Phenotypic Architecture and Genetic Polymorphims Associated with Social Behaviour in Zebrafish

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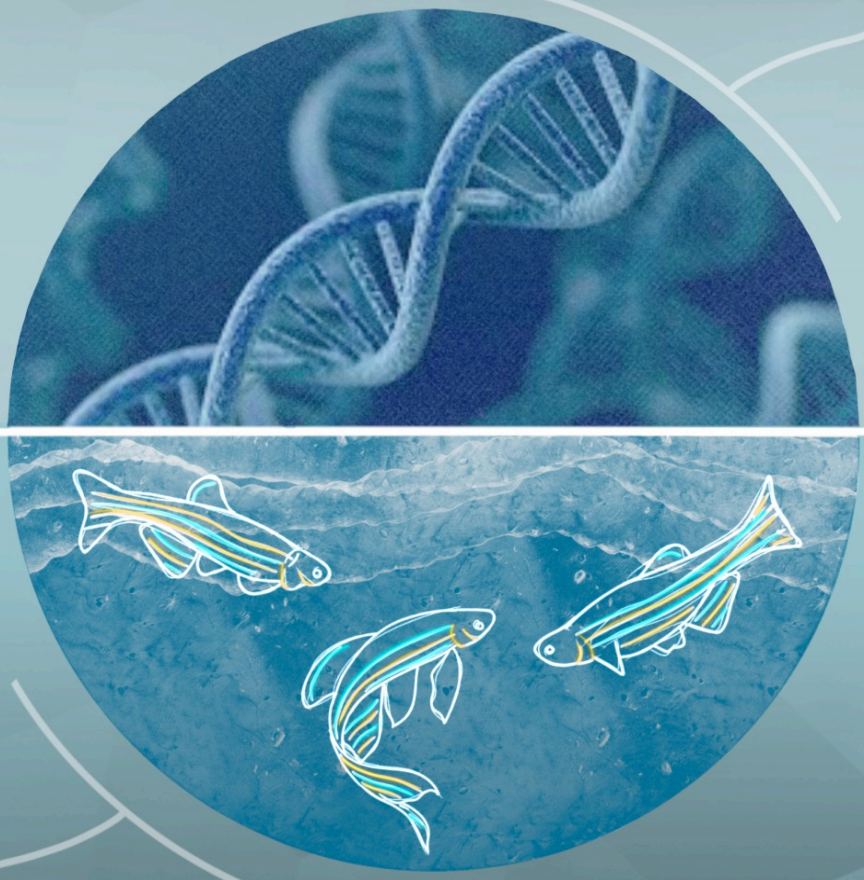

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PHENOTYPIC ARCHITECTURE AND GENETIC POLYMORPHISMS ASSOCIATED WITH SOCIAL BEHAVIOUR IN ZEBRAFISH

CLÁUDIA CRISTIANA DA CRUZ GONÇALVES

A DISSERTATION
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TABLE OF CONTENTS

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List of Figures

List of Tables

Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
ACTH	Adrenocorticotropic hormone
AH	Anterior Hypothalamus
ASD	Autism Spectrum Disorder
AVP	Arginine Vasopressin
BlAMY	Basolateral Amygdala
BNST	Bed Nucleus Stria Terminalis
CG	Central Gray
CRF	Corticotropin-releasing Factor
DA	Dopamine
GABA	Gamma-aminobutyric Acid
GWAS	Genome Wide Association Studies
HIP	Hippocampus
HPA	Hyphotalamic-pituitary-adrenal
LS	Lateral Septum
meAMY	Medial Extended Amygdala
mPOA	Medial Preoptic Area
MRS	Mesolimbic Reward System
Nacc	Nucleus Accumbens
OT	Oxitocyn
OTR	Oxytocin Receptor
PAG	Periaqueductal Gray
PCA	Principal Component Analysis
QTL	Quantitative Trait Locus
SBN	Social Behaviour Network
SDMN	Social Decision-Making Network
SNP	Single Nucleotide Polymorphisms
Str	Striatum
T	Testosterone
VMH	Ventromedial Hypothalamus
VP	Ventral Pallidum
VTA	Ventral Tegmental Area

Summary

Social behaviour is fundamental for the survival and reproduction of organisms, and most animals are social to some degree. It is generally recognized that many neuropsychiatric diseases are associated with some form of social deficit or are accompanied by social impairments. There is also evidence that actual and perceived social isolation are both related with increased mortality risk. Given that social behaviour is central in both humans and other animals' lives, many researchers with different backgrounds have been actively engaged in the challenge of understanding the nature of this highly complex and dynamic phenomenon.

Social behaviour that independently evolved multiple times across animals is an extremely diverse behavioural category, influenced by multiple factors (genes, hormones, environment, ecology, development, life history trait, etc.) requiring a multidisciplinary approach, integrative analysis and standardized terminologies. However, despite its great diversity (both between and within species), there are similarities namely at mechanistic and functional level, which allows organizing social behaviours in functional modules, similar to those used in gene ontology categories.

In this sense and to better understand the relationship between different social behaviours and how they are organized, we investigated the phenotypic architecture and genetic polymorphisms associated with social behaviour in zebrafish. Although the genome of humans and other species is amply known, the relation between genotype and phenotype is still an unsolved puzzle, specially for complex traits, i.e., those that depend on the small effects of many genes.

With a strong tendency to aggregate, zebrafish have been a

useful model to study social behaviour, and, considering their phylogenetic position, it enables the investigation of this phenomenon in the most successful evolutionary radiation among vertebrates (teleost fishes) that also exhibit a diverse range of social systems. Furthermore, zebrafish is suitable for genetic studies, since their genome is well characterized, and more than 70% of the genes have human orthologues.

The main goal of this thesis is to investigate the phenotypic architecture of social behaviour in zebrafish and to explore the genetic polymorphisms associated with different components of social behaviour. For this purpose, we started by phenotyping different aspects of social behaviour (i.e. motivational component = social tendency; and cognitive component = social recognition), as well as a non-social cognitive ability (i.e. object recognition), and anxiety, in a large number of individuals from a diverse genetic background (i.e. six different zebrafish laboratory strains (AB, TU, WIK, TL, 5D, Leo). For this purpose, we have used three behavioural paradigms and a total of four tests: (1) the shoal preference test, which evaluates the individual's tendency to spontaneously associate with conspecifics; (2) the social recognition test, that assess the individual's ability to recognize conspecifics; (3) the non-social recognition test, that assess the individual's capacity to discriminate between different objects; and (4) the open-field test, that measures individual's general levels of anxiety.

The results of the behavioural tests revealed that fish from all six strains exhibited a strong preference to associate with conspecifics as well as the presence of social and non-social memory. Further, the strains varied in their level of social preference and in their open-field behaviour, showing different levels of anxiety. A principal component analysis (PCA) was done in order to explore the pattern of phenotypic correlations among these behavioural traits, indicating three factors (i.e.

principal components) of behavioural variation: (1) a general investigation motivational factor, which loads social tendency from the social preference test, social exploration from the social recognition test and object exploration from the object recognition test); (2) a general cognition factor, which loads social discrimination from the social recognition test and object discrimination, from the object recognition test; and (3) an anxiety factor, which loads thigmotaxis score and proximity to the wall measures, from the open field-test. These results indicate that the motivational and cognitive domains of sociality are not linked to each other, and neither seem to be specific to social traits, since both show phenotypic correlations with non-social traits, supporting a general domain hypothesis for social traits. Anxiety is also discrete from social and cognitive domain, indicating an independence for the evolution of social traits from anxiety, which could be predicted from a pedration avoidance hypothesis for the evolution of sociality. We also addressed the adaptive vs. constraints hypotheses for the evolution of the different components of sociality and our results show that the correlations between traits vary across different populations (strains), supporting the adaptive hypothesis, and hence, the independence (instead of a constraint or dependency) in the evolvability of the motivational and cognitive components of sociality.

Finally, genetic data shows associations between some of our candidate SNPs (single nucleotide polymorphisms) and the behaviours tested. There were 29 genes associated with the motivational factor, 5 genes with anxiety and none with the cognitive factor. The SNPs associated with the motivational component include SNPs also associated with each of the traits it encompasses (social tendency, social exploration and object exploration), and represent genes that in literature are implicated in social behaviour (serotonin receptors, dopamine receptors, oxytocin) and neural plasticity (neurexins,

neuroligins, shank3a, etc.). Moreover, the SNPs associated to the motivational component are different from those associated with anxiety.

In summary, we show that sociality is modular and integrates both a motivational and a cognitive component, and that each of these components is not domain specific but rather shared with other non-social domains. Moreover, the genetic polymorphisms associated with the studied traits also show a specific association of candidate “social” genes with the motivational but not with the cognitive component, further supporting their independence. Thus, our work provides important insights into the phenotypic architecture of quantitative traits and genetic polymorphisms associated, opening up the possibility of raising new questions involving the complexity of social behaviour.

Resumo

O comportamento social é fundamental para a sobrevivência e reprodução dos organismos e a maioria dos animais apresenta algum grau de sociabilidade. É geralmente reconhecido que muitas doenças neuropsiquiátricas estão associadas a alguma forma de défice social ou são acompanhadas por perturbações na esfera das intereções sociais.

Há também evidências de que o isolamento social, real ou percebido, está relacionado com o aumento do risco de mortalidade. Dado que o comportamento social é central, tanto na vida dos humanos quanto na de outros animais, muitos investigadores de diferentes domínios do conhecimento têm-se empenhado ativamente no desafio de compreender a natureza desse fenómeno altamente complexo e dinâmico.

O comportamento social, que evoluiu várias vezes de forma independente no reino animal, é uma categoria comportamental extremamente diversificada e influenciada por vários fatores (*v.g.*, genes, hormonas, ambiente, ecologia, ontogenia, características de história de vida, etc.), exigindo assim uma abordagem multidisciplinar, uma análise integrativa e terminologias padronizadas. Apesar da grande diversidade de comportamentos sociais (tanto entre espécies como dentro da mesma espécie), existem semelhanças nomeadamente ao nível funcional e dos mecanismos subjacentes, o que permite organizar os comportamentos sociais em módulos funcionais análogos aos utilizados nas categorias de ontologia genética.

Nesse sentido, e para se compreender melhor a relação entre as diferentes categorias de comportamentos sociais e como estão organizadas, investigámos a arquitetura fenotípica e os polimorfismos genéticos associados ao comportamento social no peixe-zebra. Embora

o genoma humano e o de outras espécies sejam amplamente conhecidos, a relação entre genótipo e fenótipo é ainda pouco conhecida, principalmente no que diz respeito às características fenotípicas complexas, ou seja, aquelas que dependem dos efeitos moderados de muitos genes.

Com uma forte tendência para se agrupar (formar cardumes), o peixe-zebra tem sido um modelo útil para estudar o comportamento social, permitindo também, atendendo à sua posição filogenética, a investigação deste comportamento na mais bem-sucedida radiação evolutiva entre os vertebrados (os peixes teleósteos), que também apresenta uma gama bastante diversificada de sistemas sociais. Além disso, o peixe-zebra é adequado para estudos genéticos, uma vez que o seu genoma está bem caracterizado e mais de 70% dos seus genes possuem ortólogos humanos.

O principal objetivo desta tese é investigar a arquitetura fenotípica do comportamento social no peixe-zebra e explorar os polimorfismos genéticos associados a diferentes componentes do comportamento social.

Para esse efeito, começámos por fazer a fenotipagem de diferentes aspetos do comportamento social (i.e. componente motivacional = tendência social e componente cognitivo = reconhecimento social), bem como da capacidade cognitiva não social (ou seja, reconhecimento de objetos) e da ansiedade, em um grande número de indivíduos de linhagens genéticas diferentes (i.e., seis estirpes diferentes de peixe-zebra de laboratório – AB, TU, WIK, TL, 5D, Leo).

Usámos três paradigmas comportamentais e um total de quatro testes: (1) o teste de preferência de cardume, que avalia a tendência do indivíduo para se associar espontaneamente a conspecíficos; (2) o teste de reconhecimento social, que avalia a capacidade do indivíduo de

reconhecer membros da mesma espécie; (3) o teste de reconhecimento não social, que avalia a capacidade do indivíduo discriminar diferentes objetos; e (4) o teste de campo aberto, que mede os níveis gerais de ansiedade do indivíduo.

Os resultados dos testes comportamentais revelaram que os peixes de todas as seis linhagens têm uma forte preferência em associar-se a conspecíficos, tendo também revelado a presença de memória social e não social. Além disso, as estirpes variaram no seu nível de preferência social e no seu comportamento em campo aberto, mostrando diferentes níveis de ansiedade.

Foi feita uma análise de componentes principais (ACP) a fim de explorar o padrão de correlações fenotípicas entre esses traços comportamentais, o que indicou três fatores (ou seja, componentes principais) de variação comportamental: (1) um fator motivacional de comportamento exploratório geral, que engloba a tendência social do teste de preferência social, a exploração social do teste de reconhecimento social e a exploração de um objeto do teste de reconhecimento de objeto); (2) um fator de cognição geral, que engloba, por um lado, a discriminação social do teste de reconhecimento social e, por outro, a discriminação de objetos do teste de reconhecimento de objetos; e (3) um fator de ansiedade, que abarca a medida de tigmotaxia e a medida de proximidade da parede, a partir do teste de campo aberto.

Esses resultados indicam que os domínios motivacional e cognitivo da socialidade não estão ligados entre si e nem parecem ser específicos dos traços sociais, uma vez que ambos apresentam correlações fenotípicas com traços não sociais, apoiando a hipótese de domínio geral para traços sociais. A ansiedade constitui também um domínio diferente do social e cognitivo, indicando a independência da

evolução dos traços sociais da ansiedade, o que pudemos prever a partir da hipótese de evolução social de proteção contra predadores.

Também abordámos a hipótese adaptativa vs. a hipótese dos constrangimentos (ou dependência) para a evolução dos diferentes componentes da socialidade, tendo os nossos resultados mostrado que as correlações entre comportamentos variam entre diferentes populações (linhagens), o que apoia a hipótese adaptativa, ou seja, a independência na evolução dos componentes motivacionais e cognitivos de sociabilidade. Finalmente, os dados genéticos mostram associações entre alguns dos SNPs (*Single Nucleotide Polymorphisms* – Polimorfismos de nucleotídeo único) candidatos e os comportamentos testados. 29 genes estão associados ao fator motivacional, 5 genes à ansiedade e nenhum gene está associado ao fator cognitivo.

Os SNPs associados ao componente motivacional incluem SNPs também associados a cada uma das características que ele engloba (tendência social, exploração social e exploração de objetos) e representam genes que, de acordo com a literatura, influenciam o comportamento social (*v.g.*, recetores de serotonina, dopamina e oxitocina) e a plasticidade neural (neurexinas, neuroliginas, shank3a, etc.). Além disso, os SNPs associados ao componente motivacional são diferentes dos associados à ansiedade.

Concluindo, mostrámos que a socialidade é modular e integra um componente motivacional e um componente cognitivo, e que cada um desses componentes não é específico de um domínio, mas sim compartilhado com outros domínios não sociais. Além disso, os polimorfismos genéticos associados às características estudadas também mostram uma associação específica de genes "sociais" candidatos com o componente motivacional, mas não com o cognitivo, o que reforça a sua natureza independente. Assim, este trabalho fornece importantes pistas sobre a arquitetura fenotípica de características

quantitativas e polimorfismos genéticos associados, abrindo a possibilidade de se levantarem novas questões envolvendo a complexidade do comportamento social.

Author Contributions

Cláudia Gonçalves and Rui Oliveira designed all experiments presented in this thesis and established the behavioural protocols, together with Magda Teles. Cláudia Gonçalves performed all behavioural experiments, tracking data analysis and collect fin samples. Ricardo Leite processed all fin samples. Cláudia Gonçalves, together with Susana Varela, Kyriacos Kareklas, Magda Teles and Rui Oliveira executed the data analysis.

The study presented in chapter 2 will be part of a paper already submitted. Cláudia Gonçalves and Rui Oliveira wrote the paper with contributions from all the other authors.

Chapter I

General Introduction

1. Introduction

Sociality is ubiquitous in the animal kingdom and it affects the evolution of a wide range of phenotypes, including morphological, behavioural, and life history traits. Social behaviour is not a uniform trait, requiring the integration of diverse level of analysis, an objective framework and standardized terminologies. The work described in this thesis is an integrative study that uses zebrafish as a model organism to investigate the phenotypic architecture and genetic polymorphisms associated with social behaviour.

This chapter begins by presenting the conceptual framework of social behaviour and the characterization of the field. Next, background on two fundamental components of social behaviour (motivational and cognitive component) at two different levels, the ultimate (section 2) and proximate level (section 3) are covered. In the latter sections, the characterization of zebrafish as a model organism and its social behaviour, as well as the relevance of its use in social neuroscience is described.

1.1 Social Behaviour

Social behaviour can be defined as any behaviour directed towards another animal, usually a conspecific, but can also be an heterospecific (Nunes et al., 2017; Simeonovska-Nikolova, 2007). This type of behaviour serves many purposes and is critical to the survival and reproduction of individuals. Social behaviour ranges from a simple response to approach conspecifics, widely present in animal taxa, to more elaborated forms of interaction, such as mating and parental care, existing in many species, or cooperation and altruism, which are highly structured interactions, occuring only in a more limited number of species (Raulo & Dantzer, 2018).

Social groups present a stunning diversity in size, frequency of interaction, group composition and stability, degree of cohesion and coordination, as well as forms of cooperation and competition between members (Ebensperger & Hayes, 2016; Kappeler, 2019). Accordingly, social behaviour is not a uniform phenotypic trait, having evolved in distinct directions. As such, the neurobiological pathways underlying social behaviour may share a common basis or diverge in important ways, with no species being representative of group living in a general sense, which can limit the translational potential of animal models (Beery, 2019).

An interesting aspect of social behaviour is that it is present in very different groups and closely related species may vary considerably in their social organization (Ward & Webster, 2016). An exemple is the hymenopteran lineage that has evolved to great diversity of gradients of social organization as is the case of social insects that vary in their degree of sociability (e.g. communal, sub-, quasi-, semi-, para-, pre-, and eu-social (Linksvayer, 2010)

Sociality is, therefore, considered the pinnacle of biological complexity, and understanding the complex and dynamic nature of social behaviour has been a challege since it is also influenced by a multitude of elements, such as genetic, developmental, nervous system connectivity, physiological state, and physical and social environment (Anholt & Mackay, 2009).

Furthermore, the social environment is continously changing, and social behaviour must be highly dynamic and flexible to respond to these changes. Thus, our understanding of the mechanistic bases of social behaviour is often limited compared to other behaviours, specially in vertebrates that exhibite a significant level of social behaviour plasticity when compared to invertebrates (Rubenstein & Hofmann, 2015).

In the case of vertebrates, they show a marked inter and intraspecific variation, as they tend to have a slower pace of development and a longer life span, providing more time for learning and more possibilities of establishing stable relationships and complex social networks. Also, their complex brains with variable cognitive abilities allow them to create different strategies to navigate their complex social environments (Kappeler et al., 2013). At a proximal level, the high genetic redundancy (genes performing the same functions) and interconnectivity of neural circuits involved in social behaviours, makes its study very complex. Therefore, the study of social behaviour demands an interdisciplinary approach, comparative studies, integrative conceptual analysis and innovative methods for its broad understanding (Cacioppo & Decety, 2011).

The concept of modularity has been used to reconcile the seemingly dissimilar behaviour systems across species trying to find common explanation for this diversity (Singh et al., 2008). Modularity refers therefore, to a pattern of organization of elements (behaviours, morphological characters or genes, proteins, etc.) into sub-components highly coordinated and semi-independent of others sub-components (Klingenberg, 2008). In the relatively new field of evolutionary developmental biology (evo-devo) the modular concept of gene toolkit is central and has led to important advances in understanding the diversity of morphologies across species. The evo-devo approach combined with progress in genomics, promises to bring to light important aspects of the evolution of social behaviour (Toth & Robinson, 2007). Also, the genomic revolution and rapid advances in neuroscience and endocrinology created unprecedented opportunities for the investigation of causal relationships along the “genes-brain-behaviour” axis (Anholt & Mackay, 2009). There are many other exciting directions that the field is taking, namely the investigation of the role of genetic polymorphisms on

social behaviour traits and the influence of epigenetic changes (Janecka et al., 2017; Palumbo et al., 2018). There is also an emerging substancial scientific literature on behavioural syndromes, or "personality", in animals, which include sociality (Gosling, 2001; Sih & Del Giudice, 2012; Webber & Willis, 2020) and socially driven emotion-like behaviour (Ferretti et al., 2019; Scheggia & Papaleo, 2020).

Traditionally the study of the proximate mechanisms of social behaviour has focused primarily on mammals (primates, voles, rats) and birds, and on eusocial insects (bees and ants). But a wide variety of model systems has been emerging in the last decades that range from microorganisms to cetacens (Fox et al., 2017; Gibbs et al., 2008; Strassmann et al., 2011; Tyack & Clark, 2000), including zebrafish, which popularity as a model organism has been growing in many fields, including social neuroscience (Oliveira, 2013). However, the choice of appropriate model organisms depends on several factors, namely on the reseach question and what tools are available to dissect this question.

Although humans have very complex and on certain aspects unique social abilities in comparison to other animal groups, animal research has played a significant role for identifying general principles of behavioural control (Snowdon, 1999). The striking developments in social neuroscience research have generated considerable optimism regarding the understanding of the fundamentals of human psychiatric disorders, such as autism and schizophrenia, opening the way for new therapies. Considering that social interactions are central to human life and most phychiatric disorders involve some disruption of normal social behaviour, these studies are of paramount importance, both clinically and for society (Young, 2008). The vibrant field of social neuroscience is now prepared to tackle some of the most important questions related to behaviour, however, there is a need of more conceptual consensus in the field (Stanley & Adolphs, 2013).

In a markedly interdisciplinary field as social neuroscience, reliable communication between scientists from different areas is crucial to cover the field and to avoid semantic confusion (Cacioppo & Decety, 2011; West et al., 2007). Thus, it is timely to characterize the phenotypic architecture of social behaviours and to try to create an ontological system that fosters its study across species (aka comparative phenomics) (Gkoutos et al.,2012).

2. Components of social behaviour

Despite the striking diversity of social behaviour phenotypes, there are two key behavioural mechanisms underlying social living: a motivation to approach conspecifics (social tendency) that promotes the formation of social groups, and the cognitive ability to recognize different conspecifics (social recognition) that permits individuals to selectively adjust the expression of their behaviour to different individuals of the social group depending on previous interactions (Mateo, 2004; Ward et al., 2020).

These two basic behavioural mechanisms are therefore crucial for goal-oriented social behaviours and hence to normal social development. To better understand these mechanisms, it is important to distinguish their ultimate causes (evolutionary history and ecological function) from its proximate or mechanistic causes (how the behaviour arises in animals) (Mayr, 1961).

In this section, the motivational and cognitive components will be described using the ultimate perspective, that is, the why questions.

2.1 Motivational Component (Social Tendency)

Motivation is essential for the initiation and maintenance of behaviours and is therefore, both activational, arousal and goal-directed. Animals express motivation to meet their basic survival needs namelly water, food, sex, and social interaction (Duffy, 1957; Simpson & Balsam, 2016).

Social interaction has fitness consequences, thus, animals time and coordinate their behaviour with others to gain potential benefits (Jolles et al., 2020). Thus, according to social selection an individual fitness depends not only on its own phenotype but also on the phenotype of its social partners (Weidt et al., 2008). In this way, animals have developed a variety of traits to optimise group living.

One of the fundamental aspects that differentiates social behaviour is that it is maintained by social attraction between group members, transcending mere attraction for the same resource (Ward & Webster, 2016). So, positive social interactions (that bring animals together), including social bonds and many forms of affiliation, dominate the behaviour repertoire of many vertebrates (Carter & Keverne, 2002; Raulo & Dantzer, 2018). The tendency for animals to group together is widespread throughout the animal kingdom, and animals tend to form shoals, swarms, flocks and herds, coveys, communities, clusters, or colonies of conspecifics individuals (Reiczigel et al., 2008). However, different species tend to interact in differents ways and/or in different temporal and spatial scales. At one extreme, there are species such as the polar bear and the leopard, among others, that seem to have a solitary lifestyle with scarce interactions with conspecifics, joining a group almost exclusively at certain moments of their lives: to breed, to take care of their offsprings or when a big source of food attracts many conspecifics (Majolo & Huang, 2018). In the other extreme there are animals that spend their entire lives in the company of others, showing

great tolerance to conspecifics, forming sometimes colonies of thousands or even millions of individuals, as is the case of many insects and bats (Beery, 2019; Keller & Chapuisat, 2017). There are also species as the desert locust that can exhibit extreme phenotypic plasticity swiching from solitary behaviour, that avoids other locusts, to active gregarious behaviour, forming dense swarms (Ott & Rogers, 2010). In brief, the distinction between group-forming and solitary species is to a large extent artificial, since all animals engage in social interactions and even the so-called solitary species can switch between periods of solitary living to group-living, and they can also, during the solitary phase, maintain communication at a distance with other individuals using olfactory and auditory signals (Larsen et al., 1986). Therefore, some researchers prefer to use the term social behaviour rather than social species (Kutsukake, 2009; Majolo & Huang, 2018). The terminology “facultative” and “obligatory” social species is also used to refer to weakly/plastic and highly social species respectively (Boomsma, 2013; Vidya, 2009) However, it can create an unrealistic dichotomy. Also, there are researchers that tend to use the term “social” only for complex forms of social interaction involving stable associations, hierarchical structure and one or more forms of cooperation. Less structured groups, with unstable associations, are hence called gregarious (Ebensperger & Hayes, 2016). Additionally, groups that present an extreme form of sociality involving altruistic behaviours are called eusocial. The solitary form of life is considered the ancestral state that evolved to group living independently in various taxa, and the transition to sociality has been considered one of the major transitions in evolution (Smith, 1995). However, there are studies that have shown that social behaviour can be lost giving rise to species that are secondarily solitary (see Wcislo & Danforth, 1997).

Social tendency varies not only among species but also within species. The age, day period, seasonal factors, developmental stage, resource availability, influence an animals' social tendency (Schradin, 2013). For instance, certain species of fish and amphibians are social in their early life, but solitary in their adult stage (Ward & Webster, 2016). As mentioned above, desert locusts can be either solitarious (prefering isolation) or gregarious (attracted to conspecifics) in response to population density (Topaz et al., 2012). Other species may be social only at certain times of day. Resource availability also influences social tendency. In a co-feeding experiment conducted in two primate species (red-fronted and ring-tailed lemurs) with both wild and captive groups, that aimed to measure social tolerance, it was concluded that the level of social tolerance is species-specific and modulated to a certain degree by environmental conditions, such as food availability (Fichtel et al., 2018).

Also, some individuals of the same species are consistently more sociable than others, presenting a different "personality", also called behavioural syndrome, copyng style or temperament (Bergmüller & Taborsky, 2010; Koolhaas et al., 1999; Réale et al., 2007). In a study conducted in four different species of shrews: *Neomys fodiens*, *Sorex araneus*, *Sorex minutus* and *Neomys anomalus*, that differ in sociality and ecology, individuals were tested for their solitary activity and their social activity and agonistic behaviour within and between species' in dyadic encounters. The results suggest that the most social shrew (*Neomys anomalus)*, exhibits stronger differences in personality types, supporting the hyphotesis that social niche can influence the evolution of animal personalities (von Merten et al., 2017).

At the ultimate level, there are both benefits and costs to living in groups, and theoretically, the formation of a group occurs when the benefits for an individual to associate with others outweigh the costs (Kutsukake, 2009). Associations between conspecifics, tend to optimize

the access to resources and at the same time to reduce predation risk and energy loss.

Enhanced access to resources may occur by different processes, including the possibility to get information about resources from other group members (either conspecifics or heterospecifics, in the case of interspecies association; aka information center hypothesis). Such information can regard the location, the amount and quality of the resources, such as food, habitat and nest sites. Other benefits involve elevated foraging efficiency as well as group defence of valuable resources (aka resource-defense hypothesis) (Ebensperger & Hayes, 2016; Majolo & Huang, 2018). Living in groups also can increase access to mating partners and mating opportunities (Ebensperger & Hayes, 2016).

Likewise, social interaction allows a more efficient management of energy in the sense of reducing heat and water loss through huddling (thermoregulation) and also by reducing energy demands with shared participation in tasks (Vanthournout et al., 2016). Similarly, groups of animals that travel or move together (as fish or birds) spend less energy (Marras et al., 2015).

However, the reduction of the risk of predation is considered the primary benefit of group living, or the major ecological factor selecting for group living (Groenewoud et al., 2016), although this view has also been challenged since group living seems to evolve both in species with low and high vulnerability to predation (Varela et al., 2007). Social living increases group vigilance that allows members to detect predators more efficiently (aka many eyes hypothesis), dilutes an individual's risk of being attacked (aka selfish herd hypothesis), and reduces the efficiency of predators' attacks by confusing them. Moreover, group living also enables individuals to organize active defence strategies against predators. Additionaly, the presence of social partners can buffer the

response to stressors, such as exposure to predators (aka social support hypothesis or social buffering) (Faustino et al., 2017).

On the other hand, social relationships bring costs, such as increased probability of parasitic infection and disease transmission, infanticide, overcrowding stress and competition for limited resources and paradoxically increased vulnerability to predation due to groups being more conspicuous to predators than solitary individuals (Majolo & Huang, 2018; Varela et al., 2007). These benefits and costs can change across environments and may also be dynamic across the developmental stages of an organism.

Although researchers on sociality have traditionally focused on the relative fitness benefits and costs of group associations (adaptive hypothesis), sociality can also be directed or restricted by constraints on the course of evolution (aka Constraint Hypothesis; (Ebensperger & Hayes, 2016). Gould & Lewontin, (1979) defended in their seminal paper that constraints exert even greater pressure in delimiting pathways of change than the selective force. Constraints can arise from genetic architecure, development, or life history (Arnold, 1992). Due to genetic constrains, selection is not able to optimize all aspects of a given trait. For exemple, genetic correlations as in pleiotropy, in which a single allele affects different traits, can result in evolutionary interdependence between multiple traits, genetic trade-offs between different fitness components and reduced capacity for adaptation (Connallon & Hall, 2018). Lack of genetic variation can also constraint evolution since natural selection works on existing variation in a population. Given that traits tend to evolve from previously existing traits, adaptations are constrained by development or animal life history. In terms of life history natural selection tends to maximize individual fecundity and survival. However, intrinsic trade-offs and other types of constraints may limit life history traits, and hence fitness (Partridge, 1991).

However, constraints can also promote variation that may be implicated in positive effects (Fitch, 2012). In order to avoid connoting the term “constraint” only with negative effects, the expression “developmental bias” has been increasingly used, which can include negative and positive effects (Arthur, 2002). Constraints have been also implied to explain the stable coexistence of different social behavioural phenotypes within populations, for exemple life history trade-off has been implicated in the evolution of animal personalities (Santostefano et al., 2017). The evolutionary role of developmental bias has been studied from different perspectives and has been controversial, due to the difficulties of operationalization and due to conceptual ambiguity (Arthur, 2002). In this way, the integration of genetic analysis into the field of social evolution tends to provide unifying ideas, since it favors not only a mechanistic understanding of behaviours but also the interpretation of the function and evolution of behavioural traits (Wolf et al., 2007).

Today, the integrated approach of modern evolutionary development biology (evo-devo), which considers both evolutionary and developmental genetics, allows experimental analysis of developmental bias and the possibility to identify shared genetic or developmental pathways across morphological traits that contribute to the development of complexity and diversity (Brakefield, 2006). In this way, it may be possible to define the relative importance of natural selection and developmental bias.

2.2 Cognitive components

All animals combine information from the external environment with information stored in memory and information about their internal physiological state to produce behaviour (Rubenstein & Hofmann, 2015). Cognition refers to the mechanisms involded in the acquisition, storage

and processing of information by the nervous system (Sih & Del Giudice, 2012). Taking into account that conspecifics constitute a major component of the environment of social species, most of their decisions are affected by conspecifics (Reiczigel et al., 2008). Living in groups requires, among other cognitive abilities, recognising different group members, understanding social rules within groups, predicting the behaviour of others, and making decisions. Here we describe the basic cognitive processes underlying social abilities including: the capacity to collect information from others (social attention), recognizing conspecifics (social recognition or social memory), learning from others (social learning) and making decisions regarding social interactions (social decision making).

2.2.1 Social Attention

Social attention is an important behavioural mechanism to extract key information from others by observing relevant individuals (Klein et al., 2009). This information can provide immediate adaptive benefits, being fundamental in learning, memory, communication, and decision-making processes (Nunes et al., 2017). Thus, animals have evolved to detect and process certain kinds of social stimuli that leads to the identification and pursuit of receptive mates and potential allies as well as predators or other social threats (Ghazanfar & Santos, 2004).

The body shape and biological motion (aka animacy cues) are two important features that can transmit information about the presence of conspecifics (Nunes et al., 2020). In general, motion animacy cues attract visual inspection, and moving animals attract more visual attention because they change their status (behaviour, trajectory, location) more frequently than other environmental components (e.g. plants or rocks), requiring frequent reinspection (Altman et al., 2016;

Klein et al., 2009; New et al., 2007). In a study where bioinspired robots mimicking zebrafish's visual features were used, the robots successfully attracted both isolated individuals and small shoals (Polverino et al., 2012).

Paying attention to the attention of others, that is, following an individual's gaze is also an important feature to obtain valuable information about predators, food or for understanding their intentions, interests, goals, or affective states (Itakura, 2004).

A study about social attention in rhesus macaques and humans shows that both reflexively shift their attention to where another individual is looking in a visual target detection task (Deaner & Platt, 2003). Animals also tend to attend to emotional cues rather than neutral cues. Thus, attention to threat reflects trait affect and animal emotion and welfare can be assessed through attention bias that is sensitive to changes in the emotional state (Howarth et al., 2021).

Likewise, animals attend preferencially to novel stimuli when compared to familiar stimuli. Novelty seeking correlates, therefore, with other behaviours such as anxiety-like behaviour, emotionality and cognitive functions, and has been widely used to mesure these behaviours (Redolat et al., 2009).

2.2.2 Social Recognition

Social recognition is the ability to recognize individual conspecifics or categories of conspecifics, such as social hierarchical status, sex, age, reproductive state, or any possible aspect of the phenotype (Kogan et al., 2000; Ward et al., 2020). It is necessary for directing the appropriate behaviour towards the appropriate individual, hence it constitutes the foundation for the formation of all social relationships (Insley et al., 2003). Social recognition is a form of memory with fitness benefits in the

domains of reproduction, territorial defense, establishment of dominant hierarchies, and cooperation (Ferguson et al., 2002).

At the ultimate level, social recognition is adaptive because it reduces the energetic costs of aggressive competition by enabling to target aggression appropriately, favoring greater stability of hierarchies and productivity of the group (Gherardi et al., 2012). It also enables to obtain an equilibrium between inbreeding and outbreeding when selecting a mate (Insley et al., 2003). The ability for parents to identify their own offspring has also obvious advantages, since it allows them to reduce the amount of care or avoid providing care to unrelated offspring (Svensson et al., 2010). Likewise, recognition and avoidance of unfamiliar individuals could also be advantageous in the way that excludes conspecifics that may carry novel pathogens (Choleris et al., 2012).

Social recognition is based on salient social cues, relying on multiple sensory modalities and different species vary substantially in their recognition capacities (Insley et al., 2003). In humans and other primates, individual recognition depends mainly on visual and auditory cues (Ferguson et al., 2002). In most other animals, olfactory cues play an important role in social recognition, with chemical signals (pheromones) providing direct information about sex, social status, individuality, and physical condition (Shelley et al., 2006). Insects can distinguish between nestmate and non-nestmate based on olfactory cues (Signorotti et al., 2014). Chemical cues are also of particular relevance in discrimination among aquatic species due to its important role in information signaling, especially in turbid or deep environments with limitations to vision (Ward et al., 2007). However, vision can be of great importance to fish in case of short-range detection (Douglas, 1990). In freshwater fish, social recognition relies in a combination of visual and chemical cues (Brown, 1994). Vocal signals are the most

commonly used cues in avian recognition systems (Sharp et al., 2005) and electric cues in electric fish (Metzner & Heiligenberg, 1991).

Living in groups requires both general and specialized cognitive skills (Choleris et al., 2012). Some species limit their social recognition to a basic categorization of animals (conspecific vs heterospecific, kin vs non-kin, adult vs young, male vs female, familiar vs unfamiliar, etc.) while others reach a high degree of specificity being able to identify particular individuals (Tibbetts & Dale, 2007; Ward & Webster, 2016). The latter, true individual recognition occurs in stables groups, with repeated interactions among group members, and has been shown to occur in mammals and birds (Colgan, 1989; Insley et al., 2003). However, in large groups, with thousands of individuals, there is little value for an accurate discrimination between individuals. Thus, the specificity of social recognition capacities depends on the complexity of social interactions and group size (Ward et al., 2020).

The process of social recognition is commonly divided into three steps. The first one consists in the emission of cues (e.g. odors, sounds, colors) by a “sender”. This process is not intentional (as in the case of communication), as it can occur involuntarily through physiological mechanisms (Gherardi et al., 2012). The next step, the perceptional component, involves the detection of these cues by another individual (receiver), which will allow the receiver to allocate the sender to a social category (Ward et al., 2020). Finally, the third step involves a behavioural response by the “receiver” towards the “sender".

Social recognition is typically measured using a one-trial memory test, a simple experimental paradigm based on the natural tendency of animals to explore novel stimuli. This test is entirely based on the animal’s spontaneous behaviour and it does not use a training phase or any learning rules that involve primary reinforcement, such as food or aversive stimuli (Ennaceur, 1988). It evaluates, therefore, a preference

between a previously presented conspecific (familiar) and a novel one (Antunes & Biala, 2012). This preference is quantifyed by the time spent exploring the familiar vs. the new conspecific. The one-trial recognition test was used in this thesis to measure both social and non-social memory.

2.2.3 Social Learning

Social learning is a type of learning that is based on the observation of, or interaction with, another animal or its products, and that does not require direct reinforcement (Gariépy et al., 2014; Heyes, 1994). The adaptive advantages of social learning extend to the most varied contexts such as predator avoidance, intoxication avoidance (food choice), tool use, movement patterns, mate-choice and courtship, parental care, competition, problem solving strategies, etc. (Gariépy et al., 2014). Social learning can be divided in two types: learning from others and learning about others. Learning from others includes collecting information about threats, strategies to escape predators and any strategies regarding the environment exhibited by conspecifics; learning about others includes collecting information about the conspecifics themselves, namelly mate quality, competitive abilities, etc. (Nunes et al., 2017).

At the ultimate level social learning enhances survival and reproduction since it saves time, energy, and reduces individual risk during the learning process (Lind et al., 2019). Copying behaviour from others allows preventing the costs associated with individual trial-and-error learning, while developing behaviour repertoires that lead to flexible and hence adaptive behaviour. The benefits of social learning for juveniles are of particular relevance and many of the things that juveniles have to learn can be learned quickly. A special type of rapid

social learning is imprinting (Galef & Laland, 2005). However, the use of informations from others may not always be advantageous since it may be inappropriate, outdated or maladaptive (Laland & Williams, 1998).

Imitation and associative learning are processes of learning that have been suggested as a basis for social learning. Imitation occurs when an observer learns through observation of the behaviour of a model individual and the consequence of that behaviour (Bandura, 1969). Associative learning is a process that modifies the behaviour of the observer individual by associating one stimulus with another, or one stimulus with a given behaviour or shorter sequences of behaviours (Heyes, 1994). Associative learning plays an important role in animal cognition research which has long been dominated by conditioning experiments (Dickinson, 2012). Classical conditioning is a form of associative learning in which the animal pairs two stimuli and the subsequent response is modified. In operant conditioning, the individual pairs its own behaviour with the consequence of that behaviour (Tükel, 2020).

Social learning is present in most animals (from vertebrate to invertebrate) and zebrafish, for example, is able to use social information to change behaviours related to risk-taking, as demonstrated in a study where “shy” wild zebrafish become “bolder” after interacting with “bold” domesticated zebrafish (Zala et al., 2012).

2.2.4 Social Decision-Making

Animals need to constantly make decisions that are crucial to their fitness. In social species, many of these decisions must be taken together with other group members. Consensus decision-making is therefore needed to preserve the group and promote cohesion. This

strategy is common in animals and it is present in many different behavioural contexts (Conradt & Roper, 2005). For instance, Mountain Gorillas seem to exchange grunts to assess their readiness to depart from a resting area and start travelling or feeding, resulting in a coordinated group movement. In this case, signaling readiness has the advantage of preventing separation from the group or spending time and energy in false starts (Harcourt & Stewart, 1994).

The dynamics of social interactions lead to different decision-making patterns and the analysis of these behaviours requires detailed observation of both individual and social behaviour combined with mathematical modelling (Deneubourg & Goss, 1989).

3. Proximate Mechanisms of Social Behaviour

For a long period, the study of social behaviour at the molecular level was considered too complex and somehow intractable (Cacioppo et al., 2010). However, with the genomics revolution and the advance in animal models of social cognition, this ideia has been changing. Today from a clinical and translational research perspective, there is an increasing interest in identifying the biological substrates that guide social behaviour and hence to understand the fundamentals of neuropsychiatric disorders (Porcelli et al., 2019).

Given that animal's lives are replete with social interactions, many genetic, cellular, hormonal, neural and cognitive mechanisms have evolved to support their social organization (Leser & Wagner, 2015). Social behaviour is therefore regulated as a function of both internal states and external environmental conditions. This regulation is achieved by the coordinated action of these mechanisms integrating numerous signals in a complex decision process (Simpson & Balsam,

2016). Here, we will address the genetic, neuromolecular, and neural circuits underlying social behaviour.

3.1 Gene Expression

Gene expression represents the major process by which the genotype generates the phenotype. This process occurs in a sequence of complex, interrelated and highly regulated events (i.e. transcription, and translation), resulting in a functional product (RNA and protein) used to perform organismal functioning and behaviour (Naumova et al., 2013). Genes do not control behaviour directly, but rather make use of RNA and proteins, acting at different times and levels to affect the brain and hence the expression of behaviour (Robinson et al., 2008).

Genes are differentially expressed in different tissues, and in humans brain tissue is characterized by one of the highest levels of gene expression compared to other tissues and cell types (de la Grange et al., 2010; Roth et al., 2006). It is thought that the brain expresses about fifty percent of all the genes of the genome at a given point of time (Jia et al., 2014; Sweatt, 2009). There is also a high variability of gene expression in different brain tissues, namelly the cerebellum, which seems to have the most distinguishable gene expression pattern when compared to other brain areas (Roth et al., 2006).

Genes are stably inherited over generations; however, new experiences can change the brain through the interplay with the environment and learning process (Volgin et al., 2018).The relationship between genes, brain and social behaviour has complex entanglements, having long puzzled researchers and sparked controversy (Robinson et al., 2008). However, there is evidence that environmental stimuli can elicit rapid and dramatic changes in gene expression (Cummings et al., 2008; Simões et al., 2015). The identification of genes affecting social

behaviour can help us to better understand the development and maintenance of sociality as well as to create models and therapies for psychiatric human disorders (Johnsson et al., 2018). Despite the progress made to date in elucidating the genetic and molecular basis of social behaviour, the identification of genes affecting social behaviour represents a real challenge (Godinho & Nolan, 2006). Yet, their identification encompasses a myriad of possibilities. Due to pleiotropic effects, one gene can act on more than one single trait, and due to epistasis, many genes can interact to affect one single trait. Additionally, different genes function in different tissues at different times during the ontogeny of an organism (Sokolowski, 2001). On the other hand, researchers are faced with the difficulty of defining and quantifying behaviours, especially social behaviour that is quite diverse (Johnsson et al., 2018). The study of eusocial insects brought important contributions to the understanding of genetic control of social behaviour and phenotypic plasticity, largely because of the high diversity in the degree of sociality across species as well as the highly plastic but stereotypical transitions between castes and other life history stages (e.g. workers and nurses in honey bees, (Weitekamp et al., 2017). So far, few specific genes that are involved in a given social behaviour trait or psychiatric disorders have been conclusively identified (Johnsson et al., 2018).

Social behaviour phenotypes typically depend on the effect of many genes (polygenic control). However, in some cases, a single gene can have a pronounced effect on social behaviour (Nipitwattanaphon et al., 2013). Remarkably, Oxitocyn (OT) and vasopressin (AVP) family receptor genes have been widely associated to social behaviour and cognition across species and to some of the most common mental disorders in humans related with impairements in social function (Aspé-Sánchez et al., 2016). Other important genes for social behaviour are

those encoding for dopamine reward pathways, serotonergic emotional regulation, or sex hormones (Ebstein et al., 2010). The study of interactions between OT and AVP receptor genes with other genes and pathways have also provided important insights into the genetic basis of social behaviour (Aspé-Sánchez et al., 2016); please see section 3.2. for more details about the role of these receptor genes on social behaviour, as well as the effects of their interactions).

Genetic analysis allows the identification of genes involved in specific traits including complex traits (a trait in which does not occur a one-to-one relationship between genotype and phenotype) by studing organisms where gene function is altered. In general there are two approaches for understanding the function of a gene: forward genetics (from phenotype to gene) and reverse genetics (from gene to phenotype) (Bućan & Abel, 2002), see figure 1.1.

Forward genetics, also called direct genetics, starts from the study of an altered phenotype (mutant) or a trait and then the isolation and identification of the DNA sequence change in the genome that underlies this mutant phenotype or feature of interest (Peters et al., 2003). Mutants used for this purpose can occur naturally or be artificially induced using chemicals or X-radiation (Anholt & Mackay, 2009). Forward genetic screening requires therefore a given phenotype to investigate the causal mutations, genes, or QTLs (quantitative trait loci), using genetic mapping, positional cloning, fine mapping, among other genomic approaches (Sahu et al., 2020). Forward genetics is very useful specially in cases where genes are either unknown or not cloned (Takahashi et al., 1994).

Reverse genetics, goes in the opposite direction, and aims to identify what phenotypes are controlled by a particular known DNA sequence from the genome. In this way, genes are manipulated to assess their effects on a particular trait. It starts with a particular gene

sequence that is altered and the associated changes in the phenotype are studied (Peters et al., 2003). There are several techniques used in reverse genetics to manipulate genes and produce mutant organisms such as Knockout/ genome editing, over expression, site directed mutagenis, transcriptome, anti-sense RNA, and RNAi. This approach enables the study of the function of a family of genes in different organisms for which no forward genetics mutants have been identified yet. With the development of genome projects and the availability of genes with defined sequences, this approach has made dramatic progress.

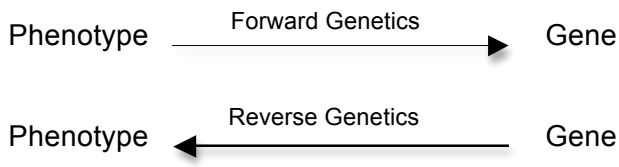

Figure 1.1 Forward and reverse genetics

Forward and reverse approaches are complementary and sometimes researches use a combination of both to confirm their studies.

In this thesis a genetic forward approach was used following a candidate genes association study. This kind of studies evaluate genetic variation (polymorphisms) associated with phenotypic traits based on a priori hypothesis about the function of a selected gene, or a group of pathway-related genes (Alghamdi & Padmanabhan, 2014). A large number of such studies, including genome-wide association studies

(GWAS), have tried to associate behaviours, mental disorders and biochemical endophenotypes with genetic polymorphisms (Albert, 2011).

Candidate gene studies usually focus on the analysis of a relatively small number of pre-specific genes, while GWAS looks for genetic variants covering the entire genome (Modena et al., 2019). Candidate genes studies depend on a set of markers that are identifiable regions in the genome (locus), where the DNA sequence differs between individuals (i.e. is polymorphic) (Collins et al., 2016). Some polymorphisms are functional, which means that they have a significant effect on the gene product, while others are neutral (i.e. have no effect being simply useful markers; (Daly & Day, 2001). Single nucleotide polymorphisms (SNPs) are the simplest type of polymorphism resulting from a single base substitution. The SNPs located in protein-coding RNA genes are functional and those located in non-coding regions are neutral. SNPs are of special interest to researchers, since they are relatively frequent in the genome and millions of SNPs have been identified and mapped (Collins et al., 2016).

A major advantage of the candidate gene approach is that studying candidate genes increases the plausibility of detecting trait-associated genes. It can also be implemented relatively fast and cheaply than GWAS and may allow the identification of genes with small effects. Regarding the disadvantages, this approach is limited to what is already known about the biology of the trait of interest, excluding information of novel genes that may also influence the trait of interest (Kwon & Goate, 2000). The primary critical step in conducting candidate gene studies is, therefore, the choice of a potentially contributing gene for the trait or disease under investigation. Candidate genes are usually chosen based on evidence that they are biologically relevant for the trait of interest, or if variants of this gene have an overall impact on the fuction of the gene, or even if the polymorphisms of the candidate gene are frequent enough

in the population to conduct satisfactory statistical analysis (Wang et al., 2013). Once the candidate(s) gene(s) and suitable polymorphisms (markers) are selected, gene variants are checked by observing their occurence in random test subjects (cases) that have the trait and the selected control subjects (Patnala et al., 2013). Both groups are then genotyped for the selected marker to look for consistent differences.

3.2 Neuroendocrine regulation

The regulation of social behaviour has been linked to a set of hormones and neurohormones that might evolve independently across distantly related species (Rubenstein & Hofmann, 2015). Hormones are a major mechanism ensuring coordination between different individuals as social interactions trigger quick responses in circulating hormones which modulate neural circuits through widely distributed hormone receptors in the brain (Adkins-Regan, 2005; Oliveira & Oliveira, 2014).

Three categories of molecules have consistently been shown to play a central role in the regulation of social behaviour (Rubenstein & Hofmann, 2015): nonapeptides, steroid hormones and biogenic amines (particularly monoamines). An important aspect of neuroendocrine regulation is the reciprocity between the neuroendocrine systems and the social behaviour. Hormones affect behaviour, but behaviour can, in return, feed back and affect hormone levels. Thus, these two systems influence and are influenced by each other (Oliveira, 2004).

3.2.1. Nonapeptides

Nonapeptides are an ancestral nine amino-acid neuropeptide family that includes oxytocin (OT) and arginine vasopressin (AVP) in mammals.

The word oxytocin is derived from the Greek, means “quick birth” for its well-documented role in many aspects of female reproduction, namely uterine-contration during labor and milk ejection during lactation (Lee et al., 2009). While vasopressin (from vasopressor, i.e. “causing the constriction of vessels”), also called antidiuretic hormone, owes its names from its roles on blood pressure and the regulation of peripheral fluid balance (Gruber, 2014). These two closely related neuropeptides are synthesized in the hypothalamus and secreted by the posterior lobe of the pituitary gland in none overlapping or occasionally juxtaposed areas that remain separated in distinct sets of neurons (Stoop, 2012). OT and AVT can act as neurohormones, when released peripherally by the pituitary gland or as neuromodulators, when released centrally in target brain regions (Lieberwirth & Wang, 2014).

These peptides share a common ancestry across both vertebrates and invertebrates, and it has been proposed that they are derived from a VP-like peptide by gene duplication in jawless fish (cyclostomes) about 500 million years ago (Kochman, 2013). Thus, there might be variation in the exact structure of this neuropeptide across species. For exemple, vasopressin/oxytocin are the names used for the peptide sequences found in mammals, vasotocin/mesotocin are the designation for those found in birds, amphibians, and non-avian reptiles, while vasotocin/isotocin are the peptides sequences found in teleost fishes (Donaldson & Young, 2008), (see figure 1.2). However, this nomenclature is ambiguous and might lead to confusion, as each peptide discovered, with minimal difference in their gene structure and sequence, receives a different name, even if it has the same function. And, as more species are studied, exceptions appear as is the case of the elephant shark and the ratfish (two non-mammalian species) which present the exact same oxytocin nonapeptide sequence (Wircer et al., 2016).

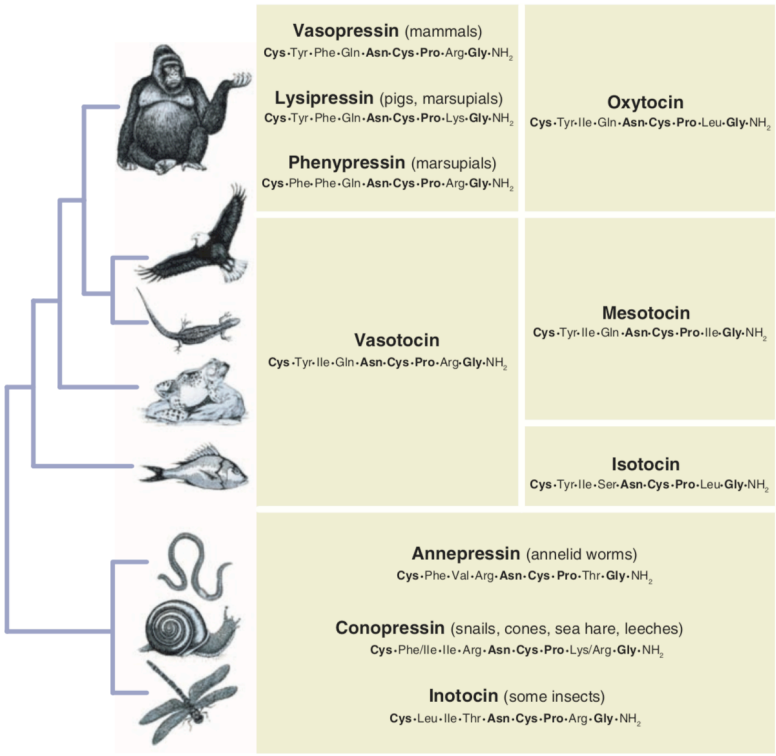

Figure 1.2 Oxytocin and Vasopressin homologs, In (Donaldson & Young, 2008)

From what is known so far, all vertebrate species, with the exception of cyclostomes, contain at least one OT family peptide and one AVP family peptide, whereas invertebrates have only one oxytocin/vasopressin homolog (Kanda et al., 2005). However, many groups particularly cartilaginous fishes, present secondary duplications expressing three or four nonapeptides (Goodson, 2008). Secondary duplication is also present in marsupials, which express three different VP homologs (VP, lysipressin and phenypressin) in addition to both mesotocin and OT. In general, eutherians express only VP and OT, although lysipressin replaces VP in pigs (Goodson, 2008).

These two nonapeptides exert their actions through the activation of a family of G-protein-coupled receptors belonging to the rhodopsin

class (see figure 1.3). In mammals, four nonapeptide receptors have been identified: one OT receptor labeled OTR with the gene named OXTR, and three AVP receptors named V1a, V1b and V2 (Ocampo Daza et al., 2012). Zebrafish has two oxytocin receptor orthologue genes, the OT receptor (oxtr) and the OT receptor-like (oxtrl), and two vasopressin zVP-1 receptors (Landin et al., 2020).

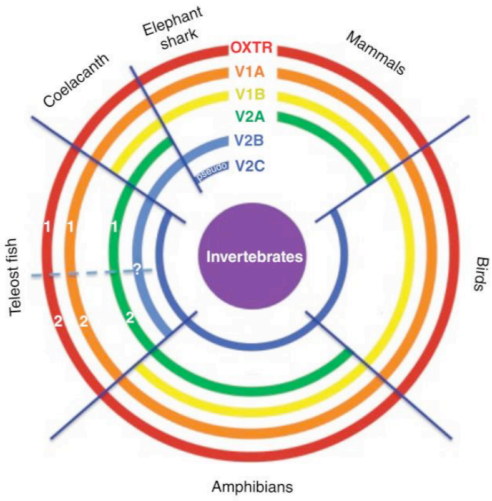

Figure 1.3 The evolutionary development of OT and AVP receptors.
Each concentric circle represents one family of receptors. The dashed line in the teleosts represents a lineage-specific duplication generating two different paralog genes refered as 1 and 2 (Wircer et al., 2016).

The knowledge about the interaction between the receptors of these peptides is sparse and it is not clear if they developed distinct functions or if there is functional redundancy (Landin et al., 2020). It is thought that at least some functions of OT- and AVP- like peptides on social behaviour might be the result of interactions between the receptors (Albers, 2015). However, a study in medaka fish provides evidence that

oxtr1, but not oxtr2, plays a role in mate choice in a sex-specific manner, suggesting that the receptors may have distinct functions (Yokoi et al., 2020). Although these peptides share high sequence and receptor similarities across distant taxa, the distribution of their target receptors varies largely both within and across species (Johnson & Young, 2015).

The function of nonapeptides has been studied across several organisms including humans using pharmacological and genetic approaches. In addition to influencing numerous aspects of physiology, this nonapeptide family has repeatedly been implicated in the modulation of social behaviour, including affiliation (pair bonding, parental behaviour, allogrooming), aggression, and social cognition, namely social recognition and social learning (Lieberwirth & Wang, 2014).

Species from the genus *Microtus* (voles), have been of particular importance for the study of affiliative behaviour, since they exhibit a high level of inter-specific variation in this particular behaviour, despite their similarity in appearance and numerous aspects of non-social behaviour (Insel & Shapiro, 1992). For instance, prairie voles (*Microtus ochrogaster)* are monogamous with bonds that last long after mating, extensive bi-parental care of offspring, selective aggression towards unfamiliar conspecifics and grief-like behaviour following partner loss. In contrast, montane voles (*Microtus montanus*) are polygamous with separation after mating and limited male parental care (Johnson & Young, 2015). In parallel, substancial differences have been observed in the neuroanatomic distribution of OT receptors (OTR) and AVP receptor (AVPR1a) between these two closely related species. The differences concentrated mainly in specific mesolimbic reward areas including the prefrontal cortex (PFC), ventral pallidum (VP), nucleus accumbens (NAcc) and lateral septum (Insel & Shapiro, 1992; Johnson & Young, 2015). Also, in prairie voles, blockade of OTR or AVPR1a in these areas

prevents pair bonding, particularlly in the NAcc or PFC (Johnson & Young, 2015).

Similarly, blockade of OTR and AVPR1 homologs in monogamous cichlid fishes leads to a significant reduction in both affiliative behaviour towards the pair mate and aggression towards neighbors during bond formation (Oldfield & Hofmann, 2011). In birds (zebra finches), blockade of nonapeptide receptors by an OT antagonist reduces susbtantially the time spent with large groups and familiar social partners, and central infusion of mesotocin produces opposite effects (Goodson et al., 2009). Domestic dogs nasally sprayed with OT showed higher social orientation and affiliation towards both conspecifics (dog partners) and humans (their owners) than when sprayed with placebo (Romero et al., 2014). In humans, it has been demonstrated that plasma OT levels are significantly higher in new lovers than in singles, suggesting increased activity of oxytocinergic system during the initial stages of romantic attachment (Schneiderman et al., 2012). Also, intranasal OT administration in humans (both sexes) increases the ratings of perceived trustworthiness and attractiveness, suggesting that OT may increase affiliative behaviour towards unfamiliar individuals (Theodoridou et al., 2009). Also, first time and experienced marmoset fathers show increased VR1a and V1a receptor-labeled dendritic spines (Kozorovitskiy et al., 2006).

Given that, sociality comes with specific cognitive abilities that enable the required information processing about others (social recognition), as well as information from others (social learning) it is not surprising that this nonapeptide system is also involved in social cognition. Central pathways for AVP and OT have been linked to different forms of memory and learning as well as complex social behaviours, especially in mammals (Winslow & Insel, 2004). For example, in rats centrally injected AVP facilitates consolidation of

olfactory information and improves conspecific recognition (Le Moal et al., 1987). Male mice mutant for a functional OT gene fails to develop social memory (but not spatial memory) and injection of OT in these mutant mice restores social recognition memory (Ferguson et al., 2000). Expressing V1aR in the lateral septum of V1aR knockout mice also restores social recognition (Bielsky et al., 2005). Both OT and AVP prolong the recognition of social odors in hamsters via acting on OTRs but not V1aRs and this effect is limited to social stimuli (Song et al., 2016). Zebrafish OTR mutants exhibited deficit for both social and objects recognition but not in shoal preference and object approach (Ribeiro et al., 2020). Also, the two zOT zebrafish receptors are implicated in social behaviour regulation and this effect is independent of anxiety-like behaviour (Landin et al., 2020).

3.2.2 Steroid Hormones

Steroids are a group of hormones derived from cholesterol that regulate many physiological processes particularly those involved in reproduction and in the stress response, also influencing a range of social behaviours (Tennenhouse et al., 2017; Whirledge & Cidlowski, 2019). These hormones are mainly produced in the gonads (testes and ovaries) and the fetoplacental unit during pregnancy and by the adrenal cortex or interrenal tissue, in fish and amphybians (Adkins-Regan, 2005). Based on their receptors, steroid hormones are generally classified into four categories: corticoids, androgens, estrogens and progestins (Oliveira & Gonçalves, 2008). Gonads usually release more androgen, estrogen and progestins, while adrenals release more corticoids. However, these hormones can be produced in different locations. For instance, androgens and estrogens, besides being produced in gonads, are also sinthetized in the adrenal cortex and in the brain (Adkins-Regan, 2005).

Testosterone (T), the principal circulating androgen in males, is secreted almost exclusively in the testes and in females is produced in ovaries, adrenal glands, and fat cells. Similarly, estrogen, which is the primary sex hormone controlling the female reproductive development, is predominantly produced in the ovaries and in males is produced in adipose, skin, brain, and bone, which convert testosterone to estrogen by aromatase action (Cooke et al., 2017).

It is thought that steroidogenesis and the enzymes regulating their biosynthesis are conserved across vertebrates (Bauer et al., 2000). However, not all species produce the exact same steroids. For exemple, in the case of primates and teleost fish, cortisol is the predominant glucocorticoid, whereas birds and most rodents produce corticosterone (Adkins-Regan, 2005). Phylogenetic studies that examined the primary amino acid sequence of 73 steroid receptors from a range of jawed vertebrates and jawless fish (sea lamprey) concluded that there were two serial gene duplication events of an ancestral steroid receptor about 450 million years ago, before the divergence of the lineages (Whirledge & Cidlowski, 2019).

The effects of steroid hormones can be mediated by the modulation of gene expression (slow mechanism) as well as by fast nongenomic mechanisms through membrane-associated receptors and signaling cascades (Lösel & Wehling, 2003). The multiple interactions between steroid hormones and other signaling molecules lead to a high level of complexity and panoply of effects (Whirledge & Cidlowski, 2019).

Concerning affiliative behaviour there is evidence that hormones of the hyphotalamic-pituitary-adrenal axis (a major stress response pathway) are implicated in social bonding, even if indirectly. For example, mammalian birth (a markedly stressful experience) is closely followed by high level of adrenal activity and subsequent release of peptides namely OT, favoring the formation of social attachment (Carter

& Keverne, 2002). Also, monogamous prairie vole (of both sexes) reproductively naïve that never experienced cohabitation before, showed a rapid decline in corticosterone followed their first encounter with a stranger conspecific (Carter et al., 1995). Likewise, participation in grooming tends to reduce stress (level of glucocorticoids) in female baboons (Wittig et al., 2008). This shows that the intervention of these hormones seems to reduce the stress or fear of social contact, allowing individuals to engage in social behaviour.

There is strong evidence that the OT receptor system is generally steroid dependent (Gimpl et al., 2002). In rats, it has been demonstrared that estrogens as well as progesterone regulate OT receptor distributions in the hypothalamus (Schumacher et al.,1992), while in prairie voles (females) steroid dependent increases in OT receptors only occurs in the anterior olfactory nucleus (Carter et al., 1995). In vertebrates, arginine vasopressin AVP family is an important element of the HPA, playing a role in the stress response. HPA axis activation is mediated by the release of neuropeptides such as AVP and corticotropin-releasing factor (CRF), which stimulates the release of adrenocorticotropic hormone ACTH (Beurel & Nemeroff, 2014).

Many hormones including sex steroids (particularly estrogen) and glucocorticoids have been systematically associated with the regulation of cognitive processes such as memory and learning (Frick et al., 2015). For exemple, androgen dependent vasopressinergic system has been implicated in social recognition in male rats (Bluthe et al., 1990), and post-training estradiol injection enhances memory in ovariectomized rats trained in a hidden platform water maze task (Packard & Teather, 1997). Likewise, implants of estradiol in gonadectomized adult male rats enhances their performance in a win-shift radial maze-learning task (Luine & Rodriguez, 1994). The win-shift component of the test refers to the alternation of rewarding arms during the trial and test phase and this

strategy is commonlly used to assess spatial memory in rodents (see (Gaffan & Davies, 1981).

Studies in humans demonstrated that high levels of gonadal steroids secreted at the luteal phase of the menstrual cycle might facilitate performance on cognitive and motor tasks in young healthy women (Hampson, 1990). Similarly, suplementation of testosterone in healthy older man resulted in a significant enhancement of spacial cognition, but not of other cognitive domains, which is possibly mediated by the aromatization of testosterone to estrogen (Janowsky et al., 1994). The effects of steroid hormones on memory and learning are therefore very complex, varying according to the steroid, the task performed and the individuals' sex and age (Frick et al., 2015).

3.2.3 Monoamines

The monoamine neurotransmitters are a class of molecules released at the synapses, that contain one amine chemical group (Meriney & Fanselow, 2019). Depending on the specific precusor, this group is classified into two categories: catecholamines and indolamines. Catecholamines include dopamine, noradrenaline, and adrenaline, while indolamines include serotonine and histamine (Wong & Gjedde, 2009). The major representatives of monoamines with a recognized role in social behaviour and cognition in vertebrates are dopamine and serotonin (Libersat & Pflueger, 2004).

Monoamines act on distributed networks of neurons in the central nervous system as well as in the periphery and their synaptic receptor targets have highly conserved functions in synaptic transmission across taxa (Meriney & Fanselow, 2019).

Monoamines modulate multiple social behaviours in both vertebrates and invertebrates, including mechanisms of group cohesion and affiliation (Bacqué-Cazenave et al., 2020; Hewlett, 2018).

In humans, impairement in the monoaminergic system, especially in dopaminergic and serotonergic systems can lead to neurologic and neuropsychiatric disorders such as schizophrenia and Parkinson's disease (Libersat & Pflueger, 2004).

Serotonin or 5-hydroxytryptamine (5-HT) is a major neuromodulator of the central nervous system in vertebrates and is well known for its effects on anxiety, mood, aggressive and defensive behaviour, as well as on cognition, behaviour flexibility and body function such as temperature, sleep and circadian rhythm, blood pressure, appetite, hormones, motor activity, etc. (Bacqué-Cazenave et al., 2020). There are seven different serotonin receptor classes (5-HT1 to 5-HT7). Each class includes sub-classes (e.g., 5-HT1-A, 5-HT1-B, etc.) that usually differ in terms of localization and downstream signaling (Feldman, 2004). Serotonin receptors are found outside as well as within the central nervous system. In the nervous system they are more concentrated in certain regions such as various limbic structures, the striatum and the medial orbitofrontal cortex. Interestingly, this set of areas has a great resemblance with the so-called social brain, responsible for social cognition and social decision-making (Siegel & Crockett, 2013). In zebrafish, different types of 5-HT receptors such as 5-HT1 and 5-HT2 are involved in the control of behaviour including swimming behaviour ((Brustein et al., 2003).

Typically, the effects of 5-HT are more in the sense of enhancing or attenuating the neuronal responses for calibrating most behaviours, rather than to elicit or to stop a specific behaviour (Bacqué-Cazenave et al., 2020). Injection of serotonin 1A agonist in adult male titi monkeys resulted in a decrease of pair-bond behaviour (Larke et al., 2016). Also,

the serotonin transporter knockout mouse shows high levels of anxiety, decreased prosocial behaviour and decision-making inhibition (Kalueff et al., 2010). Many studies have shown the contribution of different serotonin receptors in learning and memory, in particular by interacting with dopaminergic, glutamatergic, cholinergic and GABAergic systems (Buhot et al., 2000; Meneses, 2001; Perez-García & Meneses, 2008).

Dopamine (DA) has also been associated with motivation but plays also a role in an array of other behavioural processes such as mood, stress and addiction. DA actions are mediated by five differents receptor subtypes (D1–D5), categorized into two principal groups: D1-like receptor type and D2- like receptor type (Jaber et al., 1996). However, zebrafish have eight dopamine receptors, drd1, 2a-c, 3, 4a-c, of which drd1 is the homolog of the mammalian D1-like group and the remaining receptors belong to the D2-like group (Fonseka et al., 2016). DA (acting via D2 receptor) has been implicated in the regulation of partner preference in female and male prairie voles (Aragona et al., 2003; Wang et al., 1999). Similarly, the mesolimbic dopaminergic pathway has been involved in zebrafish pair formation (Banerjee et al., 2013). In young rats, dopaminergic neurotransmission in the nucleus accumbens (NAc) has a relevant role in social play behaviour (Manduca et al., 2016). Dopamine also revealed to be an inducer of grooming behaviour in cockroaches (Weisel-Eichler et al.,1999).

3.3 The Social Brain

The neural circuits that implement the cognitive abilities underlying social skills have been named the social brain. It allows animals to detect, appraise and respond to others according to their behaviour, through a process of social decision-making (Rogers-Carter & Christianson, 2019).

Dealing with the social world is very challenging because it is more unpredictable than the physical environment, requiring the capacity for flexible responses, also known as behavioural flexibility.

It is thought that the size and composition of social groups and the dynamic of social relationships has driven the evolution of cognitive abilities and brain size (Silk, 2007). According to the social brain hypothesis, larger brains with higher encephalization indexes have evolved for thousands of years in response to the complexity of social interactions, rather than in response to other ecological domains (Dunbar, 1998). However, more than the size of the social group, the stable or enduring forms of pairbonding might be the critical factor that might lead to the evolution of increased cognitive abilities and larger brain size (Dunbar & Shultz, 2007). It has also been proposed that brains that allows for higher behavioural flexibility evolved as a response to rapid changes in the environment, when direct genetic control over the phenotype is outpaced by the rate of environmental change (Taborsky & Oliveira, 2012). In agreement with this, Damásio (1994) pointed out that developing cognitive abilities, and ultimately a mind, has given organisms a new way of adapting to changes in the environment that could not been addressed by adaptation by natural selection on genetic variation over generations. Indeed, although social behaviour is partially controlled by genes, behaviour flexibility often relies on cognitive abilities that allow individuals to adjust their behaviour to specific situations and optimize social interactions (Taborsky & Oliveira, 2012). Social interactions impose cognitive demands that are so unique and it is thought that brain structures underlying it might have evolved in specific modules (domain specific hypothesis), whose functions are separated from those of other non-social modules (Adolphs, 2009; Ferguson et al., 2000; Song et al., 2016; Wersinger et al., 2004). In vertebrates a set of evolutionary conserved brain areas that are

interconnected with each other, have been identified that together functioning as a network, regulate social behaviour. This circuit has become known as the Social Decision-Making Network, which integrates the mesolimbic reward system and the social behaviour network (O'Connell & Hofmann, 2012).

3.3.1 Social Decision-Making Network

The Social Decision-Making Network (SDMN) is described as a network of forebrain and midbrain structures that are conserved across the five major vertebrate lineages (mammals, birds, reptiles, amphibians, and teleost fish) that regulate and implement social behaviour (Rogers-Carter & Christianson, 2019; Teles et al., 2015). A social behaviour network (SBN) of brain structures that regulate elementary social behaviour in mammals was first introduced by Newman (Newman, 1999). It included six limbic regions: the lateral septum (LS), the bed nucleus stria terminalis/medial extended amygdala (BNST/meAMY), the medial preoptic area (mPOA), the anterior hypothalamus (AH), the ventromedial hypothalamus (VMH), and the periaqueductal gray/central gray (PAG/CG) (Newman, 1999). These areas express sex steroid hormone receptors and are involved in multiple forms of social behaviour such as affiliation, parental care, aggression, mating, social recognition etc. (Newman, 1999). About one decade later, O'Connell and Hofmann observed that anatomical nodes of the mesolimbic dopamine reward system express various of the receptors and genes involved in the SBN acros many vertebrates and hence, proposed that it works together with the SBN forming a wider framework, the social decision-making network, (see figure 1.4) (O'Connell & Hofmann, 2011).

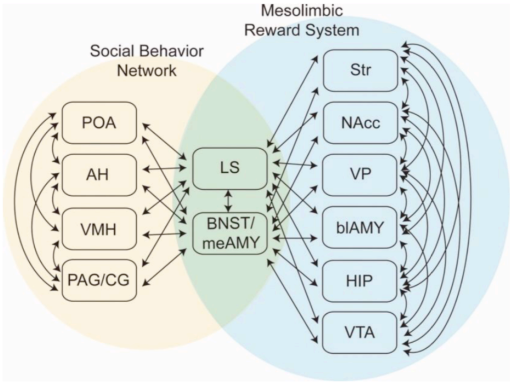

Figure 1.4 The Social Decision-Making Network

Brain regions interactions between the social behaviour network (on the left) and the mesolimbic reward system (on the right), as well as their shared nodes (on the center). Arrows show anatomical connections between these brain regions.
POA= Preoptic Area, AH= Anterior Hypothalamus, VMH= Ventromedial Hypothalamus, PAG/CG= Periaqueductal Gray/ Central Grey, LS= Lateral Septum, BNST/meAMY= Bed Nucleus Stria Terminalis/ Medial Extended Amygdala, Str= Striatum, NAcc= Nucleus Accumbens, VP= Ventral Pallidum, blAMY= Basolateral Amygdala, HIP= Hippocampus and VTA= Ventral Tegmental Area (O'Connell & Hofmann, 2011).

The mesolimbic reward system is the brain circuit responsible for the assessment of the relative value of the social stimuli (via dopaminergic signaling) and the consequence of the social behaviour, i.e executes processes that result in an individual's motivation to respond to their environment. Besides domapinergic signaling, mesolimbic structures also express genes associated to gonadal hormones and oxytocin and vasopressin, which are all implicated in decision-making processes across many species. This system originates primarly in the ventral tegmental area (VTA) and projects to the nucleus accumbens (NAcc), being a part of complex circuits also involving the hippocampus (HIP), basolateral amygdala (blAMY), ventral pallidum (VP), striatum (Str), lateral septum (LS) and the bed nucleus of stria terminalis/medial amygdala (BNST/meAMY) (O'Connell & Hofmann, 2011).

These two circuits (SBN and Mesolimbic Reward System) that compose

the SDMN are anatomically linked by connections between numerous brain regions and share two nodes: the LS and the BNST. They complement each other since the mesolimbic reward system is responsible for the individual's motivation to respond to the external stimuli, whereas the SBN executes specific behaviours in response to mesolimbic inputs (Rogers-Carter & Christianson, 2019).

4. Zebrafish Model System

Zebrafish *(Danio rerio)* is a small tropical freshwater teleost fish commonly used as an aquarium species for its beautiful skin pattern, ease of caring and good tolerance to a broad range of environmental conditions, such as life in captivity and limited space, as well as relatively high fish density (Kinth et al., 2013). Since the 1980s, zebrafish started to be used in biological research and increasingly received great attention from the scientific community and within a few decades, it has become one of the most important model organisms (Bradbury, 2004). Additionally, zebrafish is a prolific breeder, presenting external fertilization with transparent embryos and its quick development allows *in vivo* monitoring of different processes during its early-stage development. It also presents a small size, low husbandry cost, easy of genetic and other experimental manipulation, rich behaviour repertoire and relatively high physiological and genetic homologies with mammals (Stewart et al., 2014).

In this section we will describe important biological features of this model and present the advantages and limitations of using it in social neuroscience.

4.1 The Organism

Zebrafish belongs to the family Cyprinidae, which contains about 44 species, from the order Cypriniformes (McCluskey & Braasch, 2020; Spence et al., 2007). The term *Danio* means “of the rice field”, from the Bengali language, which refers to the habitat where it is commonly found, and the common name zebrafish is related to their skin colour pattern, which resembles the zebra pattern with horizontal stripes (and not vertical) along both sides of their body and in the caudal and anal fins (figure 1.5 A-B) (Spence et al., 2007). However, due to a spontaneous mutation in the zebrafish connexin 41.8 gene, some zebrafish present rows of spots instead of the alternated light and dark stripes (figure 1.5 C-D) (Watanabe et al., 2006). This spotted pattern, that resembles a leopard, gives the name to this zebrafish strain (Leo). Another mutant with changes in pigmentation is the Tupfel long-fin (TL) (Fig.6D), which is a double spontaneous mutant, with a recessive pigment (similar to Leo) and a dominant long-fin mutation (Audira et al., 2020).

The laboratory strains used nowadays in biomedical research are domesticated strains that have been removed from the wild for many generations. Examples of commonly used wild-type zebrafish strains include: AB, TU, WIK, TL, 5D, and Leo.

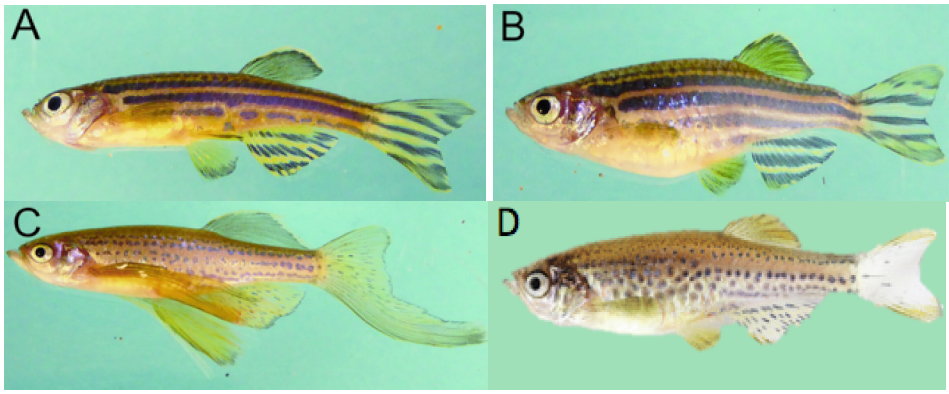

Figure 1.5 Zebrafish (*Danio rerio*): (A) male, (B) female, (C) Tupfel long-fin TL strain and (D) Leopard Leo strain (adapted from Meyers, 2018).

Zebrafish have a classical fusiform shape, laterally compressed, with an upward directed mouth and a small size, normally two to four centimeters long (total length at maturity in laboratory condition) (Spence, 2011). The features that allow us to distinguish it from other species are the five to seven dark stripes that run from gill to tail, the two pairs of barbels and an incomplete lateral line at the pelvic fin level (Spence et al., 2007).

Zebrafish are diurnal, having a day-time activity and night-time rest cycle. In captivity they can live, on average, two to three years, with some living up to 5 years (Gerhard et al., 2002). Zebrafish larvae are almost completely transparent with some rows of individual melanophores and iridophores (Nüsslein-Volhard & Singh, 2017). The visible morphological differences between males and females are minimal and dissection is necessary to properly distinguish the sexes if they have not reached sexual maturity (see figure 6 A and B). Mature females have a round belly when it is filled with eggs, are in general slightly larger than males and have a small genital papilla. The males are slender and have larger anal fins. The color pattern of the two sexes

is similar but males have a brighter golden appearance at sexual maturity (Nüsslein-Volhard & Singh, 2017).

4.2 Geographical Distribution and Habitat

It is assumed that zebrafish is native to the floodplains of Southeast Asia, most likely from India and Bangladesh (Spence, 2011). Data concerning the geographic distribution of this species is scarce and not very precise, indicating a wide distribution over the Indian subcontinent, extending to different regions of India, Bangladesh, Nepal, Myanmar, and Pakistan (Arunachalam et al., 2013).

Zebrafish's natural habitats are typically slow-moving or standing water bodies protected by aquatic vegetation and sedimentary substrate (e.g., rice paddies, lakes, ditches, ponds, and irrigation canals connected to paddy fields (Arunachalam et al., 2013). The Monsoon climate, characteristic of these geographical regions, with a remarkable contrast between rainy and dry seasons, has a significant impact on habitat parameters (Harper & Lawrence, 2011). The temperature may vary between 12 and 39 ºC and the water chemistry and abundance of food resources can also change significantly between seasons (Parichy, 2015). Rice cultivation and jute production has also a significant impact on the zebrafish habitat. However, zebrafish have shown relative resistance to anthropomorphic disturbance (Parichy, 2015). Records concerning their vertical distribution showed that normally they occupy the whole of the water column, mainly in open water near aquatic vegetation (Spence et al., 2006).

4.3 Diet, Competitors and Predators

Zebrafish are omnivorous, with a diverse diet that includes mainly

zooplankton and insects, but also phytoplankton, vascular plants, filamentous algae, detritus, arachnids, spores, and invertebrate eggs. They can also eat theirs own eggs and larvae (Parichy, 2015).

The main predators of zebrafish most commonly reported in the wild are snakeheads and freshwater garfish. However, there are potential predators that are not often mentioned, such as nocturnal catfish and birds like the Indian pond heron. There are also many predators of larval zebrafish like Mastacembelids, adonate larvae, and dragonflies (Spence et al., 2007).

4.4 Zebrafish Perception

Zebrafish is guided mainly by its vision in the crucial task of capturing prey and detecting predators (Fleisch & Neuhauss, 2006). Zebrafish starts to capture prey as early as 4 days postfertilization (Muto & Kawakami, 2013). Thus, the visual system needs to develop fast and it is not surprising that the first postmitotic retinal cells appear at about 28 hours postfertilization and its visual system becomes functional in only 72h postfertilization (Richardson et al., 2017). Interestingly, zebrafish eyes are quite large if we consider its overall size (Zimmermann et al., 2018).

Given that zebrafish is a diurnal species, their eyes are rich in cones, with a cone density similar to that of humans (Richardson et al., 2017). Many fish are tetrachromatic, including the zebrafish, which possesses a rich color vision (Park et al., 2016).

Zebrafish is capable of recognizing conspecifics and predators using both visual and olfactory cues (Barcellos et al., 2015; Madeira & Oliveira, 2017). When a zebrafish is injured, occurs the release of an alarm substance that elicits an alarm response in conspecifics, in order to prepare them to avoid and cope with danger. Non-injured fish also

communicate predation risk to conspecifics by releasing chemicals into the water which in turn induce cortisol increase in conspecifics, and might be the result of an adaptive indicator of potencial risk (Barcellos et al., 2015).

4.5 Zebrafish Social Behaviour

Zebrafish is a gregarious species that typically forms mixed-sex shoals from small (4-12 fish) to large groups (few hundreds) in the wild depending on environmental conditions (Suriyampola et al., 2016). There is evidence that zebrafish prefers to associate with larger and more active shoals (Pritchard et al., 2001). The presence of conspecifics acts as a social buffer against stressors (Faustino et al., 2017) and might play a rewarding role, acting as a positive reinforcement in associative learning tasks and increasing brain dopamine levels (Al-Imari & Gerlai, 2008; Saif et al., 2013).

Sociality develops early in zebrafish and it tends to form social groups throughout their lives. However, larvae seem not to display a shoaling preference until they reach the juvenile stage, when they acquire a social preference that remains stable during adulthood (Engeszer et al., 2007).

Although zebrafish behaviour was initially considered to be merely reflective, further studies have shown considerable complexity and a rich behaviour repertoire (territoriality, fight and escape, mating strategies, tranfer of information, social buffering, group decisions, learning, memory, etc.) (Oliveira, 2013).

Differences at genetic, physiological, morphological and behavioural level, have already been reported between wild zebrafish and different laboratory strains as well as among laboratory strains (Lange et al., 2013; Meyer et al., 2013; Van Den Bos et al., 2017;

Vignet, 2013). These differences are due to their independent origin (scientific stock centers, labs, pet shops or directly from the wild), specific mutations, such as in TL, and because of domestication to the laboratorial environment (Suurväli et al., 2020). An example of this variation is reported in a recent study that shows that TL presents higher shoal cohesion than the AB strain (Séguret et al., 2016). Another study comparing the same strains (TL and AB) showed that AB presents higher cortisol levels in response to the inhibitory avoidance test than TL (Gorissen et al., 2015). The inhibitory avoidance is a behavioural paradigm in which the animal learns to avoid moving from a lighted into a dark compartment in order to prevent an aversive stimulus (normally electric shocks) (Gold, 1986).

Zebrafish also presents sex differences in terms of behaviour and physiological responses. There is evidence of sex differences in the pattern of brain cell proliferation, with males exhibiting higher cell proliferation in cerebellum, whereas females express more adrenoreceptors in the lateral zone of dorsal telencephalon and fewer adrenoceptors in the cerebellum (Ampatzis & Dermon, 2007).

4.6 Zebrafish in Social Neuroscience

The use of zebrafish to study behaviour has increased significantly over the last decades and numerous tools have been developed for this purpose. Many behaviour paradigms initially developed for rodents (open-field, T-maze, light/dark box, one trial recognition test, etc.) have been successfully adapted to zebrafish, which presents associative learning in different sensory modalities. Zebrafish also has the advantage of being diurnal, similarly to humans, responding to visual stimuli in a robust and easily manner, while rodents, for example, are predominantly nocturnal and highly sensitive to environmental changes

(light, odors, sound, temperature, etc.) (Geng & Peterson, 2019). Also, zebrafish behaviour can be genetically and pharmacologically manipulated in both adults and larvae. Zebrafish possess similarities in brain function with other vertebrates and share all major neurotransmitters, hormones and receptors with mammals (Levin & Cerutti, 2009) (Kalueff et al., 2014). For these reasons, zebrafish has been used to study normal and pathological behaviour as well as to model a wide range of human brain disorders, from anxiety to neurodegerenation (Khan et al., 2017).

The zebrafish genome has been completely sequenced and more than 70% of the genes have human orthologues. Furthermore, a zebrafish brain atlas is available for both larvae and adults, as well as a range of research tools, including electrophysiological and optogenetic tools, quantification of immediate early genes and target genes using in situ hybridization, forward and reverse genetic methods, conditional transgenic strains as well as a range of WT strains with distinct behavioural phenotypes and genetic background (Levin & Tanguay, 2011).

As with others model organisms, zebrafish also has its limitations. For exemple, certain brain areas, such as the cerebral cortex, are not developed as in mammals, or some structures in the central nervous system are still difficult to map to their mammalian counterparts. Also some behaviours, like parental care, are not known in zebrafish (Miller, 2011). Furthermore, the rapid ascention of this model in behavioural neuroscience and the increasing number of zebrafish strains used in labs, brought some concerns related to their indiscriminate use in behaviour tests (Van Den Bos et al., 2017). It is known that zebrafish strains used in research may have diverse breeding histories and origins, that gives them a unique genetic background that may result in behavioural variability (Vignet, 2013).

Despite having approximately the same number of chromosomes pairs as humans (25 compared against 23), zebrafish presents duplication in the genome (as all theleost fish) (Glasauer et al., 2014) and significant rearrangements in the order of loci within chromosome segments when compared to the human genome (Postlethwait, 2000; Stewart et al., 2014). However, the advantages of using zebrafish in social neuroscience still outweigh the limitations, and zebrafish is therefore a powerful model to study social behaviour traits.

5. Aims of the Thesis

The overall objective of this thesis is to investigate the phenotypic architecture of social behaviour and their associated genetic polymorphisms in zebrafish. The specific aims are the following:

1- To assess if there is an association between the motivational and cognitive components of sociality, namely between social tendency and social recognition, which would support the evolution of a sociality syndrome;

2- To examine if social and non-social cognitive abilities (i.e. social vs. object recognition) are independent from each other, or if they co-vary supporting a general domain factor;

3- To investigate if there is an association between social tendency (or a putative sociality syndrome) and anxiety trait, which would support the evolution of sociality within the scope of a response to predation;

4- To explore if the phenotypic correlations found between motivational and cognitive components of sociality are fixed or vary across different

populations (lab strains), in order to test the constraint vs. adaptive hypothesis for their evolvability;

5- To investigate if there are associations between candidate SNPs and the different social behaviours measured (social tendency, social and non-social memory and anxiety), in order to characterize the genetic basis of the observed phenotypic correlations.

6- To explore if there are SNPs specifically associated with social and non-social behaviours or anxiety.

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Chapter II

Phenotypic Architecture of Sociality and its Associated Genetic Polymorphisms in Zebrafish

Abstract

Sociality is often seen as a single phenotypic trait, but it relies on motivational and cognitive components implemented by specific causal mechanisms. Hence, these components may have evolved independently, or may have been linked by phenotypic correlations driven by a shared selective pressure for increased social competence. Furthermore, these components may be domain-specific or of general domain across social and non-social contexts. Here we have characterized the phenotypic architecture of sociality in zebrafish, which has been increasingly used as a model organism in social neuroscience. For this purpose, we have behaviorally phenotyped zebrafish from different wild type lines in four tests: social tendency, social and non-social recognition, and open-field test. Our results indicate that: (1) sociality has two main components that are independent from each other (social tendency and social recognition), hence not supporting the occurrence of a sociality syndrome; (2) both social traits are phenotypically linked to non-social traits (non-social exploration and non-social memory, respectively), forming two general behavioural modules, general inspection and general recognition, and suggesting that sociality traits have been co-opted from general-domain motivational and cognitive traits. Moreover, the study of the association between genetic polymorphisms (i.e. single nucleotide polymorphisms, SNPs) and each behavioural module further supports this view, since several SNPs from a list of candidate “social” genes, are statistically associated with the general inspection (motivational), but not with a general recognition (cognitive), behavioural module. The SNPs associated with general inspection are widespread across different chromosomes and include neurotransmitters, neuromodulators, and synaptic plasticity genes, suggesting that this behavioural module is regulated by multiple genes,

each of them with small effects. Together, these results support the occurrence of general domain motivational and cognitive behavioural modules in zebrafish, which have been co-opted for the social domain.

Keywords:
Phenotypic correlations, Single Nucleotide Polymorphisms (SNPs), social tendency, social recognition, social cognition, zebrafish

2.1 Introduction

Sociality is ubiquitous among animals, with animal aggregations and the formation of social groups occurring across most animal taxa (Ward and Webster, 2016). From a causal perspective sociality relies on two elementary behavioral mechanisms: (1) a motivation to approach conspecifics (social tendency) that leads to the formation of social groups; and (2) the cognitive ability to recognize different conspecifics (social recognition) that allows individuals to selectively adjust the expression of their behaviour to different individuals they encounter. Given the fundamental role of these two behaviours for sociality, one can predict them to be selected together during social evolution, leading to a phenotypic correlation between them. On the other hand, each of these traits relies on different endophenotypes, with social tendency requiring a motivational (i.e. goal-directed) response, and social recognition requiring a cognitive ability (i.e. encoding, storing and recalling information about conspecifics in order to discriminate them), each implemented by different proximal mechanisms. For example, in mammals social recognition is hippocampus-dependent (for a review see Okuyama and Teruhiro, 2018), whereas social tendency relies on mesolimbic reward circuits (e.g. Dolen et al., 2013; Gunaydin et al., 2014). Moreover, social recognition may reflect a general domain cognitive ability, that evolved to allow animals to discriminate different entities, social or not (e.g. edible vs. non-edible food), in the environment, rather than a domain-specific trait selected by sociality (e.g. Heyes and Pearce, 2015; Varela et al., 2020). In this case a phenotypic correlation would be expected between social recognition and non-social (e.g. object) recognition. Similarly, social tendency may reflect a general domain response to threat perception in the environment, since cohesiveness in animal aggregations is known to

increase with perceived danger (i.e. aka defensive aggregation; e.g. rats: Bowen et al., 2012; zebrafish: Kleinhappel et al., 2019). In this case a phenotypic correlation would be expected between social tendency and behavioural measures of anxiety/stress. Thus, the phenotypic architecture of sociality can be characterized by the pattern of phenotypic correlations among these behavioural traits.

The evolution of correlated traits can be explained by two alternative hypotheses: (1) the constraint hypothesis, that postulates the occurrence of shared proximal mechanisms such as a pleotropic effect of a gene, or a hormone with multiple target tissues; or (2) the adaptive hypothesis, that proposes that positive correlations between traits only occur in environments that favour them, such that selection can break apart maladaptive combinations of traits; These two hypotheses generate different predictions that can be tested by comparing the patterns of correlated characters across different populations of the same species. The constraint hypothesis predicts traits to be correlated across populations irrespective of ecological conditions, whereas the adaptive hypothesis predicts correlations between traits to vary between populations depending on local conditions. Thus, these two scenarios also have different evolutionary consequences, with the correlated traits acting as an evolutionary constraint in the first case and the correlation being itself an adaptation in the latter. Although, this rational has been used to study the evolution of behavioural syndromes (aka personality) (e.g. Bell, 2005), to the best of our knowledge, it has not been applied yet to analyze the evolution of correlated social behaviour traits. These two hypotheses can also be tested by assessing if the genetic architecture of correlated traits is shared or not. Given the complexity of social behaviour traits they are expected to be under the influence of multiple genes, with small effects of each of them. In fact, several genes involved in neurotransmission (e.g. dopamine, serotonin; Sören et al.,

2010; Gunaydin et al., 2014; Gunaydin & Deisseroth, 2014; Walsh et al., 2018), neuromodulation (e.g. oxytocin; Donaldson and Young, 2008; Goodson and Thompson, 2010; Goodson, 2013) and synaptic plasticity mechanisms (e.g. neuroligins/ neurexins; Sudhof 2008; Grayton et al., 2013; Rabaneda et al., 2014; Hornberg et al., 2020) have been reported to influence social behaviour in multiple ecological domains across a wide range of vertebrate taxa. Moreover, these "social" genes are expressed in brain regions that together form an evolutionary conserved social decision-making network in vertebrates (O'Connell and Hofmann, 2011, 2012). Therefore, the question is to what extent these candidate genes show specific or shared patterns of association with the motivational and cognitive components of sociality discussed above.

Enough variation in both social tendency and social recognition occurs across species and between individuals of the same species, which should allow to test the abovementioned hypotheses. The tendency to associate with conspecifics varies considerably among species, ranging from weakly social species, in which social interactions only occur at specific times (e.g. breeding), to highly social species, in which individuals stay all their lives in close proximity and interacting with others. Similarly, variation in social recognition ability also occurs across species, from basic levels of recognition (e.g. conspecific vs. heterospecific), to increasingly more elaborate ones with high degree of specificity (e.g. kin vs. non-kin; particular individuals) (Tibbetts and Dale, 2007). Moreover, variation in both social tendency and social recognition also occur within species, both intra- (e.g. with age and life-history stage) and inter-individually.

In this study we aim to characterize the phenotypic architecture of sociality in zebrafish (*Danio rerio*) by characterizing social tendency, social recognition and object recognition across multiple laboratory zebrafish populations that have evolved separately in captivity for

multiple generations and by characterizing the genetic polymorphisms of candidate “social” genes associated with these behavioural traits. In zebrafish, isogenic strains are not viable due to inbreeding depression (Mrakovcic and Haley 1979). Hence, laboratory zebrafish populations differ from those of other model organisms in that they are recurrently outcrossed to maintain diversity (Nasiadka and Clark 2012). As a result, laboratory zebrafish populations contain significant but varying levels of genetic diversity (Brown et al., 2012; Balik-Meisner et al., 2018; Brown et al. 2012). In parallel, zebrafish strains (e.g. AB, TU, WIK) have already been shown to vary in many behaviours, including locomotor activity, anxiety traits, stress reactivity, learning abilities and shoaling (e.g. Oswald and Robison, 2008; Egan et al. 2009; Sackerman et al. 2010; Barba-Escobedo & Gould 2012; Lange et al., 2013; Mahabir et al., 2013; Maximino et al., 2013; Vignet et al., 2013; Vital and Martins, 2013; Liu et al., 2014; Gorissen et al., 2015; Séguret et al., 2016). The paralleled variation genetic diversity and several behavioural phenotypes, provides the rationale that constitutive genetic variation may contribute to the observed behavioural variability.

Here we specifically aim to test: (1) if there is an association between social tendency and social recognition, supporting the evolution of a sociality syndrome; (2) if social and non-social cognitive abilities (i.e. social vs. object recognition) are independent from each other, or if they co-vary supporting a general domain factor; (3) if there is an association between social tendency (or a putative sociality syndrome) and anxiety trait; (4) if the phenotypic correlations found are fixed or vary across strains (populations), in order to test the constraint vs. adaptive hypothesis; (5) to what extent the genetic architecture of each of these behavioural traits is shared or not, which would provide evidence for genetic pleiotropic effects underlying a putative sociality syndrome. For the latter, we have assessed the association between known single

nucleotide polymorphisms (SNPs) in zebrafish for a set of candidate "social" genes (see methods for details) and each behavioural trait.

2.2 Methods

2.2.1 Zebrafish strains and housing conditions

Zebrafish were raised in the Fish Facility of the Instituto Gulbenkian de Ciência under laboratory conditions. The following strains were used in this study:

1. AB, was established by George Streisinger and Charline Walker in the Oregon labs, from two strains, A and B, purchased by George Streisinger at different times from a pet shop in Albany, Oregon, in the late 1970s. The original A and B strains probably originated from a hatchery in Florida. The AB strain has been screened for lethal-free embryos by in vitro fertilization and selected females subsequently used to establish the current AB strain (Streisinger et al. 1981; Chakrabarti et al. 1983). This procedure reduced the number of lethal mutations in this line, which has been used as the primary background for most of the transgenic and mutant strains that are currently available.

2. TU (*Tüebingen*), originated from a pet store in *Tüebingen* and was selected during the 1990s at the Max-Planck in *Tüebingen* to remove embryonic lethal mutations from the background before being used by Sanger for the zebrafish sequencing project (Mullins et al. 1994; Nusslein-Volhard, 2002).

3. WIK (Wild India Kolkata) was derived from a wild catch of a single pair in India near Kolkata. The WIK strain is very polymorphic relative to the TU strain and was first described as WIK11 (Rauch et al. 1997).

4. TL (*Tüpfel* long fin), was derived from a cross between an AB with a spotted phenotype and a TU resulting in a long-finned phenotype. This line is homozygous for leot1, a recessive mutation causing spotting in adult fish (aka tup), and for lofdt2, a dominant mutation causing long fins.

5. 5D (5D Tropical), was derived at Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University in 2007, from a commercial breeding facility (5D Tropical Inc., Florida), to generate a *Pseudoloma neurophilia* (Microsporidia) free strain (Kent et al., 2011).

6. Leo (Leopard), is a wild type strain commonly available in pet shops, which displays a spotted adult pigment pattern instead of striped. This strain is homozygous for a spontaneous mutation in the gene leopard (leo), *leot1* (Frankel, 1979; Haffter et al. 1996; Watanabe et al. 2006).

A total of 164 experimentally naive adult zebrafish of both sexes, aged 6-8 months, were used in this study as focal subjects (AB: M = 8, F = 14; TU: M = 9, F = 12; WIK: M = 12, F = 4; TL: M = 13, F = 10; Leo: M = 7, F = 10; 5D M = 32, F = 33). Focal fish were raised and housed separately from fish used as stimuli to prevent effects of prior familiarity. Fish used as stimuli were of the same strain as the focal fish. Housing was in groups of 35 fish kept in 3.5 L aquaria of a recirculating system (ZebraTec, 93 Tecniplast), with water parameters set at 27-28 °C, 7.5 ± 0.2 pH and ~ 900 µSm, and <0.2 ppm nitrites, <50 ppm nitrates and 0.01-0.1 ammonia. Daily photoperiods were alternated between 14h light and 10h dark and feeding occurred twice-daily and included a combination of live (*Paramecium caudatum; Artemia salina*) and processed dry food (GEMMA Micro).

2.2.2 Experimental setup and procedures

The behaviour of each experimental fish was assessed in four different tests: (1) a shoal preference test to measure social tendency; two one-trial recognition tests using either objects (2) or conspecifics (3) as stimuli to measure non-social and social recognition/exploration, respectively; and (4) an open-field test to measure the anxiety trait. Excluding the open-field, all setups included an experimental tank (30 L x 15 W x 15 H cm) and two adjacent tanks (15 L x 15 W x 7.5 H cm) with a stimulus-holding compartment having a viewing side of 10 cm in the shoal preference test and of 5 cm in the social and object recognition tests, the difference accounting for the different visual target areas offered by a shoal *vs.* an individual or an object. Water depth was kept constant at 9cm in all tanks. For the open-field test a round tank with a 22cm diameter was used and water level was kept at 6 cm depth. Experimental set-ups for each test are illustrated in Figure 2.1 (a-e) (see in result section page 103).

All tests occurred during the light period between 09:00 and 19:00, before which fish were kept overnight in an aquarium with individual compartments for identification purposes. These compartments were separated by fine mesh that allowed visual and chemical access to neighbours and minimized stress from isolation. In the experimental tanks, external stimuli were visually blocked by opaque, non-reflective stickers and opaque covers obscured adjacent stimulus containers prior to the onset of recordings during the shoal preference and recognition tests. Behaviour during tests was recorded using a black and white mini surveillance camera (Henelec 300B) suspended above the experimental tank and relaying the image to a laptop kept at a distance to reduce disturbance of fish by the experimenter. Video acquisition was done with Pinnacle Studio 14 (Corel

Corporation, Ottawa, Canada) software (www.nchsoftware.com). During recording, lighting in the room was kept at conditions that reduce water-surface reflection in the videos, and extra lighting was provided by an infrared lightbox placed under the experimental tank in order to facilitate video tracking during the data collection stage. Between tests, water in the experimental tank was changed to eliminate olfactory cues.

Before tests, focal fish were netted from their individual overnight compartment and immediately placed in the experimental tank. For the shoal preference test, fish were first given 10 minutes to acclimatize to the experimental tank and then tests were initiated by removing the opaque covers and allowing fish visual access to the two adjacent containers, one empty (control) and the other holding a mixed-sex shoal (Figure 2.1c), for 10 minutes. The side of presentation of each stimulus was counterbalanced between focal individuals to control for side biases.

For the open-field test (Figure 2.1b), animals were placed in the centre of the circular tank and recorded for 10 minutes.

Recognition tests (Figure 2.1 d-e) were comprised of two phases: an acquisition phase and a probe-test phase, and the experiment included a 10 minutes initial acclimation period before the acquisition phase and a 10 minutes interval before the probe-test phase. Both phases were initiated by removing opaque covers and allowing fish visual access to two adjacent containers. During the acquisition phase, animals were presented with two novel stimuli for 10 minutes: two conspecifics for the social test and two objects (0.5 ml eppendorf tubes of the same colour) for the non-social test. During the probe test, animals were presented with one of the stimuli from the acquisition phase (familiar) and a novel stimulus (a new conspecific or a differently coloured eppendorf tube) for 10 minutes. For the non-social recognition test, the size of the eppendorf tube was matched to the average

zebrafish size to control for size-dependent prey or predator directed responses and, based on preliminary preference tests, were coloured with colours of equal preference by the fish (either green or red for all strains, except for Leo that instead show no preference between purple and blue). The side of each stimulus (novel or familiar) during probe tests was counterbalanced across animals, to control for side biases, and the colour used for the familiar or novel stimulus was randomised, to control for colour biases.

Videos were analysed using a commercial videotracking software (EthoVision XT, Version 11.5, Noldus Information Technology) and behavioural measures were extracted from each test. Regions of Interest (ROI) marked were kept at an average body length distance from the target location (grey regions in Figure 2.1 a-b). Social tendency during the shoal preference test was quantified by the proportion of time in ROIs spent near the shoal, social and non-social discrimination during the conspecific and object recognition tests was measured by the proportion of time in ROIs spent near the preferred stimulus (familiar or novel), while the overall time spent in ROIs near both stimuli was used as a measure of exploration. Anxiety in the open-field test is typically exhibited by thigmotaxis (i.e. the propensity to avoid exposed areas), which was measured as the proportion of time spent within the ROI near the periphery following first entry (to control for any initial freezing in the centre), while the average distance (in cm) from the wall was used to quantify the edge or wall orienting tendency associated with fear-induced thigmotaxis (Kalueff et al., 2013).

2.3 Genetic polymorphisms analysis

At the end of the behavioural phenotyping, animals were anesthetized by immersion into an athyl 3-aminobenzoate methanesulfonate salt solution (MS222) 100-200mg/L, a fin clip was collected from the caudal fin of each experimental fish, and preserved in a digestion mix (PK, 10mg/ml, Lysis solution [Fermentas #K0512], TE buffer) until further processing. Subsequently, DNA was extracted from preserved fin clips using DNA extraction kit [Fermentas #K0512] with some adjustments to the protocol provided by manufacturer. Briefly, samples were thawed at room temperature and placed in a thermomixer for approximately 20h with shaking (700 rpm) at 50ºC. After, chloroform was added in a 1:1 ratio and the samples gently mixed by inversion. Samples were then centrifuged at 18506g (13200 rpm in Eppendorf 5430R centrifuge) for 7 minutes and the upper aqueous phase transfer to a new 1.5 ml tube 800 µl (720µl H_2O + 80µl of precipitation solution [Fermentas #K0512]) was added to each tube, mixed gently by inversion for 2 minutes and centrifuged again for 10 minutes at (18506 g). The supernatant was removed, the DNA pellet dissolved in 100µl NaCl 1.2M solution [Fermentas #K0512], and 300µl of freezer cold 100% ethanol (-20ºC) was added to allow DNA to precipitate over night at -20°C. In the day after, samples were centrifuged for 10 minutes (18506g) and the ethanol removed. To wash the pellet, 200 µl of freezer cold 70% ethanol was added to each sample and centrifuged for 10 minutes (18506 g). Finally, the pellet was allow to dry for 15-30min at 37ºC and 30µl of DNAse-free sterile H2O was added. To access the concentration and quality of the DNA, samples were quantified in the Nanodrop (Thermo Scientific, Nanodrop 2000) and the ratios 260/280 and 260/230 listed.

We built a list of candidate genes to test their association with the behaviour traits, based on evidence from the literature for their

involvement in the regulation of social behaviour. This gene list included genes for: neurotransmitter systems (e.g. dopamine, serotonin), neuromodulators (e.g. oxytocin, AVT, NPY), neuroplasticity (e.g. bdnf, neurexins, neuroligins), and genes linked to autism (e.g. shank3a). To obtain candidate SNPs for the genes of interest, all germline variations from this species were downloaded from http://ftp.ensembl.org/pub/release-104/variation/gvf/danio_rerio/ in the form of a GVF file. The GVF file was filtered to keep only SNPs in locus of interest and which evidence was sustained by frequency observations to increase probability of variation. Sequences were extracted with Ensembl's Biomart tool using the "Zebrafish Short Variants (SNPs and indels excluding flagged variants) (GRCz11)" dataset. Several iterations of Assay Design 4.0 (Agena Biosciences), which designs multiplexed MassEXTEND® assays for Mass Spectrometry detection, were run to accomplish an even distribution on the genes of interest. Four multiplexes were designed with 38, 36, 35 and 35 assays. Agena Biosciences iPlex(®) Kit, MassARRAY(®) platform and Typer software v.4 were used following manufacturer's standard protocols and procedures, for the genotyping reactions, acquisition of genotypes and inspection of results, respectively. 139 SNPs in locus of interest were successfully sequenced, but we had to remove 7 for lack of variation between the 164 tested zebrafish (the final list of SNPs is available in Table 2).

2.4 Statistical Analysis

In order to confirm that all strains express social tendency and are able of social and object recognition, one-sample *t*-tests ($\mu \neq 0.5$ vs. >0.5) were used to test if the scores of social tendency, object discrimination

and social discrimination were significantly different from chance levels for each sex and for each strain. Next we extracted behavioural modules that aggregate correlated behaviours by carrying out a factor analysis using principal component extraction (PCA) followed by varimax rotation, based on the correlation matrix of all behavioural measures (social tendency, social discrimination, social exploration, object discrimination, object exploration, thigmotaxis and edge-orienting). The analysis identified three main components (Cs) to which we call behavioural modules: general inspection, general recognition and anxiety (see the results' section for more details). Then, Linear Mixed Models (LMM) were used to assess the effects of sex, strain, the interaction of the two and the fish ID as a random covariate on the scores each behavioural module, followed by Tukey post-hoc tests. These analyses were carried out in the statistical software Minitab ® (Minitab Inc., State Collage, PA, USA).

The remaining analysis were carried out in the statistical software R, version 4.0.4 (R Core Development Team 2021). To test if the behavioural modules are differently related for with each zebrafish strain, we computed Pearson correlations matrices between the three PC scores across each strain. All p-values were corrected for multiple testing with Benjamin and Hochberg's method. Heatmaps were used for visual representation of the behavioural correlation matrices for each strain. The R packages Hmisc (Harrell 2020) and "ggplot2" (Wickham 2016) were used for computing the correlations and building the heatmaps, respectively. The quadratic assignment procedure (QAP) correlation test, with 5000 permutations (Borgatti et al., 2013), was used to assess the association between any two phenotypic (i.e. behavioural modules) correlation matrices between different zebrafish strains on UCINET 6 (Borgatti et al., 2002). Given that the null hypothesis of the

QAP test is that there is no association between matrices, a significant p-value indicates that the correlation matrices are similar.

To check whether the genetic distance between subjects (using their genetic data from the list of 132 SNPs) are structured by strain or represent a uniform population, we performed a hierarchical clustering analysis, using the "philentropy" package (Drost 2018). We computed the jaccard distance between all subjects, which is the proportion of the similar genetic distances between subjects over the total genetic distances. With the genetic distances' matrix, we performed the hierarchical clustering with complete-linkage, which calculates the maximum distance between clusters before merging. Then, we plotted the hierarchical cluster in a dendrogram using the "dendextend" package (Galili 2015). We found a structed population with 5 clusters, corresponding to 4 of the 6 different strains, with the 5^{th} cluster merging the TU and WIK strains together (see results section for more details). Therefore, we decided to include strain as a covariate in the analyses of SNP-behaviour associations (see below).

To assess the associations between genetic polymorphisms and behaviour, we tested each of the 132 SNPs independently against each behavioural phenotype (the 7 behaviours and 3 PC scores). We did not include 3 zebrafish subjects in this analysis because their sample call rate was bellow 5%, that is, they lacked genetic information for most SNPs. For the behaviours that followed a linear distribution (general inspection, general recognition, anxiety and edge-orienting) we used linear models (LM) implemented with the R "base" package. For the behaviours that were proportions (social tendency, social discrimination, social exploration, object discrimination, object exploration and thigmotaxis), we used generalized linear models (GLM) with beta regression implemented with the "betareg" package (Cribari-Neto & Zeileis 2010). In all models, the behaviours were the response variables,

SNP was the explanatory variable and strain was a co-variate. SNPs were integers, where 1 represented the heterozygote case and 0 and 2 the homozygotes. For example, for SNP rs180151563, 0 represents the genotype AA, 1 the genotype CA and 2 the genotype CC. For some SNPs there were only two of the three possible conditions. Strain represents the different origins of the zebrafish subjects that we tested. It was also an integer, varying between 1 and 6, where 1 represented the 5D strain, 2 the AB strain, 3 the Leo strain, 4 the TL strain, 5 the TU strain and 6 the WIK strain. For each statistical model, we used the summary () function in R to extract the p-value of the SNP, which was corrected for the strain effect. Because we run 132 independent tests for each SNP, we corrected the p-values with the false discovery rate (FDR) adjustment method.

For some of the SNP-behaviour associations that remained significant after FDR adjustment, we used the “ggplot2” (Wickham 2016) and “ggpubr” (Kassambara 2020) packages to draw boxplots for the given phenotype, broken down by the SNP genotype. Over the boxplots, we added dot plots broken down by strain to help visualizing the strain effect on zebrafish behaviour. For a more comprehensive comparison of the SNP-behaviour associations, we plotted the significant associations by behavioural categories using Venn diagrams, with the “VennDiagram” package (Chen 2018).

2.5 Ethics statement

All experimental procedures were reviewed by the institutional internal Ethics Committee at Instituto Gulbenkian de Ciência and approved by the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Portugal; permit number 0421/000/000/2017).

2.6 Results

2.6.1 Phenotypic architecture of sociality in zebrafish

Scores of social tendency (i.e. preference for shoal over empty tank), as well as object and conspecific discrimination scores (i.e. preference between a novel and a familiar stimulus) were all significantly different than chance for individuals of both sexes and for all strains tested (one-sample *t*-test: $\mu \neq 0.5$, $P < 0.001$; see Table S1; Fig. 2.1 f-h), indicating that social affiliation and social and object recognition abilities are present in males and females across zebrafish strains.

To assess the phenotypic architecture of sociality we performed factor analysis using principal component extraction followed by varimax rotation based on the correlation matrix between measures extracted from 4 separe tests of social and associated behaviours (sampling adequacy: KMO > 0.5; sphericity: Bartlett's $\chi^2_{21} = 253.76$, $P < 0.001$; determinacy of multicollinearity: $\rho = 0.754$). The analysis identified 3 components (C) with eigenvalues ≥ 1 (Fig. 2.1i and Table 1). C1 shows a strong loading of social tendency measured in the social preference test and of social and object exploration measured in the social and object discrimination tests, respectively, suggesting the occurrence of a general inspection behavioural module that is expressed both in social and non-social contexts. C2 shows a strong loading of thigmotaxis and edge-orienting measured in the open-field test, corresponding to an anxiety behavioural module. Finally, C3 shows a strong loading of object and social discrimination, measured in the object and social discrimination tests, respectively, suggesting the occurrence of a General Recognition behavioural module that is expressed both in social and non-social contexts.

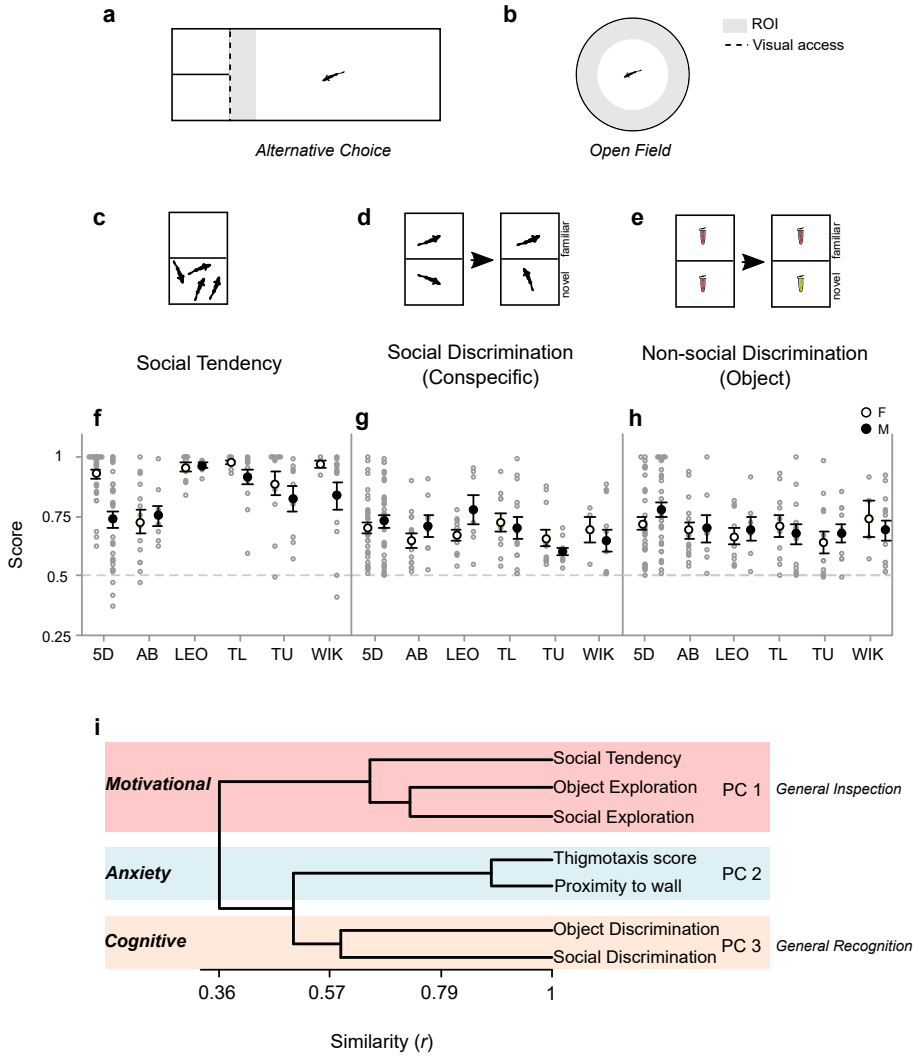

Figure 2.1 Social and associated behaviours in zebrafish. (**a**) Across strains, a two alternative-choice set-up was used to measure social preference and recognition abilities (**b**) and an open field test of measuring anxiety-driven thigmotaxis towards the periphery and edges. Regions of interest (ROI) were set within 1 standard body-length from target locations or stimuli. (**c**) Social tendency was measured by interaction preferences towards a shoal. Recognition in both the social (**d**) and non-social (**e**) context we

measured by the ability to discriminate between a familiar and a novel stimuli. (**f**) Males (full circles) and females (open circles) of all strains (5D, AB, LEO, TL, TU, Wik) exhibited above chance (dashed line) preference for shoal over an empty tank (social tendency, **f**) and discrimination between a novel and familiar stimulus in both a social (conspecific; **g**) and non-social (object; **h**) context [bars indicate 95% CI]. Behavioral measures exhibited different degrees of correlation (*r*), illustrated in the cladogram as degrees of association (**i**), based on which factorial analysis revealed three principal components (PC): PC1 aggregates social tendency and social and object exploration corresponding to a motivational component of sociality; PC2 aggregates thigmotaxis and distance to wall measured in the open field test, corresponding to an anxiety component; PC3 aggregates object and social discrimination, corresponding to a general-domain cognitive component.

Table 1. Loadings extracted by the varimax rotaton of principal components from the correlation matrix of behaviours across tests, for zebrafish of all strains. Bold type indicates the strongest contributors (coefficient > 0.5) to each component (C)

			Component loadings [a]			
Test	Behaviour	Parameters	PC 1 *General Inspection*	PC 2 *Anxiety*	PC 3 *General Recognition*	Comm. [b]
SP	Social tendency	Proportion total ROI time spent with shoal	**0.725**	-0.033	0.180	0.559
SR	Social discrimination	Proportion total ROI time spent with preferred conspecific	0.113	-0.031	**0.852**	0.739
	Social Exploration	Proportion test time spent in ROI of both conspecifics	**0.762**	0.085	-0.224	0.639
OR	Non-social discrimination	Proportion total ROI time spent with preferred object	-0.363	0.139	**0.619**	0.535
	Non-social exploration	Proportion test time spent in ROI of both objects	**0.754**	-0.042	-0.111	0.583
OF	Thigmotaxis	Proportion time spent within ROI of wall, after first entry (control for initial freezing in centre)	0.107	**0.932**	0.084	0.888
	Edge-orienting	Average distance from wall (cm)	0.112	**-0.941**	0.009	0.897
		Eigenvalue [c]	*1.845*	*1.784*	*1.211*	*4.840*
		% Variance explained	*26.4*	*25.5*	*17.3*	*69.1*

[a] Correlation between PC and variable values

[b] Communalities: Proportion of variable variance explained by all PCs

[c] Variance of transformed data used for each PC

To test if the behavioural modules described above (i.e. general inspection, general recognition, and anxiety) can evolve differently from each other in each zebrafish strain – which represent different laboratory populations established by different wild type founders and that have evolved independently from each other in somewhat similar laboratory conditions - we computed correlation matrices between individual scores for each module (varimax rotated PC scores) for each of the different zebrafish strains. We then used the quadratic assignment procedure (QAP) correlation test to compare the correlation matrices of the different strains. The results identified a single significant negative correlation ($r = -0.9988$, $p = 0.0002$) between 5D and WIK correlation matrices. Thus, none of the correlation matrices were similar between each other (Fig. 2.2), supporting the adaptive hypothesis that predicts different patterns of phenotypic correlations in different populations.

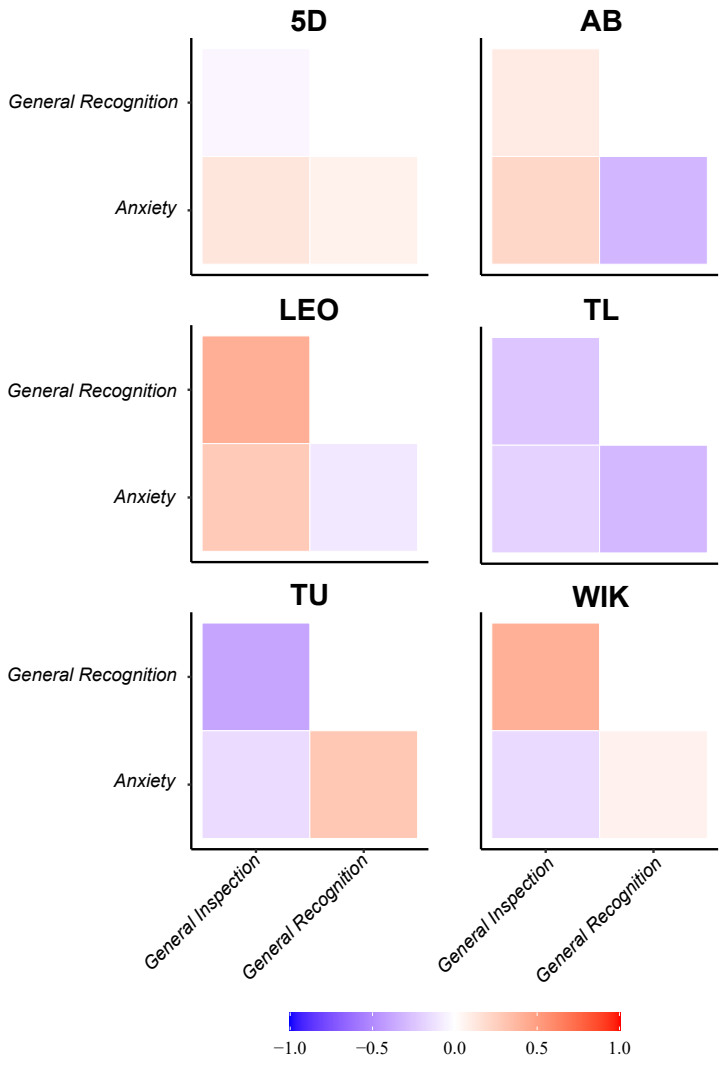

Figure 2.2 Phenotypic correlation matrices for the behavioural modules General Inspection, Genral Recognition and Anxiety across six different zebrafish laboratory strains (i.e. populations). Color code represents correlation (*r*) values.

2.6.2 Genetic polymorphisms associated with behavioural modules

To assess if the different behavioural modules identified above were linked by a shared genetic architecture, we have investigated the association between a set of genetic polymorphisms (SNPs) in a list of candidate “social” genes and each of the measured behaviours and PC behavioural modules. Given the fact that we have phenotyped individuals from 6 different wild type strains, we checked for structured genetic variation by computing the genetic distance between the phenotyped individuals for the SNPs under study. We found that genetic variation for the SNPs of interest is highly structured with individuals from the same wild type strains clustering together (Fig. 2.3a).

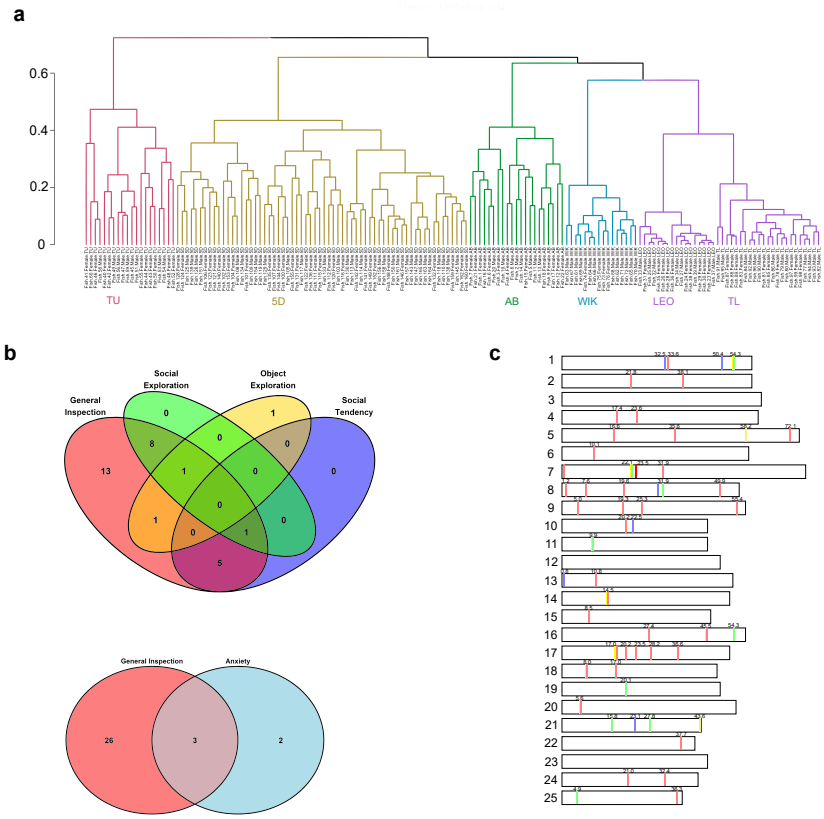

Figure 2.3 Genetic clustering and behavioural associations. (**a**) Hierarchical clustering of genetic distances (Jaccard distance) between the sampled individuals indicates the occurrence of 5 major clusters that overall match the 6 wild type strains used (pink cluster: TU; gold cluster: 5D; green cluster: AB; blue cluster: Wik), with Leo and TL included in the purple cluster but subsequently segregated from each other in two lower order clusters. (**b**) Venn diagrams representing the number of SNPs the General Inspection component shares with its constitutive behaviors (social tendency, social exploration, and object exploration) and the Anxiety component. (**c**) Chromosome mapping of the SNPs that are significantly associated with the General Inspection component and its constitutive nehaviours, following the color code used by the Venn diagrams, and with the position of each SNP on each chromosome is given in bp.

Therefore, we have used the strain as a covariate in the model that assessed the association between each SNP an each of the behavioural modules. Out of the 132 SNPs that showed variation in our sampled individuals 52 (which mapped to 29 genes) were significantly associated with General Inspection, none with General Recognition and 8 (which mapped to 5 genes) with anxiety (Table 2).

Table 2. Lists of genes with SNPs associated with the behavioural modules general inspection (and its contributing behaviours) and anxiety. Abbreviations: 5HTR=serotonin receptor; D = dopamine receptor; Cyp19a1b = cytochrome P450, family 19, subfamily A, polypeptide 1b; Nrxn = neurexin; Nlgn = neuroligin; Npas1 = Neuronal PAS Domain Protein 1; NPY = neuropeptide Y; Nr4a3= nuclear receptor subfamily 4, group A, member 3; Nr4a2a= nuclear receptor subfamily 4, group A, member 2a; Dkk2 = dickkopf WNT signaling pathway inhibitor 2; itprid1 = ITPR interacting domain containing 1; MECP2 = methyl CpG binding protein 2; Syngap1b = synaptic Ras GTPase activating protein 1b; Tsc2 = TSC complex subunit 2; Chd7 = chromodomain helicase DNA binding protein 7.

Gene name	SNP name	General Inspection	Social Tendency	Social Exploration	Object Exploration	Anxiety
5HTR-1aa	rs180146258	association	association			
5HTR-1aa	rs180146259	association		association		
5HTR-2cl1	rs180151790	association		association	association	
5HTR-2cl2	rs180074553				association	
5HTR-3a	rs180073160	association				
5HTR-3a	rs180073162	association				
5HTR-3a	rs180073164	association				
5HTR-3a	rs180168240	association	association			
5HTR-3b	rs180168236	association				
5HTR-3b	rs180168238	association				
5HTR-7b	rs180131627	association				
5HTR-7b	rs180131628	association				
5HTR-7c	rs180162109	association				
5HTR-7c	rs40616624	association				
5HTR-7c	rs40650859	association				
Chd7	rs180043369					Association
Cyp19a1b	rs180038734	association		association		
D2b	rs180032799	association				
D2b	rs180107813	association				
D2b	rs180173350	association			association	
D3	rs180060870	association				
D3	rs180060872	association				
Dkk2	rs180052655	association	association			
GnRH-2	rs40618151	association		association		
itprid1	rs180062152	association				
MECP2	rs180034118	association		association		
MECP2	rs180034123	association				
nlgn-1	rs180124055	association				
nlgn-1	rs180124079	association		association		
Nlgn2a	rs180151551	association		association		Association
Nlgn2a	rs180151563	association				
Nlgn2b	rs180131390	association	association			
Nlgn2b	rs180109431	association				
Nlgn4xa	rs180050066	association	association			
Npas1	rs180107067	association				
NPY	rs180080888	association		association		
Nr4a2a	rs180134986					Association
Nr4a3	rs180101713	association				
Nrxn-1b	rs180110916	association				
Nrxn-1b	rs180110942	association	association			
Nrxn-2a	rs180168558	association		association		

Nrxn-2b	rs180174009	association		
Nrxn-3b	rs180149774			Association
oxytocin	rs180034306	association		Association
oxytocin	rs180034305			Association
Shank3a	rs179558694	association		
Shank3a	rs180084393	association		
Shank3a	rs180084400	association		
Shank3a	rs180084434	association		
Syngap1b	rs180104498	association		
Tryoptophan H2	rs180086458	association		
Tryoptophan H2	rs180086462	association		
Tsc2	rs180053194	association		
Tsc2	rs180053196	association		
Tsc2	rs180053204	association	association	
Tsc2	rs180053446	association	association	
Tsc2	rs180055121	association		
TyrosineH2	rs180036401			Association
TyrosineH2	rs180036402			Association
Vasotocin	rs180174456			

Regarding the 3 behaviours that loaded to the General Inspection behavioural module, 6 SNPs (mapping to 6 genes) were associated with social tendency, 11 (mapping to 10 genes) with social exploration, and 3 (mapping to 3 genes) with object exploration. Of these 20 SNPs associated with these behaviours that load to General Inspection, only one (mapping to the serotonin receptor gene 5HTR 2cl2) is not also associated with General Inspection (Fig. 2.3b; Table 2). Moreover, of the 29 SNPs associated with General inspection, 16 are also associated at least with one of the behaviours that constitutes these behavioural module (Fig. 2.3b; Table 2). However, there is a reduced overlap between the SNPs associated with these different behaviours: only one SNP affects both social tendency and social exploration (matching the gene 5HTR-1aa), and only another SNP affects both social exploration

and object exploration (matching the gene 5HTR-2cl1) (Fig. 2.3b; Table 2).

The SNPs associated with the general inspection behavioural module are widely distributed across the zebrafish genome being absent only from chromosomes 11, 12, 19, 21 and 23 (Fig. 2.3c). However, one can find SNPs associated with behaviours that load to General Inspection module in some of these chromosomes; SNPs associated with social exploration in chromosome 11, 19 and 21; and SNPs associated with social tendency and with object exploration in chromosome 21 (Fig. 2.3c).

The list of SNPs associated with the General Inspection module include genes involved in neurotransmission (e.g. serotonin and dopamine receptors), neuromodulation (e.g. NPY, oxytocin), synaptic plasticity (e.g. neuroligins, neurexins) and epigenetic marking (e.g. methyl CpG binding protein 2) (see Fig. 2.4 for arbitrarily selected illustrative examples).

2.7 Sex and strain differences in behavioural modules

Although it was not the central question of this study, the occurrence of sex and wild type strain (aka strain) differences in the expression of the behavioural modules identified above can be informative when choosing strains to run specific behavioral tests in zebrafish. Therefore, we have also tested for both sex and strain differences in General Inspection, General Recognition and Anxiety (Fig. 2.5).

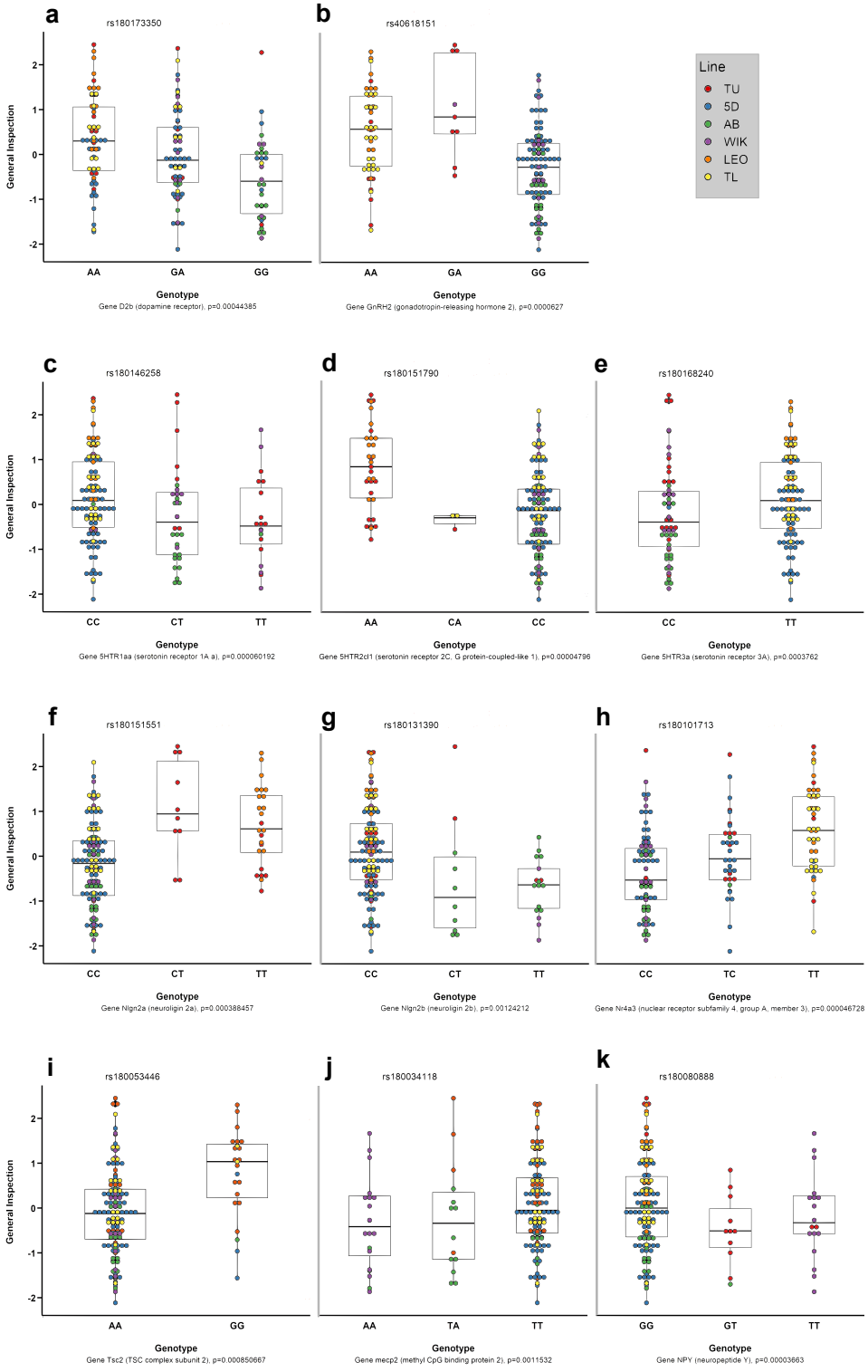
a
rs180173350
General Inspection
AA
GA
GG
Genotype
Gene D2b (dopamine receptor), p=0.00044385
b
rs40618151
AA
GA
GG
Genotype
Gene GnRH2 (gonadotropin-releasing hormone 2), p=0.0000627
Line
TU
5D
AB
WIK
LEO
TL
c
rs180146258
CC
CT
TT
Genotype
Gene 5HTR1aa (serotonin receptor 1A a), p=0.000060192
d
rs180151790
AA
CA
CC
Genotype
Gene 5HTR2cl1 (serotonin receptor 2C, G protein-coupled-like 1), p=0.00004796
e
rs180168240
CC
TT
Genotype
Gene 5HTR3a (serotonin receptor 3A), p=0.0003762
f
rs180151551
CC
CT
TT
Genotype
Gene Nlgn2a (neuroligin 2a), p=0.000388457
g
rs180131390
CC
CT
TT
Genotype
Gene Nlgn2b (neuroligin 2b), p=0.00124212
h
rs180101713
CC
TC
TT
Genotype
Gene Nr4a3 (nuclear receptor subfamily 4, group A, member 3), p=0.000046728
i
rs180053446
AA
GG
Genotype
Gene Tsc2 (TSC complex subunit 2), p=0.000850667
j
rs180034118
AA
TA
TT
Genotype
Gene mecp2 (methyl CpG binding protein 2), p=0.0011532
k
rs180080888
GG
GT
TT
Genotype
Gene NPY (neuropeptide Y), p=0.00003663

Figure 2.4 Illustrative examples of SNPs associated with the General Inspection component: (**a**) D2b; (**b**) GnRH2; (**c**) 5HTR1aa; (**d**) 5HTR2d1; (**e**) 5HTR3a; (**f**) Nlgn2a; (**g**) Nlgn2b; (**h**) Nr4a3; (**i**) Tsc2; (**j**) MECP2; (**k**) NPY. Individuals of the different lines are represented by different colours according to color code indicated in the figure legend.

General Inspection presented a main effect for both sex and strain, but not for the interaction between them (sex: $F_{1, 163} = 6.70$, $p < 0.05$; line: $F_{5, 163} = 11.04$, $p < 0.001$; sex*line: $F_{5, 163} = 1.22$, $p = 0.304$), with females having significantly higher scores than males, and LEO having the best performance which was significantly higher than that of WIK, 5D, and AB, with 5D being also significantly higher than AB (Fig. 2.5 a).

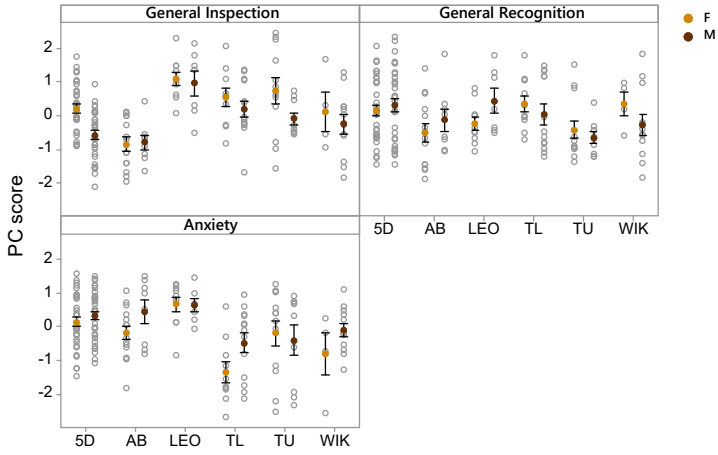

Figure 2.5 Comparison of General inspection, General Recognition and Anxiety across strains and sexes (orange=females; brown = males). Individual scores ranged between 0 and 1, with 0.5 indicating no preference between stimuli at chance levels (dotted line). Bars indicate 95% Confidence Intervals with Bonferroni correction and means that do not share a letter are significantly different [$P < 0.05$].

General recognition only presented a significant main effect for strain (sex: $F_{1, 163} = 0.01$, $p = 0.994$; line: $F_{5, 163} = 2.64$, $p < 0.05$; sex*line: $F_{5, 163} = 1.04$, $p = 0.396$), with 5D having significantly higher performance than TU, and all other strains having intermediate, and not statistically different, performances.

Anxiety presented a main effect for both sex and strain, but not for the interaction between them (sex: $F_{1, 163} = 4.75$, $p < 0.05$; strain: $F_{5, 163} = 8.32$, $p < 0.001$; sex*strain: $F_{5, 163} = 1.21$, $p = 0.396$), with males having significantly higher scores than females, and among strains Leo having the highest score which was significantly higher than that of TU, Wik, and TL, with the latter (TL) being significantly lower than 5D and AB

2.8 Discussion

In this study we have characterized the phenotypic architecture of sociality in zebrafish. We have behaviourally phenotyped males and females of six different wild type laboratory strains in 4 behavioural tests (social tendency, social and object discrimination and open-field) and showed that social tendency (i.e. preference to associate with conspecifics) and the ability to discriminate between conspecifics (social recognition) is present in both sexes of all strains tested. A factorial analysis identified three main behavioural modules: (1) general inspection, which includes social tendency measured in the social preference test and social and object exploration, measured in the social and object discrimination tests, respectively; (2) general recognition, which includes social and object discrimination, measured in the social and object discrimination tests, respectively; and (3) anxiety, which include the behavioural measures of thigmotaxis and edge-orienting taken in the open-field test. Therefore, the motivational (social tendency)

and cognitive (social recognition) aspects of sociality are not phenotypically correlated, a result that does not support the occurrence of a sociality syndrome, which could be predicted by shared selective pressures on these two traits for the evolution of sociality. Moreover, the fact that both social tendency and social recognition are phenotypically correlated with similar non-social behaviours (i.e. object exploration and object recognition, respectively), integrating two general-domain behavioural modules (general inspection and general recognition), supports the hypothesis that these behaviours are not domain specific and have been evolutionarily co-opted from general-domain motivational and cognitive traits. These results are in agreement with a recent study showing that in zebrafish both social recognition and object recognition, but not social tendency, are oxytocin-dependent, hence suggesting a common proximate mechanism indicative of a general-domain cognitive trait (Ribeiro et al., 2020). Finally, it is worth noting that even though sociality has been proposed to be promoted by predator pressure as a defensive mechanism (Groenewoud, 2016), anxiety forms an independent behavioural module from those where social traits are included.

Even with the motivational and the cognitive components of sociality being part of two different behavioural modules, a shared selective pressure on both for the enhancement of social competence could result in a physiological linkage between the two behavioural modules; for example, due to the evolution of a common neuromodulator that phenotypically integrates the independent neural mechanisms underlying general inspection and general recognition. In fact, even though that social affiliation and social memory have been shown to rely on separate neural circuitry, some neuromodulators, such as oxytocin have been shown to regulate both mechanisms [e.g. (Fergunson et al., 2001, Resendez et al., 2020)] opening the possibility for the evolution of

physiological constraints that phenotypically link these two domains. We tested the constraint hypothesis, which predicts traits to be correlated across populations irrespective of ecological conditions (Bell, 2005; Dochtermann and Dingemanse, 2013), in our data set by comparing the matrices of phenotypic correlations among the three behavioural modules extracted from the factor analysis across the six wild type strains used in this study. Given that these wild type laboratory strains have been established independently from different founders collected in the wild and have been evolving independently from each other in similar stochastic laboratory environments, they can be seen as independent representative populations of this species (despite living in artificial environments). Contrary to the prediction of the constraint hypothesis, the phenotypic correlation matrices were not similar between any pair of zebrafish laboratory strains studied. In fact, there was only one significant QAP correlation between the 5D and WIK matrices, but it was a negative correlation suggesting an asymmetric structure of the matrices. Therefore, our data supports the alternative adaptive hypothesis, that proposes that positive correlations between traits are the result of historical selection favouring particular trait combinations (i.e. selection-induced linkage disequilibrium, Saltz et al., 2017; Royauté et al., 2020), such that the evolution of different combinations between the different behavioural modules is not physiologically or genetically linked, and hence divergence of the correlation matrices between populations is unconstrained.

The study of the association between a set of genetic polymorphisms (SNPs), in candidate genes that have been implicated in social behaviour in vertebrates (“social genes”), and the behavioural modules that emerged from our factor analysis indicates that only the general inspection (motivational) module is associated with SNPs in the “social genes”, further supporting the lack of genetic linkage between

this module and the general recognition (cognitive) module. Thus, the "social genes" studied here seem to be associated with a general domain motivational component of social behaviour, rather than with a general domain cognitive component, which probably relies on memory related genes not included in our "social genes" list. Moreover, our results also indicate a low overlap in the genetic polymorphisms association (3 out of 29 SNPs) between the general inspection and the anxiety modules, which suggests that despite these two behaviorual modules relying on motivational mechanisms they have significantly different genetic architectures.

Interestingly, all except one of the genetic polymorphisms (5HTR 2cl2) associated with the three behaviours that loaded to the general inspection behavioural module, are also associated with this behavioural module indicating an agreement between phenotypic (i.e. behavioural correlations) and the genetic (i.e. genetic polymorphisms) data supporting the occurrence of this behavioural module. The genetic polymorphisms associated with these behaviours include neurotransmitter and neuromodulator systems known to modulate motivational states, such as serotonergic (social tendency is associated with, 5HTR1aa, 5HTR3a and social exploration with 5HTR-1aa, 5HTR-2cl1) and neuropeptidergic pathways (social exploration is associated with GnRH2 and NPY), as well as genes involved in synaptic plasticity, such as the neuroligin/neurexin system (social tendency is associated with *Nrxn1b*, *Nlgn2b*, and *Nlgn4xa*, and social exploration with *Nlgn1*, *Nlgn2a*, and *Nrxn2a*) and epigenetic marking (social exploration is associated with the methyl CpG binding protein 2).

On the other hand, the genetic polymorphisms associated with object exploration include less "social genes" (only 3), which are restricted to the serotonergic and dopaminergic neurotransmitter pathways (5HTR-2cl1, 5HTR-2cl2, D2b). Thus, even within a behavioural module it is

possible to observe a significant partitioning of the genetic associations with the different component traits of that module. This conclusion is further supported by the fact that there are only 2 SNPs, in the same gene (5HTR-1aa) that are associated both with social tendency and social exploration and only another SNP in one gene (*5HTR-2cl1*) associated both with social and object exploration.

The SNPs associated with the General Inspection behavioural module are distributed across 20 of the 25 chromosomes that constitute the zebrafish genome, being absent only from chromosomes 11, 12, 19, 21 and 23. However, one can find SNPs associated with behaviours that load to the general inspection module in chromosomes that do not contain SNPs associated with the behavioural module itself (e.g. SNPs associated with social exploration in chromosome 11,19 and 21, and the SNPs associated with social tendency and with object exploration in chromosome 21). In a previous study that aimed to identify quantitative trait loci (QTL) in zebrafish for behavioural and morphological traits, QTLs for social tendency have been identified when using one of the two statistical methods used (genetic algorithm mapping vs. interval mapping) in chromosomes 18 and 24 (Wright et al., 2006). In our study, variation in social tendency is associated with SNPs located in chromosomes 1(#2), 8, 10, 13 and 21. However, the General Inspection module, where social tendency is included, has associated SNPs on chromosomes 18 and 24. Hence, this mismatch between the QTL results and our results presented here can be due either to a false detection of these QTLs by the genetic algorithm mapping method, given the lack of support from the interval mapping method in the previous study, which led the authors not to claim these QTLs themselves (Wright et al., 2006); or to an indirect association through the link between social tendency and the general inspection module. Either way, our results show that the SNPs associated with both the general inspection module

and the behaviours that constitute this module are widespread across the genome, supporting a many genes (each with small effects) genetic architecture for these traits.

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Supplementary Material

Table S1.

One-Sample T:

Test of μ = 0.5 vs > 0.5

Variable	N	Mean	StDev	SEMean	95%LB	T	P
Social tendency_5D-F	33	0.925	0.103	0.0179	0.8948	23.70	0.000
Social discrimination_5D	33	0.697	0.139	0.0242	0.6557	8.13	0.000
Object discrimination_5D	33	0.719	0.158	0.0275	0.6725	7.96	0.000
Social tendency_5D-M	32	0.736	0.192	0.0340	0.6786	6.95	0.000
Social discrimination_5D	32	0.728	0.156	0.0276	0.6812	8.26	0.000
Object discrimination_5D	32	0.777	0.154	0.0272	0.7311	10.18	0.000
Social tendency_AB-F	14	0.726	0.174	0.0475	0.6435	4.86	0.000
Social discrimination_AB	14	0.646	0.114	0.0304	0.5925	4.81	0.000
Object discrimination_AB	14	0.691	0.128	0.0343	0.6306	5.58	0.000
Social tendency_AB-M	8	0.750	0.122	0.0432	0.6676	5.77	0.000
Social discrimination_AB	8	0.708	0.124	0.0440	0.6248	4.73	0.001
Object discrimination_AB	8	0.698	0.155	0.0549	0.5941	3.61	0.004
Social tendency_LEO-F	10	0.954	0.060	0.0191	0.9185	23.73	0.000
Social discrimination_LE	10	0.669	0.077	0.0243	0.6241	6.93	0.000
Object discrimination_LE	10	0.666	0.108	0.0340	0.6031	4.86	0.000
Social tendency_LEO-M	7	0.960	0.029	0.0109	0.9390	42.09	0.000
Social discrimination_LE	7	0.773	0.158	0.0598	0.6568	4.56	0.002
Object discrimination_LE	7	0.697	0.124	0.0470	0.6054	4.19	0.003
Social tendency_TL-F	10	0.975	0.024	0.0074	0.9616	63.90	0.000
Social discrimination_TL	10	0.722	0.130	0.0410	0.6470	5.42	0.000
Object discrimination_TL	10	0.710	0.149	0.0472	0.6234	4.45	0.001
Social tendency_TL-M	13	0.913	0.116	0.0322	0.8558	12.84	0.000
Social discrimination_TL	13	0.698	0.168	0.0465	0.6152	4.26	0.001
Object discrimination_TL	13	0.677	0.155	0.0429	0.5999	4.11	0.001
Social tendency_TU-F	12	0.886	0.170	0.0490	0.7976	7.87	0.000
Social discrimination_TU	12	0.657	0.120	0.0346	0.5944	4.53	0.000
Object discrimination_TU	12	0.643	0.163	0.0469	0.5588	3.05	0.006
Social tendency_TU-M	9	0.820	0.160	0.0533	0.7205	6.00	0.000
Social discrimination_TU	9	0.600	0.053	0.0176	0.5677	5.70	0.000
Object discrimination_TU	9	0.678	0.123	0.0409	0.6022	4.36	0.001
Social tendency_WIK-F	4	0.966	0.034	0.0169	0.9262	27.59	0.000
Social discrimination_WI	4	0.693	0.105	0.0526	0.5695	3.67	0.017
Object discrimination_WI	4	0.742	0.152	0.0761	0.5629	3.18	0.025
Social tendency_WIK-M	12	0.835	0.204	0.0589	0.7290	5.68	0.000
Social discrimination_WI	12	0.647	0.150	0.0434	0.5690	3.38	0.003
Object discrimination_WI	12	0.690	0.151	0.0436	0.6120	4.37	0.001

N = Sample size
StDev = Standard Deviation
SEMEan = Standard Error of the Mean
95%LB = 95% Lower Bound
T = Statistical T test result
P = P-value

Chapter III

General Discussion

6.1 Overview of empirical findings

The principal goal of this thesis is to investigate the phenotypic architecture and genetic polymorphisms associated with social behaviour in zebrafish.

To achieve this goal we started by phenotyping two basic elements of social behaviour: (1) the motivational component that is the tendency to approach and spend time with others, called affiliation; and (2) the cognitive component or social recognition that is the ability to distinguish individuals in social groups. In order to test if sociality involves domain specific or general domain factors, a non-social cognitive ability (i.e object discrimination) was also phenotyped. Anxiety was as well considered in this study since it has been suggested that predator avoidance is a major ecological factor selecting for group living (Groenewoud et al., 2016).

Phenotypic variations for these three variables (social tendency, memory and anxiety) were tested between sex and across six different wild type zebrafish laboratory strains: Tuebingen (TU), Tupfel Long Fin (TL), Leopard (Leo), AB, WIK (Wild India Karyotype), and 5D. The inclusion of information about phenotypic variation across strains allowed us to test alternative hypothesis for the evolution of correlated traits (i.e. constraints vs. adaptive hypothesis). We also used different strains to capture as much of genetic variation as possible in zebrafish, to increase the chance of finding phenotypic variation associated with genetic polymorphisms.

For the phenotyping we used three behaviour paradigms. We explored first the social tendency that was assessed via a shoal preference paradigm, which has been widely used to measure the motivation of fish to approach conspecifics, where the time spent close

to the shoal against an empty tank is recorded and taken as a measure of preference. Findings indicate a strong social preference in all strains. It is known that shoaling is an important behaviour in zebrafish (both in the natural habitat and in laboratory, Miller & Gerlai, 2011). Zebrafish shoal early in their development and engage in this type of behaviour for most of their waking hours (Engeszer et al., 2007, Miller & Gerlai, 2011). Although specific preferences for shoaling in zebrafish seem to be learned, shoaling tends to maintain relatively stable over the lifespan (Engeszer et al., 2004, Miller & Gerlai, 2007). Studies concerning shoaling in zebrafish have been focused mainly on the choice of shoal mates (Kraus & Prichard 1999, Severino & Gerlai, 2008; Pyon, 2011; Snekser et al., 2010) and also on shoaling tendency (Wright et al., 2003). Some differences in shoaling tendency have been reported across strains. Wright et al. (2006) compared shoaling preference in laboratory and wild-caught zebrafish strains and found that wild-derived populations demonstrate stronger tendency to shoal than laboratory strains (AB and WIK). Between the laboratory strains tested they found that the AB strain has the lowest tendency to shoal, which is in accordance with our results. However, the effect of strain in social tendency is conflicting since another study demonstrated stronger social preference in AB (Barba-Escobedo & Gould, 2012).

To assess memory, we used a one trial recognition test, a common behaviour paradigm without conditioning that tests an individual's capacity to discriminate between a familiar stimulus and a novel one. This test was used to evaluate both social memory (conspecifics as stimuli) and non-social memory (objects as stimuli). In both tests, animals across strains exhibited significant discrimination between stimuli (significantly above chance level, 0.5). Zebrafish is capable of recognizing conspecifics and predators using both visual and olfactory cues (Madeira & Oliveira, 2017; Barcellos et al., 2014). Barba-

Escobedo & Gould (2012) repported strain differences in social discrimination, with AB and golden strains showing social discrimination but not the WIK strain.

Anxiety was tested in an open-field test, a widely used paradigm to investigate anxiety-related behaviour by measuring wall-hugging tendencies. Differences in anxiety level were found between strains and sex, with Leo being the most anxious strain. In another study comparing anxiety levels between different zebrafish strains, Leo together with the albino strain, also revealed the highest levels of anxiety (Egan et al., 2009). Other evidence shows that Leo presents lower level of serotonin in the brain and higher anxiety (Maximino et al., 2013).

In order to explore the phenotypic architecture of zebrafish social behavioural traits, a principal component analysis (PCA) was done using measures across tests for all strains and sex. Results indicated three major factors or principal components: (1) a general investigation motivational factor, which loads social tendency from the social preference test, social exploration from the social recognition test and object exploration from the object recognition test; (2) a general cognition factor, which loads social discrimination from the social recognition test and object discrimination from the object recognition test; and (3) an anxiety factor, which loads thigmotaxis score and proximity to the wall measures, from the open-field test. Thus, we concluded that phenotypic modules of sociality (motivational and cognitive domains) are not phenotypically linked to each other and each of them is part of a general domain behavioural module. Also, anxiety was not related to either the social or the cognitive behavioural modules, indicating its independence from the social traits, which could have been predicted from a pedration avoidance hypothesis for the evolution of sociality. Here, we also addressed the adaptive vs. constraints hypotheses for the evolution of the different components of sociality and

our results show that the phenotypic matrices of correlations for the behavioural modules extracted from factorial analysis, vary across the different populations (strains), supporting the adaptive hypothesis, and the independent evolvability of the motivational and cognitive components of sociality.

Finally, we found an association between some SNPs and some of the behaviours tested. There were 29 genes with SNPs associated with the motivational factor, 5 genes with anxiety and none with the cognitive factor. The SNPs associated with the motivational component included SNPs also associated with each of the traits it encompasses (social tendency, social exploration and object exploration), and represent genes implicated in neurotransmission (serotonin receptors, dopamine receptors), neuromodulation (oxytocin, NPY) and synaptic plasticity (neurexins, neuroligins, shank3a). Moreover, most of the SNPs associated with the motivational component are different from those associated with anxiety. In brief, there is an association between some of the candidate "social genes" and the motivational component of sociality but not with the cognitive component of sociality further supporting their independence.

6.2 Specific aim 1: Sociality syndrome hypothesis

Animals usually present consistent variation in behaviour that can be observed over time and across different contexts (Sih et al., 2004). In humans, this variation has long been termed personality, whereas in other animals it was initially seen as noise in the dataset (Schradin, 2013). Nevertheless, the study of personality-like behaviour in animals is not new since this phenomenon has been explored in some primates, domesticated animals, and laboratory rodents (Sih et al., 2004). In the last decades, there has been a growing interest in investigating

behavioural syndromes and the closely related concepts of personality, temperament and coping styles in several organisms using an evolutionary and ecological perspective (Bergmüller & Taborsky, 2010; Koolhaas et al., 1999; Réale et al., 2000).

The concept of behavioural syndrome was defined by Sih et al. (2004) as suites of behavioural traits that are correlated across contexts and situations. This approach allows correlating behaviours that have historically been studied separately. A common example of a behavioural syndrome is the positive correlation between boldness and aggressiveness that has been identified in several species (Dingemanse et al., 2007; Dochtermann & Jenkins, 2007; Kortet and Hedrick, 2007). Behavioural syndromes are therefore important for the study of both inter- and intra-specific behavioural variation and have been seen as a bridge that may integrate proximate mechanisms with the ecology and evolution of behaviour (Sih & Bell, 2008). The term behavioural type is used at an individual level (individuals with a particular combination of behaviours X and Y) while the term behavioural syndrome is used at the population level (Bell, 2007). The study of behavioural syndromes is also important to understand species distribution, their response to the environment and speciation rates (Sih et al., 2004).

It has been suggested that the evolution of sociality across different animal taxa involves a sociality syndrome (Settepani et al., 2016). There is a potentially relevant behavioural syndrome that has not been explored as far as we know, which represents a correlation between social tendency and social recognition (two fundamental components of sociality). Sociality involves a set of motivational and cognitive skills that foster the reward value of interacting with others (social affiliation/social tendency) and the ability to discriminate between others (social recognition), which may have been selected together during social evolution, leading to a phenotypic correlation between

them. In this sense, we aimed to assess if there is an association between social tendency and social recognition in zebrafish, forming a sociality syndrome. However, our findings indicate that social tendency is not linked to social recognition, suggesting that they have not been selected together.

Accordind to Ward & Webster (2016) despite that social recognition constitutes the foundation for social organization of populations and species, the formation of animal groups usually does not require true social recognition, but rather a basic form of attraction towards conspecifics, along with habitat and resource preference. A study on chemical social recognition in wild populations of three-spined sticklebacks (*Gasterosteus aculeatus*) shows that these fishes, which usually associate with conspecifics from the same habitat as themselves, can change and update their preference according to environmental changes of different locations. This suggests that these fishes use self-reference (i.e. own habitat and diet experience) to mediate their choices rather than social recognition based on previous social interactions (Ward et al., 2007). Moreover, despite shoaling in zebrafish being a spontaneous behaviour that appears early in development, specific shoaling preferences develop much later (Engeszer et al. 2004, Engeszer et al. 2007). These evidences further suggests that social tendency and social recognition may not have been selected together, which explains the independence of these two traits in our study. This also suggests that social recognition may not be always at the basis of group formation but is fundamental for group organization. Thus, social experience and social environment seem to play an important role in shaping an individual's memory ability (Moretz at al. 2007). For instance, a study in which two different species of bees were used (social and solitary species), revealed that the social species had better learning and memory skills than the solitary one in a non-

social task (Dukas & Réal, 1991). One possible explanation is that social bees live in colonies with labor division and task specialization, leading to greater performance in the task in which they specialize, while solitary bees have to balance this activity with others activities, leading to poorer performance (Dukas & Réal, 1991). It makes sense that in species in which cooperation/task specialization is present (e.g. cooperative breeders) there is better discrimination (memory) than in non-cooperative species, as is the case of zebrafish.

6.3 Specific aim 2: Is sociality a domain-specific or a general domain trait?

Social interactions impose cognitive demands that are so unique requiring rapid identification of social stimuli such as recognition of others and their disposition (emotional states), vast capacity of memory integration, anticipation and evaluation of others' behaviours (Adolphs, 2009). Accordingly, it has been suggested that cognitive processes and brain mechanisms that subserve social behaviours are domain-specific (Spunt & Adolphs, 2017). In this sense, the term social cognition has been used to refer to cognitive processes that are involved in social behaviour (Zuberbuhler & Byrne, 2006). Social specific domain theory has been actively debated in social neuroscience and has been used to explain many cognitive phenomena. Today, the degree of this apparent specialization, how it evolved and what is it role is still remains an important, and intricate question (Spunt & Adolphs, 2017). At present, the concept of a social specific domain has received support from brain imaging and lesion studies in humans, and there is also evidence of sophisticated brain specialization for social information processing in rodents (Bielsky I et al., 2004, Wersinger et al., 2004, Okuyama et al.,

2016, Raam et al. 2017). However, it is not clear to what extent social cognition is domains specific or domain general. Recent studies reveal that the amygdala that has been consistently associated to social behaviour regulation and recognition of emotional facial expressions has a more abstract role that is not specific to the social domain (Gothard, 2020). Likewise, the fusiform face area in the human brain that is considered as a specialized structure for face recognition, is also activated by non-face objects provided that individuals acquire substantial expertise with this objects (Gauthier et al., 2000).

In order to have insights on this question, we proposed to examine if social and non-social cognitive abilities (i.e. social vs. object recognition) are independent from each other, or if they co-vary, hence supporting a general domain factor. Our results show that the motivational and cognitive domains of sociality are not linked to each other, and neither seem to be specific to social traits, since both show phenotypic correlations with non-social traits serving similar function for non-social objects, hence supporting a domain general hypothesis for social traits. These results are consistent with a recent study with zebrafish, that showed that both social recognition and object recognition, but not social tendency, were oxytocin-dependent (Ribeiro et al., 2020). Furthermore, recent studies have highlighted the multidimensional response properties of neurons that can operate in multiple process in a stimuli or context-dependent way, being able to contribute to a large number of cognitive functions (e.g. social and non-social related; Gothard, 2020, Nieuwenhuys & Puelles, 2016; see also Pessoa et al., 2019). Oxytocin for example, has been shown to regulate both affiliative and cognitive mechanisms allowing the evolution of physiological constraints that phenotypically link these two domains. This type of organization may allow vertebrates to have high behavioural

flexibility, necessary to cope with the complex and changing social environment involving many agents (Pessoa et al., 2019).

6.4 Specific aim 3: Is there an association between social tendency and anxiety?

Animals's survival largely depends on the expression of appropriate behaviour and physiological responses to threats. Anxiety can, to a certain extent, prepare the individual to detect and deal with threats, but high levels of anxiety can be maladaptive (Marks & Nesse, 1994; Bateson et al., 2011). A study with rats shows that moderate stress can enhance social support-seeking behaviour and positive behaviour towards conspecifics, while high level stress tends to anulate the positive behavioural effects of moderate stress (Muroy et al., 2016). Another study demonstrated that under stressful conditions, that triggered the release of oxytocin, rodents tended to approach and maintain closeness with familiar conspecifics (Olff et al., 2013). There are also evidences that high or chronic levels of stress may disrupt the development of the brain, while acute activation of the body's stress response can be adaptive, increasing survival chances (Muroy et al., 2016).

Predator avoidance has been considered a main selective force for group living (Groenewoud et al., 2016). Nevertheless, social interactions can also present costs, being itself a source of anxiety mainly in the context of conflict and competition with conspecifics (Majolo & Huang, 2017). Thus, avoiding others, as well as approaching others are two basic behavioural processes, present in all species being fundamental for their survival (Oliveira, 2013). In this way, these two behavioural processes have been suggested to be related and, in this work, we

checked if there is an association between social tendency and anxiety in zebrafish.

Zebrafish is considered a good model to study both acute and chronic anxiety, being sensitive to a range of environmental challenges such as, novelty stress, alarm substance, exposure to predator, as well as anxiogenic and withdrawal drugs (Jonathan et al., 2011). In zebrafish, the presence of conspecifics also acts as a social buffer against threats (Faustino et al., 2017). Other researchs have reported correlations between boldness and anxiety (Hamilton et al., 2021, Hope et al., 2020) but the occurrence of an assotiation between social tendency and anxiety is not clear (Muroy et al., 2016).

Our results show that anxiety is discrete from the motivational and cognitive behavioural modules, indicating independence for the evolution of social traits from anxiety, which have been predicted from a predation avoidance hypothesis for the evolution of sociality.

A recent study reported that moderate early life stress enhances zebrafish working memory, however it did not influence social and anxiety responses (Fontana et al., 2019). Similarly, predictable chronic stress in rats increased resilience by improving memory (Parihar et al., 2011). However, unpredictable chronic stress in juvenile and adult rats has the opposite effects, promoting resilience in the former and vulnerability in the latter (Ricon et al., 2012). Interpretation of anxiety behaviour reveals a complex pattern that can be influenced by imprinting, epigenetics, type of anxiety and by the behavioural test used.

6.5 Specific aim 4: Adaptive vs. constraint hypotheses

The acquisition of similar phenotypes in similar environments has been suggested as evidence that natural selection has produced evolutionary

adaptation. On the other hand, the expression of similar phenotypes in different environments by the same genetic or developmental pathway, has been pointed out as an indicator of constraints on adaptation (Losos, 2011). Accordingly, the evolution of correlated traits can be explained by two alternative hypotheses: (1) the Adaptive Hypothesis, which predicts correlations between traits to vary between populations depending on local conditions; and (2) the Constraint Hypothesis, which predicts traits to be correlated across populations irrespective of ecological conditions. We tested these two hypotheses for the evolution of different components of sociality. We used six different strains (as a repeated evolution event) that evolved independently from each other, but in similar conditions since they are all laboratory strains (domesticated populations). We tested if the phenotypic correlations matrices vary across strains or are similar. The results show that the patterns of correlations between traits vary across different populations (strains), supporting the adaptive hypothesis, and the independence in the evolvability of the motivational and cognitive components of sociality.

6.6 Specific aim 5: Genetic basis of phenotypic correlations

As genomic information on social animals becomes more available, there are growing opportunities to examine the genetic basis of social behaviour (Thompson & Richards, 2016).

Here we investigate the occurrence of associations between candidate SNPs and the different social behaviours tested (social tendency, social and non-social memory and anxiety) to characterize the genetic basis of the observed phenotypic correlations. The results demonstrate associations between some of the candidate genes (via their SNPs) and the behaviours tested. From 132 candidate genes, we found 29 genes associated with the motivational factor, 5 genes with

anxiety and none with the cognitive component. The fact that we did not find any genes associated with the cognitive factor can be explained by the fact that our list of candidate genes was based on previous evidence of their involvement in social behaviours and not directly in cognition. This specific association of candidate "social" genes with the motivational but not with the cognitive component is in agreement with the results at the phenotypical level and further supports their independence.

The SNPs associated with the motivational component included SNPs also associated with each of the traits it encompasses (social tendency, social exploration and object exploration), and some of these SNPs are present in more than one trait from the principal component. These SNPs map into genes involved in neurotransmitter and neuromodulator pathways, and in neural plasticity. Here we will focus on the most representative, namely serotonin receptors, dopamine receptors, oxytocin, neurexins, neuroligins and *shank3a*.

Many serotonin receptors (5HTR-1aa, 5HTR-3a, 5HTR-3b, 5HTR-7b, 5HTR-7c, 5HTR-2cl1) are associated with the motivational component. It is well recognized that serotonin plays a central role in shaping social responses and the serotonergic pathway is also recognized by its highly response to the social stimuli (Kiser et al., 2012). Serotonin system affects therefore the sensitivity to social stimuli and promotes as well the flexibility to the adaptation to social environment condition (Kiser et al., 2012). This could explain why this key neurotransmitter is implicated in the motivational component of social behaviour. Dopamine (DA) and dopaminergic receptors are equaly recognized for their role in social interactions and they are specially involved in motivation, reward and hedonic states (Liu et al., 2017).

The nonapeptide Oxytocin, the gene *shank3a* (a synaptic

scaffolding protein), as well as genes encoding neuroligins (cell adhesion molecules at synapses) have been implicated in the ASD (Autism Spectrum Disorder) phenotype (Andari et al., 2010; Gauthier et al., 2009). A prominent characteristic of ASD is a functional deficit in social behaviour, including disinterest, aloofness or unexpected forms of communication (Anisman et al., 2018). Difficulties in changing routines or adjusting to new situations are also common symptoms (Kapalka, 2010).

The gene *Shank3,* located in the cromossome 22q13.3 is considered one of the most promising ASD candidate genes (Peça et al., 2011). *Shank3* is a member of the *Shank* gene family, which encodes a synaptic scaffolding protein, involved in maturation of dendritic spines and induction of functional dendritic spines (Massrali et al., 2019). Mutation in this gene has been directly associated with some types of intellectual disability and mainly to ASD (Durand et al., 2007, Berkel et al., 2010). It is, therefore, very interesting that the homologue of this gene in zebrafish has been found to be implicated in the motivational component of behaviour.

Neuroligins and neurexins are synaptic cell-adhesion molecules important for coordinating synaptic connectivity in the brain (Dean & Dresbach, 2006). Mutations in the genes encoding neuroligin-3 and -4, such as mutation in genes encoding direct interaction partners of neuroligins (*neurexins* and *shank*), as well as other proteins involved in synaptic mechanisms, are also directly associated with ASD (Corthals et al., 2017). The transmembrane proteins neurexins are also implicated in regulating locomotor activity and social behaviour and is implicated in both social tendency and social exploration (Grayton & Collier, 2013), which makes sense to be among the list of our genes.

Finally, our results also reported that genes associated with anxiety are different from those associated to the motivational

component, which reinforces the results of our phenotypic correlations.

6.7 Final Remarks

Sociality is a striking phenotypic innovation that evolved independently many times (Doody et al., 2012). Thus, social behaviour is present in very different taxonomic groups, and closely related species may vary considerably in their social tendency and social organization (Ward & Webster, 2016). For instance, the most sophisticated form of social interaction (eusociality) may be present in simple societies (ex. *Lasioglossum albipes*) with few members and basic division of labor, as well as in sophisticated societies (ex. *Apis mellifera*) that contain thousands to millions of workers with highly specialized division of labour (Yan et al., 2014). Curiously, complex forms of social behaviour are not exhibited only by animals that possess well-developed brains and nervous systems, as humans, but also in invertebrates, such as the species-rich group of eusocial insects. As Shell & Rehan (2017) claim "advanced eusociality thus does not represent a necessary evolutionary eventuality for social species, but rather indicates the far extreme of fundamentally flexible social organization". Clearly, the flexibility and complexity of social organizations is not limited to primates or high vertebrates, but is widespread to many distantly related taxa. Nowadays, it is acknowledged that the basic organization of the brain is shared across vertebrates, contrasting with the triune brain's concept of MacLean that defended the gradual acquisition of layers of brain structures through evolution (MacLean, 1990, Pessoa et al., 2019). In the nineteenth century, Darwin already argued that differences between humans and other behaviour was quantitative and on a continuum rather than qualitative. However, the evolution of different combinations of

social behaviours might mean that these behaviours have different underlying mechanisms that can be combined in distinct ways (Goodson, 2013). Therefore, the study of social behaviour is very challenging and tracing both the phenotypic and genetic architecture of social behaviour becomes imperative for understanting the organization of the mechanisms underlying it. Researchers who engage in the study of social behaviour use generally an integrative approach that combine perspectives from multiple research domains and multiple species (Cacioppo & Decety, 2011). Recent advances in neuroscience provided the necessary toolkit for a better view of the mechanisms involved in social interactions (Blumstein et al., 2010).

Research on social insects has undoubtedly made a major contribution to the unification of two previously separate research sub-fields, molecular biology and social behaviour (Thompson & Richards, 2016). However, zebrafish is today a model with great potential for the study of social behaviour, presenting several strains (domestic and trasgenic) with interesting possibilities to establish genetic and evolutionary studies. Our study demonstrates that from both the phenotypic and genetic points of view, there is an independence of the basic components of social behaviour, which suggests that they have evolved separately. But, these components do not constitute a close system, since social interactions contribute to a greater social complexity when interactions take multiple forms and occur in different contexts, using a flexible combination of traits.

This study provides important insights on how phenotypic variation across modes of social organization is reflected at the molecular level, taking another step for understanding the complexity of social behaviour.

6.8 Limitations and future directions

One potential limitation of this study is that we did not use a full coverage of the genome, (i.e, we used a candidate gene approach) to study the associations between genetic polymorphisms and behavioural traits, and hence we do not have a genome wide view of the genes and pathways associated with the studied social traits. Also, our candidate gene list did not cover genes that are directly linked to cognition, decreasing the chances of finding genes linked to that component. In future studies it would be important to include in the list of candidate genes, genes related to cognition and try to increase the coverage of the SNP array.

Taking into account that anxiety implies epigenetic processes and is complex to assess, it would also be important to have additional measures of anxiety in future studies. Also the introduction of wild-caught strains in future studies may be relevant in order to answer other questions about the evolution of social behaviour.

The literature on neural architecture suggests that neurons apparently specialized in certain stimuli response (e.g. social stimuli) are likely to respond to other types of stimuli (e.g. non-social stimuli) when these become behaviourally relevant in the context of a complex task (see Putnam & Gothard, 2019). Comparing neural, phenotypic and genetic architecture data may provide further insights into the social specific vs. domain general hypotheses, as well as into the complexity and flexibility of social behaviour.

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3

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Phenotypic Architecture and Genetic Polymorphims Associated with Social Behaviour in Zebrafish

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