant males (mean body mass ± SEM: 48.03 g ± 1.99 g; mean standard length ± SEM: 11.23 cm ± 0.16 cm) were phenotyped for their behavioural profile (see below: personality tests), lightly anaesthetized (MS-222, Pharmaq; 300 ppm) to be weighed and measured, and then introduced into experimental tanks (Figure 1). Each experimental tank (40 x 50 x120 cm) was divided into 3 compartments with transparent and holed partitions so that chemical and visual contact was possible between compartments. Each compartment contained a dominant male and 4 females. Each side compartment held a focal male. After 1 week of habituation, focal males were allowed to interact with a male (see below) introduced to their compartment. The intruder male was removed 3 min after the first aggressive behavior of the focal male towards the intruder. The experiment was run for several weeks to obtain several sampling time points after intrusion. Focal males were randomly removed from the tank at 2, 5, 15, 30, 60 or 90 min after intrusion to collect blood and returned to the experimental tank. A sampling time point of 0 min corresponds to a week where there was no intrusion. Intruder males were isolated individually also in experimental tanks with 4 females per compartment; they were also territorial males but selected from different stock tanks from those of focal males. Focal males were always larger than intruders (mean body mass ± SEM: 25.31 g ± 0.96 g; mean standard length ± SEM: 9.07 cm ± 0.14 cm) to ensure the focal male's social advantage. Focal males were subjected to a different intruder each week to control for possible familiarity cues and promote more aggressiveness from focal fish towards stranger intruders (Aires et al., 2015). Interactions between focal and intruder males were video recorded for subsequent behavioral analysis. In the next-to-last week of experiment, focal males were injected with sGnRH (1000 μg/kg; sGnRH analog, (Des-Gly^{10},D-Ala^{6},Pro-NHEt^{9})-LHRH (salmon); Bachem #H-7525) and sampled after 60 min to measure the maximum physiological level of each male's androgens. In the final week of experiment, focal males were phenotyped for behavioral profiles (personality tests) using the same behavioral protocol that was used before the start of the experiment to ascertain trait consistency over time, a main requisite of personality. To reduce behavioral or hormonal fluctuations associated with natural circadian rhythm, personality tests

were always conducted in the afternoon, while the territorial intrusions were always conducted in the morning.

sGnRH dose was selected based on a dose-response pilot experiment where four different doses and a saline control were tested in male fish. We selected the dose that produced the highest significant increase in circulating androgens above baseline levels (Supplementary Figure S1).

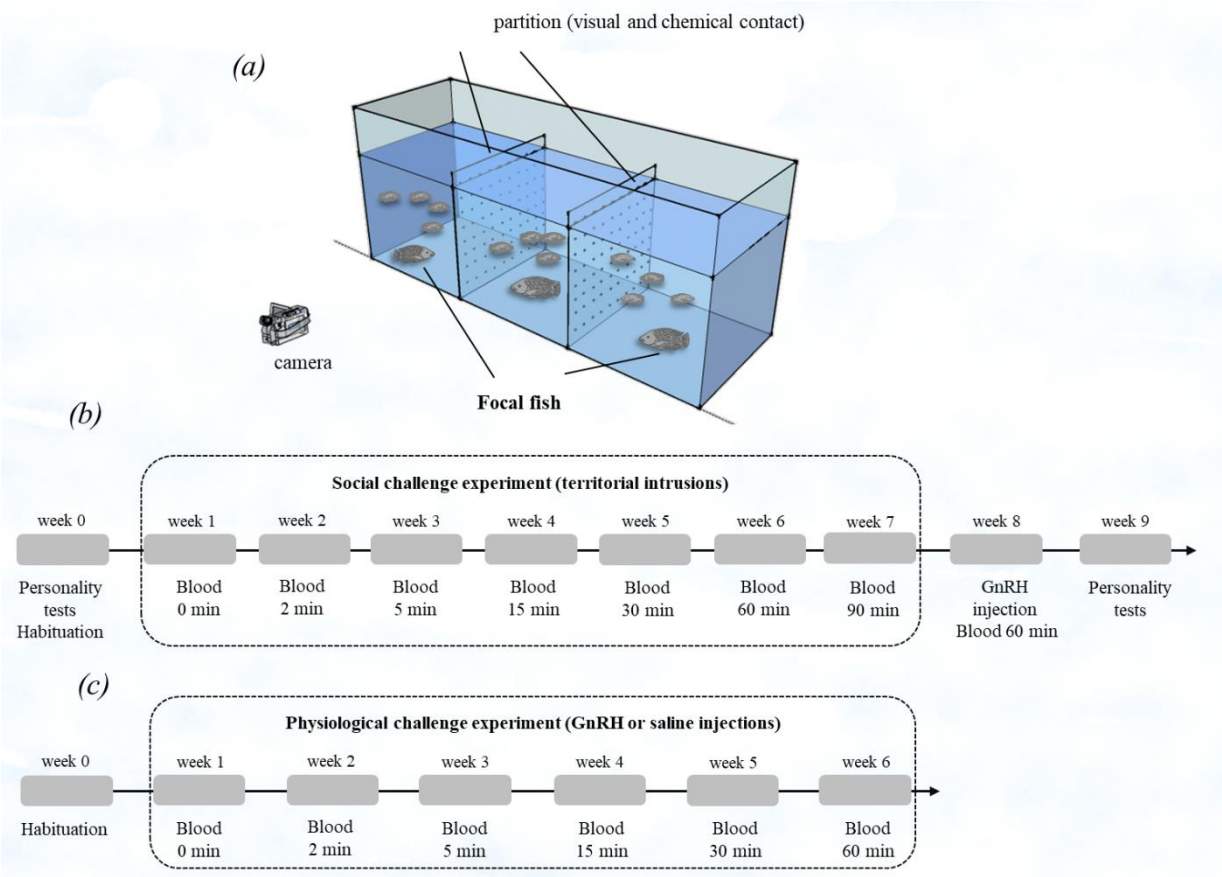

Figure 1. Experimental design. *(a)* 3D diagram of the experimental setup. Experimental tanks were divided in compartments by partitions which allowed chemical and visual contact between them. Each compartment contained a dominant male and 4 females. Each side compartment held a focal male. *(b)* Timeline of the Social challenge experiment (within-subject design). In the first week of experiment, focal males were phenotyped for behavioral profiles (personality tests). In the following weeks, focal males were exposed to territorial intrusions and their blood sampled at 2, 5, 15, 30, 60 and 90 min after the intrusion. In week 8, focal males were injected with sGnRH and sampled after 60 min to measure the androgen's physiological maximum of each fish. In the final week of experiment, focal males were phenotyped for behavioral profiles (personality tests). *(c)* Timeline of Physiological challenge experiment (within-subject design).

Focal males were i.p. injected either with sGnRH (GnRH treatment group) or with a saline solution (control group) once a week and blood was sampled 2, 5, 15, 30 or 60 min after the injection.

2.2.2. Personality tests

To determine if the androgen response to social interaction is related to behavioral types, we tested individuals on two personality dimensions: exploration-avoidance and aggressiveness (Réale et al., 2007). The focal male was subjected twice (in the first and the last week of the main experiment, see above) to a battery of behavioral tests to assess individual variability and trait consistency. In each session, the focal male was exposed to 4 behavioral tests: (1) open field, (2) novel object, (3) mirror test and (4) net restraining (Figure 2). Tests were performed consecutively in the same order, but the order of males used in each session was random. The experimental arena consisted on an unfamiliar circular white tank (external diameter: 54 cm; filled to a depth of 12 cm; 20 l of water) with a webcam (Logitech webcam C170) placed overhead for a top-down view. A video camera (Sony DCR-SR58E) was placed on the side to enable better discrimination of aggressive behaviors in the mirror test. To minimize disturbance from the surroundings, opaque divisions were placed around the arena. The experimental arena was cleaned at the end of each individual session and the water was replaced with clean maturated water.

Open Field Test (OF) – The male was carefully placed in the arena. After 30 s of acclimation, the test phase was initiated and the male was tracked using commercial video tracking software (EthoVision® XT 8.0, Noldus Inc. the Netherlands). Two zones were defined for assessing thigmotaxis (a wall-seeking spatial strategy associated with anxiety, (Champagne et al., 2010) and exploratory behavior in a novel but limited environment: a 10 cm outer zone (near the arena walls) and the remaining inner area as the centre zone. The following behaviors were recorded for 10 min: total time in movement, total distance moved, total time spent in the centre zone of the arena, latency to enter the centre zone, number of times spent in the centre zone of the arena, and distance moved in the centre zone. Males that never entered the centre zone were given a maximum latency of 600 s.

Novel Object Test (NO) – Immediately after the OF assay, a weighted red object was introduced in the arena using a fishing line. To keep the object novel, males were presented with a red Lego brick (3x3x3 cm) in one trial and a red ball of similar dimensions (4 cm diameter) in the other trial. This test was conducted to estimate neophobia/neophilia behavior,

a particular dimension of exploration-avoidance temperament trait (Réale et al., 2007). Two zones were defined for assessing behavioral measures: the novel object zone was a circular 10 cm radius zone around the object; the remaining area. The following behaviors were recorded for 10 min: total time in movement, total distance moved, total time spent in the novel object zone, latency to enter the novel object zone, number of times spent in the novel object zone and distance moved in the novel object zone. Males that never entered the novel object zone were given a maximum latency of 600 s.

Mirror Test (MT) – At the end of the NO, the novel object was gently removed using the fishing line. The male was given 2 min to settle, then a mirror (dimension: 21 x 30 cm) was placed in the arena leant in a way that prevented males from going behind it during the trial. Since fish do not recognize themselves in mirror, they fight their own image as if it is a conspecific intruder (Oliveira et al., 2005). This test was conducted to assess the male's agonistic reaction in a standardized assay. Two zones were defined to obtain behavioral measures: the mirror zone was a 10 cm-width area near the mirror; the remaining area. The following behaviors were recorded for 5 min: total time in movement, total distance moved, total time spent in the mirror zone, latency to enter the mirror zone, number of times spent in the mirror zone and distance moved in the mirror zone. Additionally, the number and duration of aggressive behaviors (lateral displays, frontal displays, bites, tail beating) and latency to attack were analyzed using Observer XT software (Noldus technology, version 5, Netherlands). Males that never entered the mirror zone were given a maximum latency of 300 s.

Net Restraining Test (NR) – At the end of the MT, the male was held in a net, out of water, for 1 minute. The following behaviors were recorded: number of escape attempts and the total time spent in escape attempts. This is an assay that has been used to evaluate escape behavior in other fish species (e.g., gilthead sea bream, *Sparus aurata L.*, in Arends et al., 1999; Castanheira et al., 2013; flatfish Senegalese sole, *Solea senegalense,* in Silva et al., 2010; Nile tilapia, *Oreochromis niloticus,* in Martins et al., 2011).

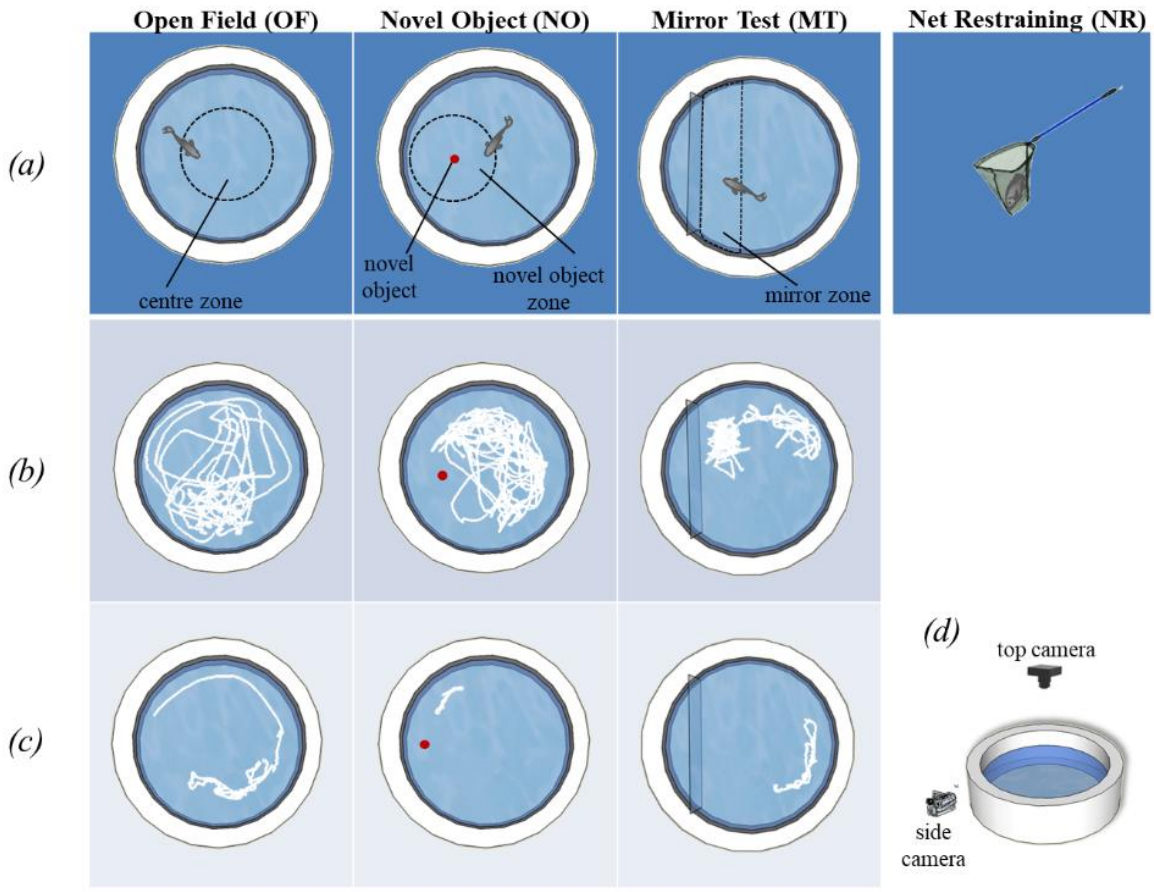

Figure 2. Personality tests. *(a)* Behavioral testing procedures: open field (OF), novel object (NO), mirror test (MT) and net restraining (NR). *(b)* and *(c)* representative examples of video-tracking of: *(b)* individual with high level of exploration in OF and NO and that fought with the mirror; and *(c)* individual with low level of exploration in OF and NO and that did not fight with the mirror. *(d)* 3D diagram of the experimental arena.

2.3. Physiological challenge experiment

This study was conducted to characterize the time course of the androgen response to a GnRH challenge. On the first day of experiment, focal males were lightly anaesthetized (MS-222, Pharmaq; 300 ppm) to be weighed and measured, and then introduced into the same experimental tanks used in the Social Challenge Experiment (Figure 1). Sixteen focal dominant males (mean body mass ± SEM: 51.65 g ± 2.93 g; mean standard length ± SEM: 11.80 cm ± 0.22 cm) were used. Focal males were arbitrarily assigned to the GnRH treatment group (n = 8) or to the control group (n = 8). All males had intraperitoneal injections once a week, treatment group (G group) with a GnRH analog (100 μg/kg), controls (V group) with a saline vehicle solution (0.9% sodium chloride). The experiment was run for several weeks to obtain samples

at several time points after the injection. Focal males were randomly removed after 2, 5, 15, 30 or 60 min after injection to collect blood and returned to the experimental tanks. The sampling time point of 0 min corresponds to a week where there was no injection.

2.4. Behavioral observations

Behaviour of focal males during territorial intrusions (main experiment) was analyzed using Observer XT software (Noldus technology, version 5, Netherlands), based on the ethogram provided by Baerends and Baerends-van Roon (1950). Relevant behavioral patterns were identified to measure male aggressiveness towards the intruder, the neighbor or the females (i.e. bites, displays, buttings, chasing, tail beating). Other behaviors (nipping, swimming, floating, courting, glass interactions) were also quantified. We recorded the frequency and latency of the reported behaviors, as well as the attack latency (i.e., time between the beginning of the recording period and the first aggressive behavior). Similarly, for personality tests the aggressive behaviour of focal males during the Mirror Test (MT) and the time spent performing escape attempts in the Net Restraining Test (NR) was analyzed using the same software. Other variables measured in the personality tests were obtained with EthoVision XT 8.0 (Noldus Inc. the Netherlands).

2.5. Blood sampling

Males were anaesthetized (MS-222, Pharmaq; 450 ppm) and blood was collected from the caudal vein using heparinized 25-gauge needles. Blood sampling always took place within 4 min of the induction of anaesthesia to prevent possible effects of handling stress on steroids levels (Foo and Lam, 1993). Blood samples were centrifuged (10 min, 3000 *g*, 4ºC) and plasma was stored at - 20ºC until further processing.

2.6. Hormone assays

11-ketotestosterone (KT), testosterone (T) and cortisol (F) free steroids were extracted from plasma samples by adding diethyl-ether (Merck). Samples were then agitated for 20 min, centrifuged (5 min, 163 *g*, 4ºC) for phase separation and kept at -80 ºC for 15 min to freeze the water phase and separate the ether fraction (containing the free steroid). This process was repeated twice to obtain higher extraction efficiency. The ether fraction was evaporated with a Speedvac (Savant SC1101) and the dried organic phase was re-suspended in phosphate buffer.

Steroid concentrations were measured by radioimmunoassay using a testosterone antibody from Research Diagnostics Inc (#WLI-T3003, rabbit anti-testosterone) and cortisol antibody from Fitzgerald (#20-CR-50, rabbit anti-cortisol). The antibody used for the 11-ketotestosterone assay was kindly donated by D. E. Kime and the corresponding specificity table was published in Kime and Manning (1982). Reactive markers used in radioimmunoassays for testosterone and cortisol were from Amersham Biosciences ([1,2,6,7-3H] Testosterone, #TRK402-250 µCi; [1,2,6,7-3H] Cortisol, #TRK407-250 µCi) while 11-ketotestosterone marker was produced in-house from marked cortisol (Kime and Manning, 1982). Samples collected from each male were run in the same assay. Inter-assay variabilities were 7.9 % for KT, 8.0 % for T and 11.9 % for F. Intra-assay variation coefficients were 8.0 %, 4.9%, 4.7%, 0.4% and 5.2 % for KT; 11.5%, 5.5%, 5.1, 4.3% and 8.8% for T; 4.9 % and 11.2 % for F.

2.7. Data analysis

Normality of the data was tested by analyzing skewness and kurtosis values (Kline, 1998) and running Shapiro-Wilk tests. If necessary, variables were log transformed to meet parametric assumptions. Homocedascity was confirmed with Levene's test.

The androgen scope of each individual, defined as the androgen responsiveness score, was assessed by dividing KT baseline levels for KT physiological maximum (GnRH induced levels). Individuals that had values above the mean were defined as low androgen responders (LR), because they had a lower scope for response; while those that had an androgen responsiveness score below the mean were considered the high androgen responders (HR) because they had a greater scope of response. We used KT in this calculation since it has been reported as the main androgen in teleost fish (Borg, 1994) and found to respond to social interactions in this species (Hirschenhauser et al., 2004). For the Social challenge experiment, hormone levels (KT, T and F) were analyzed using a Linear Mixed Model (LMM) with time and type of androgen response (HR vs LR) as fixed effects and focal male as a random effect. Planned comparisons were used to compare steroid levels relative to baseline and the p-values were adjusted for multiple testing using the Benjamini & Hochberg (1995) procedure.
Plots of residuals, fitted values and estimated random effects were used to confirm assumptions of LMM.

Body condition index in the beginning and at the end of the experiment was calculated for each male by dividing its actual weight by its expected weight. Expected weight was assessed by the weight-length relationship obtained in a field study for *O. mossambicus* (Silva,

1985). T-tests were used to compare body mass, body size and body condition index for the two groups (LR and HR males).

We measured repeatability of behavioral variables, for which we obtained multiple measurements (personality tests and territorial intrusions). Repeatability, more generally referred to as the intra-class correlation (ICC), is defined as the proportion of phenotypic variation explained by differences between individuals (Dingemanse and Dochtermann, 2013; Lessells and Boag, 1987): Repeatability=$V_{IND}/(V_{IND}+V_e)$, where $V_P = V_{IND}+V_e$ is the phenotypic variation, composed of the between-individual variance V_{ID} and the within-individual variance V_e. V_e represents the 'residual error' due to errors in measurements and general environmental variance. On the other hand, repeatability aims to measure the total variation that is reproducible, that is, the consistency of each trait in the population, allowing comparison across studies for the same trait and across traits in the same study (Dingemanse and Dochtermann, 2013; Harrison et al., 2018). To verify that behavioral responses reflected personality traits, we used the rtpR package (Stoffel et al., 2017) to calculate repeatability. With this package, uncertainty is measured via parametric bootstrapping and likelihood ratio tests are used for significance testing. The number of parametric bootstrap iterations for confidence interval estimation and statistical significance was set to 1000. We have not calculated repeatabilities for variables accounting for aggressive behaviors in the Mirror Test since in the second trial, none of the males fought with the mirror.

T-tests were used to compare behavioral measures that were repeatable, and assess if any of the behaviors were significantly different for the two groups (LR and HR males).

Behavioral variables of the OF were reduced with Principal Component Analysis (PCA) and different component loadings were obtained with the variable principle normalization method. Since variables were preferentially loaded on the first PCA dimension, only one component was selected which described 68.8% of the variance of behavior on the OF. This PCA component was interpreted as describing males as more or less exploratory (Supplementary Table S1). Behavioral variables of the territorial intrusion experiment were also reduced with PCA and different component loadings were obtained with the variable principle normalization method. The first PCA dimension was loaded with behavioral variables related to aggression towards intruders and females (29.7 % explained variance), while the second component was related to aggression towards the neighbor (18.5 % explained variance) (Supplementary Table S2). For these PCA analyses we used the average of the behaviors between the trials. A Pearson correlation between PCA scores of the OF and PCA scores of territorial intrusions was used to examine the relationship between exploration and aggression.

For the Physiological challenge experiment, hormone levels were analyzed using a Linear Mixed Model (LMM) with time and treatment (GnRH or saline injected) as fixed effects and focal male as a random effect. Planned comparisons were used to compare steroid levels relative to baseline and the p-values were adjusted for multiple testing (Benjamini & Hochberg 1995).

Effect sizes were computed for LMM tests (omega-squared, ω^2) and for post-hoc tests (Cohen's d).

Since we were analyzing individual variability we decided not to remove any apparent outliers or extreme values. Degrees of freedom may vary between the analyses due to missing values because of technical problems (i.e. with blood collection, RIA or video recordings).

Statistical analysis was performed using IBM SPSS® statistics v.21, STATISTICA v.10 (StatsoftInc), and R (R Core Team, 2015) with the following packages: nlme (linear mixed model), multcomp (planned comparisons), rtpR (repeatability), sjstats (effect sizes).

2.8. Ethics statement

In this study, we have kept aggressive interactions to a short period (3 min) and no signs of physical injuries were observed during any of the staged interactions. Experimental procedures used in this study were conducted in accordance with the institutional guidelines for the use of animals in experimentation and were approved both by the internal Ethics Committee of ISPA and by the Portuguese Veterinary Authority (Direção Geral de Alimentação e Veterinária, Portugal; permit number 0421/000/000/2013).

3. Results

Social challenge experiment

There was inter-individual variation in baseline levels (KT: 8.0-fold; T: 7.2-fold; F: 39.6-fold), physiological maximum (KT: 4.6-fold; T: 7.7-fold) and in the androgen scope (KT: 18.9-fold; T: 6.6-fold) of focal males (Figure 3). Following this variation, males were grouped into low responders (LR; n = 7) or high responders (HR; n = 13) according to their androgen responsiveness score (see details in Methods). There was no significant variation in body size between LR and HR males ($t(18) = 1.767$, $p = .094$), but there was in body weight (beginning of experiment: $t(17) = 3.089$, $p = 0.007$; end of experiment: $t(18) = 2.100$, $p = 0.050$). Body

condition index was also significantly different between LR and HR fish at the beginning (t(17) = 3.261; p = 0.005; mean (LR) ± SE = 69.91 ± 4.995; mean (HR) ± SE = 59.91 ± 6.655 values) and at the end of the experiment (t(18) = 2.099, p = 0.050; mean (LR) ± SE = 61.97 ± 7.004; mean (HR) ± SE = 55.22 ± 6.795).

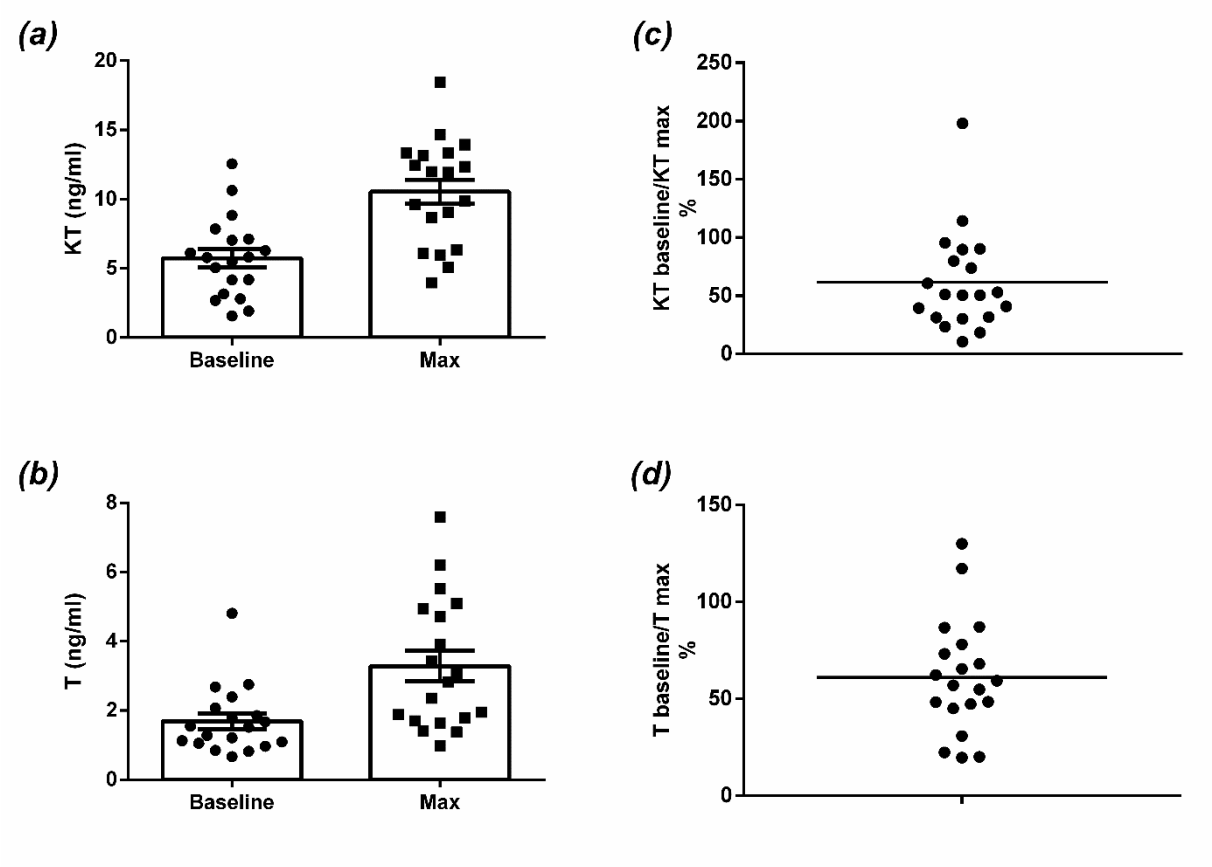

Figure 3. Individual variation in androgens. *(a)* 11-ketotestosterone (KT); *(b)* testosterone (T); *(c)* androgen scope for KT (KT baseline/KT max); *(d)* androgen scope for T (T baseline/T max); where baseline corresponds to the week with no intrusion and max to the week of GnRH injection. Histograms in *(a)* and *(b)* show mean ± standard error of the mean (SEM), in *(c)* and *(d)* the line shows the mean. Individual values shown as symbols.

The levels of KT, T and F changed significantly with time (KT: $F_{(7,117)} = 6.855$, $p < 0.0001$, T: $F_{(7,116)} = 7.296$, $p < 0.0001$, F: $F_{(6,98)} = 23.718$, $p < 0.0001$, Table 1), but were not significantly affected by whether the fish were HR or LR (KT: $F_{1,18} = 0.071$, $p = 0.793$, T: $F_{(1,18)} = 0.104$, $p = 0.751$, F: $F_{(1,18)} = 0.395$, $p = 0.538$; Table 1). However, the interaction between these factors was significant for KT ($F_{(7,117)} = 2.629$, $p = 0.015$, Table 1). HR fish had a significant increase above baseline of KT 2 min and 5 min and then again at 90 min after the

territorial intrusion (Table 1, Figure 4a). There was no difference in KT or T in LR fish after territorial intrusions (Table 1, Figure 4a and b).

Table 1. Effect of time and type of fish (LR vs HR) on hormone levels after territorial intrusion (Social challenge experiment). Main effects (LMM), interactions, effect sizes and planned comparisons between the baseline and the other time points.

Comparisons	**Main effects**			**Planned Comparisons**					
				LR			**HR**		
	F	**p**	**ω^2**	**z**	**p**	**d**	**z**	**p**	**d**
KT			.564						
Time	6.855	**<.0001**							
Fish Type	.071	.793							
Time x Fish Type	2.629	**.015**							
0 min vs 2min				-.869	.490	.613	2.660	**.043**	.810
0 min vs 5 min				-.885	.490	.392	2.579	**.043**	.806
0 min vs 15 min				-1.723	.136	.994	1.939	.123	.734
0 min vs 30 min				-1.708	.136	1.137	.693	.570	1.413
0 min vs 60 min				-1.862	.125	1.186	2.281	.063	.849
0 min vs 90 min				.264	.853	.370	2.502	**.043**	.942
0 min vs GnRH				.038	.970	.021	6.859	**<.0001**	.982
T			.673						
Time	7.296	**<.0001**							
Fish Type	.104	.751							
Time x Fish Type	1.413	.207							
0 min vs 2 min				-.902	.734	.364	.670	.782	.227
0 min vs 5 min				-.701	.782	.248	1.369	.734	.415
0 min vs 15 min				-.022	.996	.007	.946	.734	.278
0 min vs 30 min				-.548	.817	.193	.285	.988	.053
0 min vs 60 min				-.005	.996	.002	1.144	.734	.381
0 min vs 90 min				-.115	.996	1.010	2.153	.219	.745
0 min vs GnRH				1.106	.734	.377	6.258	**<.0001**	1.564
F			.698						
Time	23.718	**<.0001**							
Fish Type	.395	.538							
Time x Fish Type	1.160	.334							
0 min vs 2 min				3.533	**.002**	1.029	7.547	**<.0001**	1.750
0 min vs 5 min				3.118	**.005**	1.430	3.199	**.005**	.956
0 min vs 15 min				2.330	**.048**	.796	2.116	.069	1.246
0 min vs 30 min				.319	.750	.036	1.002	.422	1.102
0 min vs 60 min				-.922	.428	.467	-.562	.627	.098
0 min vs 90 min				-1.451	.220	1.802	-1.787	.127	.209

11-ketotestosterone (KT); testosterone (T); cortisol (F); LR – Low responder fish; HR – High responder fish; z: z-test estimate; ω^2: effect size estimate (omega squared); d: effect size estimate

(Cohen's d); p: p-value after multiple comparison adjustment; statistically significant values are in bold.

GnRH injection elicited a significant increase of both androgens only in HR fish (Table 1, Figure 4a and b). F levels were higher than baseline between 2 min and 15 min for LR and between 2 min and 5 min for HR (Table 1, Figure 4c).

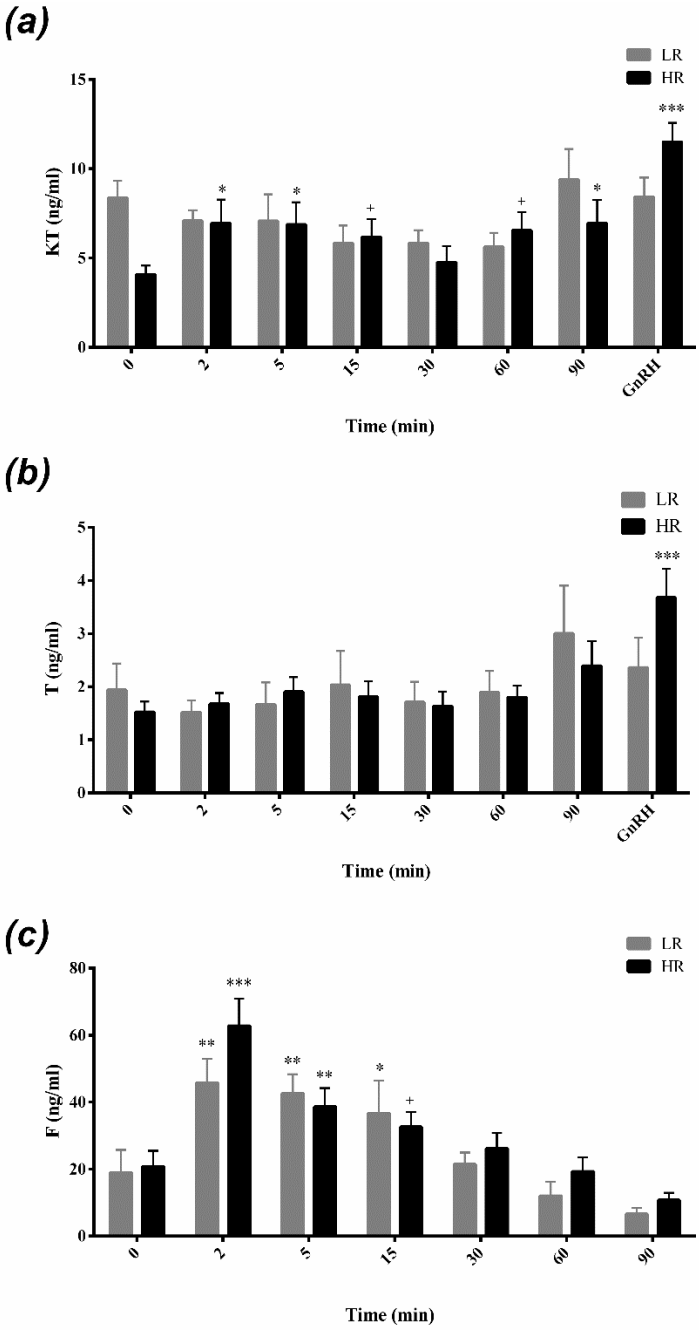

Figure 4. Temporal pattern of the hormonal response to territorial intrusions for Low Responder (LR) and High Responder (HR) fish (see methods for details) in Social **challenge**

experiment. Values are mean ± standard error of the mean (SEM). *(a)* 11-ketotestosterone (KT); *(b)* testosterone (T); *(c)* cortisol (F); $^{+}$ non-significant trend $p<.10$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

For personality tests, only behavioral variables from the Open Field (OF) were significantly repeatable, namely the distance moved in the centre zone, total distance moved and total time in movement, with repeatability scores higher than 0.57 (Supplementary Table S3).

For territorial intrusions, most of the behavioral variables, either aggressive or non-aggressive were significantly repeatable (Supplementary Table S4). The most repeatable behavior was time spent in chases with a score of 0.587 (Supplementary Table S4).

Several repeatable behavior variables differed significantly between LR and HR males. In the OF, the total distance moved was significantly different between males (LR moved more than HR; Table 2). Other behaviors measured in the OF showed a non-significant tendency of LR to move more in the center of the arena and to spend more time in movement than HR (Table 2). During territorial intrusions, the total number of behaviors (measured as a proxy of activity), aggressive behaviors towards both the intruder and the neighbor and the aggressive behaviors towards the intruder were significantly different, with LR displaying a higher number of these behaviors than HR (Table 2). Similarly, aggressive behaviors towards the neighbor show a non-significant trend of LR to be more aggressive than HR (Table 2).

The PCA score of the OF was significantly negatively correlated with the second PCA score of the territorial intrusions ($r = -.466$, $p = 0.039$, $n = 20$), suggesting that individuals with higher scores of exploratory behavior were less aggressive towards the neighbor.

Table 2. Statistical values for the differences between behavior of LR and HR fish in personality tests and over the territorial intrusions.

	Mean LR	Mean HR	t (df)	p	d
Open Field Test					
Distance moved in the centre zone	841.222	450.677	1.896 (18)	.074	.834
Total distance moved	1714.852	824.268	2.095 (18)	**.050**	1.000
Total time in movement	221.362	114.180	1.994 (18)	.062	.981
Territorial intrusions					
Total number of aggressive behaviors (males)	35.090	26.400	2.550 (18)	**.020**	1.237
Total time spent in aggressive behaviors (males)	203.119	179.333	2.067 (18)	.053	.928
Total number of aggressive behaviors (intruder)	28.281	21.405	2.210 (18)	**.040**	1.080
Total time spent in aggressive behaviors (intruder)	171.867	152.838	1.736 (18)	.100	.822
Total number of aggressive behaviors (neighbor)	6.810	4.995	1.839 (18)	.082	.609
Total time spent in aggressive behaviors (neighbor)	31.252	26.495	.339 (18)	.738	.151
Total number of aggressive behaviors (females)	1.938	1.808	.181 (18)	.858	.091
Total time spent in aggressive behaviors (females)	3.267	1.854	.470 (18)	.644	.216
Total number of non-aggressive behaviors	1.457	1.764	-.354 (18)	.727	.160
Total time spent in non-aggressive behaviors	5.062	15.051	-1.231 (18)	.234	.598
Total number of behaviors	38.486	29.972	2.518 (18)	**.021**	1.156

LR – Low responder fish; HR – High responder fish; t: t-test estimate; d: effect size estimate (Cohen's d); p: p-value; statistically significant values are in bold.

Physiological Challenge Experiment

The levels of KT changed significantly with time ($F_{(5,67)} = 3.862$, $p = 0.004$, Table 3) but were not significantly affected by treatment with GnRH or control (saline) ($F_{(1,14)} = 0.462$, $p = 0.508$, Table 3). However, the interaction between these factors was significant ($F_{(5,67)} = 9.568$, $p < 0.0001$, Table 3). The levels of T changed significantly with time ($F_{(5,68)} = 7.851$, $p < 0.0001$, Table 3) but were not significantly affected by treatment ($F_{(1,14)} = 3.380$, $p = 0.087$, Table 3). The interaction between these factors was significant ($F_{(5,68)} = 11.529$, $p < 0.0001$, Table 3). Fish injected with GnRH significantly decreased KT, 2 min and 5 min after the injection and then significantly increased at 60 min, compared to the baseline (Table 3, Figure 5a). For the control group, KT significantly decreased 15 min after the injection and remained below the baseline even after 60 min (Table 3, Figure5a 5). For T, there was a significant increase of T only for GnRH-treated group 30 min and 60 min time points (Table 3, Figure 5b).

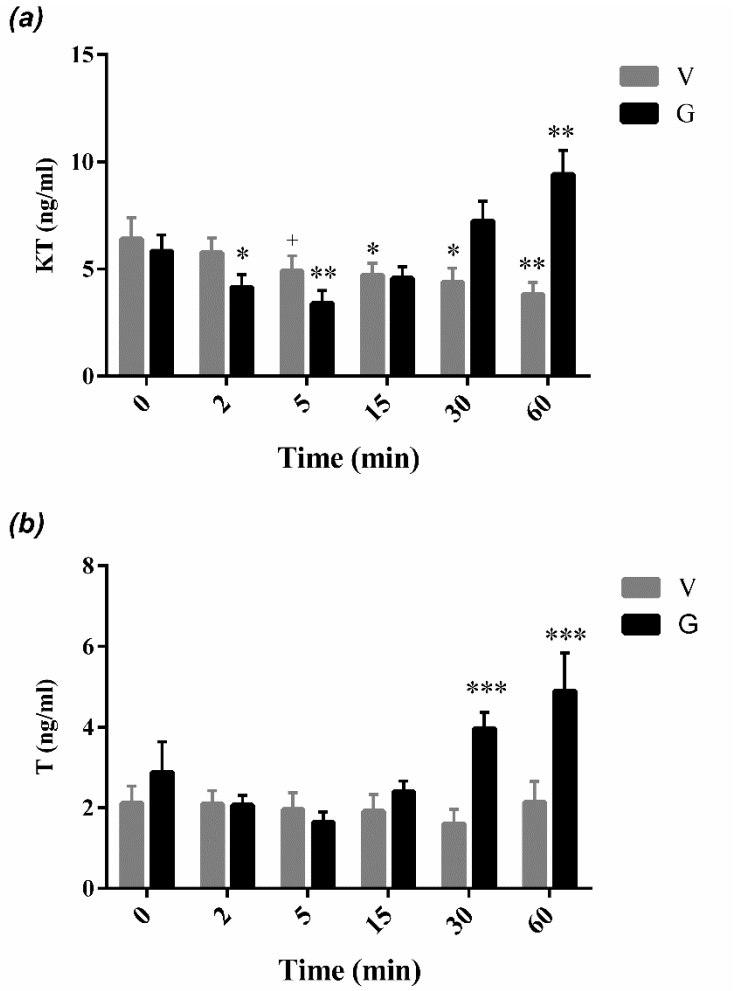

Figure 5. Temporal pattern of the hormonal response of fish injected with vehicle (V) or with GnRH (G) in the Physiological challenge experiment. Values are mean ± standard error of the mean (SEM). *(a)* 11-ketotestosterone (KT); *(b)* testosterone (T); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 3. Effect of time and treatment (GnRH vs saline) on hormone levels (Physiological challenge experiment). Main effects (LMM), interactions, effect sizes and planned comparisons between the baseline and the other time points.

Comparisons	**Main effects**			**Planned Comparisons**					
				GnRH group			**Saline group**		
	F	**p**	**ω^2**	**z**	**p**	**d**	**z**	**p**	**d**
KT			.608						
Time	3.862	**.004**							
Treatment	.462	.508							
Time x Treatment	9.568	**<.0001**							
0 min vs 2 min				-2.170	**.050**	.861	-.833	.405	.264
0 min vs 5 min				-3.152	**.005**	1.248	-1.956	.072	.611
0 min vs 15 min				-1.637	.127	.673	-2.198	**.050**	.737
0 min vs 30 min				.843	.405	.335	-2.643	**.021**	.842
0 min vs 60 min				3.188	**.005**	1.482	-3.533	**.004**	1.479

T			.820					
Time	7.851	**<.0001**						
Treatment	3.380	.087						
Time x Treatment	11.529	**<.0001**						
0 min vs 2 min			-.425	.936	.105	.199	.936	.057
0 min vs 5 min			-2.345	.055	.682	-2.205	.055	.184
0 min vs 15 min			.723	.783	.362	-.071	.943	.236
0 min vs 30 min			4.148	**.0002**	1.683	-2.266	.055	.945
0 min vs 60 min			5.161	**<.0001**	1.774	-.218	.936	.054

11-ketotestosterone (KT); testosterone (T); z-test estimate; ω^2: effect size estimate (omega squared); d: effect size estimate (Cohen's d); p: p-value after multiple comparison adjustment; statistically significant values are in bold.

4. Discussion

Our results show that the androgen response to territorial intrusions varies between dominant males of the Mozambique tilapia and is related to their scope for response (i.e. the difference between baseline and maximum physiological levels for each fish). Individuals that have a lower scope for androgen response did not increase androgens after a territorial intrusion but were more aggressive and exploratory. Males with a higher scope for response have fewer aggressive and exploratory behaviors and exhibit two waves of KT response, one soon after the aggressive challenge (2-15 min) and other about an hour later (60-90 min). Moreover, subjecting fish to a GnRH challenge elicits an androgen increase 30-60 min after the injection.

In the case of the Mozambique tilapia, a polygynous species where males do not show parental care, the Challenge Hypothesis predicts a low androgen response to social interactions, when compared to species with other mating systems (Wingfield et al., 1990). We found that androgen responsiveness was rather variable, even though males had the same social rank, since baseline and physiological maximum levels differed largely between individuals. Therefore, males with KT baseline levels very close to their maximum (Low responder fish, LR) were not capable of significantly increasing androgen levels either to social or physiological challenges. Interestingly, other males (High responder fish, HR) had a higher scope for response and exhibited two peaks of KT levels, probably playing different roles. The quick response of KT may be related to the necessity to deal with the ongoing fight, regulating the male's behavior during the interaction (Marler et al., 2005). Another explanation for this short-term response can be a stress-provoked response to intrusion that has been described in acute stress events (e.g. androgen elevation 3 min after confinement in sockeye salmon, *Onchorhynchus nerka*: Kubokawa et al., 2001), which is confirmed by the elevated levels of cortisol soon after the social challenge. Several authors have proposed that fast androgen responses may enable swift

and flexible behavioral responses to social challenges (Kempenaers et al., 2008; Oliveira, 2004). On the other hand, the delayed KT response suggests its involvement in the modulation of future interactions, possibly as a result of the outcome of the interaction (e.g. winner/loser effects) (Hsu et al., 2011; Oliveira et al., 2009; Oyegbile and Marler, 2005). From an overall perspective, we verified that KT increased in some individuals but decreased in others, hence these changes would cancel out each other at the population level. These results further support the idea that endocrine studies should focus on individual data rather than on the mean value of an heterogeneous group of individuals (Bennett, 1987; Williams, 2008). For instance, in a breeding blue tit population, *Cyanistes caeruleus,* T levels varied 200-fold (Kempenaers et al., 2008). Even within the same social status, animals can have distinct endocrine profiles, baseline and environment hormone reactions (e.g., Alcazar et al., 2016; Virgin and Sapolsky, 1992). Moreover, to compare traits or physiological measures obtained in different situations the same individuals should be used in within-subject designs (Bennett, 1987; Williams, 2008). In addition, not only the magnitude but also the temporal pattern of the androgen response can vary, which means that the variation of response with time for each individual is much more relevant than single 'snapshots' of the hormonal variation (Kempenaers et al., 2008).

At the behavioral level, we found several behaviors to be quite variable between individuals but consistent within individuals. Regarding personality tests, behavior variables were repeatable only in the Open field (OF) test. This demonstrates that exploratory behavior is consistent in this species within a two months interval, even when subjecting males to repetitive behavioral and experimental manipulations. The other tests, Novel Object (NO), Mirror Test (MT) and Net Restraining (NR), seemed to be influenced by the underlying factors and could not be used to characterize consistent behavioral profiles. For example, a lack of consistency across contexts (social vs isolation) of the NO test in this species has already been reported (Galhardo et al., 2012), while a lack of consistency across time has been found for other species (e.g., Gilthead seabream, *Sparus aurata*: (Castanheira et al., 2013). Supporting consistency of exploratory behavior and not of other traits in other taxa, are long-term personality studies carried in zebra-finches, *Taeniopygia guttata*, which found fearlessness and exploration the most repeatable traits within and across life phases, contrary to struggling rate (comparable test to the NR), aggression or boldness (David et al., 2012; Wuerz and Krüger, 2015).

In line with our results, temporal consistency in agonistic behavior has been previously found for other species (e.g., Australian lizard, *Egernia whitii*, While et al., 2010; bluefin killifish, *Lucania goodei*, McGhee and Travis, 2010; sheepshead swordtail, *Xiphophorus*

birchmanni, Wilson et al., 2013). However, most of the behaviors, either aggressive or not, during the several sessions of territorial intrusions were found to be repeatable. Interestingly, a study in European seabass, *Dicentrarchus labrax*, performed coping screening tests in different contexts (isolation vs group) and repeated them within various time intervals (up to 629 days) and found that, in opposition to individual tests, group-based tests were consistent across contexts and time (both short- and long-term) (Ferrari et al., 2015). Together these data suggest that social context promotes behavioral consistency at the individual level.

In our experiment we found a moderate negative correlation between aggressive behavior towards the intruder and exploration, that could be defined as a 'behavioral syndrome' (Bell, 2007; Sih et al., 2004) and could mean that the traits are regulated by a common neuroendocrine, genetic or neurobiological mechanism (Coppens et al., 2010; Sih et al., 2004). The literature offers contradictory findings from several authors which attempted to explore the relationship between androgens and behavior, yet using baseline androgen levels (e.g. positive relation of exploration, boldness and aggression with T baseline levels in the mangrove rivulus, *Kryptolebias marmoratus*, Chang et al., 2012; positive relation between aggressive behavior and baseline T levels in male rats (Tryon Maze Dull S-3 rats), Schuurman, 1980; negative relation of exploration and boldness with T baseline levels in male great tits, *Parus major*, van Oers et al., 2011; negative relation of aggression with T baseline levels in male Australian lizards, *E. whitii*, While et al., 2010; no differences in T baseline levels between rats, *Rattus norvegicus*, selected for high or low levels of aggression, Everts et al., 1997). In the current study, individuals with a lower scope for KT response presented higher exploratory, activity, aggressiveness and body condition indices, in opposition to individuals with a higher scope of response. This set of results links relative levels (baseline versus physiological maximum), rather than absolute levels, of KT to exploration, activity and male-male aggressive behavior.

The results obtained for the stimulation of the HPG axis by treating animals with GnRH suggest that the two waves of KT response could be mediated by different physiological mechanisms. The late (90 min) response of androgens to social interactions seems to agree with the temporal response of the HPG axis, whereas the short-response does not. Research in mammals has confirmed the existence of a direct neural pathway responsible for the regulation of gonadal functions, including testosterone secretion (Mayerhofer, 2007; Selvage et al., 2006). The involvement of this pathway in the androgen response to social stimuli has never been explored. However, it is a mechanism, independent of the pituitary release of gonadotropins into circulation and its transport to the gonads, which seems compatible with the quick response we observed. In the Nile tilapia (*Oreochromis niloticus*), a close related species, nerve bundles

have been identified in the testis close to Leydig cells (Nakamura and Nagahama, 1995), which may be controlling androgen release. Further studies on this question are needed to assess if the early and late androgen responses are mediated by direct neural pathways vs. HPG axis.

In summary, we found that the androgen response to social challenges varies between males depending on their scope for response, and when present it presents two peaks that seem to be regulated by different physiological mechanisms: an early response probably mediated by direct neural pathways followed by a late endocrine response mediated by the HPG axis. We suggest that determining the temporal pattern of the androgen response to social interactions and considering individual variability may be the key to understanding contradictory results of the Challenge Hypothesis.

Author Contributions

ASF and RFO designed the experiment; ASF and AR performed behavioral experiments; ASF processed samples and analyzed the data; ASF and RFO wrote the paper.

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Data Availability Statement

The datasets generated for this study are available on request to the corresponding author.

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CHAPTER 5

Forebrain transcriptional response to transient changes in circulating androgens in a cichlid fish

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Abstract

It has been hypothesized that androgens respond to the social interactions as a way to adjust the behaviour of individuals to the challenges of the social environment in an adaptive manner. Therefore, it is expected that transient changes in circulating androgen levels within physiological scope should impact the state of the brain network that regulates social behavior, which should translate into adaptive behavioural changes. Here, we examined the effect that a transient peak in androgen circulating levels, which mimics socially driven changes in androgen levels, has on the forebrain state, which harbors most nuclei of the social decision-making network. For this purpose, we successfully induced transient changes in circulating androgen levels in an African cichlid fish (Mozambique tilapia, *Oreochromis mossambicus*) commonly used as a model in behavioral neuroendocrinology by injecting 11-ketotestosterone or testosterone, and compared the forebrain transcriptome of these individuals to control fish injected with vehicle. Forebrain samples were collected 30 min and 60 min after injection and analyzed using RNAseq. Our results showed that a transient peak in 11-ketotestosterone drives more accentuated changes in forebrain transcriptome than testosterone, and that transcriptomic impact was greater at the 30 min than at the 60 min post-androgen administration. Moreover, several genes involved in the regulation of translation, steroid metabolism, ion channel membrane receptors or even genes involved in epigenetic mechanisms were differentially expressed after 11-ketotestosterone or testosterone injection. Thus, this study identifies specific genes that may mediate changes in the brain after social interactions which will influence future behavior.

Keywords: Cichlid, Challenge Hypothesis, androgens, brain, RNAseq

1. Introduction

Androgens are essential for reproduction. They influence morphology and physiology traits and have a pivotal role in the modulation of reproductive and also aggressive behaviors (Oliveira 2004). In turn, the social environment is known to modulate the circulating levels of androgens (Oliveira 2004, 2009). The Challenge Hypothesis has been proposed to explain androgen changes throughout the life history of an animal due to environmental (e.g. photoperiod) and social cues (Wingfield *et al.* 1990; Goymann *et al.* 2007). According to this hypothesis, circulating androgens have their lowest levels in the non-breeding stage, while in the breeding season quite dynamic patterns are found. Herein, androgens vary between a breeding baseline (triggered for instance by day length) and a physiological maximum elicited by sexual interactions or aggressive confronts with conspecifics (Goymann 2009). So, the social modulation of androgens could be a proficient way of increasing androgens only when necessary, preventing extended high levels (and potentially harmful) in circulation. Indeed, despite the inherent benefits of androgens on the fitness of an animal, elevated levels of androgens are relevant drawbacks. They interfere with paternal care and pair bonding, are energy-consuming and have been associated to immunosuppression and oncogenic effects (Wingfield *et al.* 2001; Oliveira 2004).

At a functional level, these socially driven changes in circulating steroid levels have been recognized to influence subsequent behaviors (Oliveira 2009). For instance, after a fight winner and loser effects (i.e. animals which experience victory have a higher probability of winning subsequent matches and defeated animals are more likely to lose subsequent fights, respectively) have been described in many species, including teleost fish (Hsu *et al.* 2006). Interestingly, the winner effect seems to be mediated by androgens. In studies using the California mice (*Peromyscus californicus*) males that experience prior winnings have a transient increase of androgen levels (Oyegbile and Marler 2005) and are more aggressive in next fights (Trainor and Marler 2001). Furthermore, injecting androgens in castrated California mice males after winning a fight induces an increase in aggression in subsequent agonistic encounters in opposition to vehicle-injected males (Trainor *et al.* 2004). Moreover, in an African cichlid fish (Mozambique tilapia, *Orechromis mossambicus*) the administration of androgen antagonists blocks the winner effect (Oliveira *et al.* 2009). Altogether, these results demonstrate that androgens have a role in the integration of past social encounters, regulating aggression in future interactions (Wingfield 2005). Actually, it is the integration of information related to the social environment with internal features, such as previous social experiment and

organism condition, which allows individuals to respond adaptively to permanently changing social environments (Taborsky and Oliveira 2012; Oliveira and Oliveira 2014). Hormones, such as androgens, seem to be major players in this process acting as neuromodulators (Oliveira 2009).

On the other hand, there is a growing body of literature that recognizes the influence of the Social Decision Making Network (SDMN), a set of brain areas that together control social behavior (Goodson 2005; O'Connell and Hofmann 2011). The SDMN is constituted by interconnected core nodes whose concerted activity patterns correlate with the expression of distinct social behaviors, such as aggressive, mating or parental behaviors (e.g. Newman 1999; O'Connell *et al.* 2012; Maruska *et al.* 2013). These brain nodes are mainly located in the forebrain and express sex-steroid and neuropeptide receptors, allowing the neuromodulation of the network by these hormones, and in specific androgens (Goodson 2005; O'Connell and Hofmann 2011; Oliveira 2012). Moreover, the SDMN seems to be evolutionarily conserved in all taxa (O'Connell and Hofmann 2012), and it has been extensively studied in non-mammalian species such as birds, reptiles and also teleost fish (e.g. Teles *et al.* 2015; Roleira *et al.* 2017; Kabelik *et al.* 2018; Eswine *et al.* 2019). Thus, this could be the explaining mechanism by which transient changes in circulating androgens - induced by social interactions - influence future behaviors. Also, changes in gene expression patterns of this network should result in contrasting brain transcriptomes and consequently different behavioral patterns (Cardoso *et al.* 2015), which highlights the relevance of transcriptomic studies in disclosing these rapid shifts in the state of the neural network.

The aim of our study was to elucidate the mechanisms on how androgens affect behavior by investigating the effect of a physiological and transient increase of androgens in the brain. For this purpose, we characterized brain gene expression temporal patterns after pharmacologically manipulating animals' hormonal states. We used Mozambique tilapia, an African cichlid fish with a lek-mating system (Fryer and Iles 1972). In this species, males exhibit two contrasting phenotypes. Dominants are usually larger, dark colored and establish territories to which they attract females and mate; while subordinates are faded color similarly to females and are not able to hold territories (Oliveira and Almada 1998). In the Mozambique tilapia, androgens influence social behavior and also respond to the social environment (Oliveira 2009). In this study, we injected dominant male fish either with 11-ketotestosterone (KT) or testosterone (T) and compared with a group injected with vehicle solution. Since the expression of androgen receptors in the forebrain of teleost fish is broad (e.g. *P. notatus*,

Forlano *et al.* 2010; *Carassius auratus*, Gelinas and Callard 1997; *A. burtoni*, Harbott *et al.* 2007; Munchrath and Hofmann 2010), we collected forebrain samples at different sampling times after treatment injection and analyzed their transcriptome profiling with RNAseq.

2. Materials and methods

2.1. Animals and housing

O. mossambicus adult males from a stock held at ISPA were used in this experiment. Fish were maintained in glass tanks (120 x 40 x 50 cm, 240 l) with a fine gravel substrate. Tanks were supplied with a double filtering system (gravel and external biofilter) and constant aeration. Water quality was analyzed twice per month for nitrites (0.2–0.5 ppm), ammonia (<0.5 ppm, Pallintest kit) and pH (6.0 - 6.2). Fish were kept at a temperature of 26 ± 2 ºC, a 12L:12D photoperiod, and fed with commercial cichlid floating sticks. Ninety-nine focal dominant males (weight: mean body mass ± SEM: 44.64 g ± 1.00 g; mean standard length ± SEM: 11.23 cm ± 0.12 cm; age: 2.5 - 3 years old) were used in this study. There was no difference in body size or weight between treatments (see below; $t(18) = 1.767$, $p = .094$).

Males' social status was monitored several times per week and territorial males were identified based on nuptial black coloration and exhibition of reproductive behaviour, including territory defense and digging of a spawning pit in the substrate, for at least 1 week (Oliveira and Almada 1996).

2.2. Experimental setup

Subjects were lightly anaesthetized (MS-222, Pharmaq; 300 ppm) to be weighted and measured and then individually housed in experimental tanks. Each experimental tank (50 × 25 × 30 cm, 40 L) had opaque lateral walls to prevent male's visual contact with adjacent tanks. After 1 week of isolation, focal males were arbitrarily assigned to one of the following treatments: intra-peritoneal (i.p.) injection with (1) 11-ketotestosterone (KT-treated group); (2) testosterone (T-treated group); or (3) vehicle (V-treated group). Focal males were injected, returned to experimental tanks and sampled 15, 30 or 60 min after injection to collect blood (sample size of 8-12 per group) and/or brain. A control group, similarly isolated for one week but not injected, was sampled for blood to measure baseline androgen levels. To reduce hormonal

fluctuations associated with natural circadian rhythm, the experiment was conducted in the morning.

11-ketotestosterone dose (Steraloids, 0.02 μg/g BW) was selected based on a pilot experiment where three different doses were tested in castrated male fish. We selected the dose that produced a significant physiological increase in circulating levels (Figure S1) similar to the one observed for this species in male-male interactions (Almeida *et al*. 2014). Testosterone (Steraloids) concentration used was also 0.02 μg/g based on a previous study (Huggard *et al.* 1996). Stock hormones were dissolved in 100 % ethanol to a concentration of 0.5 mg/ml and then diluted in saline solution (0.9% sodium chloride) until their final concentration. Vehicle solution consisted in 0.05% ethanol in saline solution.

2.3. Blood sampling

Males were anaesthetized (MS-222, Pharmaq; 450 ppm) and blood was collected from the caudal vein using heparinized 25-gauge needles. Blood sampling always took place within 4 min of the induction of anaesthesia to prevent possible effects of handling stress on steroids levels (Foo and Lam 1993). Blood samples were centrifuged (10 min, 3000 *g*, 4ºC) and plasma was stored at – 20ºC until further processing.

2.4. Hormone assays

11-ketotestosterone (KT) and testosterone (T) free steroids were extracted from plasma samples by adding diethyl-ether (Merck). Samples were then agitated for 20 min, centrifuged (5 min, 163 *g*, 4ºC) for phase separation and kept at -80 ºC for 15 min to freeze the water phase and separate the ether fraction (containing the free steroid). This process was repeated twice to obtain higher extraction efficiency. Ether fraction was evaporated with a speedvac (Savant SC1101) and the dried organic phase was re-suspended in phosphate buffer. Steroid concentrations were measured by radioimmunoassay using a T antibody from Research Diagnostics Inc (#WLI-T3003, rabbit anti-testosterone). The antibody used for KT was kindly donated by D. E. Kime and the corresponding specificity table was published in Kime and Manning (1982). The reactive marker used for T was from Amersham Biosciences ([1,2,6,7-3H] Testosterone, #TRK402-250 μCi) while KT marker was produced in-house from marked cortisol (Kime and Manning 1982). Inter-assay variabilities were 5.3 % for KT and 8.2 % for

T. Intra-assay variation coefficients were 2.4 %, 2.1 % and 7.6 % for KT and 8.9 %, 8.2 % and 4.5 % for T.

2.5. Tissue processing and RNA extraction

We randomly selected 5 focal males for brain analysis from each one of the following experimental treatments: KT-group (2 sampling time points: 30 min and 60 min), T-group (2 sampling time points: 30 min and 60 min) and V-treated group (2 sampling time points: 30 min and 60 min). In total we sacrificed 30 individuals, with an overdose of MS-222 (Pharmaq; 800 ppm). These sampling times take in consideration the time course of the socially driven androgen response in *O. mossambicus* which shows two peaks, an earlier one at 5-15 min and a late one at 60-90 min, and aim to assess the effects of the early androgen peak on brain state. Although no data is available for *O. mossambicus* on the time lag between the circulating and brain androgen peak in response to social interactions, it is known from other species that steroids in the brain peak 20-30 min later than in plasma (Droste *et al.* 2008; Remage-Healey *et al.* 2008). After sectioning of the spinal cord, forebrain area (olfactory bulbs, telencephalon and diencephalon) was dissected under a stereomicroscope (VWR SZB200) and collected in 500 µl of Qiazol lysis buffer (RNeasy Lipid Tissue Mini Kit, Qiagen). Samples were stored at -80 ºC until RNA extraction. Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen) with some protocol adjustments. Briefly, samples were homogenized with a pellet pestle motor (Kontes) and added 100 µl of chloroform. Incubation times were increased in order to maximize RNA recovery and in the end samples were diluted in 50 µl of RNase-free water. DNase digestion was performed to guarantee samples free of DNA contamination. RNA quantity was assessed using a Nanodrop spectrophotometer (Thermo Scientific) and RNA integrity was confirmed using Bioanalyzer (Agilent). RNA was stored at -80ºC until processing.

2.6. Library preparation, RNA sequencing and reference genome mapping

cDNA was generated with SmartSeq2 protocol (Picelli *et al.* 2014) and libraries were prepared with an optimized Nextera protocol (Baym *et al.* 2015).

RNA libraries of the 30 samples were pooled and sequencing was performed by the Centre for Genomic Regulation (CRG, Barcelona, Spain). cDNAs were amplified according to the Illumina RNA-Seq protocol and sequenced in three lanes using the Illumina HiSeq 2500 v4 system as paired-end 75-bp reads so that 200-300 million reads per sample could be achieved.

Quality of the data was checked with FASTQC software (Andrews 2010). Cutadapt (Martin 2011) was used to remove low quality reads and adapter sequences. The clean reads were mapped onto the Nile tilapia, *Oreochromis niloticus*, reference genome (Oreochromis_niloticus.Orenil1.0.92) using Hisat2 (Kim *et al.* 2015). Quality control of alignments was ascertained with Qualimap (Okonechnikov *et al.* 2016) and the table of counts was generated with HTSeq (Anders *et al.* 2015).

2.7. Data analysis

2.7.1. Hormone analysis

Normality of steroid data was tested by analyzing skewness and kurtosis values (Kline 1998) and running Shapiro-Wilk tests. Hormone variables were log-transformed to meet parametric assumptions. Outliers were identified using a generalized extreme studentized deviate procedure ($p = 0.05$, maximum number of outliers set to 20% of the sample size) and removed from data. Homocedascity was confirmed with Levene's test. Hormone levels (KT, T) were analyzed using planned comparisons to compare steroid levels between each time-point (15, 30 and 60 minutes) and the baseline (no-injection group) for each treatment (KT-, T- or V-treated groups). P-values were adjusted using the Benjamini and Hochberg (1995) procedure. Effect sizes were computed for planned comparisons (Cohen's d). Statistical analysis was performed using R (R Core Team, 2015) and STATISTICA v.10 (StatsoftInc).

2.7.2. Differential gene expression analysis

Gene counts were imported to R, and edgeR package was used for gene expression analysis (Robinson *et al.* 2010). We filtered genes with very low levels of expression levels and retained genes expressed in at least 3 samples. An exploratory analysis was performed by multidimensional scaling (MDS) to check relative similarities among replicates. One of the samples from the V-treated group was identified as an outlier and excluded from further analyses.

Differentially expressed (DE) genes were determined for each experimental group (KT- and T-treated groups) using the V-treated group as a reference. Counts were normalized using the TMM normalization method and the generalized linear model (GLM) likelihood ratio (LR) test for significance was implemented in edgeR (Robinson *et al.* 2010) for each time point

separately (30’ and 60’). P-values were adjusted for multiple testing using false discovery rates (FDR) with the Benjamini and Hochberg (1995) procedure. The threshold for DE genes was set to FDR < 0.1 and a fold change > 1.1. The RNAseq produced a total number of clean reads that ranged between 21.44 and 62.07 million reads. About 8.56 to 26.15 million reads were mapped onto the genome. For visualization of the global expression patterns of DE genes among treatment groups, a hierarchical clustering analysis was performed for each time point. The reliability of the hierarchical cluster was assessed by 1,000 bootstrap resampling of the expression values using the R package pvclust (Suzuki and Shimodaira 2006). Heatmaps were produced with the hclust function in R, adapted to produce a hierarchical clustering of Z-transformed expression values using Euclidean distance with complete linkage. A Principal Component Analysis (PCA) was also conducted to cluster samples by groups using DE genes.

Tilapia gene annotation and gene ontology terms were obtained from the ENSEMBL BIOMART database. GO term enrichment for genes detected as differentially expressed were evaluated in GOstats v2.42.0 (Falcon and Gentleman 2007), using a ‘conditional’ hypergeometric test with a P-value < 0.05. This method accounts for the hierarchical relationships of GO terms, and hence, a formal correction for multiple testing cannot be applied due to the implicit dependence between neighbouring GO terms, which do not comply with the independence of tests needed for correction of the p-values.

The relative contribution of GO enrichment data in terms of GO classes they represent was visualized using the GO slim vocabulary and the web tool CateGOrizer (Zhi-Liang *et al.* 2008).

2.8. Ethics statement

Experimental procedures used in this study were conducted in accordance with the institutional guidelines for the use of animals in experimentation and were approved both by the internal Ethics Committee of ISPA and by the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Portugal; permit number 0421/000/000/2013)

2.9. Data Availability

Raw sequence reads were deposited in BioProject portal at NCBI (BioProject ID PRJNA591471).

3. Results

Hormone levels

The levels of KT and T changed significantly with time and treatment (Table 1). Androgen treated fish (either KT-treated or T-treated) had a significant increase above baseline of the injected androgen 15 min and 30 min but not 60 min after administration (Table 1, Figure 1). There were no differences in either KT or T in fish injected with vehicle (V-treated group) (Table 1, Figure 1).

Table 1. Effect of time and treatment (KT, T or vehicle) on circulating hormone levels. Planned comparisons and effect sizes between the baseline and the other time points for each treatment.

	Planned Comparisons					
Comparisons	**Androgen-treated group**			**Vehicle group (V)**		
	t	**p**	**d**	**t**	**p**	**d**
KT						
0 min vs 15 min	**-4.681**	**<.0001**	**2.089**	-.3361	0.7378	.104
0 min vs 30 min	**-3.722**	**.001**	**1.857**	-1.362	0.355	.514
0 min vs 60 min	-1.126	.396	.578	.7978	.513	.101
T						
0 min vs 15 min	**-6.482**	**<.0001**	**3.449**	.6376	.5258	.416
0 min vs 30 min	**-2.715**	**0.025**	**1.486**	.2003	.9461	.339
0 min vs 60 min	-1.400	.332	.212	.0678	.9461	.200

11-ketotestosterone (KT); testosterone (T); t-test estimate; d: effect size estimate (Cohen's d); p: p-value after multiple comparison adjustment; statistically significant values are in bold.

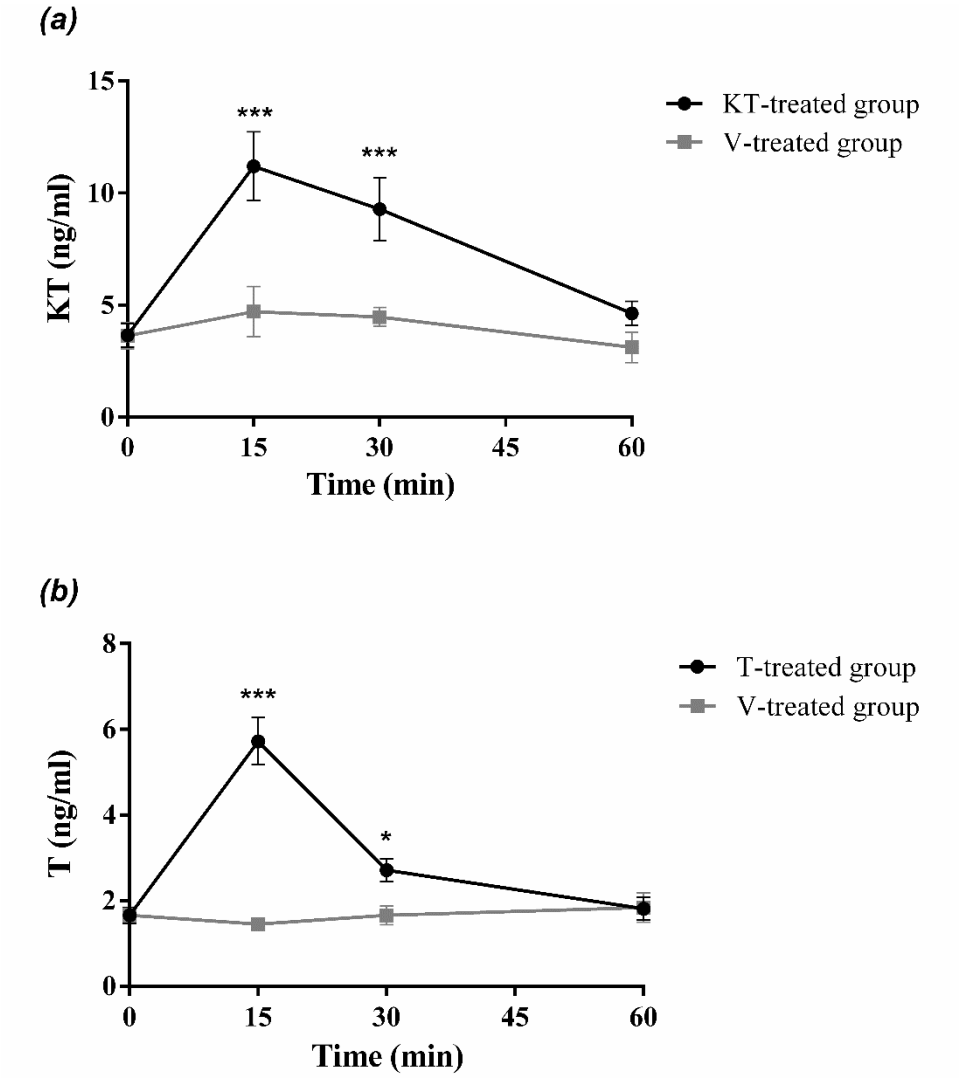

Figure 1. Temporal pattern of androgen circulating levels of fish injected with vehicle (V-treated group), 11-ketotestosterone (KT-treated group) or testosterone (T-treated group). Sample size for each point: 8-11 individuals. Values are mean ± standard error of the mean (SEM). *(a)* 11-ketotestosterone (KT) levels of V and KT-treated groups; *(b)* testosterone (T) levels of V and T-treated groups; * $p < 0.05$; *** $p < 0.001$.

Forebrain genomic response at 30 min after androgen administration

A total of 319 differentially expressed (DE) genes was observed in the KT-treated group compared with the V-treated group, of which 104 were up-regulated and 215 down-regulated (Figure 2c, Table S1). In the T-treated group, 101 DE genes were found compared with the V-treated group, of which 26 were up-regulated and 75 down-regulated (Figure 2c, Table S2). Eighteen genes were DE both in the KT- and T-treated groups relative to the V-treated group. Hierarchical clustering shows that although all V-treated individuals clustered together according to their DE genes, KT-treated and T- treated individuals did not cluster according to

their DE genes (Figure 2a). Principal component analysis showed that that the treatments tend to separate, with the first component explaining 59.4 % of the variance and separating the 3 treatments (Figure 2b), whereas the second component describes 18.5 % of the variance in DE genes and allows separation between the V-treated and the androgen treated groups.

The GO analysis (Tables S5 to S8) found different biological processes, cellular components and molecular functions enriched by DE genes between KT- and T-treated groups. For up-regulated DE genes, KT-treated group had enrichment of processes related to metabolism, carbohydrate metabolism, cell and catalytic and transporter activity, while T-treated group had a predominant enrichment of processes related to metabolism, development and cell differentiation (Figure 3, Tables S5 and S7). The vast majority of down-regulated DE genes of the KT-treated group were associated to metabolism and cell organization, cell and intracellular and binding and catalytic activity, while for the T-treated group, these genes were associated to transport, ion transport, cell and transporter activity (Figure 4, Tables S6 and S8).

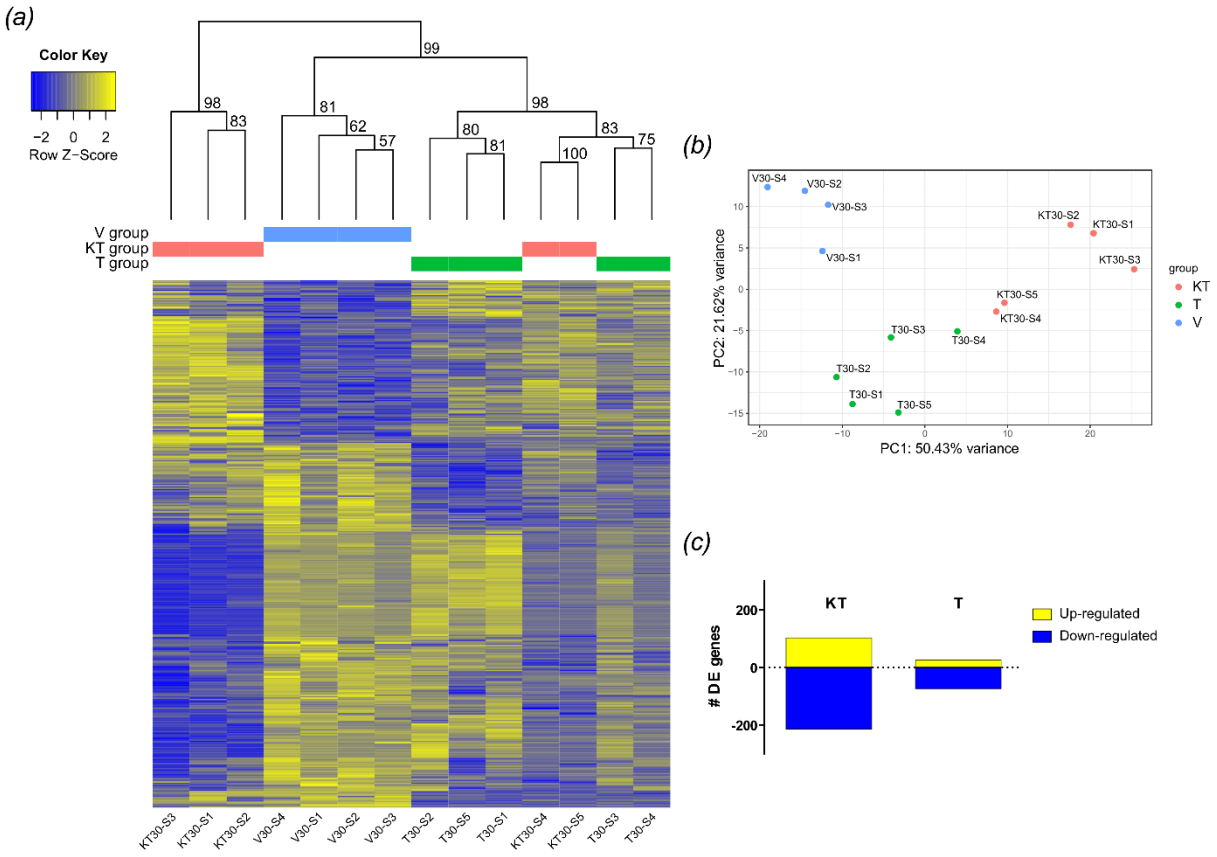

Figure 2. Differences in forebrain gene expression patterns of fish injected with vehicle (V-treated group), 11-ketotestosterone (KT-treated group) or testosterone (T-treated group) at 30 min post-injection: *(a)* Heatmap of differentially expressed genes. Intensity of color indicates relative expression levels of each gene (rows) in each treatment (columns), with blue representing downregulated transcripts and yellow upregulated transcripts. For each cluster obtained with hierarchical clustering, unbiased p-values (value between 0 and 1 but here in %) can be seen above the heatmap. These values were calculated via multiscale bootstrap resampling, indicating how strong the cluster is supported by data. *(b)* Principle Component Analysis (PCA) of DE genes of fish from the three treatment groups. *(c)* Number of differentially expressed genes of fish injected with 11-ketotestosterone (KT-treated group) or testosterone (T-treated group) using a vehicle group (V-treated group) as a reference group.

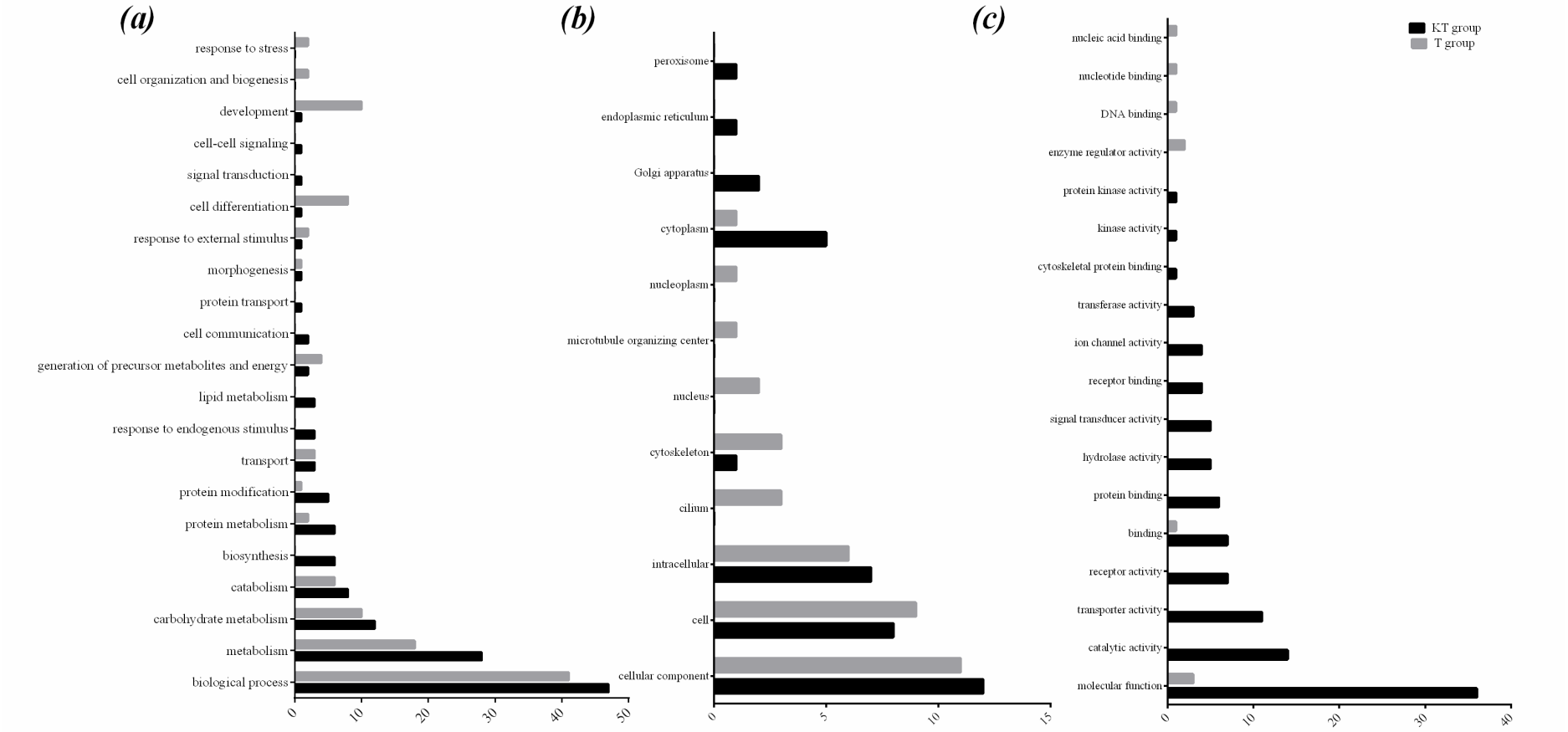

Figure 3. Differences between KT-treated and T-treated fish at 30 min post-injection in the representation of DE genes in the gene ontology (GO) classes for each ontology: (a) Biological Process, (b) Cellular Component and (c) Molecular Function. Enriched GO terms were obtained for upregulated transcripts for each treatment group and mapped to a total of 127 GO slim ancestor terms with CateGOrizer.

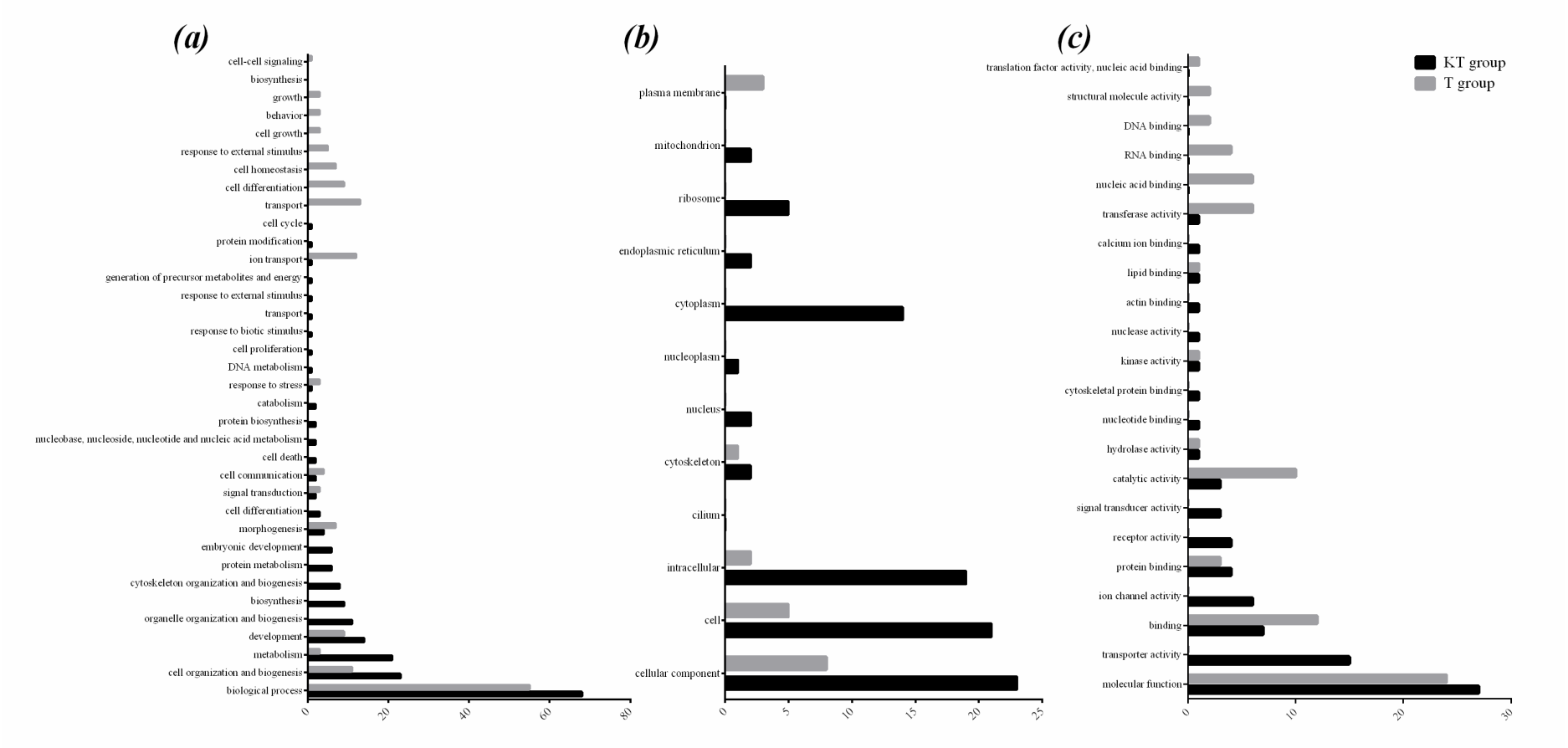

Figure 4. Differences between KT-treated and T-treated fish at 30 min post-injection in the representation of DE genes in the gene ontology (GO) classes for each ontology: (a) Biological Process, (b) Cellular Component and (c) Molecular Function. Enriched GO terms were obtained for downregulated transcripts for each treatment group and mapped to a total of 127 GO slim ancestor terms with CateGOrizer.

Forebrain genomic response at 60 min after androgen administration

Only 1 differentially expressed (DE) gene was observed in the KT-treated group compared with the V-treated group, which was down-regulated (Table S3). In the T-treated group, 8 DE genes were found compared with the V-treated group, 1 up-regulated and 7 down-regulated (Table S4). The DE gene observed in the KT treated-group was not present in the list of DE genes obtained for the T-treated group. Hierarchical clustering shows that although all except two individuals clustered following their treatment according to their DE genes (Figure 5a). Principal component analysis shows that that the groups tend to separate, with the first component explaining 65.0 % of the variance and separating all groups. The second component describes 12.5 % of the variance of DE genes (Figure 5b).

The GO analysis (Tables S9 to S11) identified different biological processes, cellular components and molecular functions enriched by DE genes between KT- and T-treated groups. The vast majority of down-regulated genes of the KT-treated group were associated to ion transport and transporter activity, while for the T-treated group, these genes were associated to metabolism and catalytic/hydrolase activity (Figure 6, Tables S9 and S11).

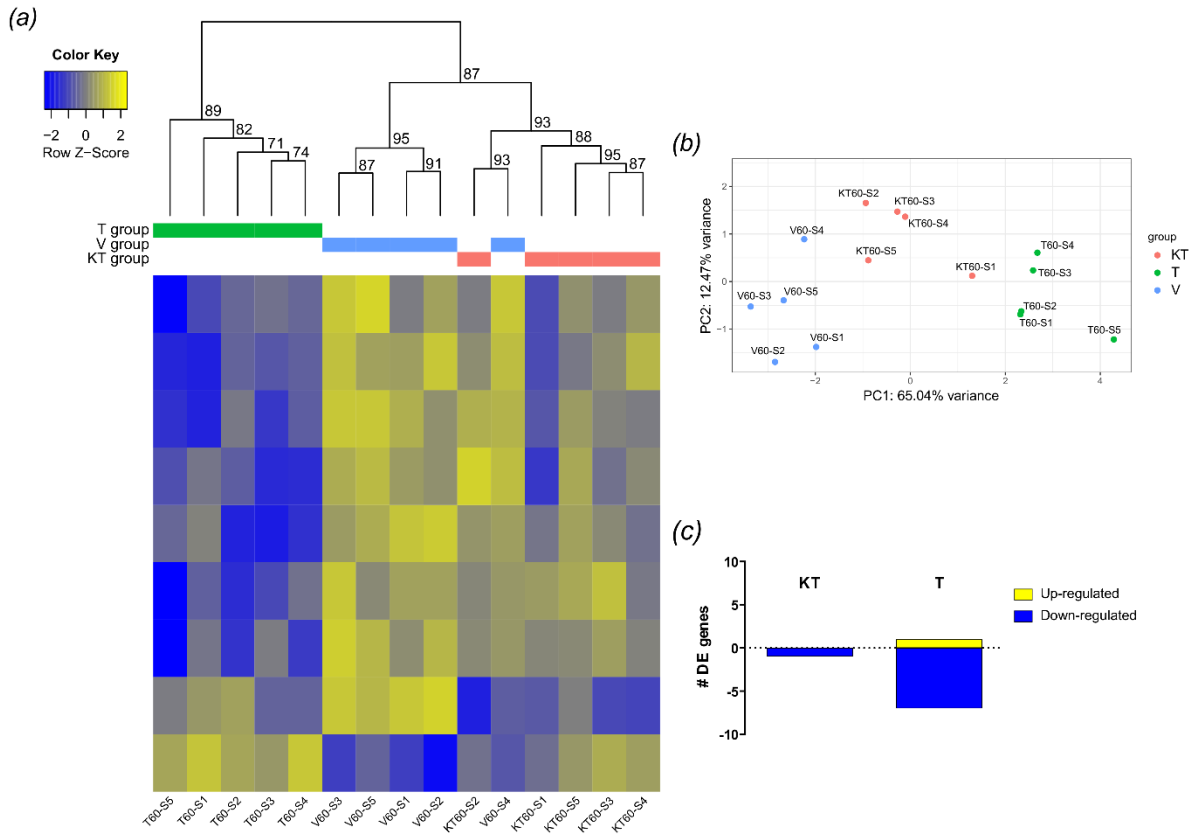

Figure 5. Differences in brain expression patterns of fish injected with vehicle (V-treated group), 11-ketotestosterone (KT-treated group) or testosterone (T-treated group); sampling

time point of 60 min. *(a)* Heatmap of differentially expressed genes. Intensity of color indicates relative expression levels of each gene (rows) in each treatment sample (columns), with blue representing downregulated transcripts and yellow upregulated transcripts. For each cluster obtained with hierarchical clustering, unbiased p-values (value between 0 and 1 but here in %) can be seen above the heatmap. These values were calculated via multiscale bootstrap resampling, indicating how strong the cluster is supported by data. *(b)* Principle Component Analysis (PCA) of DE genes of fish from the three treatment groups. *(c)* Number of differentially expressed genes of fish injected with 11-ketotestosterone (KT-treated group) or testosterone (T-treated group) using a vehicle group (V-treated group) as a reference group.

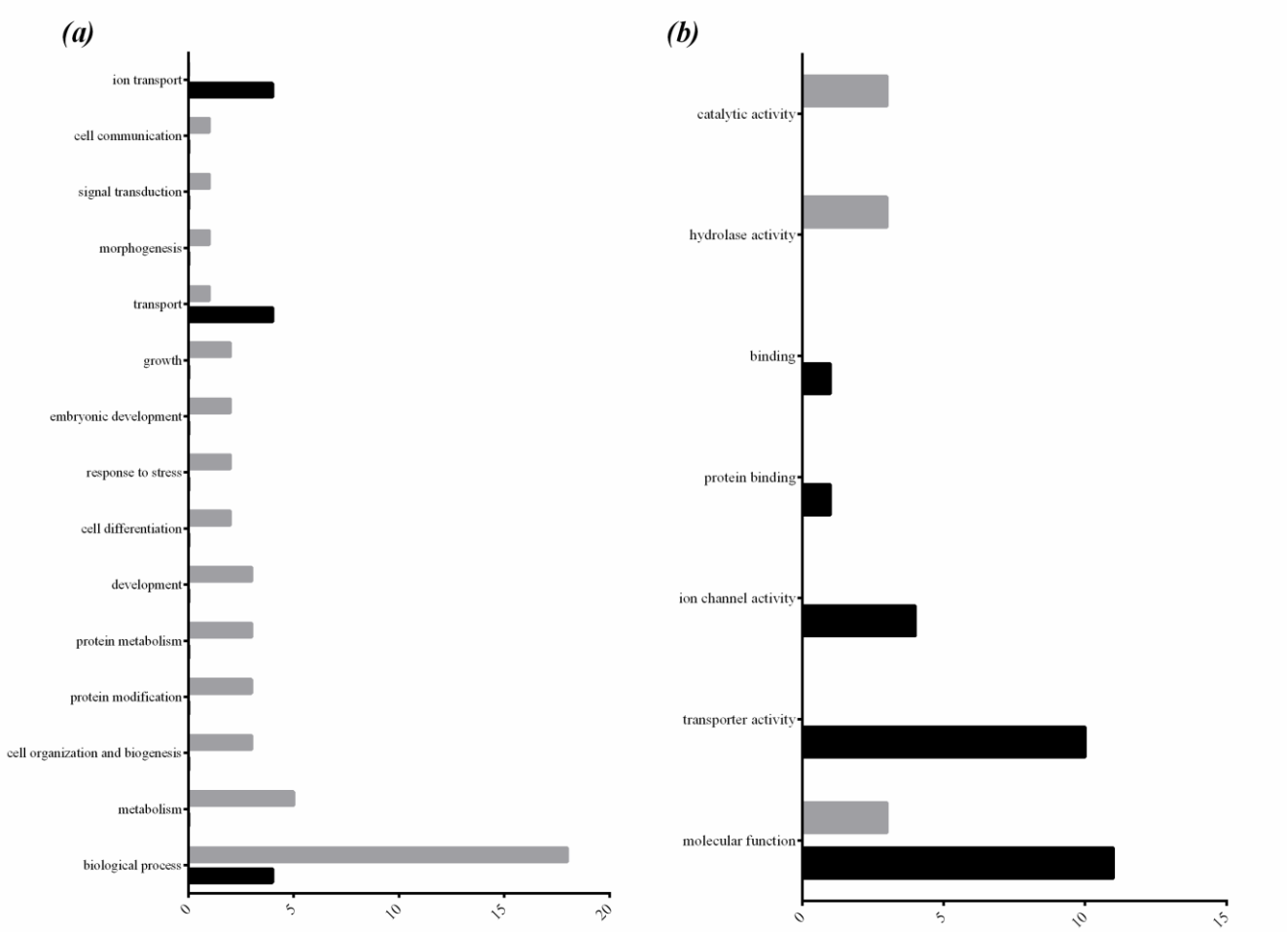

Figure 6. Differences between KT-treated and T-treated fish at 60 min post-injection in the representation of DE genes in the gene ontology (GO) classes for each ontology: (a) Biological Process and (b) Molecular Function. Enriched GO terms were obtained for downregulated transcripts for each treatment group and mapped to a total of 127 GO slim ancestor terms with CateGOrizer.

4. Discussion

Our results show that a physiological and transient increase of circulating androgens, which mimics the transient androgen response to social interactions, induces significant changes in the pattern of forebrain gene expression in Mozambique tilapia territorial males. Individuals injected with KT experienced a transient increase of KT levels and had a higher number of genes differentially expressed relative to vehicle-treated fish, than individuals injected with T which also had a transient increase of T levels. Moreover, in both androgen treatments there were more genes differentially expressed in the forebrain 30 min after the injection than in 60 min after the injection. Together these results indicate that transient changes in circulating KT have a higher impact in changes in the forebrain transcriptome, which may underly adaptive behavioural responses to social challenges.

A growing body of research has adopted genomic scale gene expression studies to unravel the brain mechanisms associated to social interactions (e.g., mating behavior, Lawniczak and Begun 2004; affiliative interactions: Shpigler *et al.* 2019; agonistic interactions: Oliveira *et al.* 2016; social eavesdropping: Lopes *et al.* 2015; mate choice: Cummings *et al.* 2008). Specifically the transcriptomic response to social challenges posed by brief territorial intrusions have been described in a comparative maner across taxa (i.e. in the house mouse, *Mus musculus*, the threespined stickleback, *Gasterosteus aculeatus*, and in the honey bee, *Apis mellifera*) and genes related to hormones are commonly affected (Rittschof *et al.* 2014). On the other hand, the effect of chronic exposure to androgens on the brain during development or in adulthood has been documented (Peterson *et al.* 2013; Ghahramani *et al.* 2014). However, to our knowledge, the specific effect of an acute and transient peak of androgens, like the one observed in response to social interactions, on the brain, has not been investigated.

In teleost fish, KT is considered the main circulating androgen since it has a higher impact than T on spermatogenesis, secondary sex characters and sexual behavior (reviewed by Borg, 1994). In several teleost species, including the Mozambique tilapia, KT responds to social interactions, contrary to T (e.g. Hirschenhauser et al., 2004, Oliveira et al., 1996). The present study confirms KT as more effective than T in producing significant changes in brain transcriptome. Interestingly, in this study both androgens are shown to induce the differential expression of several (>100) genes in the brain of the Mozambique tilapia. However, different sets of genes are DE-expressed in KT and T treated fish. For the KT-treated group (30 min sampling time point), several genes involved in the regulation of translation (e.g. ribosomal proteins) or steroid metabolism (dehydrogenase/reductase, cholesterol 24-hydroxylase) were

differentially expressed but many other genes were affected. For instance, kisspeptin-2, a gene known to modulate gonadotropin secretion (Nile tilapia, Park *et al.* 2016) and estrogen receptor membrane were downregulated, while the immediate early gene *c-fos* was up-regulated. On the other hand, it is known that steroids can induce changes in a question of minutes or even seconds through nongenomic mechanisms, typically involving intracellular second messengers (mostly calcium changes) and signal transduction cascades (Michels & Hoppe, 2008). So far, it has been described the activation of membrane receptors, hormone-binding globulin receptors, protein kinases or the regulation of voltage- and ligand-gated ion channels and transporters (reviewed in Michels and Hoppe 2008). Importantly, some of these mechanisms affect gene transcription (Foradori *et al.* 2008). In this study, we detected the up-regulation of ion channel membrane receptors (glycine receptors, *glra2*, *glrbb*; G-protein coupled receptors, *gprc5bb*; glutamate receptors, *grik1b*), also of auxiliary proteins of glutamate receptors of the AMPA-subtype (cornichon and pentraxin, Greger *et al.* 2017) and protein kinases (e.g. *mapk11*), probably to be used in these rapid androgen effects. Also, and as already mentioned, androgens can have oncogenic effects, thus, several of the reported DE genes for the KT-treated group are associated with tumors (e.g. phosphoglycerate mutase 1, cathepsin Z, ephrin, Pernicova *et al.* 2011; Beauchamp and Debinski 2012; Hitosugi *et al.* 2012) while others are involved in neuroprotection (e.g. *mapk11*, Nguyen *et al.* 2005) or neuronal growth (limbic system associated membrane protein, Pimenta *et al.* 1995), supporting previous evidence for the opposition between neuroprotective and neuroendangering roles of androgens (Foradori *et al.* 2008). For the T-treated group (30 min sampling time point), secretagogin, a tumour marker (Birkenkamp-Demtroder *et al.* 2005) is up-regulated, while programmed cell death 1 and death effector domain-containing 1, genes involved in apoptosis, are down-regulated (Inohara *et al.* 1997; Sharpe *et al.* 2007). However chromatin- interacting genes were up-regulated (barrier-to-autointegration factor-like protein (Oh *et al.* 2015), suggesting the existence of epigenetic mechanisms underlying an increase of plasma testosterone. Together these results suggest that KT and T play distinct roles in the regulation of brain molecular processes.

The current study also intended to explore the dynamics of gene expression. The importance of characterizing the temporal dynamics of brain activity in behavioral genomics has been highlighted by some authors (Rittschof and Hughes 2018; Renn and Aubin-Horth 2019). A time course study of the transcriptomic response in the threespined stickleback after short territorial intrusions has found several sets of genes whose expression profile changed in concert together, originating different gene clusters with different temporal expression patterns (Bukhari *et al.* 2017). Moreover, genes belonging to each cluster had a similar function

(Bukhari *et al.* 2017). This work supports the hypothesis that multiple waves of transcription are produced in response to the social environment, with a first genomic response more related to stimulus perception, followed by a second wave of genomic response responsible for the behavioral response, then recovering and finally adjusting future behavior (Aubin-Horth and Renn 2009; Bell and Aubin-Horth 2010; Clayton 2013). The existence of waves of gene expression is also supported in the honey bee with a similar behavioral paradigm (Shpigler *et al.* 2017). Likewise, our results emphasize that gene expression is dynamic and that selecting only a single sampling time point may miss the peak of transcriptomic response since at 60 min after androgen administration very few genes were differentially expressed. In contrast, at 30 min post-treatment a significant wave of transcription has been detected with most of the DE genes being down-regulated, in line with the results of Bukhari et al. (2017), suggesting that individuals respond first by down-regulating brain activity and afterwards up-regulating it. An important aspect to highlight is that in our study, we focused on a large brain area, the forebrain, that encompasses most nuclei that make up the social decision making network. Therefore, we captured the overall response of this network to transient androgen changes but we do not provide detail on putative regional differences across this network in the neurogenomic state of each of its nodes. In another transcriptomic study conducted in male threespined stickleback (*Gasterosteus aculeatus*), it was found that several genes were up-regulated in the diencephalon and down-regulated in other brain areas in response to a territorial challenge (Sanogo *et al.* 2012). These results confirm the idea that each brain region has its own distinct neurogenomic response, and even if the same genes are differentially expressed in different regions, they can have in fact opposite regulatory directions (Sanogo *et al.* 2012). Therefore, with ongoing methodological developments and the reduction of sequencing costs, future studies should gain from the characterization of the transcriptomic response of each of the brain nuclei that together make up the SDMN. Nevertheless, the present study features substantial elements responsive to androgens, specially 30 min after an androgen rise in the blood and can be seen as a first proof of principle for the mode of action of socially driven changes in androgens on subsequent social behavior.

In summary, our findings suggest that a transient rise of circulating androgens, such as the one observed after social interactions elicits relevant transcriptional changes, part of a integrative process of adjusting future behaviors and promoting adaptive and socially competent behaviors.

Author Contributions

ASF and RFO designed the experiment. ASF and AR performed the experiment. ASF processed samples. ASF and SDC analyzed the data. ASF and RFO wrote the paper with contributions from AR and SDC.

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CHAPTER 6

General Discussion

1. Overview of results

This thesis intended to explore the neural and endocrine mechanisms which regulate social behavior in the cichlid fish, *O. mossambicus*. To accomplish this, we proposed to investigate several of the components of social behavior, examining how the brain controls behavior, assessing how hormones act in the brain and influence behavior and conversely how social behavior affects hormones.

First, we focused on the Social Decision Making Network (SDMN), adding further evidence of the existence of a diffuse network, instead of localized brain regions that control social behavior (CHAPTER 2). Moreover, we tried to understand if the pattern of activation of the SDMN changes when individuals assess the outcome of social interactions or if it is just dependent on the expression of social behavior *per se*. Interestingly, all treatments elicited distinct patterns in the SDMN, even though we were not able to induce the perception of outcome in one of the experimental treatments. So, the perception of the outcome of the social interaction is not a necessary condition to trigger a SDMN response, suggesting that, at least in agonistic interactions, the mutual assessment of relative fighting behavior drives these acute changes in the state of the SDMN.

In CHAPTER 3, we manipulated the AVT system to evaluate its specific role in the behavior of our model species. We obtained a consistent effect of AVT on the behavior of males towards females but not towards males. Interestingly, we found that the blockage of V1A receptors in gonad-intact fish affected their aggressiveness towards females, suggesting an interaction between androgens and AVT in the regulation of this behavior in the Mozambique tilapia.

CHAPTER 4 determined the temporal pattern of androgen levels in response to territorial intrusions and related it to inter-individual variation in terms of the behavioral phenotype of each individual. We found distinct patterns of androgen response to social interactions due to underlying individual differences in the scope for response that were in turn associated with aggressive and exploratory behavior. In addition, we analyzed the temporal pattern of androgen levels after a GnRH injection. Altogether, our results suggest that different mechanisms may regulate the androgen response to social interactions.

Finally, we examined the specific effect that a temporary peak of androgen circulating levels may exert in the brain (CHAPTER 5). It has been hypothesized that androgens respond to the social environment as a way to adjust the behaviour of individuals to future interactions in an optimized manner. For this purpose, we treated fish with androgens and studied brain

transcriptomic changes with the RNAseq technique. Our results showed that 11-ketotestosterone elicited more accentuated changes than testosterone and that transcriptomic impact was greater at the 30 minutes than at the 60 minutes' sampling point. Moreover, this study unveiled relevant genes in the brain whose expression is affected by a surge of circulating androgens.

2. The Social Decision Making Network

Since Newman (1999) introduced the concept of the Social Behavior Network (SBN) in mammals, a considerable amount of literature has examined the regulation of social behavior by a dynamic network of brain nuclei across different vertebrate taxa, including teleost fish (Goodson, 2005; Teles, Almeida, Lopes, & Oliveira, 2015). Challenging the dogma of neural localization of behavioral function, the SBN brought a fresh perspective by stating the existence of core nodes that together control social behavior, such that the state of the network better explains the behavioral output than the activity of a single node *per se* (Newman, 1999). All of these brain nuclei are reciprocally interconnected with each other, such that differential activation of the nodes creates dynamic patterns responsible for multiple behaviors. Later, O'Connell and Hofmann (2011) proposed the Social Decision Making Network (SDMN), a wider model which included the Mesolimbic Reward System.

In the Mozambique tilapia, the existence of the SDMN has already been supported in the scope of the "audience effect" phenomenon (Roleira, Oliveira, Lopes, & Oliveira, 2017). In our work, by using agonistic interactions, we showed that different behavioral states are paralleled by different neurogenomic states of the SDMN as captured by the pattern of expression of immediate early genes in the sampled nodes of the SDMN.

In zebrafish, and as already pointed out (see section 2 of CHAPTER 1), it has been shown that mirror fighters as well as winners and losers of real fights have distinct brain activation profiles (Teles et al., 2015) suggesting that the assessment of fight outcome is the key explaining factor underlying the existence of shifts in functional connectivity between the nodes of the SDMN, in the scope of aggressive interactions. Another study, in the same species, addressed this issue by staging agonistic interactions and assessing its broad effect on the brain with a genome-wide microarray chip (Oliveira et al., 2016). In this work, the brain transcriptome of fish that fought against mirrors and also the brains of winners and losers that participated in real fights were examined. Interestingly, mirror fighters presented a transcriptomic profile similar to that of non-interacting fish, while winners and losers displayed

striking changes in their brain transcriptomes. These results suggest that neurogenomic responses associated to changes in social status, i.e., in the perception of the fight outcome, depend on the mutual assessment of fighting ability (Oliveira et al., 2016).

In the present thesis, we failed to induce the perception of outcome in one of the treatments (MD treatment) because both fish equally engaged in the fight. Probably, this could have been avoided by using one-way mirrors to avoid the opponents to interact with the focal fish. Notwithstanding this limitation, it allowed us to show that the pattern of activation of the SDMN changes not due to the assessment of the fight outcome nor it is just dependent on the expression of aggressive behavior. Instead, the mutual assessment of relative fighting behavior drives these acute changes in the neuromolecular restructuring of the SDMN.

Interestingly, the consistent expression of behaviors, associated with winning or losing (i.e., behavioral states) are not correlated with IEG expression in brain nuclei (Teles et al. 2015 and present study). Similarly, in males of the brown anole lizard, *Anolis sagrei*, it was also found that the pattern of activation of the SDMN was mostly independent of the specific induced behaviors, either in reproductive or aggressive contexts, although the activity of some nodes did correlate with behavior (Kabelik et al., 2018). These findings strengthen the idea that the activity of the SDMN vary with social context but the relationship between behavioral output and network activity is not straightforward.

Also, our results showed that even a short-term social interaction of 2 min triggers rapid and relevant changes in the pattern of activation and functional connectivity of the SDMN, enabling individuals to perceive their social environment (e.g. acquiring information relative to social status) and optimize their behavior accordingly in ongoing interactions (social competence, Oliveira, 2009).

Furthermore, androgens are known to respond to social interactions and, as stated by the challenge hypothesis, this response has an adaptive role in fine-tuning behavior to a competitive and demanding social environment. Contrary to our expectations, there was a lack of response of androgens in these experimental treatments that will be discussed below (section 4 of this chapter).

3. Vasotocin modulation of social behavior

Importantly, this neural network is modulated by steroids and nonapetides (Goodson, 2005). In the present work, we examined the effect of AVT in contexts that elicit courtship and aggression. The manipulation of AVT levels significantly affected the behavior of males

towards females and highlighted the involvement of V1A receptors but no effect was detected in the behavior towards males.

There are several possible explanations for this result. Different AVT subpopulations have been hypothesized to have distinct roles. Greenwood et al. (2008), proposed that gigantocellular AVT neurons modulate courtship and aggression, whereas parvocellular AVT neurons control subordinate behavior. These inferences were based on the fact that *A. burtoni* dominant males have higher AVT expression levels on gigantocellular neurons and lower AVT expression on parvocellular neurons while subordinates show the opposite pattern. In addition, parvocellular AVT expression rates in subordinates correlated with fleeing behavior and gigantocellular AVT expression in dominants correlated with gonadosomatic index (Greenwood et al., 2008). Another study, in the same species, observed increased activation of magnocellular cells in agonistic behaviors (and not in courtship) and emphasized the key role of magnocellular AVT neurons in aggression contrary to gigantocellular neurons (Loveland & Fernald, 2017). This study is in accordance with a previous one in zebrafish which established the same relationship between behavioral phenotypes and AVT subpopulations (Larson, O'Malley, & Melloni, 2006).

In the case of the Mozambique tilapia, anatomical and chemical differences are also evident between dominants and subordinates. Subordinate males have larger cell body areas of magnocellular and gigantocellular AVT neurons and submissive behavior correlates both with soma size of AVT cells in all three nuclei and the number of AVT magnocellular neurons (Almeida & Oliveira, 2015). Also, there is less AVT in the pituitary of dominant individuals when compared to subordinates (Almeida, Gozdowska, Kulczykowska, & Oliveira, 2012). These results further support the idea that in this model species distinct AVT subpopulations may exert contrasting modulatory effects responsible for increased behavior plasticity, which are not possible to distinguish with the experimental procedure used in the present work.

On the other hand, the existence of other mechanisms influencing the AVT system (Albers, 2012) may explain the lack of effect we obtained on aggressive behavior. For instance, serotonin (5-HT) is an important neuromodulator involved in the regulation of the HPG axis, stress response and also aggressive behavior (see Prasad, Ogawa, & Parhar, 2015 and Winberg & Thörnqvist, 2016 for comprehensive reviews of the serotonin role on teleosts' reproduction and behavior). It has been shown to modulate aggression in several species, including teleosts. In the rainbow trout (*O. mykiss*) and Atlantic cod (*Gadus morhua*), the increase of the serotonin precursor L-tryptophan in the fish diet reduces aggression (Höglund, Bakke, Øverli, Winberg, & Nilsson, 2005; Winberg, Øverli, & Lepage, 2001) Similarly, in the bluehead wrasse, *T.*

bifasciatum, treatment with fluoxetine, a selective 5-HT reuptake inhibitor responsible for increasing synaptic concentration of 5-HT levels (Stahl, 1998), decreases territorial aggression (Perreault, Semsar, & Godwin, 2003). Yet, in the Siamese fighting fish, *Betta splendens*, serotonin injections decrease aggression in mirror experiments but either fluoxetine or the increase of L-tryptophan in their diet has no effect on aggressiveness (Clotfelter, O'Hare, McNitt, Carpenter, & Summers, 2007). Moreover, in zebrafish, treatment with fluoxetine has no effect on aggressive behavior (Winberg & Thörnqvist, 2016) but using a specific antagonist for 5-HT1A receptors increases aggression (Filby, Paull, Hickmore, & Tyler, 2010). Although generalizability of these results is not straightforward, it is clear that serotonin is a key player in the regulation of social behavior. Furthermore, it seems to act in concert with other neuromodulators, such as AVT. For instance, in the bluehead wrasse, fluoxetine inhibits AVT expression of preoptic neurons without affecting their size (Semsar, Perreault, & Godwin, 2004), while icv injections of AVT in the rainbow trout increase serotonergic activity in the hypothalamus and in the telencephalon (Gesto, Soengas, Rodríguez-Illamola, & Míguez, 2014). Even though studies in teleosts on this matter are scarce, investigations carried in rodents are conclusive. Experiments have shown that, in general, aggression is promoted by AVP and inhibited by 5-HT, respectively through V1A and 5-HT1B receptors (Ferris et al., 1997; Ferris, Stolberg, & Delville, 1999; Ferris & Potegal, 1988). Importantly, in the Syrian golden hamster, these receptors colocalize in the anterior hypothalamus, a relevant area in the context of aggression, which is characterized with many AVP and 5-HT fibers and putative terminals (Ferris et al., 1997). Herein, putative 5-HT synapses on AVP neurons were also described (Ferris et al., 1997). Moreover, treatment with fluoxetine decreases aggressiveness but also impairs the effect of AVT on aggressive behavior (Ferris et al., 1997), suggesting that serotonin modulates the activity of AVP neurons. Altogether these cases support a relevant interaction between serotonergic and vasotocin system that is worth to be explored in further research, especially in teleosts.

Another significant aspect to consider is the role of androgens on the AVT/AVP system. Semsar and Godwin (2003) used the sex-changing fish, *T. bifasciatum*, to investigate this matter. In *T. bifasciatum* species, whenever large females gain social dominance, a female-to-male sex-change occurs, which is characterized by behavioral and morphological alterations. As already pointed out earlier (CHAPTER 1), in this species, AVT influences courting behavior (Semsar, Kandel, & Godwin, 2001). However, castration of females or males has no effect on behavior neither elicited changes on mRNA AVT levels (Semsar & Godwin, 2003). Yet, the same authors detected differences in the AVT immunoreactive soma size of gigantocellular

neurons (Semsar & Godwin, 2003). Hence, in this species, it seems that AVT exerts its action but independently of gonadal hormones, which is not surprising since ovariectomized females still undergo the behavioral sex change (Godwin, Crews, & Warner, 1996). An important issue emerging from these findings is that androgens had a clear morphological effect on AVT neurons suggesting that it is possible that in the Mozambique tilapia, the same phenomenon may have occurred, and in turn influenced behavior. Clearly, future studies should address putative morphological effects of castration on the AVT system of this species.

4. The androgen response to social interactions

The Challenge Hypothesis (Wingfield, Hegner, Dufty, Jr., & Ball, 1990) has been instrumental in providing a conceptual framework for numerous studies on the subject of social neuroendocrinology. Currently with more than 1900 citations, its influence on the field is unquestionable.

However, the generalizability of this model has been subject to certain limitations. Initially, the Challenge Hypothesis was proposed based on comparative data from bird species, but currently has been tested across all vertebrate taxa, including teleost fish (Hirschenhauser & Oliveira, 2006; Hirschenhauser, Taborsky, Oliveira, Canário, & Oliveira, 2004; Oliveira 2004). Yet, many published studies, including in birds, were unsuccessful in obtaining the expected androgen response in social interactions (e.g., rodents: Fuxjager & Marler, 2010; dwarf mongooses: Creel, Wildt, & Monfort, 1993; amphibians: de Assis, Navas, Mendonça, & Gomes, 2012; fish: Ros, Vullioud, Bruintjes, Vallat, & Bshary, 2014; reptiles: Baird, Lovern, & Shine, 2014; birds: Moore et al., 2004).

A very recent review and meta-analysis on birds of Goymann et al (2019) proposes an updated version of this model, in which female-male interactions have the pivotal role in determining male androgen responses. As such, in species with parental care behavior, males would have low androgen levels that actually should not respond to male-male interactions (to avoid paternal care suppression), but should rise due to the presence of reproductively active females (Goymann et al., 2019). Conversely, in the case of species without parental care, the same authors agree that these males should exhibit high androgen levels if females are available and also that androgen circulating levels would be able to increase in response to male challenges, because of the reduced costs associated to androgens in these mating systems (Goymann et al., 2019). This is not evident though in the case of some polygynous species

where males don't defend their fry (lizards: (Baird et al., 2014); rodents: (Fuxjager et al., 2010), but of course, they could be already at their maximum androgen physiological level as pointed by Goymann et al (2019).

Apart from this relevant and fresh perspective of the Challenge Hypothesis that should be tested in other taxa, other factors may explain the variability observed in the androgen response male-male interactions. In the present work, we demonstrated that the androgen response to social interactions is dependent on the individual's intrinsic features. Actually, we showed that even within the same species, individuals may have distinct patterns of hormonal response. Thus, in the case of the Mozambique tilapia, a polygynous species, some individuals may hold baseline androgen levels close to their physiological maximum and consequently they lack an androgen response to social interactions (as Goymann et al. 2019 highlighted), while others have a considerable scope of response. Moreover, we found that these physiological characteristics were correlated with behavioral traits such as exploration and aggressiveness.

An important feature that was not assessed in this study was whether androgen measurements were repeatable. Nevertheless, other studies already addressed this issue. For instance, a study in male dark-eyed juncos, *J. hyemalis*, found that testosterone response to the GnRH challenge, but not baseline levels, consistently varied between individuals (Jawor et al., 2006). Similarly, in males of eastern bluebirds, *S. sialis*, both baseline and GnRH-induced testosterone levels were repeatable (Ambardar & Grindstaff, 2017). So, further studies are recommended to confirm the repeatability of androgen variation in the Mozambique tilapia.

Still, the insights gained from our study may be of assistance to study behavior and/or physiology. Here, we raise important questions about the necessity to consider each fish as a unique individual. As a consequence, we should think carefully before disregarding those values that seem outliers. As Williams (2008) argues: 'individuals with such 'extreme phenotypes' could be very informative in understanding links between mechanism and phenotypic variation'. The use of repeated measures designs is also a strong recommendation. A study carried in zebra finches, *Taeniopygia guttata*, is a good illustration of this point. The authors compared the daily energy expenditure between nonbreeding and egg-laying life stages and found intra-individual variation ranging between -33 and 46% (Vézina, Speakman, & Williams, 2006). Importantly, at the population level, these changes were undetectable (similarly to our study). Additionally, this variability was repeatable (Williams, Vezina, & Speakman, 2009) and correlated with locomotor activity and reproductive effort (i.e. size and mass of clutch) (Vézina et al., 2006). Taken together, it is clear that researchers should expand

their vision beyond the "Golden Mean" and research carried in the scope of the Challenge Hypothesis should take this in consideration.

These findings may help us to understand why in the study of CHAPTER 2, we didn't find any response in androgen levels in fish that fought with real opponents. We can infer that we may have missed the increase of androgens, characteristic of social interactions, because we selected a time point of plasma collection in-between the two peaks of response or because of the inter-individual variation in the scope of the androgen response, which diluted any differences that might exist.

Other issue to address is the putative existence of two waves of androgen response to social interactions that may be regulated by different mechanisms (Oliveira, 2009). One hypothesis is that, like in the stress response, the androgen response to social interactions could occur at two different time scales (i.e. immediate neural mediated response followed by subsequent endocrine response within minutes). Research in mammals has confirmed the existence of a neuronal pathway responsible for the regulation of gonadal functions, such as testicular development (Nagai, Murano, Minokoshi, Okuda, & Kinutani, 1982) and testosterone production (Frankel & Ryan, 1981), independent of the pituitary gland (reviewed in Mayerhofer, 2007). Retrograde tracing studies, in Sprague Dawley rats, also showed that neurons from testes are connected by a multi-synaptical pathway with several brain areas, such as the paraventricular nucleus of the hypothalamus, central amygdala or the A5 noradrenergic group of the brainstem (Lee, Miselis, & Rivier, 2002). These fibers contact Leydig cells in the testis and regulate testosterone secretion (Selvage, Parsons, & Rivier, 2006; Selvage & Rivier, 2003). Electron-microscopy and immunohistochemical studies describe nerve bundles with neurotransmitters containing vesicles in testis close to Leydig cells (rat: Rauchenwald, Steers, & Desjardins, 1995; cat: Wrobel & Gürtler, 2001; piglet: Wrobel & Brandl, 1998; man: Nistal, Paniagua, & Abaurrea, 1982; Okkels & Sand, 1940; Prince, 1996). Testis innervation demonstrates to be essential for the gonads well-functioning (e.g., Gerendai, Nmeskeri, & Csernus, 1986). Moreover, it seems to be relevant in stress situations, where the sympathetic nervous system is required and the HPG functions appear non-essential (Frankel & Ryan, 1981; Sapolsky, 1986). Future studies will need to be undertaken to assess if the androgen response to social interactions is exclusively mediated by the hypothalamic-pituitary-gonadal (HPG) axis or if there is also a direct neural pathway controlling androgen release by the gonads.

Thus, a specific set of studies would be necessary: 1) blockage of the HPG axis, may be accomplished through ablation of LH and FSH cells in adults (e.g. transgenic NTR/mCherry line, Curado, Stainier, & Anderson, 2008) to confirm the existence of androgen production

independent of LH and FSH; 2) blockage of the sympathetic nervous system via local injury of nerves innervating the gonad or by chemical ablation (e.g. using a Cre-TH line crossed with the diphtheria toxin receptor (DTR) and then injecting diphtheria toxin (DT) locally (Zeng et al., 2015) or alternatively using a brain-sparing DT (Pereira et al., 2017); TH: tyrosine hydroxylase is one of the enzymes responsible for the synthesis of norepinephrine) to verify if this nerves are necessary for the androgen production; 3) activation of the sympathetic nervous system through local optogenetic stimulation is a common technique (e.g. ChR2/TH line, Zeng et al., 2015). Neither of these tools is available in the Mozambique tilapia, whereby using other model species would be recommendable. All these tools are available in mice.

5. Transcriptional regulation of brain gene expression after an androgen response

Rna-seq technology is currently used as a large-scale approach to access the transcriptome of a given cell or tissue allowing considerable advances on the study of biological processes (Qian, Ba, Zhuang, & Zhong, 2014). In CHAPTER 5, our study was designed to determine the effect of an androgen increase in the brain. Thus, we pharmacologically manipulated animals' hormonal states to gain insight into the molecular mechanisms associated with an androgen response. We were able to identify many genes that responded to a transient increase of androgens in the blood. The challenge is now to uncover how this set of genes is involved in orchestrating changes in behavior that allow individuals to respond appropriately to the social environment (Robinson, Grozinger, & Whitfield, 2005). Further research is encouraged, in particular, studies which adopt an integrated perspective, analyzing genome, transcriptome, epigenome and using 'reverse genomics' (Harris & Hofmann, 2014; e.g. using genetic tools such as interference RNA (iRNA) or CRISPR genome editing) to confirm the actual contribution of the putative genes that were found.

Interestingly, in this work, we obtained considerable individual variation, especially between individuals of the KT-treated group, which suggests that the same variation in androgen levels may elicit different brain transcriptomic responses and subsequently behavior outputs for different individuals. Thus, it would be interesting to explore, in the future, the link between androgens, behavior and personality as studied in CHAPTER 4, but at the brain level (Bell & Aubin-Horth, 2010). It is recommended, however, to perform further studies in brain macroareas or even minor areas to improve the resolution of the analysis.

Importantly, it is crucial to highlight that hormones affect behavior not only by changes in plasma steroid levels but also through changes in the number, affinity and specificity of

hormone receptors (Fuxjager et al., 2010), which may differ among species and individuals (Ball & Balthazart, 2008). For example, in *A. burtoni*, mRNA expression levels of several hormone receptors differ between males of different social ranks (Korzan, Fernald, & Grone, 2014). Interestingly, receptors expression respond acutely to changes in the social environment, as in the case of the teleost mangrove rivulus fish, *K. marmoratus*, where the expression levels of androgen receptors change in response to fights, and this effect seems to be dependent on the fight outcome and baseline androgen levels (Li, Earley, Huang, & Hsu, 2014). Moreover, androgens can bound to hormone-binding proteins that regulate their bioavailability (Oliveira, 2009; Zeginiadou, Koliais, Kouretas, & Antonoglou, 1997) or presumably be produced in the brain (Baulieu, 1998; Pradhan, Solomon-lane, Willis, Grober, & B, 2014; Schmidt et al., 2008), although this has not been confirmed in teleosts. Additionally, androgens can be converted in other steroids by specific enzymes (e.g. aromatase) as already mentioned (Cornil, Ball, & Balthazart, 2012; Roselli, Liu, & Hurn, 2009). Thus, hormonal modulation of behavior consists of an intricate system with several levels of control.

6. Final remarks

Having an appropriate response to other individuals, independently if they are potential mates or competitor conspecifics, is life-decisive. Adaptive behavior results from the continuous feedback between the nervous system, the body and the environment (Oliveira & Oliveira, 2014). In this thesis, we used different perspectives to uncover a few more pieces of the complexity associated with social behavior and the mechanisms by which it is regulated. Hormones, such as vasopressin and androgens, exert potent modulatory influence on social behavior by acting on a core of brain areas which together control behavior. On the other hand, the social environment influence hormones. Yet, neither the precise mechanisms by which the brain and the gonad articulate the androgen response nor how among-individual variation can influence androgen and behavioral responses to the social environment have been well-studied. These questions and others were raised along with the results obtained with this thesis and seem to be worthwhile to explore.

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APPENDIX A

Neuroendocrinology of Social Behavior in Teleost Fish

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Abstract

This review addresses the role of hormones on the regulation of social behavior of teleost fish. It is proposed that hormones regulate social behavior by acting upon an evolutionary conserved network of brain regions that controls the expression of the different types of social behavior from aggression, to mating to parental care. Evidence for the endocrine regulation of these different types of social behaviors is presented with a special focus on sex steroids and nonapeptides of the vasotocin/isotocin family. Finally, the role of some hormones as pheromones that influence social interactions is also discussed.

Keywords

Social brain; sex steroids; isotocin; vasotocin; aggression; mating; parental care

1. Introduction

Animals need to interact with other members from the same species in order to survive and reproduce successfully and the set of behaviors used in these social interactions are labelled social behaviors. From a functional perspective these social behaviors can be classified into major groups according to the type of interaction in which they are expressed, namely, aggressive, mating, parental and pro-social. Interestingly, despite their different functions, all these types of social behaviors seem to share a common underlying mechanism, composed of a neural network, recently named the social decision-making network (O'Connell and Hofmann, 2012a, 2011), whose overall state parallels the expression of the specific type of social behavior expressed in a given moment in time, such that the state of the network is a better predictor of social behavior than the activity of a specific brain region (Goodson and Kabelik, 2009; Teles et al., 2015). Moreover, the fact that all nodes in this network express receptors for steroid hormones and for neuropeptides opens the possibility for its neuromodulation and concomitantly for the regulation of its behavioral output by these agents. Thus, by acting on the social decision-making network hormones can regulate the expression of social behavior and integrate it with the organismal state of the individual. Hormones can also regulate the social behavior of animals other than the one in which they are produced when they act as hormonal pheromones. In such cases hormones are released into the environment and act on receptors located in sensory tissues of other individuals triggering changes on their behavior. In this review we will use the conceptual framework sketched above to illustrate how hormones regulate social behaviors in teleost fish.

2. The social decision-making network

2.1. Short-history of the concept

In 1999, Newman challenged the neuroscientific community by proposing the existence of a core set of brain areas that collectively regulate social behavior in mammals. Each one of these areas is reciprocally connected with the others, contains sex steroid hormone receptors and it is involved in the activation or regulation of several social behaviors. It was designated as Social Behavior Network (SBN) and it is composed of six limbic areas: the Lateral Septum (LS), the Medial Extended Amygdala (medial amygdala, meAMY and bed nucleus of the stria terminalis,

BNST), the Medial Preoptic Area (POA), the Anterior Hypothalamus (AH), the Ventromedial and Ventrolateral Hypothalamus (VMH), all localized in the forebrain, and the Midbrain Periaqueductal Gray and Tegmentum (PAG/CG), lying in the midbrain. Her model was based on a considerable amount of behavioral, neuroanatomical and neuroendocrine evidences from diverse studies in rodents and other mammals, which used electrical stimulation, neuropharmacological manipulations, specific brain lesions and detection of immediate early gene expression (IEG). Together, these data show that common areas jointly influence sexual, parental or even aggressive behavior, counteracting the idea of one area (or even a separate mini-circuit) is determining a specific behavior. Instead, all these areas represent the nodes of a neuroanatomical network, whose dynamic activation patterns are responsible for multiple behaviors. For instance, male sexual behavior would be the result of successive behavioral responses such as sniffing, mounting, ejaculation or grooming, which altogether are activated by this integrated circuit and modulated by environmental stimuli and sex steroids. Newman also highlighted that species and sex differences in social behaviors are a consequence of brain organization and connectivity divergences, influenced by variations in hormone sensitivity along development, on this central network.

Later, Goodson (2005) expanded the same framework to other non-mammalian vertebrates describing important evidences for birds and teleost fish, and providing foundations for the evolutionarily conservation of the SBN in vertebrates. He also contributed to a better insight into this network by adding the role of peptidergic neuromodulation such as arginine vasotocin (AVT, homologue of mammalian arginine vasopressin) or isotocin (IT, homologue of oxytocine) on social behavior and specifically as an integrating component of SBN.

More recently, O'Connell and Hofmann (2012a, 2011) proposed that social behavior would be regulated by an even wider network. The Social-Decision Making Network (SDMN) would include SBN and the Mesolimbic Reward System. The latter system is the brain circuit responsible for the evaluation of the salience of a stimulus (via dopaminergic signaling) prior to the behavioral response, which in turn is elicited by the former network. Thus, the reinforcing/rewarding component of social behavior as a substantial feature of an individuals' adaptive response to the environment is the main argument of the authors. The mammalian mesolimbic reward system is constituted by the Striatum (Str), the Nucleus Accumbens (NAcc), the Ventral Pallidum (VP), the Basolateral Amygdala (blAMY), the Hippocampus (Hyp), the Ventral Tegmental Area (VTA), and the LS and the meAMY/BNST, overlapping with nodes of the SBN. The authors performed a comparative analysis of the two neural circuits in five vertebrate lineages: mammals, birds, reptiles, amphibians and teleost fish. Putative brain

homologies were described based on neuronal connections, the presence of steroid hormone receptors, gene expression, neurochemistry, developmental and behavioral studies. Concurrently, they provided a very useful resource to study the neural substrates responsible for social behavior in vertebrates and a relevant framework to make species comparisons.

Nevertheless, attention must be drawn to the fact that some proposed homologies are not complete, instead they are only partial. In that sense, in a recent review, Goodson and Kingsbury (2013) proposed the inclusion of the paraventricular nucleus of the hypothalamus (PVN) within the mammalian POA node (i.e. POA/PVN), in order to comprehend vasopressin-oxytocin nonapeptides neurons crucial for the regulation of social behavior. With this incorporation the POA/PVN mammalian node would be similar to the anamniotes POA node.

On the other hand, for some non-mammalian species homologies of the SDMN nodes are not clear and functional studies are still missing, especially for the mesolimbic reward system (Goodson and Kingsbury, 2013). So, despite being strongly supported in mammals, for other taxa the SDMN must be cautiously evaluated and tested.

2.2. Homologies between teleost fish and mammalian brain areas in the SDMN

In teleosts, the SBN is assumed to be composed by the ventral (Vv) and lateral (Vl) part of ventral telencephalon, supracommisural part of the ventral pallium (Vs), POA, ventral tuberal region (vTn), anterior tuberal nucleus (aTn), all localized in the forebrain, in addition to the PAG, lying in the midbrain (O'Connell and Hofmann, 2011). On the other hand, the Mesolimbic Reward System is presumably constituted by the dorsal (Vd) and central (Vc) part of the ventral telencephalon, the medial part of the dorsal telencephalon (Dm), the lateral part of the dorsal telencephalon (Dl), the posterior tuberculum (TPp) on the midbrain, and also Vv/Vl and Vs, that are also nodes of the SBN (O'Connell and Hofmann, 2011), Figure 1.

Both Vv and Vl seem to be homologous of mammalian LS region. Cholinergic neurons were detected only in this telencephalic area. It is reciprocally connected to several important nuclei and it expresses sex steroid receptors (see Wullimann and Mueller, 2004; O'Connell and Hofmann, 2011 for more details). It is also involved in reproductive behavior since electrical stimulation of Vv in Sockeye salmon, *Oncorhynchus nerka* elicits females' digging and spawning while Vv and Vs ablation in the goldfish *Carassius auratus* impairs male ejaculation (Kyle and Peter, 1982; Satou et al., 1984). Vs is putatively homologous of meAMY/BNST based on developmental evidences, as well as neurochemical and connectivity similarities (see Wullimann and Mueller, 2004; O'Connell and Hofmann, 2011 for further details), even though

some consider that ventral telencephalon (Vp) should also be included within this node (Goodson and Kingsbury, 2013).

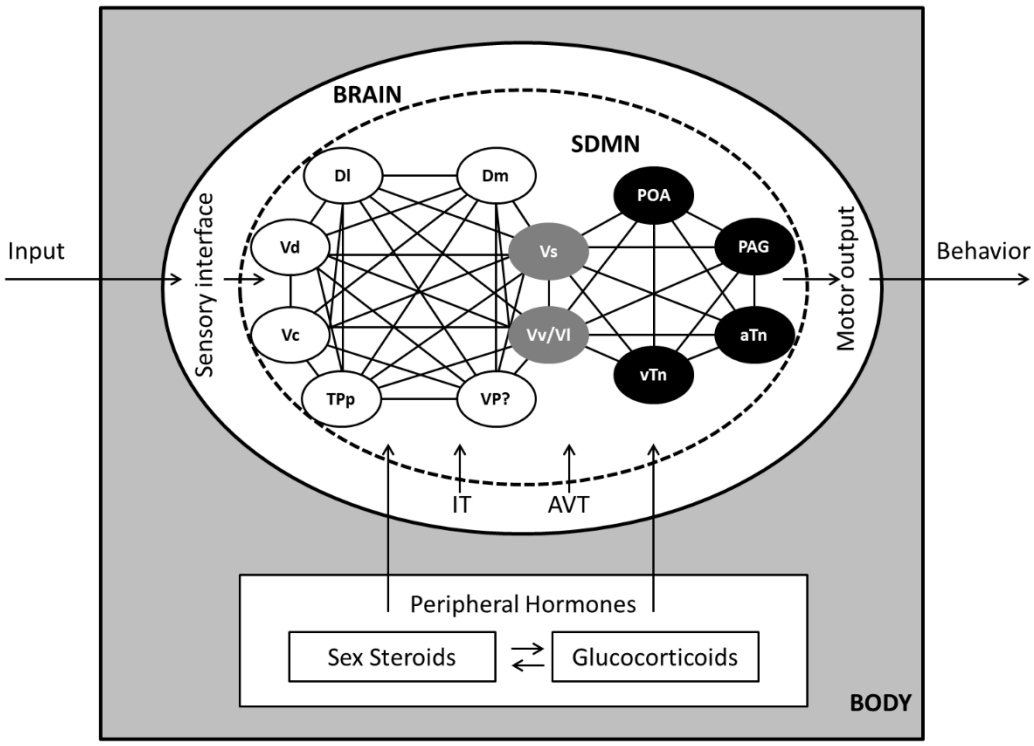

Figure 1. Representation of the interaction between hormones and the Social-Decision Making Network (SDMN) within teleosts' social behavior: putative nodes of the Mesolimbic Reward System in white - dorsal (Vd) and central (Vc) part of the ventral telencephalon, medial part of the dorsal telencephalon (Dm), lateral part of the dorsal telencephalon (Dl), posterior tuberculum (TPp) -, and the Social Behavior Network in black - Medial Preoptic Area (POA), ventral tuberal region (vTn), anterior tuberal nucleus (aTn), and Midbrain Periaqueductal Gray (PAG). Ventral and lateral (Vv/Vl) part of ventral telencephalon and supracommisural part of the ventral pallium (Vs), overlapping nodes of the SBN and the Mesolimbic Reward System, are in grey. A homologous for the mammalian Ventral Pallidum (VP) node has not yet been identified.

The teleost POA homology is well established. This node that is imperative for the regulation of sexual, parental and aggressive behaviors (Demski and Knigge, 1971; Macey et al., 1974; Satou et al., 1984; Wong, 2000) is localized in the hypothalamus along the third ventricle, just like in mammals. It is divided in three subregions according to cell size: parvocellular, magnocellular and gigantocellular and it is reciprocally connected with the

telencephalon and other hypothalamic regions (reviewed in O'Connell and Hofmann, 2011). vTn is the putative homologous of AH because it is localized between the POA and the ventral hypothalamus, it receives and sends projections to several hypothalamic regions and it expresses sex steroid receptors (O'Connell and Hofmann, 2011 reviewed this information). However, functional studies are yet not available to confirm this homology. aTn was proposed to be the teleost equivalent of VMH, although only a subset of aTn cells are actually homologous (Goodson and Kingsbury, 2013). Lying in the ventrocaudal region of the hypothalamus, it is reciprocally connected with several parts of the telencephalon and contains sex steroid hormone receptors (reviewed in O'Connell and Hofmann, 2011) but functional studies are as well limited. PAG is also present in teleosts, located near the torus semicircularis and receiving and sending projections to several other nuclei (see O'Connell and Hofmann, 2011 for more information). Functionally, it is associated with social communication, specifically, in sound production of the plainfin midshipman *Porichthys notatus* (Kittelberger et al., 2006). Vc is comparable to Str in mammals, while Vd seems homologous to NAcc, but this is only supported by neurochemical studies and some hodological evidences (consider Wullimann and Mueller, 2004; O'Connell and Hofmann, 2011 for more details). Unfortunately, a homologous for the mammalian VP node has not yet been identified. Dm is the putative homologous of blAMY based on developmental, tract tracing and lesions studies, demonstrating its implication in emotional learning, as shown in mammals (reviewed in O'Connell and Hofmann, 2011; Portavella et al., 2002). The homologous of Hyp is considered to be the Dl mainly because of its involvement in spatial learning (Dl lesions of *C. auratus* impair map-like memory representations, Rodríguez et al., 2002), besides some of its hodological features (reviewed in O'Connell and Hofmann, 2011). Finally, even though amphibians and teleosts lack a midbrain dopaminergic population, TPp, located in the ventral diencephalon, has been suggested to present homologies to VTA, the A10 dopaminergic cell group, because of its dopaminergic ascending projections to Vd and conserved neurochemical patterns (see O'Connell and Hofmann, 2011 for details). However this seems to be refutable as a recent study on the zebrafish, *Danio rerio*, 'projectome' underlines that posterior tuberculum cells (DC2 and DC4-6 cell groups), seem homologous to A11 mammalian dopamine neurons based on transcription factor conservation and projection patterns (Tay et al., 2011). Actually ascending projections to telencephalon are scarce (and only from DC2 and DC4 neuronal cells), while the most important dopaminergic connections between the subpallium and the ventral diencephalon are descending (Tay et al., 2011). Consequently, the existence of a mesolimbic reward system in fish is questionable since the connection between the VTA and forebrain

regions is considered the core of the dopaminergic reward system (Bromberg-Martin et al., 2010; Spanagel and Weiss, 1999).

2.3. Functional analysis of the SDMN in teleost fish

A recent study in zebrafish functionally tested the SDMN concept. In this study, IEGs expression was determined along selected nodes of the SDMN while animals were in different behavioral states (Teles et al., 2015). The aim of this work was to test for functional brain specialization or alternatively for functional connectivity and for example determine whether expression of social behavior is explained by the activation of a single node or by the overall combination of the activity in the various nodes. They quantified IEGs expression in the brains of winners and losers of agonistic fights, as well as in individuals that only experienced mirror fights and compared them with a reference non-interacting group. IEGs transcription patterns of *c-fos* and *egr-1* as measured by qPCR were used as markers of neuronal activity in the Vv, Vs, POA, Dm and Dl. The similarity of IEGs activation between groups and areas showed that there were no patterns of localized activity in a specific nucleus. Instead socially driven behavioral states demonstrated patterns of functional connectivity across the nodes. The notion of a SDMN is therefore supported in a teleost fish.

On the other hand, a considerable number of studies centered on the behavioral responses of teleost fishes have documented the activation of specific SDMN nodes, hence establishing their involvement in the regulation of social behavior. For instance, Desjardins et al (2010) focused on how mate information impacts female neural activity. In this work, they measured IEG (*c-fos* and *egr-1*) expression levels by qPCR in gravid females of the Burton's mouthbrooder cichlid, *Astatotilapia burtoni*, that saw their preferred males winning or losing a fight. They looked specifically to the Vv (LS), POA, vTn (AH), aTn (VMH), PAG, Dm and Dl. Results demonstrate that the POA and VMH, two nuclei known to be involved in the control of reproduction, are highly activated when females see their preferred male winning, whereas IEG response in the mammalian LS homologue region (a nucleus associated with anxiety) is elicited when females see the male losing.

O'Connell et al (2013) directed their interest to how individuals integrate social information. These researchers presented *A. burtoni* males with different social stimuli and discovered that visual information (seeing a female or a male) is sufficient to elicit *c-fos* transcription in dopaminergic neurons of Vc, and this transcription is significantly correlated to aggressive behavior in the case of exposure to an intruder male. These data suggest that Vc

seems to be involved in assessing stimulus visual valence. Another interesting survey was also carried out in this species. Since *A. burtoni* males can reversibly switch between dominant and subordinate status and rapidly present distinct phenotypes, investigators examined IEG levels in several brain areas of males ascending or descending in social status, as compared with control individuals (Maruska et al., 2013a, 2013b). In socially ascending males, both *c-fos* and *egr-1* levels were higher than in control males in all the SDMN nuclei (Vv, Vs, POA, vTn, aTn, Dm and Dl) (Maruska et al., 2013b). Descending males presented different activation patterns for *c-fos* and *egr-1* across the same areas. *c-fos* expression levels were increased in the Vs, POA and aTn by comparison with controls while *egr-1* mRNA levels were higher in the Vv, Vs, vTn, Dm and Dl (Maruska et al., 2013a). Another relevant study used the monogamous cichlid *Amatitlania nigrofasciata* as a model to study the influence of isotocin in parental care (O'Connell et al., 2012). The authors compared males housed with their mate (control males), single fathers who had the mate removed or lone males (mate and offspring removed), and quantified *c-fos* expression in Vv, POA and the central part of the dorsal telencephalon (Dc). At the behavioral level, only single fathers increased paternal care immediately after removal of their mate and they also presented significantly higher IEG activity levels in Vv compared to lone males, as well as increased *c-fos* expression in the parvocellular POA isotocin neurons. Together these data suggest that isotocin promotes paternal care after mate removal and that Vv and POA are important brain areas in this process. Finally, a very interesting study with *P. notatus*, where reproductive behavior is intimately associated with social acoustic signals, measured *c-fos* activation in several brain nuclei including vTn, aTn and TPp (Petersen et al., 2013). The authors report a significant increase of IEG expression in aTn and TPp of males exposed to acoustic signals of other males compared to control males, showing the importance of these nuclei in social reproductive communication in this species.

3. Endocrine regulation of social behavior in teleost fish

The pioneering work of Arnold Berthold on the endocrine regulation of sexual behavior in animals demonstrated the influence of a "blood-borne product" released in peripheral glands on behavior (Berthold, 1849). Likewise, early studies on the endocrine regulation of behavior in fish focused on the role of hormones produced in the periphery, mainly gonadal steroids, prostaglandins and corticosteroids, and there is now substantive information on the role of these hormones as modulators of social behavior. However, in some cases, the expression of social behavior seems to be independent from hormones produced in the periphery and the role of

brain-synthesized hormones, in particular neurosteroids and neuropeptides, has been receiving increasing attention (for a review see Gonçalves and Oliveira, 2010; Oliveira and Gonçalves, 2008).

Regardless of the source, still little is known on how molecular and cellular mechanisms of hormonal action in the brain modify social behavior in fish. The recent concept of the SDMN is useful as it allows studies on the neuroendocrine modulation of social behavior to be focused in relevant brain areas. As described above, recent work has addressed the interplay between social environment and activation of the nodes of the SDMN using IEG as proxies of neuronal activity (e.g. Desjardins et al., 2010; Teles et al., 2015). However, studies investigating the regulatory role of hormones in the functionality of the SDMN are still scarce in spite of the fact that major modulatory effects of hormones in the SDMN are expected, as evidenced by the widespread distribution of hormone receptors in its nodes. In particular, estrogen (*P. notatus*, Forlano et al., 2005; *Micropogonias undulatus*, Hawkins et al., 2005; *D. rerio*, Menuet et al., 2002; *A. burtoni*, Munchrath and Hofmann, 2010; *Dicentrarchus labrax*, Muriach et al., 2008), progesterone (*A. burtoni*, Munchrath and Hofmann, 2010), androgen (*P. notatus*, Forlano et al., 2010; *C. auratus*, Gelinas and Callard, 1997; *A. burtoni*, Harbott et al., 2007; Munchrath and Hofmann, 2010), vasotocin (*A. burtoni*, Huffman et al., 2012; rock hind, *Epinephelus adscensionis*, Kline et al., 2011), and isotocin (*A. burtoni*, Huffman et al., 2012) receptors are widely distributed along the areas of the SDMN (see also Diotel et al., 2011).

Below we review the evidence for the endocrine modulation of different types of social behavior in fish, addressing their possible mechanisms of action at the brain level.

3.1. Aggressive behavior

Different categories of hormones, notoriously gonadal steroids, corticosteroids and nonapeptide hormones, have been associated with the regulation of aggressive behavior in fish. For gonadal steroids, early evidence demonstrated that male dominance correlated with circulating androgen levels, in particular with the non-aromatizable androgen 11-ketotestosterone (11KT, for a review see Oliveira and Gonçalves, 2008; Oliveira et al., 2002). Classical castration-androgen replacement experiments suggested an effect of gonadal androgens in aggression and a meta-analysis confirmed that exogenous administration of androgens promotes aggression in fish (Hirschenhauser and Oliveira, 2006). In one of the best studied models in this respect, the cichlid *A. burtoni*, a change from a submissive to a dominant status in males increases the expression of the GnRH1 gene and the concomitant production of its peptide in neurons of the

POA, inducing gonadal development and a surge in plasma 11KT levels within 30 minutes (Francis et al., 1993; Maruska and Fernald, 2010; White et al., 2002). Interestingly, in this species a submissive experience seems to have more rapid and more profound physiological consequences than a dominant one as aggressive behaviors were reduced more promptly in males undergoing a descent in social status than they emerged in animals ascending in social status (White et al., 2002). Similarly, in zebrafish males, losing a fight induces a more pronounced change in future aggressive behavioral displays and in the neurogenomic state of the whole brain than winning (Oliveira et al., 2011; Oliveira et al., 2016).

Aggression in females has been much less investigated although, interestingly, the role of androgens in the modulation of aggressive displays seems to be more consistent than for males. In the Mozambique tilapia, plasma testosterone (T) levels in females peak during a phase of the reproductive cycle that matches increased aggression (Oliveira and Canário, 2000). In the cichlid *Neolamprologus pulcher*, females and males jointly defend a territory all year round and females were shown to be more aggressive than males in response to a territorial intrusion and to experience a higher increase in androgen levels (including 11KT, usually undetected in females, Desjardins et al., 2006). Similarly, in the blue acara, *Andinoacara pulcher*, T administration increased aggressive behavior in females (Munro and Pitcher, 1985) and daily injections of T to females of the Siamese fighting fish *Betta splendens* for a period of nine weeks increased aggression directed towards males, although it decreased aggression directed towards females (Badura and Friedman, 1988). Finally, in all-female groups of *A. burtoni*, it was observed that females start to exhibit typical male behaviors, including territorial and aggressive displays (Renn et al., 2012). In this experiment, dominant females had higher T levels when compared with subordinates and T levels correlated with aggressive displays like chasing and threatening.

In birds and mammals, the central effects of androgens on aggressive behavior seem to depend on the aromatization of T into estradiol (E2) (Trainor et al., 2006). In fish, however, in spite of aromatase being abundant in nuclei of the SDMN (e.g. Forlano et al., 2001), there are conflicting results on the role of aromatization in aggression. Several lines of evidence suggest a direct action of androgens in the expression of aggressive behavior in fish: 1) androgen receptors are widely distributed in nuclei of the telencephalon and diencephalon, including in areas of the SDMN (e.g. Harbott et al., 2007); 2) differences in plasma androgen levels between aggressive and less aggressive phenotypes seem to be more evident for the non-aromatizable androgen 11KT than for T (Oliveira, 2005) and the peripheral administration of 11KT has been shown to promote aggression in teleosts (e.g. Rodgers et al., 2013); 3) in some species

peripheral administration of estrogens has an inhibitory effect on male aggression (e.g. three-spined stickleback, *Gasterosteus aculeatus*, Bell, 2001; *B. splendens*, Clotfelter and Rodriguez, 2006; *D. rerio*, Colman et al., 2009; Filby et al., 2012; peacock blenny, *Salaria pavo*, Gonçalves et al., 2007; *A. pulcher*, Munro and Pitcher, 1985; sand goby, *Pomatoschistus minutus*, Saaristo et al., 2010) and; 4) whole brain aromatase activity was shown to be inversely correlated with aggression in females of the sex-changing blue-banded goby, *Lythrypnus dalli* (Black et al., 2005), suggesting that a higher availability of T (or a decrease in E2 synthesis) promotes aggression. Contrarily, in the cichlid *A. burtoni*, E2 administration increased male aggression (O'Connell and Hofmann, 2012b) and pharmacologically blocking aromatization with Fadrozole decreased these behaviors in the same species (Huffman et al., 2013) and also in the weakly electric fish *Gymnotus omarorum* (Jalabert et al., 2015). This would suggest that aromatization of T into E2 is needed to promote male aggressive displays in these species. Clearly, more data is needed to interpret the divergent results across-species and understand what the general pattern in fish is.

The above data suggests that a dominance experience (e.g. winning fights) activates the hypothalamic-pituitary-gonadal axis, increasing the secretion of gonadal androgens that in turn act on different tissues to promote the expression of a dominant phenotype, including increased aggression, via effects on the brain (and the reverse for submissive experiences). This feedback between the environment and behavior, translated via neuroendocrine modulation by androgens, would allow animals to dynamically adjust their behavior and physiology to a particular social context (Oliveira, 2004). However, the hypothesis that high levels of aggression are maintained through a positive effect of gonadal androgens in the brain is contradicted by other studies. The majority of studies where males were gonadectomized during the breeding season, thus reducing the circulating levels of androgens, failed to found a significant effect on male aggression (reviewed in Gonçalves and Oliveira, 2010). As an example, in the Mozambique tilapia gonadectomy impaired the expression of reproductive behaviors in males, including nest building and courtship displays, but did not affect aggressive behavior towards a conspecific male (Almeida et al., 2014). Also, in the social sex-changing bluehead wrasse *Thalassoma bifasciatum*, gonadectomy did not prevent female-to-male behavioral change, including an increase in the expression of aggressive behavior, when females were given an opportunity to occupy a vacant territory (Godwin et al., 1996). Finally, variation in aggressive behavior has been shown to occur in immature individuals and animals outside the breeding season, questioning the hypothesis of aggression being modulated by gonadal androgens in these contexts. For example, in the *G. omarorum* high levels of aggression

were decoupled from 11KT levels outside the reproductive season (Jalabert et al., 2015) and in the damselfish *Stegastes nigricans*, also a year-round territorial species, androgen levels did not increase when an aggressive challenge was presented to males (Ros et al., 2014).

The contradictory results found for the effect of sex steroids on aggressive behavior have driven the search for alternative modulators of aggression. A pathway that has also been shown to relate with the neuroendocrine modulation of aggression in fishes is the hypothalamic-pituitary-interrenal axis. In the above mentioned study in *S. nigricans*, where androgens failed to respond to an aggressive challenge, cortisol levels in the plasma increased after males were presented with intra and interspecific challenges and its concentration was strongly correlated with aggressive behaviors (Ros et al., 2014). Also, in the cichlid *A. pulcher*, cortisol administration increased aggression towards a model intruder (albeit not towards a mirror image, Munro and Pitcher, 1985). In juvenile rainbow trout, *Oncorhynchus mykiss*, cortisol administration failed to promote aggression one hour after being administered but pharmacological blockage of GR and MR receptors reduced aggression levels, suggesting that basal levels of cortisol were contributing to aggressive behavior via activation of intracellular GR and MR receptors (Schjolden et al., 2009). Nevertheless, prolonged (48h) exposure to cortisol reduced aggression, suggesting a complex time-dependent effect of this hormone in aggressive behavior (Øverli et al., 2002).

AVT and IT have also been implicated in the regulation of aggression, and more generally social behavior, in fishes (reviewed in Godwin and Thompson, 2012). AVT neurons occur in the POA and project to the neurohypophysis, releasing AVT into circulation when activated, but also project to many other brain regions, including the ventral telencephalon, thalamus and mesencephalon (for details on the neuroanatomy of the AVT system in fish see Godwin and Thompson, 2012; Huffman et al., 2012; Thompson and Walton, 2013). Within the POA, three subpopulations of AVT neurons can be identified; parvocellular, magnocellular and gigantocellular, and they have been suggested to play different roles in osmoregulation and modulation of behavior (Greenwood et al., 2008). Receptors for both AVT and IT have been found throughout nuclei of the SDMN, suggesting a direct neuromodulatory action of these neuropeptides in nodes of this brain network (Huffman et al., 2012; Lema, 2010).

Following studies in mammals that associated AVP with increased expression of aggressive behavior, AVT has also been implicated in the regulation of aggression in fishes, although with inconsistent results between species. A positive effect of AVT on aggression has been described for some species, as for example nonterritorial phase males of *T. bifasciatum* (Semsar et al., 2001), males of the damselfish *Stegastes leucosticus* (Santangelo and Bass,

2006), and males of *A. nigrofasciata* (Oldfield and Hofmann, 2011) while a supressive effect of AVT on aggression has been described for other species, as for example territorial phase males of *T. bifasciatum* (Semsar et al., 2001), males of the brown ghost knifefish, *Apteronotus leptorhynchus* (Bastian et al., 2001), males of Amargosa pupfish, *Cyprinodon nevadensis amargosae* (Lema and Nevitt, 2004; but see Lema et al., 2015) and males and females of *D. rerio* (Filby et al., 2010). These contradictory results may have different, but not necessarily exclusive, explanations. In studies with butterfly fish of the genus *Chaetodon*, it was shown that a territorial species had larger AVT-immunoreactive somata within the POA area and higher AVT fibre densities within a number of telencephalic nuclei than a non-territorial species and that aggression correlated positively with the number of POA gigantocellular AVT cells and negatively with the number and size of POA parvocellular AVT cells (Dewan and Tricas, 2011; Dewan et al., 2008). In the *C. nevadensis amargosae*, telencephalic *pro*AVT mRNA levels were found to be elevated in subordinate males and to correlate with aggression. These males also had higher $V1a_1$ receptor transcript levels in the telencephalon and hypothalamus, as compared with dominant males. On the other hand, the levels of *pro*AVT mRNA were four fold higher in the hypothalamus of dominant males, which also had higher levels of hypothalamic $V1a_2$ receptor transcript abundance (Lema et al., 2015). This may be interpreted as hypothalamic AVT playing a role in the expression of aggressive behavior in dominant males via the activation of the $V1a_2$ receptor, while AVT action in forebrain targets would promote aggression only in subordinate animals. Greenwood et al. (2008) showed an opposite pattern of AVT mRNA expression in *A. burtoni* parvocellular and magnocellular subpopulations of AVT cells, with territorial males having higher levels of expression of AVT in the gigantocellular layer but lower levels in the parvocellular layer, as compared with non-territorial males. The authors suggested that gigantocellular neurons might be more related with the modulation of dominance related traits, including the expression of aggressive behavior, while parvocellular cells may relate to the activation of the stress axis or submissive behaviors. Following this model, Godwin and Thompson (2012) suggest that AVT projections from the POA may regulate "sociosexual circuits", including those related with aggression, by modulating neuronal action in central brain regions, including nodes of the SDMN. On the other hand, AVT would also be able to promote submissive and escape behaviors by producing peripheral changes that feedback to the brain. These effects could be induced either directly, via modulation of hindbrain autonomic nuclei that regulate peripheral states, or indirectly, as for example through the demonstrated capacity of AVT to stimulate the stress axis (Baker et al., 1996). As a consequence, peripheral, or even central, administration of AVT may have

variable effects in behavior as it activates the targets of multiple subpopulations of AVT neurons. In addition, and as demonstrated in mammalian models, the effects of AVP/AVT in behavior are greatly dependent on tissue sensitivity which may vary across phenotypes, developmental stages or seasons due to variation in the abundance of its receptors (e.g. Walton et al., 2010). Future studies manipulating the AVT system using techniques like optogenetics, transgenics or others, may offer an opportunity to investigate the exact function of the different subpopulations of AVT neurons and subtypes of receptors in the modulation of aggression, and other categories of social behavior, in fish.

The effect of IT on aggressive behavior in fish has been less investigated. In the plainfin midshipman, IT administered to the POA-anterior hypothalamus elicited fictive aggressive vocalizations in a neurophysiological preparation of parasitic (sneaker) males but not of territorial males (Goodson and Bass, 2000). On the other hand, IT administered to males of the beaugregory damselfish had no effect in aggressive displays (Santangelo and Bass, 2006) and similar results were found in females and males of the cichlid *N. pulcher* (Reddon et al., 2012). Further studies are needed before a role for IT in the modulation of fish aggression can be established.

Finally, hormones involved in somatic growth, in particular the growth hormone (GH) and somatostatin (SS), have also been proposed as modulators of aggressive behavior in fish. These hormones are synthetized at the level of the hypothalamus projecting to somatotropes in the pituitary. In *A. burtoni*, SS seems to inhibit the expression of aggressive behavior in a dose-dependent fashion and independently of any potential effect in gonadal androgen secretion (Trainor and Hofmann, 2006). In the rainbow trout, peripheral administration of GH was shown to increase male aggression but this was interpreted as an indirect effect as GH also increased swimming activity that promoted agonistic encounters (Jönsson et al., 1998). Later, Jönsson et al. (2003) confirmed this hypothesis by administering GH directly into the third ventricle of juvenile rainbow trout and observing also an increase in swimming activity. Interestingly, in the *A. burtoni* study, only chasing behavior and not threatening behavior was affected by SS. As SS is known to inhibit the release of GH, the inhibitory effects of SS in aggressive displays could be explained by a decrease in general locomotor activity induced by a reduction in GH levels. This data is also contradictory to the findings of Hofmann and Fernald (2000) showing that dominant males have larger SS immunoreactive neurons in the POA as compared with subordinate animals, suggesting that SS administration should increase aggression if it is directly related with the endocrine regulation of these behaviors.

3.2. Mating behavior

The brain regions associated with the expression of sexual behaviors have been extensively described in vertebrates, including fish (for a related review see Forlano and Bass, 2011). Notoriously, the POA and anterior hypothalamus are known to be central brain regions for the control of reproduction as they contain the GnRH neurons that command the release of the gonadotropins LH and FSH from the pituitary, regulating gonadal development and secondarily gonadal steroid secretion. These regions also synthesize neuropeptides relevant for reproduction and are rich in sex steroid receptors. Early studies highlighted the role of this area in reproduction by showing that electrical stimulation of the POA induced reproductive behaviors in male bluegill sunfish *Lepomis macrochirus* (Demski and Knigge, 1971), results later confirmed in the hime salmon *Oncorhynchus nerka* (Satou et al., 1984). As mentioned above, females of the cichlid *A. burtoni* observing their preferred male winning fights activated the POA (as measured by IEG expression) and also the Vm, another area implicated in female sexual displays in vertebrates (Desjardins and Fernald, 2010). In a study in female Medaka *Oryzias latipes*, mating induced widespread *c-fos* expression in the POA, telencephalon, optic tectum and cerebellum (Okuyama et al., 2011), suggesting the implication of a widespread set of brain nuclei in female mating behavior. In addition to these central brain regions, hormones can also modulate reproductive displays by acting in sensory or effector systems. As an example, in female midshipman E2 acts in the inner ear's sacculus to increase the degree of temporal encoding of the frequency content of male vocalizations (Sisneros et al., 2004), thus synchronizing female phonotaxis and receptivity with maturation of the ovaries (for a review see Sisneros, 2009).

Both female and male reproductive behaviors are expected to be coordinated with gonadal function and thus hormones of gonadal origin, in particular sex steroids for males and sex steroids and prostaglandins for females, have been seen as main candidates for endocrine regulation of reproductive behaviors in fish.

The main androgens detected in fish plasma are T, 11KT and 11β-hydroxytestosterone (Borg, 1994). Males have usually higher plasma levels of 11KT than females while T levels often do not differ between sexes (Borg, 1994; Lokman et al., 2002). The impact of manipulating androgen levels in male reproductive displays is highly variable (see Oliveira and Gonçalves, 2008 for a review). While gonadectomy is effective in reducing plasma androgen levels (e.g. Almeida et al., 2014; Gonçalves et al., 2007; Salek et al., 2001), in some cases a concomitant reduction in reproductive behaviors occurs (e.g. *O. mossambicus*, Almeida et al.,

2014; *G. aculeatus*, Hoar, 1962; *M. americana*, Salek et al., 2001) while in others they are maintained (e.g. *G. aculeatus*, Páll et al., 2002; *T. bifasciatum*, Semsar and Godwin, 2003). Also, exogenous administration of androgens either to gonadectomised or intact males has variable effects on male mating displays (for a list of studies see Oliveira and Gonçalves, 2008). For example, androgen administration to gonadectomised males of *M. americana* restored sexual displays, with 11KT being more effective than T (Salek et al., 2001), while 11KT administration to intact males of the rock-pool blenny *Parablennius parvicornis* failed to promote sexual behavior (Ros et al., 2004). Finally, pharmacologically blocking androgen receptors decreased male nesting behavior in *G. aculeatus* (Sebire et al., 2008) and male courtship displays in the guppy *Poecilia reticulata* (Baatrup and Junge, 2001), in agreement with the hypothesis that male mating behaviors are directly facilitated by androgens. The administration of androgens to juveniles, parasitic "sneaker" males or females, all phenotypes with lower plasma androgen levels than males, likewise produced variable results. In the peacock blenny, T implants inhibited female-like displays in castrated parasitic males but failed to promote nesting male behaviors (Gonçalves et al., 2007), while in *C. auratus* T and 11KT implants given to intact females induced the full-suite of male sexual behaviors (Stacey and Kobayashi, 1996).

The variable results obtained between studies of the effects of androgens on male sexual behavior surely have multiple causes, including differences in hormone concentrations, type of androgens and antiandrogens used, hormone delivery mode, species-specific differences, season when experiments were performed or duration of exposure to the hormone, just to mention a few. Nevertheless, the overall pattern suggests that androgens have a positive effect on male sexual displays also in fish.

The central effects of androgens on male displays and in particular their potential action in the nodes of the SDMN are still poorly understood. In mammals, aromatization of T into E2 in the brain plays a crucial role in the regulation of male sexual behavior (reviewed in Ball and Balthazart, 2004; Baum, 2003). However, although aromatase is abundant in the areas of the SDMN and partly co-localizes with androgen receptors (Forlano et al., 2010; e.g. Gelinas and Callard, 1997; Harbott et al., 2007), the evidence for aromatization playing a role in the activation of male sexual displays in fish via local conversion of T into E2 is less obvious than in birds or mammals. In fact, in some studies the non-aromatizable 11KT seems to have a more effective role in the induction or recovery of male sexual displays than the aromatizable T (e.g. Stacey and Kobayashi, 1996) and pharmacologically blocking aromatization with Fadrozole was shown to inhibit male displays in *P. reticulata* (Hallgren et al., 2006) but not in *A. burtoni*

(Huffman et al., 2013). Also, exposure to estrogens or xenoestrogens generally reduces male sexual displays (e.g. *P. reticulata*, Bayley et al., 1999; *C. auratus*, Bjerselius et al., 2001; Colman et al., 2009; *D. rerio*, Pradhan and Olsson, 2015). Furthermore, while androgens masculinize the electric organ discharge signal in ghost knifefishes, estrogens feminize it (reviewed in Smith, 2013). In zebrafish, E2 seems to feminize the male brain and 11KT to masculinize the female brain, as assessed by gene transcriptomic profiling (Pradhan and Olsson, 2015), further supporting a direct action of androgens on male reproductive behavior.

Male sexual displays are often more elaborated than female displays and consequentially there are more published studies testing the effect of endocrine manipulations in sexual behavior in males than in females. The regulation of female sexual behavior was initially hypothesized to be controlled by gonadal hormones and determined by the mode of reproduction (Stacey, 1981). In internal fertilizers, sexual behavior and fertilization are temporally dissociated and sex steroids were proposed to regulate female displays. By contrast, in external fertilizers female sexual behavior was considered to be mostly restricted to oviposition, which may be regarded as homologous to parturition. Thus ovarian prostaglandins, which induce uterine contractions in mammals and oviposition in fishes (Jalabert and Szöllösi, 1975), were proposed to modulate female spawning behaviors. This idea was originally proposed based mainly on data for *P. reticulata*, an internal fertilizer, and *C. auratus*, an external fertilizer, but new evidence suggests that the sex steroid and prostaglandins pathways may actually be complementary. In a sex-role reversed population of the peacock blenny, an external fertilizer, females court males with very elaborate displays (Gonçalves et al., 1996), providing an opportunity to test the effects of endocrine manipulations in female sexual behavior. Ovariectomy was effective in quantitatively reducing the expression of female courtship displays and nuptial colouration two weeks after the removal of the ovaries but still a majority of ovariectomized females (9 out of 13) courted at least once a nesting male, showing that removal of the ovaries does not completely suppress the expression of sexual behavior (Gonçalves et al., 2014). Interestingly, both the steroid E2 and the prostaglandin PGF2α restored female sexual displays (but not nuptial coloration), although PGF2α was more effective than E2 in this respect. In the same population, parasitic males that mimic female displays occur and the removal of the testes in these males has the paradoxical effect of promoting even further the expression of female-like behaviors (Gonçalves et al., 2007). This is probably explained by the fact that androgens suppress female-like displays in sneaker males (Gonçalves et al., 2007; Oliveira et al., 2001) and removing the testes reduces circulating androgen levels, releasing this inhibition (Gonçalves et al., 2007). Because E2 levels are very low in sneaker males and E2

administration does not promote female-like displays (Gonçalves et al., 2007), other neuroendocrine mechanisms are proposed to regulate these behaviors (see below).

Prostaglandins have been shown to promote female displays in other externally fertilizing species, including the paradise fish, *Macropodus opercularis* (Villars et al., 1985), the black acara *Cichlasoma bimaculatum* (Cole and Stacey, 1984), the barb *Puntius gonionotus* (Liley and Tan, 1985), the cichlid *A. burtoni* (Kidd et al., 2013) and, notoriously, the goldfish *C. auratus* (reviewed in Kobayashi et al., 2002). Recently, Juntti et al. (2016), confirmed this role of PGF2α in *A. burtoni* by showing that the expression of a putative PGF2α receptor in areas like the vagal lobe and POA increase during spawning and that the activation of this receptor is needed for spawning behavior to occur. This evidence suggests that ovarian prostaglandins act in external fertilizing teleost species, and probably also in amphibians, as a short-duration endogenous messenger to synchronize sexual behavior with the presence of mature oocytes in the ovaries. However alternative explanations exist including the possibility of direct neural communication between the gonads and the brain via the vagal nerve, which would induce neural synthesis of PGF2α and the activation of female reproductive displays Juntti et al. (2016), or the activation of brain PGF2α synthesis by other ovarian hormones. For example, in mammals E2 has been found to promote the synthesis of prostaglandins both in the uterus (PGF, Ham et al., 1975) and in the POA (PGE2, Amateau and McCarthy, 2002). It seems possible that the above-described positive effects of both E2 and PGF2α in the activation of sexual displays in ovariectomized females of *S. pavo* could occur via a direct effect of PGF2α in the brain and to a stimulatory effect of E2 in the neural synthesis of PGF2α. Studies investigating in further detail the interconnection between sex steroids and prostaglandins in fish, in particular the effects of gonadal steroids in brain PGF2α synthesis and receptor expression, and the modulation by PGF2α of SDMN nodes look like promising venues for future research.

Nevertheless, evidence that the expression of sexual behavior in fish does not rely on hormones of gonadal origin was obtained by Godwin et al. (1996) who showed that in the sex-changing wrasse *T. bifasciatum* females could rapidly occupy a territory left vacant by the removal of the dominant male and express male courtship and spawning displays in the absence of gonads. Hypothalamic abundance of proAVT mRNA in the brain of these females during sex-change increases fourfold when compared with non-changing females (Godwin et al., 2000) and is higher in ovariectomized dominant females than in subordinate females (Semsar and Godwin, 2003), suggesting that AVT may be a key peptide regulating the transition into male sexual displays in this species. However, the effect of AVT seems to be context-dependent

as AVT administration failed to induce male sexual behaviors when a dominant male was present (Semsar and Godwin, 2004). Interestingly, 11KT administration also promoted male displays in subordinate ovariectomized females but did not change AVT hypothalamic levels (Semsar and Godwin, 2004, 2003), suggesting that gonadal androgens modulate male reproductive displays via a pathway independent from AVT. The positive effects of AVT on male sexual displays are confirmed by studies in other species (e.g. *A. leptorhynchus*, Bastian et al., 2001; white perch, *M. americana*, Salek et al., 2001). For example, in the gymnotiform *Brachyhypopomus gauderio*, AVT stimulated the production of electric signals used for courtship by direct action on the hindbrain pacemaker cells (Perrone et al., 2010). However, in the cichlid *A. burtoni* blocking the V1a receptor inhibited aggression and stimulated courtship displays in subordinate males that ascended to become dominant, suggesting that AVT impairs male reproductive displays in this context, although the same manipulation did not produce any effects in stable dominant or subordinate fish (Huffman et al., 2015).

AVT has also been shown to promote female displays. In a sex-role reversed population of the peacock blenny *Salaria pavo*, AVT mRNA levels were higher in the courting morphs, i.e. in females and in female-mimicking parasitic males, than in non-courting nesting males (Grober et al., 2002). Accordingly, AVT administration promoted sexual displays in females and in parasitic males but not in nesting males (Carneiro et al., 2003).

Taken together, these results suggest that AVT promotes sexual behavior in fishes and following the model proposed by Godwin and Thompson (2012) this is probably achieved via modulation of central brain areas, including nodes of the SDMN, by AVT projections from the POA.

3.3. Parental care

The wide diversity of modes of reproduction in fishes extends to the patterns of care to eggs or juveniles and examples of no care, paternal, maternal, biparental or even alloparental care can be found (Breder and Rosen, 1966). This offers an excellent opportunity to study the proximal mechanisms of parental care in vertebrates but not much is known on the brain substrates of parental care in fish. In mammals, the POA, thalamus, BNST and the LS have been associated with the expression of paternal care (for a review see Dulac et al., 2014). In fish, the putative homologue for the lateral septum is the Vv but an equivalent area to the BNST is still ambiguous. Also, Dc, the central part of the dorsal telencephalon, has been suggested to relate with paternal care in bluegill sunfish (Demski and Knigge, 1971). In the only study so far

investigating the neural substrates of parental care in fish using IEG, it was shown in the cichlid *A. nigrofasciata* that parental males have a higher activation of the Vv, but not of the POA or Dc, as compared with non-parental males (O'Connell et al., 2012).

Paternal care is more common than maternal care (Breder and Rosen, 1966) and thus the endocrine regulation of parental behavior has been mainly investigated in males. A conceptual framework for the relationship between androgens and parental care was proposed by Wingfield (1990), following the observation in birds that male androgen levels decrease during the parenting phase as compared with the mating phase, even in animals that continue to reproduce after the initiation of parental care (Wingfield, 1984). This hypothesis postulates that androgen levels should be lower during the paternal phase, when social instability is usually reduced, as compared to the mating phase, when males need to compete for territories and sexual partners. In fish, androgen levels drop during the parental phase thus supporting this prediction of the challenge hypothesis (Oliveira et al., 2002). However, there are many exceptions to this pattern (e.g. Rodgers et al., 2006; Ros et al., 2003) and exogenous administration of androgens to parenting males failed to have the expected suppressive effect in parental behavior in some species (T propionate implants in *L. macrochirus*, Rodgers et al., 2012; e.g. 11KT implants in *P. sanguinolentus parvircornis*, Ros et al., 2004). In the context of the challenge hypothesis, the regulation of paternal behavior by androgens is interpreted as a trade-off between parental behavior and androgen-induced aggression. The decrease in androgen levels postulated to occur during the parental phase would decrease the frequency of aggressive behaviors, releasing more energy and time to parental duties. Androgens may thus be better seen as secondary modulators of parental behaviors, more related with aggressive displays, while other hormones are expected to have a more direct regulatory action on specific parental care displays.

Prolactin (PRL) has been suggested as a prime candidate for the endocrine modulation of both paternal and maternal behavior across vertebrates (e.g. Bachelot and Binart, 2007; Schradin and Anzenberger, 1999), including fish (reviewed in Whittington and Wilson, 2013). The first study on the effects of this hormone in fish paternal behavior were conducted in the ocellated wrasse *Symphodus ocellatus* where PRL administration was shown to promote egg fanning behavior in nesting males (Fiedler, 1962). These results were corroborated by studies in other species, both in males (e.g. blue discus, *Symphysodon aequifasciata*, Blüm and Fiedler, 1965; *L. macrochirus*, Kindler et al., 1991; *G. aculeatus*, Páll et al., 2004) and females (e.g. *S. aequifasciata*, Blüm and Fiedler, 1965). However, there are also studies where the expected positive effect of PRL on parental behavior was not observed. In the cooperatively breeding

cichlid *N. pulcher,* PRL mRNA levels in the pituitary were not elevated in breeding females as compared with non-breeding females and PRL administration to both males and females did not promote parental behavior (Bender et al., 2008). In the Nile tilapia *Oreochromis niloticus*, pituitary and plasma levels of the two PRL isoforms described in fish also did not differ between female mouthbrooding eggs and non-incubating females (Tacon et al., 2000). Nevertheless, PRL II showed high variation during the mouthbrooding period and the authors did not exclude a possible role of PRL in the regulation of maternal behavior. In a comparative study between a monogamous and a polygynous species of cichlids of the genus *Herichthys*, gene expression levels of PRL and of a PRL receptor in brain macroareas were not associated with paternal care (Oldfield et al., 2013).

Interestingly, sex steroids have been found to interact with PRL, raising the possibility of modulation of parental behavior by sex steroids occurring indirectly via effects in PRL. Estrogens have been found to promote the secretion of PRL from pituitary glands (e.g. *O. mossambicus* Barry and Grau, 1986; rainbow trout *O. mykiss*, Williams and Wigham, 1994). Onuma et al. (2005), report variable effects of E2, T and 11KT in PRL mRNA expression levels in pituitary cell cultures of Masu salmon depending on gender and time in the reproductive season. During the pre-spawning stage, E2, T and 11KT increased the expression of PRL mRNA while opposite effects were detected during the spawning stage, suggesting that sex steroids may regulate both positively and negatively PRL secretion. This study also highlights the importance of integrating variation in tissue sensitivity to modulatory agents to understand their mode of action.

Similar to what has been described for oxytocin in mammals, IT has also been found to regulate paternal care in fish. In the monogamous cichlid, *A. nigrofasciata*, *c-fos* expression was higher in POA parvocellular IT neurons in fathers that in non-fathers and the administration of an IT receptor antagonist blocked paternal care (O'Connell et al., 2012). Interestingly, IT fibers and IT receptors are present in the Vv, a brain area observed to be more activated in fathers than in non-fathers, raising the possibility of stimulatory effects of IT on parental behavior occurring via modulation of neuronal signals in this brain region (O'Connell et al., 2012).

3.4. Prosocial behavior

The idea that social bonding, or affiliative behavior, can be regulated by evolutionary conserved brain and neurochemical systems across vertebrates is relatively new. In fact, since the ground-

breaking research on the role of AVP and oxytocin in the regulation of social behavior in rodents (reviewed in Young et al., 2011), evidence has been accumulating showing that these nonapeptides, and their nonmammalian homologues, play an important role in social bonding, affiliative behavior and attention to social stimuli also in other taxa, including fish (reviewed in Godwin and Thompson, 2012). In goldfish, intracerebroventricular administration of AVT and IT has opposite effects in social behavior, with AVT reducing approach behavior towards a conspecific and IT increasing the duration of this behavior (Thompson and Walton, 2004). The inhibitory effects of AVT seemed to be mediated by AVT cell projections from the POA to the hindbrain (Thompson and Walton, 2009). This was confirmed by showing that the behavioral effects of AVT infusions into the 4th ventricle were more potent than the effects of infusions into the 3rd ventricle (Thompson et al., 2008). Accordingly, it was demonstrated that seasonal changes in behavioral responsiveness to AVT are associated with changes in hindbrain sensitivity to AVT, as measured by the expression of the V1a AVT receptor in this brain region (Walton et al., 2010). In the cichlid *N. pulcher*, peripheral administration of IT seemed to increase responsiveness to socially relevant information during aggressive contests as IT-treated fish fought in accordance with the size of the opponent while control animals fought according to their intrinsic aggressive levels (Reddon et al., 2012). In the monogamous cichlid *A. nigrofasciata*, the peripheral administration of an AVP/IT receptor antagonist to males reduced affiliative behavior although it did not prevent pair-bonding nor did it disrupt pair bonding after pairs had been established (Oldfield and Hofmann, 2011). In the cleaner wrasse, *Labroides dimidiatus*, AVT inhibited interspecific cleaning activities while it did not affect other social conspecific behaviors (Soares et al., 2012). In the same study, IT administration failed to affect social behavior. In contrast with previous studies, in *N. pulcher* IT administered intraperitoneally reduced affiliative behavior and in a second study brain IT levels were found to be negatively correlated with these behaviors (Reddon et al., 2015).

Information on the brain areas targeted by AVT or IT to regulate social bonding is still very scarce but Godwin and Thompson (2012) suggest forebrain nodes of the SDMN to be likely candidates for AVT modulation; indeed motor output pathways descending from these regions show dense AVT innervation and project into multiple central targets. New studies selectively manipulating subpopulations of AVT cells will be able to test this hypothesis.

4. Hormones as social semiochemicals

When hormones are released into the environment, either actively or passively, they convey information about the sender that becomes potentially available to other conspecifics, and thus can be seen as social semiochemicals (i.e. chemical cues of conspecific origin). If released hormones have evolved to convey information about the sender and trigger a specific and adaptive response in the receivers, then they can be viewed as pheromones (Sorensen, 2014; Wyatt, 2010). Thus, the evolution of hormonal pheromones has been proposed to follow a cue-signal continuum, where different evolutionary states can be recognized (Sorensen and Scott, 1994; Sorensen and Stacey, 1999; Wisenden, 2014; Fig 2). In the ancestral state, senders release hormones into the environment but potential receivers are unable to detect them. In a second state, receivers evolved the capacity to detect and respond adaptively to the hormone, hence they are now spying on senders based on the hormone that acts as a chemical cue for the internal state of senders. Finally, a third evolutionary state may evolve if the selective pressure imposed by spying on senders leads to the evolution of specialized production and/or release of the hormonal pheromones, which becomes a signal (i.e. true pheromone) according to animal communication terminology (Wisenden, 2014).

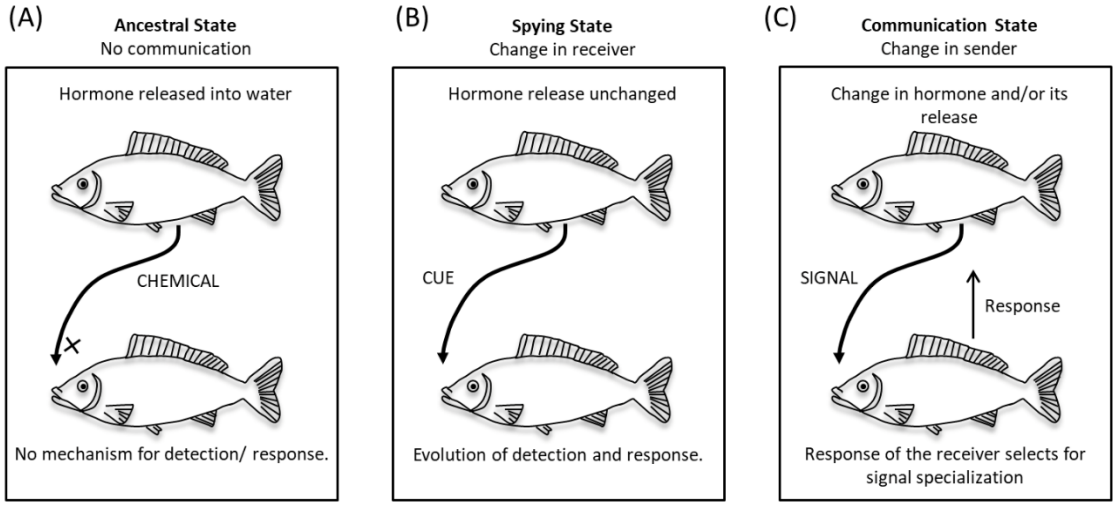

Figure 2. Stages in the evolution of chemical signaling in fish: (A) ancestral state, in which fish release a hormone that is not detected by conspecifics; (B) spying state, where conspecifics are able to detect and respond adaptively to the hormone; (C) communication state, characterized by a response of the receiver which selects for signal specialization.

An apparently easy way to discriminate between spying and true signaling would be to look for the occurrence of specialized structures in the production and/or release of pheromones. Many such structures have been described among teleost fish, such as the seminal vesicles in catfish (*Clarias gariepinus*) that release female attractants (Resink et al., 1989), the hypertrophied mesorchial glands in the testes of gobies that secrete steroids that attract females (e.g. Colombo et al., 1980; Murphy et al., 2001), or anal glands in blennies, which consist of a transformation of the first rays of the anal fin into a sex-pheromone producing gland (Serrano et al., 2008a, 2008b). Interestingly, in the case of both gobies and blennies i.e., in species in which male alternative reproductive tactics occurs and both territorial and sneaker males are present, only territorial males develop the glands that produce female attractants (blennies: Gonçalves et al., 1996; gobies: Locatello et al., 2002), suggesting a secondary loss of the pheromone-producing tissue in sneakers. However, the absence of such specialized structures cannot by itself rule out the ability to release/store pheromones and thus apparent cases of chemical spying in fish, may just reflect our failure to detect these more subtle specializations. A good example of such scenario is the goldfish (*Carassius auratus*), where, despite lacking any obvious specialization for production and release of pheromones, females release sequentially two hormonal pheromones: (1) a preovulatory pheromone, consisting of progestins (17,20-beta-dihydroxy-4-pregen-3-one and its sulphated form) and androstenedione, that has a primer effect on sperm production in males; and (2) a post-ovulatory pheromone, consisting of prostaglandins (Prostaglandin F2-alfa and 15-keto-PGF2-alfa), that triggers male courtship and makes ovulated females attractive to the males (reviewed in Stacey and Sorensen, 2002). Given that these female pheromones are the result of passive release into the water of sex hormones and their metabolites involved in female ovulation (progestins and androstenedione) and in the regulation of female reproductive behavior (prostaglandins), and there are no specialization for the production and/or their release in the scope of chemical communication, this system has initially been considered a classic example of males spying on reproductive state of females in order to increase their reproductive success (Stacey and Sorensen, 2002; Wisenden and Stacey, 2005). However, subsequently it has been found that these hormones are mainly released through the urine and that female goldfish increase the frequency of pulses of urine when in the presence of a male, in particular if in the presence of oviposition substrate (Appelt and Sorensen, 2007), indicating a specialization in the sender for the release of the signal, compatible with a true signaling scenario. This form of control of signal release is also present in other species where hormonal pheromones are released through the urine and males adjust the urination rate in response to the presence of receptive/ pre-ovulatory females (e.g.

Mozambique Tilapia, *O. mossambicus*, Almeida et al., 2005; Burton's mouthbrooder, *A. burtoni*, Maruska and Fernald, 2012; swordtail, *Xiphophorus birchmanni*, Rosenthal et al., 2011) . Thus, the role of hormones as pheromones may be more common than initially thought.

Most of the examples of hormonal pheromones provided above are related to reproduction and to their effect on the behavior and physiology of the opposite sex. However, evidence has accumulated indicating a role for chemical cues in other aspects of fish social behavior such as intra-sexual aggression, parental care, and affiliative behaviors (Keller-Costa et al., 2015; e.g. Sorensen and Baker, 2014). Unfortunately, for most of these other cases of chemical communication the chemical identity of the cue/signal is not know, and thus hormones cannot be directly implicated. One particular function that has been the focus of recent research is the role of chemical communication in the regulation of male-male aggression in cichlids (Keller-Costa et al., 2015). In the Mozambique tilapia, males also release urine during agonistic interactions in pulses of short duration and those that become subordinate stop releasing urine (Fig.3; Barata et al., 2007). If urination is surgically prevented agonistic interactions escalate and more overt aggressive behaviors are expressed (Keller-Costa et al., 2012). Moreover, in stable dominance hierarchies the olfactory potency of the urine is correlated with the male's social rank (Barata et al., 2007) and urine of dominant and subordinate males triggers different patterns of gene expression in olfactory brain regions of male receivers (Simões et al., 2015a).

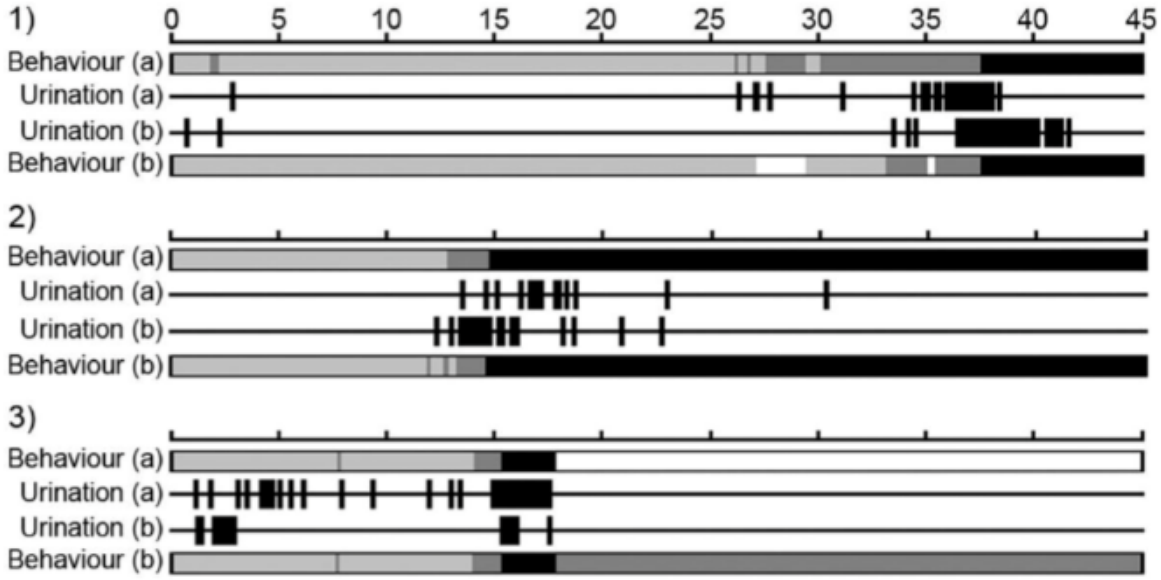

Figure 3. Examples (1–3) of behavior of two territorial tilapia males (a) and (b) interacting for 45 min (submissive: white; not aggressive: light grey; aggressive displays: dark grey; highly aggressive: black) and their corresponding release of urine pulses (urination). In (1), male (a)

started aggressive behavior and urination (time point around 25 min), subsequently male (b) initiated aggressive displays and then agonistic interaction escalated to high symmetrical aggression. In (2), both males increased their urination frequency and aggressivity almost at the same time (within 10–15) and maintained agonistic behavior until the end of experiment, although urine pulses decreased significantly. In (3), after the release of several urine pulses from both males and a short period of symmetrical high aggression, male (a) became submissive whereas male (b) continued with agonistic displays; both males stopped urine release at this time point (around 18 min) (adapted from Barata et al., 2007).

Together these results strongly suggest the presence of a chemical signal in the urine used in social status assessment in this species. Interestingly, dominant males also store large volumes of urine (Barata et al., 2007), having hypertrophied urinary bladders (Keller-Costa et al., 2012), which indicates a specialization for urine release supportive of the evolution of a true dominance pheromone rather than just a dominance cue. The chemical identity of this putative dominance pheromone has not been established yet, but it is known to have multiple components, present both in polar and non-polar urine fractions (Keller-Costa et al., 2016) and to be distinct from the sex pheromone that has primer physiological effects on females (Keller-Costa et al., 2014). Similar evidence for the occurrence of putative dominance pheromones has also been collected for two other cichlid species (Burton's mouthbrooder, Crapon de Caprona, 1980; Maruska and Fernald, 2012; Nile tilapia, Giaquinto and Volpato, 1997; Gonçalves-de-Freitas et al., 2008), suggesting that pheromones may be also be a widespread phenomena in this teleost family.

5. Conclusions

To conclude, it is now clear that hormones exert powerful modulatory effects on social behavior by acting on a core of forebrain and midbrain areas that underlie the expression of these behaviors in fishes. However, the exact mechanism through which hormones change the functional connectivity of the SDMN to affect behavior remains poorly understood and this is a promising area for research. Studies manipulating hormone levels and investigating the effect in the neurogenomic states and neuronal output of nodes of the SDMN will help to elucidate how hormones modulate the expression of social behaviors. Also, different hormonal systems are known to interact with each other, and studies addressing the cross-talk between endocrine systems are valuable. Finally, hormones may also act on social behavior by acting as pheromones and their role in the regulation of social interactions has started to be unveiled.

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APPENDIX B

Integrative Neurobiology of Social Behavior in Cichlid Fish

In press:

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Abstract

Social behavior encompasses the interactions between individuals of the same species, fundamental to their survival and reproduction. The study of these kinds of behavioral patterns and the unraveling of its underpinnings is a fascinating research area. However, to fully understand social behavior it is essential to integrate the various components underlying social interactions. From a mechanistic point of view, we ought to grasp specifically how the brain controls behavior, through the concerted action of its neural circuits, cells, genes and molecules, and also how the social environment feedbacks and impacts the brain. On the other hand, this pursuit of knowledge on the proximate factors which determine social behavior is pivotal to achieve valuable insights on its ultimate causes. Performing comparative studies across different species, taking in consideration developmental, ecological or life history features, has been a growing concern. A considerable amount of literature has been published on these matters using cichlids as model systems. Cichlids can give an important contribution to the field due to their amazing diversity and complexity of behavioral patterns and mating strategies. Here, we review the current state of knowledge on the neural basis of social behavior specifically focusing on studies carried out with cichlid fish.

Introduction

The aim of this chapter is to provide a comprehensive review of the neurobiology of social behavior in cichlids. First, we begin to present cichlid fish as emerging vertebrate models on the study of social behavior, highlighting some of their remarkable features that can be explored to acquire new sights in the field. Next, the Social Decision Making Network, a neural network which involves a set of core brain areas interconnected with each other that together control social behavior and are modulated by steroids and nonapeptides, is introduced and its application to cichlid research is discussed. The third section examines the Social Brain hypothesis proposed to explain animal cognition and the relation between sociality and brain size. An overview of the notable cognitive adaptations for social living in cichlids is also included. The following section discusses the neuroendocrine regulation of social behavior, by exploring how steroids and nonapeptides act in the brain and influence behavior and conversely how social behavior affects hormones. Finally, the neurogenomics of social behavior, namely the discovery of the specific genes and pathways which regulate behavior acquired with high throughput genomic tools, is addressed and several studies in cichlids are reported.

1. Cichlids as Models for the Study of Social Behavior

The Cichlidae family is distinguished for being the most species-rich family of vertebrates, with more than 3,000 species distributed widely along American, African and Asian continents (Kocher 2004). Cichlids are subject of particular interest on their explosive and diverse speciation since around 2,000 species evolved in a short period of time (Kocher 2004; Seehausen 2006). Also, the parallel evolution of adaptive phenotypes, either in closed and distant related lineages, and all the underlying mechanisms involved along the process of evolution are central themes on cichlids (Henning and Meyer 2014).

Besides phenotypic diversity such as color patterns, body shapes or head morphology, these fish are characterized by diverse social systems. Mating systems vary between: 1) monogamy, when a male and a female form a mating pair establishing a pair bond, e.g. *Tropheus moori* (Egger et al. 2006) and *Pelvicachromis taeniatus (Langen et al. 2013)*, 2) polygamy, when a mate has several partners but with possible selection of the partner at each spawning, e.g. Neol*amprologus brichardi* (Limberger 1983), 3) polygyny, when males mate with several females but females only mate with one partner, e.g. *Lamprologus callipterus* (Sato 1994), 4) polyandrous, when females mate with several males but a male spawns with only a female, e.g. *Julidochromis transcriptus* (Kohda et al. 2009), or 5) promiscuous, when males fertilize eggs from several females and the eggs of one female can be fertilized by several males, e.g. *Mchenga eucinostomus* (McKaye 1983). Another appealing facet is their reproductive strategies (Taborsky 2001) as they can monopolize mates or resources by defending spawning sites or nests in lek-systems, e.g. *Lethrinops c.f. parvidens* (Kellogg et al. 2000), perform sneaking fertilizations by parasitic males, e.g. *Oreochromis mossambicus* (Oliveira and Almada 1998a) and adopt mutualistic cooperative breeding systems , e.g. *N. brichardi* (Taborsky 1984a). There are cases where fertilization occurs externally, e.g. *Pseudocrenilabrus multicolor* (Mrowka 1987) or orally by females that suck the male sperm into the buccal cavity where they keep the eggs, e.g. *Thoracochromis wingatii* (Wickler 1962). Then parents can incubate eggs in nests, e.g. *L. callipterus* (Sato 1994), caves, e.g. *P. taeniatus* (Thünken et al. 2007) or in their mouths as female mouthbrooders, e.g. *Tropheus moori* (Egger et al. 2006), or male mouthbrooders, e.g. *Xenotilapia flavopinnis* (Kuwamura et al. 1989). Finally, parental care systems range between biparental, e.g. *P. taeniatus (Langen et al. 2013)*, unipaternal, e.g. Saint Peter's fish, *Sarotherodon galilaeus* (Balshine-Earn 1997) or unimaternal, e.g. *Pseudocrenilabrus multicolor* (Mrowka 1987). There are also species that can display several of these forms of care, e.g. *S. galilaeus* (Schwanck and Rana 1991; Balshine-Earn 1997). Figure

1 presents a phylogenetic comparative analysis among some of the Lake Tanganyika cichlids, which integrates behavioral traits (form of care and sex of the care provider) as an example of this behavioral diversity.

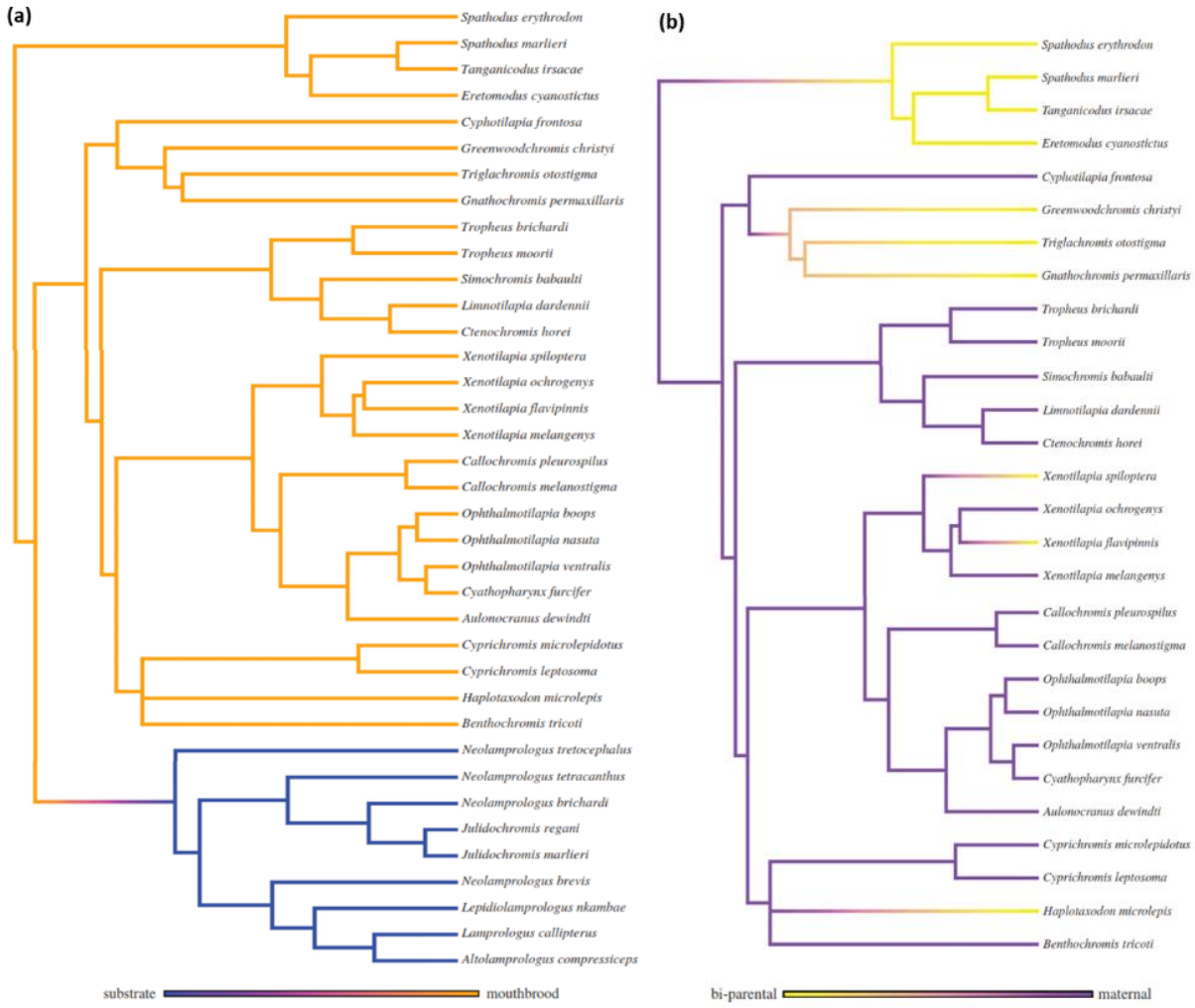

Figure 1. A molecular phylogeny of some of the Lake Tanganyika species with possible character transitions in (a) the form of care (substrate guarding in blue and mouthbrooding in orange) and (b) the sex of the care provider (bi-parental care in yellow and maternal care in purple). Adapted from Tsuboi et al. 2015.

In addition to this vast and unique repertoire of social behavior, the advantage of having five cichlid genomes and transcriptomes released, namely *Oreochromis niloticus*, *Neolamprologus brichardi/pulcher*, *Maylandia zebra*, *Haplochromis nyererei*, and *Astatotilapia burtoni* (Brawand et al. 2014) and the recent development of powerful tools applicable in cichlidae species, such as high-throughput sequencing (e.g. RNA-seq), transgenics (Juntti et al. 2013; Golan and Levavi-Sivan 2013; Ma et al. 2015) with particular

emphasis on CRISPR/Cas9 mutagenesis technique (Juntti et al. 2016), has projected cichlids to the spotlights. In the near future, we expect exciting developments within cichlids research and in particular in social behavior.

2. The Cichlid Social Brain: Social Complexity and Brain Evolution

Traditionally, social behavior repertoire was considered to be determined by specific and differential brain areas or mini-circuits. However, a growing body of literature recognizes that social behavior is regulated by a broader and dynamic brain network. Newman (1999) was the first to introduce the concept of the Social Behavior Network (SBN) in mammals, which states the existence of a set of brain areas that together control social behavior. The SBN is composed by six nodes: the Medial Extended Amygdala (meAMY/BNST), the Lateral Septum (LS), the Medial Preoptic Area (POA), the Anterior Hypothalamus (AH), the Ventromedial and Ventrolateral Hypothalamus (VMH) and the Midbrain Periaqueductal Gray and Tegmentum (PAG/CG). It is important to bear in mind that other unspecified areas are also relevant for characterizing social conducts yet each one of the SBN areas is a core node, reciprocally interconnected with the others, expresses sex steroid hormone receptors and has an established role in the activation or regulation of several types of social behavior. Diverse studies using brain lesions, electrical manipulation, neuropharmacological manipulations, and immediate early gene expression provided solid evidence for the common involvement of these specific areas on reproductive, parental or even aggressive behavior. SBN is thereby defined as an integrated neuroanatomical network in which the dynamic activation patterns of the nodes are responsible for multiple behaviors modulated by social environment and sex steroids. For instance, a sequence of temporal behavioral responses such as sniffing, mounting, ejaculation or grooming (the typical repertoire of male rodents' reproductive behavior), would be the result of the activation of this circuit, modulated either by external factors (environment) and intrinsic components (sex-steroids). Also, the key determinant factors of species and sex, are responsible for ascertaining brain's organization and connectivity across a common central network, which in turn are shaped by hormones throughout development and lifetime. As a consequence, a vast array of social behavior patterns which are species-specific arises, as well as pronounced dissimilarities among males and females within the same species (Newman 1999).

In 2005, Goodson (2005) suggested the extension of this model to non-mammalian vertebrates. He presented several evidence to support that birds and teleost fish also have a SBN, and particularly an amazing evolutionarily conservation of the mechanisms which

regulate social behavior in vertebrates. Furthermore, he introduced neuropeptide modulation, namely arginine vasotocin (AVT, the mammalian homologue of arginine vasopressin) and isotocin (IT, the homologue of oxytocin), as an important component of the SBN by enabling additional plasticity and diversity to social behavior.

However, an individuals' adaptive response is an integration of internal physiological cues and external stimuli being evaluated. The evaluation of the salience of a stimulus is assumed to be regulated by the Mesolimbic Reward System (via dopaminergic signaling) and is pivotal on social behavior. In that sense, O'Connell and Hofmann (2011) proposed that social behavior would be concurrently regulated by two neural circuits: the SBN and the Mesolimbic Reward System, constituting the Social-Decision Making Network (SDMN). The mesolimbic reward system is a composite of eight areas: the Striatum (Str), the Nucleus Accumbens (NAcc), the Ventral Pallidum (VP), the Basolateral Amygdala (blAMY), the Hippocampus (Hyp), the Ventral Tegmental Area (VTA), and the LS and the BNST/meAMY, overlapping nodes of the SBN. With this study, they provided a comparative analysis of these two networks in five major vertebrate lineages: mammals, birds, reptiles, amphibians and teleost fish. Based on hodology, neurochemical profiles, development, gene expression, presence of steroid hormone receptors and behavioral functional studies, the authors presented putative brain homologies. Their aim was achieved - to provide a useful resource to study the neural substrates responsible for social behavior in vertebrates and a relevant framework to make species systematic comparisons.

Although the SDMN model is consistent and strongly supported in mammals, its application to other non-mammalian species is refutable. Some of the proposed homologies are not straightforward or are only partial, and functional studies are lacking, particularly for the mesolimbic reward system, raising apprehension in its extrapolation to other taxa, such as cichlids (Goodson and Kingsbury 2013). One example of such is the POA node, where the anamniotes correspondent comprehends vasopressin-oxytocin nonapeptides neurons, whereas in amniotes those cells are within the paraventricular nucleus of the hypothalamus (PVN). Thus, some propose the mammalian node as POA/PVN, thereby including these peptidergic neurons, so important on behavioral modulation (Goodson and Kingsbury 2013). The same authors endorse the SDM as a workable framework that is not yet an evidenced and confirmed model.

2.1. Comparing Teleost Fish and Mammals: SDMN Brain Homologies

Teleost SBN is presumably constituted by the supracommisural part of the ventral pallium (Vs), the ventral (Vv) and lateral (Vl) parts of ventral telencephalon, the POA, the ventral tuberal nucleus (vTn), the anterior tuberal nucleus (aTn), all localized in the forebrain, and the PAG, lying in the midbrain (O'Connell and Hofmann 2011). The Mesolimbic Reward System is assumed to be composed by the central (Vc) and dorsal (Vd) parts of the ventral telencephalon, the medial part of the dorsal telencephalon (Dm), the lateral part of the dorsal telencephalon (Dl), the posterior tuberculum (TPp) on the midbrain, and also the Vv/Vl and the Vs, concurring nodes of the SBN (O'Connell and Hofmann 2011). Figure 2 represents a schematic diagram of the SDMN in teleosts and Table 1 summarizes the putative mammalian correspondence for each teleost brain nuclei.

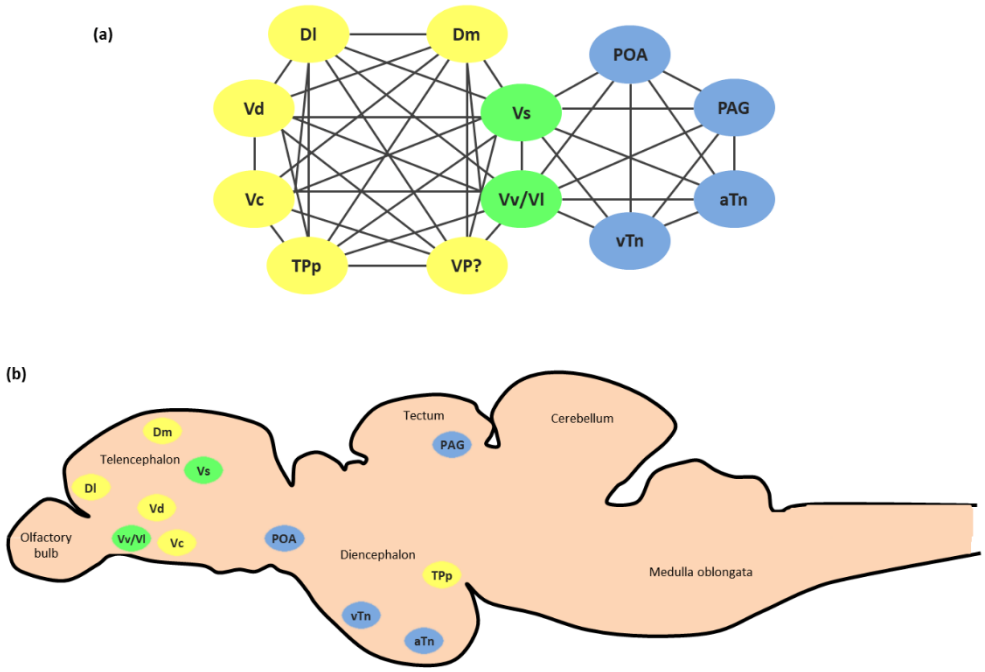

Figure 2. The Social-Decision Making Network (SDMN). (a) Representation of the interaction between hormones and the SDMN within teleosts social behavior: putative nodes of the Mesolimbic Reward System in yellow - dorsal (Vd) and central (Vc) part of the ventral telencephalon, medial part of the dorsal telencephalon (Dm), lateral part of the dorsal telencephalon (Dl), posterior tuberculum (TPp) -, and the Social Behavior Network in blue - Medial Preoptic Area (POA), ventral tuberal region (vTn), anterior tuberal nucleus (aTn), and Midbrain Periaqueductal Gray (PAG). Ventral and lateral (Vv/Vl) part

of ventral telencephalon and supracommisural part of the ventral pallium (Vs), overlapping nodes of the SBN and the Mesolimbic Reward System, are in green. A homologous for the mammalian Ventral Pallidum (VP) node has not yet been identified. (b) Schematic diagram of a sagittal section of a teleost brain representing the SDMN brain nodes.

Table 1. Putative mammalian correspondence for each teleost brain nuclei of the Social-Decision Making Network (SDMN).

	Abbrev.	Teleosts SDMN Brain Nuclei	Putative Mammalian Correspondence	
Social Behavior Network	POA	Medial preoptic area	Medial preoptic area	
	vTn	Ventral tuberal nucleus	Anterior hypothalamus	
	aTn	Anterior tuberal nucleus	Ventromedial and ventrolateral hypothalamus	
	PAG	Periaqueductal gray	Periaqueductal gray and tegmentum	
	Vs	Supracommisural part of the ventral pallium	Medial extended amygdala (bed nucleus of the stria terminalis and medial amygdala)	
	Vv/Vl	Ventral (Vv) and lateral (Vl) parts of ventral telencephalon	Lateral septum	**Mesolimbic Reward System**
	Vc	Central part of the ventral telencephalon	Striatum	
	Vd	Dorsal part of the ventral telencephalon	Nucleus accumbens	
	Dm	Medial part of the dorsal telencephalon	Basolateral amygdala	
	Dl	Lateral part of the dorsal telencephalon	Hippocampus	
	TPp	Posterior tuberculum	Ventral tegmental area	

The fact that teleost telencephalon suffers an eversion during development - instead of an invagination like all other vertebrates - renders homology determination an arduous task (Wullimann and Mueller 2004). Nevertheless, grounded on neuron connectivity, neurochemical profiles, development, gene expression, presence of steroid hormone receptors and functional studies, O'Connell and Hofmann (2011) present the following brain homologies:

- The Vs is the putative homologous region of the meAMY/BNST, due to conserved expression patterns of developmental genes, hodological features and neurochemical studies (reviewed in O'Connell and Hofmann 2011). Goodson and Kingsbury (2013)

though indicate chemoarchitecturally evidence to consider that the postcommissural nucleus of the ventral telencephalon (Vp) is combined with the Vs and suggest that the Vs/Vp is even homologue to the entire subpallial amygdala.

- The Vv and Vl are comparable to the mammalian LS region since cholinergic neurons were only detected here. This area is reciprocally connected to other nuclei and it expresses sex-steroid receptors (see Wullimann and Mueller 2004; O'Connell and Hofmann 2011 for more details). Its relationship with reproductive behavior is supported by some studies – the Vv and the Vs ablation of *Carassius auratus* impairs males' ejaculation while stimulation of the Vv in females' *Oncorhynchus nerka* elicits digging and spawning (Kyle and Peter 1982; Satou et al. 1984). However, besides the fact that the Vv present some similarities with other regions, the Vv/Vl are exclusive of subpallial origins while LS has important pallial components involved in the process information between the SDM and the mesolimbic system. Thus, relevant questions arise with this homology (Goodson and Kingsbury 2013).
- The teleost POA is localized in the hypothalamus along the third ventricle and is divided into three subregions accordingly to cell size: parvocellular, magnocellular and gigantocellular. Like in mammals, it is reciprocally connected with telencephalon and other hypothalamus regions (reviewed in O'Connell and Hofmann 2011). Several studies report its role in sexual, parental and aggressive behaviors (Demski and Knigge 1971; Macey et al. 1974; Satou et al. 1984; Wong 2000).
- The vTn was proposed to be the teleost correspondence of AH since it is localized between the POA and the ventral hypothalamus, it receives and sends projections to several hypothalamic regions and expresses sex-steroid receptors (O'Connell and Hofmann 2011 revised this information). Despite Goodson and Bass (2000) proposed preoptic area–anterior hypothalamus region as a regulatory component of reproductive vocalizations in *Porichthys notatus*, other functional studies are yet not available.
- The homologous of aTn is considered to be the VMH, however, only a subset of aTn cells are actually homologous (Goodson and Kingsbury 2013). It is localized in the ventrocaudal region of the hypothalamus, receiving and sending projections to several parts of the telencephalon and contains sex-steroid hormone receptors. O'Connell and Hofmann (2011) provide further details. Functional studies are as well limited.
- The PAG is located near the torus semicircularis. It is reciprocally connected with several other nuclei and contains sex-steroid hormone receptors (see O'Connell and Hofmann

2011, for more information). Functionally similar to mammals, it is as well associated with social communication, e.g., *P. notatus* (Kittelberger et al. 2006).

- The Vc seems homologous of the Str in mammals, essentially based on neurochemical studies (consider Wullimann and Mueller 2004 and O'Connell and Hofmann 2011, for more details).
- The Vd has been suggested to present homologies to the NAcc due to hodological evidence such as ascending dopaminergic projections or the presence of dopamine receptors and GABA immunoreactivity but at the neurochemical level presents similarities also to the Str (consider Wullimann and Mueller 2004 and O'Connell and Hofmann 2011, for more details). More studies are needed to fully comprehend Vc and Vd nuclei.
- Unfortunately, a teleost homology for the mammalian VP node has not yet been identified.
- The Dm seems to match the blAMY based on hodology, neurochemistry and lesions studies connecting this region to emotional learning, suchlike in mammals, as reviewed in Portavella et al. (2002) and O'Connell and Hofmann (2011).
- The Hyp is the putative homologous of the Dl due to tract tracing evidence and lesions studies in *C. auratus* showing its relevance in spatial learning; reviewed in Rodríguez et al. (2002) and O'Connell and Hofmann (2011).
- Lastly, the TPp homology is controversial. Amphibians and teleosts lack a midbrain dopaminergic population, however, TPp, located in the ventral diencephalon, seems homologous to mammalian VTA and/or substantia nigra because of its dopaminergic ascending projections to the striatum region and gene expression profiles (see O'Connell and Hofmann 2011 for details). On the other hand, a study on zebrafish uncovered that posterior tuberculum cells seem homologous to A11 mammalian dopamine neurons, contrary to what happens in the VTA, constituted by A10 dopamine neurons (Tay et al. 2011). Based on transcription factor conservation and projection patterns they showed that ascending projections to telencephalon are scarce, while the most important dopaminergic connections between the subpallium and the ventral diencephalon are as a matter of fact descending. Consequently, the existence of a mesolimbic reward system in fish is questionable (Goodson and Kingsbury 2013) since the VTA is considered a primary component of the mesolimbic dopamine system (Spanagel and Weiss 1999; Bromberg-Martin et al. 2010).

2.2. The Cichlid Social Decision Making Network (SDMN)

Endocrine modulation of the SDMN, and subsequently of social behavior, in cichlid fish, is supported by in situ hybridization studies in the Burton's mouthbrooder cichlid, *Astatotilapia burtoni* which report the expression of estrogen, progesterone, androgen, arginine vasotocin and oxytocin receptors (Harbott et al. 2007; Munchrath and Hofmann 2010; Huffman et al. 2012; O'Connell et al. 2012; Loveland and Fernald 2017; Weitekamp et al. 2017), widely distributed along the areas of the SDMN.

So far, most of the published studies documenting the activation of the SDMN network, specifically in cichlids, used *A. burtoni* as a fish model. One example of this is the work undertaken by Maruska et al (2013a, b). Since *A. burtoni* males can reversibly switch between dominant and subordinate status and rapidly present distinct phenotypes, the authors cleverly used a paradigm to manipulate social rank. Then, they measured by qPCR brain immediate early genes (IEG), the first genomic response to a stimulus, as markers of neuronal activity, in several brain areas of males either ascending or descending in social status and compared with control individuals. For social ascending males, both *c-fos* and *egr-1* levels were higher in all the studied SDMN nuclei (Vs, Vv, POA, vTn, aTn, Dm and Dl) (Maruska et al. 2013b). Surprisingly, descending males presented different activation patterns for *c-fos* and *egr-1* across the same areas, namely, increased *c-fos* expression levels in the Vs, POA and aTn while *egr-1* mRNA levels were higher in the Vv, Vs, vTn, Dm and Dl (Maruska et al. 2013a). Additionally, hormone levels are affected in minutes, which suggest that the SDMN is involved in integrating social information along with hormonal states, to favor social transitions (Maruska et al. 2013a, b).

On the other hand, Desjardins et al (2010) studied how social information regarding potential mates affects females at the brain level. They induced different neural states in gravid females by exposing them to fights where their preferred males won or lost. Then, they examined IEG, *c-fos* and *egr-1*, expression levels in the Vv (LS), POA, vTn (AH), aTn (VMH), PAG, Dm and Dl, selected nodes of the SDMN. Results demonstrate that reproductive nuclei, namely POA and VMH (see Sakuma and Pfaff 1979, for a supporting example on the VMH role in mammals' reproductive behavior), show highest IEG expression when females see their preferred males winning. In contrast, the mammals LS homologue region, which is a nucleus associated with anxiety in mammals (Degroot et al. 2001) is highly activated when females see the male losing.

O'Connell et al (2013) focused on how individuals process social cues, by presenting *A. burtoni* males with different sensory stimuli (visual and/or chemical) in distinct social context. They found that visual information (seeing a female or a male) is sufficient to elicit *c-fos* transcription in dopaminergic neurons of Vc, compared to the neutral (control) condition. Interestingly, in the case where males were exposed to an intruder male stimulus, the elicited genomic response is significantly correlated to aggression but not with motor activity. The authors suggest that the Vc can be involved in assessing stimulus visual valence.

Other researchers evaluated the influence of the nonapeptide isotocin in parental care by using the monogamous cichlid *Amatitlania nigrofasciata* (O'Connell et al. 2012). They quantified *c-fos* expression by in situ hybridization technique to compare biparental males housed with their mate (control males), single fathers with the mate removed or lone males with mate and offspring removed. They directed their interest to Vv, POA and the central part of the dorsal telencephalon (Dc) and also in the co-localization of *c-fos* and isotocin in the POA. Single fathers increased paternal care immediately after mate removal and also presented significantly higher IEG activity levels in the Vv compared to lone males, as well as increased *c-fos* expression in the parvocellular preoptic isotocin neurons. In summary, these results show that isotocin is involved in paternal care by promoting parental behavior after mate removal and that Vv and POA are important underlying brain areas.

Finally, Roleira et al. (2017) analyzed, by qPCR, the patterns of brain activation of Mozambique tilapia (*O. mossambicus*) males subjected to territorial intrusions, in the presence or absence of a female audience and tested the SDMN hypothesis. Focused on studying the mechanisms underlying the audience effect phenomenon (see 3.1 section for more details on this subject), they verified that, besides the increase of the territorial defense behaviors by focal males in the presence of females, contrasting social contexts originated different behavioral states represented by distinct patterns of functional connectivity across the SDMN nodes. In particular, no localized activity (i.e. immediate-early genes expression *c-fos* and *egr-1*) of any of these nodes (Dm, Dl, Vv, Vs, POA, aTn, CG) was attributed to either of the treatments but instead different clusters of brain areas and corresponded densities of connections, supporting the SDMN model (Roleira et al. 2017). These cases and others, e.g. in the teleost fish *Danio rerio* (Teles et al. 2015), support the SDMN hypothesis and its involvement in the regulation of social behavior.

3. Cognitive Adaptations for Social Living

Social cognition is the process of acquiring information and also to manage, store and apply it whenever is necessary, particularly in the context of social relationships (Dukas 2004). The term embodies a manifold of concepts such as perception, social learning, memory, attention or decision making (Dukas 2004), which allow an individual to apprehend social information and adopt proper behavioral responses.

'The Machiavellian intelligence hypothesis' (Byrne and Whiten 1988, 1997) was initially proposed to explain the evolution of cognitive abilities in primates as a result of social complexity. The main idea is that selective forces acted preferentially upon individuals with advantageous social strategies, such as, manipulation and deception, which allow them to have more successful competitive interactions with others. Increased fitness is achieved when an individual benefits at the expense of others (manipulation); occasionally both parts gain (cooperation), while in other situations group members are unaware of the loss involved (deception). Clearly, the cognitive capacities of recognizing conspecifics and recall relative status, affiliations or even past events are essential for one to adopt the above-mentioned Machiavellian strategies (Byrne 1997). With this hypothesis, the authors also suggested that social cognitive abilities are related to size or structure of the brain, based on the fact that primates have larger brains and enhanced cognitive skills compared with other animals (Byrne and Whiten 1988, 1997). However, Dunbar (1992, 1995) was the first to test this hypothesis showing that primate group size correlates with relative neocortical volume. As a consequence, the 'Social brain hypothesis' (Dunbar 1998) as an alternative label was adopted, which posits that complex societies require more social cognitive competences and consequently larger brains, specifically neocortex tissue, to process the increasing degree of information involved. Interestingly, relative brain size is specifically associated with pair bonding, i.e., larger brains correlate with monogamy, suggesting that this was the main factor underlying brain evolution (Dunbar and Shultz 2007). Both the Machiavellian intelligence hypothesis and the social brain hypothesis have been applied to other vertebrate taxa, namely fish (Bshary 2006, 2011; Dunbar and Shultz 2007).

For instance, Pollen et al (2007) compared closely related species of cichlids from the Ectodini clade of Lake Tanganyikan that differed in habitat preference and social organization. They obtained correlations between habitat features and brain size, cerebellar size, medulla and olfactory bulb, while only telencephalon and hypothalamus correlated with sociality. The telencephalon is larger whereas the hypothalamus is decreased in monogamous species. These

results endorse the ‘mosaic evolution model’ of the brain because suggest that selection acted differentially on distinct brain regions and that both environmental and social characteristics acted as selective pressures (Barton and Harvey 2000). Within this model, each brain region is functionally different, i.e., is responsible for a distinct set of behaviors and evolution acts only in the regions underlying the cognitive traits being selected (favoring its enlargement) since brain tissue is metabolic costly (Isler and van Schaik 2006). In opposition, the ‘developmental constraints model’ defends that if selection acts on the brain, it would induce a change in whole brain structure (Finlay et al. 2001). Likewise, a more extensive study on 43 cichlid species of this lake corroborated the mosaic evolution model since brain structures showed variation in their patterns of evolution despite some contribution of concerted evolution (Gonzalez-Voyer et al. 2009a). Another study, focused on 39 species from the Lake Tanganyikan, evidenced that brain size is correlated with diet and female brain size correlates significantly with care type (Gonzalez-Voyer et al. 2009b). Specifically, in species where only the females care for the fry females had larger brains than females that are helped by males in the care of the young (biparental care) (Gonzalez-Voyer et al. 2009b). However, other analyses report that after controlling for brain size, only cerebellum and hypothalamus from males are actually negative-associated with female-only care (Gonzalez-Voyer and Kolm 2010). In the same study, cerebellum volume seems decreased with sexual selection, hypothalamus is negatively associated with mating competition and telencephalon size shows sexual dimorphism and is negatively correlated with mating competition (Gonzalez-Voyer and Kolm 2010).

Having in mind the Social brain hypothesis, Reddon and colleagues (2016) compared several related cooperative cichlid species from Lake Tanganyika and other non-cooperative cichlids to test if cooperation is translated in larger brains. Typically, in cooperatively breeding species, fish live in groups with a dominant breeding pair and several conspecifics that act as helpers. Helpers defend the territory against predators and other conspecifics and participate in brood care. This apparent altruistic behavior has costs (e.g. slower growth rate) and benefits (e.g. lower mortality, higher parental reproductive success, increased probability of acquiring territories) (Taborsky 1984b; Balshine-Earn et al. 1998). However, although cooperation behavior is complex requiring several cognitive competences, there were no differences in whole brain mass between cooperative and independently breeding species (Reddon et al. 2016).

Sylvester et al (2010, 2013) centered their investigations in the mechanisms responsible for brain development comparing rock- and sand-dwelling cichlids from Lake Malawi (East Africa), fish that display differentiated brains and behaviors even though genetically similar

(Loh et al. 2008). Rock-dwellers (‘mbuna’ species) are more territorial and aggressive, live in complex habitats and eat algae; sand-dwellers (‘non-mbuna’ species) are seasonal-lek breeders and eat small prey utilizing acute vision. Interestingly, rock-dwelling cichlids have larger telencephalon and olfactory bulbs whereas sand-dwelling cichlids exhibit enlarged optic tecta, thalamus and eyes (reviewed in Sylvester et al. 2011). By manipulating gene expression patterns of specific developmental genes (ventral Hedgehog - Hh - and dorsal Wingless - Wnt) which integrate different signal transduction pathways, (Sylvester et al. 2011) in cichlids and in zebrafish, they were able to change telencephalon patterning (pallial/subpallial organization from mbuna to non-mbuna proportions, and vice-versa, Figure 3) and show that variations in early development are responsible for changing brain structures (Sylvester et al. 2010, 2013).

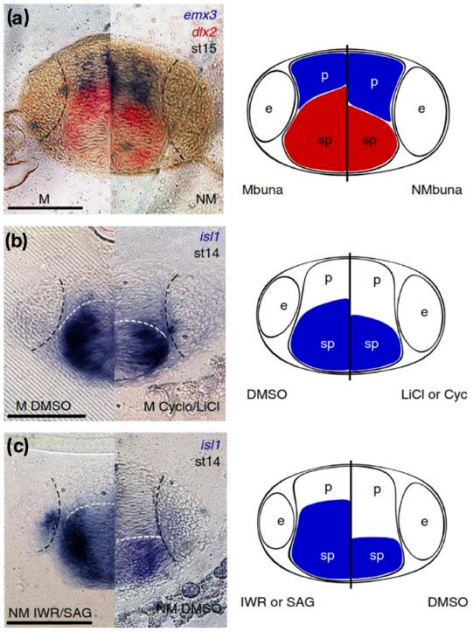

Figure 3. Study of the mechanisms responsible for brain development in cichlid rock (‘mbuna’, M) versus sand (‘non-mbuna’, NM) dwellers (Sylvester et al. 2013). Left panels are transverse sections of the telencephalon, and right panels are schematics of the data represented on the left. Black-dotted lines represent the position and size of the eyes in each section. Scale bar, 100 mm. (a) A ‘splitscreen’ double ISH of *emx3* (blue) and *dlx2* (red) used to visualize the pallium and subpallium in mbuna (M), left, and non-mbuna (NM), right, showing the difference in pallial/subpallial proportions. (b) Expression of the subpallial marker *isl1* is depicted, and an expansion of the pallium (above dotted white line) in cyclopamine- or LiCl-treated mbuna (right side) versus DMSO control mbuna (left) is showed. LiCl is an activator of the Wnt pathway, whereas cyclopamine is an antagonist of the Hh pathway. (c) An expansion of the subpallium

(below dotted white line) in SAG- or IWR-treated non-mbuna (left side) versus DMSO control non-mbuna (right) is showed. SAG is an Hh agonist and IWR is a Wnt antagonist.

Further studies that address not only variation in size but also the complexity and connectivity of different brain areas in cichlids are expected to highlight which brain features are responsible for such complex systems of social organization.

3.1. Social Skills in Cichlids

In fish, a considerable amount of literature has been published on social cognition and distinguished the primary elements of social cognition (Bshary et al. 2002, 2014; Brown et al. 2011; Oliveira 2013; Bshary and Brown 2014). The following section presents a collection of these cognitive adaptations for social living in cichlids, including but not limited to individuals' recognition, counting abilities, and transitive inference.

The ability of individuals to recognize others has been studied in some cichlids. In the cooperative breeder *N. pulcher,* by using playback videos, it was shown that these fish court their mates and fight against other conspecifics (Balshine-Earn and Lotem 1998). Other authors used manipulated digital images and showed that *N. pulcher* can distinguish between familiar and unfamiliar conspecifics based on visual cues. Specifically, fish use facial features to discriminate among individuals but not other body characteristics (Kohda et al. 2015). Amazingly, this ability takes less than 0.5 sec (Kohda et al. 2015). Moreover, within this species, it was also shown that individuals can distinguish between kin and non-kin and preferably associate with kin (Hert 1985; Le Vin et al. 2010). This could be a way to improve (indirect) fitness by helping to raise kin and also to avoid inbreeding (Pusey and Wolf 1996). This species is also able to recognize predators only by using visual information (O'Connor et al. 2015b). In *Neolamprologus multifasciatus*, other cooperative species, territorial males are more aggressive to strange males than to strange females, while females exhibit the reverse (Schradin and Lamprecht 2000). Interestingly, the cave breeding fish, *P. taeniatus*, recognizes their own odor (Thünken et al. 2009). This species can clearly distinguish between its own odor in comparison with an odor of an unfamiliar conspecific and additionally prefer the cave with its own scent over one with a familiar kin odor (Thünken et al. 2009). The authors suggest that this ability could probably confer them advantages in localizing their own cave territory and to avoid intrasexual competition (Thünken et al. 2009).

Another interesting phenomenon is the 'dear enemy' effect, evidenced in territorial species, where dominant individuals show less aggressivity to familiar conspecifics than to

unfamiliar ones (*sensu* Fisher 1954). The latter individuals induce an increased aggressive response as no previous interactions occurred between them and no previous information on their competitive abilities is available. These intruders are thus seen as potentially more threatening since they may try to control the territory if the resident does not counteract rapidly and efficiently (Temeles 1994). The dear enemy effect has been tested in several taxa, e.g. mammals, birds, reptiles, amphibians, and also in fish (Ydenberg et al. 1988; Temeles 1994). In cichlids, it has been confirmed in *A. nigrofasciata* (Leiser and Itzkowitz 1999), *N. pulcher* (Frostman and Sherman 2004) and *O. mossambicus* (Aires et al. 2015) attesting to the capacity of cichlids to distinguish between familiar and unfamiliar conspecifics, as a way to adopt more optimized social responses. Interestingly, a study on *N. pulcher* has shown that low-ranking subordinates are more aggressive to a subordinate conspecific visitor than higher-ranking fish when compared with control groups (Ligocki et al. 2015b), showing that social behavior towards conspecifics is modulated according to the perception that an individual has of others. This can be seen as beneficial to the group by the dominants but perceived as a threat by subordinates.

Besides individual recognition, remembering past interactions could be advantageous to individuals, for instance as a mechanism to recall other males physical condition. Thus, the duration of memory of dominance relationships was addressed in a study with *J. transcriptus* (Hotta et al. 2014). The authors staged fights with paired-sized males and assessed winner and loser individuals and then loser males were able to interact with the winner male or with other males, 3, 5 or 7 days after the first trial. Results show that loser males only displayed subordinate behavior in the presence of the winner male and not to other rival males in day 3 and day 5 and that subordinate behavior disappeared at day 7 (Hotta et al. 2014). This study reports that subordinate males recall a fight up to 5 days maybe as a way to avoid confrontations with a ‘stronger’ male.

Amazingly, cichlids can distinguish the social rank of conspecifics by direct experience but also make predictions about the relation between individuals that were never compared by using the information available on known relationships (Grosenick et al. 2007). For instance, if A is more dominant than B (A>B), and B has a higher social rank than C (B>C), then A is hierarchically above C (A>C). This more complex social cognitive mechanism is named transitive inference. *A. burtoni* males were tested for the ability to infer hierarchy by watching pairwise fights of size-matched males. Only visual information was available. Bystanders were able to discriminate individuals and specifically the dominance hierarchy (A>B>C>D>E) by preferring to associate with the losers (Grosenick et al. 2007). Transitive inference was also

supported in a highly social cichlid fish, *J. transcriptus*, (Hotta et al. 2015a, b). This cognitive ability would able fish to avoid engaging in costly aggressive interactions with conspecifics that are 'stronger' than them.

Another cognitive skill described in fish is their counting abilities. Freshwater tropical angelfish, *Pterophyllum scalare*, are able to discriminate between shoals ranging in size ratio from 4:1, 3:1 and 2:1 (Gómez-Laplaza and Gerlai 2011). Interestingly, the relative size of the shoals instead of the absolute numerical difference between them was the main mechanism underlying this preference, since they were not able to distinguish more similar shoals (e.g. 1.7:1 ratio). This suggests that angelfish make relative comparisons between shoal sizes, always preferring to stay closer to the larger shoal (Gómez-Laplaza and Gerlai 2011). The competence to distinguish more from less seems conserved in cichlids and provides a means for species to benefit from shoaling (e.g. by acquiring increased protection against predators or efficacy in foraging).

Even though social learning has been reported in several teleost fish as a way to apprehend information regarding antipredator, foraging, migration behaviors among others (reviewed in Brown and Laland 2003), in cichlids, studies are scarce. Barks and Godin (2013) tested if juvenile convict cichlid *Amatitlania nigrofasciata* could learn to distinguish novel visual cues as a threat or non-threat, by using social information from conspecifics, but without success. On the other hand, Alcazar et al. (2014) found that older males have more fighting abilities than younger ones, and in some cases, younger animals were larger. The authors consider age as a proxy of social experience (i.e. more social interactions) and suggest that social learning is a major advantage of agonistic competition.

Other social skills present in animals that live in communication networks are 'eavesdropping' and 'audience effects'. When an individual directly communicates/signals to another, further animals may receive this information as well. Hence, communication involves a signaler, a receiver and also bystanders (McGregor 1993). These animals, which are not directly involved in the interaction, compose an audience, and their presence, influences the signaler behavior ('audience effects'). This means that signalers can strategically change the salience or intensity of their signals according to the presence of bystanders. This has been already tested in several teleosts (Doutrelant et al. 2001; Dzieweczynski et al. 2005, 2014; Plath et al. 2008; Plath and Schlupp 2008) and is a phenomenon that is also dependent on the composition of the audience (Doutrelant et al. 2001).

In the cichlid *A. burtoni*, Desjardins and colleagues (2012) showed that non-territorial males act more aggressively and court females when the territorial male is not watching them.

They also tested for the effect of different audiences on the behavior of pairs of size-matched dominant males fighting each other and verified that when a larger male is in the audience, focal males decrease aggression, whereas a gravid female induces an increase in aggressive behavior, compared with controls. This confirms that *A. burtoni* males change their behavior if they are being observed but also that there is a fine-tune modulation of the behavior according to whom is watching, possibly to avoid unnecessary agonistic interactions and optimize reproductive opportunities (Desjardins et al. 2012). A study based on a similar paradigm focused on brain IEG *egr-1* activation pattern in several areas of the SDMN, Vs, Vv, POA, vTn, aTn, PAG, Dm, Dl, of both the signaler and bystander, with different relative sizes (Desjardins et al. 2015). Results obtained with qPCR show that nuclei involved in reproduction and aggression, Dm, POA and Vv, are differentially expressed in males that are fighting but surprisingly also in the males that are watching them, in comparison with control individuals. Furthermore, both when the audience was composed of larger males and when fighting males were larger, the Vv, a nucleus associated with anxiety was activated in the fighting males and observer males respectively. Interestingly, the patterns of brain gene expression (namely in the POA and the Vv) between fighters and observers are more similar than controls, suggesting that the same circuit is activated whenever social behavior is expressed but also when social information is received and that the SBN plays a pivotal role in cichlids social cognition (Desjardins et al. 2015).

In the cooperative breeding cichlid *N. pulcher* subordinates vary in their response to a predator according to the presence or absence of neighbors, specifically, they increase their aggressive behavior if another group of conspecifics is watching them, probably as a way to signal their ability to group survival (Hellmann and Hamilton 2014). On the other hand, bystanders that use the available social information are called eavesdroppers. Eavesdropping is the ability of individuals to indirectly collect information about others just by watching social interactions and use this information in their subsequent behaviors. This social skill is increasable advantageous as it is a way to acquire valid information, for instance on opponents' fighting abilities or potential mates, which involves no costs, since the animals don't engage in dangerous interactions. Eavesdropping has already been reported in several teleosts, proving the importance of this skill in social behavior (Oliveira et al. 1998; Doutrelant and McGregor 2000; Earley and Dugatkin 2002; Abril-de-Abreu et al. 2015) and also in the convict cichlid *A. nigrofasciata* (van Breukelen and Draud 2005). *A. nigrofasciatus* is a monogamous species where male and female establish a bond that sometimes is broken by one of the individuals to get access to another mate. Within this study, females were presented to other males (rival

males), with the same size or larger than their mates and the divorce rate was evaluated. Then, rival males were allowed to interact with male mates in the presence of the female eavesdroppers. The authors found that there was an increased rate of divorce (50%) when rival males were larger than mates and females watched the interaction between the males, whereas the treatment with decreased divorce (0%) was the situation where males were similar in size and females did not eavesdrop (van Breukelen and Draud 2005). This study shows that these females tend to divorce when a higher quality male is available but they need to evaluate their relative condition by an eavesdropping tactic.

Besides the examples already mentioned, other very interesting social skills have been reported in teleost fish such as cooperative hunting (Strübin et al. 2011; Vail et al. 2013), manipulation and deception (Bshary and Oliveira 2015) or collective cognition (Sumpter et al. 2008; Ward et al. 2011). In the future, we expect to understand further the neural circuits underlying these social cognitive processes in fish as more neurogenomic tools become available.

4. Neuroendocrine Regulation of Social Behavior

The relationship between hormones and behavior has been a matter of interest for several centuries. The initial paradigm established hormones as directly responsible for behaviors, grounded in classical experiences of castration and androgen replacement studies (see Oliveira 2004, for historical background). However, experiments showing that hormones rather increase the probability of the individuals to express behaviors instead of switching on and off behaviors altered this simplistic concept (e.g. Albert et al. 1993). Currently, it is well recognized that hormones act as modulators of the neural mechanisms underlying behavior (Oliveira 2009). On the other hand, intensive studies in the last decades have focused on the influence of social interactions on hormones. Actually, social environment surprisingly feedbacks on neuroendocrine mechanisms, i.e., social interactions are responsible for changing hormone levels which, in turn, modulate perceptive, motivational and cognitive mechanisms and ultimately subsequent social behavior (Oliveira 2004).

Mazur (1985) was the first to propose a reciprocal relationship between androgens and dominant behavior. Later, “The Challenge Hypothesis” (Wingfield et al. 1990) was presented to explain the adaptive nature of the androgen response to social interactions, and it has been characterized across all vertebrate taxa (Hirschenhauser and Oliveira 2006). The Challenge Hypothesis generates a number of predictions regarding the patterns of androgen social

responsiveness according to mating system and parental care types, which have been extensively tested in the last decades and in particular in cichlids (see Oliveira 2004, for a review in this topic).

Nevertheless, social modulation of neuroendocrine activity is not restricted to androgens (Goodson 2005; Summers et al. 2005; Godwin and Thompson 2012). The next sections will present examples of these interactions between hormones and social behavior, centering our discussion on sex steroids (11-ketotestosterone, KT; testosterone, T; estradiol, E2), cortisol (F) and the neuropeptides AVT and IT.

4.1. Hormones Action on Behavior

As mentioned earlier, sex steroids receptors (AR, ER, PR) are distributed throughout the telencephalon and the diencephalon in specific areas related to social behavior, proving to be major actors in the regulation of these behaviors (*A. burtoni*, Munchrath and Hofmann 2010).

Reproductive behavior, for instance, seems to be intimately associated with sex steroids since castration studies in males abolishes spawning pit digging, nuptial coloration and courtship (*Pseudocrenilabrus multicolor*, Reinboth and Rixner 1970; *Sarotherodon melanotheron*, Levy and Aronson 1955; *A. burtoni*, Francis et al. 1992**;** *O. mossambicus*, Almeida et al. 2014a). Exogenous administration of AR agonists also supports this association by promoting nest building behavior or courtship (*A. burtoni*, (O'Connell and Hofmann 2012); *A. nigrofasciata*, (Sessa et al. 2013) while AR antagonists decrease courtship (O'Connell and Hofmann 2012); *A. nigrofasciata*,(van Breukelen 2013). Estrogens also seem to play a complex role in nest building behavior because either ER agonists or antagonists promote nest building behavior (Sessa et al. 2013). Other researchers claim, however, that gonadectomized males maintain reproductive behavior repertoire (*Hemichromis bimaculatus*, Noble and Kumpf 1936; *Andinoacara latifrons*, Aronson et al. 1960, *Sarotherodon melanotheron and Oreochromis upembae*, Heinrich 1967), which suggests that sex steroids influence on behavior is species specific. Furthermore, a more recent study on *A. burtoni* provided solid evidence that prostanglandin PGF2α is necessary and sufficient to induce reproductive behavior in *A. burtoni* females (Juntti et al. 2016). Since the injection of PGF2α in females induces spawning behavior, they generated mutants that had no expression of the putative PGF2α receptor (Ptgfr), by using CRISPR/Cas9 technology. Results show that female mutants are not able to express final stages of reproductive behavior inasmuch as PGF2α acts presumably on the POA and the

vagal lobe acting as a signaler of fertility into the brain where this receptor is expressed regulating sexual behavior (Juntti et al. 2016).

Aggression is also modulated by sex steroids since androgen-treated fish increase aggressive behavior (e.g., *A. burtoni*, Fernald 1976; *Andinoacara pulcher*, Munro and Pitcher 1985) whereas blocking androgen receptors lowers aggression levels (e.g., *A. nigrofasciata*, Sessa et al. 2013). However, apparently, there are independent neural circuits controlling reproductive and aggressive behaviors. For instance, in *A. nigrofasciata*, van Breukelen (2013) studied, both in the laboratory and in the field, the effect of androgens on pre-spawning courtship and aggression by using flutamide as a blocker of androgen receptors. Results show that flutamide was responsible for a significant decrease on the courtship behavior of males treated with flutamide silastic implants comparing to control or sham males. However, aggression towards conspecific males was not affected by this androgen receptor antagonist, supporting evidence for a decoupling between courtship and aggression in terms of neuroendocrine mechanisms. Castration experiments also corroborate this idea. A work with *O. mossambicus* gonadectomized males show that these animals suffer a profound decrease of circulating androgens and stop expressing reproductive behaviors, yet aggression is not affected (Almeida et al. 2014a). These observations and the possible enzymatic conversion of testosterone to estrogen, support the argument that androgens moderate aggressive behavior directly or via aromatization to estrogen. O'Connell and Hofmann (2012) concluded that in *A. burtoni* androgens are associated with reproductive behavior while estrogens moderate aggression by comparing the effects of agonists and antagonists for each sex steroid receptor. Studies focused on aromatase, the enzyme responsible for converting estradiol in testosterone, show a correlation between aromatase mRNA levels and aggression in *A. burtoni* (Huffman et al. 2013). Treating fish with fadrozole (aromatase inhibitor) decreases aggression and E2, increases T and increases brain aromatase expression in POA (Huffman et al. 2013). However, it has no effect in reproductive behavior (Huffman et al. 2013).

AVT and IT are also involved in social interactions, as it has been demonstrated in several investigations although with contrasting results. In the cooperative breeder, *N. pulcher*, IT regulates dominance interactions since IT treated fish increase submissive behavior (Reddon et al. 2012; Hellmann et al. 2015) but correlates negatively with affiliative behavior (Reddon et al. 2015). Moreover, IT may inhibit grouping behavior since injecting males with an oxytocin receptor antagonist increased grouping preference and an exogenous isotocin dose-dependent injection decreased this behavior (Reddon et al. 2014). Interestingly, IT treated males also increase responsiveness to social information, i.e, they are more aggressive to larger opponents

(*N. pulcher*; (Reddon et al. 2012). O'Connor et al (2016), however, reported a positive correlation between IT and both affiliative and submissive behaviors in the cooperative breeder. In *A. nigrofasciata*, blocking of IT inhibits parental care and removal of the mate induces over-expression of the immediate early gene *c-fos* on IT neurons localized in the POA (O'Connell et al. 2012). In the same study, IT does not seem to influence affiliative behavior toward the mate; however, blocking both AVT and IT receptors decreases this behavior while the pair bond is forming (Oldfield and Hofmann 2011). On the other hand, aggression seems to be related to higher expression levels of whole brain AVT in *N. pulcher* and *Telmatocromis temporalis*, (O'Connor et al. 2016), whereas in *A. burtoni* dominant males, AVT injections elicits loss of status and reduces aggression which in turn are not changed when males are treated with an AVT antagonist (Huffman et al. 2015). Actually, AVT and IT (and its receptors) expression in the whole brain seem species-specific (O'Connor et al. 2015a) as well as the relation between IT and behaviors (O'Connor et al. 2016). Ramallo and colleagues (2012) provided a detailed characterization of the vasotocinergic system in *Cichlasoma dimerus*: AVT neuron projections are found mostly in the forebrain and the hindbrain while AVT stimulates the production of gonadotropins (LH and FSH) on pituitary extracts *in vitro* and androgens on testis culture. They also detected AVT mRNA and peptide in the testis thus showing the influence of AVT in the HPG axis as a neuromodulator in the central nervous system and playing a role as a neurohormone at a peripheral level.

The interaction between stress and glucocorticoids on fish survival, physiology or reproductive capacity has been reported for several years (reviewed in Schreck 2010). However, cortisol produced by the HPI (Hypothalamus – Pituitary – Interrenal) axis also acts in the regulation of social interactions. Munro and Pitcher (1985) treated *A. pulcher* males with cortisol and fish seemed to increase submissive behavior. Another indirect evidence of corticosteroids modulating behavior is exemplified by a study in which *A. burtoni* males were presented to a video playback of a male displaying aggressively (Clement et al. 2005). In this case, non-territorial males with intermediate levels of F showed more direct aggression than subordinate individuals with high or low F, in turn characterized by increased displaced aggression. The authors concluded that the behavioral response of subordinate males was moderated by cortisol levels and suggest the existence of an optimal cortisol value that would promote advantageous in social challenges. Another study reports that corticosteroid receptors gene expression is sex-differentiated once males express higher levels of GR2 and MR in the liver, and the latter is correlated with submissive behavior in *N. pulcher* (O'Connor et al. 2013).

Table 2 summarizes the effects of hormonal manipulations on the social behaviors described above.

4.2. Social Feedback on Neuroendocrine Mechanisms

In social species, individuals should be socially competent, i. e., they should optimize their behavior according to a constantly changing and challenging environment. To do so, individuals integrate information about the social environment they live with internal cues and respond in a more adaptive manner (Oliveira 2009). Steroid hormones play a central role in this adaptive and embodied mechanism since social interactions elicit quick plasma hormonal responses that modulate neural mechanisms through widely distributed steroid receptors (Oliveira and Oliveira 2014). For instance, males' exposure to social stimuli, either a female or a conspecific male, induce a plasma androgen increase (*O. mossambicus,* Borges et al. 1998; *N. pulcher, Lamprologus callipterus, Tropheus moorii, Pseudosimochromis curvifrons, O. mossambicus*; Hirschenhauser et al. 2004, 2008; *A. nigrofasciata,* Sessa et al. 2013). A study on female mate choice revealed that *A. burtoni* males change their reproductive and aggressive behavior, as well as androgen levels, according to female physiology (hormone release) and/or behavior and in turn females choose mates that release more androgens into water (Kidd et al. 2013). Interestingly, visual information is per se sufficient to influence hormone systems since in *A. burtoni* seeing a dominant and larger male suppresses dominant behavior of a smaller male and is responsible for a decrease in KT levels and a gene expression increase of CRF, GnRH and AVT (Chen and Fernald 2011). Agonistic interactions also elicit an androgen increase in *O. mossambicus* spectators (Oliveira et al. 2001). Unexpectedly in some situations, there are no hormonal responses; fish fighting with their image in the mirror display very aggressive behaviors, however, there are no changes in androgens (Oliveira et al. 2005). In this case, the evidence of a decoupling between agonistic behavior and androgens reveals that fish appraisal (interpreted by the fight outcome) seems to be necessary to induce an endocrine change. However, this result seems to be species-specific (*A. burtoni*, Desjardins and Fernald 2010, and *Pundamilia* spec., Dijkstra et al. 2012), raising the debate on the adaptive function of androgens changes. It seems that hormonal responses resulting from the perceived outcome of agonistic interactions would affect subsequent social interactions rather than affecting the current dispute between individuals. Probably, androgens fluctuations are a way for individuals to take into account their potential to gain further interactions and maintain social status/dominance avoiding prejudicial defeats (Oliveira 2009). Indeed, animals winning social conflicts have a higher probability of winning subsequent interactions with other conspecifics while losing a

fight has the opposite effect. This has been called the Winner/Loser effect (Hsu and Wolf 1999) and has been seen in *O. mossambicus* (Oliveira et al. 2009). Yet, the winner effect is blocked when individuals are treated with an androgen antagonist (Oliveira et al. 2009).

Other important evidence on social environment influencing hormones is that plasma androgen levels vary with social status. Dominant males typically have higher levels of androgens (KT and/or T) than subordinate males (*A. burtoni*, Parikh et al. 2006; *N. pulcher*, Desjardins et al. 2008; *O. niloticus*, Pfennig et al. 2012; *C. dimerus*, Morandini et al. 2014).

In *N. pulcher*, non-territorial aggregation males have higher T and lower KT and helpers have higher F (Bender et al. 2008). Another study in the same species has shown that female breeders have higher levels of T than helper females or even males (Desjardins et al. 2008), suggesting that androgens may promote parental care. Looking at brain gene expression patterns dominant/breeder females are very similar to dominant males, evidence for a masculinization at the molecular and hormonal level of these females (Aubin-Horth et al. 2007). The keynote here is that steroid levels are a consequence of social status. Oliveira et al (1996) demonstrated that urinary sex steroids levels after group formation are good predictors of social establishment; KT increased in territorial males and decreased in non-territorial males and no changes were reported in T levels when compared to levels prior to hierarchical establishment (see also Almeida et al. 2014b). On the other hand, social challenges raise differential hormonal responses according to individuals' social status. In *N. pulcher*, agonistic interactions elicit higher plasma levels of T and similar KT levels in dominant females than subordinate females, and in contrast higher levels of KT and equivalent levels of T in dominant males compared to subordinate males (Taves et al. 2009). Likewise, androgen levels of males socially isolated differ in their response according to their previous social status; dominant males decrease KT and subordinates show a tendency to increase KT whereas F varies depending in the prior social context (*O. mossambicus*, Galhardo and Oliveira 2014).

In turn, androgens modulated by social status determine for instance expression of secondary behavioral (e.g. nuptial coloration, spawning pit volume) and morphological traits (e.g. mandible width, dorsal fin height) specifically in territorial males (*O. mossambicus*, Oliveira and Almada 1998b). Dominant males typically have larger GSI (gonadosomatic index) than non-territorial males (*O. mossambicus*, Oliveira and Almada 1999; *O. niloticus*, Pfennig et al. 2012; *C. dimerus*, Alonso et al. 2012; *A. nigrofasciata*, Chee et al. 2013). However, subordinate males are still reproductively active despite differences in testis structure (Pfennig et al. 2012).

Table 2. Effects of hormonal manipulations on social behavior: - decrease; + increase; 0 no effect; ND not described/not applicable.

Species	Hormonal manipulation	Plasma Hormones	Nest/spawning pit building	Nuptial coloration	Courtship displays	Paternal care	Agression	Mating/ Affiliative behavior	Reference
Astatotilapia burtoni	Castration	- T - KT - E2	ND	ND	ND	ND	ND	ND	Francis et al 1992
Oreochromis mossambicus	Castration	- T - KT	-	-	-	ND	0	ND	Almeida et al 2014
Aequidens pulcher	Androgens added to water T E2 F	ND	ND	ND	ND	ND	+ - -	ND	Munro and Pitcher 1985
Astatotilapia burtoni	Injections T	ND	0	+	0	ND	+	ND	Fernald, 1976
Astatotilapia burtoni	Injections E2 DHT 17α-20β-P ER antagonist AR antagonist PR antagonist	ND -E2 and -T (SUB) 0 E2, T, P (DOM) 0 E2, T, P (DOM/ SUB) 0 E2, T, P (DOM/ SUB)	ND	ND	0 (DOM) + (DOM) + (DOM) 0 (DOM) - (DOM) - (DOM)	ND	+ (DOM/SUB) 0 (DOM/SUB) 0 (DOM/SUB) - (DOM) 0 (SUB) 0 (DOM/SUB) 0 (DOM/SUB)	ND	O'Connell and Hofmann, 2012

Table 2 - Effects of Hormonal manipulations on social behaviour: - decrease; + increase; 0 no effect; ND not described/not applicable (cont.).

Species	Hormonal manipulation	Plasma Hormones	Nest/spawning pit building	Nuptial colouration	Courtship displays	Paternal care	Agression	Mating/ Affiliative behaviour	Reference
Astatotilapia burtoni	PR antagonist	0 E2, T, P (DOM/ SUB)			- (DOM)		0 (DOM/SUB)		O'Connell and Hofmann, 2012
Astatotilapia burtoni	Injections Aromatase inhibitor	+T -E2	0	ND	0	ND	- (DOM)	ND	Hufmann et al 2013
Amatitlania nigrofasciata	Silastic implants AR antagonist	ND	ND	ND	-	ND	0	ND	van Breukelen 2013
Amatitlania nigrofasciata	Injections ITR antagonist	ND	ND	ND	ND	-	0	0	O'Connell et al, 2012
Amatitlania nigrofasciata	Injections ITR + AVTR antagonist	ND	0	ND	ND	ND	-	-	Oldfield and Hofmann, 2011
Neolamprologus pulcher	Injections IT	ND	ND	ND	ND	ND	+ submission	0	Reddon et al, 2012
Neolamprologus pulcher	Injections IT ITR antagonist	ND	ND	ND	ND	ND	ND	- (high/low dose) +	Reddon et al 2014

Androgens likewise modulate color patterns in *A. burtoni* territorial males since KT levels (and aggression) are higher in yellow territorial males than in blue territorial males (Korzan et al. 2008). A flexible behavioral strategy seems to underlie this color changing ability. Other very interesting illustration is what is observed in *A. burtoni* females. Sometimes they adopt a male typical appearance and behavior, namely courtship behavior and aggressive territorial defense mostly towards other females (Renn et al. 2012). This intriguing behavior is associated with higher T levels and a non-significant trend to higher E2 comparatively to subordinate females (Renn et al. 2012). Data on the mentioned study cannot infer on the ultimate function of this apparently hormonal modulated behavior but one can speculate that this observed behaviorally plasticity could confer them adaptive advantages in competition for males.

Importantly, hormone effects on behavior can occur not only by changes on plasma steroids but also through changes in the density of hormone receptors (Fuxjager et al. 2010). For instance, in *A. burtoni*, differences in mRNA expression levels of several hormone receptors have been found between males of different social ranks (Korzan et al. 2014). This is also evident in an interesting study with the teleost mangrove rivulus fish, *Kryptolebias marmoratus*, where the expression levels of androgen receptors change in response to fights, and this effect seems to be dependent on the fight outcome and baseline androgen levels (Li et al. 2014)**.** Alternatively, androgens can also bound to hormone binding proteins that regulate their bioavailability (Zeginiadou et al. 1997; Oliveira 2009) or produced in the brain *de novo* from cholesterol (Baulieu 1998; Schmidt et al. 2008; Pradhan et al. 2014), though studies confirming this in cichlids are still missing. Finally, androgens can be even converted in other steroids by specific enzymes (e.g. aromatase) as already mentioned (Roselli et al. 2009; Cornil et al. 2012). In *A. burtoni*, for instance, subordinate males have more aromatase expression than dominant males, indicating a probable compensatory mechanism to low sex steroid levels in plasma (Huffman et al. 2013). A remarkable example on the reciprocity between hormones and behavior is the social regulation of reproductive plasticity in *A. burtoni*. A considerable number of studies in the last years provided a very detailed picture of how social environment impacts dramatically an individual. *A. burtoni* is an African maternal mouth-brooding species with a lek-breeding system. Males present two distinct phenotypes, which can rapidly reverse due to changes in the social environment. Dominant males are brightly colored (yellow or blue) with a black eye-bar, establish territories and attract females. In contrast, subordinate males show more faded coloration, school with females, fail to establish territories and typically do not reproduce. Dominant males have an up-regulated HPG axis comparatively to subordinate males

(reviewed in Maruska and Fernald 2014) namely, higher levels of gonadotropins and expression of GnRH1 receptors at the pituitary; higher levels of plasma sex steroids (KT, T and E2) and gonadotropins LH and FSH. Testes have larger GSI and increased expression of gonadotropins, androgens, estrogens, glucocorticoids and mineralocorticoid receptors and steroidogenic acute regulatory protein. However, once a territory is available, subordinate males ascend in social rank and as expected circulating androgens rise but also profound changes occur at the level of behavior, brain and reproductive system within different time frames (reviewed in Maruska and Fernald 2014).

In a more recent study, researchers have focused on the effect of social interactions on the AVT system. By assessing the levels of expression of the immediate-early gene *egr-1* by *in situ* hybridization technique, they showed that agonistic and courtship interactions induce differential patterns of activation of AVT neurons (Loveland and Fernald 2017).

Furthermore, AVT seems to be regulated by social status. In *O. mossambicus*, subordinate males have larger cell body areas of AVT neurons in magnocellular POA and gigantocellular POA and submissive behavior correlates with soma size of AVT cells in all three nuclei (parvo-, magno- and gigantocellular) and AVT cell number in the magnocellular POA (Almeida and Oliveira 2015). Ramallo et al (2012) compared dominant and subordinate males in *C. dimerus* soon after establishing hierarchy concluding that subordinates have larger AVT parvo-cellular neurons in the POA than dominant males, pointing to a putative role of these neurons in submissive behavior. AVT brain levels of *N. pulcher* detected by HPLC-FL are higher in subordinate than in dominant males and IT correlates negatively with affiliative behavior (Reddon et al. 2015).

In *O. mossambicus*, on the other hand, Almeida et al (2012) used the same method to quantify the levels of both AVT and IT in several macro-dissected brain areas and the pituitary gland. Results show that the pituitary is the area with more concentration of the neuropeptides and the olfactory bulbs is the brain area with more abundance of AVT. Subordinate AVT pituitary levels are higher than those of dominants whereas dominant hindbrain IT levels are significantly higher compared to subordinates, suggesting a potential involvement of AVT in social stress in subordinate fish and of IT in the regulation of dominant behavior at the level of the hindbrain. A lack of correlation between AVT and IT levels suggests a decoupling between AVT and IT neuroendocrine systems at the CNS level. Moreover, the authors propose an independent control of hypophysial and CNS nonapeptide secretion. In other study, KT levels and V1a2 (AVT) receptor expression levels in the hypothalamus are more associated with territoriality and social dominance than with pair bonding (*Herichthys cyanoguttatus* and

Herichthys minckleyi, Oldfield et al. 2013). Greenwood and colleagues (2008) studied AVT expression in *A. burtoni*. Results show that whole brain AVT expression is higher in territorial than in non-territorial males however in the posterior POA territorial males have higher levels of AVT expression but in the anterior POA AVT expression is lower than in non-territorial males. This last evidence may be related to AVT influencing the stress response in non-territorial males, which usually present higher F levels than dominant males (Fox et al. 1997). O'Connor et al (2015a) studied IT and AVT and their receptors in several cichlids species and showed that there were differences in whole-brain gene expression between social and non-social species, providing evidence for species-specific gene expression patterns relative to social behavior.

Another important component of social species is their social system and a more recent study conducted by Reddon et al. (2017) specifically addressed this matter. These authors characterized IT and AVT neurons, by immunohistochemistry, of several cooperative cichlid species with contrasting social systems. By comparing 4 highly social cooperatively breeding species with 4 other less social independent breeders, all of *Neolamprologus* genus, they verified that the first (higher social) group had less parvocellular isotocin neurons in the preoptic area than the other (less social), and that these two sets of fish could be distinguished only by the size and number of isotocin neurons (Reddon et al., 2017). They also report no distinction on vasotocin neurons. In summary, nonapeptides respond in a dynamic way to changes in social status and different social ranks parallel differences in nonapeptides. Although the differences seem to be species-specific, researchers should be aware of the influence that each social living system can have on nonapeptides. Given the great diversity on the social organization of cichlids, it would be interesting to compare how nonapeptides affect or can be affected by other types of social system (e.g. type of mating or parental care systems).

Fish can also perceive the social environment as a stressor and, as a consequence, a cascade of different physiological and behaviorally responses occur, including HPI axis activation and subsequent production of corticosteroids (Barreto and Volpato 2006; Galhardo and Oliveira 2009). In social species, and in particular in cichlids, a negative correlation between cortisol and dominance has been reported for both sexes in *A. burtoni* (Fox et al. 1997) and *C. dimerus* (Alonso et al. 2012; Morandini et al. 2014) that can be related to chronic stress in subordinate males or social stability. However, in *N. pulcher*, dominant males have higher F levels and F correlates with social behavior only in subordinate males (Mileva et al. 2009). The authors explain these cortisol plasma levels arguing that dominant status in this species is

difficult to achieve and maintain, or, in other words, dominant males are subjected to a higher allostatic load as proposed by Goymann and Wingfield (2004). Another possible interpretation pointed out is that a reproductively active fish could have higher cortisol levels since corticosteroids are associated with spawning or gametes production in other species. In the same species, subordinate female cortisol levels depend on the social context. When these females live in groups where the dominant breeding pair is aggressive to each other, they have higher cortisol levels whereas lower levels are detected in females engaging in more social and non-agonistic interactions with dominant females (Ligocki et al. 2015a). The necessity of looking into the whole social 'picture' and not to only direct interactions to one focal individual is therefore highlighted. Additionally, in *A. burtoni*, besides androgen receptors, also glucocorticoid receptors mRNA are expressed in GnRH1 neurons in POA, responsible for regulating reproduction; territorial males have higher expression levels of ARα, MR, GR1a and GR2 while non-territorial males have GR1b higher levels of mRNA (Korzan et al. 2014). Finally, Earley et al (2006) studied agonistic interactions between *A. nigrofasciatus* males but no cortisol differences were detected between winners and losers. Corrêa et al (2003) also reported no cortisol distinction between dominants and subordinates (*O. niloticus*).

5. Neurogenomics of Social Behavior

Neurogenomics is a recent and exciting avenue which started to be pursued after the achievement of several genome-scale projects (Boguski and Jones 2004). Sequencing of several cichlids genomes and transcriptomes (Brawand et al. 2014), as well as the increasing advance on molecular biology and other genomic resources, plus their complex repertoire of social behavior, has launched cichlids as promising neurogenomic models for the study of social behavior. The aim is to understand what is the molecular basis of social behavior, i.e., to unravel the genes and pathways which regulate behavior as well as other development and physiology features underlying social interactions ('sociogenomics', Robinson et al. 2005). Again, the interaction between sociality and brain is reciprocal, so studies also seek to address how the social environment impacts genes (Robinson et al. 2005).

Social plasticity is a key characteristic of cichlids where the same genotype produces diverse behavioral phenotypes, which are distinguished by the expression of specific behavioral profiles. At the molecular level, each behavioral state corresponds to a different neurogenomic state, depicted by a distinct pattern of gene expression and consequently brain transcriptome (Cardoso et al. 2015). Differential gene expression, specifically along the several nodes of the

SDMN, allows multiple combinations of neural states, by changing the weight of each node and the strength of the connections between them (Cardoso et al. 2015). On the other hand, most of these nodes express receptors for neuromodulators and steroid hormones, responsible for the social regulation of the network (Munchrath and Hofmann 2010).

Thereafter, most neurogenomic research is based on transcriptomics studies in which brain gene expression is measured to see how it correlates with the behavior of interest. Several genes are then identified as candidate genes responsible for social behaviors. Afterwards, it is especially necessary to perform 'reverse genomic' studies where the putative genes are experimentally tested to ascertain their actual contribution to the behavioral phenotype (Harris and Hofmann 2014). This can be accomplished, for instance, by manipulating gene expression (e.g. pharmacology, transgenics, siRNA), performing brain mapping of gene expression or identifying direct targets of novel transcription factors (Harris and Hofmann 2014).

5.1. Microarray Studies

Microarray hybridization analysis was the first technique to measure brain gene expression on a genome-wide scale (Zhang et al. 2009; Wong and Hoffmann 2010; Qian et al. 2014). The underlying principle consisted in using nucleic acid probes, representing genes of interest fixed in a solid surface (microarray), and incubating them with fluorescently labeled cDNA that in turn hybridizes with the elements in the chip (Zhang et al. 2009). This means that the knowledge of the genes' sequence is required, limiting its production to a few (model) species (Zhang et al. 2009). Hence, heterologous microarrays were widely used to assess mRNA levels of closely related species (Renn et al. 2004). Next, we will describe several studies which applied microarrays to unveil social behavior at the molecular level.

The first transcriptomics study in a cichlid species was performed in *N. pulcher*. It used a heterologous microarray with ~4500 elements constructed from a cDNA *A. burtoni* library (Aubin-Horth et al. 2007) previously validated for other cichlid species (Renn et al. 2004). The authors compared dominant and subordinate individuals from both sexes at the behavior, hormonal and molecular levels. Four genes were differentially expressed between dominant and subordinate brains, independently of sex: AVT, a myelin-basic protein, a CD-59 protein and one unknown gene. AVT, for instance, had higher expression levels in dominant compared to subordinate individuals. Interestingly, dominant females had similar brain expression profiles to males, independently of status, and significantly higher levels of AVT expression than dominant males. Female dominant behavior and testosterone levels were also high and

similar to dominant males while KT levels were markedly lower. Taken together, these findings indicate that dominant breeder females are very similar to males at the hormonal and molecular level and that molecular and endocrine functions are separately (modular) organized and likely sex-regulated (Aubin-Horth et al. 2007).

Renn et al (2008) investigated whole brain gene expression in dominant and subordinate males as well as in females of *A. burtoni*. By comparing the different phenotypes with a homologous microarray array, they identified several gene candidates from neuroendocrine pathways associated with dominance or reproduction behavior. A number of genes already known to be involved in social dominance were confirmed along with the microarray analysis, namely GnRH1, galanin, AVT or brain aromatase, all upregulated in dominant males. Transcription factors, cellular metabolism genes, cell-cycle regulators, genes encoding structural proteins or neuropeptides were also detected and associated with the behavioral phenotypes. For instance, in dominants, higher expression levels of genes coding for structural proteins such as tubulin and actin, and genes involved in axonal growth, neuromodulin and neuroserpin, suggest increased brain structural reorganization within this phenotype. The authors also report increased expression of the neuropeptides somatotropin, prolactin and somatolactin and proopiomelanocortin in dominant males, probably related to gonad maturation and growth. A significant finding to emerge from this study was the upregulation of GABA receptor in dominant males while kainite-type glutamate receptors are upregulated in subordinate males, suggesting different regulating mechanisms of dominance status and novel research targets to explore (Renn et al. 2008).

In (2009), Machado et al published a comparative study between two cichlid species with different mating strategies. Whole brain transcriptome from both males and females of *X. flavipinnis,* a monogamous species, and the polygyny *Xenotilapia melanogenys* were compared with the same array of *A. burtoni*. They also performed a meta-analysis combining their results with the two previously reported studies to compare sex-specific gene patterns. Data reveal that sex-specific gene profiles show great variation between species, supporting the idea that the mating system is responsible for major brain transcription changes. Comparative genomic studies across species are of extreme relevance because they can shed light on the ultimate causes (function and evolution) of behavior besides the proximate mechanisms usually approached (Wong and Hoffmann 2010).

Schumer and colleagues (2011) performed as well a comparative study between two closed related cichlid species, *Julidochromis marlieri* and *J. transcriptus*. While the latter species is characterized by a social system where males are dominant, territorial and larger than

females, the former species shows the opposite pattern. Thus, they investigated if the same pattern of gene expression was responsible for similarities on behavioral phenotypes, namely between *J. marlieri* females and *J. transcriptus* males. They used a heterologous microarray chip (*A. burtoni* array with ~20 000 features) to compare brain transcripts of both females and males of *J. marlieri* and *J. transcriptus* and obtained a set of genes related to aggression similarly expressed in both species. IT and parvalbumin are examples of potential regulators of this behavior since the corresponding genes were highly expressed on both *J. marlieri* females and *J. transcriptus* males. The results suggest that the mechanisms underlying aggressive behavior are conserved between species (Schumer et al. 2011).

O' Connell and Hoffman (2012) performed an investigation already described in the previous section. Since ER antagonist administration influenced aggression of *A. burtoni* males, independent of social status, they decided to analyze transcription patterns in POA, comparing individuals injected with ER antagonist or with vehicle. POA expresses 56% more genes in dominant males than in subordinate males. This is however not unexpected since subordinate males express lower levels of estrogen receptors (O'Connell and Hofmann 2012). Only four unknown genes were similarly regulated between these two phenotypes, when ER antagonist was administered.

A study on *O. mossambicus* examined the effect of social odors in the transcriptomes of specific brain areas, namely, the olfactory bulb (OB) and the posterior part of the dorsal telencephalon (Dp, the homolog for the mammal olfactory cortex) (Simões et al. 2015). The authors used a microarray of *A. burtoni* to assess gene transcript patterns of dominant males subjected to urine from dominant and subordinate individuals, female conditioned water (either pre- or post- ovulated) or a blank control. They also recorded electro-olfactograms in dominant males subjected to the different stimuli to measure olfactory potency. One interesting finding is that hierarchical gene expression profiles are different between males and females in the olfactory bulb, meaning that this brain area seems to discriminate males from females while olfactory epithelium allows individuals differentiation within each sex, evidenced by electro-olfactograms results (Simões et al. 2015). Likewise, transcriptomes of OB and Dp are considerably different for every social stimulus presented, indicating that olfactory system can discriminate social status and reproductive condition (see Figure 4, Simões et al. 2015). Several gene candidates were also uncovered, such as somatotropin, somatostatin, brain aromatase, GnRH1, pro-opiomelanocortin alpha 2, differentially expressed in olfactory bulb or olfactory epithelium. Markers of neural activity, *egr-1* and cytochrome C oxidase were, in turn, down-regulated in olfactory bulb area by fish subjected to male chemical cues when compared to fish

stimulated with female odors. Hence, Simões et al (2015) hypothesize a role of olfactory modulation on memory consolidation of social odors.

More recently, Renn et al (2018) performed an interesting comparative transcriptomic study. The authors used a second generation *A. burtoni* microarray to compare, at the molecular level, cichlids of the tribe Ectodini from Lake Tanganyika, but with different mating systems (polygyny vs monogamy). They analyzed field samples of females and males of four closely related species, which evolved independently, and they obtained a set of genes that seem to be associated with monogamy, independent of species or sex. This comparative study seems to support the hypothesis that although these species went through independent evolutionary transitions from polygyny to monogamy, similar changes in brain gene expression patterns have occurred (Renn et al. 2018).

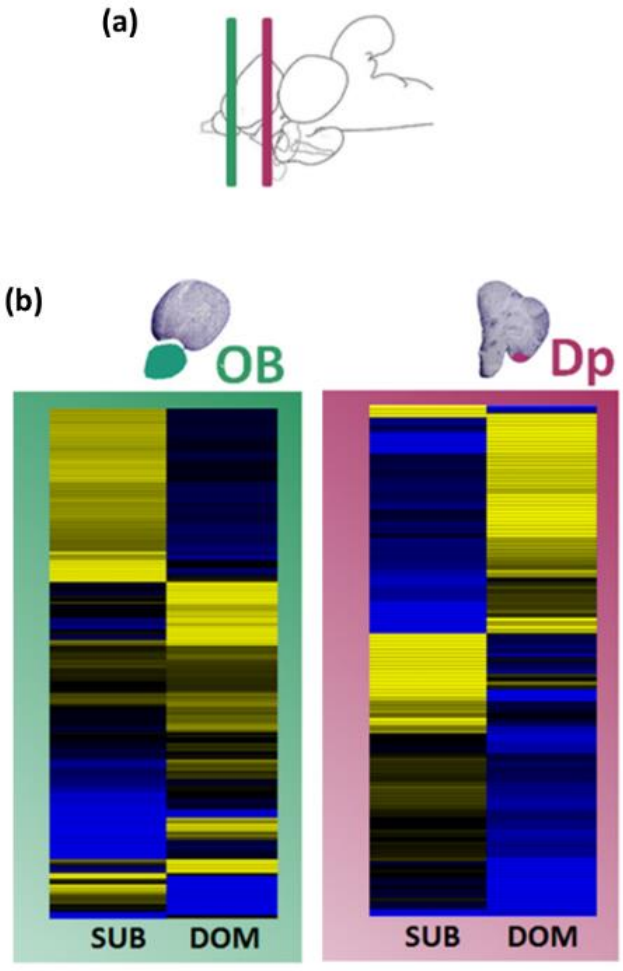

Figure 4. Neurogenomics of social behavior in *O. mossambicus* (Simões et al. 2015). (a) A sagittal view of a tilapia's brain cut by two lines (green and violet) representing the location of the coronal cuts depicted illustrating the specific areas sampled olfactory bulb (OB) and

posterior part of the dorsal telencephalon (Dp), respectively. (b) Hierarchical clustering of significantly different expressed genes ($P < 0.01$) for the comparison of dominant and subordinate male olfactory cues in OB and Dp. The heatmaps (blue – down-regulated, yellow – up-regulated) show estimated gene expression levels.

5.2. New Approaches to Neurogenomics

Microarray technique has however been replaced by more advanced technology. Recent progress has introduced the powerful next-generation mRNA sequencing, 'RNAseq', which is based on deep-sequencing and quantitative analysis (see Qian et al. 2014, for a detailed description and several applications on fish transcriptomics). Besides being more sensitive, it enables large quantity of sequence information obtained in an unbiased manner and not only gene expression information (Qian et al. 2014). It is possible to discover unknown transcribed regions, detect different gene isoforms, splicing sites or UTRs (Qian et al. 2014). Currently, the costs of RNAseq are becoming increasingly accessible and are no longer a limiting factor. So far, there are a few published articles on cichlids with RNAseq analysis. Kasper et al (2018) compared telencephalon transcriptomic patterns of alloparental egg care helpers (cleaners) and non-helpers (non-cleaners) of *N. pulcher*. In this species, the expression of these social phenotypes is not dependent on heritable genetic variation but is shaped during ontogeny and affected by social and ecological constraints (Kasper et al. 2018). Results showed that in the absence of the clutch, only the neural differentiation gene *irx2* is significantly different between cleaners and non-cleaners suggesting its involvement in the differentiation of these social phenotypes (Kasper et al. 2018). On the other hand, in the presence of the clutch, three genes, involved in neuroplasticity, hormonal signaling and cell proliferation, were simultaneously up-regulated in cleaners and non-cleaners, which seem to be commonly involved in the perception and integration of the clutch stimulus (Kasper et al. 2018). Two other studies focused on monogamy and bower building in cichlid fishes used RNA-seq to disentangle the associated evolutionary mechanisms (York et al. 2018; Young et al. 2019).

Finally, we would like to highlight epigenetics as an alternate neurogenomic mechanism of social plasticity. All the above-mentioned examples rely on transient changes in gene expression responsible for changing neurogenomic states of the brain. On the other hand, epigenetics is related to functional modifications of the genome in response to environmental information, without any change of DNA sequence (reviewed in Zhang and Meaney 2010; Roth 2012). These modifications are responsible for regulating gene expression and leading to

changes in physiology, cognition and behavior (Zhang and Meaney 2010; Roth 2012). There are several epigenetic processes, including DNA methylation, binding of non-coding RNA or histone modifications that can influence gene expression and ultimately behavior, since early-life to adulthood (Zhang and Meaney 2010; Cardoso et al. 2015). A study focused on DNA methylation of GnRH1 in *A. burtoni* showed differences in the methylation state of this gene throughout development and also after gestational crowding of the respective mouthbrooding mothers (Alvarado et al. 2015). Fry from crowded mothers had a GnRH1 promoter hypomethylated and higher transcription levels of GnRH1 compared with control mothers, attesting social control of GnRH1 through epigenetic mechanisms (Alvarado et al 2015). Lenkov and colleagues (2015) manipulated methylation state of subordinate individuals of the same species by injecting them with DNA methylating and de-methylating chemicals. Fish with higher methylation states were found to have more probability to ascend social status in opposition with lower methylation levels' individuals. Although associated with long-lasting and irreversible changes in behavior (Cardoso et al. 2015), epigenetics might be associated to more transient changes in social behavior such as reversible transitions between social ranks, promising to be a deep field to explore. In the near future, we hope to disentangle how and which genes, molecular pathways and neural circuits regulate social behavior.

Conclusions

In summary, in this review we show that social behavior is amazingly diverse, complex and dependent on several internal and external factors. Indeed, only an effective integration of these several building blocks that compose social behavior would allow the achievement of a thorough understanding. Using cichlids as study models on social behavior can give an important contribution to the field due to their extraordinary social diversification. Unfortunately, just a small number of cichlid species have been studied extensively but including a broader range of species in future research would be profitable. In addition, the increasing availability of highly-developed molecular and genomic tools will certainly contribute to the rapidly expanding of the field.

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APPENDIX C

Supplementary Information CHAPTER *2*

Supplementary Material

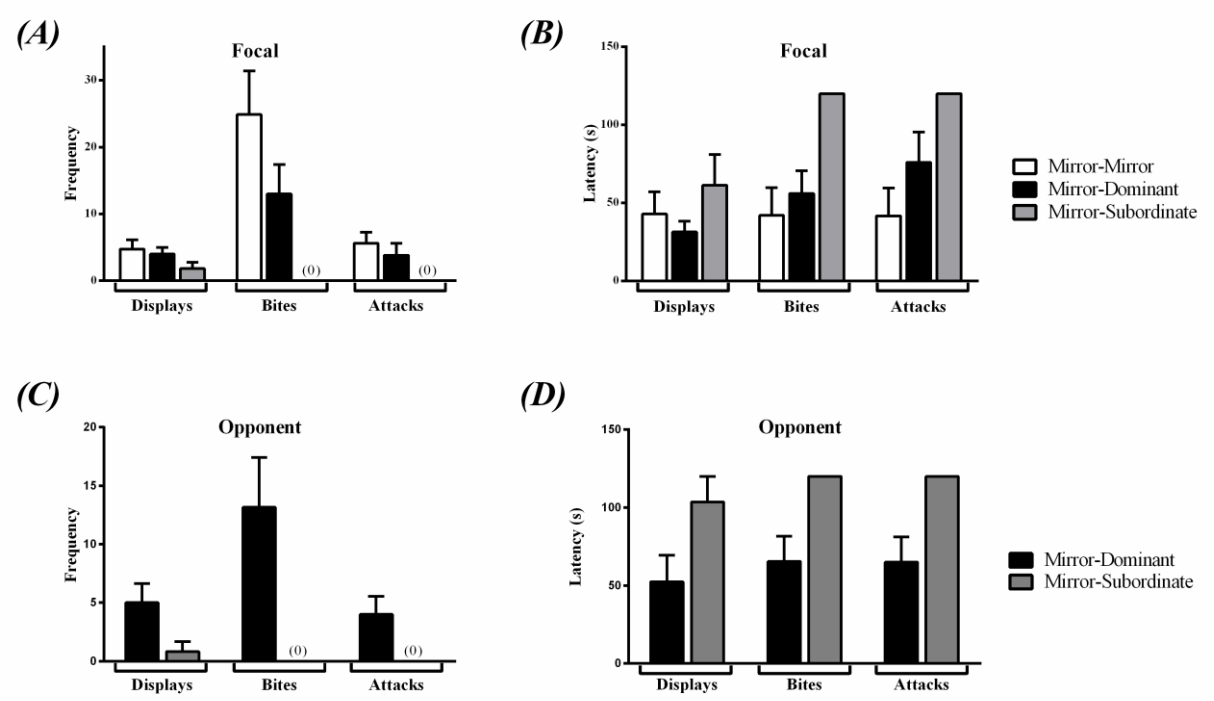

Figure S1. Behavioral measurements for the focal fish - (A) frequency and (B) latency - and the opponent fish - (C) frequency and (D) latency - for each experimental condition.

Table S1. Primer sequences and annealing temperatures (Ta) for the genes studied.

Gene	Primer sequences	Ta (ºC)
c-fos	Fw: 5'-CCGTGGACACTCTGGGATA-3' Rv: 5'-AAGGAGGCACTTGATGCTGT-3'	61
egr-1	Fw: 5'-CTCTGGGCTGATAGGCAATGTT-3' Rv: 5'-TGAGATGAGGACGAGGAGGTAGAA-3'	60
gnrh1	Fw: 5'- TATCCTCAGAATGGCTGCAA -3' Rv: 5'- GTTGTCCAGATCCCTCTTCC-3'	55
eef1A	Fw: 5'- AGCAAGTACTACGTGACCATCATTG -3' Rv: 5'- AGTCAGCCTGGGAGGTACCA -3'	61

Fw: forward primer; Rv: reverse primer.

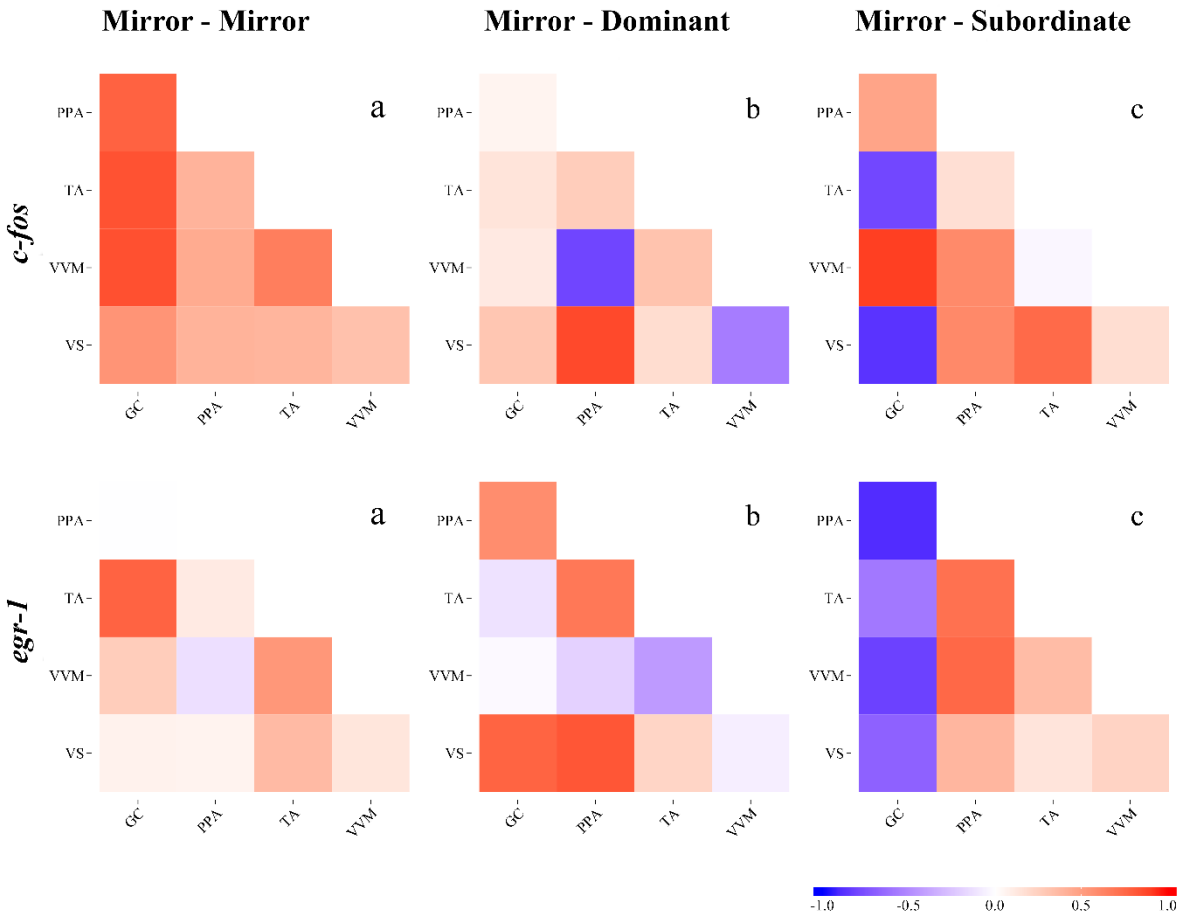

Figure S2. Functional connectivity in the SDMN network for all the experimental treatments, as measured by Pearson correlations between pairs of brain nuclei for *c-fos* and *egr-1*. Color scheme represents *r* values from −1 (blue) to 1 (red). GC, central gray; PPa, anterior part of the periventricular preoptic nucleus; TA, nucleus anterior tuberis; VVm, medial part of the ventral subdivision of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon. Different letters indicate significantly different patterns of IEG expression in brain nuclei between treatments using the QAP correlation test.

APPENDIX D

Supplementary Information CHAPTER *4*

Supplementary Material

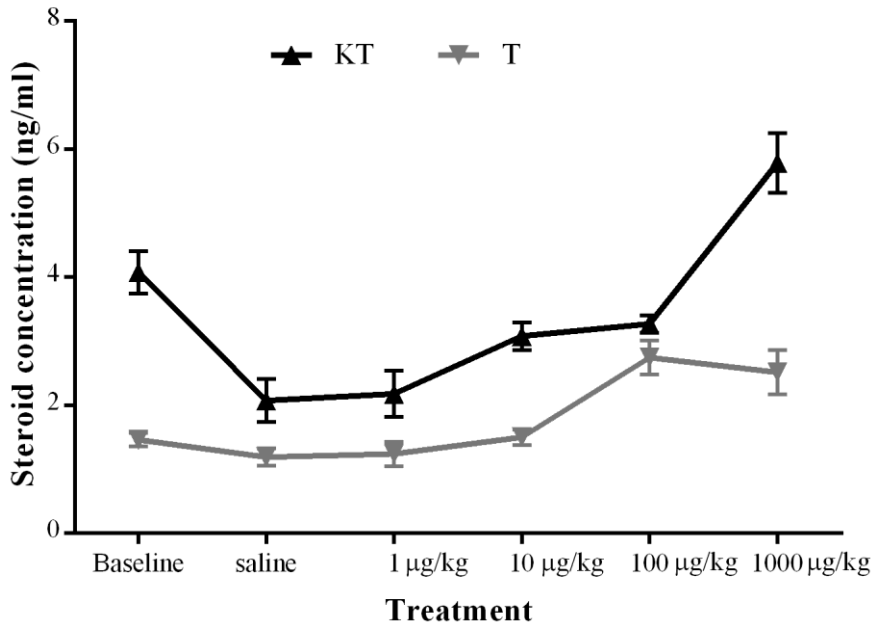

Figure S1. Dose-response experiment to test which concentration dose of GnRH (1 µg/Kg, 10 µg/Kg, 100 µg/Kg, 1000 µg/Kg) is responsible for a significant increase of 11-ketotestosterone (KT) and testosterone (T) in male fish (n=7-9 per group). Saline group corresponds to fish injected with saline solution (n= 9). The higher concentration (1000 µg/Kg) was confirmed to produce a significant increase of both androgens levels in plasma relative to baseline non-injected fish (n=32).

Table S1. Principal component analysis of behavioral variables of the OF.

Behavioral variables	Component loading	
	PC1	PC2
Time spent in the centre zone	.623	.530
Latency to enter the centre zone	-.600	-.642
Number of times spent in the centre zone	.860	-.104
Distance moved in the centre zone	.948	-.095
Total distance moved	.939	-.295
Total time in movement	.925	-.279
Eigenvalue	4.127	.879
% of variance explained	68.79	14.64

Table S2. Principal component analysis of behavioral variables of territorial intrusion experiment.

Behavioral variables	Component loading	
	PC1	PC2
Number of bites (intruder)	.693	-.180
Number of chases (intruder)	.836	.123
Time spent in chases (intruder)	.852	.161
Number of tail beats (intruder)	-.104	.575
Number of buttings (intruder)	.801	.156
Number of lateral displays (intruder)	.062	.228
Time spent performing lateral displays (intruder)	-.345	-.432
Number of frontal displays (intruder)	-.295	.296
Time spent performing frontal displays (intruder)	-.105	.279
Latency to attack intruder (first aggressive behavior)	-.736	.249
Number of displays (neighbor)	.287	.681
Time spent performing displays (neighbor)	.046	.774
Number of times near the neighbor's partition	-.370	.498
Time spent near the neighbor's partition	-.586	.509
Number of bites (females)	.312	-.599
Number of chases (females)	.648	-.564
Time spent in chases (females)	.763	-.420
Number of times nipping at the surface	-.723	-.136
Time spent nipping at the surface	-.686	-.289
Number of times near lateral glass wall	-.504	-.320
Time spent near lateral glass wall	-.531	-.350
Number of times swimming	-.393	-.633
Time spent in swimming	-.415	-.453
Eigenvalue	6.841	4.249
% of variance explained	29.74	18.48

Table S3. Repeatability (R) estimation for each variable measured in personality tests. SE – standard error.

Behavior variable	R	SE	p
Open Field Test			
Time spent in the centre zone	0.031	0.138	0.49
Latency to enter the centre zone	0	0	0.5
Number of times spent in the centre zone	0	0.131	1
Distance moved in the centre zone	0.617	0.151	0.001
Total distance moved	0.577	0.154	0.003
Total time in movement	0.651	0.136	0.0006
Novel Object Test			
Time spent in the novel object zone	0	0.131	0.5
Latency to enter the novel object zone	0	0.143	1
Number of times spent in the novel object zone	0.067	0.146	0.427
Distance moved in the novel object zone	0	0.154	0.5

Total distance moved	0.114	0.166	0.344
Total time in movement	0.093	0.154	0.381
Mirror Test			
Number of times spent in the mirror zone	0.33	0.196	0.079
Time spent in the mirror zone	0	0.095	1
Latency to enter the mirror zone	0	0.155	1
Distance moved in the mirror zone	0	0.159	0.5
Total distance moved	0	0.158	1
Total time in movement	0	0.155	1
Net Restraining Test			
Number of escape attempts	0.199	0.18	0.217
Total time spent performing escape attempts	0	0	1

p: p-value after multiple comparison adjustment; statistically significant values are in bold.

Table S4. Repeatability (R) estimation for each variable measured during the several intrusions that each focal fish was subjected. SE – standard error.

Behavior variable	R	SE	p
Aggressive behaviors towards the intruder			
Number of bites	0.44	0.111	<.0001
Number of chases	0.519	0.105	<.0001
Time spent in chases	0.587	0.101	<.0001
Number of tail beats	0.212	0.095	0.004
Number of buttings	0.266	0.104	0.0006
Number of lateral displays	0.107	0.078	0.083
Time spent performing lateral displays	0.208	0.094	0.005
Number of frontal displays	0.213	0.098	0.004
Time spent performing frontal displays	0.244	0.099	0.001
Total number of aggressive behaviors	0.281	0.105	0.0003
Latency to attack (first aggressive behavior)	0.392	0.103	<.0001
Aggressive behaviors towards the neighbor			
Number of displays	0.159	0.089	0.023
Time spent performing displays	0.152	0.086	0.026
Number of times near the neighbor's partition	0.198	0.095	0.007
Time spent near the neighbor's partition	0.209	0.095	0.005
Total number of aggressive behaviors	0.27	0.103	0.0004
Total time spent performing aggressive behaviors	0.104	0.075	0.099
Aggressive behaviors towards females			
Number of bites	0.299	0.11	0.0001
Number of chases	0.366	0.106	<.0001
Time spent in chases	0.462	0.11	<.0001
Non-aggressive behaviors			
Total number	0.368	0.108	<.0001
Total time spent	0.256	0.102	<.0001
Total behaviors			
Total number of behaviors	0.214	0.098	0.004

p: p-value after multiple comparison adjustment; statistically significant values are in bold.

APPENDIX E

Supplementary Information CHAPTER *5*

Supplementary Material

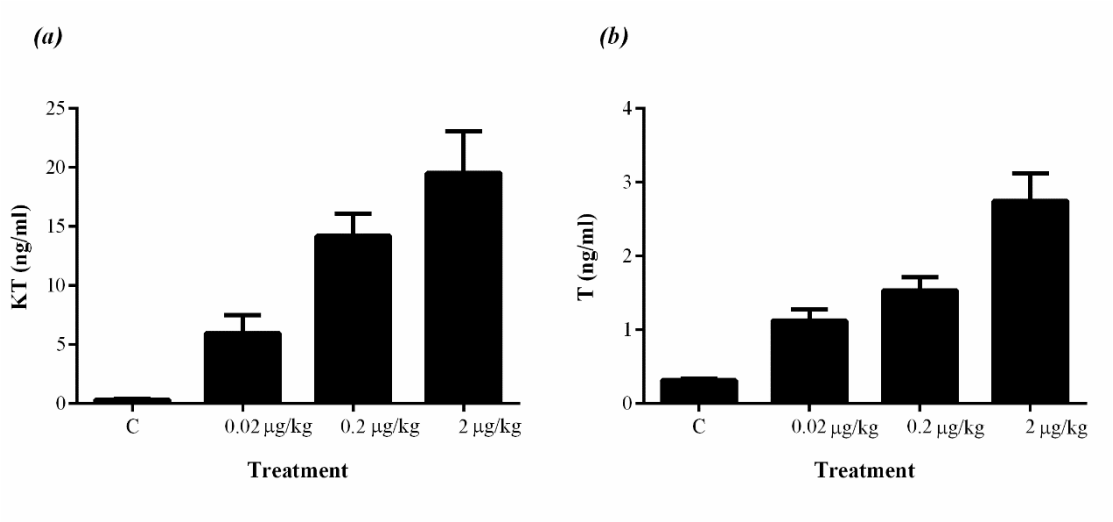

Figure S1. Dose-response experiment to test which concentration dose (0.02 µg/g, 0.2 µg/g, 2 µg/g) is responsible for a significant increase of androgens in male castrated fish. *(a)* 11-ketotestosterone (KT) and *(b)* testosterone (T). Males were castrated and isolated in individual tanks. One week after operation, fish were injected with KT or T and returned to experimental tanks. Sixty min after injection, blood was collected (n=7-8 per treatment). The control treatment (n=5), similarly isolated for one week but not injected, was sampled for blood to measure baseline androgen levels. The lowest concentration (0.02 µg/g) was confirmed to produce a significant increase of both androgens levels in plasma relative to control fish.

Table S.1. List of differentially expressed genes between KT- and V-treated groups for the 30' time point. Positive log2FoldChange (logFC) indicates an up-regulation of the transcript for KT-treated individuals, whereas a negative log2FoldChange indicates a down-regulation relative to the reference group. FDR - False discovery rate.

gene_id	gene_description	gene_name	logFC	FDR
ENSONIG00000016380	-		-8.997	0.000
ENSONIG00000006279	palmitoyltransferase ZDHHC3	zgc:77880	-1.321	0.000
ENSONIG00000016075	ribosomal protein S27a	rps27a	-0.897	0.000
ENSONIG00000013990	C-X-C motif chemokine 10		1.290	0.000
ENSONIG00000015497	-		-0.812	0.000
ENSONIG00000016967	trafficking protein particle complex subunit 6B		0.573	0.000

ENSONIG00000025501	-		-4.226	0.000
ENSONIG00000000803	delta-type opioid receptor	oprm1	0.714	0.001
ENSONIG00000006283	ribosomal protein L23	rpl23	-0.747	0.001
ENSONIG00000018515	ribosomal protein S24	rps24	-1.004	0.001
ENSONIG00000023302	-		-0.767	0.001
ENSONIG00000000435	ribosomal protein S12	rps12	-0.891	0.001
ENSONIG00000008663	transporter 1, ATP binding cassette subfamily B member	tap1	0.890	0.001
ENSONIG00000000900	60S ribosomal protein L19	rpl19	-0.720	0.001
ENSONIG00000018877	-		0.623	0.002
ENSONIG00000002634	fatty acid binding protein 11a	fabp11a	-0.813	0.002
ENSONIG00000002488	ribosomal protein L36A	rpl36a	-1.220	0.003
ENSONIG00000003387	ribosomal protein L12	rpl12	-0.996	0.003
ENSONIG00000004031	retinoid-binding protein 7	rbp7b	-2.375	0.003
ENSONIG00000006510	-		7.804	0.003
ENSONIG00000012509	ribosomal protein L17	rpl17	-0.829	0.003
ENSONIG00000018863	ribosomal protein S15a	rps15a	-1.098	0.003
ENSONIG00000023151	-		-0.652	0.003
ENSONIG00000009685	-		-0.767	0.003
ENSONIG00000011225	spleen associated tyrosine kinase	syk	0.515	0.004
ENSONIG00000001057	somatostatin 1, tandem duplicate 1	sst1.1	-0.942	0.004
ENSONIG00000007508	ribosomal protein L37	rpl37	-1.321	0.004
ENSONIG00000006539	ribosomal protein L18	rpl18	-0.821	0.005
ENSONIG00000014692	ribosomal protein, large P2	rplp2	-1.163	0.005
ENSONIG00000020785	tubulin folding cofactor C	tbcc	0.521	0.005
ENSONIG00000025951	-		7.305	0.005
ENSONIG00000000019	exportin 1 (CRM1 homolog, yeast) a	xpo1a	0.449	0.005
ENSONIG00000014130	ribosomal protein L21	rpl21	-1.094	0.005
ENSONIG00000022008	small nucleolar RNA, H/ACA box 74B	RF00090	-2.504	0.005
ENSONIG00000003560	ribosomal protein L13a	rpl13a	-0.653	0.005

ENSONIG00000019760	lysine (K)-specific methyltransferase 2Ba	kmt2ba	-0.683	0.005
ENSONIG00000016166	ribosomal protein S19	rps19	-0.852	0.006
ENSONIG00000019064	ribosomal protein S26	rps26	-1.070	0.006
ENSONIG00000001300	ribosomal protein S11	si:dkey-159f12.2	-0.982	0.006
ENSONIG00000009071	transmembrane protein 169	tmem169b	0.579	0.006
ENSONIG00000015488	ARFGEF family member 3	arfgef3	-0.785	0.007
ENSONIG00000002672	ribosomal protein L30	rpl30	-0.817	0.007
ENSONIG00000010495	ribosomal protein S15	rps15	-0.684	0.007
ENSONIG00000012247	ribosomal protein L31	rpl31	-0.974	0.007
ENSONIG00000015448	ribosomal protein L14	rpl14	-1.010	0.007
ENSONIG00000017676	60S ribosomal protein L26	rpl26	-0.776	0.007
ENSONIG00000000006	ribosomal protein S16	rps16	-0.781	0.007
ENSONIG00000005440	ribosomal protein S25	rps25	-0.897	0.007
ENSONIG00000012015	Rho family GTPase 3	rnd3a	0.419	0.008
ENSONIG00000001184	glycine receptor, beta b	glrbb	0.360	0.009
ENSONIG00000004876	ribosomal protein L23a	rpl23a	-0.726	0.009
ENSONIG00000005934	ribosomal protein L13	rpl13	-0.829	0.009
ENSONIG00000007401	ribosomal protein S18	rps18	-0.783	0.009
ENSONIG00000014266	ribosomal protein L24	rpl24	-0.882	0.009
ENSONIG00000016242	frizzled related protein	frzb	-0.694	0.009
ENSONIG00000017987	60S ribosomal protein L22		-0.931	0.009
ENSONIG00000019509	ras-related protein Rab-37		0.455	0.009
ENSONIG00000021883	RNA, 7SK small nuclear	RF00100	-1.635	0.009
ENSONIG00000022844	-		-0.996	0.009
ENSONIG00000022881	-		-1.839	0.009
ENSONIG00000006857	ribosomal protein L8	rpl8	-0.588	0.009
ENSONIG00000014744	ribosomal protein S10	rps10	-0.645	0.009

ENSONIG00000016452	G protein-activated inward rectifier potassium channel 1	kcnj19b	0.420	0.009
ENSONIG00000010857	-		-0.797	0.010
ENSONIG00000017838	ribosomal protein S14	rps14	-0.969	0.010
ENSONIG00000024746	-		-2.169	0.010
ENSONIG00000000629	peptidyl-prolyl cis-trans isomerase	ppiab	-0.666	0.010
ENSONIG00000024667	-		2.425	0.010
ENSONIG00000001662	-		-2.055	0.010
ENSONIG00000017358	ribosomal protein L32	rpl32	-0.870	0.011
ENSONIG00000004296	ribosomal protein S23	rps23	-0.963	0.012
ENSONIG00000010625	calcium-binding protein 7		0.381	0.012
ENSONIG00000020134	charged multivesicular body protein 3	chmp3	0.605	0.012
ENSONIG00000018086	-		-0.465	0.012
ENSONIG00000026856	-		-2.506	0.013
ENSONIG00000000434	survival motor neuron domain containing 1	smndc1	0.371	0.013
ENSONIG00000014882	ribosomal protein S21	rps21	-1.172	0.013
ENSONIG00000019959	potassium channel tetramerization domain containing 5	kctd5a	0.415	0.013
ENSONIG00000024770	-		-2.051	0.013
ENSONIG00000007486	ribosomal protein L28	rpl28	-0.684	0.014
ENSONIG00000013655	tetratricopeptide repeat protein 39B	ttc39b	0.509	0.015
ENSONIG00000012496	protein phosphatase 1 regulatory subunit 3B	ppp1r3b	2.313	0.015
ENSONIG00000014275	ornithine carbamoyltransferase	otc	-1.482	0.015
ENSONIG00000007736	ribosomal protein L36	rpl36	-0.995	0.016
ENSONIG00000012814	peptidylprolyl isomerase B	ppib	-0.415	0.016
ENSONIG00000011175	Rho guanine nucleotide exchange factor (GEF) 28a	arhgef28a	-1.001	0.017
ENSONIG00000011286	ribosomal protein L7a	RPL7A	-0.549	0.017
ENSONIG00000001431	FAT atypical cadherin 4	FAT4	-0.736	0.018
ENSONIG00000020185	proto-oncogene c-Fos	fosaa	0.778	0.018

ENSONIG00000018356	ribosomal protein S8	rps8a	-0.569	0.018
ENSONIG00000006321	S100 calcium binding protein A10b	s100a10b	-0.945	0.019
ENSONIG00000001111	VIP peptides	VIP	0.609	0.020
ENSONIG00000016564	CD40 molecule, TNF receptor superfamily member 5	cd40	0.474	0.020
ENSONIG00000001704	kisspeptin-2	kiss2	-1.578	0.020
ENSONIG00000012741	ubiquitin A-52 residue ribosomal protein fusion product 1	uba52	-0.865	0.021
ENSONIG00000009185	calcium channel, voltage-dependent, beta 2b	cacnb2b	0.522	0.022
ENSONIG00000009739	ribosomal protein L27	rpl27	-0.853	0.022
ENSONIG00000012669	cell division cycle 26	cdc26	-1.239	0.022
ENSONIG00000012073	-		-0.831	0.022
ENSONIG00000017688	G protein-coupled receptor, class C, group 5, member Bb	gprc5bb	0.366	0.022
ENSONIG00000018767	-		0.869	0.022
ENSONIG00000003384	C-C motif chemokine 2		-0.519	0.024
ENSONIG00000002300	contactin-3	cntn3a.2	-0.708	0.025
ENSONIG00000018790	interferon-induced protein 44		1.956	0.027
ENSONIG00000019723	S100 calcium binding protein A10a	s100a10a	-0.780	0.028
ENSONIG00000000615	ribosomal protein L22	rpl22	-0.643	0.028
ENSONIG00000005846	serine/threonine-protein phosphatase PP1-beta catalytic subunit	PPP1CB	0.386	0.028
ENSONIG00000006465	dual specificity protein phosphatase 3		0.477	0.028
ENSONIG00000012752	ribosomal protein S9	rps9	-0.577	0.028
ENSONIG00000001263	ribosomal protein S3	rps3	-0.713	0.029
ENSONIG00000005609	ATM interactor	atmin	0.422	0.029
ENSONIG00000007988	ribosomal protein L38	rpl38	-1.154	0.029
ENSONIG00000008360	heme binding protein 1		0.417	0.029
ENSONIG00000025968	-		3.444	0.029
ENSONIG00000004628	periostin	postna	0.923	0.029

ENSONIG00000005624	glutamate receptor ionotropic, kainate 1	grik1b	0.638	0.030
ENSONIG00000014956	NIPBL, cohesin loading factor		-0.519	0.031
ENSONIG00000005229	neuropeptide FF-amide peptide precursor		-0.684	0.031
ENSONIG00000012455	-		-0.870	0.031
ENSONIG00000022297	-		-1.301	0.033
ENSONIG00000000786	synapse differentiation-inducing gene protein 1-like	syndig1l	0.339	0.033
ENSONIG00000017968	ribosomal protein S20	RPS20	-0.762	0.033
ENSONIG00000010985	cytochrome b5 reductase 2	cyb5r2	-0.748	0.035
ENSONIG00000009861	mutS homolog 6	msh6	-0.457	0.036
ENSONIG00000014384	ribosomal protein L6	rpl6	-0.543	0.036
ENSONIG00000001481	-		-0.764	0.036
ENSONIG00000001755	cornichon family AMPA receptor auxiliary protein 2	cnih2	0.440	0.037
ENSONIG00000007608	G protein subunit gamma 10	gng10	-1.017	0.037
ENSONIG00000022344	-		1.523	0.037
ENSONIG00000004628	periostin	postna	0.923	0.029
ENSONIG00000005624	glutamate receptor ionotropic, kainate 1	grik1b	0.638	0.030
ENSONIG00000014956	NIPBL, cohesin loading factor		-0.519	0.031
ENSONIG00000005229	neuropeptide FF-amide peptide precursor		-0.684	0.031
ENSONIG00000012455	-		-0.870	0.031
ENSONIG00000022297	-		-1.301	0.033
ENSONIG00000000786	synapse differentiation-inducing gene protein 1-like	syndig1l	0.339	0.033
ENSONIG00000017968	ribosomal protein S20	RPS20	-0.762	0.033
ENSONIG00000010985	cytochrome b5 reductase 2	cyb5r2	-0.748	0.035
ENSONIG00000009861	mutS homolog 6	msh6	-0.457	0.036
ENSONIG00000014384	ribosomal protein L6	rpl6	-0.543	0.036
ENSONIG00000001481	-		-0.764	0.036

ENSONIG00000001755	cornichon family AMPA receptor auxiliary protein 2	cnih2	0.440	0.037
ENSONIG00000007608	G protein subunit gamma 10	gng10	-1.017	0.037
ENSONIG00000022344	-		1.523	0.037
ENSONIG00000004302	inositol hexakisphosphate kinase 1	ip6k1	-0.751	0.038
ENSONIG00000008577	neuronal pentraxin receptor a	nptxra	0.447	0.038
ENSONIG00000008686	neuronal cell adhesion molecule	NRCAM (1 of many)	-0.432	0.038
ENSONIG00000008992	ribosomal protein L9	rpl9	-0.790	0.038
ENSONIG00000009211	vigilin	hdlbpb	-0.419	0.038
ENSONIG00000011864	ribosomal protein L10a	rpl10a	-0.505	0.038
ENSONIG00000015706	cytochrome c oxidase copper chaperone	cox17	-0.698	0.038
ENSONIG00000019363	HIRA interacting protein 3	hirip3	-1.638	0.038
ENSONIG00000019693	PHD finger protein 5A	phf5a	-0.857	0.038
ENSONIG00000000770	ATR serine/threonine kinase	atr	-0.742	0.038
ENSONIG00000007120	2'-deoxynucleoside 5'-phosphate N-hydrolase 1	dnph1	-0.614	0.040
ENSONIG00000007506	profilin-1	pfn1	-0.462	0.040
ENSONIG00000011721	transmembrane protein 184C	TMEM184C (1 of many)	0.360	0.041
ENSONIG00000022415	-		-1.359	0.041
ENSONIG00000023000	-		-1.036	0.041
ENSONIG00000002986	60S acidic ribosomal protein P2	rplp2l	-0.730	0.041
ENSONIG00000003284	kelch domain-containing protein 8A	klhdc8a	0.508	0.041
ENSONIG00000022734	-		-1.726	0.041
ENSONIG00000000829	ribosomal protein S29	rps29	-1.163	0.041
ENSONIG00000015837	K(lysine) acetyltransferase 6A	kat6a	-0.510	0.041
ENSONIG00000024474	-		0.508	0.041
ENSONIG00000007788	trio Rho guanine nucleotide exchange factor	TRIO	-0.448	0.041

ENSONIG00000009369	-		-0.464	0.041
ENSONIG00000017630	eukaryotic translation elongation factor 1 beta 2	eef1b2	-0.797	0.041
ENSONIG00000017661	60S ribosomal protein L22-like 1	rpl22l1	-0.698	0.041
ENSONIG00000018192	interferon-induced protein with tetratricopeptide repeats 1		2.496	0.041
ENSONIG00000019243	ribosomal protein L5	rpl5b	-0.615	0.041
ENSONIG00000003849	lysosomal alpha-glucosidase	si:ch73-12o23.1	0.324	0.043
ENSONIG00000015660	ADP-ribosylation factor GTPase activating protein 2	arfgap2	0.410	0.043
ENSONIG00000009122	keratin, type I cytoskeletal 18	krt18a.1	-0.332	0.043
ENSONIG00000009780	ER membrane protein complex subunit 10	emc10	-0.369	0.043
ENSONIG00000007346	dehydrogenase/reductase X-linked		0.548	0.044
ENSONIG00000011852	ribosomal protein S4 X-linked	rps4x	-0.704	0.044
ENSONIG00000007641	kelch like family member 8	klhl8	0.378	0.044
ENSONIG00000012756	leukocyte receptor cluster (LRC) member 8	leng8	-0.443	0.044
ENSONIG00000025397	-		-0.626	0.045
ENSONIG00000005058	family with sequence similarity 168 member A	fam168a	-0.401	0.046
ENSONIG00000002309	fatty acid-binding protein, heart	fabp3	-0.402	0.046
ENSONIG00000004350	tumor protein, translationally-controlled 1	tpt1	-0.760	0.046
ENSONIG00000007904	arginine and glutamate rich 1	arglu1a	-0.428	0.046
ENSONIG00000009081	defender against cell death 1	dad1	-0.549	0.046
ENSONIG00000019041	protein kinase C delta	prkcda	0.345	0.046
ENSONIG00000008980	polypeptide N-acetylgalactosaminyltransferase 13	galnt13	0.374	0.047
ENSONIG00000007360	zinc finger protein 423	si:ch211-216l23.1	-0.417	0.051
ENSONIG00000025158	-		-1.412	0.051
ENSONIG00000005941	RNA binding motif protein 33a	rbm33a	-0.470	0.052

ENSONIG00000012005	family with sequence similarity 3 member A	fam3a	0.348	0.052
ENSONIG00000007284	calpain 8	capn8	-1.576	0.052
ENSONIG00000007292	fatty acid synthase	fasn	-0.357	0.052
ENSONIG00000007358	CCR4-NOT transcription complex, subunit 1	cnot1	-0.476	0.052
ENSONIG00000011301	calcium-binding mitochondrial carrier protein SCaMC-2-B	slc25a25b	0.359	0.052
ENSONIG00000002436	si:ch211-221f10.2	si:ch211-221f10.2	-0.792	0.053
ENSONIG00000000709	kinesin family member 17	kif17	1.184	0.054
ENSONIG00000004857	-		-0.458	0.054
ENSONIG00000007578	SVOP-like	svopl	1.446	0.054
ENSONIG00000008244	-		-1.542	0.054
ENSONIG00000017753	ribosomal protein L11	rpl11	-0.519	0.054
ENSONIG00000024680	-		-0.500	0.054
ENSONIG00000003062	glyoxalase I	glo1	-0.427	0.054
ENSONIG00000006746	endothelin-1		-1.341	0.054
ENSONIG00000018130	-		-0.775	0.054
ENSONIG00000018366	phosphoglycerate mutase 1		0.305	0.054
ENSONIG00000000248	small nuclear ribonucleoprotein polypeptides B and B1	snrpb	-0.840	0.055
ENSONIG00000002903	TM2 domain containing 3	tm2d3	0.335	0.055
ENSONIG00000017025	signal transducing adaptor molecule 2	stam2	0.368	0.055
ENSONIG00000011302	galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 1	B3GAT1 (1 of many)	0.513	0.056
ENSONIG00000019438	ribosomal protein lateral stalk subunit P0	rplp0	-0.532	0.057
ENSONIG00000017923	ribosomal protein L10	rpl10	-0.503	0.057
ENSONIG00000001326	fatty acid-binding protein, brain	fabp7a	-0.614	0.057
ENSONIG00000008652	corepressor interacting with RBPJ, 1	si:ch73-167c12.2	-0.499	0.057

ENSONIG00000016533	inter-alpha-trypsin inhibitor heavy chain family member 6	itih6	1.841	0.057
ENSONIG00000013940	glutathione S-transferase theta-1	gstt1a	-1.163	0.058
ENSONIG00000014529	ribosomal protein S6	rps6	-0.448	0.058
ENSONIG00000017705	La ribonucleoprotein domain family member 4B	LARP4B	-0.654	0.058
ENSONIG00000018968	capping actin protein, gelsolin like	capgb	-0.690	0.058
ENSONIG00000019224	mago homolog, exon junction complex subunit	magoh	-0.485	0.058
ENSONIG00000003773	retinal Mueller cells isomerohydrolase	rpe65b	0.594	0.058
ENSONIG00000013848	dpy-19 like 4	DPY19L4	0.396	0.058
ENSONIG00000011361	glucosamine-6-phosphate deaminase 2		0.404	0.059
ENSONIG00000022376	-		-0.522	0.059
ENSONIG00000013283	transmembrane protein 208	zgc:77041	-0.446	0.060
ENSONIG00000017694	polycystic kidney disease 1a	pkd1a	-0.569	0.060
ENSONIG00000001626	small integral membrane protein 8	smim8	-0.601	0.061
ENSONIG00000012302	attractin	atrn	-0.444	0.062
ENSONIG00000006305	ephrin-A1b	efna1b	0.374	0.063
ENSONIG00000001074	cholesterol 24-hydroxylase	cyp46a1.4	0.436	0.063
ENSONIG00000015876	synuclein, gamma b (breast cancer-specific protein 1)	sncgb	-0.382	0.064
ENSONIG00000015052	coiled-coil serine-rich protein 1	ccser1	-0.525	0.065
ENSONIG00000007366	cerebellin 1 precursor	cbln1	0.336	0.065
ENSONIG00000016034	glycine receptor alpha 2	glra2	0.311	0.065
ENSONIG00000015528	ribosomal protein L27a	RPL27A	-0.723	0.066
ENSONIG00000002351	C-C motif chemokine 17		0.376	0.066
ENSONIG00000018030	cullin associated and neddylation dissociated 1	cand1	-0.383	0.066
ENSONIG00000000180	mitogen-activated protein kinase 6	mapk6	-0.458	0.066
ENSONIG00000000059	TTK protein kinase	ttk	1.080	0.067
ENSONIG00000009676	glutathione S-transferase	gsta	-1.229	0.067
ENSONIG00000012780	complement component C7	c7b	-0.867	0.067

ENSONIG00000026732	-		-2.168	0.067
ENSONIG00000014432	potassium voltage-gated channel interacting protein 3	KCNIP3	0.328	0.068
ENSONIG00000012720	transcription factor 4	TCF4	-0.493	0.068
ENSONIG00000005772	protein FAM162B	fam162a	-0.304	0.068
ENSONIG00000010223	transglutaminase 2, like	tgm2l (1 of many)	-0.540	0.068
ENSONIG00000012808	tight junction protein 2a (zona occludens 2)	tjp2a	-0.424	0.068
ENSONIG00000016692	limbic system associated membrane protein	LSAMP	0.407	0.068
ENSONIG00000017545	NADH:ubiquinone oxidoreductase subunit B6	ndufb6	-0.494	0.068
ENSONIG00000018462	cathepsin Z		0.341	0.068
ENSONIG00000023379	-		-0.525	0.068
ENSONIG00000002046	ATP synthase peripheral stalk subunit d	atp5pd	-0.422	0.069
ENSONIG00000007133	QKI, KH domain containing, RNA binding b	qkib	-0.415	0.069
ENSONIG00000007470	ribosomal protein L18a	rpl18a	-0.502	0.069
ENSONIG00000012074	-		0.363	0.069
ENSONIG00000015413	spectrin beta chain, non-erythrocytic 1	SPTBN1 (1 of many)	-0.374	0.069
ENSONIG00000001063	dihydropyrimidinase-related protein 5	dpysl5a	0.406	0.072
ENSONIG00000009933	mitogen-activated protein kinase 11	mapk11	0.358	0.072
ENSONIG00000008464	ring-box 1	rbx1	-0.582	0.074
ENSONIG00000002078	-		-0.732	0.074
ENSONIG00000006862	spectrin, beta, non-erythrocytic 1	sptbn1	-0.340	0.074
ENSONIG00000007495	stress-associated endoplasmic reticulum protein 1	zgc:85858	-0.667	0.074
ENSONIG00000014288	telomeric repeat binding factor 1	terf1	-1.639	0.074
ENSONIG00000024931	-		1.084	0.074
ENSONIG00000026416	-		-0.585	0.074

ENSONIG00000003175	solute carrier family 7 member 6	slc7a6	1.143	0.075
ENSONIG00000012048	nucleosome assembly protein 1 like 1	nap1l1	-0.432	0.075
ENSONIG00000015698	NADH:ubiquinone oxidoreductase subunit AB1	ndufab1b	-0.405	0.075
ENSONIG00000007254	sulfotransferase family 2, cytosolic sulfotransferase 3	sult2st3 (1 of many)	1.265	0.076
ENSONIG00000005218	glycerophosphodiester phosphodiesterase domain-containing protein 5	gdpd5b	0.326	0.076
ENSONIG00000010120	methylsterol monooxygenase 1	msmo1	-0.368	0.076
ENSONIG00000012603	LIM domain transcription factor LMO4.1	lmo4a	0.365	0.076
ENSONIG00000007537	major facilitator superfamily domain containing 8	mfsd8	0.388	0.077
ENSONIG00000013963	RNA binding motif protein X-linked 2		-0.551	0.077
ENSONIG00000014713	histocompatibility minor 13	hm13	-0.340	0.077
ENSONIG00000016081	DnaJ heat shock protein family (Hsp40) member B12	dnajb12b	0.366	0.077
ENSONIG00000017697	ring finger protein 44	rnf44	0.352	0.077
ENSONIG00000017893	zinc finger, DHHC-type containing 15b	zdhhc15b	0.337	0.077
ENSONIG00000019507	calmodulin 2		-0.449	0.077
ENSONIG00000020215	ribosomal protein L35	rpl35	-0.875	0.077
ENSONIG00000022509	-		-0.632	0.077
ENSONIG00000023816	-		-0.759	0.077
ENSONIG00000020161	CLOCK-interacting pacemaker a	cipca	0.404	0.077
ENSONIG00000026982	-		1.201	0.078
ENSONIG00000003110	-		0.937	0.078
ENSONIG00000015838	non-specific cytotoxic cell receptor protein 1	nccrp1	-0.654	0.078
ENSONIG00000021392	-		-1.129	0.079
ENSONIG00000002576	profilin 2	pfn2	-0.429	0.079
ENSONIG00000010307	ribosomal protein L15	rpl15	-0.423	0.079
ENSONIG00000013855	-		1.692	0.079

ENSONIG00000016072	bone morphogenetic protein receptor, type IAa	bmpr1aa	-0.972	0.079
ENSONIG00000018638	-		0.302	0.079
ENSONIG00000023958	-		-0.560	0.079
ENSONIG00000017312	vacuolar protein sorting 13 homolog D (S. cerevisiae)	vps13d	-0.493	0.079
ENSONIG00000002956	transmembrane protein 165	tmem165	0.376	0.080
ENSONIG00000003182	myosin XVAb	myo15ab	-1.020	0.080
ENSONIG00000015902	cytochrome c oxidase subunit 7A2 like	cox7a2l	-0.563	0.080
ENSONIG00000024232	-		-1.651	0.080
ENSONIG00000004248	POZ/BTB and AT hook containing zinc finger 1	patz1	-0.496	0.080
ENSONIG00000017056	nascent polypeptide associated complex subunit alpha	naca	-0.425	0.080
ENSONIG00000018688	androgen-induced gene 1 protein	pex3	0.316	0.080
ENSONIG00000009189	signal transducing adaptor molecule (SH3 domain and ITAM motif) 1	stam	0.311	0.080
ENSONIG00000019471	ketohexokinase	khk	-0.468	0.080
ENSONIG00000002326	ribosomal protein S2	rps2	-0.405	0.082
ENSONIG00000017758	-		-0.405	0.083
ENSONIG00000002814	ribosomal protein lateral stalk subunit P1	rplp1	-0.724	0.083
ENSONIG00000003186	transmembrane and coiled-coil domains 3	tmco3	0.392	0.083
ENSONIG00000016910	lysosome membrane protein 2	scarb2a	-0.534	0.083
ENSONIG00000023353	-		-0.645	0.083
ENSONIG00000026129	-		-1.396	0.083
ENSONIG00000009689	desmoglein-2		0.571	0.084
ENSONIG00000020491	-		2.611	0.084
ENSONIG00000016893	post-GPI attachment to proteins factor 2	PGAP2 (1 of many)	0.358	0.086
ENSONIG00000017603	fibroblast growth factor 20-like	fgf20b	-1.770	0.086
ENSONIG00000009675	transmembrane protein 14A	zgc:163080	-0.503	0.086

ENSONIG00000020174	iron-sulfur cluster assembly 2	isca2	-0.748	0.086
ENSONIG00000002752	lysine (K)-specific methyltransferase 2Bb	kmt2bb	-0.411	0.087
ENSONIG00000006400	regulation of nuclear pre-mRNA domain containing 2b	rprd2b	-0.465	0.087
ENSONIG00000021138	-		1.187	0.088
ENSONIG00000003225	vacuolar protein sorting 13 homolog C		-0.439	0.088
ENSONIG00000004155	spermidine/spermine N1-acetyltransferase family member 2b	sat2b	0.550	0.092
ENSONIG00000017848	NADH:ubiquinone oxidoreductase subunit A12	ndufa12	-0.560	0.092
ENSONIG00000008480	thyrotrophic embryonic factor b	tefb	-0.456	0.093
ENSONIG00000002558	lysosomal-associated membrane protein 2	lamp2	-0.312	0.093
ENSONIG00000006986	huntingtin interacting protein K	hypk	-0.721	0.093
ENSONIG00000006460	retinol-binding protein 1		-0.669	0.094
ENSONIG00000025528	-		0.310	0.095
ENSONIG00000004497	L-amino-acid oxidase-like		4.202	0.095
ENSONIG00000003885	centrosomal protein 41	cep41	0.369	0.097
ENSONIG00000001390	heparan sulfate glucosamine 3-O-sulfotransferase 2	hs3st2	0.440	0.098
ENSONIG00000006166	sodium channel, voltage-gated, type III, beta	scn3b	0.336	0.098
ENSONIG00000012274	putative helicase mov-10-B.2	mov10b.2	1.590	0.098
ENSONIG00000002168	ribosomal modification protein rimK-like family member A	rimkla	0.337	0.099
ENSONIG00000004468	dedicator of cytokinesis 3	dock3	-0.467	0.099

Table S.2. List of differentially expressed genes between T- and V-treated groups for the 30' time point. Positive log2FoldChange (logFC) indicates an up-regulation of the transcript for T-treated individuals, whereas a negative log2FoldChange indicates a down-regulation relative to the reference group. FDR - False discovery rate.

gene_id	gene_description	gene_name	logFC	FDR
ENSONIG00000006279	palmitoyltransferase ZDHHC3	zgc:77880	-1.459	0.000
ENSONIG00000016380	-		-5.688	0.000
ENSONIG00000025501	-		-5.193	0.000
ENSONIG00000008086	myosin-6		-2.262	0.000
ENSONIG00000020090	-		-4.196	0.000
ENSONIG00000017772	-		-4.002	0.000
ENSONIG00000003458	POP4 homolog. ribonuclease P/MRP subunit	pop4	-1.153	0.002
ENSONIG00000012073	-		-1.069	0.002
ENSONIG00000017094	potassium channel subfamily K member 2		-1.158	0.002
ENSONIG00000005497	SUV3-like helicase	supv3l1	0.697	0.007
ENSONIG00000001153	solute carrier family 8 (sodium/calcium exchanger). member 1b	slc8a1b	-0.811	0.008
ENSONIG00000004915	-		-0.819	0.008
ENSONIG00000013290	monoacylglycerol lipase ABHD2-A	abhd2a	-1.028	0.008
ENSONIG00000022741	-		-2.122	0.008
ENSONIG00000022844	-		-1.070	0.008
ENSONIG00000023172	-		-1.449	0.008
ENSONIG00000007647	sodium/glucose cotransporter 4	slc5a9	-4.407	0.010
ENSONIG00000000709	kinesin family member 17	kif17	1.479	0.013
ENSONIG00000002433	granzyme E-like		-3.873	0.013
ENSONIG00000003182	myosin XVAb	myo15ab	-1.365	0.013
ENSONIG00000010239	semaphorin-4E	sema4e	-2.006	0.013
ENSONIG00000015497	-		-0.653	0.013
ENSONIG00000020134	charged multivesicular body protein 3	chmp3	0.641	0.013
ENSONIG00000021513	-	RF00026	-2.472	0.013

ENSONIG00000022575	-		-1.655	0.013
ENSONIG00000026519	-		-1.347	0.015
ENSONIG00000012496	protein phosphatase 1 regulatory subunit 3B	ppp1r3b	2.436	0.016
ENSONIG00000014736	-		-1.363	0.018
ENSONIG00000007088	-		-2.842	0.019
ENSONIG00000022565	-		-1.743	0.020
ENSONIG00000008270	single Ig and TIR domain containing	sigirr	1.471	0.021
ENSONIG00000015404	ariadne RBR E3 ubiquitin protein ligase 1	arih1	-0.506	0.021
ENSONIG00000008321	dehydrogenase/reductase SDR family member 13	dhrs13b	1.023	0.021
ENSONIG00000007149	neural EGFL like 1	NELL1	-0.450	0.028
ENSONIG00000018767	-		0.911	0.028
ENSONIG00000016129	RNA polymerase III subunit A	polr3a	-0.767	0.033
ENSONIG00000016398	ankyrin 2	ANK2	-0.455	0.035
ENSONIG00000024179	-		-4.696	0.058
ENSONIG00000000461	dysferlin. limb girdle muscular dystrophy 2B (autosomal recessive)	dysf	-0.699	0.059
ENSONIG00000005595	dachsous cadherin-related 1a	dchs1a	-0.723	0.059
ENSONIG00000010123	egl-9 family hypoxia-inducible factor 1a	egln1a	-0.868	0.059
ENSONIG00000010591	nitric oxide synthase 1	nos1	-0.395	0.059
ENSONIG00000013841	intraflagellar transport 27 homolog (Chlamydomonas)	ift27	0.548	0.059
ENSONIG00000022521	-		-1.928	0.059
ENSONIG00000025718	-		1.335	0.059
ENSONIG00000023694	-		-1.270	0.059
ENSONIG00000016835	MAGI family member. X-linked b	magixb	1.163	0.063
ENSONIG00000022509	-		-0.728	0.063
ENSONIG00000024212	-		-1.456	0.063
ENSONIG00000000238	-		-8.321	0.065

ENSONIG00000000351	calcium channel. voltage-dependent. P/Q type. alpha 1A subunit. b	cacna1ab	-0.573	0.065
ENSONIG00000008548	si:ch211-200p22.4	si:ch211-200p22.4	-0.394	0.065
ENSONIG00000013103	death effector domain-containing 1	dedd1	-0.401	0.065
ENSONIG00000013562	RING finger protein B	krcp	-1.658	0.065
ENSONIG00000015488	ARFGEF family member 3	arfgef3	-0.673	0.065
ENSONIG00000018453	semaphorin-4G	sema4ga	-0.509	0.065
ENSONIG00000020746	-		-2.204	0.065
ENSONIG00000026949	-		-3.553	0.065
ENSONIG00000022832	-		-3.161	0.066
ENSONIG00000011151	transportin 1	tnpo1	-0.525	0.068
ENSONIG00000005589	PTPRF interacting protein. binding protein 2a (liprin beta 2)	ppfibp2a	1.690	0.074
ENSONIG00000005862	rhomboid domain containing 1	rhbdd1	-1.294	0.074
ENSONIG00000007254	sulfotransferase family 2. cytosolic sulfotransferase 3	sult2st3 (1 of many)	1.412	0.074
ENSONIG00000007284	calpain 8	capn8	-1.649	0.074
ENSONIG00000008410	ubiquitin-conjugating enzyme E2 Q2	ube2q2	-0.449	0.074
ENSONIG00000008715	A disintegrin and metalloproteinase with thrombospondin motifs 20	ADAMTS 20	0.893	0.074
ENSONIG00000014459	homeobox protein orthopedia B	otpb	0.512	0.074
ENSONIG00000022678	-		-0.459	0.074
ENSONIG00000005996	solute carrier family 45. member 4	slc45a4	-1.451	0.078
ENSONIG00000012756	leukocyte receptor cluster (LRC) member 8	leng8	-0.452	0.078
ENSONIG00000023261	-		1.083	0.078
ENSONIG00000019591	solute carrier family 8 member A3	slc8a3	-0.510	0.079
ENSONIG00000020210	whirlin b	whrnb	-0.690	0.079
ENSONIG00000011691	-		-1.684	0.085
ENSONIG00000016533	inter-alpha-trypsin inhibitor heavy chain family member 6	itih6	1.924	0.085
ENSONIG00000020221	-		1.948	0.085

ENSONIG00000026398	-		-1.606	0.085
ENSONIG00000005500	-		-0.549	0.085
ENSONIG00000012642	DCC netrin 1 receptor	dcc	-0.595	0.085
ENSONIG00000014067	si:ch211-161h7.8	si:ch211-161h7.8 (1 of many)	0.646	0.085
ENSONIG00000023726	-		-1.788	0.085
ENSONIG00000009124	programmed cell death 1 ligand 1		-3.677	0.087
ENSONIG00000006903	barrier-to-autointegration factor-like protein		1.217	0.089
ENSONIG00000003179	mitochondrial elongation factor 2	mief2	0.388	0.092
ENSONIG00000005692	ryanodine receptor 3	ryr3	-0.575	0.092
ENSONIG00000007210	secretagogin		0.420	0.092
ENSONIG00000010217	methyl-CpG-binding domain protein 3	mbd3a	0.423	0.092
ENSONIG00000003823	leucine rich repeat kinase 2	lrrk2	-0.422	0.093
ENSONIG00000003800	nucleolar complex associated 4 homolog	noc4l	0.537	0.100
ENSONIG00000003892	ryanodine receptor 1	ryr1b	-0.436	0.100
ENSONIG00000003974	-		0.964	0.100
ENSONIG00000004344	DENN domain containing 6A	dennd6aa	-0.564	0.100
ENSONIG00000005665	microtubule associated scaffold protein 2		-0.372	0.100
ENSONIG00000006968	diacylglycerol kinase delta	si:dkey-172j4.3	-0.345	0.100
ENSONIG00000007421	-		-2.237	0.100
ENSONIG00000020019	myosin phosphatase Rho interacting protein	MPRIP	-0.384	0.100
ENSONIG00000020020	-		-9.106	0.100
ENSONIG00000023492	-		0.560	0.100
ENSONIG00000024417	-		0.617	0.100
ENSONIG00000024770	-		-1.707	0.100
ENSONIG00000016155	ecto-NOX disulfide-thiol exchanger 1	enox1	-0.342	0.100

Table S.3. List of differentially expressed genes between KT- and V-treated groups for the 60' time point. Positive log2FoldChange (logFC) indicates an up-regulation of the transcript for KT-treated individuals, whereas a negative log2FoldChange indicates a down-regulation relative to the reference group. FDR - False discovery rate.

gene_id	gene_description	gene_name	logFC	FDR
ENSONIG00000012591	-	-	-1.179	0.085

Table S.4. List of differentially expressed genes between T- and V-treated groups for the 60' time point. Positive log2FoldChange (logFC) indicates an up-regulation of the transcript for T-treated individuals, whereas a negative log2FoldChange indicates a down-regulation relative to the reference group. FDR - False discovery rate.

gene_id	gene_description	gene_name	logFC	FDR
ENSONIG00000001532	polycomb group ring finger 3	PCGF3	-0.710	0.002
ENSONIG00000000643	-		-1.237	0.035
ENSONIG00000012073	-		-1.149	0.035
ENSONIG00000017410	phosphodiesterase 9A	pde9a	1.758	0.058
ENSONIG00000008807	LDL receptor related protein 2	lrp2a	-0.891	0.062
ENSONIG00000001837	lysine (K)-specific demethylase 6B, b	kdm6bb	-0.520	0.072
ENSONIG00000009169	plexin A1a	plxna1a	-0.850	0.100
ENSONIG00000009994	SWT1 RNA endoribonuclease homolog	swt1	-0.660	0.100

Table S.5. Gene ontology enrichment for transcripts up-regulated between KT- and V-treated groups for the 30' time point. Conditional enrichment was obtained with unadjusted $P < 0.05$. Expected count: number of transcripts in each category expected based on the distribution of categories among all transcripts tested. Observed count: number of transcripts conferring the enrichment in each category for each module.

GO ID	Go term	Expected count	Observed Count	p-value
Biological Process				
GO:0010906	regulation of glucose metabolic process	0.0410	2	0.001
GO:0002478	antigen processing and presentation of exogenous peptide antigen	0.0051	1	0.005
GO:0002479	antigen processing and presentation of exogenous peptide antigen via MHC class I	0.0051	1	0.005
GO:0045234	protein palmitoleylation	0.0051	1	0.005
GO:0018230	peptidyl-L-cysteine S-palmitoylation	0.0051	1	0.005
GO:0051234	establishment of localization	10.4539	19	0.005
GO:0070459	prolactin secretion	0.0103	1	0.010
GO:0071312	cellular response to alkaloid	0.0103	1	0.010
GO:0071315	cellular response to morphine	0.0103	1	0.010
GO:0005981	regulation of glycogen catabolic process	0.0103	1	0.010
GO:0014072	response to isoquinoline alkaloid	0.0103	1	0.010
GO:0006706	steroid catabolic process	0.0103	1	0.010
GO:0006707	cholesterol catabolic process	0.0103	1	0.010
GO:0048255	mRNA stabilization	0.0154	1	0.015
GO:0009251	glucan catabolic process	0.0154	1	0.015
GO:0042754	negative regulation of circadian rhythm	0.0154	1	0.015
GO:0044247	cellular polysaccharide catabolic process	0.0154	1	0.015
GO:0010595	positive regulation of endothelial cell migration	0.0154	1	0.015
GO:0032881	regulation of polysaccharide metabolic process	0.0154	1	0.015
GO:0043470	regulation of carbohydrate catabolic process	0.0154	1	0.015
GO:0006044	N-acetylglucosamine metabolic process	0.0154	1	0.015
GO:0018198	peptidyl-cysteine modification	0.0154	1	0.015

GO:0090050	positive regulation of cell migration involved in sprouting angiogenesis	0.0154	1	0.015
GO:0007023	post-chaperonin tubulin folding pathway	0.0205	1	0.020
GO:0045721	negative regulation of gluconeogenesis	0.0205	1	0.020
GO:0045912	negative regulation of carbohydrate metabolic process	0.0205	1	0.020
GO:0044724	single-organism carbohydrate catabolic process	0.2360	2	0.023
GO:0060326	cell chemotaxis	0.2360	2	0.023
GO:0019318	hexose metabolic process	0.2411	2	0.024
GO:0055085	transmembrane transport	5.7296	11	0.025
GO:0010632	regulation of epithelial cell migration	0.0256	1	0.025
GO:0043467	regulation of generation of precursor metabolites and energy	0.0256	1	0.025
GO:0018095	protein polyglutamylation	0.0256	1	0.025
GO:0043535	regulation of blood vessel endothelial cell migration	0.0256	1	0.025
GO:0044262	cellular carbohydrate metabolic process	0.2513	2	0.026
GO:0002474	antigen processing and presentation of peptide antigen via MHC class I	0.0308	1	0.030
GO:0043255	regulation of carbohydrate biosynthetic process	0.0308	1	0.030
GO:1901135	carbohydrate derivative metabolic process	2.4006	6	0.032
GO:0038003	opioid receptor signaling pathway	0.0359	1	0.035
GO:0043487	regulation of RNA stability	0.0359	1	0.035
GO:0042886	amide transport	1.8569	5	0.038
GO:0006486	protein glycosylation	0.7540	3	0.040
GO:0001649	osteoblast differentiation	0.0410	1	0.040
GO:0036211	protein modification process	8.6893	14	0.045
GO:0046890	regulation of lipid biosynthetic process	0.0462	1	0.045
GO:1901616	organic hydroxy compound catabolic process	0.0462	1	0.045
GO:0070085	glycosylation	0.8053	3	0.047

Cellular Component

GO:0000164	protein phosphatase type 1 complex	0.0430	2	0.001
GO:0042825	TAP complex	0.0054	1	0.005
GO:1903293	phosphatase complex	0.1345	2	0.008
GO:0031300	intrinsic component of organelle membrane	0.1668	2	0.012
GO:0016021	integral component of membrane	29.9291	38	0.019
GO:0072357	PTW/PP1 phosphatase complex	0.0269	1	0.027
GO:0000139	Golgi membrane	0.7084	3	0.034
GO:0031463	Cul3-RING ubiquitin ligase complex	0.0377	1	0.037
GO:0005779	integral component of peroxisomal membrane	0.0377	1	0.037
GO:0044425	membrane part	31.1073	38	0.039
GO:0030173	integral component of Golgi membrane	0.0430	1	0.042
GO:0005871	kinesin complex	0.0484	1	0.047

Molecular Function

GO:0070740	tubulin-glutamic acid ligase activity	0.0047	1	0.005
GO:0004979	beta-endorphin receptor activity	0.0047	1	0.005
GO:0038047	morphine receptor activity	0.0047	1	0.005
GO:0015433	peptide antigen-transporting ATPase activity	0.0047	1	0.005
GO:0046979	TAP2 binding	0.0047	1	0.005
GO:0033781	cholesterol 24-hydroxylase activity	0.0047	1	0.005
GO:0008009	chemokine activity	0.1027	2	0.005
GO:0015276	ligand-gated ion channel activity	0.7472	4	0.007
GO:0004342	glucosamine-6-phosphate deaminase activity	0.0093	1	0.009
GO:0038046	enkephalin receptor activity	0.0093	1	0.009
GO:0032575	ATP-dependent 5'-3' RNA helicase activity	0.0093	1	0.009
GO:0005049	nuclear export signal receptor activity	0.0140	1	0.014
GO:0042287	MHC protein binding	0.0140	1	0.014
GO:0004712	protein serine/threonine/tyrosine kinase activity	0.0140	1	0.014
GO:0001664	G-protein coupled receptor binding	0.2055	2	0.018
GO:0016934	extracellular-glycine-gated chloride channel activity	0.0187	1	0.019

GO:0004082	bisphosphoglycerate mutase activity	0.0187	1	0.019
GO:0004619	phosphoglycerate mutase activity	0.0187	1	0.019
GO:0015631	tubulin binding	0.5884	3	0.021
GO:0022836	gated channel activity	1.5924	5	0.021
GO:0015018	galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase activity	0.0233	1	0.023
GO:0017080	sodium channel regulator activity	0.0233	1	0.023
GO:0016594	glycine binding	0.0233	1	0.023
GO:0008186	RNA-dependent ATPase activity	0.0280	1	0.028
GO:0015467	G-protein activated inward rectifier potassium channel activity	0.0280	1	0.028
GO:0030374	ligand-dependent nuclear receptor transcription coactivator activity	0.0327	1	0.032
GO:0016881	acid-amino acid ligase activity	0.0327	1	0.032
GO:0008889	glycerophosphodiester phosphodiesterase activity	0.0327	1	0.032
GO:0003724	RNA helicase activity	0.0374	1	0.037
GO:0022892	substrate-specific transporter activity	3.8572	8	0.038
GO:0022824	transmitter-gated ion channel activity	0.3129	2	0.039
GO:1904680	peptide transmembrane transporter activity	0.0420	1	0.041
GO:0008146	sulfotransferase activity	0.3269	2	0.042
GO:0022857	transmembrane transporter activity	4.6744	9	0.043
GO:0022838	substrate-specific channel activity	1.9286	5	0.044
GO:0005126	cytokine receptor binding	0.3456	2	0.047

Table S.6. Gene ontology enrichment for transcripts down-regulated between KT- and V-treated groups for the 30' time point. Conditional enrichment was obtained with unadjusted P < 0.05. Expected count: number of transcripts in each category expected based on the distribution of categories among all transcripts tested. Observed count: number of transcripts conferring the enrichment in each category for each module.

GO ID	Go term	Expected count	Observed Count	p-value
Biological Process				
GO:0043604	amide biosynthetic process	3.2815	62	0.000
GO:0006518	peptide metabolic process	3.4035	62	0.000
GO:0006412	translation	2.8118	57	0.000
GO:1901566	organonitrogen compound biosynthetic process	5.9866	63	0.000
GO:0009059	macromolecule biosynthetic process	20.5983	68	0.000
GO:0019538	protein metabolic process	30.7977	80	0.000
GO:0044249	cellular biosynthetic process	25.0218	69	0.000
GO:0034641	cellular nitrogen compound metabolic process	29.1126	72	0.000
GO:0044260	cellular macromolecule metabolic process	44.6112	83	0.000
GO:0071704	organic substance metabolic process	63.4247	98	0.000
GO:0043009	chordate embryonic development	3.3924	17	0.000
GO:0002262	myeloid cell homeostasis	0.8647	9	0.000
GO:0006414	translational elongation	0.2328	5	0.000
GO:0009790	embryo development	7.5165	22	0.000
GO:0002520	immune system development	3.1042	12	0.000
GO:0030218	erythrocyte differentiation	0.4422	5	0.000
GO:0051726	regulation of cell cycle	1.8292	9	0.000
GO:0048821	erythrocyte development	0.3658	4	0.000
GO:0030833	regulation of actin filament polymerization	0.6541	5	0.000
GO:0030832	regulation of actin filament length	0.6763	5	0.001
GO:0043254	regulation of protein complex assembly	0.6984	5	0.001
GO:0009987	cellular process	100.4307	116	0.001
GO:0030097	hemopoiesis	2.5870	9	0.001
GO:0008154	actin polymerization or depolymerization	0.8426	5	0.002
GO:0051693	actin filament capping	0.2328	3	0.002
GO:0072332	intrinsic apoptotic signaling pathway by p53 class mediator	0.0665	2	0.002
GO:0032970	regulation of actin filament-based process	0.8869	5	0.002
GO:0030834	regulation of actin filament depolymerization	0.2550	3	0.002
GO:1901880	negative regulation of protein depolymerization	0.2661	3	0.002
GO:0032272	negative regulation of protein polymerization	0.2661	3	0.002
GO:0097190	apoptotic signaling pathway	0.2772	3	0.003
GO:0043244	regulation of protein complex disassembly	0.3215	3	0.004
GO:0042592	homeostatic process	3.7139	10	0.004

GO:1902904	negative regulation of supramolecular fiber organization	0.3326	3	0.004
GO:0051494	negative regulation of cytoskeleton organization	0.3548	3	0.005
GO:0090066	regulation of anatomical structure size	1.6075	6	0.005
GO:0009058	biosynthetic process	0.7141	4	0.006
GO:0060216	definitive hemopoiesis	0.3880	3	0.007
GO:0042541	hemoglobin biosynthetic process	0.1330	2	0.007
GO:0048513	animal organ development	15.9199	26	0.008
GO:0043624	cellular protein complex disassembly	0.4545	3	0.010
GO:0009159	deoxyribonucleoside monophosphate catabolic process	0.0111	1	0.011
GO:1990403	embryonic brain development	0.0111	1	0.011
GO:0001502	cartilage condensation	0.0111	1	0.011
GO:0043966	histone H3 acetylation	0.0111	1	0.011
GO:0032984	macromolecular complex disassembly	0.4767	3	0.012
GO:0006334	nucleosome assembly	0.1885	2	0.015
GO:0006333	chromatin assembly or disassembly	0.1996	2	0.017
GO:0001878	response to yeast	0.2106	2	0.018
GO:0045088	regulation of innate immune response	0.2217	2	0.020
GO:0010265	SCF complex assembly	0.0222	1	0.022
GO:0035284	brain segmentation	0.0222	1	0.022
GO:0043570	maintenance of DNA repeat elements	0.0222	1	0.022
GO:0043623	cellular protein complex assembly	1.6178	5	0.023
GO:0006323	DNA packaging	0.2550	2	0.027
GO:0022900	electron transport chain	0.6763	3	0.030
GO:0097428	protein maturation by iron-sulfur cluster transfer	0.0333	1	0.033
GO:0010172	embryonic body morphogenesis	0.0333	1	0.033
GO:0044246	regulation of multicellular organismal metabolic process	0.0333	1	0.033
GO:0032965	regulation of collagen biosynthetic process	0.0333	1	0.033
GO:0035988	chondrocyte proliferation	0.0333	1	0.033
GO:0065003	macromolecular complex assembly	3.7472	8	0.034
GO:0030036	actin cytoskeleton organization	2.5277	6	0.041
GO:1901207	regulation of heart looping	0.0443	1	0.044
GO:0031145	anaphase-promoting complex-dependent catabolic process	0.0443	1	0.044
GO:0014036	neural crest cell fate specification	0.0443	1	0.044
GO:0006825	copper ion transport	0.0443	1	0.044
GO:0051493	regulation of cytoskeleton organization	0.8259	3	0.050

Cellular Component

GO:0005840	ribosome	1.4164	59	0.000
GO:1990904	ribonucleoprotein complex	2.4636	61	0.000
GO:0043228	non-membrane-bounded organelle	9.5505	66	0.000
GO:0043229	intracellular organelle	18.5136	55	0.000
GO:0044464	cell part	54.3267	86	0.000
GO:0015934	large ribosomal subunit	0.1437	6	0.000

GO:0015935	small ribosomal subunit	0.1800	6	0.000
GO:0022625	cytosolic large ribosomal subunit	0.0337	2	0.000
GO:0044446	intracellular organelle part	16.1988	30	0.000
GO:0008091	spectrin	0.0562	2	0.001
GO:0030863	cortical cytoskeleton	0.0787	2	0.003
GO:0005746	mitochondrial respiratory chain	0.1462	2	0.009
GO:0042788	polysomal ribosome	0.0112	1	0.011
GO:0019866	organelle inner membrane	1.0237	4	0.019
GO:0035145	exon-exon junction complex	0.0225	1	0.022
GO:0005854	nascent polypeptide-associated complex	0.0225	1	0.022
GO:0070776	MOZ/MORF histone acetyltransferase complex	0.0225	1	0.022
GO:0008250	oligosaccharyltransferase complex	0.0225	1	0.022
GO:0005740	mitochondrial envelope	1.6424	5	0.024
GO:0044444	cytoplasmic part	5.9309	11	0.030
GO:0005791	rough endoplasmic reticulum	0.0337	1	0.033
GO:0005853	eukaryotic translation elongation factor 1 complex	0.0450	1	0.044
GO:0031975	envelope	1.9798	5	0.048

Molecular Function

GO:0003735	structural constituent of ribosome	1.2356	59	0.000
GO:0019843	rRNA binding	0.0974	4	0.000
GO:0003723	RNA binding	5.4608	19	0.000
GO:0048306	calcium-dependent protein binding	0.0196	2	0.000
GO:0004364	glutathione transferase activity	0.0785	2	0.003
GO:0008289	lipid binding	2.3536	7	0.009
GO:0070694	deoxyribonucleoside 5'-monophosphate N-glycosidase activity	0.0098	1	0.010
GO:0004454	ketohexokinase activity	0.0098	1	0.010
GO:0031177	phosphopantetheine binding	0.0098	1	0.010
GO:0016531	copper chaperone activity	0.0098	1	0.010
GO:0003746	translation elongation factor activity	0.1569	2	0.010
GO:0004579	dolichyl-diphosphooligosaccharide-protein glycotransferase activity	0.0196	1	0.020
GO:0032137	guanine/thymine mispair binding	0.0196	1	0.020
GO:0016743	carboxyl- or carbamoyltransferase activity	0.0196	1	0.020
GO:0004128	cytochrome-b5 reductase activity	0.0196	1	0.020
GO:0008097	5S rRNA binding	0.0196	1	0.020
GO:0005200	structural constituent of cytoskeleton	0.2648	2	0.029
GO:0004462	lactoylglutathione lyase activity	0.0294	1	0.029
GO:0018024	histone-lysine N-methyltransferase activity	0.2844	2	0.033
GO:0001784	phosphotyrosine binding	0.0392	1	0.039
GO:0003691	double-stranded telomeric DNA binding	0.0392	1	0.039
GO:0016278	lysine N-methyltransferase activity	0.3236	2	0.041
GO:0051219	phosphoprotein binding	0.0490	1	0.048
GO:0016651	oxidoreductase activity	0.3530	2	0.048

Table S.7. Gene ontology enrichment for transcripts up-regulated between T- and V-treated groups for the 30' time point. Conditional enrichment was obtained with unadjusted $P < 0.05$. Expected count: number of transcripts in each category expected based on the distribution of categories among all transcripts tested. Observed count: number of transcripts conferring the enrichment in each category for each module.

GO ID	Go term	Expected count	Observed Count	p-value
Biological Process				
GO:1902908	regulation of melanosome transport	0.0007	1	0.001
GO:0021767	mammillary body development	0.0015	1	0.001
GO:0005981	regulation of glycogen catabolic process	0.0015	1	0.001
GO:0009251	glucan catabolic process	0.0022	1	0.002
GO:0044247	cellular polysaccharide catabolic process	0.0022	1	0.002
GO:0032881	regulation of polysaccharide metabolic process	0.0022	1	0.002
GO:0043470	regulation of carbohydrate catabolic process	0.0022	1	0.002
GO:0050728	negative regulation of inflammatory response	0.0030	1	0.003
GO:0021884	forebrain neuron development	0.0037	1	0.004
GO:0043467	regulation of generation of precursor metabolites and energy	0.0037	1	0.004
GO:0010906	regulation of glucose metabolic process	0.0060	1	0.006
GO:0021872	forebrain generation of neurons	0.0067	1	0.007
GO:0030212	hyaluronan metabolic process	0.0074	1	0.007
GO:0005977	glycogen metabolic process	0.0082	1	0.008
GO:0021854	hypothalamus development	0.0097	1	0.010
GO:0060341	regulation of cellular localization	0.0134	1	0.013
GO:0051904	pigment granule transport	0.0149	1	0.015
GO:0035845	photoreceptor cell outer segment organization	0.0149	1	0.015
GO:0032401	establishment of melanosome localization	0.0164	1	0.016
GO:0007034	vacuolar transport	0.0179	1	0.018
GO:0042462	eye photoreceptor cell development	0.0194	1	0.019
GO:0030203	glycosaminoglycan metabolic process	0.0201	1	0.020
GO:0051336	regulation of hydrolase activity	0.2398	2	0.023
GO:0043666	regulation of phosphoprotein phosphatase activity	0.0231	1	0.023
GO:0048666	neuron development	0.2450	2	0.023
GO:0033059	cellular pigmentation	0.0253	1	0.025
GO:0031329	regulation of cellular catabolic process	0.0253	1	0.025
GO:0031347	regulation of defense response	0.0261	1	0.026
GO:0032268	regulation of cellular protein metabolic process	0.2606	2	0.026
GO:0035303	regulation of dephosphorylation	0.0268	1	0.026
GO:0030030	cell projection organization	0.2785	2	0.030
GO:0051650	establishment of vesicle localization	0.0328	1	0.032
GO:0044724	single-organism carbohydrate catabolic process	0.0343	1	0.034
GO:0019318	hexose metabolic process	0.0350	1	0.034
GO:0044262	cellular carbohydrate metabolic process	0.0365	1	0.036
GO:0015980	energy derivation by oxidation of organic compounds	0.0432	1	0.042
GO:0046530	photoreceptor cell differentiation	0.0447	1	0.044
GO:0032101	regulation of response to external stimulus	0.0447	1	0.044
GO:0042254	ribosome biogenesis	0.0477	1	0.047
GO:0022008	neurogenesis	0.3589	2	0.047
GO:0021953	central nervous system neuron differentiation	0.0506	1	0.050
Cellular Component				
GO:0016581	NuRD complex	0.0021	1	0.002

GO:0000164	protein phosphatase type 1 complex	0.0055	1	0.005
GO:0017053	transcriptional repressor complex	0.0055	1	0.005
GO:0005871	kinesin complex	0.0062	1	0.006
GO:0005930	axoneme	0.0075	1	0.008
GO:0036064	ciliary basal body	0.0082	1	0.008
GO:0009986	cell surface	0.0089	1	0.009
GO:1903293	phosphatase complex	0.0171	1	0.017
GO:0070603	SWI/SNF superfamily-type complex	0.0329	1	0.032
GO:0044463	cell projection part	0.0342	1	0.034
GO:0005929	cilium	0.0445	1	0.044
Molecular Function				
GO:0008327	methyl-CpG binding	0.0010	1	0.001
GO:0019888	protein phosphatase regulator activity	0.0310	1	0.031
GO:0004867	serine-type endopeptidase inhibitor activity	0.0380	1	0.037

Table S.8. Gene ontology enrichment for transcripts down-regulated between T- and V-treated groups for the 30' time point. Conditional enrichment was obtained with unadjusted $P < 0.05$. Expected count: number of transcripts in each category expected based on the distribution of categories among all transcripts tested. Observed count: number of transcripts conferring the enrichment in each category for each module.

GO ID	Go term	Expected count	Observed Count	p-value
Biological Process				
GO:0070588	calcium ion transmembrane transport	0.2283	4	0.000
GO:0098660	inorganic ion transmembrane transport	1.0900	7	0.000
GO:0051209	release of sequestered calcium ion into cytosol	0.0333	2	0.000
GO:1902656	calcium ion import into cytosol	0.0359	2	0.001
GO:0051282	regulation of sequestering of calcium ion	0.0385	2	0.001
GO:0051238	sequestering of metal ion	0.0385	2	0.001
GO:0006816	calcium ion transport	0.0405	2	0.001
GO:0060401	cytosolic calcium ion transport	0.0410	2	0.001
GO:0007204	positive regulation of cytosolic calcium ion concentration	0.0436	2	0.001
GO:0032845	negative regulation of homeostatic process	0.0436	2	0.001
GO:0055085	transmembrane transport	2.8648	9	0.001
GO:0003010	voluntary skeletal muscle contraction	0.0026	1	0.003
GO:0007274	neuromuscular synaptic transmission	0.0026	1	0.003
GO:0031443	fast-twitch skeletal muscle fiber contraction	0.0026	1	0.003
GO:0006874	cellular calcium ion homeostasis	0.0769	2	0.003
GO:0006811	ion transport	2.1364	7	0.004
GO:0021960	anterior commissure morphogenesis	0.0051	1	0.005
GO:0007263	nitric oxide mediated signal transduction	0.0051	1	0.005
GO:0001778	plasma membrane repair	0.0051	1	0.005
GO:0007624	ultradian rhythm	0.0051	1	0.005
GO:0071678	olfactory bulb axon guidance	0.0051	1	0.005
GO:0072507	divalent inorganic cation homeostasis	0.1077	2	0.005
GO:0035725	sodium ion transmembrane transport	0.1334	2	0.008

GO:0030003	cellular cation homeostasis	0.1411	2	0.009
GO:0050918	positive chemotaxis	0.0103	1	0.010
GO:0006809	nitric oxide biosynthetic process	0.0103	1	0.010
GO:0055082	cellular chemical homeostasis	0.1693	2	0.012
GO:0050881	musculoskeletal movement	0.0128	1	0.013
GO:2001057	reactive nitrogen species metabolic process	0.0128	1	0.013
GO:0033292	T-tubule organization	0.0128	1	0.013
GO:0055065	metal ion homeostasis	0.1795	2	0.014
GO:0016049	cell growth	0.1924	2	0.016
GO:0033564	anterior/posterior axon guidance	0.0180	1	0.018
GO:0038007	netrin-activated signaling pathway	0.0180	1	0.018
GO:0008345	larval locomotory behavior	0.0205	1	0.020
GO:0034599	cellular response to oxidative stress	0.0231	1	0.023
GO:0050801	ion homeostasis	0.2462	2	0.025
GO:0048846	axon extension involved in axon guidance	0.0256	1	0.025
GO:0048668	collateral sprouting	0.0256	1	0.025
GO:0051234	establishment of localization	5.2269	10	0.027
GO:0001966	thigmotaxis	0.0282	1	0.028
GO:0007205	protein kinase C-activating G-protein coupled receptor signaling pathway	0.0308	1	0.030
GO:0007610	behavior	0.2796	2	0.032
GO:0048268	clathrin coat assembly	0.0333	1	0.033
GO:0016358	dendrite development	0.0333	1	0.033
GO:0006941	striated muscle contraction	0.0359	1	0.035
GO:0048644	muscle organ morphogenesis	0.0385	1	0.038
GO:0021955	central nervous system neuron axonogenesis	0.0385	1	0.038
GO:0015672	monovalent inorganic cation transport	0.7592	3	0.039
GO:0072593	reactive oxygen species metabolic process	0.0462	1	0.045
GO:0031103	axon regeneration	0.0462	1	0.045
GO:0036269	swimming behavior	0.0487	1	0.048
GO:0048644	muscle organ morphogenesis	0.0385	1	0.038
GO:0021955	central nervous system neuron axonogenesis	0.0385	1	0.038
GO:0015672	monovalent inorganic cation transport	0.7592	3	0.039
GO:0072593	reactive oxygen species metabolic process	0.0462	1	0.045
GO:0031103	axon regeneration	0.0462	1	0.045
GO:0036269	swimming behavior	0.0487	1	0.048

Cellular Component

GO:0030315	T-tubule	0.0023	1	0.002
GO:0005891	voltage-gated calcium channel complex	0.0728	2	0.002
GO:0030677	ribonuclease P complex	0.0047	1	0.005
GO:0009897	external side of plasma membrane	0.0070	1	0.007
GO:0016459	myosin complex	0.1643	2	0.012
GO:1905348	endonuclease complex	0.0164	1	0.016
GO:0034703	cation channel complex	0.2395	2	0.024
GO:1902495	transmembrane transporter complex	0.3287	2	0.042

Molecular Function

GO:0005219	ryanodine-sensitive calcium-release channel activity	0.0172	2	0.000
GO:0005432	calcium:sodium antiporter activity	0.0201	2	0.000
GO:0022890	inorganic cation transmembrane transporter activity	1.2650	7	0.000
GO:0099604	ligand-gated calcium channel activity	0.0373	2	0.001
GO:0005217	intracellular ligand-gated ion channel activity	0.0459	2	0.001
GO:0015085	calcium ion transmembrane transporter activity	0.0653	2	0.002
GO:0038006	netrin receptor activity involved in chemoattraction	0.0029	1	0.003
GO:0015491	cation:cation antiporter activity	0.0803	2	0.003

GO:0022891	substrate-specific transmembrane transporter activity	2.0969	7	0.004
GO:0004517	nitric-oxide synthase activity	0.0057	1	0.006
GO:0008331	high voltage-gated calcium channel activity	0.0057	1	0.006
GO:0005216	ion channel activity	1.1790	5	0.006
GO:0005509	calcium ion binding	1.7384	6	0.007
GO:0015297	antiporter activity	0.1291	2	0.007
GO:0005262	calcium channel activity	0.1341	2	0.008
GO:0022803	passive transmembrane transporter activity	1.2679	5	0.008
GO:0005515	protein binding	14.0645	22	0.009
GO:0004526	ribonuclease P activity	0.0115	1	0.011
GO:0001784	phosphotyrosine binding	0.0115	1	0.011
GO:0017112	Rab guanyl-nucleotide exchange factor activity	0.0115	1	0.011
GO:0005215	transporter activity	3.2558	8	0.014
GO:0051219	phosphoprotein binding	0.0143	1	0.014
GO:0022836	gated channel activity	0.9782	4	0.016
GO:0005545	1-phosphatidylinositol binding	0.0172	1	0.017
GO:0010181	FMN binding	0.0287	1	0.028
GO:0004143	diacylglycerol kinase activity	0.0344	1	0.034
GO:0003779	actin binding	0.7860	3	0.044

Table S.9. Gene ontology enrichment for transcripts down-regulated between KT- and V-treated groups for the 60' time point. Conditional enrichment was obtained with unadjusted P < 0.05. Expected count: number of transcripts in each category expected based on the distribution of categories among all transcripts tested. Observed count: number of transcripts conferring the enrichment in each category for each module.

GO ID	Go term	Expected count	Observed Count	p-value
Biological Process				
GO:0071805	potassium ion transmembrane transport	0.0130	1	0.013
GO:0006813	potassium ion transport	0.0141	1	0.014
GO:0098655	cation transmembrane transport	0.0346	1	0.035
GO:0098660	inorganic ion transmembrane transport	0.0352	1	0.035
Molecular Function				
GO:0016286	small conductance calcium-activated potassium channel activity	0.0004	1	0.000
GO:0005227	calcium activated cation channel activity	0.0011	1	0.001
GO:0005516	calmodulin binding	0.0026	1	0.003
GO:0005267	potassium channel activity	0.0093	1	0.009
GO:0015077	monovalent inorganic cation transmembrane transporter activity	0.0205	1	0.020
GO:0022836	gated channel activity	0.0227	1	0.023
GO:0046873	metal ion transmembrane transporter activity	0.0236	1	0.024
GO:0005216	ion channel activity	0.0274	1	0.027
GO:0022803	passive transmembrane transporter activity	0.0295	1	0.029
GO:0008324	cation transmembrane transporter activity	0.0339	1	0.034
GO:0022891	substrate-specific transmembrane transporter activity	0.0488	1	0.049

Table S.10. Gene ontology enrichment for transcripts up-regulated between T- and V-treated groups for the 60' time point. Conditional enrichment was obtained with unadjusted P < 0.05. Expected count: number of transcripts in each category expected based on the distribution of categories among all transcripts tested. Observed count: number of transcripts conferring the enrichment in each category for each module.

GO ID	Go term	Expected count	Observed Count	p-value
Molecular Function				
GO:0004114	3'	0.0023	1	0.002
GO:0008081	phosphoric diester hydrolase activity	0.0062	1	0.006
GO:0016788	hydrolase activity	0.0364	1	0.036

Table S.11. Gene ontology enrichment for transcripts down-regulated between T- and V-treated groups for the 60' time point. Conditional enrichment was obtained with unadjusted P < 0.05. Expected count: number of transcripts in each category expected based on the distribution of categories among all transcripts tested. Observed count: number of transcripts conferring the enrichment in each category for each module.

GO ID	Go term	Expected count	Observed Count	p-value
Biological Process				
GO:0070293	renal absorption	0.0005	1	0.000
GO:0071557	histone H3-K27 demethylation	0.0007	1	0.001
GO:0016577	histone demethylation	0.0010	1	0.001
GO:0008214	protein dealkylation	0.0012	1	0.001
GO:0061384	heart trabecula morphogenesis	0.0017	1	0.002
GO:0070988	demethylation	0.0030	1	0.003
GO:0060319	primitive erythrocyte differentiation	0.0035	1	0.003
GO:0071526	semaphorin-plexin signaling pathway	0.0047	1	0.005
GO:0031101	fin regeneration	0.0134	1	0.013
GO:0035162	embryonic hemopoiesis	0.0154	1	0.015
GO:0034101	erythrocyte homeostasis	0.0186	1	0.019
GO:0006898	receptor-mediated endocytosis	0.0199	1	0.020
GO:0048872	homeostasis of number of cells	0.0218	1	0.022
GO:0016569	covalent chromatin modification	0.0256	1	0.025
GO:0031099	regeneration	0.0290	1	0.029
GO:0030099	myeloid cell differentiation	0.0298	1	0.029
GO:0042060	wound healing	0.0365	1	0.036
GO:0048589	developmental growth	0.0477	1	0.047
Molecular Function				
GO:0071558	histone demethylase activity (H3-K27 specific)	0.0006	1	0.001

GO:0032451	demethylase activity	0.0012	1	0.001
GO:0017154	semaphorin receptor activity	0.0038	1	0.004