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The Social Brain: How social stimuli are translated into neuroendocrine signals?

José Miguel Simões

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NOTA PRÉVIA

Para a elaboração da presente dissertação, e nos termos do nº1 Artigo 41, do regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado no Diário da República nº 209, II Série de 30 de Outubro de 2006, foram usados integralmente dois (2) artigos científicos publicados e dois (2) submetidos para publicação em revistas internacionais indexadas. Tendo os trabalhos mencionados sido efectuados em colaboração, o autor da dissertação esclarece que relatimente aos dois primeiros artigos apresentados, nomeadamente Chapter I (i) e (ii), participou na recolha e análise de dados, discussão dos resultados e redação dos manuscriptos. Relativamente aos restantes artigos, o autor esclarece que participou integralmente na sua concepção e execução, incluindo o delineamento das experiências, recolha e análise de dados, interpretação e discussão dos resultados, assim como na redação de todos os manuscriptos.

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Resumo

Na Natureza os animais ajustam as suas exibições comportamentais de acordo com as flutuações diárias no seu ambiente social. Mas de que forma este ambiente social influencia o comportamento exibido pelos indivíduos e que alterações acontecem a nível cerebral decorrentes destas variações no ambiente social? E, por outro lado, quais os mecanismos fisiológicos, moleculares e genéticos na génese destas modificações?

O comportamento social é um traço ubíquo no Reino Animal, sendo que a maioria dos animais vivem (senão na sua totalidade, pelo menos uma parte da sua vida) em ambientes sociais. De forma sucinta, podemos definir o comportamento social como o conjunto de ações decorrente da interação entre dois ou mais indivíduos, mais commumente da mesma espécie. Desta forma, o comportamento social pode ser visto como um fenótipo interativo: pois depende em parte das interações localmente estabelecidas com outros indivíduos para a sua expressão. O estudo do comportamento, também denominado Etologia, teve ao longo da história um papel de destaque, que culminou com a atribuição do Prémio Nobel em Medicina e Fisiologia em 1973, para três distintos cientistas, Konrad Lorenz, Niko Tinbergen e Karl von Frisch, pelos seus trabalhos de excelência na área. Estes contribuíram de forma definitiva para uma maior compreensão das bases do comportamento social, ao focarem a sua investigação em comportamentos familiares ou de grupo, comportamentos agonísticos e de corte, e na comunicação entre indivíduos no seio de um grupo. Para Tinbergen, o estudo biológico do comportamento animal deve integrar um conjunto de abordagens multi-disciplinares devido ao elevado grau de complexidade dos padrões comportamentais exibido pela maior parte das espécies. Desta forma, Tinbergen sugere responder a quatro perguntas mutuamente exclusivas que exploram as explicações proximais e distais para as causas e origens de determinado padrão comportamental (dito de uma forma simplificada: como? e porquê?). As questões proximais prendem-se com o estudo dos mecanismos que permitem o indivíduo executar determinado comportamento, incluindo mecanismos sensoriais e endócrinos que

regulam o comportamento. Como a origem e desenvolvimento destes mecanismos podem ser moldados por experiências com o meio social, ou até mesmo aprendidos através da observação de conspecíficos, é importante também considerar a ontogenia destes mesmos comportamentos. Por outro lado, as questões distais focam-se na evolução dos mecanismos supracitados, mais concretamente tentando perceber a função de determinados comportamentos na sobrevivência do indivíduo, e encontrar explicações para a evolução dessa mesma função através da sua história filogenética.

O estudo do comportamento animal assenta no pressuposto que existe uma flexibilidade comportamental (mais ou menos) extensa e intrínseca a cada indivíduo, de tal forma que o mesmo indíviduo pode expressar comportamentos distintos em resposta a ambientes sociais semelhantes, dependendo unicamente do seu meio interno. A expressão de comportamentos parece então depender da percepção do indivíduo do seu meio social, da sua experiência social prévia e, naturalmente, do seu estado interno. Um indivíduo socialmente apto tem de ser capaz de avaliar corretamente o seu ambiente social, ajustando o seu comportamento de forma a maximizar o rácio entre custos e benefícios de se envolver numa interação, optimizando, desta forma, a sua regulação e distribuição energéticas. A esta capacidade dos indivíduos para alterar o seu comportamento em função da informação disponível acerca do seu ambiente social chamamos competência social. Este conceito, remete-nos novamente para a ideia de flexibilidade comportamental referida no início deste paragráfo. Esta plasticidade nas respostas comportamentais pode também ser adaptativa, auxiliando os indivíduos a lidarem com desafios inerentes à variabilidade, e por vezes imprevisibilidade, do seu ambiente, mas por outro lado pode acarretar custos intrínsecos. Os custos associados a esta plasticidade comportamental são mensuráveis quando consideramos, por exemplo, genótipos com fenótipos equivalentes em dois ambientes distintos, diferindo somente em termos de plasticidade e fitness. Exemplos destes custos prendem-se com a

aquisição de informação do ambiente, ou custos de manutenção de mecanismos sensoriais ou regulatórios, ou finalmente custos de produção fenotípica.

Esta plasticidade comportamental depende, naturalmente, de uma plasticidade dos circuitos neurais subjacentes ao comportamento social, que é conseguida através de uma regulação da expressão génica cerebral. A *Social Behavior Network* (literalmente, a rede (neural) do comportamento social, SBN) integra um conjunto de nódulos neurais responsáveis pela regulação de comportamentos sociais (seja agressão, corte ou comportamento parental). Inicialmente descrita para mamíferos (e posteriormente alargada para aves e peixes), esta rede inclui: o septo lateral, a área pré-óptica, o hipotálamo anterior, o hipotálamo ventromedial, a amígdala medial e a substância cinzenta periaquedutal. Em comum estas áreas partilham 3 aspectos-chave: (1) estão reciprocamente interconectadas, (2) contêm recetores de hormonas gonadais e (3) são ativados, ou têm uma importante função regulatória, em resposta a comportamentos sociais. Esta rede parece codificar a informação de uma forma dinâmica, de tal forma que determinado perfil comportamental parece ser melhor explicado pelo perfil de ativação da rede na sua globalidade, do que pela atividade individual de cada nódulo. Conceptualmente, a pluralidade de combinações de ativação dos diferentes nódulos possível, parece explicar a diversidade de comportamentos exibida entre espécies e até mesmo entre indivíduos.

Os mecanismos neurais subjacentes à plasticidade comportamental podem então atuar de duas formas: (1) provocando alterações estruturais nos circuitos da SBN, o que conduz a mudanças comportamentais que ocorrem lentamente, mas que são dramáticas e duradouras; (2) ou modulando bioquimicamente a atividade nodal, o que provoca alterações comportamentais significativamente mais rápidas, mas transientes e muito mais subtis do que no primeiro caso.

Como foi dito anteriormente, uma consequência direta da ativação destes circuitos neurais é um aumento na expressão génica, que a juzante afeta a integração da informação

social. Estes mecanismos genéticos parecem exercer lentamente um efeito sobre os circuitos neurais existentes, e apesar de não terem um correlato comportamental imediato, estão dependentes das experiências do indivíduo com o meio. Actualmente, considera-se que estes mecanimos dependentes da atividade neuronal devem-se a três processos: (1) fosforilação de proteínas específicas, como o CREB (*cAMP response element-binding*), que regulam determinadas vias de sinalização ou atuam diretamente em *immediate-early genes* (IEG); estes IEG são genes de resposta rápida que são ativados de forma transiente face a um estímulo externo, antes mesmo de existir síntese proteica, e que (2) promovem alterações na expressão de uma cascata de módulos de genes co-regulados no cérebro; finalmente, (3) a transcrição de microRNAs controla a tradução de proteínas sinápticas que, em última instância, modifica o estado neurogenómico do cérebro em resposta aos estímulos sociais iniciais. Em suma, a ativação de IEG, em resposta a um estímulo externo vai orquestrar a integração das respostas genómicas e da informação social disponível, co-regulando conjuntos de genes cuja co-expressão conduz a uma expressão génica comportamentalmente induzida, e que resulta na exibição de diferentes fenótipos sociais.

Deste modo, diferentes estados neurogenómicos emergem em resposta a diferentes estímulos externos, orquestrados por diferentes vias de sinalização na interface entre o ambiente e o genótipo do indivíduo. Naturalmente, estas pistas ambientais são traduzidas em informação biológica relevante, enquanto pistas internas, ou de índole fisiológica, são integradas simultaneamente com a experiência prévia do indivíduo. As exibições comportamentais decorrentes destes processos tendem a ser adaptativas, resultando na evolução de estratégias flexíveis, optimizadas para responder às alterações do ambiente social.

A investigação nesta área do conhecimento tem sofrido avanços metodológicos em anos recentes, com o desenvolvimento de novas técnicas genómicas. Estas permitem um estudo mais aprofundado do impacto das flutuações no ambiente social no genoma de um indivíduo, e têm contribuído de forma definitiva para alimentar o debate do “inato vs. adquirido” (ou

"nature vs. nurture"). A possibilidade de estudar alterações da expressão génica ao nível do genoma completo (ou parcialmente completo), através de análises trancriptómicas, veio cimentar a ideia de um genoma que responde de forma dinâmica aos estímulos externos. Adicionalmente, os genes, o comportamento social e o cérebro parecem também interconectados, de tal forma que o ambiente social influencia a expressão génica a nível cerebral, o que resulta em alterações na expressão de comportamentos; por outro lado, variações genéticas podem alterar a função cerebral e também os comportamentos sociais. Ao integrarmos o estudo destas alterações genéticas a nível genómico, com conceitos de neurobiologia, etologia e biologia evolutiva poderemos compreender melhor o papel da plasticidade nesta interação dinâmica entre os genes e o ambiente que esculpe o nosso comportamento e o nosso cérebro.

Os objectivos desta tese são então: (1) por um lado compreender como estímulos ambientais podem conduzir a variabilidade fenotípica – estudando como a informação social regula a expressão génica em áreas cerebrais relevantes para o comportamento social, que, por sua vez, vão ativar respostas neuroendócrinas que promovem alterações no perfil comportamental dos indivíduos induzidas pelo meio social onde estão inseridos; (2) por outro lado, compreender como estímulos sociais mais simples (como estímulos sensoriais) podem modular padrões de expressão génica em zonas cerebrais específicas para o seu processamento.

Como espécies modelo foram utilizadas duas espécies de teleósteos, um dos *taxa* mais diversos e plásticos entre os vertebrados, com mais de 20,000 espécies descritas, que englobam uma diversidade de estruturas sociais, sistemas de acasalamento e prestação de cuidados parentais única no Reino Animal. Os peixes ósseos oferecem-nos tal riqueza fenotípica, que poderão contribuir de forma definitiva para uma melhor compreensão da função e evolução da variabilidade dos mecanismos proximais (i.e. cérebro, hormonas e genes) envolvidos no comportamento social. Para isto elegemos duas espécies-modelo: o peixe-zebra

(*Danio rerio*) – uma espécie-modelo importante na área da genética, devido à extensa anotação do seu genoma e a possibilidade de utilização de organismos mutantes e transgénicos; e a tilápia de Moçambique (*Oreochromis mossambicus*) – uma espécie de ciclídeo utilizada como modelo em estudos comportamentais e neuroendócrinos, e que apresenta um repertório comportamental invulgarmente extenso e complexo, em que estímulos sensoriais multimodais são frequentemente exibidos em diferentes contextos (como agressão e corte).

No primeiro capítulo desta tese foi catalogado o repertório agonístico do peixe-zebra e estudadas a estrutura e dinâmica deste tipo de interação (Capítulo I.I). De seguida, indivíduos da mesma espécie foram expostos a diferentes interações sociais, cujo desfecho era manipulado (*e.g.* indivíduo vencia ou perdia uma luta), de forma a perceber o impacto destas experiências sociais na modulação da expressão de estados neurogenómicos no cérebro destes peixes (Capítulo I.II). No segundo capítulo, foi desenvolvido um mapa estereotáxico em 3 dimensões, do cérebro de *O. mossambicus* recorrendo a cortes histológicos e imagens de ressonâncias magnéticas (Capítulo II.I). Este trabalho possibilitou a localização de áreas de interesse estudadas na última secção do Capítulo II, onde se caracterizou o efeito da modulação molecular e fisiológica da maquinaria responsável pelo processamento de estímulos olfativos em duas áreas cerebrais (bolbos olfativos e palium olfativo). Para tal foram utilizados odores representativos de diferentes fenótipos sociais apresentados por ambos os sexos nesta espécie, nomeadamente: entre machos dominantes e subordinados e fêmeas antes e após a desova.

Palavras-chave: Comportamento Social, Plasticidade, Cérebro, Genómica, Teleósteos

Abstract

Animals continuously fine-tune the expression of social behaviors according to daily fluctuations on their social environment. But how does the social environment influence brain and behavior and what are the underlying physiologic, molecular and genetic mechanisms? Behavioral flexibility depends on neural plasticity of circuits underlying social behavior, which is achieved by social regulation of brain gene expression. Different neurogenomic states emerge in response to different external stimuli and switches between states are orchestrated by signaling pathways interfacing the social environment and the genotype.

The goal of this thesis is to understand how social environment influences brain genomic transcription: (1) during a complex social interaction in zebrafish and (2) after stimulation with context-specific social olfactory stimuli in the Mozambique tilapia.

Zebrafish, *Danio rerio*, has long been used as a model organism in developmental biology and genetics. Despite of their limited behavioral repertoire, the available genetic tools make it a promising model for the study of social behavior. In contrast, the Mozambique tilapia, *Oreochromis mossambicus*, has a rich behavioral repertoire in which visual and chemical information are conveyed to conspecifics, although having limited brain anatomy information and less genetic tools available.

Our research suggests that the outcome of a single social interaction in zebrafish has consequences for subsequent behavior and significant impact on their brain transcriptome. These responses to social interactions seem to involve cognitive appraisal of stimuli, since the objective structure of the event does not trigger a genomic response but rather the appraisal the individual makes of the event. In tilapia, different chemical social cues not only affect neural activity of the olfactory epithelium but also elicit specific patterns of gene activation in brain areas related to olfactory processing. This reinforces the idea of an extensive transcriptional plasticity of teleost genomes, especially in response to rapid changes in social environment.

Key-words: Social behavior, Plasticity, Brain, Genomics, Teleost.

Para os meus Pais,

Contents

General Introduction

Why Social Behavior?

Social behavior is ubiquitous in nature, as the vast majority of animals live partly (or fully) in social environments (Komdeur, 2010). In broad terms, social behavior is any behavior caused by or affecting another individual, usually of the same species. Thus, it is best described as an interacting phenotype: a phenotype that depends at least in part on interactions with social partners for its expression (Bshary, 2011).

The study of social behavior has been of keen interest throughout history and ethologists such as Konrad Lorenz, Niko Tinbergen and Karl von Frisch, dedicated part of their research to understanding the basis of social behavior, by investigating group and family life, fighting, communication, display behaviors and mating. For Tinbergen the biological study of behavior, known at the time as Ethology, must integrate several different approaches due to its high complexity. To address this, he proposed that we address four mutually exclusive questions exploring both proximate and ultimate explanations for the cause and origin of that behavioral pattern (Tinbergen, 1963), sometimes known as 'how' and 'why' questions. Proximate questions about behavior ponder on how an individual is able to perform a certain activity: what mechanisms within the animal enable it to behave in that way (Tinbergen's Causation, nowadays called Mechanism). The proximate causes of behavior embrace both sensory and endocrine mechanisms that regulate behavior, which can be modified by individual experience. Consequently, it is important to understand how learning modifies a certain behavior and thus the proximate origins of that behavior must be considered as well (Tinbergen's Ontogeny). On the other hand, ultimate questions about behavior focus on why animal species evolve proximate systems that allow them to behave in a certain way? In other words, the ultimate cause of a behavior must help to understand how that behavior helps the

individual to survive and breed (Tinbergen's Survival Value, also known as Function). Again, if we consider the ultimate origin of a given behavioral pattern we have, in essence, to examine its evolutionary history by comparing how that behavior varies across a group of closely related species (Tinbergen's Evolution, or Phylogeny) (Tinbergen, 1963).

Studying social behavior in all these different perspectives could prove as fruitful as time consuming; hence, not surprisingly there is still a lack of integrative studies approaching both the proximate and ultimate causes of social behavior. Nonetheless, this field of research has seen some breakthroughs, in recent years, since the pioneering papers of Hamilton (Hamilton, 1964) and Maynard Smith and Price (1973), and the landmark syntheses of Wilson (1975) and Trivers (1985) on sociobiology and social evolution.

The study of social behavior has thrived despite of not always following these theoretical constructs. The staggering diversity and beautiful complexity of repertoires exhibited across taxa in the natural world has always intrigued and fascinated scientists. The observation of naturally behaving animals and their unusual complex social lives nurtured new theories to explain several social phenomena and also social evolution. For instances, due to its particular characteristics, social behavior was used as a rationale to explain the degree of cephalization within primate species: according to this theory, species living in social groups with more frequent and complex interactions should present larger brains than species living in partial or total social isolation – the argument being that different primate species can have bigger brains not only to cope with the demands of living in social groups but also to socially maneuver and manipulate other individuals (Byrne and Whiten, 1989). In fact, primate social systems are seemingly more complex than those of other species and can involve processes such as tactical deception and coalition-formation, which are rare or occur only in simpler forms in other taxonomic groups (Dunbar, 1998). This hypothesis was in its early stages dubbed the Machiavellian Intelligence hypothesis (Byrne and Whiten, 1989), a name that was

later replaced by: the Social Brain hypothesis (SBH) (Dunbar, 1998). Although this proposal was initially focused on primates, since then several scientists have been attempting to generalize it to all vertebrate taxa, as an explanation for brain evolution, with rather inconclusive results. Recent analyses suggest that it takes a very different form in other mammals and birds than it does in anthropoid primates (Dunbar and Shultz, 2007). In primates there is an apparent quantitative relationship between brain size and social group size, *i.e.*, the number of potential dyadic relationships (interpreted as one index of social complexity) is proportional to group size and correlates with brain size. In other taxonomic groups of social mammals and birds, group size does not consistently correlate with brain size (*e.g.* Beauchamp & Fernández-Juricic (2004); Shultz & Dunbar (2007)) rather taking a more qualitative relationship: pairbonded species, especially those living in lasting (if not lifelong) monogamous relationships present the largest brains when phylogenetic, life history and ecological variables are ruled out (Dunbar and Shultz, 2007). Nonetheless, this still reflects the continuous effort linked with cognitive demands behind behavioral coordination and synchrony necessary to maintain stable pairbonded relationships (Shultz and Dunbar, 2007). A broader interpretation of the SBH theory is that socially living individuals face cognitive demands that individuals living in isolation do not, and to maintain group cohesion there is a need to coordinate between the individual and the group requirements (Dunbar and Shultz, 2007). Thus, ecological problems are solved socially and the need for mechanisms that enhance social cohesion drive brain size evolution.

In this theoretical construct, relationships (especially long lasting ones) are still cognitively costly and carry functional demands in terms of fitness, which can arise from poor mate choice decisions and more immediately from behavioral coordination for example (Dunbar, 2009). In this regard, the topic of niche construction should not be overlooked in terms of fitness consequences. Niche construction is the process whereby an animal through its daily routine, activities, choices, metabolism, etc., modifies its own and/or other ecological

niches (Laland and Sterelny, 2006). The most remarkable characteristic of niche construction is not the organism-driven modification to its environment but rather the modification of the relationship between an organism and its relative niche, a trait that reinforces the active and dynamic role of the organism driving evolutionary and co-evolutionary events (Laland and Sterelny, 2006). Applying this idea to social behavior, Bergmüller & Taborsky (2010) suggest that niche specialization within a social group (social niche specialization) can have a rather decisive influence on the way an individual behaves. The authors speculate that behavioral responses to a given stimuli within a group are not all alike since individuals tend to adopt a consistent behavioral traits, when compared to other individuals in a group, which results from adopting a particular social role. This conceptual framework offers an interesting explanation about the evolution of animal personality differences between individuals (in a social context) emerging from the dynamic effects of social interactions: behavioral consistency avoids conflicts deriving from niche overlap (resource-wise, for example), thus choosing behavioral strategies that reduce conflict with other members has consequences on their Darwinian fitness (Bergmüller and Taborsky, 2010).

In summary, unlike the interactions with the physical environment, social behavior is a special case of interplay between the genome and the environment, where the conspecifics represent an environmental factor that can influence and modify the organisms' gene expression and subsequent behaviors. In recent years, several researchers have focused on this interaction between the organisms' behavior and their environment. In fact, the most exciting studies of proximate influences on behavior examine the interactions between the genome, development and the environment (Bshary, 2011). These findings have reignited the spark on the everlasting nature versus nurture debate by emphasizing the role of the environment in shaping not only behavioral traits but also activating an array of molecular processes which could ultimately lead to individual genomic adjustments.

Social Plasticity

In social species, individuals are expected to fine-tune their behavior according to their social context and previous social experiment. A socially apt individual optimizes energy allocation by correctly adjusting its social behavior and maximizing the ratio between benefits *vs.* costs involved in engaging in social interactions (Oliveira, 2009). There are numerous examples of these social performance attributes, where individuals extract social information from the environment (by eavesdropping, for example) in order to efficiently adjust their own behavior to the behavior of the group (*e.g.* dear-enemy and audience effects: (Aires et al., 2004; Doutrelant et al., 2001; Oliveira et al., 1998), bystander effect: (Oliveira et al., 2001); and winner-loser effect: (Oliveira et al., 2009)). This ability is often denominated as social competence and is considered a performance trait that has an impact on the Darwinian fitness of the individual (Oliveira, 2009). In other words, social competence can be defined as the ability of an individual in a given social context to optimize its behavioral exhibits as a function of the information given by said social environment (Taborsky and Oliveira, 2012). This concept suggests a continuous interaction between the individual and the social environment, in which the individual's behavioral output is dependent on its perception of the social context, its previous social experience and its internal state. Consequently, underlying the notion of social competence is behavioral plasticity, which enables the same individual to exhibit different behavioral elements in response to the same social stimulus, based only on their internal state (Oliveira, 2009). It has been shown that these plastic responses can be adaptive, by allowing the individuals to cope with the challenges of a variable environment, but similarly they carry costs when comparing to the constitutive expression of the trait (Pigliucci, 2001; Pigliucci, 2005). Plasticity costs are evaluated by comparing the fitness of genotypes with equivalent phenotypes, but differing in plasticity and fitness, within two environments (Callahan et al., 2008). Some examples of plasticity costs may include: information acquisition costs – naturally the process of being plastic entails an information acquisition cost (collected from the environment) (Sih, 1992); maintenance costs – if a plastic organism requires the maintenance

of sensory and regulatory machinery that fixed development does not require (Dewitt et al., 1998; Tienderen, 1991); production costs – when the cost of producing a given phenotype is greater for plastic genotypes than for fixed genotypes producing the same phenotype (Dewitt et al., 1998).

Behavioral plasticity can be better illustrated using the concept of "reaction norm" (RN), which is the representation of a given phenotypic trait value in relation to an environmental continuum (Fig. 1). In other words, plasticity can be described as a function describing how a given phenotype (or behavior) changes over an environmental gradient within a single individual, which is characterized by the trait elevation (average level of behavior) and slope (behavioral plasticity) (Dingemanse and Wolf, 2010).

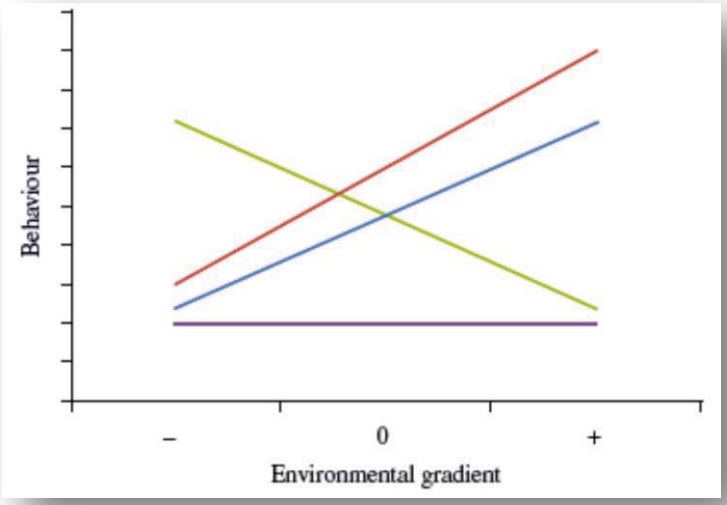


Figure 1 – Behavioral reaction norms of four different individuals to the same environmental gradient. An example could be the variation in the frequency of grooming behavior with social hierarchy. Subject 1 does not seem to modulate its behavior according to social hierarchy, whereas subject 2 and 3 increase and subject 4 decreases the frequency of grooming when in higher social ranks. The absolute value of the slope of each line represents the individual behavioral plasticity (subject 1 is less plastic than the remainder subjects). Differences in the trait mean represent different behavioral profiles (subject 1 grooms less than the others).

(in: Oliveira 2012)

At the population level, it is easy to imagine individuals with different RN, but where the average individual exhibits limited behavioral plasticity, since it does not express the full range of behavioral trait values present in the population (Dingemanse et al., 2010). Genotypes or individuals present behavioral plasticity if their RN is non-horizontal. The advantage of using the concept of RN to study plasticity is that it incorporates information on how an animal behaves on average and how its behavior is modulated over an environmental gradient, identifying the precise form of the relationship between response value and environmental condition (Dingemanse et al., 2010).

These notions suggest that a single genotype can be modulated by the social environment, resulting in particular phenotypes. But how does a single genome orchestrate complex forms of behavior? And what is the role of the social environment on this behavioral regulation? Behavior is the organism's first response to environmental fluctuations, which often results in gene expression changes derived from said behavioral interactions. This genetic modulation, acting at both the physiological and evolutionary time scales, might provide a possible mechanism for how behavioral plasticity might drive rapid behavioral evolution through changes in gene regulation (Bell and Robinson, 2011).

Recently, methodological advances have contributed to this area, with the development of several genomic approaches, which provide the tools to study the impact of fluctuations of the social environmental gradient on the individual's genome, providing new lines of debate on the contribution of "nature *vs*. nurture" (Hofmann, 2003). The possibility of studying genome-wide gene expression changes, using transcriptomic analyses, has already shown that the genome responds dynamically to external stimuli (Robinson et al., 2008). Reinforcing this idea is the notion that genes, social behavior and the brain seem to be interconnected: as the social environment influence gene expression at the brain level, which results in changes in the behavioral output; but also that genetic variations alter brain function and social behavior

(Robinson et al., 2008). By integrating this genomic-wide gene approach, with concepts of neurobiology, ethology and evolutionary biology it is possible to better understand the role of plasticity on the dynamic interaction between genes and the environment in sculpting brain and behavior (Hofmann, 2003). On this note, Oliveira (2012) suggested an integrative framework embracing the proximate mechanisms (gene modules, hormones and neural circuits) and the ultimate (evolutionary) consequences of social plasticity. As discussed previously, for the social context of an individual to promote changes in its behavior, the neural network underlying social behavior must exhibit the capacity for neural plasticity in order to explain different behavioral outputs, depending on the motivational state of the animal and its previous experience, in response to the same inputs (Oliveira, 2009). The neural mechanisms underlying this behavioral plasticity can be categorized depending on the time scale in which they operate: slow and long lasting motivational changes with dramatic behavioral consequences are usually due to a structural rewiring of neural circuits; whereas fast and transient motivational variations, which reflect smooth changes in the expression of behaviors, are usually explained by modulation of existing neural networks via biochemically switching different nodes (Zupanc and Lamprecht, 2000). Knowledge regarding these proximate mechanisms on the basis of social plasticity seems fundamental to comprehend its costs, limits and evolutionary consequences and their contribution to the dynamics of selection (Oliveira, 2012).

Neural circuits of Social Behavior and Plasticity

The notion of a changing social environment and consequent behavioral modulation, suggests that the neural networks underlying social behavior must also be plastic (Hofmann, 2003). Newman (1999), proposed an interconnected network of limbic areas that collectively regulates all social behaviors in mammals: the Social Behavior Network (SBN). The nodes that comprise this network regulate multiple forms of social behavior (*e.g.* aggression, courtship, parental behavior, etc.) and, in mammals, include (Fig.2): the extended medial amygdala (meAMY), the lateral septum (LS), the preoptic area (POA), the anterior hypothalamus (AH), the ventromedial hypothalamus (VMH) and the periaqueductal gray (PAG/GC) (Newman, 1999). These nodes fulfill three key criteria: (1) each one is reciprocally interconnected with all of the others; (2) they are all populated with neurons that contain gonadal hormone receptors; (3) each has been identified as being activated or to have an important regulatory function in more than one social behavior (Newman, 1999).

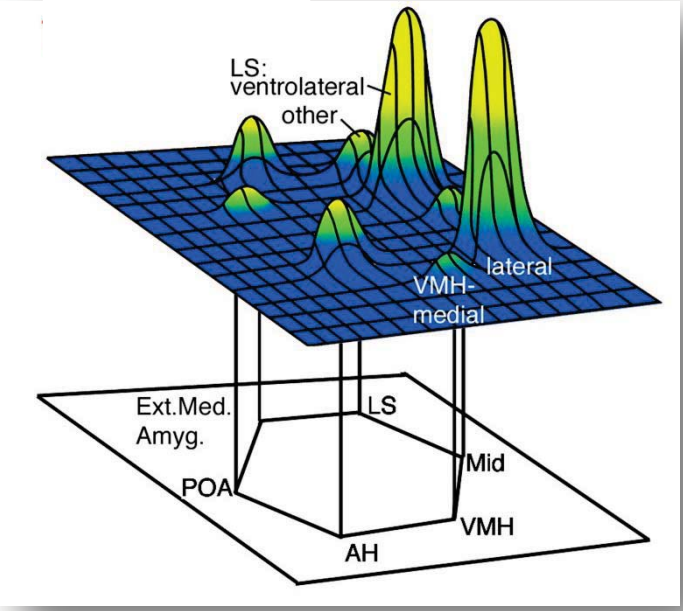


Figure 2 – Schematic representation of immediate early gene responses within the social behavior network following exposure to a same-sex conspecific in a songbird species.

(Adapted from: Goodson 2005)

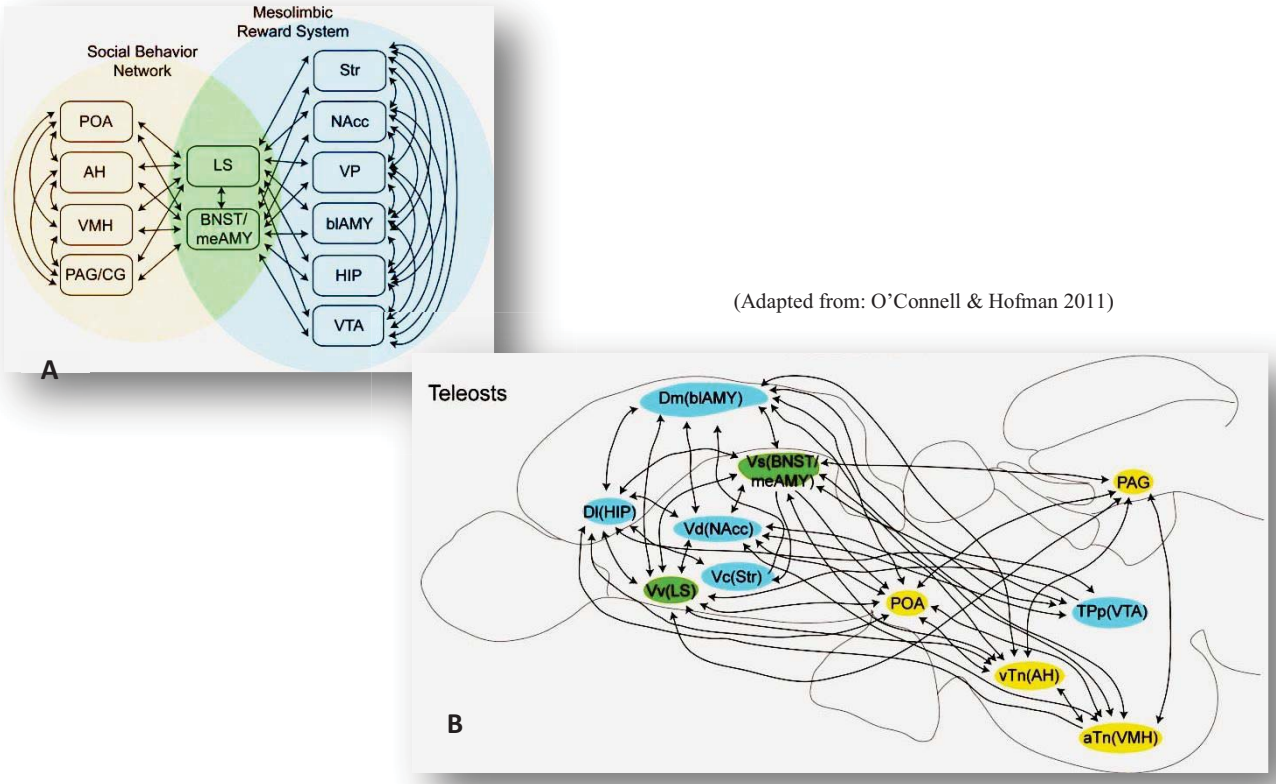
The brain circuitry regulating social behavior in non-mammalian vertebrates is extensively similar to those in mammals, as well as, other hodological features and

neuropeptide distributions which are likewise very similar across taxa (Goodson, 2005; O'Connell and Hofmann, 2011; O'Connell and Hofmann, 2012a). These observations strongly suggested that the SBN might be an evolutionarily conserved feature of the vertebrate brain and Goodson (2005) described the homologous of the mammals' SBN in the basal forebrain and midbrain of birds and teleost fish by using neuronal markers, proxies of neural activation such as Immediate-Early Genes, to measure brain expression of different social behaviors (Fig. 3), widening the scope of experiments on phenotypic variation to include the extraordinary social diversity of teleost fish and songbirds.

Reproductive behavior is a typical example within the SBN framework due to extensive work on the influence of sex steroids on brain and behavior. For instances, in rodents the POA has a central role on male sexual behavior (Hull and Dominguez, 2006), while in females the lordosis circuit seems to be regulated by the VMH (Malsbury et al., 1977). Additionally, in male rats, lesions of the LS facilitate male sexual behavior while inhibiting female sexual behavior (Kondo et al., 1990). Besides sexual behavior, also some aspects of parental care seem to be regulated by nodes of this network, since lesioning the AH in female rats facilitates maternal behavior (Bridges et al., 1999). Other nodes, like the BNST, are involved with not only reproductive behavior but also with reinforcing adaptive behaviors by mediating motivational behavior (Delfs et al., 2000). Finally, social stimuli are also processed and integrated in parts of the network: the amygdalar complex, which includes the meAMY, is involved with sensory integration, especially social odor recognition while the PAG/CG plays a role in the expression of species-specific behavior (Bharati and Goodson, 2006; Mos et al., 1982), as well as, in the context of vocal communication (O'Connell and Hofmann, 2011).

This network apparently encodes information dynamically, in such a way that is the overall profile of activation across different loci that best characterizes a given behavioral pattern, rather than the activation of a single node (Oliveira, 2012). Considering this attribute

of the SBN, it is possible to hypothesize that different combinations of node activation can give rise to an almost infinite repertoire of behaviors and explain some of the behavioral plasticity between individuals (personality) and species: at the individual level this can be due to temporary variation in node activation weights; at the intraspecific level, genetic and epigenetic differences can accommodate different social phenotypes; at the interspecific level, evolution can promote fluctuation on weights between nodes (Goodson and Kabelik, 2009).



(Adapted from: O'Connell & Hofman 2011)

Figure 4 – The social decision-making network.
A: Interactive nodes of the networks regulating social decision-making: the social behavior network (left) and mesolimbic reward system (right)
B: Sagittal view of a teleost brain highlighting the connectivity between nodes of the social decision-making circuit

Mammals			**Teleosts**
anterior hypothalamus	**AH**	**vTn**	ventral tuberal region
basolateral amygdala	**blAMY**	**Dm**	medial part of the dorsal telencephalon
bed nucleus of the stria terminalis/medial amygdala	**BNST/meAMY**	**Vs**	supracommissural part of the ventral pallium
hippocampus	**HIP**	**Dl**	lateral part of the dorsal telencephalon
lateral septum	**LS**	**Vv**	ventral part of the ventral telencephalon
nucleus accumbens	**NAcc**	**Vd**	dorsal part of the ventral telencephalon
periaqueductal gray/central gray	**PAG/CG**	**PAG/CG**	periaqueductal gray/central gray
preoptic area	**POA**	**POA**	preoptic area
striatum	**Str**	**Vc**	central part of the ventral telencephalon
ventromedial hypothalamus	**VMH**	**aTn**	anterior tuberal nucleus
ventral pallidum	**VP**	**?**	?
ventral tegmental area	**VTA**	**TPp**	posterior tuberculum

However, to be adaptive, social behavior must be reinforcing (or rewarding) in some way. Additionally, social decision-making requires stimulus salience to be evaluated prior to an adaptive behavioral response can be carried out (O'Connell and Hofmann, 2011).

Recent literature, strengthened the idea that the reward system, which includes the midbrain dopaminergic system, is the neural circuit responsible for evaluating the salience of an external stimuli, regulating appetitive behavior (Wickens et al., 2007). O'Connell and Hofmann (2011) proposed integrating the SBN, which in conjunction with sex steroids and neuropeptide hormones regulates social behavior, and the mesolimbic reward system, which evaluates stimuli salience via dopaminergic signaling, in a social decision-making network (Fig. 4). This larger framework is intimately concerned with regulating and implementing adaptive behavioral outputs in response to salient environmental challenges and opportunities and is extremely conserved across vertebrates, which suggests that the diversity of social behavior in this taxon might be explained by variations on a conserved neural and gene expression network (O'Connell and Hofmann, 2012b). Nevertheless, some authors consider that this expanded model is still lacking on sufficient data for non-mammalian species in terms of a few of the homologies proposed and also some of the SDM components lack supporting social behavior data in amphibians, reptiles and fish (Goodson and Kingsbury, 2013).

Hormone and neuromodulator receptors are expressed in all nodes of these neural networks (Caldwell and Young, 2006; Goodson, 2005; Munchrath and Hofmann, 2010; Skuse and Gallagher, 2009), allowing a local regulation of their activity by endocrine and neuromodulatory means. The two major classes of neuromodulators acting upon social behavior are monoamines and neuropeptides. Catecholamines, such as epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine, are produced by the hydroxylation and decarboxylation of tyrosine and are usually released in the peripheral and central nervous systems during stress (fight-or-flight response). Other monoamines, like

serotonin, are known modulators of motivation and aggression in both vertebrates and invertebrates, incrementing the relevance of their fundamental role as an evolutionary ancient signaling mechanism between motivational states (Kravitz, 2000). Nonapeptides from the arginine vasotocin family (AVT/AVP system – vasopressin is the neurohypophysial hormone found in most mammals) are regulated by gonadal steroids and have been also documented as regulators of social behavior in vertebrates (Goodson and Bass, 2001). In addition, some steroid hormones, like estrogen, have been proposed to have a direct nongenomic effect on neuronal activity since their production can be modulated by calcium-dependent phosphorylation in presynaptic terminals by the aromatization of testosterone (Balthazart et al., 2006). In fact, steroid hormones play a major role as indicators of internal states and are known to respond to social challenges in many species (Hirschenhauser and Oliveira, 2006; Oliveira, 2004) and regulate mechanisms of behavioral plasticity. Reinforcing the importance of these molecules on behavioral plasticity, steroid receptors are present in the different nodes of the SBN, suggesting their role as neuromodulators (Oliveira, 2009).

Biochemical switching mechanisms are known to be able to modulate the response of a given neural network under similar stimulation regimes. This phenomenon is commonly due to the interaction of neuroactive molecules with specific neural circuits, acting on its functional properties and resulting in either excitatory or inhibitory states (Oliveira, 2009). These molecules typically do not modulate behavior directly and are rather responsible for tuning ongoing neural activity in order to stimulate behavior exhibition adapted to a specific context (Libersat and Pflueger, 2004). In other words, information seems to be processed in the Central Nervous System in two different time frames. In a shorter time frame, at the scale of seconds or even milliseconds, action potentials are generated (or not) based on post-synaptic integration of excitatory and inhibitory potentials generated in response to an external stimulus. The sum of this neuronal activity results in an immediate behavioral response to that given stimulus.

Genetic mechanisms of Social Behavior and Plasticity

A direct consequence of the activation of specific neural circuits is a burst in gene expression, which in a larger time frame (between minutes [in the case of mRNA] to hours [in the case of proteins]) will also affect neural integration of information (Fig. 5). Unlike in the latter case, the effects of these genetic mechanisms are not translated in an immediate behavioral correlate but rather in a slow modification of the existent neural circuitry in an experience-dependent fashion (e.g. MAPK cascade; Sweatt, 2004; Thomas and Huganir, 2004). These socially-driven neuroplasticity biochemical mechanisms are usually due to 3 neuronal-activity dependent processes (Aubin-Horth and Renn, 2009; Oliveira, 2012; Wolf and Linden, 2012): (1) specific proteins, like cAMP response element-binding (CREB) are activated (through phosphorylation) and either regulate intracellular signaling pathways or act directly on immediate-early genes (IEG), which are activated transiently and rapidly in response to external stimuli, before any new proteins are synthesized and can encode other transcription factors or synaptic proteins; (2) the activation of IEG promotes the change in expression of a cascade of co-regulated gene modules in the brain; and finally (3) the transcription of microRNAs regulate the translation of synaptic proteins that will ultimately modify the neurogenomic state of the brain in response to the initial stimuli. To sum up, IEG activation following an external stimuli orchestrate integrated genomic responses to social information, co-regulating gene sets, which co-expression leads to behaviorally driven gene expression that results in the exhibition of different social phenotypes (Oliveira, 2012).

Environmental cues processed by the nervous system are translated into relevant biological information whereas internal physiological cues and the individual's prior experience are simultaneously integrated. As discussed previously, behavioral actions resulting from this process tend to be adaptive, resulting in the evolution of flexible strategies optimized to respond to the social environment.

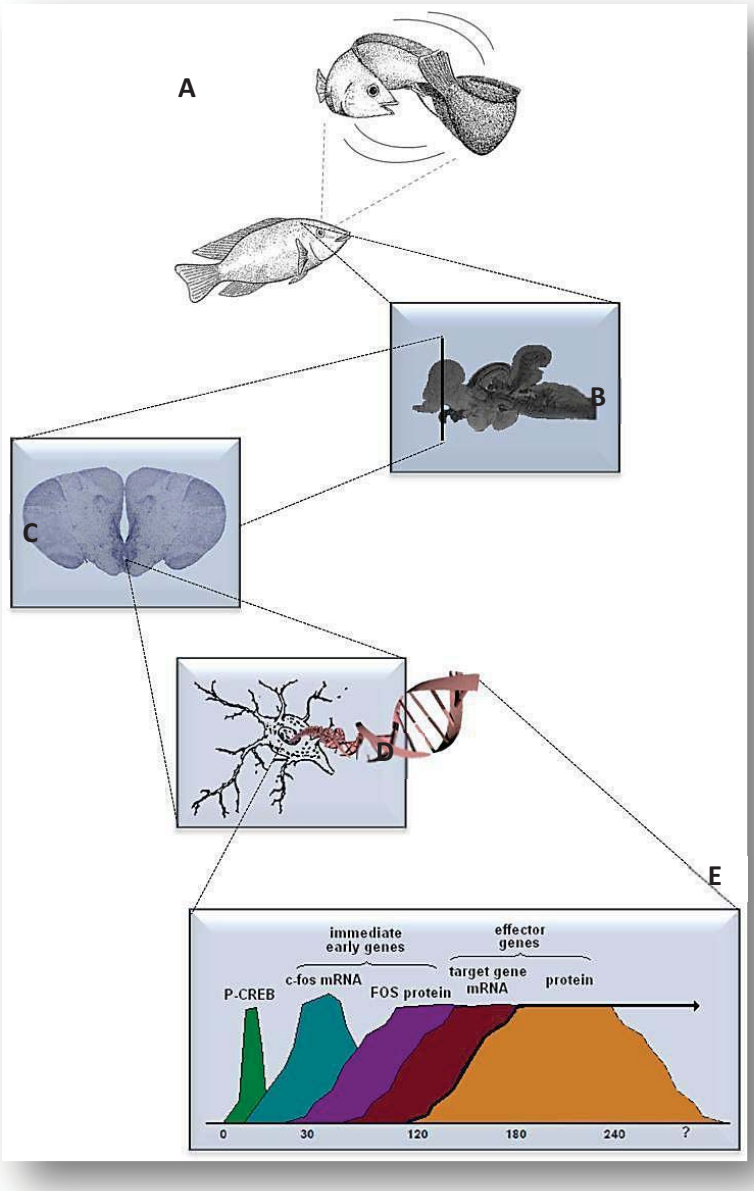


Figure 5 – Social plasticity mechanisms:
A: social animals modulate their behavior based on previous social experiences or by observing others,
B: valence and salience of this information is encoded in specific neural networks;
C: in the nodes (or nuclei) of this network,
D: gene expression profiles are affected in response to such external changes (generating new neurogenomic states)
E: which is prompted by the activation of neuronal activity-regulated transcription factors (e.g. p-CREB) which activates a molecular cascade, including the regulation of IEG and effector genes

(in: Oliveira 2012)

Recently, numerous papers in several different taxa have described the influence of social environment on the structure and activity of the genome (Robinson et al., 2008). In the honey bee (*Apis mellifera*), data shows that caste differentiation (between workers/queen), a key feature in social insects, is shaped by heritable traits but also by fluctuations in the regulation of molecular pathways linked to several life-history traits: such as metabolism, nutrition, and reproduction (Evans and Wheeler, 2001; Smith et al., 2008). Similarly, aggression-related genes in this species are under both inherited and environmental influences, which can vary with age, exposure to alarm-cues and the colony environment

(Alaux et al., 2009). In teleosts, the study of gene expression signatures of life history transitions has also been a focus of interest: in salmonids, alternative life history traits have also been studied profusely and a number of studies show alterations in brain expression profiles dependent of alternative reproductive and migratory tactics (Aubin-Horth and Renn, 2009; Aubin-Horth et al., 2005b) and their interaction with the rearing environment (Aubin-Horth et al., 2005a). All this data on the impact of the social environment on the genome unravels new possibilities concerning how adaptive behavior may evolve.

Objectives and thesis structure

This thesis has as two major goals: (1) on one hand to study how environmental inputs may produce phenotypic variation, by studying how social information regulates gene expression in brain areas relevant for social behavior that in turn activate integrated neuroendocrine responses that promote socially driven changes in behavioral profiles; and (2) on the other to understand how discrete social stimuli modulate patterns of gene expression across context-specific brain areas within short time scales.

Teleost fish are the most diverse and plastic taxa in terms of social behavior among vertebrates. This taxon includes more than 24,000 described species exhibiting diverse types of social organization, mating systems and parental care types (Helfman et al., 1997), offering unique opportunities to study both the evolution and the function of the variation in proximate mechanisms (i.e. brains, hormones, genes) involved in social behavior. To accomplish our objectives we chose two model species of teleost: the zebrafish and the Mozambique tilapia. The zebrafish is an established genetic model system with an extensive genome annotation database and several other genetic tools available (such as mutants and transgenic organisms). Nonetheless, behavioral data for this species was limited and behavioral paradigms rarely used outside the context of addiction (Echevarria, 2010; Gerlai et al., 2000). On the other hand, the Mozambique tilapia has been used as a model organism in behavioral and neuroendocrine studies and presents an extensive behavioral repertoire with multimodal signals. Due to the elevated number of social stimuli and the social unpredictability of context-based behavior in a semi-natural setting (aquaria) with freely behaving animals we decided to use a novel approach, based on olfactory stimulation, which allowed for a finer control and measure of socially-driven responses in the brain of the Mozambique tilapia. On the down side, information regarding the brain anatomy on this species was scarce.

In a first set of experiments (Chapter I.I), we characterized the behavioral repertoire of the zebrafish, *Danio rerio* during agonistic interactions and studied the structure and dynamics

of these interactions. Subsequently (Chapter I.II), the animals were exposed to different types of social interactions, in order to manipulate social experience (winning vs. losing a staged fight) and assess how these different social experiences modulated the expression of different neurogenomic states in the brain using the zebrafish.

Secondly, we used the African cichlid fish *Oreochromis mossambicus* because of its complex social behavior that can be replicated in semi-naturalistic conditions in the lab, and includes visual and chemical communication. Despite their rich behavioral repertoire, only partial information relative to their brain anatomy was available, thus we developed a three-dimensional stereotaxic atlas of the brain of the Mozambique tilapia using MRI combined with a histological map as a guiding reference to label smaller brain nuclei (Chapter II.I). Finally, we designed an experiment to understand how different social contexts regulate the molecular and physiological machinery operating in specific brain areas (the olfactory bulb and the olfactory pallium) (Chapter II.II). The goal of the present study was then to investigate how this highly complex social environment can affect gene expression at the brain level.

Model Species

The Zebrafish

Zebrafish (*Danio rerio*, Fig. 6) has been widely used as a model organism in developmental biology and genetics and in recent years has also been emerging as a new neurobehavioral model (Gerlai, 2003; Grunwald and Eisen, 2002; Guo, 2004). The success of zebrafish in biomedical research is related to their combination of advantages when compared to other already established model systems. When compared to classic invertebrate genetic model organisms such as *Drosophila melanogaster*, or *Caenorhabditis elegans*, zebrafish is a vertebrate and therefore are more closely related to humans. On the other hand, when compared to other vertebrate models (*e.g.*, rodents, anurans or songbirds) they are much smaller (adults are 3–4 cm long), have a short inter-generation time (3 months), and breed in large numbers (hundreds of embryos/female/week), and therefore a large number of animals can be easily maintained in a relatively small space, which is a prerequisite for large-scale biomedical research. Moreover, zebrafish have transparent embryos that develop externally allowing for observation of different structures and systems during development and for early genetic manipulation.

Figure 6 – The Zebrafish, *Danio rerio*.
Female on top and male on the bottom (Adapted from: http://www.aquapage.eu)

Finally, mutations in zebrafish produce phenotypes that copy many human disorders and several genes are being identified that are evolutionarily conserved and have homologs in mammals, including humans (Barbazuk et al., 2000; Lieschke and Currie, 2007; Woods et al., 2000). Recent studies in zebrafish combining molecular genetics with behavioral analyses have allowed the identification of genes involved in neuronal circuits underlying specific behaviors and mechanisms involved in neuropathogenesis (Guo, 2004; Sison et al., 2006). Complex behaviors that are goal directed (e.g., escape from predators) or emotion-related (e.g., aggression, anxiety, and fear) have also started to be characterized in adult zebrafish, and the first results suggest conserved regulatory mechanisms with mammals (Norton and Bally-Cuif, 2010), including shared modulatory neurotransmitter systems (Panula et al., 2006) and homologous brain areas (Wullimann and Mueller, 2004).

The Mozambique Tilapia

The African cichlid fish, *Oreochromis mossambicus* (Fig. 7), is an established model system to study neuroendocrine mechanisms underlying socially mediated behavioral changes. The Mozambique tilapia is a maternal mouth-brooder cichlid displaying a lek-breeding system, with a highly complex social repertoire which includes multimodal signals such as: visual (e.g. Baerends and Baerends-Van Roon, 1950) acoustic (Amorim et al., 2003) and chemical signals (Barata et al., 2007). Depending on the social environment, males can exhibit two distinct behavioral phenotypes: territorial (T) and non-territorial (NT). T individuals adopt darker colorations and establish breeding territories, digging nests on sandy bottoms to where they attract and actively court mates (Oliveira and Almada, 1996; Oliveira and Almada, 1998a). On the other hand, NT males present lighter colorations and are non-territorial, often shoaling with females, while they wait for their opportunity for social ascension. In this species, changes between these behavioral phenotypes have been shown to activate a cascade of molecular processes and a variety of biological pathways which include neuropeptides and

steroid hormones (*e.g.* Oliveira et al., 1996; Almeida et al., 2012; Oliveira and Canário, 2000). In addition, it is known that Mozambique tilapia T males are able to store urine in their bladders, in contrast to NT, and modulate their rate of urination depending on the social environment. An increase in this rate can be seen during agonistic encounters (Barata et al., 2007) or in the presence of pre-ovulatory (PRE) females (Barata et al., 2008). On the other hand, the olfactory potency of the urine measured with electro-olfactogram is different between T and NT males (Barata et al., 2008) and PRE and post-ovulatory females (Miranda et al., 2005).

Figure 7 – The Mozambique tilapia, *Oreochromis mossambicus*. Female on the left-hand side and male on the right (Adapted from: http://www.aquapage.eu)

Females also express a high degree of behavioral plasticity involving transitions between life-history stages, namely during the mouthbrooding phase (Oliveira and Almada, 1998b). Sexually active females visit breeding areas and follow courting males to their nests, engage in courtship rituals, stimulating the male genital papillae and collect their released sperm to ensure the fertilization of the eggs inside the mouth. After spawning, females leave the lek and live in isolation in shallow waters for 20-22 days while they mouthbrood the eggs and care for

the fry (Bruton and Boltt, 1975; Fryer and Iles, 1972). While mouthbrooding, females postpone their next ovulation until the fry are released. During this period, females become also more aggressive, defending the brood against predators and conspecifics (Oliveira and Almada, 1998b).

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Chapter I – How social behavior influences whole brain transcriptome of the zebrafish?

(i) Fighting Zebrafish: Characterization of Aggressive Behavior and Winner–Loser Effects

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Fighting zebrafish: characterization of aggressive behavior and winner-loser effects

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Abstract

Aggression is a key component of the behavioral repertoire of animals that impacts on their Darwinian fitness. The available genetic tools in zebrafish make this species a promising vertebrate neurogenetic model for the study of neural circuits underlying aggressive behavior. For this purpose, a detailed characterization of the aggressive behavior and its behavioral consequences is first needed. In this paper we establish a simple protocol that reliably elicits the expression of fighting behavior in zebrafish dyads and characterized it. The agonistic behavior expressed during dyadic fighting behavior has a temporal structure, indicating the existence of an underlying architecture prone to genetic manipulation. Social interactions have consequences for subsequent behavior with a potential fitness impact, which stresses the validity of this species for the study of aggression. These effects of experience seem to be mediated by different mechanisms in winners and losers. Winners increase the probability of winning subsquent fights without changing their fighting behavior, suggesting the existence of social status cues. On the other hand, losers decrease the probability of winning subsequent fights by decreasing their motivation to escalate fights. Together these results are a first step to the development of a quantitative framework for the study of aggressive behavior in zebrafish.

Introduction

Zebrafish (*Danio rerio*) has been widely used as a model organism in developmental biology and genetics and in recent years has also been emerging as a new neurobehavioral model [1-3]. The success of zebrafish in biomedical research is related to their combination of advantages when compared to other already established model systems. When compared to classic invertebrate genetic model organisms such as *Drosophila melanogaster* or *Caenorhabditis elegans*, zebrafish is a vertebrate and therefore are more closely related to humans. On the other hand, when compared to other vertebrate models (i.e. rodents) they are much smaller (adults are 3–4 cm long), have a short generation time (3 months), and breed in large numbers (hundreds of embryos / female / week) and therefore a large number of animals can be easily maintained in a relatively small space, which is a pre-requisite for large-scale biomedical research. Moreover, zebrafish have transparent embryos that develop externally allowing for visualization of different structures and systems during development and for early genetic manipulation. Finally, mutations in zebrafish produce phenotypes that copy many human disorders and several genes are being identified that are evolutionarily conserved and have homologs in mammals including humans [4-6].

Recent studies in zebrafish combining molecular genetics with behavioral analyses have allowed the identification of genes involved in neuronal circuits underlying specific behaviors and mechanisms involved in neuropathogenesis [3, 7]. Zebrafish models of brain function and disease have started to be developed including insomnia and sleep disturbances [8-10], movement disorders [11], autism [12], neurodegenerative diseases [13], cognitive impairment during ageing [14], nicotine and alcohol addiction [2, 15, 16, 17]. Complex behaviors that are goal-directed (e.g. escape from predators) or emotion-related (e.g. aggression, anxiety and fear) have also started to be characterized in adult zebrafish and the first results suggest conserved regulatory mechanisms with mammals,[18] including shared modulatory neurotransmitter systems [13, 19] and homologous brain areas [20].

Aggression serves various adaptive functions, such as the establishment of dominance relationships and hierarchies and the competition for key resources such as food, shelter or mates and territories [21], and therefore plays a major role in Darwinian fitness. Despite its biological relevance and the large body of literature dedicated to the study of aggression there is not yet an established vertebrate neurogenetic model organism for its study that would allow the use of powerful genetic tools for the dissection of the neural circuits involved, and for the understanding of how they are activated by social cues and regulated by humoral factors (but see [22] for the development of a neurogenetic model of aggression in fruit flies and [23, 24] for previous work on knock-out mice for candidate genes in the serotonergic system). For reasons mentioned above zebrafish can play such a role. For that purpose one needs first to establish that aggressive behavior is present and has a temporal structure (i.e. its sequence is not random, suggesting an underlying regulatory mechanism prone to genetic dissection), and that it has consequences for the animals (i.e. subsequent behavior is shaped by previous interactions). Since zebrafish is a gregarious species that exhibits shoaling behavior in captivity, only recently its aggressive behavior has attracted the interest of researchers. Several studies have now demonstrated that both male and female zebrafish exhibit aggressive behavior (see [25] and [19] for recent reviews), that stereotyped behavioral patterns can be observed and described in detail during agonistic interactions (e.g. [15, 26]), that territoriality and dominance hierarchies can be present [27-29] and that neuropeptides (i.e. AVT) and steroids are associated with aggressive behavior [30-32].

The main goals of this paper are (i) to establish a behavioral paradigm under which male zebrafish would consistently express fighting behavior; (ii) to characterize the structure (i.e. temporal pattern) of fighting behavior in male dyads; and (iii) to study the effects of social experience (i.e. winning/losing effects) on subsequent fights. Together these goals will contribute to the establishment of male-male fights in zebrafish as a standardized behavioral paradigm for the study of the genetics of aggression.

Material and methods

Subjects and maintenance

The individuals used in this experiment belong to a F2 generation population bred at Instituto Gulbenkian Ciência (IGC), which derived from wild-type (AB) zebrafish (*Danio rerio*) acquired from Zebrafish International Resource Center (ZIRC). Prior to the experiment, animals were kept in 8,0L tanks (30x22x15cm) with a sex ratio of 2 females per each male. Fish were kept at 26±2^{o}C on a 14D:10L and fed twice daily with freshly hatched brine shrimp, in the morning, and with commercial food flakes, in the afternoon. In this study, the average male size was 28.1±1.7mm (standard length, SL).

Experimental procedure

One of the main aims of this study was to establish a reliable behavioral paradigm to study aggression in zebrafish. Although mirror image stimulation (MIS) has been widely used as an aggression test for zebrafish, it does not elicit the full agonistic repertoire and the brain activation pattern and hormonal response associated with MIS differ significantly from those triggered by a fight with a live opponent [33-35]. Therefore, we focused on dyadic fights between size matched males. Since we wanted to create the simplest situation possible in which zebrafish would express their agonistic repertoire, in pilot studies we tested if male dyads would fight in the absence of a limited resource (e.g. shelter, mate, food) after a period of social isolation. A previous study [30] has already used successfully an isolation-induced aggression paradigm with zebrafish, using a social isolation period of 5 days. In our pilot studies we have established that 24h of social isolation was enough to promote the consistent expression of aggressive behavior in male dyads and this is the behavioral paradigm that we have used in this study.

Twenty two male dyads were formed with individuals matched for standard length (size difference < 1 mm, which is on average 3,6 % of body size) within each dyad. Subjects were individually recognized by fin clips on the extremities of the caudal, dorsal or anal fins, which

were conspicuously distinguishable. Each pair was placed in a 700 ml polycarbonate tank (18 x 10 x 9 cm) visually, but not chemically, isolated by a removable opaque PVC partition and allowed to acclimate overnight. After one day in isolation, the opaque divider was removed and the fish were left to interact for 30 min, a duration that exceeded the necessary time to determine a clear winner of the contest. Following each interaction, the fish were separated again by placing back the opaque partition. Behavioral interactions were videotaped and were subsequently observed in detail. After this first interaction, both fish were separated into two new tanks and paired up with two other fish, matched for size, but with no prior fighting experience (i.e. naïve individuals), separated by an opaque partition. After a 1h acclimation period, the opaque partitions were removed and the experienced and naïve fish could interact for 30 min (see Fig.1). These second interactions were also videotaped for subsequent behavioral analysis.

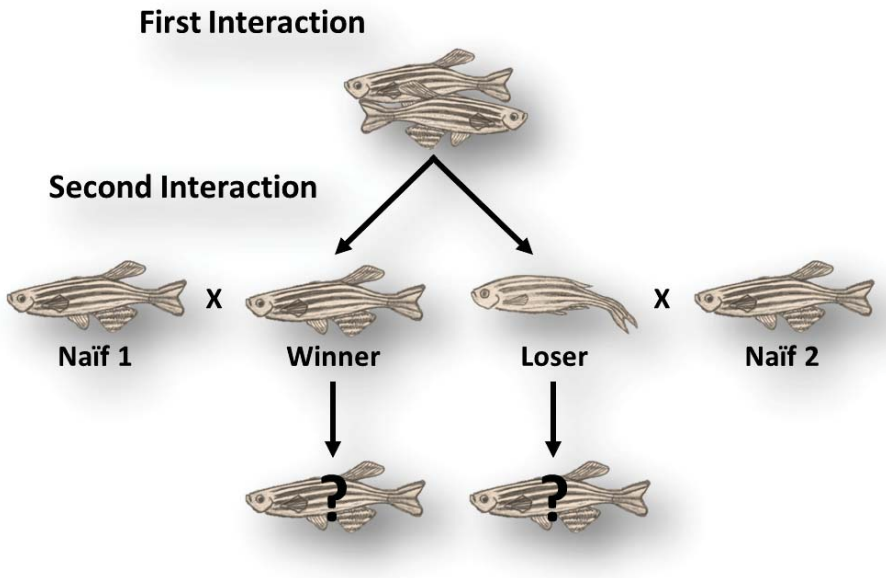

Figure 1 – Diagram illustrating the self-selection protocol used to test experience effects in the agonistic behavior of male zebrafish. During the first interaction, two naïve fish matched for size and previously isolated for 24 h are paired up. One hour after the resolution of the first interaction, during which each fish is kept in social isolation, the winner/loser of the first interaction is again paired up against a naïve size-matched opponent.

Behavioral observations

Video recordings (Sony KDL X200, Tokyo, Japan) were analyzed using the software Observer XT (Noldus, *Wageningen, The Netherlands*). An experienced observer analyzed the behavioral interactions and identified all agonistic behaviors according to the ethogram presented in Table 1 and determined, based on the asymmetry of aggressive behavior (i.e. who attacks and who is submissive in the later part of the interaction) the winner and loser of each interaction. The ethogram used in this study was build based on ca. 20 h of *ad libitum* observations of male zebrafish fights that have been previously videotaped. Stereotyped behavioral patterns that were consistently present in the interactions were described in detail.

Table I. Ethogram of aggressive behavior of male zebrafish during dyadic interactions.

Behavioral pattern	*Description*
Displays	In short distance of the opponent, usually less than one body length, fish erects its dorsal and anal fins and flares its body flank toward the opponent.
Circle	Two fish approach one another in opposite directions and with erected fins, and in an antiparallel position circle each other usually ascending in the water column. It can last from a few seconds to minutes.
Strike	The fish swims rapidly toward the opponent but no physical contact occurs between them.
Bite	Fish opens and closes its mouth in contact with the body surface of its opponent, usually near the more ventral or posterior parts of the body.
Chase	Similar to Strike behavior but with an active pursuit by the aggressor. This behavior stops when one subject stops chasing, and/or the other fish adopts a Freeze behavior.
Retreat	Fish swims rapidly away from the opponent in response to a strike or a bite.
Flee	Continued escape reaction in response to a Chase. Fish swims rapidly away from the aggressor.
Freeze	Fish stays immobile with all fins retracted near the bottom or near the surface of the aquaria and with the caudal region downward.

Frequency, latency and duration of agonistic behavior were registered using a behavior sampling with continuous recording (*sensu* [36]). After detailed scrutiny of behavioral patterns exhibited during interactions a switching point in the interaction was identified, where symmetric aggressive behaviors (e.g. displaying, circling) gave place to asymmetric ones (e.g. attack/flee, chase). This was considered the point at which the resolution of the fight took place, and since after the establishment of an asymmetry we have never observed a status reversal (i.e. the attacker becoming the attacked), one of the fish clearly could be considered the winner of the interaction. Based on this fight resolution point we defined two phases in the

fight: (1) a pre-resolution phase; and (2) a post-resolution phase. In the former phase, all behaviors from both subjects were quantified until the fight resolution point; in the latter phase, due to the consistency of the behavioral patterns exhibited by the fish (i.e. chase/flee, bite), only the last 5min of the 30 min sampling were analysed.

Eleven dyads for which the identity of the fish (i.e. the fin clips) were recognizable in the video-images of the three interactions (i.e. first interaction between emerging winner and loser, the second interaction between the previous winner and the naïve male, and the second interaction between the previous loser and the naïve male) were used for detailed analysis behavioral analyses. The observer had to identify each animal in frames where clips were clearly observable and then track each animal individually along the video recording of each behavioral trial. When there were doubts on the identity of the fish during the video-analysis the observer had to reverse the video until an unmistakable image was found and then progress again with the analysis. When it was not possible to solve the identity of the subjects in a given part of the trial and therefore it was not possible to track individually the two opponents along the whole session, these trials were dropped from the analysis.

For the other dyads in which the identity of the fish could not always be followed in the videos, but could be assessed at the beginning and at the end, only the outcome of the fight (i.e. identity of the winner and of the loser) and the identity of the initiator of the fight were collected.

Statistical analysis

In order to characterize the structure of the behavioral sequences present in zebrafish fights a transition matrix was build indicating the frequency with which each behavioral pattern followed and was followed by each other behavior of the zebrafish agonistic repertoire within each individual. The diagonal was kept at zero since we considered that each behavior pattern could not be followed by itself. This behavior sequence matrix was analysed using a

first-order Markov chain analysis to identify non-random transitions between behavioral elements (i.e. non-random temporal associations between behavioral patterns[37]). Only data from the first interaction was used for this sequential analysis. These analyses were performed using a collection of freeware programming functions developed by Robert Huber (Bowling Green State University, OH, U.S.A.) for the analysis of behavioral data (Java Grinders Library v.4.0 "Essential Equipment for Ethology", available on the Internet at http://caspar.bgsu.edu/~software/Java/).

To study the effect of the phase of the fight (pre- vs. post-resolution) and the status of the fish (putative winner vs. putative loser) on the expression of different aggressive behaviors, the frequency and duration (when appropriate) of each behavior pattern in the first interactions was analysed using a repeated measures ANOVA. To study the impact of previous experience on subsequent behavior the frequency and duration (when appropriate) of each behavior pattern, the latency for the first interaction and fight duration were compared between the first interaction (i.e. putative winner vs. putative loser) and each of the two second interactions (i.e. prior winner vs. naïve and prior loser vs. naïve), using an ANOVA model with the phase of the fight (pre-resolution vs. post-resolution) as a repeated factor and status (winner vs. loser) as an independent variable, followed by planned comparisons using contrast analysis. Apart from these behavioral variables we have also computed a composite measure of fight escalation as follows: (1) Escalation index = overt aggression / overt + ritualized aggression = (bite + chase + strike) / (bite + chase + strike) + (display + circle)

All descriptive and inference statistics were run on the statistical software package STATISTICA v. 8.0 (StatSoft 2007). Differences between proportions of second fights won vs. lost by previous winners vs. losers of first fights were tested by computing the qui-squared value for the resulting 2 x 2 contingency table. All tests were two-tailed and used a significance value of $p < 0.05$.

Results

Agonistic behavior in zebrafish

The behavioral patterns observed during male-male fights in zebrafish are described in Table 1. Dyadic fights among male zebrafish have two distinct phases. The first phase consists mainly of mutual assessment behaviors, with fish assessing each other by exhibiting display, circle and bite behaviors (see Table 1) in order to determine the other fish's relative fighting ability. This phase starts with the first interaction of the behavioral trial (latency for first interaction = 70.6 ± 164.9 ms) and lasts on average for 379.2 ± 331.0 ms, until the first chase/flee is observed which marks the point of the resolution of the fight. In the second phase, that occurs after the fight's resolution, all agonistic behaviors are initiated by the winner (e.g. bite, chase and strike), while the loser tries to flee and displays submission and freezing postures. At the end of fights losers usually stay near the bottom or top of the tank adopting a submissive posture. During the 30 min of the behavioral trial, male zebrafish displayed agonistic behavioral patterns at a rate of 1.19 behavior/s. Bite was the most frequent behavior, representing roughly 65% of all behaviors exhibited (N = 5769 behavioral acts) by the fish in the first phase. In the post-resolution phase only the winner of the interaction exhibited Bite behavior, but it represented approximately 50% of its behavioral output (N = 2842 behavioral acts). All other behaviors, though less frequent in number, represent part of a complex and highly structured behavioral sequence, which characterizes zebrafish agonistic behavior. To better understand and describe these sequences, a behavior transition matrix was analysed and non-random transitions between behavioral elements were identified. This analysis reveals a temporal structure in male zebrafish fights (i.e. behavioral sequences are non-random; behavioral sequence matrix $X^2 = 2242.8$, $p << 0.0001$) with assessment behaviors (i.e. display and circling) significantly associated with each other and with bite that is also then significantly associated with chase and strike that correspond to the

asymmetric phase of the fight (Fig. 2). A set of behaviors associated with losing the fight (i.e. freeze, flee and retreat) also appear significantly associated among themselves (Fig. 2).

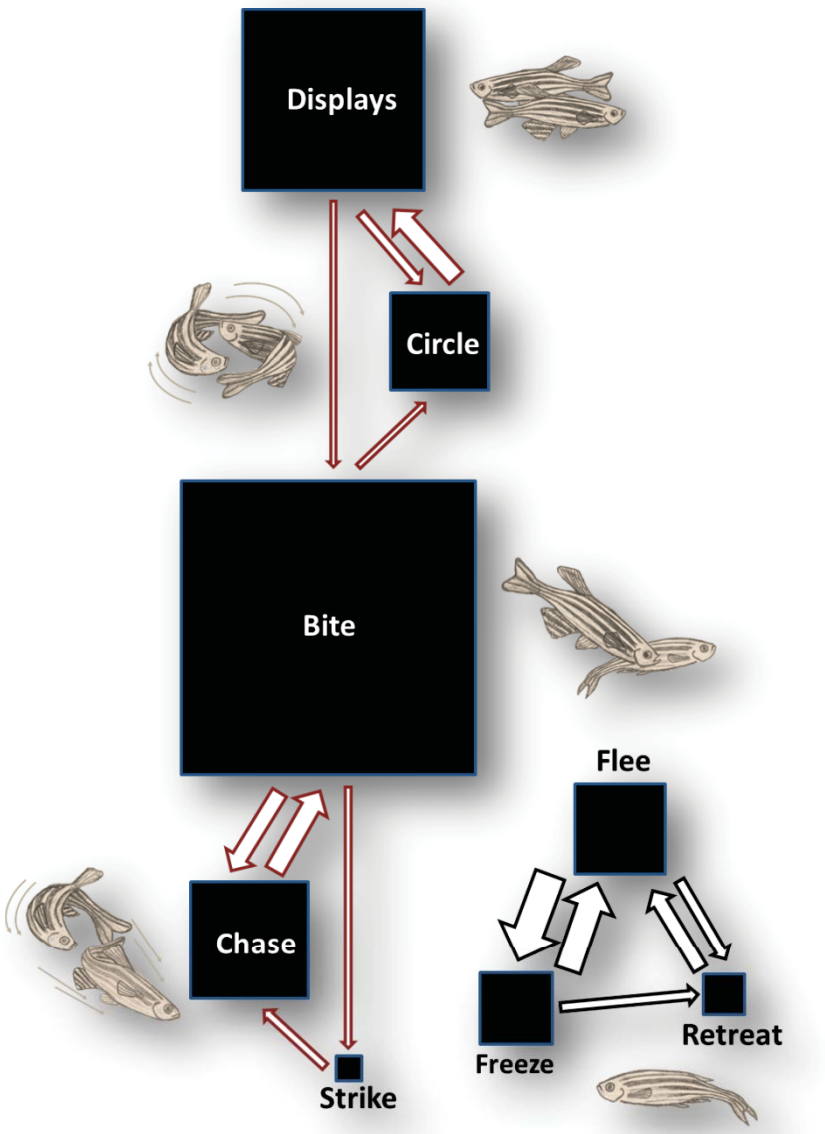

Figure 2 –Temporal structure of fighting behavior in male zebrafish dyads using a first-order Markov chain analysis. The size of each box is proportional to the relative frequency of occurrence of each behavioral pattern. Transitions between behaviors whose frequency is significantly higher than chance levels are depicted as arrows, and their size indicates the degree of significance.

Temporal dynamics of the fights and early predictors of success

To further study the effect of the phase of the fight (pre- vs. post-resolution) and the status of the fish (putative winner vs. putative loser) on the expression of different aggressive behaviors, the frequency and duration (when appropriate) of each behavior pattern and the

composed measure escalation index were analysed using a repeated measures ANOVA (Table 2).

As expected behaviors associated with mutual assessment were significantly more frequent and had a longer duration in the pre-resolution phase of the fight (Table 2) and a non-significant trend for an increase in the frequency of Chase, Freeze, Flee and Retreat was also observed from the pre- to the post-resolution phase (Table 2). The phase of the fight had no main effect on the escalation index. Social status has a main effect on the expression of bite and flee, with the former being more frequent in winners and the latter in losers, and on escalation index that is higher in winners (Table 2).

Table II. Effect of the phase of the fight (pre- vs. post-resolution) and of fish status (winner vs. loser) on the expression of aggressive behaviors. Frequency, duration and escalation index were analysed using a repeated measures ANOVA, with the phase of the fight as a repeated measure (R1; pre- vs. post-resolution phase) and status (winning vs. losing) as an independent variable. Apart from the main effects and the interaction between the two factors results of contrast analysis for the winner loser comparison in the pre- and post-resolution phases s also given. All significant results ($p < 0.05$) are underlined.

		R1		*Status*		*R1*Status*		*Preresolution*				*Postresolution*			
N = 11		*F*	p	*F*	P	*F*	P	*Winner* ($\bar{x} \pm SEM$)	*Loser* ($\bar{x} \pm SEM$)	*F*	p	*Winner* ($\bar{x} \pm SEM$)	*Loser* ($\bar{x} \pm SEM$)	*F*	p
Frequency	Bite	0.97	0.34	9.05	0.007	4.15	0.055	3.56 ± 0.18	2.57 ± 0.09	0.21	0.65	10.82 ± 0.35	0.04 ± 0.00	7.98	0.010
	Chase	2.35	0.14	4.31	0.051	6.86	0.016	0.16 ± 0.01	0.31 ± 0.02	0.38	0.54	1.33 ± 0.05	0.00 ± 0.00	6.74	0.017
	Circle	15.01	0.0009	0.00	1	0.00	1	0.21 ± 0.01	0.21 ± 0.01	0.00	1	0.02 ± 0.00	0.02 ± 0.00	0.00	1
	Displays	14.36	0.0012	0.003	0.95	0.07	0.79	0.59 ± 0.02	0.55 ± 0.01	0.03	0.87	0.05 ± 0.00	0.07 ± 0.00	0.41	0.53
	Flee	4.10	0.056	5.07	0.036	5.25	0.03	0.07 ± 0.00	0.05 ± 0.00	0.14	0.71	0.00 ± 0.00	1.20 ± 0.05	5.19	0.03
	Freeze	3.10	0.09	2.68	0.12	2.98	0.09	0.03 ± 0.00	0.00 ± 0.00	2.83	0.11	0.04 ± 0.00	1.20 ± 0.05	2.84	0.11
	Retreat	1.15	0.29	1.89	0.18	3.11	0.09	0.09 ± 0.01	0.04 ± 0.00	0.49	0.49	0.00 ± 0.00	0.42 ± 0.02	2.71	0.12
	Strike	0.69	0.42	0.90	0.35	2.36	0.14	0.01 ± 0.00	0.07 ± 0.01	0.71	0.41	0.23 ± 0.02	0.00 ± 0.00	1.77	0.19
Duration	Circle	22.59	0.0001	0.00	1.00	0.00	1.00	0.028 ± 0.009	0.028 ± 0.009	0.00	1.00	0.002 ± 0.001	0.002 ± 0.001	0.00	1.00
	Displays	13.83	0.0014	0.06	0.81	0.06	0.80	0.602 ± 0.25	0.689 ± 0.25	0.06	0.81	0.016 ± 0.01	0.017 ± 0.01	0.007	0.93
	Escalation index	0.01	0.94	11.27	0.003	0.85	0.001	0.71 ± 0.09	0.64 ± 0.09	0.31	0.59	0.36 ± 0.09	0.98 ± 0.09	21.58	0.0002

Note: $\bar{x} \pm$ SEM = average ± standard error of the mean; F = value of the F statistic.

We have also performed planned comparisons to test if differences in the expression of aggressive behavior were already present in the first phase of the fight between individuals that subsequently became winners vs. losers. None of these planned comparisons was significant for the pre-resolution phase indicating that neither the frequency of any of the

behaviors measured in the early stage of the fight nor fighting escalation at this phase are good predictors of fight outcome (Table 2).

On the other hand, similar planned comparisons comparing the frequency of each behavior and of fight escalation between winners and losers in the post-resolution phase of the fight revealed that winners express significantly more bites and chases and a higher escalation index, and losers more flee in the advanced stage of the fight (Table 2). Interestingly, the escalation index significantly increased in winners and decreased in losers from the pre- to the post-resolution phase (losers: $F_{1,20}$: 7.19, $p < 0.05$; winners: $F_{1,20}$=6.60, $p < 0.05$).

Since fish were matched for standard length within each dyad, size was also not a predictor of the fight outcome (SL of winners = 2.804 ± 0.035 mm; SL of losers = 2.800 ± 0.038 mm, t (1,22) = -0.25, p = 0.80). Being the first to engage in the interaction, which could be seen as a proxy of aggressive motivation, was also similar between individuals that became winners and individuals that became losers (6 winners vs. 7 losers, qui-square = 0.077, P = 0.78).

Experience Effects

Most of the winners of the first interaction also won the second interaction against a naïve individual (85.71%), whereas only a very small percentage of fish that lost the first interaction won the second fight (4.55%), suggesting the presence of both winner and loser effects in zebrafish (X^2 = 28.7, $p < 0.0001$; Fig. 3). In order to investigate the behavioral mechanisms that may account for these winner/loser effects we investigated the variation in motivation and persistence induced by the previous fight, by comparing the first fights [i.e. winner vs. loser, (WL)] and second fights [i.e. winner vs. naïve (WN) and loser vs. naïve (LN)].

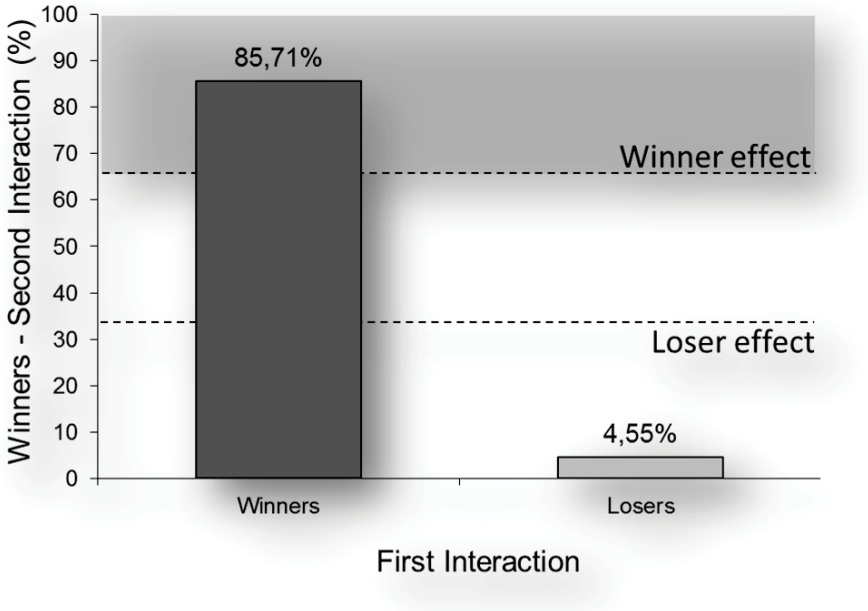

Figure 3 –Experience effects in zebrafish as indicated by the percentage of victories/defeats of previous winners and losers in the second fights. Dashed lines indicate the cut-off values for the detection of winner/loser effects calculated considering the variation in intrinsic fighting ability according to ref. [44]

As a proxy of fighting motivation of the dyad we compared the latency for the first interaction between WL and WN and between WL and LN and the identity of the initiator of the fight. As a proxy of fighting persistence in the dyads, we compared the latency for the resolution of the fight between WL and WN and between WL and LN. Although there is a trend for second fights to start sooner than the first fight, no significant differences were found for the latency for the first interaction for the planned comparisons described above (N = 11; WL/WN: $t = 0.74$, $p = 0.47$; WL/LN: $t = 1.45$, $p = 0.17$; WN/LN: $t = -1.62$, $p = 0.13$; Fig. 4a). There was no effect of prior experience on taking the initiative to start the second fight, since 3/11 previous winners and 3/11 previous losers initiated the second fights. The time needed to reach the resolution of the fight tends to be shorter in second fights (Fig.4b), but the difference is only significant for the WL vs. LN comparison ($t = 2.55$; $p < 0.05$).

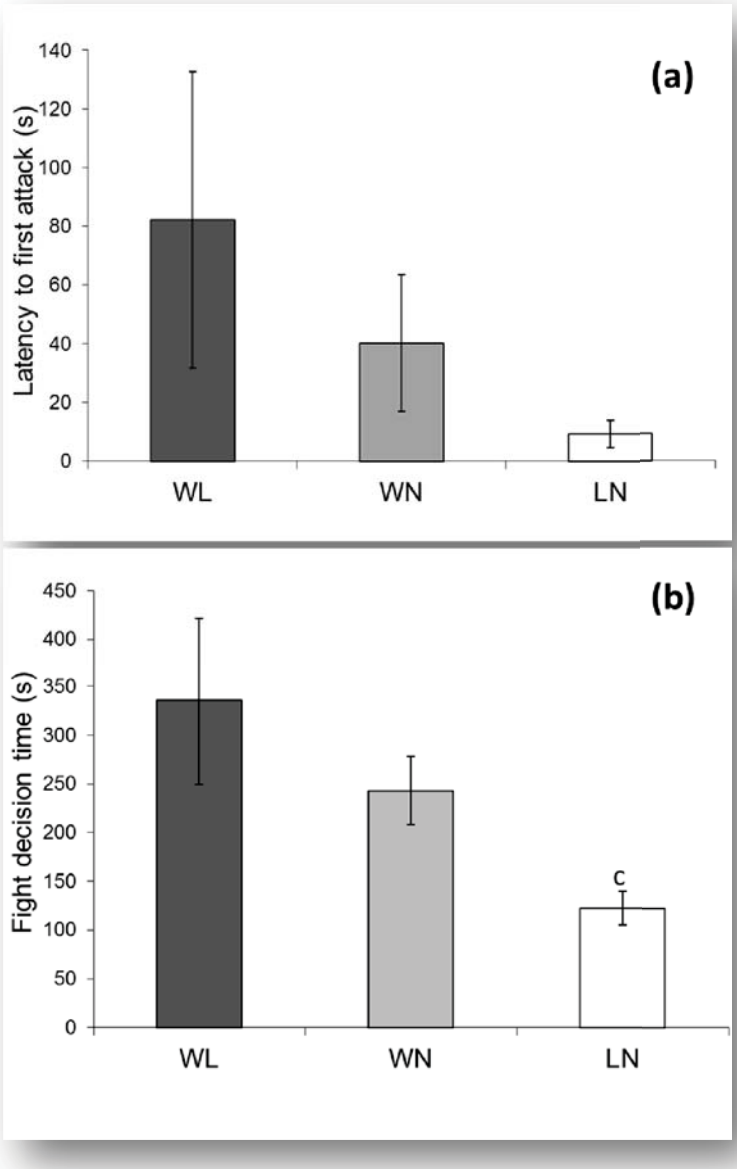

Figure 4 – Comparison of latency to first attack (a) and fight resolution time (b) between the first interaction (winner vs.loser [WL]; dark gray) and the second interactions (WN, prior-winner vs. naïve, light gray; LN, prior-loser vs. naïve,
white).

Moreover, the time needed for the resolution of the fights in the two second interactions is significantly shorter for those involving the previous loser than for those involving the previous winner (i.e. WN/LN: $t = 2.86$; $p < 0.05$). The escalation index does not change significantly in winners between the first and the second fight (ANOVA repeated

measures, contrast effect: $F_{1,20} = 0.08$, $p = 0.77$; Fig. 5), but it decreases significantly in losers (ANOVA repeated measures, contrast effect: $F_{1,20} = 5.84$, $p < 0.05$; Fig. 5). As a consequence, the escalation index of previous winners and previous losers is significantly different in subsequent fights against naïve opponents (ANOVA repeated measures, contrast effect: $F_{1,20} = 9.49$, $p < 0.01$).

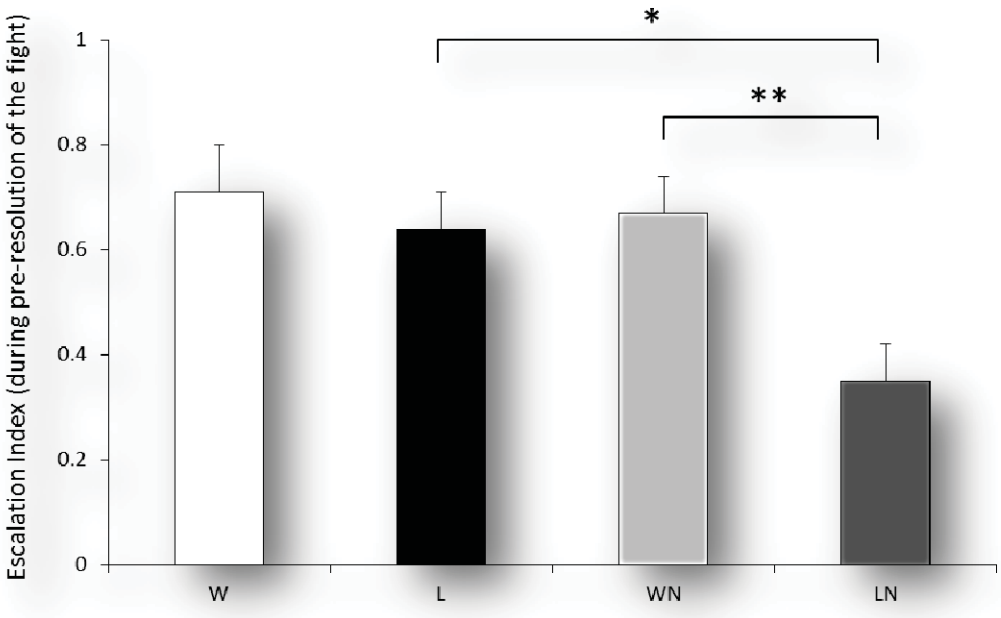

Figure 5 – Comparison of escalation behavior in the pre-resolution phase between winners (W, white) and losers (L, black) of the first fight, and between the focal males in the second interaction (WN, prior-winner vs. naïve, light gray; LN, prior-loser vs naïve, dark gray). * indicates $p<0.05$; ** indicates $p<0.01$.

Discussion

In this paper we have described a simple behavioral paradigm under which male zebrafish consistently expressed fighting behavior. Dyads of 2 males that have been previously isolated for 24h consistently expressed fighting behavior when exposed to each other, even in the absence of a limited resource to promote competition, such as food, shelter or a potential mate. In a previous study [30], 5 days of social isolation have been used to promote aggression in zebrafish. Our study indicates that 24h of social isolation is enough to promote the expression of aggressive behavior in zebrafish. Social isolation has been reported to increase

aggression in different species including fish (e.g. [38]). Different mechanisms may explain the effect of social isolation on aggressiveness, including an increased sensitivity to external stimuli that may act as releasers of aggressive behavior, or forgetting prior social experiences that are the basis for dominance hierarchies that regulate social interactions in social networks. Whatever the mechanisms involved, for the purpose of this study, the key result is that a short period of social isolation consistently promoted the expression of aggressive behavior in dyads of male zebrafish. So far the study of aggression in zebrafish has mainly used either standardized mirror image stimulation tests (e.g. [15, 39-41]) or groups of 3 or more individuals (e.g. [27-29], [42-44]). Although the mirror test can be seen as a standardized test that elicits heightened aggressive responses [45, 46] it has recently been shown that it triggers different hormonal, and brain activation patterns from those elicited by a real opponent ([33-35] RF Oliveira et al, unpublished data for zebrafish). Therefore, the use of mirror image stimulation as a behavioral paradigm to study aggression should be taken with caution, and the use of real opponent fights is advisable, especially when studying the proximate mechanism of aggressive behavior. It should also be mentioned here two major technical and analytical challenges of analyzing zebrafish aggression that we have faced:

(1) Due to the high speed with which some of the behavioral patterns are performed by the fish a frame-by-frame analysis was recurrently needed; this is very time-consuming and in some cases a higher time resolution would have been helpful. In this respect a high-speed camera (with image acquisition rates staring at ca. 200 frames per second) would be a major improvement when compared to regular video cameras (with acquisition rates of 25 to 30 frames per second depending on which video signal standard is being used, PAL or NTSC respectively).

(2) The small size of zebrafish is a limitation to the identification of particular individuals during behavioral trials either in real time or in video-recordings. We have used fin clips to individually tag subjects. Despite being very efficient to identify individuals in stock tanks this

method proved inefficient to identify individuals in the video-recordings, as illustrated by the fact that we had to drop the video-analysis of almost 50% of our behavioral trials due to the lack of a clear identification of each individual along the whole session. We have recently replaced with good results the use of fin clips by fluorescent elastomer tags (Northwest Marine Technology Inc. WA, USA) that are implanted beneath transparent or translucent tissue and remain externally visible. Although this is a promising solution for individually tagging zebrafish in behavioral assays we still have to confirm if the different colors are having an effect on the behavior of the fish.

The qualitative aspects of the behavioral patterns observed in our experiment, are consistent with other ethograms previously published for this species (e.g. [25, 28-30]). The sequence analysis based on transition matrices of behavioral patterns expressed by each contestant allowed to identify a complex and highly structured aggressive behavior in zebrafish, indicating that the expression of the different behavioral patterns that make up the agonistic repertoire is not random and that there are decision rules underlying their expression. This is a particularly relevant finding since it makes aggressive behavior in this species suitable for quantitative analysis and allows for the study of the impact of selected mutations or other genetically or pharmacologically induced changes in behavior.

Zebrafish fights lasted for ca. 5 min until a clear asymmetry was established and a clear winner and a clear loser could be established. Before fight resolution contestants mainly expressed mutual assessment behaviors such as displays and circling, and biting. After the resolution point, winners mainly expressed chase and bites and subordinates flee. Biting is present in both phases (i.e. before and after resolution but its frequency increases in the post-resolution phase where physical aggression becomes more frequent. Contrary to other studies (e.g. [47]), the initiative to start the interaction was not a good predictor of fight outcome, and there was no behavior pattern whose expression in the pre-resolution phase was an indicator

of who would win the contest. Therefore, we have failed to identify a key fight parameter used by fish to decide when to give up and retreat from fighting.

Data presented here on sequential fights shows that a recent victory increases and a recent defeat reduces the probability of winning a subsequent fight, suggesting the occurrence of winner and loser effects in male zebrafish. This is an important result since it establishes that fight outcome has behavioral consequences that may impact in the individuals Darwinian fitness.

Since we have used a self-selection protocol and did not impose winning and losing experiences to our focal fish it can be argued that the winner/loser effect observed is due to uncontrolled sources of inter-individual variation in intrinsic fighting ability. To control for this possibility we have used size matched males, and *a posteriori* we also controlled for fighting motivation by registering which male took the initiative to start the fight. Moreover, we have used a null hypothesis against which to test the effects of prior experience that is not equiprobability of winning/losing the second encounter, but having prior winners/losers winning/losing at least two-thirds of subsequent interactions against a size-matched naïve opponent, which is the probability estimated by [48] of a random individual in a population to have higher/lower intrinsic fighting ability than neutral opponents.

Two behavioral mechanisms have been advanced to explain the effects of prior experience on future fighting success: (1) changes in self-assessment of fighting ability (i.e. resource holding power, *sensu* [49]) induced by the fighting outcome (i.e. perceived increase in winners and perceived decrease in losers of own fighting ability); and (2) social cues that signal the winner/loser status to conspecifics allowing them to respond differentially to winners vs. losers of a previous fight [50-52]. These two mechanisms are not mutually exclusive and evidence for both is present in the literature. In support of the former, after a recent win or loss individuals change their contest behavior accordingly in standard aggression tests and increase the probability of initiating a new fight (see [51] for a review). In support of the latter

it is known that fish collect information from observed interactions between third parties and that they respond differentially to individuals that they have observed winning/losing a previous interaction [53]. Moreover, a number of social cues are known to signal social status. For example, both in crayfish and in cichlid fish social dominance is signaled through odorants present in the urine [54, 55].

In our study the likelihood of starting a second fight was not affected by the outcome of the first fight (i.e. same numbers of previous winners vs. losers took the initiative to start the second fight). On the other hand, the escalation index does not change significantly in winners between the first and the second fight, but it decreases in losers so that in second fights previous winners express more escalated fighting behavior than previous losers. Similarly the fight resolution time decreased in the second fights and was significantly shorter in the LN fights than in WN fights. Together these results suggest that the effects of previous experience might be different in winners and losers: while escalation decreases in losers in subsequent fights suggesting an experience driven change in the self-assessment of their own fighting ability, the behavior of winners does not seem to change significantly in the subsequent fight, and therefore experience effects in winners may be relying on social cues that signal a recent winning that naïve opponents in the second fights are responding to. These cues can be behavioral, pheromonal, or other. In another teleost fish (i.e. tilapia, *Oreochromis mossambicus*) it has been recently shown that dominant individuals release more urine than subordinates during agonistic encounters and that the urine of dominants can be discriminated from that of subordinates at the levels of the olfactory organ with that of the dominants eliciting a higher olfactory response [55, 56]. Since in zebrafish olfactory communication is also well developed and used in social context (e.g. [57, 58]) it is possible that experience-induced changes in social status or motivation to engage in a contest are signaled through olfactory cues.

The fact that second interactions involving the loser of the first interaction have a short latency for the first interaction and a shorter resolution time suggest that the loser effects has a higher impact that the winner effect. This is in accordance with the relative magnitude of winner and loser effects reported in the literature (for review see [50]). These results are also consistent with a heightened decision to retreat in previous losers, an effect that is consistent with previous work in other species [59, 60, 61]. An increased likelihood to give up appears to be the real explanation for the behavioral changes, however, this is not spelled out specifically.

In summary, in dyadic fights male zebrafish express highly structured behavior and the outcome of these fights have an impact on their subsequent behavior. Given the available genetic and genomic tools for this species, these results support the use of zebrafish as a neurogenetic model for the study of the neural and hormonal mechanisms of aggressive behavior in a vertebrate model.

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(ii) Cognitive appraisal of fight outcome is needed to activate socially driven transcriptional changes in the zebrafish brain

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Cognitive appraisal of fight outcome is needed to activate socially driven transcriptional changes in the zebrafish brain

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Abstract

Group living animals must be able to express different behavior profiles depending on their social status. This implies that the same genotype may translate into different behavioral phenotypes through socially driven differential gene expression. Here we show for the first time that what triggers the switch between status-specific neurogenomic states is not the objective structure of the social interaction but rather the subjects' appraisal of its outcome. For this purpose we had male zebrafish fight either a real opponent or their own image on a mirror. Massive changes in the brain transcriptome were observed in real opponent fighters, which experience either a victory or a defeat. In contrast, mirror fighters, which had no information on fight outcome despite expressing aggressive behavior, failed to activate a neurogenomic response. These results indicate that, even in cognitively simple organisms such as zebrafish, neurogenomic responses underlying changes in social status rely on cognitive appraisal.

One Sentence Summary

Cognitive appraisal of fight outcome, rather than mere exposure to a fight, triggers rapid changes in brain transcriptome

Main Text

Dominance hierarchies are ubiquitous in animal groups and play a key role in the regulation of social interactions between individuals competing for resources (e.g. potential

mates), such that individuals of different social status commonly express different sets of behaviors (aka behavioral states) that match their competitive ability. Typically dominant individuals express competitive and resource monopolization behaviours (e.g. courtship behaviour) that will potentially increase their Darwinian fitness, whereas subordinates refrain from direct competition for resources, thus avoiding costly social interactions (e.g. potential eviction from the group) in which they would have a low probability of success (1). However, this competition avoidance behavior of subordinates is only adaptive if it allows them to gain fitness advantages later on, for example by taking over a vacant dominant role. Thus, it is important for subordinate individuals to be able to identify opportunities for social ascend and to rapidly switch their behavior profile accordingly.

Despite the well known genetic influences on aggressive behavior (e.g. (2) social status depends to a great extent on group composition (i.e. relative competitive ability of group members) and on social factors (3, 4) and the same individual must be able to switch between different social statuses. Hence, the same genotype must accommodate the expression of multiple social phenotypes, and this should be accomplished by socially driven changes in gene expression in the brain that would lead to distinct transcriptome profiles across the social behavior neural network (aka neurogenomic states, (5, 6) corresponding to the status-specific behavioral states mentioned above. Previous studies have established this mapping of socially dependent behavioral states onto neurogenomic states (e.g. (4, 7)), and rapid responses to social interactions have also been described (8–11). However, the specific cue that signals changes in social status and triggers the switch between neurogenomic states has remained elusive. There are at least two potential cues of social status readily available in a social interaction: (A) the own aggressive behavior expressed by the individual; and (B) the behavior expressed by the opponent. Theoretically, animals may use either of these or a combination of the two to infer their social status and trigger genomic and behavioural changes accordingly. For example, if only sensing its own behavior individuals could trigger a dominant state above a certain threshold of expressed aggressiveness; or if only sensing the opponent's behavior they could trigger the dominant state in response to observed submissiveness. However, given that social status is not an individual attribute but rather a relational trait, we hypothesized that it would be adaptive for an animal to switch its status specific neurogenomic state only when faced with reliable information on relative competitive ability in comparison to other group members. Thus, we predicted that an assessment of relative fighting ability, which incorporates both expressed behavior and perceived behavior of the opponent, must be a necessary condition to activate this switch.

Here we used zebrafish to test this hypothesis by manipulating their perception of fight outcome and assessing its effects on the brain transcriptome profile. For this purpose we compared, using a genome-wide microarray gene chip, the neurogenomic response to social interactions between fish that fought a real opponent and fish that fought their own image on a mirror. Fish do not recognize themselves on a mirror and attack their own image as if it is an intruder (12). Mirror fights usually elicit similar levels of aggressive behavior to those of real opponent fights (13), but since submissive behavior is never expressed by one of the opponents (i.e. the mirror image replicates the behavior of the focal fish) the former have no outcome and the expression of aggressiveness is decoupled from the experience of winning or losing a fight. Size matched male zebrafish were socially isolated for 5 days before being exposed to a short term (aprox. 30 min) social interaction which consisted either in a mirror fight or in a real opponent fight. Aggressive behavior was quantified and the identity of the winner and the loser of the real opponent fights were noted. A reference group remained in social isolation and did not experience any social interaction. Therefore, the experimental manipulations generated 4 phenotypes regarding social experience: mirror fighters (M), winners of a real opponent fight (W), losers of a real opponent fight (L); and socially isolated fish (I). These 4 phenotypes differed among themselves in the combination of behavior expressed and behavior perceived in the opponent: W expressed aggressive behavior and perceived submissive behavior in the opponent; L expressed submissive behavior and perceived aggressive behavior in the opponent; and M expressed aggressive behavior but also perceived aggressive behavior in the opponent (Fig.1). Therefore, the following predictions can be generated:

(1) If only behavioral feedback from opponent would be relevant, then M fish should have a response profile similar to that of L;

(2) If only the individuals own behavioral expression would be relevant, then M fish should have a response profile similar to that of W;

(3) If only the comparison between perceived behavior of the opponent with the expressed behavior (or any other self measure of own competitive ability) would be relevant, then M fish should not activate a response because in mirror interactions they equal each other, and therefore no change in social status would be experienced by the subject.

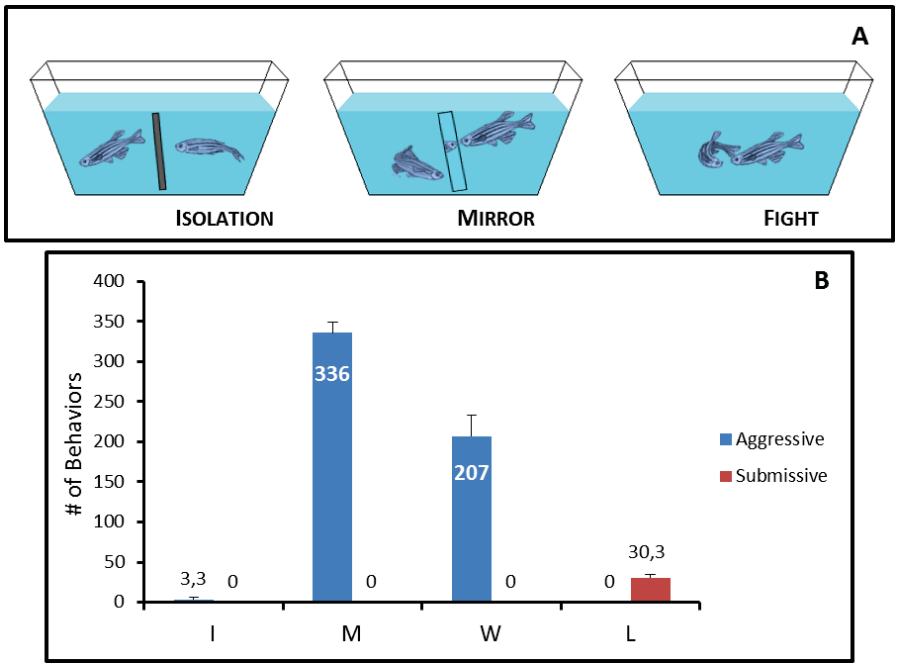
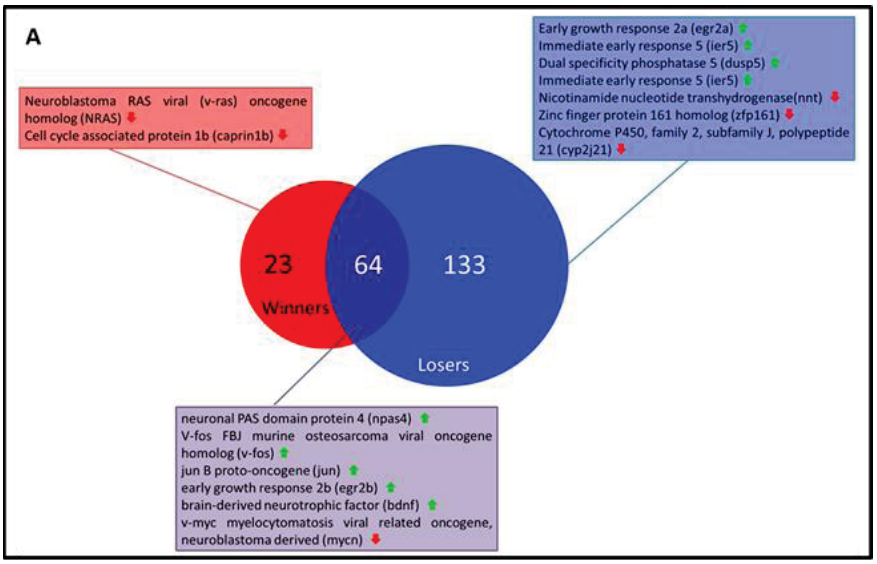


Figure 1 – Behavioural paradigm used to promote different social experiences in zebrafish. (A) Experimental set-up used to promote the four social experiences: (left panel) control group (no social interaction); (middle panel) mirror elicited fight (animals fought their own image on the mirror) and (right panel) real opponent fights (animals fought a real opponent and experienced a victory or a defeat), (B) Behavioural profiles of each social phenotype (i.e. socially isolated, mirror fighters, winner and losers) as illustrated by the frequency of aggressive and submissive behaviors (average ± S.E.M.; N=3 for each condition) expressed in the last 10 min of each type of social treatment.

Contrasting each social treatment (i.e. W, L or M) with the reference group (i.e. I) revealed 210 differentially expressed genes across all social experiences. Real opponent interactions elicited 197 differentially expressed genes in losers and 87 differentially expressed genes in winners, of which 64 were differentially expressed both in winners and losers (Fig. 2A). Thus, there were 133 genes associated with switching social status towards subordinate, 23 genes associated with becoming dominant and 64 genes associated with fighting. Among these socially regulated genes the ones with the highest fold-changes included activity-dependent immediate early genes (e.g. c-fos; early growth response 2, egr-2), neural plasticity genes [e.g. brain-derived neurotrophic factor, bdnf; neuronal Per Arnt Sim (PAS) domain protein 4, npas4], genes involved in immune function (e.g. suppressor of cytokine signaling 3a, B-cell translocation gene 2, jun B proto-oncogene) and genes involved in hormone metabolism (e.g. nuclear receptor subfamily 4, group A, member 1) (Fig. 2A; see supplementary material Table S1 for complete list of differentially expressed genes). Of particular interest was the

differential expression of bdnf and of npas4 since both regulate experience-dependent synaptic plasticity and memory formation. Bdnf is a neurotrophic factor involved in neuronal differentiation and survival and in synaptic plasticity mechanisms underlying learning and memory (14); npas4 has been recently involved in the regulation of the formation of GABAergic inhibitory synapses (15) and on the formation of contextual memory in the hippocampus (16). A gene ontology (GO) analysis of these differentially expressed genes indicated that they were not randomly distributed across the genome but were clustered in functional units in relation to molecular function, biological processes, or cellular component (see supplementary material Table S2 for significant enrichment scores of GO terms).



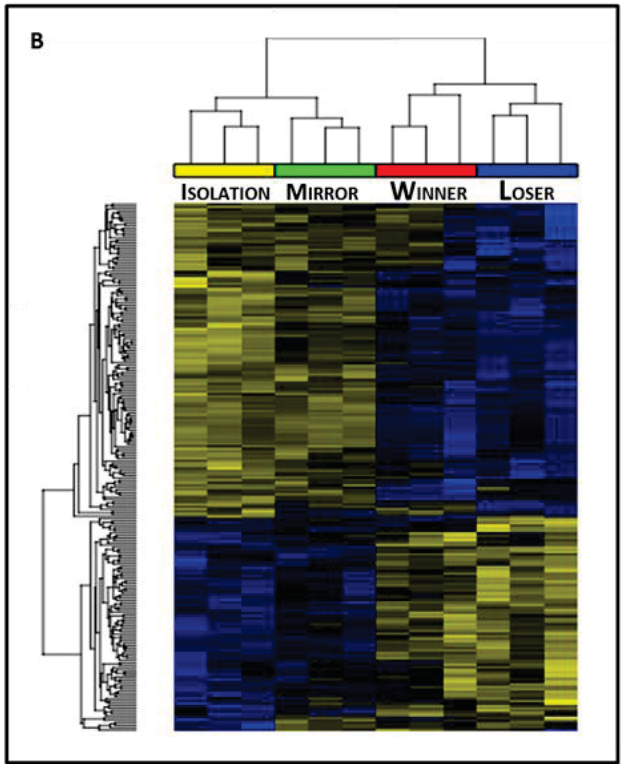


Figure 2 – Venn diagram and hierarchical cluster of socially regulated genes after a fight. A) Venn diagram showing the relationship between social status (winners [pink] and losers [blue]) and the number of differentially expressed genes. This diagram also indicates some of the genes that respond exclusively to the fight (purple), genes that were common between the two social statuses. Some examples of these genes are highlighted in the Venn diagram box. Information regarding the direction of this regulation is given by the arrows: upregulated: ⬆; downregulated: ⬇. B) The hierarchical clustering represents significantly different expressed genes ($P<0.01$) with confidence values of cluster nodes calculated using bootstrapping. The heatmap (blue – down-regulated, yellow – up-regulated) shows estimated gene expression levels for each social phenotype elicited: isolation (yellow); mirror (green); winners (red) and losers (blue).

Similarly, a signaling pathway analysis, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) as reference, showed a significant enrichment (i.e. more differentially expressed genes than would be expected by chance) of the mitogen-activated protein kinases (aka MAPK) signaling pathway (-3.9% of genes of enriched pathway; Benjamini adjusted p-value <0.05). Together, these results indicate that short-term interactions that induce changes in social status trigger activity-dependent gene pathways involved in neural plasticity.

In contrast, and in accordance with our hypothesis, mirror fights did not elicit a single differentially expressed gene. This result should be interpreted with caution. Since we were analyzing a large data set with 14,900 probes we have used a false discovery rate of 10% to control for false positives. Therefore, the lack of response of mirror fighters at the transcriptome level does not mean that they would not show any differential gene expression if tested univariately (e.g. using a candidate gene approach), but rather that the fold change of putatively differentially expressed genes in mirror fighters was below the threshold for distinguishing them from baseline gene expression levels found in the reference group. Nevertheless, the fact that mirror fighters showed a different pattern of gene activation from either those of winners or losers supports our hypothesis that cognitive appraisal of the fight outcome is necessary to induce major changes in the brain transcriptome which are potentially associated with the observed change in social state (i.e. becoming dominant/ subordinate). It should be stressed here the consistency of the transcriptome profiles induced by each social experience as revealed by the hierarchical cluster analysis of individuals according to their gene expression data (Fig. 2B). Indeed, all individuals from each social treatment were grouped together in individual clusters and higher order clusters subsequently grouped the cluster of the winners together with that of the losers, and the cluster of the mirror fighters with that of the socially isolated individuals. This indicates that the brain transcriptome profile of zebrafish closely reflects their recent acute social experiences.

The divergent changes in the brain transcriptome profile observed here between winners and losers of real opponent fights nicely match the socially driven changes in behavioral state that have been described previously for zebrafish, according to which winners of a single interaction significantly increase their probability of winning a subsequent interaction (winner effect), whereas losers decrease this probability (loser effect) (17). Therefore, the rapid changes in gene expression in the brain related to neural plasticity are closely associated with the shift between behavioural states characteristic of different social status.

The broader implication of our results is that cognitive appraisal, which has been seen as a complex cognitive ability (18), may also be playing a key role in the way animals, such as zebrafish, so far considered "simple minded" process and respond to environmental cues. This opens the way for a paradigm shift that would see model organisms with simpler nervous systems, which so far have only been used to study simple forms of associative learning (19), amenable for the study of more complex cognitive abilities (20), which can be themselves based on simpler computational abilities than initially thought.

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Supplementary Materials

Materials and Methods:

Subjects and maintenance

Zebrafish (Danio rerio) used in this experiment were wild-type (AB) acquired from Zebrafish International Resource Center (ZIRC). Prior to the experiment, fish were kept in 40L

tanks (50x30x35cm), in a 1:1 sex ratio, at 26 ± 2oC and on a 14D:10L photoperiod. Fish were fed twice a day with freshly hatched brine shrimp in the morning and commercial food flakes in the afternoon. Average fish size used was 27.1±1.7 mm (standard length, SL).

Behavioural assays

We used a modified version of an isolation-induced aggression paradigm (17), which is known to increase aggressive behaviour prolonging the fight decision time. In brief, fish were isolated five days prior to the social interaction. To test for the effects of the interaction outcome two other groups were used: a social isolation group and a mirror elicited aggression group.

Twenty-four adult males, matched for standard length (size difference < 2 mm), were exposed to one of four experimental social experiences: winning the interaction, losing the interaction, an unsolved interaction (mirror fight) or experience no interaction (reference group). Fish were always tested in pairs, to control for spurious effects of putative chemical communication that would otherwise only be present in fighting dyads. Each pair was placed in a 700 ml polycarbonate breeding tank (18x10x9 mm) isolated visually, but not chemically, by a removable opaque PVC partition for 5 consecutive days. After this period, the opaque divider was removed in all conditions, which allowed: contact between the two conspecifics in the fighting dyads; contact with the mirror in the mirror fighting treatment; and to control for stress induced just by the movement of the partition in the isolation group. In the real oponnent treatment fight duration was set to 15 min after the interaction was solved (i.e. a clear winner and loser phenotype emerged). Given that fight resolution time varied from interaction to interaction, average total interaction time in real opponent fights was 36.3±3.6min (mean±SEM). The duration of the other social treatments (mirror; isolation) was thus set to 30 min, such that all social treatments had a similar duration.

Tissue processing, RNA extraction & Gene Expression

Immediately after the social interactions fish were killed with a lethal dose of MS-222 (1000-1500 mg/l) and decapitated. Brains were rapidly collected to 500 µl of Quiazol (Qiagen) and stored at -80ºC until further processing. Total RNA was extracted according to the manufacturer's instructions (RNeasy Lipid Tissue Mini Kit, Qiagen). . RNA was then treated with DNase (RNase-free DNase set, Qiagen) to remove possible contaminations with genomic DNA and concentration and purity was estimated by spectrophotometric absorbance in a NanoDrop

ND-1000 UV-Vis Spectrophotometer (Nano-Drop Technologies). Total extracted RNA was kept at -80°C until processing.

Target Synthesis and Hybridization to Affymetrix GeneChips

RNA was processed for use on Affymetrix (Santa Clara, CA, USA) GeneChip Zebrafish Genome Arrays, according to the manufacturer's GeneChip 3' IVT Express kit user's manual. In brief, 100 ng of total RNA containing spiked in Poly-A RNA controls was used in a reverse transcription reaction (GeneChip 3' IVT Express Kit; Affymetrix) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in a 16h in vitro transcription (IVT) reaction to generate aRNA (GeneChip 3' IVT Express Kit; Affymetrix). Size distribution of the aRNA and fragmented aRNA, respectively, was assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay; 15 µg of fragmented aRNA was used in a 250-µl hybridization cocktail containing added hybridization controls. 200 µl of mixture was hybridized on arrays for 16 h at 45°C. Standard post hybridization wash and double-stain protocols (FS450_0004; GeneChip HWS kit, Affymetrix) were used on an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on an Affymetrix GeneChip scanner 3000 7G.

GeneChip Data Analysis

Scanned arrays were analyzed first with Affymetrix Expression Console software to obtain Absent/Present calls and to assure that all quality parameters were in the recommended range. Subsequent analysis was carried out with Partek Genomics Suite v. 6.6 (Partek Incorporated, St. Louis, MO). A two-way ANOVA (p-value < 0.01) was used to identify genes differently expressed taking into account batch effects (i.e. date of the microarray processing) and social treatment, after a contrast analysis between the reference group (Isolation) and the target groups (winner, loser, or mirror). To correct for multiple testing we only considered differently expressed genes after a cut-off using a false discovery rate of 10% and a minimal fold change of 1.1. Finally, hierarchical clustering of our phenotypes according to gene expression was calculated using Euclidean distances as a dissimilarity measure.

Annotation and gene ontology analysis

Gene annotation was obtained primarily from the Partek Genomics Suite software. For genes that were not annotated in the Partek software we blasted them against a locally

installed NCBI nr database (non-redundant protein database, release 2011_11) using BLASTx (v. 2.2.26) with an E-value cut-off of 10-5 , a word size of 2 and the DLOSUM62 matrix. Gene ontology (GO) analysis was also conducted using the Partek Genomics Suite v. 6.6, and the enrichment score was used to rank the most significant gene groups in the following categories: biological processes, cellular components and molecular functions (Table S2). Within each gene group we also access gene regulation (i.e. number of genes that were being up or down regulated in the group).

Confirmatory Real-time PCR

In order to validate the microarray data, the expression of the differentially expressed genes with the higher fold-changes was independently quantified by quantitative RT-PCR. The total RNA samples (also used for microarrays hybridizations) were quantified in the NanoDrop and 1-5 µg were reverse transcribed into cDNA. The synthesized cDNA (1µl) was subsequently used for quantitative PCR (QPCR). QPCR reactions (25µl) were run in a Stratagene MX3000p thermocycler in triplicate with Stratagene's SYBR green QRT-PCR Master Mix (Stratagene, Spain) and primers at 0.5 µM. Thermocycling conditions were equal for all reactions and were as follows: 5 min at 95º C, 40 cycles of 95ºC for 30 s, specific annealing temperature for each primer for 30 s and 72 ºC for 30 s. After PCR, a melting curve program from 55 to 95ºC with 0.5º C change in 10s intervals was applied and the presence of a single reaction product in each tube was confirmed. qPCR was conducted in triplicate for each individual sample and the expression of the target genes normalized to the expression of 18S to account for variations in total RNA levels between samples. Specific primer sequences (Table S3) were designed based on RNA sequences available in the zebrafish genome database (ZFIN) on Primer 3 (Premier Biosoft International, Palo Alto, CA, USA) tested for quality in the FastPCR 5.4., and PCR product sequenced to confirm the amplicon. Raw fluorescence data was submitted to PCR Miner (http://www.miner.ewindup.info/Version2; (21)) to calculate reaction efficiencies and cycle thresholds from individual wells during the reaction. For each sample, the mean CT of 18S and the target genes was calculated, and the relative initial template concentration (R0) determined from 1/1(1+E)^CT (21). The relative mRNA expression was thus given by the ratio between the target gene and 18S R0s. In order to have the same magnitude effects of microarray analysis the fold change expression for each gene was calculated. All tested genes yielded similar patterns of relative expression across treatments as the ones obtained from the microarray data (Fig. S1).

Behavioural analysis

Video recordings (Sony KDL X200, Tokyo, Japan) were analysed using the software Observer XT (Noldus, Wageningen, The Netherlands). An experienced observer analysed the behavioral interactions according to the zebrafish ethogram (17). Behaviours were divided into aggressive (bite, chase, strike) and submissive (freeze and flee),. Because we were only interested in the behavioural output resulting from the social interaction, we only analyse the pos-resolution phase of the fight where different social phenotypes (winners, losers) can be clearly identified. For the behavioural analysis of mirror fights and social isolation, the tlast 10 min of the behavioural trial were also observed. Due to the small sample size no inference statistics were computed and only descriptive data is presented. However, statistical validation of behavioural differences between the social treatments presented here in the same behavioural paradigm have been previously reported (13).

Table SI. List of significantly regulated genes after a fight. Description and accession number for each gene with a fold change greater than 1.1 are presented in descending order.

Genes	Description	Accession number	Fold change	
			Winner	Loser
npas4	neuronal PAS domain protein 4	NM_001045321	12,206	17,777
fos	v-fos FBJ murine osteosarcoma viral oncogene homolog	NM_205569	6,590	9,569
fos	v-fos FBJ murine osteosarcoma viral oncogene homolog	NM_205569	5,821	7,723
socs3a	suppressor of cytokine signaling 3a	NM_199950	5,616	6,819
nr4a1	nuclear receptor subfamily 4, group A, member 1	NM_001002173	5,117	5,842
fos	v-fos FBJ murine osteosarcoma viral oncogene homolog	NM_205569	3,969	5,698
ier1	early growth response protein 1	NM_001114453	3,442	5,533
ier2	immediate early response 2	NM_001142583	2,827	4,702
fos	v-fos FBJ murine osteosarcoma viral oncogene homolog	NM_205569	3,156	3,910
btg2	B-cell translocation gene 2	NM_130922	2,834	3,650
junb	jun B proto-oncogene	NM_213556	2,427	3,174
egr2b	early growth response 2b	NM_130997	2,301	2,907
egr2a	early growth response 2a	NM_183341	-	2,645
dhrs12	dehydrogenase/reductase (SDR family) member 12	NM_001076557	-	-2,608
plk2	polo-like kinase 2 (Drosophila)	NM_001099245	-	2,431
CH73-21G5.3	novel protein similar to hairy-related 4.2	XM_001920839	-	-2,323
mycn	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	NM_212614	-2,419	-2,209
klf7l	Kruppel-like factor 7 (ubiquitous), like	NM_001044766	-	-2,038
jdp2	Jun dimerization protein 2	NM_001002493	-	2,022
dusp5	dual specificity phosphatase 5	NM_212565	-	1,958
hirip5 / rhag	HIRA interacting protein 5	NM_001122708	-	-1,935
dusp1	dual specificity phosphatase 1	NM_213067	-	1,900
pim1	proto-oncogene serine/threonine-protein kinase pim-1	NM_001077391	1,682	1,892
cyp2j21	cytochrome P450, family 2, subfamily J, polypeptide 21	NM_201511	-	-1,886
nnt	nicotinamide nucleotide transhydrogenase	NM_214756	-	-1,834
dlg1	discs, large (Drosophila) homolog 1	NM_199526	-	1,807
cebp1	CCAAT/enhancer binding protein 1	NM_131837	-	-1,780
bdnf	brain-derived neurotrophic factor	NM_131595	1,621	1,759
nat15	N-acetyltransferase 15 (GCN5-related, putative)	NM_001082872	-	-1,744
zgc:123170	translin	NM_001025452	-	-1,735
zgc:152990	solute carrier family 20, member 2	NM_001077546	-	-1,725
lyricl	lyric-like	NM_001007135	-	-1,699
hmga1a	high mobility group AT-hook 1a	NM_213168	-1,812	-1,670
zgc:66430	testis-specific protein, Y-encoded-like 1	NM_200055	-	-1,646
foxn4	forkhead box N4	NM_131099	-	1,630
otx1	orthodenticle homolog 1	NM_131250	-	-1,621
lancl1	LanC antibiotic synthetase component C-like 1 (bacterial)	NM_001009891	-1,493	-1,607
adh8a	Alcohol dehydrogenase 8a	NM_001001946	-1,604	-1,561
rlf	rearranged L-myc fusion	XM_685832	-1,292	-1,547
zfp161	zinc finger protein 161 homolog (mouse)	NM_213536	-	-1,542
tcea2	transcription elongation factor A (SII), 2	NM_200986	-	-1,536
nfyc	nuclear transcription factor Y, gamma	NM_199639	-	-1,535
snx1	sorting nexin 1	NM_001128671	-	-1,524
lmo7a	LIM domain only 7a	NM_001128231	-	1,521
slc6a19a	solute carrier family 6 (neurotransmitter transporter), member 19a	XM_001921802	-	1,518
itpkc	inositol 1,4,5-trisphosphate 3-kinase C	XM_681366	-	-1,503
zdhhc15b	zinc finger, DHHC domain containing 15b	NM_001077781	-	-1,496
magt1	magnesium transporter 1	NM_199700	-1,468	-1,495
si:dkeyp-110e4.6	Novel protein similar to vertebrate phospholipase D family, member 4	XM_681654	-	1,492
fkrp	Fukutin related protein	NM_001042689	-	-1,491
dpf2l	D4, zinc and double PHD fingers family 2, like	NM_212696	-	-1,489
ier5	immediate early response 5	NM_001007197	-	1,484
lztr1	leucine-zipper-like transcription regulator 1	NM_001080605	-	-1,484
slc25a25	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25	NM_213257	1,524	1,473
sdad1	SDA1 domain containing 1	NM_173230	-	-1,465
or102-3	odorant receptor, family C, subfamily 102, member 3	NM_001039624	-	1,462
mc5rb	melanocortin 5b receptor	NM_173280	-	1,461
fscn1	fascin homolog 1, actin-bundling protein (Strongylo-centrotus purpuratus)	NM_001076560	-	-1,452
ccdc53	coiled-coil domain containing 53	NM_200173	-	-1,450
slc38a7	solute carrier family 38, member 7	NM_001003648	-	-1,413
fgb	fibrinogen, B beta polypeptide	NM_212774	-	1,412
mettl5	methyltransferase like 5	NM_001005949	-	-1,409
opn1mw2	opsin 1 (cone pigments), medium-wave-sensitive, 2	NM_182891	-	1,402

(*continued*)

Genes	Description	Accession number	Fold change Winner	Loser
cxxc5	CXXC finger 5	XM_681066	-	-1,402
zgc:64076	dehydrogenase/reductase (SDR family)	NM_213150	-	1,398
dhrs13	dehydrogenase/reductase (SDR family) member 13	NM_001007424	-	1,396
gpr137bb	G protein-coupled receptor 137bb	NM_001002691	-	1,391
hmga1a	high mobility group AT-hook 1a	NM_213168	-1,452	-1,388
zgc:73210	lysophospholipase II	NM_200749	-	-1,385
LOC100003487	similar to F-box only protein 34	XM_001337704	-	-1,378
zgc:153084	polynucleotide kinase 3'-phosphatase	NM_001077578	-	1,371
rab5a	RAB5A, member RAS oncogene family	NM_201485	-	-1,371
maea	macrophage erythroblast attacher	NM_199549	-	-1,367
ddx26b	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26B	NM_200434	-	1,365
LOC555852	similar to cAMP-dependent protein kinase inhibitor alpha	XR_045027	-	-1,363
hmx3	homeo box (H6 family) 3	NM_131634	-	-1,358
arglu1b	Arginine and glutamate rich 1b	NM_200162	1,357	-1,354
mchr1a	melanin-concentrating hormone receptor 1a	XM_001343108	-	1,332
hnrnpk	novel rhamnose binding lectin	NM_001100404	-	1,325
trim55b	tripartite motif-containing 55b	NM_001039982	-	1,323
ubxn4	Danio rerio UBX domain protein 4	XM_001918881	-	1,323
arl3l2	ADP-ribosylation factor-like 3, like 2	NM_200719	-	1,321
mespb	mesoderm posterior b	NM_131552	-	1,319
zgc:101663	alpha-1,3-mannosyl-glycoprotein-4-beta-N-acetylglucosa-minyltransferase C	NM_001007437	-	1,313
cnrip1a	cannabinoid receptor interacting protein 1a	NM_001003607	-1,285	-1,312
rims2	regulating synaptic membrane exocytosis 2	XM_688008	1,398	1,308
ergic2	ERGIC and golgi 2	NM_200407	-	-1,302
KNTC1	kinetochore associated 1	XM_681266	-	1,301
atp6v1b2	ATPase, H+ transporting, lysosomal V1 subunit B2	NM_182879	-	-1,300
zgc:77744	poly (ADP-ribose) polymerase family, member 16	NM_212906	-	-1,300
elf2a	E74-like factor 2a (ets domain transcription factor)	NM_001004116	-1,307	-1,296
myhz1.1	myosin, heavy polypeptide 1.1, skeletal muscle	NM_001115089	-	1,287
scd	stearoyl-CoA desaturase (delta-9-desaturase)	NM_198815	-	1,286
LOC561122	similar to X-linked neuroligin 4	XM_684526	-	-1,285
adipor1a	adiponectin receptor 1a	NM_001002467	-1,232	-1,280
picalml	phosphatidylinositol binding clathrin assembly protein, like	NM_001003741	-	1,279
six9	sine oculis homeobox homolog 9	XM_684623	-	1,275
cldn10l	claudin 10 like	NM_131771	-	1,274
cx39.9	connexin 39.9	NM_212826	1,400	1,270
phb2	Prohibitin 2	NM_199681	-	-1,262
atp6v0c	ATPase, H+ transporting, lysosomal, V0 subunit c	NM_001105136	-	-1,259
cdca5	cell division cycle associated 5	NM_001100947	-	1,257
otx5	orthodenticle homolog 5	NM_181331	-	1,242
nup43	nucleoporin 43	NM_212892	-	1,239
hoxa10b	homeo box A10b	NM_131155	1,257	1,233
Tnni2 / tnni2a.4	fast muscle troponin I / troponin I, skeletal, fast 2a.4	NM_001009901	1,346	1,229
clybl	citrate lyase beta-like	NM_001037416	-	-1,228
ap2b1	adaptor-related protein complex 2, beta 1 subunit	NM_199919	-	-1,222
rnf40	ring finger protein 40	NM_001005778	-	-1,209
pcnt1	pericentrin 1	NM_001003625	-	1,205
LOC555630	mitochondrial ribonuclease P protein 3-like	XM_678197	-	1,203
slc27a2	solute carrier family 27 (fatty acid transporter) member 2	NM_001025299	-	1,196
LOC796252	chemokine CXL-C24a	NM_001115062	-	1,188
osgepl1	O-sialoglycoprotein endopeptidase-like 1	NM_001005301	-	1,187
rap2ip	Rap2 interacting protein	NM_200149	-1,194	-1,184
ppp2cb	protein phosphatase 2, catalytic subunit, beta isoform	NM_213293	-	1,179
phf16	PHD finger protein 16	NM_201652	-	-1,173
tfip11	tuftelin interacting protein 11	NM_001002721	1,165	1,151
wu:fc54b10	MON1 homolog B (yeast)	XM_001922150	1,168	1,142
LOC100000433	hypothetical protein LOC100000433	XM_001340605	-	1,109
LOC794813	similar to Latrophilin-2 precursor (Calcium-independent x-latrotoxin receptor)	XM_001334799	-	1,106
ak2	adenylate kinase 2	NM_212596	-1,453	-
nsa2	NSA2 ribosome biogenesis homolog (S. cerevisiae)	NM_199568	-1,436	-
vps4b	vacuolar protein sorting 4b (yeast)	NM_200906	-1,429	-
LOC564494	similar to Histone H4 replacement CG3379-PC	XM_687827	1,413	-
mpv17	MpV17 transgene, murine homolog, glomerulo-sclerosis	NM_201165	1,400	-
caprin1b	cell cycle associated protein 1b	NM_213068	-1,376	-
dkey-18f23.10	cyclin T1	XM_682036	1,361	-
DKEY-79C1.2	novel protein similar to praja family protein	NM_001105116	-1,334	-
rpl7a	ribosomal protein L7a	NM_200047	-1,292	-
atp1b2a	ATPase, Na+/K+ transporting, beta 2a polypeptide	NM_131669	1,286	-

Table S2. Gene Ontology analysis of regulated genes. Several functions express significantly enriched scores in all three GO vocabularies (molecular function, biological process and cellular location) in both winners and losers. Enrichment score, p-value, number of genes in the group and gene regulation (up or down) are given for each gene group.

Gene group	Function	Condition	Enrichment score	Enrichment p-value	Genes in the group	total # genes in group	% genes in group	Regulation		GO ID
								UP	Down	
Proton transport	Biological process	Loser	7.38679	0.000619379	3	10	23.0769	3	0	15992
Regulation of transcription, DNA-dependent	Biological process	Loser	5.64758	0.00352604	18	655	2.67459	7	14	6355
ATP hydrolysis coupled proton transport	Biological process	Loser	3.83867	0.0215222	2	15	11.7647	2	0	15991
G-protein coupled receptor protein signaling pathway	Biological process	Loser	3.81177	0.022109	5	114	4.20168	1	4	7186
Dephosphorylation	Biological process	Loser	3.46778	0.0311861	3	48	5.88235	0	3	16311
Myelination of posterior lateral line nerve axons	Biological process	Loser	3.21941	0.0399788	1	2	33.3333	0	1	48932
Cortical actin cytoskeleton organization	Biological process	Loser	3.21941	0.0399788	1	2	33.3333	0	1	30866
DNA damage response, detection of DNA damage	Biological process	Loser	3.21941	0.0399788	1	2	33.3333	0	1	42769
Magnesium ion transport	Biological process	Loser	3.21941	0.0399788	1	2	33.3333	1	0	15693
Histone H4-K5 acetylation	Biological process	Loser	3.21941	0.0399788	1	2	33.3333	1	0	43981
Histone H4-K8 acetylation	Biological process	Loser	3.21941	0.0399788	1	2	33.3333	1	0	43982
Histone H4-K12 acetylation	Biological process	Loser	3.21941	0.0399788	1	2	33.3333	1	0	43983
Cellular response to gamma radiation	Biological process	Loser	3.21941	0.0399788	1	2	33.3333	1	0	71480
Amino acid transport	Biological process	Loser	3.21941	0.0399788	1	2	33.3333	1	0	6865
Transcription elongation, DNA-dependent	Biological process	Loser	3.21941	0.0399788	1	2	33.3333	1	0	6354
Mitotic sister chromatid cohesion	Biological process	Loser	3.21941	0.0399788	1	2	33.3333	0	1	7064
Nup107-160 complex	Cellular Component	Loser	5.34018	0.00479499	2	6	25	0	2	31080
Nucleus	Cellular Component	Loser	4.85945	0.00775473	30	1419	2.07039	13	20	5634
Fibrinogen complex	Cellular Component	Loser	3.21941	0.0399788	1	2	33.3333	0	1	5577
Histone acetyltransferase complex	Cellular Component	Loser	3.21941	0.0399788	1	2	33.3333	1	0	123
Clathrin coat	Cellular Component	Loser	3.21941	0.0399788	1	2	33.3333	0	1	30118
Proton-transporting two-sector ATPase complex	Cellular Component	Loser	3.21941	0.0399788	1	2	33.3333	0	1	16469
Sequence-specific DNA binding	Molecular function	Loser	9.52456	7.30E-05	15	340	4.22535	7	11	43565
Sequence-specific DNA binding transcription factor activity	Molecular function	Loser	8.46923	0.000209826	16	419	3.67816	6	13	3700
Protein dimerization activity	Molecular function	Loser	6.22975	0.00196994	4	36	10	5	2	46983
DNA binding	Molecular function	Loser	6.02211	0.00242456	19	685	2.69886	8	14	3677
Zinc ion binding	Molecular function	Loser	5.67957	0.00341503	18	653	2.68256	10	8	8270
MAP kinase tyrosine/serine/threonine phosphatase activity	Molecular function	Loser	5.61905	0.00362809	2	5	28.5714	2	0	17017
Hydrogen ion transporting ATP synthase activity, rotational mechanism	Molecular function	Loser	4.69145	0.00917338	2	9	18.1818	0	2	46933
Protein tyrosine/serine/threonine phosphatase activity	Molecular function	Loser	3.35244	0.0349989	2	20	9.09091	0	2	8138
Alcohol dehydrogenase (NAD) activity	Molecular function	Loser	3.21941	0.0399788	1	2	33.3333	1	0	4022
Regulation of transcription, DNA-dependent	Biological process	Winner	7.66551	0.000468719	11	662	1.63447	3	10	6355
Myelination of posterior lateral line nerve axons	Biological process	Winner	4.15849	0.0156311	1	2	33.3333	0	1	48932
Magnesium ion transport	Biological process	Winner	4.15849	0.0156311	1	2	33.3333	1	0	15693
Intracellular cholesterol transport	Biological process	Winner	4.15849	0.0156311	1	2	33.3333	1	0	32367
Rhombomere boundary formation	Biological process	Winner	3.87336	0.0207885	1	3	25	0	1	21654
Nucleotide phosphorylation	Biological process	Winner	3.87336	0.0207885	1	3	25			46939
Mitotic chromosome condensation	Biological process	Winner	3.65276	0.0259195	1	4	20	1	0	7076
Cilium morphogenesis	Biological process	Winner	3.32138	0.0361031	1	6	14.2857	0	1	60271
Histone H2A acetylation	Biological process	Winner	3.32138	0.0361031	1	6	14.2857	0	1	43968
SMAD protein signal transduction	Biological process	Winner	3.19039	0.0411559	1	7	12.5	0	4	60395
Histone H4 acetylation	Biological process	Winner	3.19039	0.0411559	1	7	12.5	0	1	43967
Nucleus	Cellular Component	Winner	4.45023	0.0116759	14	1435	0.966184	4	13	5634
Prefoldin complex	Cellular Component	Winner	3.65276	0.0259195	1	4	20	1	0	16272
NuA4 histone acetyltransferase complex	Cellular Component	Winner	3.32138	0.0361031	1	6	14.2857	0	1	35267
DNA-directed RNA polymerase II, core complex	Cellular Component	Winner	3.32138	0.0361031	1	6	14.2857	1	0	5665
Late endosome	Cellular Component	Winner	3.19039	0.0411559	1	7	12.5	0	1	5770
Mitochondrial intermembrane space	Cellular Component	Winner	3.19039	0.0411559	1	7	12.5			5758
Sequence-specific DNA binding transcription factor activity	Molecular function	Winner	8.0624	0.000315169	9	426	2.06897	3	9	3700
DNA binding	Molecular function	Winner	7.28001	0.000689182	11	693	1.5625	3	11	3677
Sequence-specific DNA binding	Molecular function	Winner	4.64428	0.00961644	6	349	1.69014	2	7	43565
Alcohol dehydrogenase (NAD) activity	Molecular function	Winner	4.15849	0.0156311	1	2	33.3333	1	0	4022
Protein dimerization activity	Molecular function	Winner	3.99643	0.0183812	2	38	5	0	5	46983
Phosphotransferase activity, phosphate group as acceptor	Molecular function	Winner	3.87336	0.0207885	1	3	25			16776
Nucleotide kinase activity	Molecular function	Winner	3.65276	0.0259195	1	4	20			19201
Nucleobase-containing compound kinase activity	Molecular function	Winner	3.65276	0.0259195	1	4	20			19205
Adenylate kinase activity	Molecular function	Winner	3.65276	0.0259195	1	4	20			4017
Low-density lipoprotein particle receptor binding	Molecular function	Winner	3.32138	0.0361031	1	6	14.2857	1	0	50750
R-SMAD binding	Molecular function	Winner	3.19039	0.0411559	1	7	12.5	0	4	70412
Protein phosphatase binding	Molecular function	Winner	3.19039	0.0411559	1	7	12.5	1	0	19903
Histone acetyltransferase activity	Molecular function	Winner	3.07514	0.046183	1	8	11.1111	0	1	4402

Table S3. Primer sequences used for real-time PCR validation

Target gene	Acession #	Forwar primer	Reverse primer	Product size (pb)
18s	NM_173234.1	CCGCTATTAAGGGTGTTGGA	CCCTCTCAACCTCATCCTCA	108
EGR2b	NM_130997.2	CTCCACCTGTCCCATCTC	TAGGGAACAACCCAGAGT	77
nr4a1	BC074092.1	CGGTTTCTCTGCTTTCTTGG	GCATTTGATTTGCACCATTG	229
btg2	NM_130922.1	CCACTGCCTGGCTCTTTTAG	CCGACACCAACCAATAAACC	155
Junba	NM_213556.3	GAGGGATGCTACGGACTCTG	ATGTTTTCAAAGGGCAGCAC	234
socs3	BC049326.1	CCAACACGGGTCTTCTGTG	CGAGTCACATCCATCGTCA	139
npas4	NM_001045321.1	GACACGGGTTGAGAATGGTT	GCACCAAGCACCCTGTAAAT	165

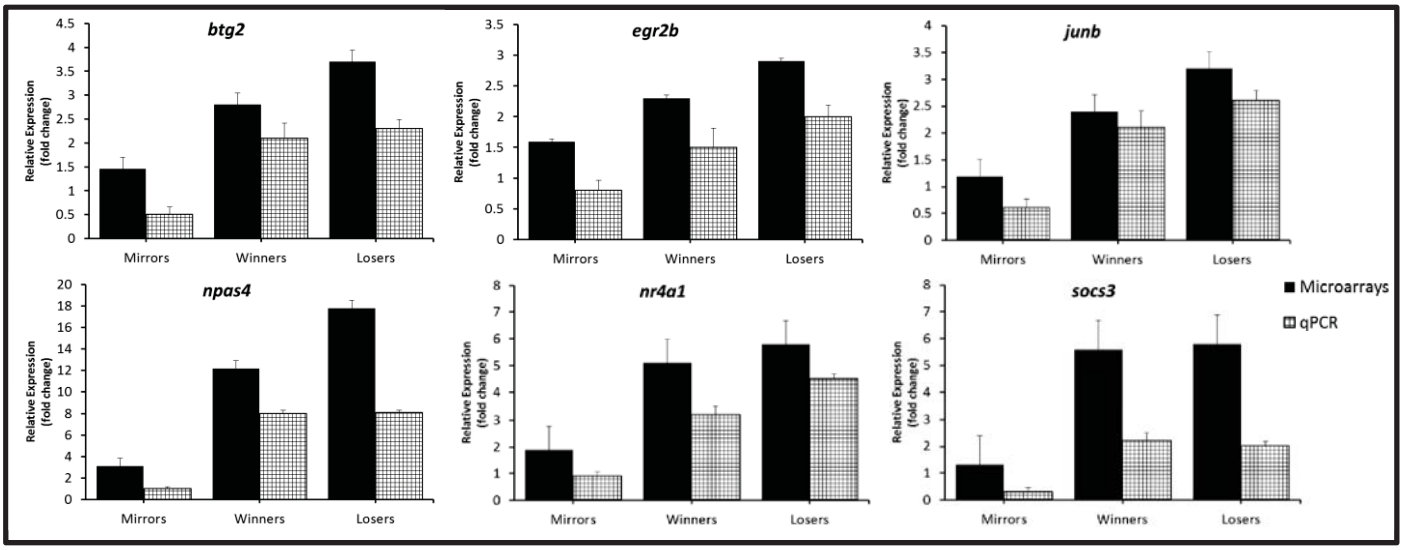

Figure S1. Comparison between the expression levels of differentially expressed genes with high fold-changes in the microarrays with their expression in confirmatory quantitative RT-PCR (qPCR). Black bars = qPCR; checkered bars = microarray technique (mean±SEM).

External Databases

The microarray data reported in this paper were archived in OMNIBUS with the reference number GSE56549: (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=oloncqgujnehpef&acc=GSE56549).

Chapter II – How a simple social stimulus can modulate the transcriptome of specific brain regions of the Mozambique tilapia?

(i) A Three-Dimensional Stereotaxic MRI Brain Atlas of the Cichlid Fish Oreochromis mossambicus

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A three-dimensional stereotaxic MRI brain atlas of the cichlid fish *Oreochromis mossambicus*

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Keywords: brain, cichlid, histology, magnetic resonance imaging, tilapia

Abstract

The African cichlid *Oreochromis mossambicus* (Mozambique tilapia), has been used as a model system in a wide range of behavioral and neurobiological studies. The increasing number of genetic tools available for this species, together with the emerging interest in its use for neurobiological studies, increased the need for an accurate hodological mapping of the tilapia brain to supplement the available histological data. The goal of our study was to elaborate a three-dimensional, high-resolution digital atlas using magnetic resonance imaging, supported by Nissl staining. Resulting images were viewed and analysed in all orientations (transverse, sagittal, and horizontal) and manually labelled to reveal structures in the olfactory bulb, telencephalon, diencephalon, optic tectum, and cerebellum. This high resolution tilapia brain atlas is expected to become a very useful tool for neuroscientists using this fish model and will certainly expand their use in future studies regarding the central nervous system.

Introduction

Cichlid fish are one of the most successful taxa in vertebrate evolution. With over 3,000 species described so far, the family Cichlidae is the most species-rich family of vertebrates offering a scope of phenotypic and behavioral variation amenable to comparative analysis that makes them a popular model for evolutionary studies (e.g. [1-6]). Cichlid fish also present a wide variation, within closely related species, of their social behavior, ranging from territorial to shoaling species, and of their mating and parental care systems, including monogamous and polygamous breeding and paternal, biparental and maternal mouth-brooding or substrate-brooding species (e.g. [1,6,7]). The complexity and plasticity of their social behavior are also remarkable(e.g. cooperative breeding, [8]; for a review of social plasticity in cichlid fish see [9] and of their cognitive abilities (e.g. transitive inference in the social domain, [10]), and recently, the impact of social complexity (i.e. dimension of social groups and existence of long-term relationships) on brain evolution in cichlids has been demonstrated [11-13]. Thus, cichlid fish offer a superb opportunity to study the neural and endocrine mechanisms underlying social plasticity and complexity and their evolution. In this regard, two African species have been mainly used in laboratory studies, the haplochromine *Astatotilapia burtoni* (e.g. [10,14,15]) and the tilapiine *Oreochromis mossambicus* (e.g. [9,16-18]). This evo-mecho approach requires the identification and precise coordinates of relevant brain areas in a three-dimensional space, which would allow their precise measurement and manipulation (e.g. experimental lesions, micro-injections) for gain and loss of function studies. However, to the best of our knowledge, only partial 2D brain atlases based on histological sections are available for these species or for any other cichlid species [19-23].

In the last two decades the use of magnetic resonance imaging (MRI) to develop digital atlases was initiated with accurate human brain atlases (e.g. [24,25]), but has been extended to non-human animals with a particular focus on mammals (e.g. mouse lemur, [26]; nemestrina monkey, [27]; mouse, [28]; rat, [29]; Rhesus macaque, [30]; marmoset monkey,

[31]). The progressive technological developments of high-magnetic field MRI techniques also allowed imaging smaller animals, without losing resolution, such as the zebrafinch [32], the zebrafish [33], and the canary [34]. The three-dimensional and digital nature of MRI brain atlases offers more visualization and computational power when compared to classical 2D atlases. Although MRI atlases have a lower resolution than histological atlases they present numerous relevant advantages related with processing and analysis of relevant CNS structures: histological atlases use paraffin- or parlodion-embedded techniques which can cause tissue shrinkage during the dehydration and processing steps; after sectioning, the rehydration and staining methods are very hard to reproduce accurately from section to section; MRI-atlases are superior when analysing and measuring volumes of longer structures (like axon tracts and cranial nerves) due to its three dimensional nature, allowing a complete overview of the studied structure [35]. Thus, MRI neuroimage databases will have a crucial role in disseminating information about brain structure and function, not only in terms of the accurate description of species-specific brain features but also as a tool for comparative studies [36].

Here, we present the first three-dimensional stereotaxic atlas of the brain of a highly social cichlid fish (Mozambique tilapia, *Oreochromis mossambicus*) using MRI combined with a histological map as a guiding reference to label smaller brain nuclei, therefore relating the soft tissue contrast obtained with MRI with the cytoarchitectonic information provided by histology.

Results

Here we present the first three-dimensional brain atlas for a cichlid fish species with complex social behavior. The Mozambique tilapia 3D brain atlas is made available online at www.ispa.pt/ui/uie/ibbg/TilapiaBrainAtlas enabling the navigation through the whole brain.

MRI data are provided in raw, Amira and Analyse formats, which will allow users to visualize the atlas as well as the delineations of brain nuclei using the software Amira, but also

other image visualization platforms, e.g. MRICro. CT images of the skull and the skull delineation are also provided at the same location.

By using MRI in combination with classic histology, we developed a detailed three-dimensional atlas of the Mozambique tilapia brain, depicting several major and minor brain structures. Using T_2-weighted and Nissl staining images in parallel for corresponding brain sections, a total of 54 brain structures (see Table 1) have been identified at an isotropic resolution of 50µm. Our sequence and specimen preparation, which included Dotarem as a paramagnetic contrast agent, enhanced the differentiation between regions in MRI images based on density, size and shape of neuronal cells. Thus, the depiction of nuclei in MRI images, is not much different from that using classic histology, since it is also possible to identify different tissue textures based on image contrast and pixel density pattern and position differences, to identify different cell agglomerations and nuclei. In contrast with classic paper histology atlases it is also possible to scroll readily between sections which provides critical insight when delimiting nuclei. Finally, with MRI one can label nuclei not only in a transverse perspective but simultaneously in all three dimensions. Nevertheless, the delineation of each nucleus was further supported by comparing MRI images to corresponding Nissl stained histological sections (Fig. 1). Therefore, all minor brain regions labelled on each MRI image, were subsequently rectified and confirmed using this comparative methodology. Although most structures are more conspicuous and detailed regarding cell morphology on the Nissl stained slides, they are nonetheless identifiable on the MRI images.

Three-dimensional rendering of the delineated structures has been computed using Amira, and the rendering images of the whole brain depicting major brain divisions as well as the 54 delineated nuclei are provided in Fig. 2. These images provide a good approximation of the shape of each structure and allow an easy estimation of the relative volume of each nucleus (Table 1).

Table I – List of brain macroareas and tracts identified, as well as, all minor brain divisions, their abbreviation and chromatic identification on the 3D MRI reconstruction.

Major Brain Divisions	Structures	Abbreviations	Colour
Telencephalon	anterior subdivision of the dorsomedial telencephalon	DMa	
	anterior part of the dorsal telencephalon	DA	
	anterior subdivision of the dorsolateral telencephalon	DLa	
	granular layer of the olfactory bulb	BO_{gra}	
	glomerular layer of the olfactory bulb	BO_{gl}	
	dorsal part of the dorsal subdivision of the dorsomedial telencephalon	DMdd	
	dorsal subdivision of the dosolateral telencephalon	DLd	
	posterior subdivision of the dorsolateral telencephalon	DLp	
	posterior part of dorsal telencephalon	Dp	
	ventral subdivision of the dorsolateral telencephalon	DLv	
	dorsal division of the dorsal telencephalon	DD	
	ventral subdivision of the ventral part of the dorsomedial telencephalon	DMvv	
	dorsal part of the ventral subdivision of the dorsomedial telencephalon	DMvd	
	ventral part of the dorsal subdivision of the dorsomedial telencephalon	DMdv	
	medial part of the ventral subdivision of the ventral telencephalon	VVm	
	dorsal part of the ventral telencephalon	Vd	
	supracommissural part of the ventral telencephalon	Vs	
Diencephalon	anterior part of the periventricular preoptic nucleus	PPa	
	posterior part of the periventricular preoptic nucleus	PPp	
	anterior thalamic nucleus	A	
	glomerular nucleus	G	
	nucleus anterior tuberis	TA	
	dorsolateral subdivision of the hypothalamus	ILdl	
	ventromedial subdivision of the inferior lobe of the hypothalamus	Ilvm	
	ventromedial thalamic nucleus	VM	
	inferior subdivision of the torus lateralis	TLAi	
	torus lateralis	TLa	
	dorsal subdivision of nucleus diffusus lateralis of the inferior lobe	DFld	
	nucleus diffusus lateralis of the inferior lobe	DFl	
	ventral subdivision nucleus diffusus lateralis of the inferior lobe	DFlv	
	nucleus diffusus medialis of the inferior lobe	DFm	
	central posterior thalamic nucleus	CP	
	lateral part of nucleus recessi lateralis	nRLl	
	periventricular nucleus of the posterior tuberculum	TPP	
	dorsal posterior thalamic nucleus	DP	
	central nucleus of the inferior lobe	CE	
	corpus mamillare	CM	
	nucleus recessi lateralis	RL	
Mesencephalon	optic tectum	TeO	
	optic tectum layer 1	TeO layer 1	
	torus semicircularis	TS	
	torus longitudinalis	TL	
Rombencephalon	eminentia granularis	EG	
	molecular layer of the lateral part of the valvula cerebelli	Val mol	
	granular layer of the lateral part of valvula cerebelli	Val gra	
	molecular layer of the medial part of the valvula cerebelli	Vam mol	
	central gray	GC	
	crista cerebellaris	CC	
	molecular layer	mol	
	molecular layer of corpus cerebelli	Ccemol	
	granular layer of corpus cerebelli	Ccegra	
Tracts	tractus opticus ventralis	oTv	
	tractus opticus	tO	
	olfactory tract	tolf	

Using the intrinsic three-axis nature of MRI-based atlases, we established a stereotaxic coordinate system. The centre *x, y,* and *z* coordinates for each structure can be found in Table 2. As a zero point of the reference frame, we propose the intersection between the mid-sagittal and the mid-horizontal planes and the anterior commissure (AC). The latter, can be easily identifiable both on MRI and Nissl histology images, and the Y/Z (rostral/caudal and dorsal/ventral) axis passing through this point corresponds to the reference axis often used by electrophysiologists.

Choosing an internal rather than an external landmark system was motivated by the fact that the shape of the fish's head may vary between sexes (males exhibit a concave dorsal head profile) and between adult and juvenile animals. Nonetheless, this approach will allow neurobiologists to accurately pinpoint different specific brain regions, when implanting cannulas or doing electrophysiology recordings. To facilitate these experimental methodologies we also imaged an entire tilapia head, where it is possible to visualize the relative position of the brain regarding its neighbouring structures (available online).

We have also collected computerized tomography (CT) images that provide relevant information concerning the bony structure protecting and surrounding the brain. Using the Amira software, a three dimensional representation of this CT information has been registered with the MRI data set and a superimposed image of both data sets is illustrated in Fig. 3. This approach allows the integration of all collected information, which provides spatial coordinates regarding structures in the brain and around it.

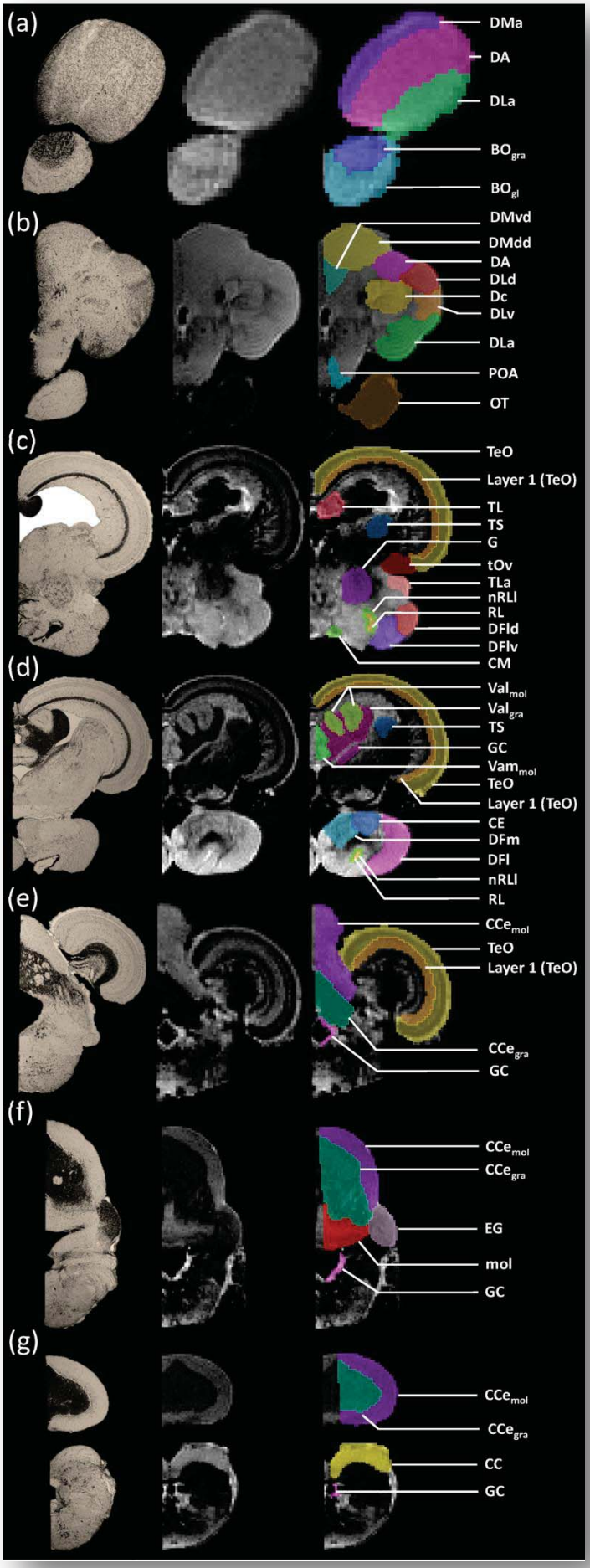

Fig. 1 – Comparison between Nissl stained histology sections (a) and MRI sections (b). On the left hand side is represented the olfactory bulbs and the beginning of the telencephalon. On the middle, we can see the end part of the optic tectum and diencephalon. Finally, on the right side is portrayed the cerebellum.

Table II – List of smaller brain divisions organized by major areas and edifying their volume and *x*, *y* and *z* coordinates. The coordinates of the structures were considered with respect to the origin at anterior commissure (in mm).

Major Brain Divisions	Structures	Volume (mm^3)	Center X	Center Y	Center Z
Telencephalon	DMa	0,197	0,432	-1,522	0,419
	DA	0,579	1,323	-1,110	1,098
	DLa	1,143	1,539	-0,954	-0,165
	BO_{gra}	0,248	0,480	-1,328	-0,543
	BO_{gl}	0,507	0,501	-1,155	-0,824
	DMdd	2,223	0,762	-0,440	1,916
	DLd	0,793	2,001	-0,615	0,970
	DLp	0,614	1,876	0,274	0,301
	Dp	0,706	1,261	0,464	-0,017
	DLv	0,269	2,160	-0,766	0,448
	DD	0,360	1,502	0,298	1,391
	DMvv	0,809	0,256	-0,333	1,049
	DMvd	0,252	0,181	0,198	1,693
	DMdv	0,607	0,797	0,278	1,402
	VVm	0,040	0,103	-0,509	-0,107
	Vd	0,050	0,186	-0,582	0,439
	Vs	0,018	0,155	-0,122	0,206
Diencephalon	PPa	0,280	0,130	0,553	-0,598
	PPp	0,017	0,070	1,693	-0,677
	A	0,049	0,142	1,698	0,150
	G	0,264	0,915	2,508	-0,934
	TA	0,239	1,356	-0,167	0,901
	ILdl	0,092	1,096	2,108	-2,181
	ILvm	0,017	0,108	1,696	-0,330
	VM	0,030	1,844	1,901	-1,286
	TLAi	0,168	1,897	1,988	-0,879
	TLA	0,257	1,861	2,721	-1,610
	DFld	0,856	1,476	3,626	-1,660
	DFl	0,262	1,484	2,705	-2,111
	DFlv	0,573	0,335	3,621	-1,534
	DFm	0,053	0,304	1,981	-0,071
	CP	0,138	0,982	2,711	-1,821
	nRLl	0,029	0,118	1,919	-0,541
	TPP	0,029	0,179	1,988	0,230
	DP	0,405	0,899	3,596	-1,126
	CE	0,124	0,358	2,605	-1,614
	CM	0,240	1,356	-0,167	0,901
	RL	0,072	1,001	2,652	-1,810
Mesencephalon	TeO	5,418	1,676	2,554	1,134
	TeO (layer 1)	1,841	1,661	2,734	1,241
	TS	0,449	1,387	2,817	0,752
	TL	0,145	0,226	2,404	1,003
Rombencephalon	EG	0,537	1,223	4,815	0,877
	Val_{mol}	0,230	0,566	3,252	1,368
	Val_{gra}	0,479	0,617	3,128	1,009
	Vam_{mol}	0,126	0,071	3,272	0,793
	GC	0,340	0,250	4,772	0,074
	CC	1,139	0,726	6,063	0,620
	mol	1,149	0,491	5,299	1,295
	CCe_{mol}	2,605	0,533	4,977	2,467
	CCe_{gra}	2,554	0,265	4,973	2,082
Tracts	tOv	0,433	1,660	2,062	-0,377
	tO	1,641	0,961	0,649	-0,933
	tolf	0,125	0,212	-2,111	-0,888

Discussion

Three-dimensional brain atlases have an enormous potential as gateways for navigating, accessing, and visualizing neuroscientific data [37]. An increasing number of recently published 3D MRI based brain atlases for emerging model organisms (e.g. zebrafinch [32], zebrafish [33] and canary [34]) highlight the advantages of using the MRI technique, despite their lower resolution when compared to classic histology and putative problems related with adjusting contrast and signal-to-noise ratio. These advantages are three-fold. First, digital MRI brain atlases, unlike classic histology sections, are not affected by shrinkage and physical distortions during sectioning and embedding of post-mortem brains.

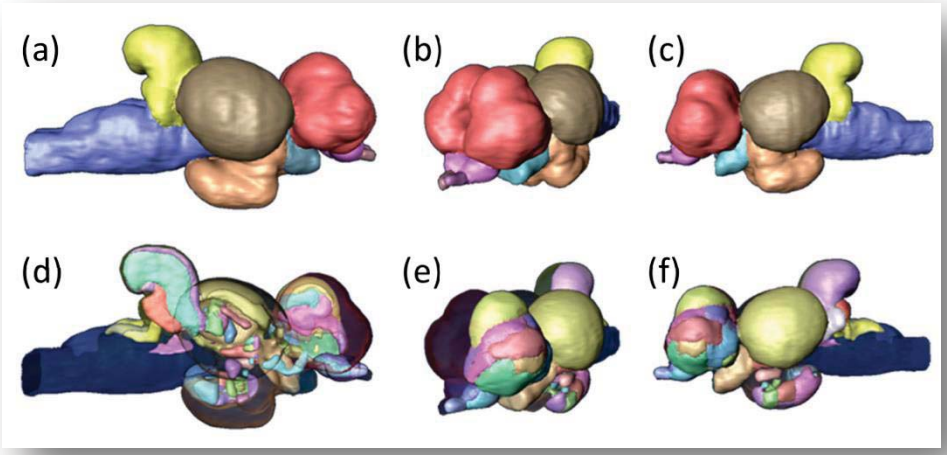

Fig. 2 – Rendering of the whole brain, depicting the major areas (a, b, c) as well as all the 54 delineated structures (d, e, f). Three different angles are presented to maximize the number of brain regions per image: (a), (d) right view; (b), (e) partial frontal view; (c), (f) left view. In the first row of images it is possible to define six major areas: telencephalon (red), olfactory bulbs (pink) and part of the olfactory tracts (purple), optic tectum (brown) and part of the optic tracts (light blue), diencephalon (orange), cerebellum (yellow) and the brain stem (blue). For a complete list of the small nuclei identified and the color code for the remaining images see Table I.

Thus, this technique provides a more precise way of processing neuroanatomical data, generating very precise stereotaxic coordinates, which can be used in electrophysiology and neuropharmacological studies. Second, and despite being limited by their resolution and contrast, MRI histology surpass the methodological constraints of classic histological sectioning techniques when analyzing complex structures [38]. It allows the morphological examination

of anatomical brain structures in a three-dimensional space, the direct visualization of shapes and volumes of different brain structures, and a computerized sectioning of complex structures at arbitrary angles [32]. To ensure a rapid progress in this area, it will require increasing contribution of neuroinformatics, akin to the growing role of bioinformatics in other areas of biology. Finally, digital MRI atlases can be very useful tools to make generalizations about localization of various brain regions, their function and spatial structure at both the macroscopic and microscopic levels and to allow the comparison between different species.

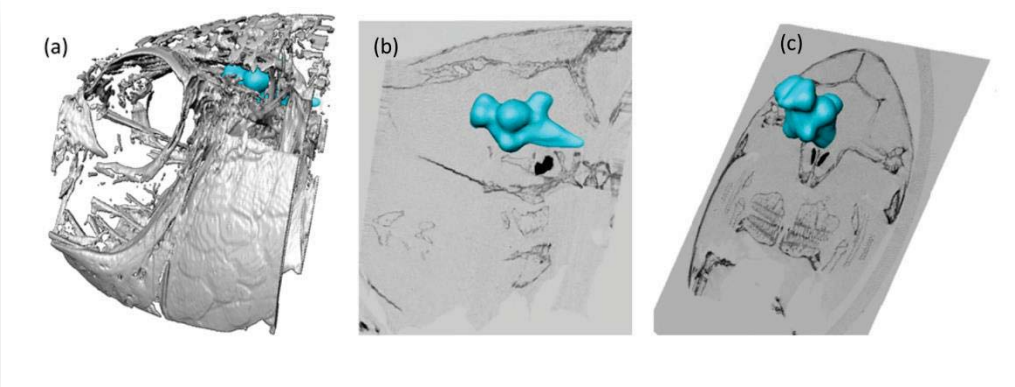

Fig. 3 – Overlap of MRI brain images (blue) with CT head data (light grey) in the Amira environment. (a) depicts a 3D reconstruction of the tilapia head based on the CT data set overlaid with a 3D tilapia brain. (b) and (c) show 2D sections of the head CT (sagittal and transverse views, respectively) and the tilapia's brain position in those perspectives.

In this paper we have managed to identify 54 brain nuclei in the brain of the Mozambique tilapia, which represents only roughly 30 % of the brain areas that have been identified in the available 2D brain atlases for this species [21,22]; where ca. 170 distinct structures have been described). The obvious reduction in the number of identifiable nuclei, due to the limitations in resolution characteristic of using the MRI technique, is surpassed by the neuroanatomical advantage of visualizing, in the same brain, volumes and shapes of different nuclei in a three dimensional space and to be able to determine their location based on a more precise coordinate system. Consequently, this provides a powerful tool for

neuroscientists to better calculate the ideal orientation of the brain for electrophysiological recordings, stereotactic injections or brain sectioning [32]. The combined use of histological and MRI images allows a better understanding of the spatial relationships of different brain structures by linking the resolution provided by the cytoarchitectural detail of classic histology, with the 3-D representations provided by the MRI technique (e.g. [31,34]).

A comparison between our 3D MRI atlas to that of zebrafish [33] shows that here we can distinguish a larger number of telencephalic and diencephalic nuclei but a lower number of the smaller nuclei located in more caudal areas (e.g. rhombencephalon, brain stem). These structures are clearly identifiable in the histological sections, but very hard to delimitate precisely in our MRI sections. This is due to the fact that we have used a less powerful MRI scanner than the one used for zebrafish (i.e. a 9.4 T that allowed an isotropic resolution of 50 µm in tilapia vs. a 16.4 T that allowed an isotropic resolution of 10 µm in zebrafish). Thus, the availability of more potent MRI scanners in the near future will play a pivotal role in the development of higher resolution 3D brain atlases for small model organisms.

Although cichlid species are excellent models for comparative social neuroscience studies, given the complexity and diversity of their social systems described above, the data published so far has used very gross neuroanatomical measures [11-13] and detailed neuroanatomical data is currently only partially available for two species [telencephalon and diencephalon of *Astatotilapia burtoni*: 19, 23; and whole brain of *O. mossambicus*: 21 and this paper]. A comparison of forebrain of these two species shows a very similar organization that is typical of percomorphs. The dorsal telencephalon of both species is divided into three highly elaborated (i.e. with many identifiable cell groups) areas, dorsolateral (Dl), dorsomedial (Dm) and dorsocentral (Dc), and two more uniform dorsal (Dd) and posterior areas (Dp). The subdivisions within each of these areas do not always match between the two species but at present it is difficult to understand to what extent these differences in nomenclature reflect real cytoarchitectural differences or different interpretations among authors. Future studies

using genetic markers may help to solve these divergencies. Two cell groups are clearly identified in both cichlid species that have not been described before in other teleost species: a granular zone in Dld (named Dl-g in *A. burtoni*) and Dcm (named Dm-2 in A. burtoni) (see sections 3/24 to 5/24 on the accompaining website to this paper). Once more, future studies are needed to establish the function of these cell groups that may represent specializations of the cichlid telencephalon. At the level of the ventral telencephalon the main cell groups described for other teleosts were also found in both species: ventral (Vv), dorsal (Vd) and supracommissural (Vs) nucleus. The diencephalon is also highly conserved in both species, with minor differences between the two species. In the hypothalamus, the diffuse nucleus of the inferior lobe in tilapia is preceded by the dorsolateral subdivion of the inferior lobe (ILdl), which will further subdivide in the dorsal and ventral subdivision of nucleus difusus lateralis of the inferior lobe, DFld and DFlv respectively. In contrast, in *A. burtoni* the diffuse nucleus of the inferior lobe (Dn) is located anatomically at the same positions of ILdl and no further divisions occur [19]. Also in the posterior tuberculum, the mammillary body lies ventrally to the preglomerular commissural nucleus (PGCn) in *A. burtoni* whereas in tilapia this structure is located ventral to the Nucleus of the posterior tuberculum (TP). In conclusion, although the three-dimensional brain atlas of tilapia presented here cannot be used accurately with other cichlid species, it offers a detailed description of a cichlid brain which, given the similarities described above between the two cichlid species studied so far, can be used with caution as a reference guide for investigators starting to work in other cichlid models.

In summary, the high resolution 3D brain atlas presented here is expected to become a very useful tool for neuroscientists already using tilapia as a model organism and will contribute to make this species more usable in future studies of the central nervous system. As a first step in this direction we have created a free access website for the tilapia 3D brain atlas and we are developing the tools that will allow the annotation by authorized visitors of the available online brain atlas with multiple information (e.g. distribution of different receptors,

neurotransmitters and neuropeptides; gene expression patterns; adult cell proliferation areas and newborn cell migration routes; etc.).

Materials and methods

Specimen Preparation

To collect MRI images, two males and two females (standard length: 10.7±1.8 mm) were perfused transcardially, first with a phosphate-buffered saline solution (PB 0.2 M), to clear the vasculature, followed by a solution of Paraformaldehyde (2 %) in Dotarem® (1 %), to fix the tissue with a paramagnetic MR contrast agent. The fish were postfixed in a mixture of PFA/Dotarem for 5 days. The day before imaging, the brains of three fish were removed from the skull and transferred to a polypropylene tube filled with Fluorinert®, a proton-free susceptibility-matching fluid and scanned with the highest resolution to enable a further identification of brain nuclei (Brain Imaging). The other perfused fish (N=1 adult male) was scanned to stereologically study the brain's position inside the head and skull (Head Imaging). Although three data sets were registered to create a model tilapia brain unfortunately, due to technical issues, the quality of the registration was limited in comparison to individual data sets and therefore, we have used a single dataset from an adult male. However, it should be stressed that the three scanned brains were visually compared, to ascertain the representativity of the data set shown, and no differences were observed.

This study was performed in strict accordance with the recommendations of the Direcção Geral de Veterinária, the Portuguese National Authority for Animal Health, and the protocol was approved by their ethics committee (Permit Number: 0420/000/000/2007). All surgery was performed under MS222 anesthesia, and every effort was made to minimize suffering.

Histological data

For the histology, four adult tilapia (2 males and 2 females; standard length: 9.6±1.1 mm) were perfused using a similar protocol to the one described above but without the MR contrast agent. After perfusion, the brains were removed from the skull, post-fixed for 1h in PFA (2 %) and transferred to a formalin solution (10 % buffer). After fixation, brains were dehydrated (Leica TP1020) and embedded in paraffin before they were cut in transverse sections at 10 µm and mounted serially on glass slides. The sections were then deparaffinised for 10 min at 70ºC, rehydrated and stained with a Nissl staining protocol. Finally, the sections were dehydrated and coverslipped with DPX mounting medium (Merck). Since there were no obvious sex differences in brain anatomy the histology figures used here represent the brain of an adult male, which is consistent with all other figures shown.

MR image acquisition

Brain Imaging - MRI scanning was performed on a 9.4T horizontal bore Magnetic Resonance Imaging system (Bruker BioSpin MRI GmbH, Ettlingen, Germany) using the standard Bruker cross coil setup, being a quadrature transmit volume coil (inner diameter 72 mm) and a quadrature receive surface coil, designed for mice brain. Horizontal images of the Tilapia brain were acquired using a fat-suppressed T_2-weighted three-dimensional RARE sequence with the following parameters: acquisition bandwidth of 33 kHz, TE/TR=30/350 ms, echo train length=2, 8 averages, a field of view of (13.5×8×10) mm^3 and an acquisition matrix of (270×160×200), resulting in a nominal spatial resolution of (50×50×50) μm^3. The total acquisition time was 12.6 hours.

Head Imaging - Images were acquired using the same MRI equipment, using the same quadrature volume coil both for transmission and receiving. For the whole head imaging was used a fat-suppressed T_2-weighted three-dimensional RARE sequence with the following parameters: acquisition bandwidth of 50 kHz, TE/TR=26/350 ms, echo train length=2, 4 averages, a field of view of (80×40×30) mm^3 and an acquisition matrix of (400x200x150),

resulting in a nominal spatial resolution of (200×200×200) μm^3. The total acquisition time was 5.8 h.

CT acquisition

In order to acquire images of the skull, the whole head of a perfused adult male was also scanned with an X-ray micro-CT system (Skyscan 1076, Belgium, focal spot size of 5µm, energy range of 20–100 keV). An image data with matrix (1649×2448×372) and resolution of (18×18×18) μm^3 was achieved.

Image post-processing

Brain and nuclei delineation was done manually using Amira software (Mercury Computers Systems, USA). Segmentation was done slice-by-slice in a transverse perspective and posteriorly confirmed systematically in the two other orthogonal views (axial and sagittal). Major brain subdivisions (Telencephalon, Diencephalon, Mesencephalon, Rhombencephalon) were first delineated, followed by structures which presented more distinct boundaries (e.g. olfactory bulbs, optic tectum and corpus cerebellis), which helped identifying smaller nuclei. In addition, histology sections were used as reference for the location and boundaries of smaller structures. Histology sections were digitised, juxtaposed to MRI images and together analysed in order to more precisely delineate all nuclei. Nuclei which did not present clear contrast differences/boundaries in the MRI were not considered, despite being histologically identifiable.

Nuclei volume measurements were calculated using the Material Statistics function in the Amira software. Uploading the MRI and nuclei delimitation data with the free software MRIcro, using the same procedures described by Poirier et al. [32], allowed to extract the stereotaxic coordinates for each nuclei.

Co-registration of CT images to the MRI brain atlas was performed with Amira, by an affine transformation of the CT data – down-sampled to (70×70×70) μm^3 – to the MRI.

Neuroanatomical analysis

There is a rich tradition in comparative neuroanatomy of fish that has prompted the emergence of different nomenclatures for brain structures of ray-finned fishes (e.g. [39-44]. In this paper we adopted the nomenclature used by [21] in the previously published 2D brain atlas of this species. This nomenclature follows the scheme proposed by [42] and [43], but introduces new terms that reflect some peculiarities of the cichlid brain.

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(ii) Social odors conveying dominance and reproductive information induce rapid neuromolecular changes in a cichlid fish

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Social odors conveying dominance and reproductive information induce rapid neuromolecular changes in a cichlid fish

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Keywords: cichlid; olfaction; olfactory bulb; telencephalon; microarray; transcriptomics

Abstract

Social plasticity is a pervasive feature of animal behavior. Animals adjust the expression of their social behavior to the daily changes in social life and to transitions between life-history stages, and such ability impacts on their Darwinian fitness. This behavioral plasticity may be achieved either by rewiring or by biochemically switching nodes of the neural network underlying the social behavior in response to perceived social information. Independently of which type of proximate mechanism underlies social plasticity, at the neuromolecular level it must rely on social regulation of gene expression, such that different neurogenomic states emerge in response to different social stimuli and the switches between states are

orchestrated by signaling pathways that interface the social environment and the genotype. In here we test this hypothesis by characterizing the changes in the brain profile of gene expression in response to social odors in the Mozambique Tilapia, *Oreochromis mossambicus*. This species has a rich repertoire of social behaviors during which both visual and chemical information are conveyed to conspecifics. Particularly, dominant males increase their urination frequency during agonist encounters and during courtship to convey chemical information reflecting their dominance status. The recording of the electro-olfactogram showed that the olfactory epithelium discerns olfactory information from dominant and subordinate males as well as from pre- and post-spawning females. We used custom-made microarrays to perform a genome-scale analysis of the brain molecular systems involved in processing olfactory stimuli.

Our results show that different olfactory stimuli from conspecifics' have a major impact in the brain transcriptome, with different chemical social cues eliciting specific patterns of gene expression in the brain. These results confirm the role of rapid changes in gene expression in the brain as a genomic mechanism underlying behavioral plasticity and reinforce the idea of an extensive transcriptional plasticity of cichlid genomes, especially in response to rapid changes in their social environment.

Background

Group living animals have to adjust the expression of social behavior to the nuances of daily social life and to transitions between life-history stages, and their ability to do so impacts on their Darwinian fitness (Oliveira, 2009). This socially driven behavioral plasticity suggests that social information should trigger changes in brain neurogenomic states that underlie different behavioral repertoires. Thus, reprogramming the functional genome in response to the social environment allows an animal to switch between adaptive behavioral states (Harris and Hofmann, 2014; Renn and Schumer, 2013). Gene expression profiling enables the study of this dynamic relationship between genotype and behavior (Hofmann, 2003) and to unveil the

genetic networks behind complex behaviors. In addition, the development of whole-genome sequencing, microarrays and other genomic resources for non-traditional model organisms, but with complex social repertoires, has provided relevant insights on how complex genotypes are translated to produce meaningful behaviors (Robinson et al., 2005; Robinson et al., 2008).

In recent years an increasing number of studies has described the influence of social environment and of social interactions on genome structure and on transcriptional and neural activity (Robinson et al., 2008). For example, caste differentiation (between workers/queen) in the honey bee (*Apis mellifera*), a key feature in eusocial insects, is influenced not only by heritable traits but also by variations in the regulation of molecular pathways linked with several life-history traits, such as nutrition, metabolism, and reproduction (Evans and Wheeler, 2001; Smith et al., 2008). The activity of aggression-related genes in this species also seems to be under both inherited and environmental influences, varying with age, exposure to alarm-cues and depending on colony environment (Alaux et al., 2009). The study of gene expression signatures of life history transitions has also been a focus in teleost fishes. For example, life history traits of salmonids have also been addressed in a number of studies showing variation in brain expression profiles related with alternative reproductive and migratory tactics (Aubin-Horth and Renn, 2009; Aubin-Horth et al., 2005b) and their interaction with the rearing environment (Aubin-Horth et al., 2005a). All the results on the impact of the social environment on genome activity highlight new possibilities concerning how social stimuli, as well as more complex interactions between conspecifics, can influence and shape gene translation into producing appropriate behavioral responses, according to external and internal cues and also to the animals' past experience.

Most of the studies discussed above characterize fixed and irreversible behavioral phenotypes, which correspond to switches between "static" neurogenomic states. But the interaction between the genome and the environment is also expected to be present in shorter time frames and to be reversible in order to accommodate labile and transient changes

in behavioral states in order for flexible adaptive behavior to evolve (Bell and Robinson, 2011; Wong and Hofmann, 2010). Behaviorally, a single interaction may have consequences for the performance of the individuals and the outcome of future interactions (e.g. winner and loser effects of agonistic interactions, Lehner et al., 2011; female mate choice, Cummings et al., 2008), but its impact on the neurogenomic state of the individuals has been scarcely characterized. However, during a social interaction social information is available and potentially exchanged through a multitude of sensory channels, which makes it difficult to isolate the relevant cues that trigger a response and to characterize the specific responses to these cues. Thus, studying simple social signals in a single sensory channel is a promising approach to start exploring the way specific social information drives genomic responses. Following this approach, in the current study we characterize the transcriptomic response to social odors in two olfactory brain regions of male Mozambique Tilapia, *Oreochromis mossambicus.* This African cichlid fish is an established model system in the study of neuroendocrine mechanisms underlying socially mediated behavioral changes (for a review see Oliveira (2009), in which the importance of chemical signaling of male social status has been described (e.g. Barata et al., 2007; Barata et al., 2008) and the olfactory system, from sensory epithelium to bulbar and extrabulbar projections, has been well characterized (Uchida et al., 2005), which allows for the identification of regions of interest in the brain.

Synopsis of the Mozambique Tilapia mating system and chemical communication

The Mozambique tilapia is an African maternal mouth-brooder cichlid displaying a lek-breeding system, with a highly complex and multimodal social repertoire, including visual (e.g. (Baerends and Baerends-Van Roon, 1950)), acoustic (Amorim et al., 2003) and chemical signals (e.g. Barata et al., 2007, Barata, Fine et al. 2008). Depending on the social environment, males can exhibit two distinct behavioral phenotypes: dominants (DOM) and subordinate (SUB). DOM individuals adopt a typical velvet black coloration and establish breeding territories on

the bottom, where they dig nests to which they attract females using courtship displays (Oliveira and Almada, 1998a; Oliveira et al., 1996). SUB males present a pale silver coloration and either move around among the breeding territories of DOM males or shoal together with females, while they wait for their opportunity for social ascension. Sneaking fertilization attempts by SUB males have also been reported (Oliveira and Almada, 1998a). Changes between these social phenotypes have been shown to activate a cascade of molecular processes and a variety of neuroendocrine pathways which include neuropeptides and steroid hormones (Almeida et al., 2012; Oliveira and Canário, 2000; Oliveira et al., 1996). Ovulated females visit male breeding arenas when ready to spawn and follow courting males to their nests, engage in courtship rituals, and collect the fertilized eggs into their mouths. After spawning, females leave the male leks and live in nursery areas located in shallow water while they mouthbrood the eggs and care for the fry (Bruton and Boltt, 1975; Fryer and Iles, 1972). During this period, females become also more aggressive, defending the brood against predators and conspecifics (Oliveira and Almada, 1998b).

Male tilapia store urine in their bladders which they use to signal social rank during agonistic interactions with other males or in the presence of pre-ovulatory females (Barata et al., 2007). Furthermore, males are able to modulate their rate of urination depending on the social environment. An increase of males' urination rate is observed during agonistic encounters (Barata et al., 2007) or in the presence of pre-ovulatory females (Barata et al., 2008). Furthermore, both the volume of stored urine and its olfactory potency, as measured by electro-olfactogram (EOG) recordings, is higher in DOM than in SUB males (Barata, Hubbard et al. 2007; Barata et al., 2008). On the other hand, females do not store urine and have a higher frequency of urination (Keller-Costa et al., 2012; Miranda et al., 2005). Additionally, females have smaller kidneys, smaller urinary bladders and the urothelial thickness of the inner surface of the bladder is also smaller than in males (Keller-Costa et al., 2012). Finally, the

odor of pre-ovulatory females elicits higher amplitude EOG responses in males than that of post-ovulatory females (Miranda et al., 2005).

The specific goal of the present study is to characterize how different social odors that convey specific information about male social status (DOM vs. SUB) and female reproductive state (pre-ovulatory, PRE vs. post-ovulatory, POST) regulate gene expression profiles in specific brain areas known to be involved in the processing of olfactory information: the olfactory bulb and the olfactory pallium. To accomplish this, we used a cDNA microarray platform, developed for another cichlid species (*A. burtoni*), that contains many known candidate genes in addition to ca. 19,000 cichlid cDNAs (Renn et al., 2004; Salzburger et al., 2008).

Materials and methods

Housing

Mozambique tilapia were housed at ISPA – Instituto Universitário, Lisboa, Portugal in mixed-sex groups which were kept in tanks with gravel substrate, which promotes nest digging by males and the establishment of territories and social hierarchies, at a temperature of 26 ± 2◦C and a 12L:12D photoperiod. Fish were fed twice daily with commercial cichlid sticks.

Stimuli collection

In different tanks, stable social groups of 10 individuals (5 males and 5 females) were left undisturbed for 5 to 8 weeks. During this period, territories were established and spawning occurred naturally. Five minute behavioral observations of each individual were done every other day and male social status and behavior was noted.

Different sampling approaches were used to collect social odors for each sex due to the intrinsic biological differences between them. Given that male tilapia store urine in their bladders, urine was collected in males by a smooth anterior-posterior massage of the

abdominal region following a procedure previously described (Oliveira et al. 1996). Urine from 3 males was pooled according to social status (DOM or SUB). Since it is very difficult to collect urine from females, female conditioned water was used instead. For this purpose females were isolated in 20-L glass tanks with dechlorinated tap water (at 27°C) for 4h (according to Miranda et al. (2005). This conditioned water was divided in two groups of 3 females each, designated as either PRE or POST, depending on the sampling point being either the day prior to their predicted ovulation day or 1-2 days after they have spawned, respectively. Female reproductive stage was determined by systematic observations of their behavior, abdomen profile and genital papilla. All samples (both female conditioned-water samples and male urine samples) were then subjected to a fractionation procedure similar to the one described in Frade et al. (2002).

Electro-olfactogram (EOG) and brain microdissection

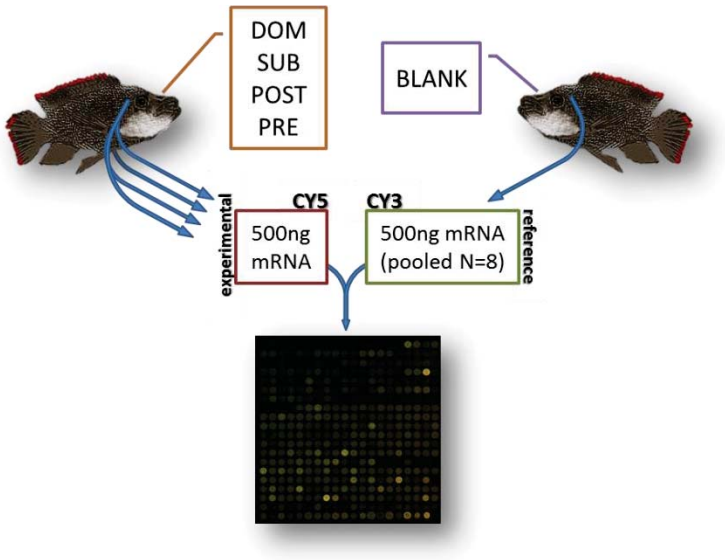
In order to characterize the responses elicited by the stimuli used in this experiment, EOGs were recorded in 33 dominant male tilapia (body mass = 182 ± 34 g) using a similar protocol to that described in Miranda et al. (2005). Briefly, each male was anaesthetized by immersion in water containing 100 mg l^{-1} MS-222 (Pharmaq, Norway) and immobilized with an intramuscular injection of gallamine triethiodide (3 mg kg^{-1} in 0.9% saline). Immobilized fish were then placed in a purpose-built V-clamp and aerated, via a mouthpiece, with water containing 50 mg l^{-1} MS-222. The right-side olfactory rosette was exposed by removal of the ring of cartilage surrounding the nostril and continuously irrigated with dechlorinated, charcoal-filtered water via a gravity-fed system (6 ml min^{-1}). The EOG was recorded using the software Axoscope (Axon Instruments, Inc., Foster City, CA, USA). The peak amplitude of the EOG was measured, blank-subtracted and normalized (using the response to the 'standard' 10^{-5} mol l^{-1} L-serine) as described by Frade et al. (2002). Blanks and standards were run twice, in the beginning and end of the recording period.

Each fish was exposed to a single olfactory stimulus, introduced into the continuous water flow via a three-way valve, for 5 s with 10 s intervals for a period of 45 min. This frequency of stimulation allowed for olfactory neurons to return to a baseline state before the next stimulation; also a pulsatile olfactory stimulation reflects the rate of urine pulses by males during social interactions (Barata et al., 2007; Miranda et al., 2005). After the olfactory stimulation, males were killed by decapitation, and the brains were rapidly dissected, embedded in Tissue-Tek® OCT™ Compound, and stored at -80°C before being sectioned coronally (200 μm) in a temperature-controlled (-18°C) cryostat. The olfactory bulbs (OB) and the putative olfactory pallium (area Dp – posterior part of the dorsal telencephalon) were then microdissected from the appropriate sections using a 27G gauge micropunch cannula (Carpenter et al., 2009).

Microarray analysis

Total RNA was extracted from both microdissected brain areas (OB and Dp) according to a standard Trizol protocol (Invitrogen) and subjected to one round of RNA amplification using Message Amp II kit (Invitrogen). Amplified RNA was analyzed for quantity and quality on the Bioanalyzer 2100 (Agilent) using the Agilent Total RNA Nano Chip assay. Samples from blank stimulations (control) collected from 8 different individuals (for both areas) were pooled and aliquoted to be used as reference in a reference based array design (see Table I and Supp. Fig. 1). mRNA (500 ng) from each experimental sample or reference were reverse transcribed using SuperScript II (Invitrogen) and labeled according to Renn et al. (2004). Following this reverse transcription, RNA was hydrolyzed and purified before being dye-coupled with Cy3 or Cy5 post-labeling Reactive Dye Pack (Amersham). A reference and experimental sample were competitively hybridized at 65^{o}C overnight to a 19K *A. burtoni* cDNA microarray (GEO platform GPL6416) constructed from brain-specific and mixed tissue libraries representing a total of 17,712 cichlid-specific features (Renn et al., 2004; Salzburger et al., 2008). This platform has

previously been shown to give biologically meaningful results in heterologous hybridizations using other cichlid species from the genus *Oreochromis* (Renn et al., 2004). Finally, microarrays were scanned with an Axon 4000B scanner (Axon Instruments) using Genepix 4.0 software (Axon Instruments). Array features were visually inspected individually and features with poor quality, that is, with a signal intensity smaller than twice the standard deviation above background, or displaying irregularities or potentially erroneous artifacts were excluded.



Supp. Fig. 1 – Hybridization design of control and reference samples. Brain samples from blank stimulations (control) collected from 8 different individuals (for both areas) were pooled and aliquoted to be used as reference in a reference based array design. mRNA (500 ng) from each experimental sample or reference were reverse transcribed and RNA was hydrolyzed and purified before being dye-coupled with Cy3 or Cy5. A reference and experimental sample were competitively hybridized overnight.

Statistical analysis

Data were processed using the LIMMA software package (v3.12.0; (Smyth, 2005) in R (v2.15.0; the R Foundation for Statistical Computing, 2012). Background-subtracted mean intensities were calculated using the minimum method and further normalized using within-array loess normalization. After this normalization step, Bayesian analysis was used to calculate gene expression levels using the ratios of intensities measured. Finally, to compare between expression profiles for the different olfactory stimulations, unsupervised hierarchical

clustering analysis were done using the *hclust* function in R/Bioconductor. The *heatmap* function in the package *gplots* was used to visualize clusters of gene expression, where only significantly expressed genes (P<0.01) across conditions were clustered. The consensus tree and confidence values were calculated via bootstrapping datasets, based on the Euclidian distanced matrix obtained for each of the 1000 permuted gene expression profile datasets.

Regarding the functional annotation of ESTs, we considered a library already compiled for another cichlid species, *A. burtoni*, and used Cytoscape (v.2.8, Smoot et al. (2011)) with the BiNGO plugin (Biological Network Gene Ontology tool, Maere et al. (2005) for the calculation of under- and over-represented GO terms and reported uncorrected hypergeometric *p*-values.

Results and Discussion

Olfactory stimulation

The overall patterns of response to social odors measured with EOG recordings (Fig. 1) were similar to those previously reported for this species (Barata et al., 2007; Barata et al., 2008; Miranda et al., 2005). The mean normalized EOG amplitude evoked by subordinate male urine at a dilution of 1:10000 was significantly smaller (0.25±0.06; N=7) than that elicited by urine samples of dominant males (0.93±0.10; N=7; P<0.01; Fig. 1). Furthermore, the mean of normalized responses to water extracts from PRE females at a dilution of 1:1000 (0.79±0.13; N=6) was significantly higher than that from POST females (0.28±0.10; N=6, P<0.01; Fig. 1).

Our results show that T and PRE stimuli elicited greater responses than SUB or POST stimuli, suggesting that males can discriminate social status and reproductive state of social partners based on olfactory cues alone. The chemical nature of the active odorants which allow for these discriminations is still unknown. Nonetheless, recent work suggests that males can assess a rival's fighting ability based on the olfactory information present in their urine (Keller-Costa et al., 2012), which might enable them to avoid time consuming and energetically costly escalated fights (Ros et al., 2006) and thus stabilize social hierarchies (Keller-Costa et al.,

2012). Thus, the EOG responses measured in the sensory neurons at the olfactory rosette suggest that they are well adapted to discriminate between urinary odorants of different male social status, which might contribute to reduce aggression and escalation of fights in a social context.

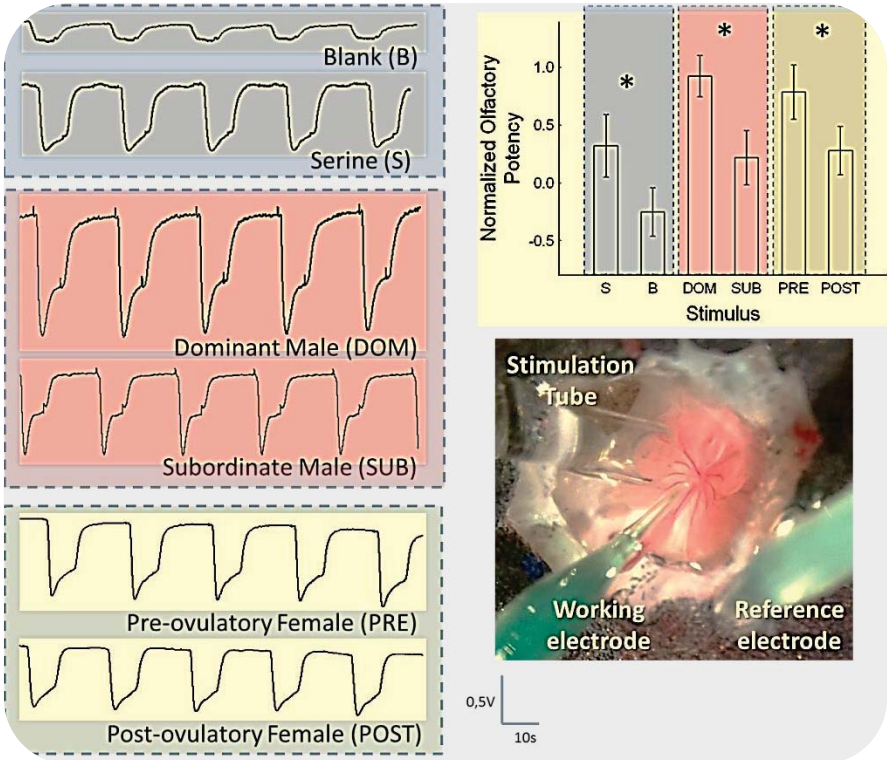


Fig. 1 – Olfactory responses of male tilapia to different stimuli. On the left hand-side, typical electro-olfactograms (EOGs) recorded in response to different stimuli: in blue – controls for normalization – serine (S) and blank (B); in pink – male urine (1:10000) – from dominant (DOM) and subordinate (SUB) males; in light green – extracts of female water (1:1000) – from pre-ovulatory (PRE) and post-ovulatory (POST) females. On the top-half on right hand side, normalized EOG amplitudes (mean ± SEM) elicited by all stimuli: S (N=6); B (N=7); DOM (N=7); SUB (N=7); PRE (N=6); POST (N=6); after 45min of stimulation (*P< 0.05). On the bottom-half, a depiction of the tilapia's olfactory rosette (40x) and the apparatus for olfactory stimulation and electrophysiological recording of olfactory evoked potentials.

Moreover, males seem to be able to discriminate between females in different stages of their reproductive cycle, probably due to specific odorants released into the water by PRE females, as previously suggested for this species (Miranda et al., 2005).

Analysis of gene expression profiles

Analysis of OB and Dp gene expression revealed hundreds of differently expressed genes after stimulation with any of the four different social stimuli (Table 1). Considering the initial more than 19K unique expressed sequence tags (ESTs) included in the analysis, over 72% hybridized with our samples (i.e. presented a signal- to-noise ratio above threshold) in both OB and Dp, confirming the usefulness of heterologous hybridization. A Bayesian analysis of gene expression levels (Townsend and Hartl, 2002) revealed that 211 of the surveyed genes in the OB showed significant differences among the 4 olfactory stimuli, whereas in Dp only 87 genes were differentially expressed ($p<0.01$; Fig. 2). No genes were found to be up- or down-regulated simultaneously in both regions, suggesting that region specific molecular processes are activated by olfactory stimulation and neural transmission. Another interesting observation concerning the number of differently expressed genes in each of these two olfactory processing centers was that at the first relay station, OB, the comparison between male and female cues seems to elicit a considerable surplus of gene regulatory activity, with more than 500 genes being differently expressed (Table 2). However, at the olfactory pallium (Dp) this number decreases substantially and the comparison between PRE and POST females emerges with almost 200 differently expressed genes (Table 2).

Table 1 –List of all significantly expressed genes organized by each one of the four olfactory phenotypes compared for both brain areas tested ($P<0.01$). Number of features annotated considered for the Gene Ontology analysis. Finally, the sample size considered for each phenotype, each comparison and each area sampled. DOM- dominant male urine; SUB- subordinate male urine; PRE- pre-ovulatory female water extract; POST- post-ovulatory female water extract.

	OB			Dp		
Olfactory phenotypes compared	Differently expressed genes	#features GO analysis	Sample sizes	Differently expressed genes	#features GO analysis	Sample sizes
DOM – SUB – PRE – POST	211	118	5-6-6-6	87	52	5-4-6-5
♂ – ♀	504	271	11-12	91	66	9-11
DOM – SUB	185	109	5-6	128	75	5-4
PRE– POST	96	56	6-6	197	172	6-5

A hierarchical cluster of these differently expressed genes in the OB and Dp revealed interesting patterns of neuromolecular activity. In both brain regions, the transcriptional response of males exposed to DOM male urine was most similar to that of males exposed to POST female water extract, and the transcriptional response to SUB male urine was most similar to the response to PRE female water extract (Fig 2).

The evidence for olfactory discrimination among stimuli in both brain regions reinforces the idea of a functional organization of the fish olfactory system with parallel pathways flowing from the sensory epithelia via the olfactory bulb into the pallium, conveying specific odor information (Hamdani and Døving, 2007; Kermen et al., 2013). Furthermore, the different brain regions seem to preferentially process certain stimuli, with sex differences in odors being mainly processed at OB and subsequent odor differentiation within each sex being processed at Dp. Cummings et al. (2008) concluded that these neuromolecular consequences drive behavioral responses in the context of female mate choice in swordtails. Unlike olfactory cues in our experiments, female choice in this species activated a suite of genes in response to classes of social stimuli: specific pathways were either up- or down-regulated when females were exposed to males or to other females. From an ecological point a view, these surprisingly similar transcriptional responses of the OB and Dp to SUB males and PRE females might be explained by the distinctive information conveyed by each behavioral phenotype and by shared valence and salience of their odors. It is possible that chemical signals emitted by SUB males are feminized, which would help to explain why DOM males are occasionally observed to direct courtship behavior towards SUB males (Oliveira and Almada, 1998a). SUB males and PRE females shoal together and share the same body coloration. When courted by DOM males SUB males exhibit female-like behaviors, which include following the DOM male to the spawning pit and getting involved in the full spawning sequence (Oliveira and Almada, 1998a).

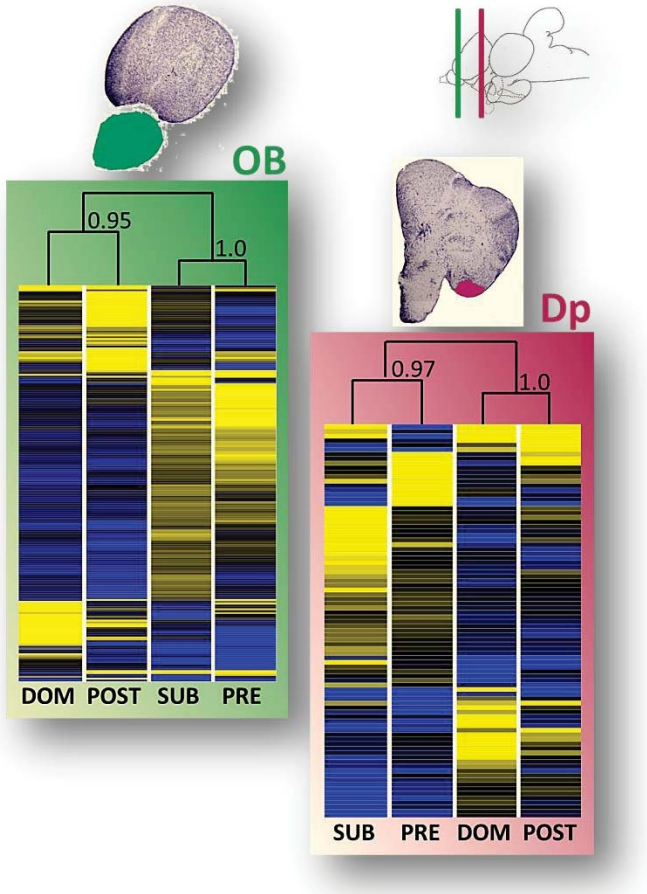


Figure 2 – Unsupervised hierarchical bootstrapped clustering of significantly different expressed genes ($p<0.01$) for all four olfactory stimuli and both brain areas sampled (OB and Dp). On the top right, a sagittal view of a tilapia's brain cut by two lines (green and violet) representing the location of the coronal cuts depicted just below illustrating the areas sampled (OB and Dp; nissl stained slices, 10 µm). On the heatmaps, blue represents significantly downregulated genes, yellow upregulated genes and black intermediate levels of expression. Confidence values of cluster nodes were calculated using bootstrapping (1000 permutations with resampling). Olfactory stimuli used in theis study: DOM- dominant male urine; SUB- subordinate male urine; PRE- pre-ovulatory female water extract; POST- post-ovulatory female water extract. Brain regions analyzed: olfactory bulb (OB), green box; posterior part of the dorsal telencephalon (Dp), purple box.

This behavior allows SUB males to remain inside the breeding aggregations in order to try sneaking egg fertilizations (Oliveira and Almada, 1998a). Despite having mature testis (Oliveira and Almada, 1998c) SUB males present lower androgen levels (Oliveira et al., 1996), lower expression of secondary sex characters (Oliveira and Almada, 1998c) and undergo androgen-dependent morphological changes in the urinary bladder and urine storage capacity,

reducing its volume to a more female-like size (Keller-Costa et al., 2012), which may also affect the composition of their urine.

The similarity between the gene expression patterns elicited by DOM male and POST olfactory signals is more difficult to explain. Both social phenotypes are usually territorial and display higher number of aggressive displays (Oliveira and Almada, 1998b; Ros et al., 2006), which might explain some similarities in chemical information. Other possible similarities of the odor bouquet released by these two groups could be related to the starvation period these fish experience or the high metabolic rates needed to endure a continuous effort like territorial defense or production of the egg batch that has just been released by POST females (Renn et al., 2009; Ros et al., 2006).

The comparison between transcriptional profiles of males stimulated with social olfactory cues with the electrophysiological data gathered from the same males but at the level of the olfactory epithelium also raises some interesting points. In our data the olfactory epithelium is more sensitive to DOM male and PRE female olfactory information but discrimination between the sexes does not seem possible (Fig.1). However, at the level of the OB the gene expression profiles suggest that males have the relevant information available that allows them to discriminate between the sexes (Fig. 1 and Fig. 3) reinforcing the importance of olfaction in African cichlids, which in other fish species also plays a major role in intra-specific communication (Sorensen, 1992), including social recognition (Gerlach et al., 2008).

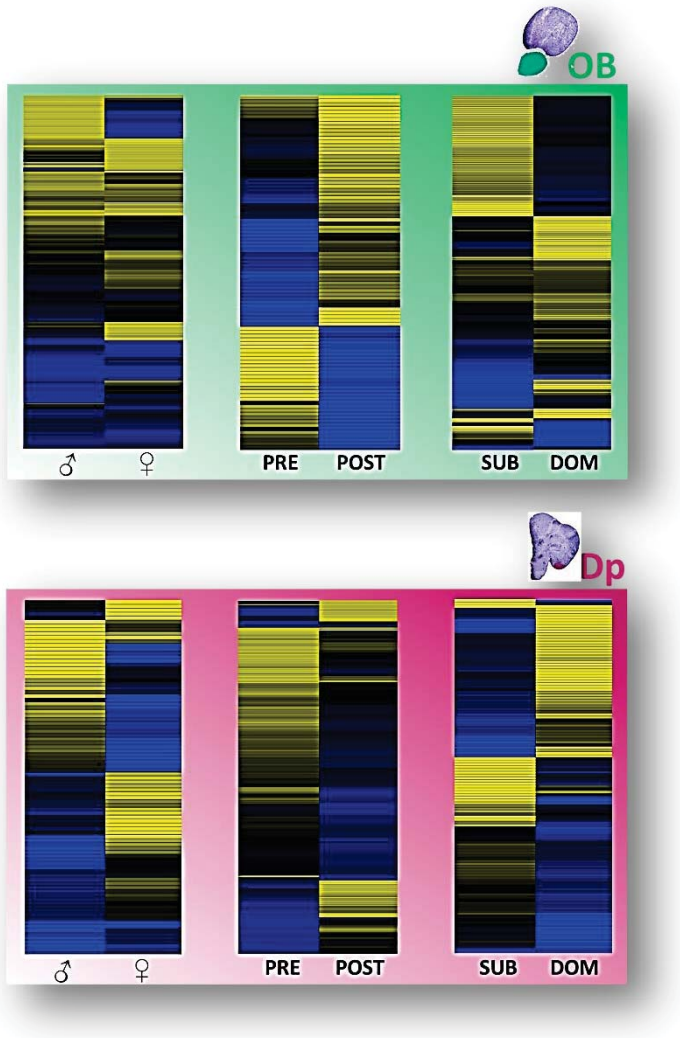


Figure 3 – Hierarchical clustering of significantly different expressed genes (P<0.01) for the comparison of three pairs of olfactory cues in both brain areas sampled (OB and Dp). Green box: olfactory bulb (OB) expression; purple box: posterior part of the dorsal telencephalon (Dp). Left panels: comparison of female (symbol) and male (symbol) cues independent of status or condition; middle pannels, comparison of pre- (PRE) and post- (POST) ovulatory female cues; right pannels: comparison of dominant and subordinate male cues. The heatmaps (blue – down-regulated, yellow – up-regulated) show estimated gene expression levels. Confidence values of cluster nodes were calculated using bootstrapping.

GO analysis

Our GO annotation scheme allowed for a categorization of a plethora of differentially expressed genes in molecular functions, biological processes and cellular components (Fig. 4), as well as, providing information about under- and over-representation of each category. In all comparisons analyzed (DOM male vs. SUB male vs. PRE female vs. POST female odors; male vs.

female odors; PRE vs. POST female odors; and DOM male vs. SUB male odors), GO terms could be applied to more than 55% of the regulated features.

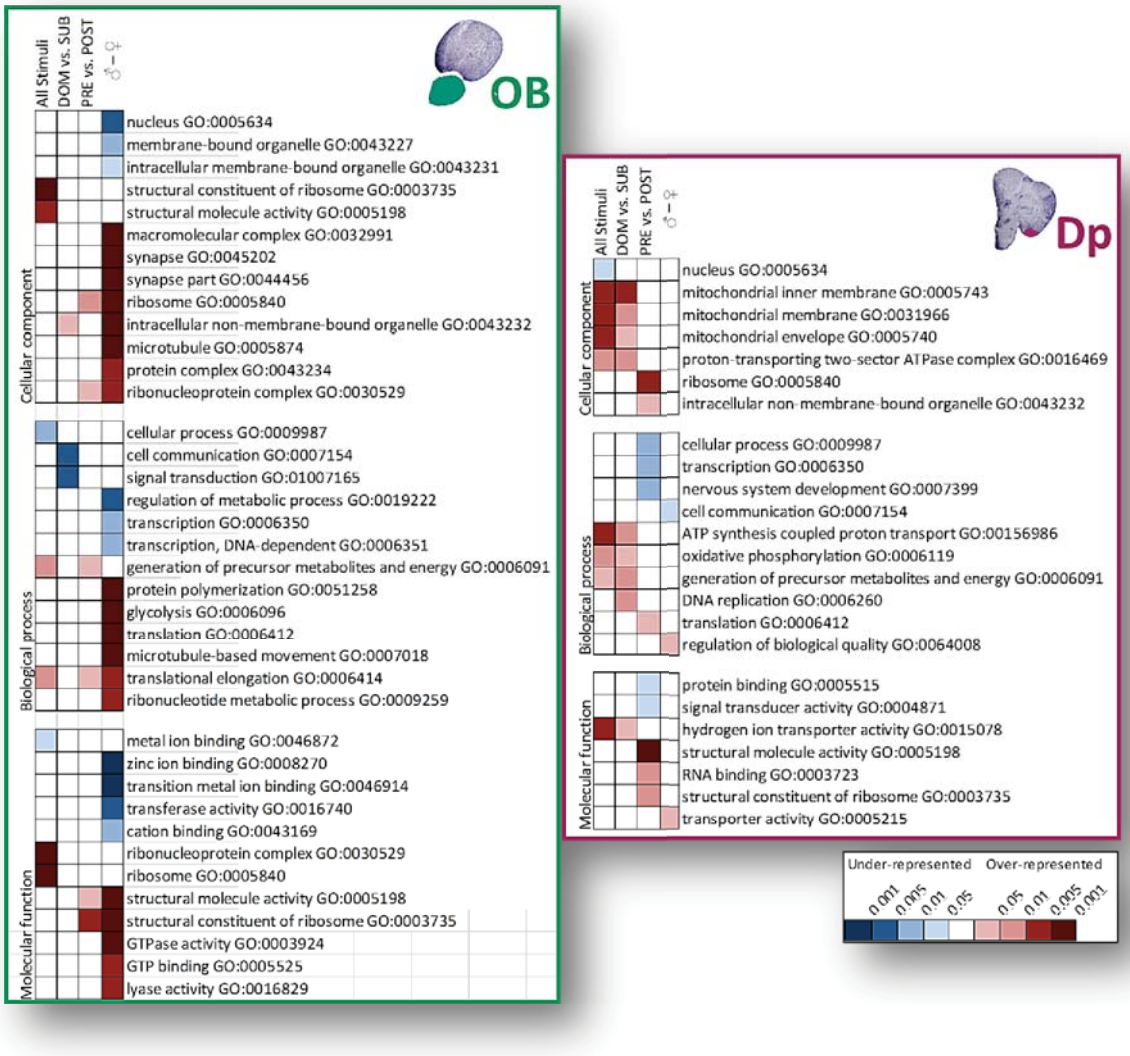


Figure 4 – Gene Ontology (GO) analysis summary for each one of the four olfactory comparisons made for both brain areas. Statistically under-represented categories are shown in blue and over-represented in red. The different GO vocabularies are shown separately: cellular component, biological process and molecular function; along with the P-values (uncorrected results of the hypergeometric test) and GO names and numbers (according to 200605 releases). DOM- dominant male urine; SUB- subordinate male urine; PRE- pre-ovulatory female water extract; POST- post-ovulatory female water extract.

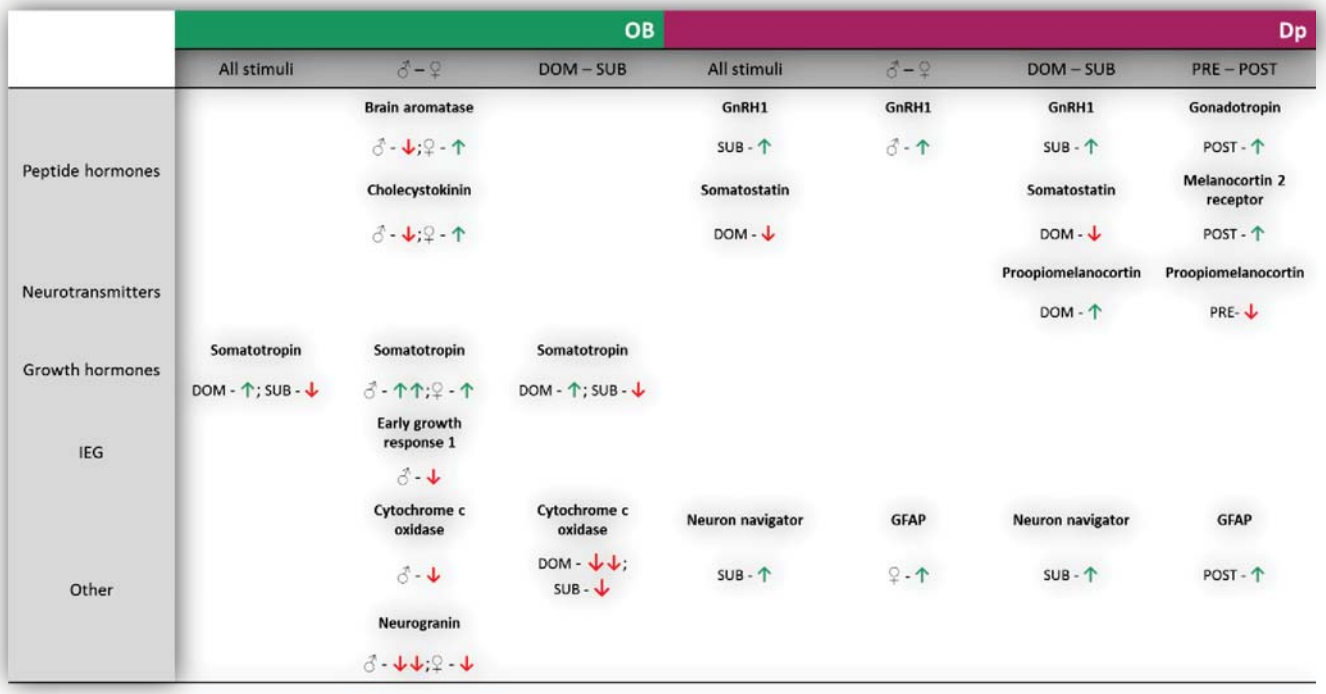
Although the results of GO analyses can be difficult to interpret, they provide a framework for developing novel hypotheses that could potentially enlighten new approaches to the molecular underpinnings of socially regulated brain function (Renn et al., 2008).

Interestingly, the functional categories expressing enriched pathways with extreme over- and under-representation are also more numerous for the distinction between males and females in the OB, and rather scarce for the same comparison at the Dp level. In the latter, the number of enriched GO terms is smaller and more evenly distributed among the remaining comparisons (DOM vs. SUB male odor and PRE vs. POST female odor). This suggests that already at the OB level, the first relay station in the olfactory circuit, information on the sex of a nearby conspecific might be filtered out, which in a social interaction would be reinforced by visual cues ascertaining this information and triggering the appropriate behavioral response.

Candidate genes

Besides activating specific molecular pathways, a number of the candidate genes are also significantly expressed in one of the two brain areas sampled from dominant males stimulated with different social odors in this experiment. Somatotropin, a member of the Growth Hormone (GH) family, is significantly up-regulated in the OB (Table 2) in response to either the odor of a DOM male or the odor of females (either PRE or POST). On the other hand, in Dp somatostatin, a known GH production inhibitor, is down-regulated after stimulation with DOM scent. Regulation of these members of the GH signaling are usually related to differential growth, a characteristically plastic trait in cichlids in response to changes in the social environment (Hofmann et al., 1999). Somatostatin is known to play an important role in the complex interplay between social behavior and somatic growth in cichlid fishes (Trainor and Hofmann, 2007) managing the allocation of energetic resources between reproduction and growth (Hofmann and Fernald, 2000). Somatostatin down-regulation only in response to the presence of an odor cue of a potentially threatening high-ranked male along with the up-regulation of somatotropin, suggests the preparation for the physical strain involved in an approaching agonistic interaction.

Table 2 – Gene Ontology (GO) analysis summary for each one of the four olfactory comparisons made for both brain areas. Statistically under-represented categories are shown in blue and over-represented in red. The different GO vocabularies are shown separately: cellular component, biological process and molecular function; along with the P-values (uncorrected results of the hypergeometric test) and List of all significantly regulated candidate genes for each one of the four olfactory comparisons made for both brain areas, organized according to presumed functional categories. Red arrows represent down-regulated genes and green arrows up-regulated genes (double arrows indicate increased extent of regulation) extracted from previously shown heatmaps.



Other candidate genes were also up-regulated in stimulated dominant males, such as: brain aromatase in the OB and gonadotropin-releasing hormone (GnRH1) and pro-opiomelanocortin (*pomc*) in Dp. The up-regulation of GnRH1 after an olfactory stimulation with SUB male odor reinforces the idea of a putative feminization of their urine discussed above, since GnRH integrates the animal's internal physiological state with incoming external cues to regulate reproduction in males. In cichlid fish, reproductive status influences the regulation of this neuropeptide and seasonal fluctuations of GnRH receptor levels in the brain can modulate olfactory processing, regulating the animal's plasticity in olfactory responsiveness (Maruska and Fernald, 2010). Although it is not known if the Mozambique tilapia has GnRH receptors in Dp, a close relative species (Nile tilapia, *Oreochromis niloticus*),

presents receptors at both OB and Dp (Gopurappilly et al., 2013; Soga et al., 2005). Interestingly, GnRH up-regulation in an extra-hypothalamic area, like Dp, can also be found in rats, where GnRH mRNA is also present both in the olfactory piriform cortex (homolog of Dp) and in the olfactory bulb (Choi et al., 1994).

egr-1 and cytochrome c oxidase (COx) were both down-regulated in OB of males stimulated with male social odor when compared with female social odor. Both genes are known markers of neural activity (Poirier et al., 2008; Wong-Riley, 1989) and the regulation of *egr-1* appears to have a pivotal role in recruiting specific neural pathways required for long-term memory processes (Poirier et al., 2008). *egr-1*-deficient mice seem to be unable to form long-term memories in behavioral tasks, such as olfactory discrimination, while their short-term memory and early-LTP remain intact (Jones et al., 2001). In zebrafish, *egr-1* activity seems to be involved in imprinting processes in early life stages and later in kin recognition, especially in the OB, since rather low basal expression levels are found in the Dp (Kress and Wullimann, 2012). In summary, *egr-1* down-regulation in the OB of DOM males in response to olfactory cues of male conspecifics, suggests a possible role of olfactory modulation on memory consolidation of social odors. Despite variation in its activity have been found to correlate with olfactory stimulations in different taxa (Déglise et al., 2003; Dorman and Moulin, 2002; Wong-Riley, 1989), little is known about COx modulation with olfactory social stimuli. In another cichlid species, *Astatotilapia burtoni*, when males were presented with visual and olfactory signals, both stimuli were needed for an androgen response in an intruder challenge paradigm but chemical stimulation alone did not induce c-Fos induction, another marker of neuronal activity (Hoffman et al., 1993), in the brain (O'Connell et al., 2013).

Conclusions

The approach we have used in the present study allows for a transcriptome-scale analysis of the molecular systems regulated by social olfactory experience. Investigating the

proximate mechanisms underlying olfactory stimulation allowed for the characterization of different genomic profiles elicited in DOM males. DOM males stimulated with different acute social cues exhibited at the more peripheral olfactory epithelium some degree of discrimination between stimuli. Nonetheless, at the brain olfactory processing centers, specific transcript patterns of activation were elicited suggesting that the olfactory system can discriminate social status and reproductive condition, as well as, its sex based solely on its chemical signature. Our findings also underscore the extensive transcriptional plasticity of the cichlid genome in response to the social environment and reinforces the importance of uncovering the molecular and cellular factors and constraints governing olfactory function. Additionally, our results also reinforce the impact of the social environment, even in short-term interactions, in the modulation of molecular switches that orchestrate signaling pathways in the brain. These measurable changes in brain genome, correspond to different neurogenomic states which in turn are expected to modulate and optimize the behavioral output expressed by the fish according to each social context (Oliveira, 2012). In summary, social odor-driven changes in brain transcriptome may provide a mechanism by which animals adjust their behavior to perceived changes in the social environment. Further studies focusing on the neuroplasticity responsible for the adaptive social behavior exhibited by cichlids might shed some light on the rapid evolution and diversification of this teleost family in the Great African lakes.

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General discussion

One of the major goals of this thesis was to investigate how social information influences the molecular cascades behind gene expression that modulate the production of meaningful behaviors in teleost fish. Previous literature focused on characterizing the fluctuations in the expression of specific candidate genes and/or their products (*e.g.* Almeida et al., 2012; Greenwood et al., 2008; Maruska et al., 2013; Trainor and Hofmann, 2007) or at the genome level (Aubin-Horth et al., 2005b; Pollen et al., 2007; Renn et al., 2004) according to a multitude of social environments. Cummings et al. (2008) concluded that these neuromolecular changes can occur even in a simple mate choice context. Based on these evidences we hypothesized that the functional genome might be more dynamic than previously thought and respond to a single social interaction and even to discrete social stimuli, such as a chemical signal. Despite the growing interest on understanding the molecular pathways behind complex behaviors the impact of social interactions at the genome level has been scarcely research in most taxa (*e.g.* Cummings et al., 2008).

In this discussion we highlighted the most important findings of this thesis and discussed its results regarding the existing literature on neurogenomics and behavioral and social plasticity. The final section concerns with future perspectives regarding the broached subjects.

Characterization of aggressive behavior in zebrafish

Aggressive behaviors are a pivotal component of the behavioral repertoire of animals. They serve numerous adaptive functions, including the establishment of dominance hierarchies and the competition for basic resources such as food, shelter or mates and territories. Zebrafish are a promising vertebrate neurogenetic model for the study of neural circuits underlying aggressive behavior and in the first section of Chapter I we described and quantified meaningful patterns of aggressive behaviour and their consequences in subsequent

interactions in order to develop a quantitative framework for the study of aggressive behavior in zebrafish.

Aggression can be defined as any overt behavior that produces aversive or noxious stimuli or harm to another organism (Olivier and Young, 2002). In zebrafish, like in most species, overt aggression is always preceded by an assessment stage, where opponents use ritualized displays before aggression escalates. These ritualized displays have been interpreted as means of assessment of fighting abilities between conspecifics, thereby preventing fight escalation and reducing the risk of physical damage (Maynard Smith and Price, 1973; Ros et al., 2006). In fact, agonistic interactions in this species seem to fit into a temporal structure that can be characterized in order to predict the probability of the subsequent behavior. This temporal architecture seems to follow the aforementioned idea of an assessment early stage, with a high frequency of agonistic displays, followed by an escalation and resolution stages, where winners and losers emerge.

But in these bouts, experience in prior contests might be fundamental, since it may provide information about potential costs of future contests (Hsu et al., 2011). Our data on sequential fights showed that a recent victory increases the probability of winning a subsequent interaction and, on the other hand, a recent defeat reduces the chance of emerging as a winner in a future fight. This interesting data set suggested the occurrence of winner and loser effects in male zebrafish, establishing that in this species fight outcome had behavioural consequences that may impact in the individuals Darwinian fitness. These effects of experience, coupled with other fighting asymmetries, like body size or prior residence, influence the establishment of dominance hierarchies (Oliveira et al., 2009).

Winner and loser effects are not uncommon in the Animal Kingdom but usually the scale of loser effects is higher and frequently lasts longer than winner effects (Hsu et al., 2006; Rutte et al., 2006). Our data reinforced this notion, by emphasizing that these effects of experience might be mediated by different mechanisms in winners and losers. Zebrafish that won an

interaction increased the probability of winning a subsequent fight with a naïf individual without changing their fighting behavior. This finding suggested that some variation in the animal's internal state occurred although it was not reflected on its behavioral expression. Losers also significantly decreased the probability of winning subsequent fights by decreasing their motivation to escalate fights shown by an increase in the latency to the first attack and a decrease in the fight decision time after a loss.

One way to explain the effects of prior experience in subsequent fighting performance is to consider that putatively it influences social cues used to signal status to other conspecifics. This in turn would result in a biased response from their opponent (depending if they are fighting a winner or a loser) since fish are known to gather information from observing conspecific interactions (Grosenick et al., 2007; Oliveira et al., 1998; Oliveira et al., 2001). If these social cues are being driven by the individual's social environment there should be regulation of brain gene expression to orchestrate these phenotypical changes (Bell and Robinson, 2011; Robinson et al., 2008; and see second part of Chapter I where we further explore this in zebrafish). Chemical signals are arguably the best candidates, as social cues, to convey this information to conspecifics about previous fighting experiences. Male pheromones in zebrafish are known to regulate reproductive success in females (Gerlach, 2006), but in other species of fish, male pheromones are known to also signal dominance (Barata et al., 2007; Maruska and Fernald, 2012; Miranda et al., 2005; Todd et al., 1967; Waas and Colgan, 1992; see second part of Chapter II where we study how these chemical social cues modulate gene expression in the Mozambique tilapia).Nonetheless, despite the ubiquity of winner and loser effects throughout the animal kingdom and its crucial influence on social structures, the ultimate and proximate causes for their existence remain unknown (Rutte et al., 2006).

Changes in brain transcriptome in fighting zebrafish

After the fights are resolved changes occur in the behavioural pattern expressed by zebrafish and ultimately winners display a different set of behaviors (chases, bites and strikes) when compared to losers (freezing and fleeing) (Oliveira et al., 2011). These changes in behavioral performance usually match the individuals' competitive ability or at least their assessment of it as the fight progresses. As social hierarchies are built and shaped, individuals must be able to readily switch between different social ranks (Oliveira, 2009), which consequently translates in an update of their behavioral displays. Underlying these behavioral states should be a genotype able to accommodate the expression of these multiple social phenotypes. These socially driven brain changes usually are accomplished by variations in the regulation of key genes, or rather genes involved in specific key signaling pathways. This gene regulated cascade of events results in distinct transcriptome profiles (neurogenomic states) reflecting the individuals' status-specific behavioral states (Wong and Hofmann, 2010). Thus, it is possible to describe singular neurogenomic patterns elicited by socially dependent behavioral states.

During a social interaction internal and external cues must be assessed in order to evaluate the costs *vs.* benefits of pursuing or ceasing the interaction (Hsu et al., 2011). The individual's assessment of the situation will influence the outcome of the fight which will later result in the gain or loss of social status. However, the specific set of internal and external cues used for this evaluation are still unknown. This appraisal will result in changes in social status and trigger the switch between the aforementioned neurogenomic states. In the second part of Chapter I we hypothesized that zebrafish cognitively appraised their fighting performance in relation to that of their opponent in order activate this switch between internal states. Internal cues such as previous fighting experience, overall condition, energy reserves and in essence the expressed behavior by the individual as the fight develops must be compared with external cues regarding information conveyed by the opponent (mostly visual or chemical information)

and their perceived fighting ability. In order to test this idea we exposed zebrafish to a mirror fight (i.e. fighting their own image on a mirror) and compared the elicited genomic profile with those of fish that experienced either a victory or a defeat.

When facing a mirror image (which zebrafish are unable to distinguish from a real fish and readily attack) behavioral feedback from the opponent matched the individual's displays and no clear winner or loser emerged and therefore no change in social status is experienced by the subject (Oliveira et al., 2005). Indeed our genomic data showed that mirror fights did not elicit a single differentially expressed gene, supporting this notion that cognitive appraisal of the fight outcome is necessary to induce major changes in the brain transcriptome.

On the other hand, transcriptomic profiles of winners and losers presented striking differences in comparison to the other social experiences (mirrors and socially isolated fish). A single short-time social interaction was sufficient to elicit a rather large number of genes being up- and down-regulated in response to these social stimuli. As mentioned before the scale of loser effects is thought to be greater than winner's (Hsu et al., 2009) and indeed in the brain of zebrafish that lost a short-time interaction the number of significantly regulated genes was larger. After a single interaction, some genes were being differently expressed in both individual's (fighting regulated genes – 30% of the total number of regulated genes) but the majority was specific for fish that experienced a defeat (60%). These social interactions which induced changes in social status also triggered activity-dependent gene pathways involved in neural plasticity, such as activity-dependent immediate early genes and genes related to learning and memory (e.g. *bdnf*, *npas4*). *bdnf* has been implicated in the differentiation and survival of neurons, as well, as an important regulator of synaptic plasticity mechanisms underlying learning and memory in adults (Cunha et al., 2010). This increased activity of genes related to memory formation, suggests that relevant information is probably being stored: related with the fight outcome, but also probably related with the fighting ability of the opponent. Additionally, a gene ontology analysis revealed that MAPK signaling pathway is

being significantly expressed. MAPK seem to have a role in the differentiation of specific cell types and regulate the proliferation of others, but are also involved in distinct forms of synaptic plasticity (Thomas and Huganir, 2004). We can argue that this regulated pathway may also indicate that these short-term interactions which induced changes in social status also triggered activity-dependent gene pathways involved in neural plasticity.

Taking into account all these evidences, may also help to better understand how a single interaction has effects in the performance of individuals in subsequent interactions in both winners and losers (Oliveira et al., 2011). In summary, our findings suggested that the brain transcriptome profile of zebrafish closely reflected their recent acute social experiences and that shifts between behavioural states characteristic of different social status were accompanied by rapid changes in gene expression in the brain and the cognitive appraisal that the individual makes of its social environment seemed to be a key factor to trigger these cascades of events.

Brain atlas of the Mozambique tilapia

Just like zebrafish, the African cichlid *Oreochromis mossambicus* has been used as a model system in a wide range of behavioural and neurobiological studies. Their remarkable social behavior, full of complex and plastic traits, multisensory signals used during both courtship and agonistic encounters makes them a suitable candidate for the study of social plasticity and behavior. Mozambique tilapia behavior has been extensively investigated in the past (*e.g.* Oliveira and Almada, 1998a; Oliveira and Almada, 1998b), along with their behavioral endocrinology (*e.g.* Oliveira and Almada, 1998c; Oliveira and Canário, 2000; Oliveira et al., 2001) and more recently the increasing number of genetic tools available for this species has provided with some insights about social regulation of gene expression (*e.g.* Almeida et al., 2012).

As one of the goals of this thesis was to understand how gene expression in brain areas relevant for social behavior promote socially driven changes in behavioral profiles, it was of key importance to develop an accurate hodological mapping of the tilapia brain to supplement the available histological data.

Three-dimensional brain atlases have a massive potential as gateways for navigating, accessing, and visualizing neuroscientific data (Essen, 2002) and present some advantages over established histological methods (see Chapter II, section I). Using magnetic resonance imaging, supported by Nissl stained brain slices, we developed a 3D high-resolution digital atlas of the Mozambique tilapia brain. The resulting images can be browsed and analyzed in horizontal, coronal and sagittal views and are freely available online at: http://www.ispa.pt/ui/uie/ibbg/TilapiaBrainAtlas/index.html. All bigger brain divisions were manually labeled, such as the olfactory bulb, telencephalon, diencephalon, optic tectum, and cerebellum. In addition, a number of smaller but relevant structures or nuclei were also labeled, using our histological data as a reference guide, amounting to over 50 identified structures in total. Using appropriate software we also made a three dimensional reconstruction of the whole brain which enriches the value of this data set. This high resolution tilapia brain atlas is expected to become a very useful tool for neuroscientists using this fish model and will certainly expand their use in future studies regarding the central nervous system, but most importantly, it was a stepping stone to identify and localize the neural circuits underlying olfactory processing in the Mozambique tilapia, which will be the subject of our next experiment.

Social odors induce rapid neuromolecular changes in the Mozambique tilapia

Similarly to zebrafish, tilapias also exhibit structured fights and depending on their social environment males can express two distinct behavioral phenotypes: dominants and subordinates. In this species visual displays during agonistic interactions are accompanied by

chemical cues *via* the urine, which conveys information regarding the males' social status (Barata et al., 2007). Analogous cues are also elicited by females to signal their sexual maturity (Miranda et al., 2005). As mentioned above changes between behavioral phenotypes activates a cascade of molecular processes and a variety of neuroendocrine pathways (e.g. Renn et al., 2008). In the second part of Chapter II, we tried to manipulate the fish's perception of its social environment using chemical signals in order to elicit the emergence of context-specific neurogenomic states and to investigate the proximate mechanisms underlying olfactory stimulation.

To characterize the changes in the brain's internal state in response to social odors of both male and female phenotypes we combined physiological and genomic approaches. We measured electrophysiological responses of the olfactory epithelium and posteriorly microdissected specific olfactory processing brain regions to hybridize with a heterologous microarray platform (Renn et al., 2004). Our electrophysiological recordings reinforce the previous notion that males can discriminate social status and reproductive state of social partners based on olfactory cues alone (Barata et al., 2007; Miranda et al., 2005). On the other hand, at the neurogenomic level, all four olfactory stimuli from conspecific males and females had a major impact in the brain transcriptome, with different chemical social cues eliciting specific patterns of gene expression in the brain. Thus, the olfactory system of male tilapias seems to be able to discriminate the social status and reproductive condition, as well as, the sex of their conspecifics based solely on their chemical signature. In goldfish, males also regulate brain gene expression when stimulated with putative sex pheromones of pre- and post-ovulatory females (Lado et al., 2013). Although none of the differently expressed genes in goldfish males match the ones in tilapia males exposed to similar stimuli in our study, this might be due to differences in sampled brain regions the tissue (telencephalon *vs.* OB and Dp) and duration of exposure to the stimuli (6h *vs.* 45min). Interestingly these authors also

collected milt from males exposed to these stimuli and concluded that changes are happening in the brain and body of the fish (Lado et al., 2013), as in preparation for a social interaction.

Our analysis also included a gene ontology analysis which suggested than information regarding the sex of the emitter can already be discriminated at the first relay station in the olfactory circuit. Finally, a number of candidate genes were also significantly regulated, suggesting that the animal's begin preparations in an anticipatory fashion according to stimuli emerging from their social environment. The evidence for olfactory discrimination among stimuli in both brain olfactory processing regions in our study supports the prevalent hypothesis of a functional organization of the fish olfactory system (Hamdani and Døving, 2007). Olfactory information flows from the sensory epithelia in the periphery reaching the brain through the olfactory nerves and progressing through the olfactory bulbs into the olfactory pallium (and other regions, see Miyasaka et al. (2014)), conveying specific odor information (Hamdani and Døving, 2007; Kermen et al., 2013). The neurogenomic patterns elicited by these cues suggested that fish could discriminate between conspecifics probably by analyzing distinctive information conveyed in these cues and further assigning valence and salience to them. If either in the presence of feminized or potentially threatening or aggressive olfactory signatures a swift switch between neurogenomic states would probably elicit the appropriate expression of behaviors in each particular context. In fact, each particular neurogenomic states is likely modulating and optimizing the individual's behavior according to each particular social context.

In summary, the results of this final study underscored once again the extensive transcriptional plasticity of fish's genome in response to the social environment and emphasized the importance of pursuing the study of the nature of these biochemical switches which orchestrate the translation of social stimuli into neuroendocrine signals and ultimately guide adaptative behavior.

Concluding remarks

The ability of fish neural networks to adapt and respond to their social environment is a notable display of plasticity. The specific neural plasticity unveiled in these studies, in response to simple agonistic interactions and even to simpler chemical social stimuli is a remarkable feature. Influenced by social stimuli, neural networks adjust gene regulated pathways in order to adjust the individual's behavior expression. The next sections explore how the social environment shapes the behavior expressed by individuals in social groups, the brain structures which, across several taxa, are used to process these social stimuli and, finally, how these nuances in the social environment ultimately drive brain gene expression to fine-tune behavioral expression.

Behavior

Social behavior is ubiquitous in nature and widespread across several taxa, including bacteria where cooperating and cheating behaviors can occur (Dunny et al., 2008). Animals must continuously integrate information from their internal and external environments in order to correctly adjust their behavior according to daily events. Usually these behaviors are directed to obtain or defend resources (such as food, shelter or mates) or to avoid danger (such as predators) and, more often than not, take place in social environments. These social environments, with whom an individual frequently (if not always) interacts, shape its behavioral expression. In several species, defending resources usually implies some sort of direct or indirect aggression. In fruitflies, *Drosophila melanogaster*, males have evolved elaborate and structured aggressive displays in defense of females and territories (Chen et al., 2002; Dow and Schilcher, 1975). These complex aggressive displays, which are comprised of a repertoire of 7 different behaviors, present a clear temporal structure much like the one reported for zebrafish (Chapter I). These displays intend to avoid a rapid escalation to overt aggression, which often leads to serious injuries. Thus, this temporal structure might be

advantageous for individuals to acquire increasingly detailed information about their opponent's fighting abilities (Chen et al., 2002) and ultimately decide whether to continue to fight or flight. In male song sparrows, *Melospiza melodia*, territorial defense is firstly performed in the form of vocalizations, which advertise male readiness to mate during their breeding season. Nonetheless, depending on the type of song the males are singing it is possible to predict the outcome of the interaction (fight or flight) since their song performance also signals aggressive escalation (Searcy and Beecher, 2009).

These interactions with the social environment have consequences not only in terms of injuries resulting from these conflicts but often reflect on behavior as well. As discussed earlier, winning or losing a social interaction affects how a zebrafish performs in future fights. Social experience can then modulate the outcome of these interactions such that winners increase the probability to win again and losers will more likely lose again (Rutte et al., 2006). In fruitflies bred specifically to present a hyperaggressive phenotype by selecting winners of fights (males tend to initiate fights sooner and retaliate more frequently), after a lost interaction males lose for a second time against a socially naïve individual (Penn et al., 2010).

In teleost fish, there are several examples of these influences of social experience in future behavior. For instances, in a mangrove North American endemic species, *Rivulus marmoratus*, fighting experience seems to be fundamental to determine winners, but only in non-escalated fights (Hsu and Wolf, 2001). Similarly, prior experience in swordtails, *Xiphophorus helleri*, has a definite impact on the probability of winning the next fight (Beaugrand et al., 1991) unless it's a highly escalated fight (Franck and Ribowski, 1989). A recent study with another mangrove species, the killifish *Kryptolebias marmoratus* explored how multiple experiences might affect future behavior. In natural settings, like these mangroves, social interactions can occur in quick succession and the performance of an animal is likely to be influenced by other experiences recently acquired. In this species, multiple experiences can reinforce each other (mostly in losing experiences), but the way they are

integrated to influence behavior is dependent on the individual's perceived fighting ability (Hsu et al., 2013). As if seen earlier, in zebrafish a single interaction can also have profound effects in the performance in future bouts.

These aggressive displays are central to the establishment and maintenance of dominance hierarchies, since the outcome of conflicts is a main factor determining dominance status. Dominance usually translates to better access to some of the critical resources aforementioned and a higher rank within these hierarchies reflects on the success of an individual (Sloman and Armstrong, 2002). Both model species of fish explored in this thesis establish these dominance hierarchies (Oliveira and Almada, 1996a; Oliveira and Almada, 1996b; Paull et al., 2010). Nonetheless, the study of these intricate social relations is better understood in cichlid fish. Lekking cichlid species, like the Mozambique tilapia, have to continuously engage in social interactions to establish territories within breeding arenas where males aggregate and build nests to attract mates. In these species, individuals can advertise their social dominance by conspicuously changing their body coloration (Baerends and Baerends-Van Roon, 1950; Fernald, 1976). Males can rapidly switch between social states depending on their success in these aggressive encounters. Chemical signals usually reinforce these visual exhibitions in cichlids and dominant males also modulate their rate of urination in the presence of rival males or potential mates (Barata et al., 2007; Barata et al., 2008; Maruska and Fernald, 2012). These phenotypical changes signal to conspecifics about the condition of the dominant male, but at the same time also signal physiological changes related with social status occurring internally. Studies in several cichlid species, firstly characterized how hormones, like androgens, might be playing a role orchestrating these changes and shaping aggressive behaviors (Fernald, 1976; Oliveira and Canário, 2000). Interestingly this myriad of cues available in the social environment of cichlids can be used by conspecifics to infer the relative strength of rivals before engaging in costly interactions (Grosenick et al., 2007; Oliveira

et al., 2001). As discussed previously in Chapter II, chemical cues seem to be sufficient to trigger these social assessment mechanisms and infer social status.

In sum, the behavior of an individual seems to be continuously shaped by interactions with their social environment. In dyad fights in zebrafish, cues about the opponent's fighting ability seem to be integrated with the fish's own ability to influence the outcome of these contests. In turn, winning or losing an interaction has consequences in the expression of future behavior and in some species, like tilapia, can even determine social status. These changes in behavior, can be perceived by other conspecifics, which integrate readily available social information (like chemical signals) to modulate their own behavioral outputs.

Brain

Previous studies suggest that the appraisal of these social interactions and stimuli should be processed in a set of limbic and cortical areas, the SBN (Newman, 1999). Social stimuli are detected by the peripheral nervous system which fires burst of actions potentials on sensory neurons that via the cranial nerves potentiate a set of neurons in the brain leading to a biochemical cascade of gene regulated signaling pathways (as seen in Chapter II). But surely the brain not only processes rather simple chemical, visual or acoustic/tactile cues, it should be able to integrate this information with previous experience to correctly evaluate the salience of the stimuli in order to express meaningful behavior.

The SBN is comprised of a series of 6 core nodes which are involved in the regulation of multiple forms of social behavior, are reciprocally connected, and contain sex steroid hormone receptors. A combination of insights from developmental studies, tract tracing and neurochemistry was used to expand this SBN (initially proposed for mammals) and identify putative brain homologies across different taxa (Goodson, 2005; Newman, 1999; O'Connell and Hofmann, 2011). The nodes that make up this network are the lateral septum (LS), preoptic area (POA), ventromedial hypothalamus (VMH), anterior hypothalamus (AH), the

periaqueductal gray/central gray (PAG/ CG), the medial amygdala (meAMY), and bed nucleus of the stria terminalis (BNST). Identifying homologies between the teleost telencephalon and other vertebrates is especially challenging since the neural tube of fish during development suffers an eversion, rather than an invagination (Wullimann and Mueller, 2004).

Half of these mammalian nodes (POA, meAMY and BNST) seem to be activated in response to aggressive stimuli in teleost fish (Goodson, 2005; O'Connell and Hofmann, 2011). The POA is known in mammals to mediate not only aggression, but also sexual behavior and maternal care. Lesions in this region in rats are known to decrease male aggression (Albert et al., 1986) and stimulation usually tends to increase their sexual behavior (Malsbury, 1971; Malsbury et al., 1977). In teleosts, this region appears to be functionally and hodologically similar (it is also known as POA) and lies in the hypothalamus, dorsally to the optic tract and alongside the third ventricle. The role of the POA seems to be highly conserved throughout vertebrate evolution and in teleosts it also plays an important role in aggression, sexual behavior and parental care (O'Connell and Hofmann, 2011).

The meAMY and the BNST in mammals share a wide network of connectivity with the hypothalamus (Dong et al., 2001). Both these areas are known to play a decisive role in mediating aggression and reproductive behavior in mammals and the body of literature dedicated to these areas is quite extensive (e.g. Coccaro et al., 2007; Miczek et al., 1974; Shaikh et al., 1986; Vochteloo and Koolhaas, 1987). For example, the meAMY has a crucial role in social odor recognition, since it receives massive projections from odor processing areas, like the vomeronasal organ (see Petrulis (2009) for a review of these neural mechanisms in Syrian hamsters, *Mesocricetus auratus*). In this species, agonistic encounters also increase immediate early gene induction in the BNST (Kollack and Newman, 1992). Developmental, neurochemical, and hodological data points to the supracommissural part of the ventral pallium (Vs) as the putative homolog of both meAMY and BNST in teleosts. In weakly electric fish, *Eigenmannia virescens*, stimulation of the POA will increase evoked courtship signals of

males (Wong, 2000). Similarly, in the bluegill (*Lepomis macrochirus*) and in red salmon (*Oncorhynchus nerka*) electrical stimulation of the POA and Vs will not only increase courtship behavior but also increment aggression (Demski and Knigge, 1971; Satou et al., 1984), whereas lesions in the same areas in the male killfish (*Fundulus heteroclitus*) will result in decreased spawning behavior (Macey et al., 1974).

Contrarily to the volume of information available for the Mozambique tilapia, several brain atlas are accessible for zebrafish (you can find a shortlist to some of these tools here: https://zfin.org/zf_info/anatomy/dict/sum.html) which include histological data (Wullimann et al., 1996) but also MRI and 3D reconstructions (Ullmann et al., 2010).

Genes

Most living systems share a set of macromolecules (nucleic acids, amino acids) for the storage, transfer and utilization of information, which is considered strong evidence for a common origin of life on Earth. But it also suggests that throughout evolutionary history, animals developed a series of mechanisms sharing the same set of building blocks.

Aggressive behavior is a complex quantitative trait, with population variations that could be attributable to multiple interacting loci with individually small effects, whose expression is dependent on the social environment (Edwards et al., 2006). Internal mechanisms coordinating the expression of these behaviors are remarkably conserved across species. For instances, a great focus of research is spent in understanding the conserved actions of hormones, in particular sex steroid hormones, as well, as neurohypophysial hormones in the regulation of aggression (Adkins-Regan, 2009; Insel et al., 1993). In zebrafish, estrogens are known to affect the dynamics of male-male aggression (Colman et al., 2009), whereas, in tilapias androgen circulating levels reflect dominance status (Oliveira et al., 1996). Recent studies in both species, suggest that the vasotocinergic system (vasotocin is the teleost homologue of vasopressin in mammals) may play a role in shaping dominant-subordinate

relationships and agonistic behavior (Almeida et al., 2012; Larson et al., 2006). Likewise, the role of cathecolaminergic systems in modulating aggressive behavior has been addressed across a wide range of animals (Bell and Hepper, 1987). Dopaminergic and serotonergic activity, for example, increase in the telencephalon of zebrafish after winning a contest (Teles et al., 2013).

All these regulatory mechanisms which are known to modulate aggressive behavior, like dysfunction of the biogenic amine systems, represent a small portion of the complex genetic architecture underlying social behavior. Interestingly, it has become increasingly evident especially with the development of genomic tools, that the activity of entire sets of genes and signaling pathways might be conserved across species (Machado et al., 2009; Smith et al., 2008).

Fruitflies are an interesting model species to better understand the genetic basis of aggression. As discussed previously, they display elaborately structured aggressive behaviors and, more importantly, were among the first organisms used for genetic analysis (only 4 chromosomes) and its genome is fully sequenced (Adams et al., 2000). In this species, the fruitless gene was thought to be involved in specifying sex differences in aggression and dominance. Sex-specific splicing of this gene can influence, for example, how a fly fights by encoding for sex-specific male/female aggressive behaviors: fruitless gene male mutants can present female-like behavior and vice-versa (Vrontou et al., 2006). Fruitless could also play a critical role in determining who a fly fights with and whether dominance relationships are formed. Despite this, a whole-genome expression profile of genetically divergent lines of fruitflies, selected for increased or decreased aggression, revealed several novel genes implicated in aggression, emphasizing how functional genomics can complement classical forward genetic screens in traditional genetic model systems. Aggressive behavior across populations can be explained in part by genetic differences (Hoffmann, 1988). In honeybees, hereditary differences in aggression are quite famous between the African subspecies, *Apis*

mellifera scutellata, and the Africanized "killer" bee, which are far more aggressive relative to the European subspecies (Hunt et al., 2007; Zayed and Robinson, 2012). A brain gene expression analysis associated with aggression in Africanized and European bees, reported hundreds of differently expressed genes, more specifically between soldier bees, followed by guards and then foragers (Alaux et al., 2009). Interestingly, the same set of genes, which are up-regulated in the highly aggressive soldiers of Africanized bees are up-regulated in European bees when exposed to an alarm pheromone (which triggers aggressive responses in defense of the colony) (Alaux et al., 2009). Hypothetically, these small variations in specific pathways, via gene regulation in space and time, can result in phenotypical novelties that may give rise to new lineages of aggressive bees in the course of evolution.

Genomic studies with the cichlid fish, *Astatotilapia burtoni,* have given vital contributions into the genomic regulation of social dominance behavior in a social context. Behaviorally this species is very similar to the Mozambique tilapia (in terms of genome there is also some similarity and for the second study of Chapter II a cDNA microarray platform developed for this species was used). In *A. burtoni*, microarray analysis showed that dominant males express higher levels of neuroendocrine-associated genes, like vasotocin and prolactin, when compared to subordinate males (Renn et al., 2008). Subordinate males of this species with an opportunity for social ascension also present a rapid genomic response. In preoptic GnRH neurons there is an increase in the induction of the IEG egr-1, as well as, the regulation of sex steroid receptors and steroidogenic acute regulatory proteins, which regulates androgen production (Burmeister et al., 2005; Maruska et al., 2013). In both studies presented here, IEG expression was regulated after social interactions, reinforcing their role as orchestrators of an integrated genomic response to social information by co-regulating different gene sets (Oliveira, 2012).

In a recent study in zebrafish, the expression profiles of specific target genes associated with aggressive behaviors were examined to unravel the neurophysiological basis of

aggression in this species (Filby et al., 2010). Much like expected, substantial differences in gene expression profiles were found mainly in the telencephalon and hypothalamus of dominant and subordinate males. This evidence fits nicely with all the aforementioned genomic studies pointing out the difference genomic profile of dominants and subordinates (Burmeister et al., 2005; Renn et al., 2008; Schunter et al., 2014) and both studies presented here. Nonetheless, in this study through the use of an integrated approach, combining gene expression profiling, behavioural analyses and pharmacological manipulations it was possible to identify candidate genes and pathways that modulate aggression in fish. The gene modules studied by these authors included the hypothalamo-neurohypophysial system, serotonin, somatostatin, dopamine, hypothalamo-pituitary-interrenal, hypothalamo-pituitary-gonadal and histamine pathways (a novel finding outside mammals) (Filby et al., 2010).

We also reported several genes that are known to regulate aggression and sexual behavior, aromatase (an enzyme that mediates brain conversion of androgens into estrogens) had lower expression in losing zebrafish and when male tilapia were stimulated with male scent. Contrasting this result, Filby et al. (2010) reported an increase in the expression of dominant females but no changes are reported for males (though isolation conditions prior to the contests are somewhat different between the two experiments which should be taking into account when comparing this types of behavioral data). Aromatase has also been reported to be differentially expressed between dominant and sneaker males and females of the blenniioid *Tripterygion delaisi* (Schunter et al., 2014) and to have a lower expression in castrated males of *Salmo salar* than in non-castrated males (Mayer et al., 1991). In *Salaria pavo* aromatase activity seems also to be suppressed in sneaker males and elevated in nesting Bourgeois males (Gonçalves et al., 2008). This higher conversion of androgens into estrogens in the brain of dominant males suggests that aromatase is a key enzyme promoting aggression males through actions in the preoptic area (Huffman et al., 2013).

GnRH was originally identified because of its essential role in the regulation of reproduction in all vertebrates. In *Oreochromis niloticus*, GnRH neuronal systems interact with olfactory pathways at the olfactory rosette modulating nest-building behavior in this species (Uchida et al., 2005). In the Mozambique tilapia, GnRH is also regulated when males are in the chemical vicinity of other males. In *A. burtoni* nonetheless, reproductive states are also being socially regulated by GnRH, such that dominant males have larger testes and more mature sperm than subordinate males (Francis et al., 1993). Despite no changes in GnRH were found in our study with zebrafish, other studies with this species reported GnRH increased activity in dominant males (Filby et al., 2010).

Finally, somatostatin which is usually known as growth hormone-inhibiting hormone because of its role in growth, has been we shown to also plays a role in controlling social behavior, namely aggression (Trainor and Hofmann, 2006; Trainor and Hofmann, 2007). In *A. burtoni*, pharmacological manipulations using somatostatin antagonists increased aggressive behavior in a dose-dependent fashion while an agonist decreased aggression. In our microarray experiment with the Mozambique tilapia, somatostatin increased when males were in the olfactory vicinity of another dominant male, similar to what was reported in *A. burtoni* (Hofmann and Fernald, 2000). Once again in our studies with zebrafish, this gene was not significantly regulated, but other studies report that is also one of the genes being overexpressed in dominants (Filby et al., 2010). Thus, somatostatin may also function to contain energetically costly processes such as somatic growth and aggressive behavior in teleost fish (Trainor and Hofmann, 2006).

In sum, the results in Chapter I suggest that a rather complex network of genes and molecular cascades might be responsible for the expression of a given number of adaptive behaviors in response to cues from the individual's social environment. On the other hand, results from Chapter II further explore how these different stimuli from the social environment can regulate specific gene modules, probably to coordinate the appropriate expression of

behaviors in response to each social stimulus. These studies demonstrate that brain gene expression is closely linked with behavior, that changes in brain gene expression mediate changes in behavior, and that the association between specific genes and behavior exists over multiple timescales, from physiological to evolutionary.

Future perspectives

The correct appraisal of the surrounding conditions is fundamental in social contexts, where aggressive behavior is common and can often lead to serious injuries and death, but also to identify opportunities (such as reproduction, access to resources, flee from predators, etc.). To survive an individual must accommodate these external stimuli along with relevant internal information to display context-appropriate behavioral responses. As in Chapter I our study only included a whole brain analysis, which might account for some of the differences with other studies, we only have a general idea that brain neurons are responding to these cues. Future research should focus on what happens in specific brain nodes of the SBN in response to certain social stimuli. More specifically what areas of the brain are responsible for appraising the social context and ultimately orchestrate the appropriate output. Probably a great number of areas and an even greater number of genes are simultaneously being activated and a global view of the brain in nonetheless needed to fully understand this network. Nowadays, with recent developments and widespread usefulness of genomic tools it is financially viable to monitor an even greater number of areas and also increase the sample size. Microdissection tools have also been updated and currently is possible to use laser dissection microscopes to more precisely collect brain tissue (e.g. O'Connell and Hofmann, 2012b). These advances promise new avenues to better understand the interaction between the genotype and the environment.

Additionally we can further test the aforementioned hypothesis that zebrafish appraise its own fighting ability against that of its opponent. This could be approached by manipulating

both internal and external social cues of the subject. Internal cues can experimentally be altered using a combination of castration and hormone replacement for androgens, pharmacological manipulations of vasotocin or use the microdialysis technique to alter cathecolaminergic systems. External stimuli can be manipulated very easily in fish since chemical stimuli alone convey information about social rank (Chapter II).

Another interesting approach is to explore the available genetic tools in zebrafish, such as transgenic (GAL4-UAS) and mutant individuals. The possibility of knocking out key target genes or to test fish lacking diffuse neuro-modulatory systems might help us understand the regulatory mechanisms behind cognitive appraisal of a given social context in zebrafish. Or, ultimately, to genetically engineer and control social behavior under the control of a heat-shock promoter whose expression could be turned on or off at different temperatures, for example, and used to express different behavioral traits using transgene methods (Shoji and Sato-Maeda, 2008).

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