Social Preference in Juvenile Zebrafish

Hande Tunbak

A thesis presented in partial fulfilment of the degree of

Doctor of Philosophy

at the **University College London**

27th March 2022

Department of Wolfson Institute for Biomedical Research

University College London

For my parents,

and the ones I love

Declaration

I, *Hande Tunbak*, confirm that the work presented in this thesis is my own. I confirm that where information has been derived from other sources has been indicated in the work.

Abstract

Social behaviours are essential for the survival and reproduction of many species, including our own. A fundamental feature of all social behaviour is social preference, which is an individual's propensity to interact with members of their species (termed conspecifics). In an average population, various social preference behaviours are readily observed, ranging from uninterested (not engaging with conspecifics) to very social (engaging with conspecifics). Individuals expressing these behaviours are typically labelled as having an asocial or prosocial, respectively. Little is known about how the underlying social circuitry gives rise to such distinct social behaviours in the population.

It is well established that adverse social experiences can impact social behaviour, including isolation during early development. Undesired social isolation (loneliness) alters behavioural patterns, neuroanatomy (e.g., brain volume) and neurochemistry in ways that resemble developmental neuropsychiatric disorders, including autism and schizophrenia. However, few studies have investigated the impact of early life isolation on social circuitry, and how this results in dysfunctional social behaviour commonly associated with these and other disorders.

In this thesis, juvenile zebrafish was used to model social preference behaviour, as it is an excellent translational model for human developmental and behavioural disorders. Population-level analysis revealed that several features of social preference behaviour could be summarised via Visual Preference Index (VPI) scores representing sociality. Using multiple behavioural parameters, comprehensive investigations of asocial and prosocial fish identified via VPIs revealed distinct responses towards conspecifics between the two phenotypes. These initial results served as a baseline for facilitating the identification of atypical social behaviour following periods of social isolation.

The impact of isolation on social preference was assessed by applying either the full isolation over the initial three weeks of development or partial isolation, 48 hours or 24 hours, before testing. Following periods of social isolation, juvenile zebrafish displayed anxiety-like behaviours. Furthermore, full and partial isolation of 48 hours, but not 24 hours, altered responses to conspecifics.

To assess the impact of social isolation on the social circuitry, the brain activities of fish were analysed and compared between different rearing conditions using high-resolution two-photon imaging. Whole-brain functional maps of isolated social phenotypes were distinct from those in the average population. Isolation-induced activity changes were found mainly in brain regions

linked to social behaviour, social cue processing, and anxiety/stress (e.g., the caudal hypothalamus and preoptic area).

Since some of these affected regions are modulated by serotonin, the reversibility of the adverse effects of social isolation on preference behaviour was investigated by using pharmacological manipulation of the monoaminergic system. The administration of an anxiolytic the drug buspirone demonstrated that altered social preference behaviour in isolated fish could be rescued by acutely reducing serotonin levels.

By investigating social preference at the behavioural and functional level in wild-type juvenile zebrafish, this work contributes to our understanding of how the social brain circuity produces diverse social preferences. Furthermore, it provides important information on how early-life environmental adversity gives rise to atypical social behaviour and the neurotransmitters modulating the circuit, offering new opportunities for effective intervention.

Impact Statement

Humans are inherently social beings, with social interactions and relationships playing essential roles in healthy development and function. However, a diverse spectrum of social preferences exists in the average population. The two extremes of this spectrum, asocial and prosocial phenotypes, represent individuals with low and high social drive, respectively. Little is known about how the social brain circuitry gives rise to individual preferences fundamental to all social behaviours. Furthermore, few studies have investigated how undesired social isolation (loneliness), particularly in early life, results in dysfunctional social behaviour commonly associated with several developmental neuropsychiatric disorders that affect millions, including autism and schizophrenia[3,4]. The recent imposition of social restrictions on a global scale due to the coronavirus COVID-19 pandemic has led to a substantial rise in mental health issues and loneliness, which is predicted to have negative consequences for many years to come. Therefore, the urgency to find effective strategies to tackle the adverse effects of social isolation has never been so high. One way to address these problems is to use social animal models to study the impact of social isolation. Within these, juvenile zebrafish are particularly suited to this purpose because they are social, have tiny optically accessible brains, and are amenable to extensive pharmacological screenings.

The pipeline developed and described in this thesis has extracted several behavioural parameters to characterise social preference behaviour. The resulting collection of scripts, functions and code has been made publicly available through publication (https://elifesciences.org/articles/55863) and online via an open-source repository (GitHub). Additional code used to prepare the figures in this thesis can be found (HandeTunbak/Social_Behaviour_Extended_Analysis (github.com)), which could be used by zebrafish research and social behaviour research communities.

Several existing protocols were enhanced to map the social brain circuitry and assess the impact of isolation; these included the improvement of existing in-*situ* hybridisation protocols to facilitate probe penetration, prevent signal saturation for precise image comparisons, and streamline the registration process to increase throughput. Subsequently, peers have requested these improved protocols at UCL and other institutes globally to be utilised in their research, thus prompting the initiation of a detailed methodology to be submitted to Bio-protocol.

Applying the abovementioned methods to the range of experimental datasets presented in this thesis revealed insights not possible with previous approaches and other animal models. For instance, isolated asocial fish were found to have entirely different functional brain patterns when

exposed to conspecifics than naturally occurring asocial fish in the average population. It is demonstrated for the first time that the functional changes caused by social deprivation are consistent with an increase in an anxiety-like state resulting in hyper-sensitisation to social stimuli, similar to the effects of isolation in humans. These findings highlight that the asocial phenotypes arising from isolation is adverse and provides crucial information on how isolation leads to impaired social preference.

Furthermore, the impact of social isolation on behaviour was found to be diminished via the acute reduction in serotonin levels through the administration of an anxiolytic drug, buspirone. Given the success in rescuing social preference behaviour in isolated fish through manipulation of the monoaminergic system, this result further demonstrates the potential of the use of juvenile zebrafish as a new model system for studying the impact of isolation on brain function, allowing the exploration of different strategies for reducing or even reversing its adverse effects.

The current findings, published in *eLife* (**see Appendix**), provide a glimpse into how prolonged periods of social isolation could impact our behaviour. It predicts that we could feel anxious upon returning to everyday life, which is already observed with lockdown lifting. Moreover, it suggests that strategies targeting anxiety may be vital in overcoming the difficulties we face returning to our regular social lives post-Covid. Since the experiments conducted in this thesis are carried out in juvenile zebrafish, these results may also serve as a foundation in which the impact of lockdown/isolation can be assessed on the young brain and additionally used to predict the implications in children in years to come.

Ultimately, the work described herein establishes a generalised framework, comprised of a battery of behavioural and functional analysis, for uncovering the mechanisms linking social isolation to social preferences and is poised to facilitate the discovery of new therapies and novel strategies in tackling the adverse effects of social isolation.

Acknowledgements

This work is the result of the efforts of many people. I may have had a significant role in it as this is my PhD, but none of this would have been possible without my family's belief in who I would become, my supervisors' scientific vision, or my friends' wisdom, both in and outside the lab. For that, I am eternally grateful.

First and foremost, I must thank Dr Elena Dreosti. You saw potential in me to become a PhD student; it has always been an honour to know I am your first PhD student; I hope I have made you proud. Thank you for taking me under your wing and allowing me to work with you. With your guidance, the world of zebrafish behaviour studies has been opened to me to explore, and you have given me all the tools to succeed. Your work ethic, tenacity and approach to scientific research is inspirational. While this opportunity has been a great learning curve, you have always gone out of your way to make things easier; I cannot thank you enough.

To Adam Dr R. Kampff, I would like to express my deepest gratitude for the scientific discussions and introducing me to coding. Without your input and support, this PhD would have lasted much longer than four years, and I would have been stuck in the many traps of zebrafish behaviour. Your advice in coding has helped me realise a further interest I am keen to follow in future. I can only hope that my scripts will be as elegantly and methodically written as yours as I follow your footsteps into becoming a coding Jedi.

I want to thank Carole Wilson; you have been a great support during my time, encouraging me to push on and look at things in another light. I am genuinely thankful for everything you have done to help me, including words of advice that still ring true to this day.

To the fish staff, Heather Callaway, Jenna Hakkesteeg, Karen Dunford, Paul Barwood, Joe Warmsley, Elise Hitchcock, for your around the clock care of my fish, you have all been exceptional in producing a mass effort to ensure my animals are consistent in size for such critical experiments on my end. I am sure I was a nuisance from day one and can only promise to continue asking you a million questions on fish welfare, just because I know you are the right people to ask, and you will never lead me astray, thank you so much!

I want to thank Steve Wilson; your advice has greatly benefited me, keeping me on track and producing high-quality research. You also deserve thanks for bringing together a community of bright individuals who share a passion for zebrafish research and support one another, helping each other grow, from which I have greatly benefitted.

This brings me to the next group of individuals to thank. I could not have completed my PhD without the fantastic team on the first floor of the UCL Anatomy Building. Each of you has played a massive part in my PhD, so it's only fair to mention you here. Even though I have bothered everyone at some point with a bombardment of my trivial questions, a specific few have suffered the most throughout my time. I want to thank those individuals by name: Ana Faro, Chintan Trivedi, Declan Lyons, and Gareth Powell.

There are a few names to bring up in my acknowledgements that were not present when I submitted my thesis, but each has a reason to be named. Renato Gomes Da Silva Martino, Ingrid Lekk, Joanna Lau Yen Na, Lisa Tucker, Niccolo Fioritti, and Pedro Henriques, for your support, advice and company have made being the only lab member in the Dreosti lab for the first three years a pleasant experience. Without you all, I would have felt more isolated than my socially deprived fish, and for this, you are greatly missed. I wish you all the best of luck and sincerely hope our paths will cross again soon!

So, to thank the students who have been there with me throughout my time working through my PhD. Through you all, I have learned how to teach and pass on my knowledge, and I can only hope your experience in the lab working with me has been thought-provoking and pleasurable and much as I have found it to be. I have learnt a lot and found the experience fulfilling with every one of you! Therefore, I would like to mention and thank, in no other order but chronological: Mireya Vazques-Prada, Hoda Abid, Eline Balavoine, Szilvia Kiraly, Wen Zeng, Franz Schmidt, and of course, the students from In2Science.

Prof Jason Rihel, you are due a specific mention due to your general wisdom and advice on approaching scientific research. I have learned so much from you, which has helped me progress in my scientific career with your guidance. Thank you for allowing me to work alongside your lab on the 2021 life paper. I am excited to see what you and your team produce in the upcoming years, as I am sure the quality of the research will be second to none.

To Elisa Clemente, I would like to thank you for being there with me right to the end of my PhD, keeping me focused, and being a positive inspiration during my writing. I regret that I only got to fully know you towards the end of my time in the Dreosti lab. I wish you the very best of luck completing your PhD and will see you on the other side.

I want to mention James J. Cox. I was able to turn to you for help when I never had the support from a thesis committee; you were every bit as good, more so even that there was a personal touch to your advice. Your help in the last few months made a tremendous difference to my approach to all aspects of my PhD, and for that, I am ever so grateful.

And of course, then there is the surprise of my life, my fiancé Stephen Wood. I am delighted that fate brought us together not long before I embarked on this journey, and now I can't even imagine a life without you. You were there from the beginning, always cheering me on. I sincerely cannot thank you enough for being there for me every step of the way of this PhD, including all the times I dragged you into work and your help and support with this thesis. I would have never taken half of the achievements I have succeeded in if it wasn't for you always believing me and pushing me. I hope that I have done you proud and am excited to live the remainder of our lives together. I know the words 'I love you can often be overused, so although I feel this way, I want to try another three instead: 'You complete me!'

Of course, I cannot forget to thank Moira and Douglas Wood. You both have been incredibly supportive through everything, opening your home and allowing me to take over the living room entirely when I came to visit just so that I can work in a quiet atmosphere and be on hand for help and advice wherever needed; you did all this without me ever having to ask. You won't know how much this has helped, but I can assure you it truly has, and I cannot thank you enough for this.

And finally, I saved a few lines for my family. All of this is for you, as you are responsible for making the pillars that support who I am today. You have all made sacrifices to help me, from staying up late, discussing ideas and statistics, to accompanying me to the lab in the dead of night to check up on fish and experiments. I sincerely hope that I have done you proud! Erol, Selma, Hale and Hakkihan Tunbak, I love you all very much! **Iyi ki varsınız!**

Contents

List of Figures

List of Tables

List of publications

First author publication

Tunbak, H, et al. 2020. "Whole-Brain Mapping of Socially Isolated Zebrafish Reveals That Lonely Fish Are Not Loners." *eLife* 9. https://elifesciences.org/articles/55863 – *see appendix for PDF.*

Named author

Kroll, F., Powell G., T. Ghosh, M., Antinucci, P., Hearn, T. J., http://orcid.org/0000-0003-3180-1401 **Tunbak H**., et al. 2020. "A Simple and Effective F0 Knockout Method for Rapid Screening of Behaviour and Other Complex Phenotypes." *eLife* 10. https://elifesciences.org/articles/59683.

Glossary

The following table describes the essential terminology used throughout the thesis.

Term	Definition
Asocial side	The area of the behavioural chamber where there are no conspecifics.
Acclimation phase	The fifteen minutes preceding the introduction of social cues are used to habituate fish to the environment.
Asocial (S-) fish	Fish with low/no social drive, VPI ≤ -0.5.
Swim bouts	A short period of intense swimming is used by juvenile fish to move around.
Conspecifics	An individual of the same species.
Inclusive fitness theory	Two components summarise the total fitness: (1) the direct fitness derived from reproduction, and (2) the indirect fitness that depends upon social interactions with relatives—also referred to as kin selection[3].
Kin	Genetically related individuals.
No Social Cue (NSC) fish	Juvenile zebrafish tested without conspecifics during the socialisation phase to control for prolonged exposure to the assay.
No-Social Preference (NSP) fish	Fish with intermediate social drive, -0.5 <VPI < 0.5.
Prosocial (S+) fish	Fish with strong social drive, VPI ≥ 0.5.
Schooling	Type of shoaling where fish swim and turn in an organised and synchronised manner.
Shoaling	Group of fish swimming together.
Social preference	The definition depends on the field of study. In this thesis, it is defined as the propensity to be near conspecifics.
Socialisation phase	The second fifteen-minute period of the assay. Typically involves the presentation of conspecifics except with NSC fish.
Sociality	The degree to which animals tend to associate in social groups.
Visual Preference Index (VPI)	A calculated value indicates the degree of social preference of a tested fish. Values range from -1 (asocial fish) to 1 (prosocial fish).
Social Behaviour Network	Set of brain structures thought to be involved in social behaviours.
Social side	The half of the assay chamber where conspecifics are located.

Chapter 1: General Introduction

What is sociality?

The definition of sociality is primarily understood as the inclination of organisms to aggregate, creating groups in which they live together and display reciprocal, cooperative behaviour[4]. Social groups can be categorised into two types in humans based on the strength of relationships observed and the duration of these interactions[5]. Of the two kinds of social groups, the one in which cohesiveness is displayed the greatest is the 'primary group', typically formed by kin (relatives), close friends and neighbours[6]. Such groups tend to be small and long-term. Secondary groups, therefore, include all other person-to-person relations not included in the first group, and these tend to extend over shorter durations[5].

A cooperative society consists of several social groups interacting in a highly organised manner. Like humans, animals can also form complex communities and create groups that can be brief or permanent, such as herds, colonies, and schools. Although many animal societies are formed by related individuals (e.g., family groups), this is not a requirement for societies. The cooperative breeding behaviour of some birds[7], for example the red-cockaded woodpecker, is representative of a non-kin society[8] in which the offspring receive additional care from non-parental group members.

Social behaviour and the types of interactions

What is social behaviour? Animals perform many activities during their lives, intending to survive and reproduce; they seek food and mates, defend themselves, and in many cases, care for their offspring and even relatives, such as the behaviour observed in African savannah elephants[9]. These activities are deemed social when they involve interactions among members of the same species (conspecifics) in a way that influences either immediate or future behaviour[10]. Although the gathering of multiple individuals increases the opportunity for social interaction, it alone is not a requirement for social behaviour. For example, the emission of pheromones by male Stenogastrinae (a subfamily of wasps) during *'patrolling flights'* to attract potential mates qualifies as social behaviour[11,12]. Although social interactions can be complex, particularly when many individuals are involved[13], they are often categorised into five main groups: cooperative, mutualistic, altruistic, parental, and aggressive[10,14].

Cooperation

The term 'cooperation' is derived from two Latin words: 'co' and 'operari', meaning 'together' and 'to work', respectively. Hence, cooperation requires the working together of two or more individuals. Cooperation is an umbrella term for behaviour encompassing social interactions

between individuals of the same species (intraspecific cooperation) or individuals of different species (interspecific cooperation)[15]. Typically, cooperative behaviours result in a net gain for all participants over time, such as the giver and recipient(s) (+/+)[16], but in its broadest sense can also include interactions which provide a benefit to recipient(s) at the cost to the giver (+/-)[17].

Cooperation is essential for social species including our own, playing an important role in an individual's ability to survive and subsequently reproduce –fitness[18]. While cooperation per se does not require complex cognition[19], for example, in bacteria and eusocial insects, some types of cooperative behaviour in more socially complex species (particularly involving time delays between investment and compensation) likely require cognitive abilities for inhibitory control and associative learning[20]. However, studies have shown that the nature of a social relationship is more important than food motivation and cognitive abilities in driving cooperative interactions. For instance, Dale et al., 2020, showed that the affiliative bond between pairs of wolves strongly influences success on a coordination task, in which pairs were required to pull simultaneously on either end of the rope to reach a reward, and the extent of prosociality shown by one individual to their partner as measured by the prosocial touch screen task, in which one individual rewards its partner by pressing the correct symbol on a touch-screen. Furthermore, in the same study, the social rank of individuals had an inverse effect on the same measures and increased inequality in the number of rewards individuals received in the inequality aversion buzzer task in which individuals alternately press a buzzer to reward the other[21]. Similarly, in humans, studies have shown that a desire for positive relationships is more beneficial than a motive for power in social dilemmas requiring cooperation[21]. Thus, successful cooperative behaviours between conspecifics may likely reflect the established or future desires for affiliative social relationships of all participating individuals.

Mutualism

Although intraspecies interactions usually have a higher impact on fitness since they are relevant for reproduction (e.g., mating and parenting) exclusively involving conspecifics and survival since they also share the same ecological niche, often competing for the same resources (e.g., food and shelter), there are notable exceptions in both fitness components. A classic example concerning reproduction is the heterospecific mating of Amazon mollies (*Poecilia formosa*), an all-female unisexual fish species which requires the presence of sperm of a male from a closely related species to trigger embryogenesis without the genetic contribution of sperm DNA for the zygote –gynogenesis[22,23]. Therefore, intraspecies interaction can be equally crucial to a participating individuals' fitness and survival.

Mutualism is an exclusive term to describe the mutually beneficial interaction between two species working in a cooperative manner, as observed in the cleaner-client relationship between BlueStreak Indo-Pacific cleaner wrasses (*Labroides dimidiatus*) and larger reef fish species, where

cleaner fish consume ectoparasites off the gills of so-called 'client' fish[24–26]. It is widely believed that mutualism comes about because one species adapts to the presence of another[27,28]. A classical experimental approach to testing for mutualism is to evaluate the performance of a species before and after its partner has been removed or, when not possible, kept at a low density[27,29–32]. Interspecific interaction in which the removal of each partner results in a decreased performance of the other confirms mutualism[28].

Research shows mutualism is frequent, and when mutualistic rewards suffice, sustaining mutualistic partnerships is vital to maintaining much of the biodiversity that drives ecosystems[33,34], especially agricultural ecosystems essential to human wellbeing[35,36].

Altruism

Unlike mutualism, altruism refers to social behaviour that benefits another individual at a cost to oneself, typically between conspecifics. For example, giving your lunch away is altruistic because although it helps someone hungry, it comes at the expense of being hungry. In the animal kingdom, a similar example of altruism can be found amongst vampire bats (*Desmodus rotundus*), which have been observed regurgitating blood meals to donate it to other members of their group who have failed to feed that night to ensure they do not starve[37,38]. Studies have shown altruistic behaviours to be imperative for the fitness of functioning societies, reducing morbidity and mortality rates[39] and promoting mental health and happiness[40] within growing populations.

Parenting

Typically, parenting behaviours involve the social interactions between parents (maternal[41], paternal[42], or both[43,44]) with offspring for feeding and general care. Many species, including ours, display shared care – alloparenting (cooperative breeding in animals), in which nonparents help care for offspring[45]. Several studies in humans and animals show that parental interactions, particularly in early life, can shape behaviour, cognitive ability and stress responses of young in later life[46,47]. For example, in humans, evidence suggests maternal care might have a protective effect on offspring behavioural problems. A study on a Japanese cohort of 982 families found that mothers who scored highly on social interaction showed lower odds of having a child with emotional symptoms and hyperactivity-inattention problems[48]. Furthermore, maternal care in humans and rats has been linked to increased expression of the N-methyl-D-aspartate (NMDA) receptor subunit, brain-derived neurotrophic factor (BDNF), both mediators of synaptic plasticity[49–51], and increase cholinergic innervation of the hippocampus, all of which enhances spatial learning and memory, and thus cognition[52–56].

Aggression

It is a common misconception that the word *'social'* implies amicable interaction through which the cooperative behaviour of individuals typically leads to a mutually beneficial end. While many social interactions are of this nature (affiliative), allowing individuals to acquire resources

such as food and shelter, social behaviour can also be agonistic, for example, displaying dominance, aggression, and fighting. Thus, the display of dominance exhibited by red deer (*Cervus elaphus*) in the form of an intimidating roar that works to warn away male competitors[57,58] is deemed equally social as the altruistic grooming behaviour of common marmosets (*Callithrix jacchus*)[59]. Aggressive behaviours have been described as hard-wired, such as the response male mice display to an intruder male[60], and learnt, such as the increase in aggressive behaviour observed in children with aggressive parents[61]. Regardless of the origin of the aggressive behaviour (i.e., innate or learnt), in many species, including our own, studies have shown that aggression is associated with increased avoidance behaviour in victims and is thus capable of shaping the likelihood of future social interactions[22,60,62].

The evolution and persistence of prosocial behaviour in nature

It is no longer a common belief that cooperative behaviours evolved for the good of the species. Instead, in line with Darwinian reasoning, it is thought that the unit of natural selection is the individual and that social behaviour is fraught with competition. Social animals often fight over territory, mates, and food throughout the animal kingdom when resources are low. Yet, surprisingly prosocial behaviours such as cooperation and altruism are also displayed among individuals. But why should an individual carry out cooperative behaviours that appear costly to perform but benefit others? Theoretical explanations for the evolution of cooperation and altruism within any population can be broadly categorised into two groups: direct and indirect fitness benefits[63].

The first of the two groups, direct fitness, concerns how the number of offspring an individual begets (personal fitness) is impacted by their behaviour[63–67]. One possibility is that although the cost of performing cooperative behaviours comes at a cost to the individual performing the behaviour, the benefits received outweigh the cost[17]. For example, through cooperative breeding found in some birds (such as the previously mentioned red-cockaded woodpecker) and mammalian species (such as in meerkats (*Suricata suricatta*)), individuals can increase the size and the success of a group through factors, such as greater successful foraging, and in doing so improving their individual prospects for survival and reproduction[68,69].

Another possibility is that some mechanism exists for enforcing cooperation by rewarding cooperators or punishing cheaters. Skinner et al., 1953, proposed the reinforcement theory stating that if a behaviour/response is coupled to a reward, that response will tend to be repeated but not when associated with a punishment[70]. Sidowski (1957; and colleagues (1956)) experimentally demonstrated these two tendencies by tasking two subjects to score as many points as possible. Paired subjects were presented with two buttons, and unknowingly to both,

the left button rewarded a point (indicated by a red light) delivered to the other individual whilst the right an electric shock. Whether or not subjects were made aware of being paired with similarly instructed individuals had little impact on the results. Subjects from both experimental conditions (informed and uninformed) readily learned to reward the other (however unwittingly) and avoid electric shocks by generating reward-reward sequences[71,72]. Later, Kelley et al., 1962, summarised the two tendencies mentioned above as 'win-stay' and 'lose-change' patterns when investigating the question of learning a reward-reward sequence without awareness of interpersonal nature by requiring subjects to respond simultaneously (rather than ad libitum as in Sidowskis experiments) to earn points[73]. Animal experiments like Sidowski's have shown comparable results, demonstrating that behavioural tendencies to repeat rewarding and not punishing actions are not limited to humans. Specifically, in an investigation conducted with pairs of rats (*Rattus norvegicus*) visually separated by an opaque divider, pairs are observed rewarding each other when one of the rats' behaviours produces non-social stimuli, such as lights or buzzers, made available to the other[74].

It is beyond the work presented here to detail the variety of ways by which reward and punishment may enforce cooperative behaviours: policing, sanctions, reciprocal altruism, indirect (reputation-based) reciprocity and strong reciprocity. Those interested may wish to refer to the work of Fehr[75], Mouden[76], and Guala[77].

The second of the two categories explaining the persistence of cooperative and altruistic behaviour, indirect fitness, concerns how the behaviour of other individuals may consequently gain a said individual's fitness that also carries the cooperative gene[63,67,78–80]. The easiest and most common way this could occur is if genes are identical by descent; for example, by helping a close relative reproduce, an individual is still indirectly passing on its own gene to the next generation. Hamilton's rule states that altruistic behaviours will be selected between individuals who share a given percentage of genes when their positive effect on reproductive potential or fitness is more significant than their direct fitness cost - 'inclusive fitness'[79]. For instance, a female wolf with a pup may nurse her full sisters' starving offspring. Since the benefit to the wolf's sister is greater than the cost to her pup (marginally reduced), the wolf gains inclusive fitness through her behaviour, increasing the potential of passing on shared genes to subsequent generations (example adapted from[81,82]).

Often, geographical factors and availability of resources may limit the dispersion of genes whereby individuals stay near their birthplace, thus increasing neighbours' genetic relatedness–population viscosity[83]. Additionally, increasing the number of individuals occupying a given space increases the opportunity for social interactions such as cooperative behaviours. Therefore, when cooperation is directed indiscriminately toward all neighbours, it is unsurprising it favours those who are related. Smith et al., 1964, coined the term 'kin selection' to describe how indirect fitness

benefits arise from cooperative behaviour preferentially directed towards related individuals[84]- albeit inadvertently.

Conversely, when cooperative behaviour is directed towards nonrelatives, it may be explained by the 'greenbeard' mechanism[79,80,85,86]. Although Hamilton proposed the greenbeard mechanism mathematically in 1964, it is named after Dawkins's 1976 illustration of Hamilton's work depicting a green beard[87]. Hamilton and Dawkins explain that a single gene (or several tightly linked genes) which causes cooperative behaviour may be recognised by other individuals through its association with a distinctive phenotypic marker (such as a green beard), thus facilitating its selection within a given population[79,85]. In other terms, individuals who share similar phenotypic traits are more likely to behave cooperatively or altruistically towards one another, increasing the likeliness of that gene being passed on to the next generation. A limitation of the greenbeard theorem is that it only works when the behaviour gene and phenotypic marker remain linked, breaking down when one of the two components is expressed without the other. For example, individuals may exhibit the phenotypic trait but not the cooperative behaviour -'falsebeards'[85]. Such individuals gain fitness advantage by reaping the benefits of others' behaviours without themselves providing any fitness benefits to others. The appearance of falsebeards in the population will lead to the decline of the phenotype until ultimately lost[85]. Between the greenbeard mechanism and kin selection (which remains to be disproven), the former is a more widely accepted theorem explaining why prosocial social behaviours, like cooperation and particularly altruism, persist in nature[65].

Shared phases of social behaviour

Although social behaviours are complex (increasing with the number of participants) and present differently across many species, they typically share a series of three phases leading up to social interaction: detection, approach, and investigation[88].

Detection

During detection, an individual aims to identify the presence and location of a social target using the unique sensory cues emitted by the target. Sensory signals may be transmitted through olfactive, acoustic, and visual modalities, and species often give prominence to one type of sensory modality when gaining information about a social target and their surroundings[89–92]. For example, olfaction in rodents is the dominant sensory modality regulating social behaviours[93,94]. Hamsters can identify the sex of a target through odours from the target's urine and faecal samples and Harderian secretions[95,96]. Upon sensing conspecifics, a rat will typically decrease its velocity, rhythmically twitch its nose and whiskers, and bob its head while slowly changing orientation[97]. The coordination of these actions allows rats to maximally sample a target's odour

and thus quickly determine its direction. Once the target is detected and its direction determined, the said individual may move in or away.

Approach

In the approach phase, various motor outputs may be observed amongst species, such as walking, running, flying, or swimming, the purpose of which is to reduce the distance between an individual and a social target. Furthermore, approach toward a social target is a consequence of a said individual's internal readiness to engage with conspecifics and is influenced by both endogenous and exogenous variables, such as the age and size of the target[91] or danger in the immediate environment[98–100], respectively.

Like humans, most animals (vertebrates and invertebrates) are socially inclined, and the presence of conspecifics and such cues is intrinsically rewarding. For instance, Mulholland et al., 2021, reported that captive chimpanzees made more overt responses for the opportunity to view conspecifics compared to nonsocial control content presented on a touchscreen. Specifically, chimpanzees performed significantly more touches to play videos of other chimpanzees and spent more time looking at both videos and photos of conspecifics than nonsocial cues[101]. Studies on rats have shown that post-partum dams are willing to give up food and cocaine to be near pups[102,103] and during social play emit high-frequency (50 kHz) vocalisations associated with other appetitive activities, including drugs abuse, such as in response to amphetamine[104–108]. The rewarding nature of conspecifics is what drives the said individual to approach and subsequently investigate and interact with the target, usually in a consummatory manner.

Investigation

During the investigation phase, an individual will continue to build on information about the social target gained in the previous stages through closer examination. Investigation plays an essential role in confirming the identity and gauging the readiness of the social target to interact, both of which help the said individual decide which type of interaction is most appropriate. This phase is as much about the social target gaining information about the approaching individual as it is about the individual acquiring information about the social target. Incorrect identification and interpretation of intent and willingness to interact amicably by both parties may result in fighting.

Focusing on the said individual, the motor output during the investigation phase involves orienting itself toward the social target for closer examination[109–113]. Like in identification, during the investigation phase, the said individual explores the social target using various senses. Sound, sight, and smell offer a means of acquiring information over longer distances, whilst touch and taste typically require a closer proximity between an individual and the social target.

In many mammals, including rodents[114] and non-human primates (New World monkeys: ring-tailed lemur (*Lemur catta*)[115], squirrel monkeys (*Saimiri sciureus*), and spider monkeys (*Ateles*

geoffroyi)[116], and Old-World species: Sumatran orangutans (*Pongo abelii*), Western lowland gorillas (*Gorilla gorilla gorilla*), Western chimpanzees (*Pan troglodytes verus*) and bonobos (*Pan paniscus*)[117]), such examination is carried out predominately via sniffing. Sniffing is often directed toward facial and anogenital areas, where pheromones are enriched[118]. In addition to smell, rodents also use visual cues to gain information about the social status of another. For instance, Wesson, 2013, reported that subordinate rats reliably decrease their sniffing frequency upon being investigated by more dominant rats. Failure to do so shortens the latency for agonistic behaviours by dominant rats. In follow on experiments where rats were rendered unable to smell through treatment of zinc sulphate ($ZnSO_4$) administered to each nostril, subordinates continued to exhibit reduced sniffing in the presence of dominant rats demonstrating the independence of this behaviour from olfaction[119].

The approach and investigation phase precedes social behaviours; and are therefore independent of it. As previously mentioned, the expression of approach and investigation can reflect an individual's internal readiness to engage with a social target even when external factors may block subsequent interactions. For instance, zebrafish show continued body orienting towards visually accessible conspecifics even when a transparent divider separates them[91,111,120]. The social preference test (typically conducted in rodents[121]) measures the relative time a test animal spends approaching and investigating one or more conspecifics or a non-social stimulus. The test provides a means to assess a given animal's propensity to engage with the social target, commonly referred to as social preference (see **Chapter 2** for how the definition of social preference varies across fields). When social interactions are blocked, as mentioned, a continued attempt by an individual to approach social targets reflects a high level of internal readiness and, therefore, preference for social interactions within the said individual.

Underlying circuitry of social behaviour

The path from detection to generating a social behavioural output requires rapid multi-step processing beginning with the binding of a ligand with the appropriate sensory neuron, followed by signal transduction across several brain areas before reaching the mesencephalic locomotor region (MLR), and finally ending with command neurons for locomotion situated in the brainstem, which activates the appropriate motor programs of social behaviour. Moreover, the processing from the point of detection to social interactions is also not direct, with feedback mechanisms existing in the system at the detection, approach, and investigation phases to guide the decision-making process for initiation and maintenance of social interaction.

Although the zebrafish model offers excellent opportunities to study the underlying circuitry of social behaviours in higher resolution, much of our current understanding comes from existing

research on rodent models, predominantly olfaction studies. Therefore, in this section, the circuitry that underlies the detection, approach and investigation phases of social behaviour are described predominantly using rodent examples. Where possible, examples are provided from zebrafish research.

Circuitry underlying detection

In many species, such as rodents, olfaction is the most crucial sensory modality for communication, although auditory and visual cues may also facilitate the localisation of conspecifics[122–124]. Odours emitted from distant targets are readily detected by olfactory sensory neurons (OSNs) located in the main olfactory epithelium (MOE). The signals from the OSNs are transferred to the main olfactory bulb (MOB) to be further distributed across multiple brain regions, including olfactory tubercle (OT), anterior olfactory nucleus (AON), cortical amygdala, piriform cortex (Pir), and the entorhinal cortex (ENT)[125]. During sniffing in rats, micro-movements such as fastened breathing, twitching of the whiskers and nose, and up and down head movements are generated by the medullary circuit, including the pre-Botzinger complex (preBotC), the core repository generator[126].

Owing to the physics of sound propagation in water, for small aquatic species including zebrafish, hearing is sufficient for social communication but much less adequate for determining the direction from which a signal comes[127]. Therefore, smell and vision have prominent roles in detecting, locating, and recognising objects and conspecifics far from the animal's body.

Upon detecting conspecific pheromones in the water, zebrafish instigate approach behaviour[128]. When simultaneously presented with water scented by siblings versus untreated water, they spend more time (i.e., observations) in water containing conspecific olfactory cues than untreated water[128]. Similarly, zebrafish use olfaction to detect and locate threats in their surroundings. For example, in teleost's including zebrafish, an alarm substance is made in the specialised epidermal club cells and enriched dorsally[129–131]. The alarm substance is non-secretory and can only be set free by mechanical damage to the fish[131,132], thus signalling the injury of conspecifics. Following detection of the alarm substance via olfaction, nearby zebrafish take evasive actions such as freezing, random darting, tightening the fish shoal, and swimming away from the predator[130,133].

Olfaction in zebrafish starts with an odour arriving at the olfactory bulb (OB, equivalent to the rodent MOB) which contains all OSNs with all the major and minor receptor repertoires expressed on a common sensory surface[134]. In the zebrafish OB, the axons coalesce to form glomeruli, similar to the situation in mammals. However, mammalian glomeruli receive input from several thousand OSNs compared to the hundreds for zebrafish[135]. From the OSNs, signals are

transferred to two telencephalic centres, the dorsal-posterior telencephalon (Dp, corresponding to the Pir in mammals[136]) and the ventral nucleus of the ventral telencephalon (Vv, corresponding to the septal areas in mammals[137]) and two diencephalic centres, the posterior tuberculum (also known as posterior tubercle, PT), right habenula (rHb), and the hypothalamus (HT)[138]. Both the PT and the rHb project to the MLR in the brain, the PT directly, and the rHb indirectly via the interpeduncular nucleus (IPN)[139,140].

As mentioned, vision also plays a fundamental role in social preference behaviour in many species, including zebrafish, which use visual cues to detect and recognise conspecifics[141]. When exposed to real or virtual conspecifics, zebrafish immediately approach to interact with the visual social stimuli[91,142]. The visual stimulation of the zebrafish retina leads to a plethora of brain areas being activated including but not limited to the entopeduncular nucleus (EN), pallium (P), pretectum (Pr), lateral tegmentum (Tg), subpallium (SPd), ventral and anterior tectum (TeOv, TeOa, respectively), posterior parvocellular preoptic nucleus (PPp), rostral medial hypothalamus (mHT), intermediate and dorsal medulla oblongata (Moi and Mod, respectively), PT, dorsal thalamus (DT), and several areas of the hypothalamus (HT, caudal; cHT, intermediate; iHT, and dorsal; dHT)[143].

Studies have shown that zebrafish are capable of learning to detour around transparent barriers to reach a group of conspecifics with performance proficiency levels comparable to those previously observed only in corvids and apes[144,145]. Control experiments suggest that zebrafish performances in such experiments are explained by using olfaction in conjunction with visual cues to locate conspecifics, unlike other tested teleost species (i.e., the guppy, *Poecilia reticulata,* redtail splitfin, *Xenotoca eiseni*, and Sarasins minnow, *Oryzias sarasinoru)* which rely predominantly on vision[145]. In a recent study looking at the role of visual and olfactory cues in social decisions of two teleost species (zebrafish and guppy), Santaca et al., 2021, demonstrated that zebrafish rely more on olfactory cues than visual cues to estimate shoal sizes but use both senses equally to discriminate between familiar and unfamiliar conspecifics[146]. These results suggest that while zebrafish may use the same sensory systems (i.e., olfaction and vision) in detection, approach and investigation phases before social interaction, there are large differences in the relative importance of the different senses in the perception of the conspecifics across the three phases.

Circuitry underlying approach

The nucleus accumbens (NAc) has been identified as a critical region mediating goal-directed approach behaviours, including toward conspecifics[147–149], in many species. For instance, in rats, bilateral inactivation of NAc impairs preferential social approach towards stressed juveniles[150]. In monogamous California mice (*Peromyscus californicus*) and mandarin voles (*Lasiopodomys*

mandarinus) administration of an oxytocin receptor antagonist into NAc decreases social approach towards conspecifics[151,152]. Conversely, in the same study on mandarin voles, oxytocin administration into the NAc increases approach[152], and in mice, enhancing serotonin release in NAc rescues social approach deficit in autism models[153]. In a recent study looking at the neuronal dynamics that underlie the formation and maintenance of bonds, Scribner et al., 2020, using miniscope imaging, reported that distinct ensembles of NAc cells are activated during approach towards partners and novel conspecifics in prairie voles (*Microtus ochrogaster*)[154]. Notably, Scribner documented that these NAc cell ensembles became active before the approach, supporting NAc's potential function in driving this behaviour[154].

The ventral tegmental area (VTA) is a heterogeneous region of diverse cell types that play distinct roles in modulating reward and aversion based on connectivity to different upstream and downstream brain structures[155–159]. One of the downstream structures of the VTA is the NAc, to which the VTA projects densely[159–161]. Consistent with the role of the NAc in approach, optogenetic activation of the VTA-NAc cells facilitates social approach behaviour[160].

Multiple regions along the main olfactory pathway connect to the NAc, as demonstrated by virus-mediated trans-synaptic tracing[162]. Specifically, injection of a modified rabies virus into the core of the NAc leads to dense labelling in the AON and Pir, suggesting that these structures can potentially send conspecific olfactory information to the NAc[162]. Indeed, studies have shown that the Pir is the primary olfactory cortex serving broad functions in odour recognition and discrimination when investigating conspecifics[163,164].

In rats, prosocial ultrasonic vocalisations (USVs), such as those emitted during play and associated with positive emotional states[165], have also been shown to activate the NAc cells and increase dopamine levels in the area[166,167]. The activation of the NAc in response to USVs is believed to be mediated by projections received from the basolateral amygdala (BLA), which receives extensive auditory inputs[168]. Electrophysiological recording found that a majority of BLA cells are responsive to social USVs[165], and activation of these cells increases reward-seeking behaviour in rats[169]. Consistent with the role of BLA-NAc cells in social approach, lesions to the BLA reduce approach behaviour[170].

Although oxytocin was previously mentioned in rodent studies, it is a highly conserved neuropeptide found in all investigated vertebrates[171,172]. In teleosts, including the zebrafish, the orthologue of oxytocin is called isotocin (zOT), and its paralogue vasopressin is often referred to as arginine vasotocin (AVT)[173]. The zOT/AVT neurosecretory system in fish consists of three main cell groups distributed along the ventral portion of the POA (gigantocellular (gPOA), magnocellular (mPOA), and parvocellular (pPOA)[174]), from which nonpeptidergic neurons project fibres to

diverse brain areas, such as the Vv, diencephalon (De), and various mesencephalic structures, and projections to the neurohypophysis[175].

Levels of zOT and AVT are implicated with the outcome of agonistic social interactions. For instance, in a study investigating the expression of zOT and AVT in brain areas following short-term agonistic social interactions, Teles et al., 2016, documented decreased expression of zOT in the OB of winners compared to losers, mirror-fighters, and controls with no social encounters, and losers showed increased expression in zOT in the De compared to the aforementioned groups[173]. A fight's outcome can significantly impact subsequent interactions since the winner of an encounter is more likely to win its next interaction[173]. In contrast, the loser decreases its probability of success since they tend to avoid direct confrontation and retreat when challenged[173], indicating the relevance of past experience in future agonistic interactions.

Like in rodents, oxytocin has also been implicated in approach behaviour in zebrafish. For example, mutant lines deficient in zOT and its receptors express a lack of motivation to approach demonstrator conspecifics they had previously observed in a distressed state compared to wildtypes[176]. Together these results support the functional role of oxytocin in approach behaviour in the zebrafish model.

Circuitry underlying investigation

Most amniotes, and to a certain degree, amphibians[177] have a primary and separate accessory system for olfaction[125]. Like the OSN, vomeronasal sensory neurons (VSN) in the vomeronasal organ (VNO) also detect odours[178]. However, signals relayed to the accessory olfactory bulb (AOB) from the VNO are subsequently transferred to different brain areas than those received at the MOB, including the bed nucleus of the accessory olfactory tract (BAOT), bed nucleus of the stria terminalis (BST), posteromedial cortical amygdaloid nucleus (PMCo) and mostly to the medial amygdala (Me) which projects to the medial hypothalamus[125,179].

In rodents, the VNO gathers information essential for the precise social identification of conspecifics, such as the sex and strain of animals[180]. The rat VNO is tuned to sex-specific cues and is used to assess whether a female is in oestrus[181]. Transgenic mice deficient in a subset of VNO cells expressing TRP2, a putative ion channel of the transient receptor potential family, lose the ability to discriminate sex, mounting intruder males and females excessively and indiscriminately[182]. Furthermore, the VNO appears necessary for initiating aggressive behaviours. For example, genetically impairing VNO cells result in the loss of maternal[183] and male-male territorial aggression, reducing both the number and latency of attacks, in mice[184]. Mice with deficits in a subset of VNO cells, i.e., expressing olfactory-specific G-protein γ8 subunit (Gγ8), show

reduced pheromone-mediated aggressiveness in both males and females, with all other socio-sexual behaviours remaining unaltered[185].

The VSN mediates recognition between kin and non-kin by detecting major histocompatibility complexes (MHC)[186]. Hence, peptide ligands of MHC class I molecules, typically shared by related individuals, activate sensory neurons in the vomeronasal epithelium and signal individual recognition[187]. In the VNO, MHC class I molecules only bind to peptides with particular amino acid residues at the anchor sites, therefore selectively activating VSNs with the same anchor residue with high specificity[188]. Studies have shown that exposing pregnant mice to male urine containing MHC class I molecules comparable to their own leads to implantation failure and abortion of her mate's offspring[189].

Despite lacking a separate VNO in addition to a main olfactory epithelium, zebrafish also use the olfactory system to recognise kin[190]. During a 24 hour time window at 6 dpf, larval zebrafish learn to differentiate between kin and non-kin odours in a process called imprinting[191–193]. Studies have shown that, like in rodents, MHC ligands are the underlying chemical cue triggering olfactory imprinting[128]. Furthermore, *In vivo* calcium imaging showed responses to MHC peptides in olfactory bulb neurons to be spatially overlapping with responses to kin odour but not food odours, suggesting MHC peptides to be part of kin odour in zebrafish[128].

In teleosts, five major populations of OSNs are present, the ciliated OSNs (cOSNs)[194], microvillous OSNs (mOSNs)[195], pear cells[140], crypt cells[135], and Kappe cells (specific to cartilaginous fish like zebrafish)[196], all located on the olfactory epithelium. Among these sensory neurons, mOSNs and crypt cells have been implicated in kin recognition. For example, in a detailed study analysing which type of OSNs detect kin odour, using phosphorylated extracellular signal-regulated kinase (pERK) expression as a readout of activation, Biechl et al., 2016, reported that a small population of mOSNs respond to kin odours[190]. Furthermore, in the same study, Biechl showed that although the total number of crypt cells does not differ in imprinted versus non-imprinted zebrafish larvae, the number of activated crypt cells is significantly higher after kin odour stimulation in imprinted compared with non-imprinted larvae, as well as compared with control larvae stimulation[190]. Research shows that crypt cells (responsive to kin odours) activate mgG2 cells in the OB, subsequently activating Otp-expressing cells in the intermediate nucleus of the telencephalon (Vi)[190]. Therefore, the Vi is amongst the brain regions previously described to be activated downstream of the OB in response to kin recognition, including the Vv, supracommissural nucleus of the ventral telencephalon (Vs) and the Dp[135,190,195,197].

Lastly, during the investigation phase, the information gained about the visual stimuli is reflected in both brain area selection and activity levels. In an elegant study tracing the neural response to virtual and real conspecifics in the brains of zebrafish, Kappel et al., 2020, reported

that significant deactivation of the EN region was a specific response to the virtual conspecific (shaped as a black dot) moving in continuous motion[143]. In the same study, Kappel reported that the virtual conspecific moving in a biological bout-like motion, critical to affiliative social behaviour[198], by contrast causes a significant deactivation in the P and to a lesser degree in the Pr, Tg, and the SPd, together with a significant activation in the TeOv, all specific in response to the movement of the stimulus[143]. Furthermore, activation of the ventral thalamus (VT) and, to lesser degrees, the TeOa, PPp and the mHT coupled with the significant deactivation of the MOi, and the lateral pallium (Pl), were specific responses to interaction with real conspecifics[143]. Kappel also reported that the activation patterns of some brain areas are also shared in response to virtual and real conspecifics, including the significant deactivation of the MOd and the substantial activation of the PT, DT, cHT, iHT, and the dHT[143]. Together, these results suggest that various visual stimuli result in unique brain activity patterns in and across multiple brain regions, the selection and activity levels of which are influenced by the features of the visual stimuli.

Notably, activated brain regions are not specific to the modality of the sensory input. Studies have shown that olfaction is one of several sensory inputs to the habenula, contributing to the regulation of fear response[197,199,200]. Similarly, the PT has been implicated in the olfactory and visual systems but is also reported to be variably activated in response to food stimuli, electric shock, and mustard oil[201]. Therefore, although the modality of a signal at the start of the neuronal cascade may differ, activating different sensory neurons, signals can be ultimately received by the same brain areas, supporting that differential activity patterns in and across brain regions modulate behavioural outputs[202].

Factors influencing social preference behaviour

Why does readiness to express social behaviour vary widely in a given population, including our own[203,204]? The short answer is that social interactions can be costly. Although conspecifics and social behaviours are intrinsically rewarding[205–207], an individual must balance these payoffs with a potentially substantial trade-off: cost[88]. For example, a high cost and low benefit scenario will typically result in a lower probability of social interaction between two conspecifics.

Many factors influence the balance of costs and benefits and, subsequently, the individual's readiness to exhibit social behaviours. These factors include:

1) internal factors of an individual, such as genes, sex, age, and reproductive state,
2) experiential factors, such as winning or losing, and
3) external factors that are related to the environment, including food availability, population density, and social isolation.

Together the above factors determine an individual's readiness to participate in social interactions by controlling the likeliness of sensory-motor transition at two time points. The first point is when an individual initiates a reaction to a social target that may be at some distance away from it; namely, the transition from the detection to the approach phase[88]. Here, internal, experiential, and external factors influence if and how quickly an individual approaches, avoids or ignores the social target upon detection[91,208–210]. The second point is when an individual initiates a reaction to the social target, i.e., transition from the investigation phase to a type of observable interaction, such as cooperation, mutualism, altruism, parental care, or aggression[88]. The appropriate motor behaviour for the proper interaction is initiated when a specific internal threshold is reached from the sensory information collected during the investigation phase. The same factors that influenced the first point can also affect the set point of the threshold that triggers the action[211–213].

Oestrus cycle modulates female readiness to approach males

Female behaviours in many species are influenced by the oestrus cycle[214–217]. For example, research has shown that the oestrus cycle influences BALB/cByj female mice performances in the tail flick, tail suspension and open field tests[217], and aggression in both female mice and women[214,215]. Similarly, in many species, female drive for sexual encounters with males is synchronised with the ovulation period[218–221], with females showing the lowest interest in males during dioestrus, which coincides with high progesterone and low oestrogen levels. In contrast, female interest in males increases during the proestrus and oestrus, corresponding to when oestrogen levels surge, and progesterone levels are at their lowest[222].

Sex hormones modulate female sexual interest by acting on multiple nodes in the circuit[218,219,223]. Dey et al., 2015, showed that during dioestrus, when the progesterone level is high, female mice VNSs are temporarily and specifically rendered 'blind' to male pheromones (major urinary proteins, MUPs) emitted in the urine[223]. Selective anosmia of females towards male-emitted MUPs is mediated by progesterone acting on non-canonical progesterone receptors, which subsequently recruits phospholipase C β2 (PLCβ2) to suppress MUP-responsive but not MUP-nonresponsive cells of the VNS[223]. Hence, the rise of the female sex hormone progesterone during dioestrus decreases a female's olfactory ability to detect a male, contributing to reduced sexual interest of females toward males.

In rodents, sex hormones such as oestradiol can act on the NAc, to increase females' preference to approach males via three different ways. First, by acting on oestrogen receptors, oestradiol can directly modulate the activity of NAc medial spiny neurons (MSN)[224]. For instance, using female rats in differing phases of the oestrus cycle, Proano et al., 2018, reported that oestradiol robustly alters the intrinsic excitability and miniature excitatory postsynaptic current

(mEPSC) of NAc MSNs, and is essential for the oestrus cycle-dependent fluctuation of the inherent properties of MSNs[225]. Second, oestradiol can also modulate MSNs through its influence on dopamine transmission. For example, studies have shown that during oestrus, when oestradiol level is high, VTA dopamine cells show a higher firing rate, and stimulation of VTA dopaminergic terminals elicits a higher dopamine release in NAc when compared to dioestrus[226]. The enhanced VTA-NAc dopamine level promotes activation of dopamine receptor 1 (D1R) MSN cells, consequently favouring female social approach toward males[160,227,228]. Third, oestradiol can act on hypothalamic neurons to enhance dopamine release, specifically in response to social cues. For example, studies show that in ovariectomized mice, oestradiol increases the excitability of the neurotensin expressing medial preoptic area (MPO^{Nts})[229] cells. Since MPO^{Nts} cells are preferentially excited by male odours, interface with the VTA, and directly induce dopamine release in the NAc, an increase in excitability of these cells during proestrus is likely to increase dopamine release in NAc to male odours, thus, promoting the social approach of females toward males.

Overall, the fluctuation of sex hormones, e.g., progesterone and oestradiol, over the oestrus cycle alters the female's interest and sensitivity to male conspecifics by changing cell responsiveness at multiple regions along the detection, approach, and investigation circuits.

Social defeat-induced social avoidance

Although social stimuli are intrinsically attractive because of their rewarding nature, negative experiences can shape and even completely override this attraction. For instance, Qi et al., 2018, reported that male mice defeated by another male showed avoidance toward the aggressor [230]. In detail, a defeated C57B6/L intruder mouse (selected for its smaller size to ensure loss) placed in the cage of a resident Kunming mouse, for a minimum period of 10 minutes, spends significantly less time in areas proximal to the resident aggressor. This avoidance behaviour persisted for seven days before gradually diminishing[230]. Several experiments have shown that one-time defeat-induced avoidance is target specific since social avoidance (accompanied with aversive responses) is observed in defeated rodents only in the presence of the dominant male that defeated them and not in the presence of unknown conspecifics[231–234].

Chronic social defeat is a modified version of the resident-intruder paradigm (mentioned above) and typically varies in the number of daily resident-intruder encounters and spans several weeks[235]. A modification of this paradigm includes housing the intruder into a compartment adjacent to the resident aggressor, separated by a transparent divider, following daily doses of social defeat to maintain sensory stimulation, thus subjecting the intruder to continuous psychological stress[60,236].

Experiments on avoidance behaviour following chronic social defeat have reported depression-like behaviours (i.e., decreased preference for attractants like sucrose (anhedonia), and reduced locomotion and exploratory activity in a novel environment) in a subset of animals, as well as social avoidance behaviour towards unknown conspecifics[204,237–241]. Currently, mounting evidence suggests that generalised social avoidance may not be so indiscriminate as initially thought, with reports that chronic social avoidance-induced social avoidance behaviour in mice does not generalise to other phenotypic characteristics than those expressed by the aggressor. For instance, Ayash et al., 2019, showed that socially defeated C57BL/6J mice scored below one on the social interaction index on subsequent social encounters with resident CD-1 mice, spending less time interacting with novel conspecifics that shared phenotypic characteristics of the CD-1 aggressor (i.e., white fur and red eyes) but not others[62].

Social avoidance behaviours resulting from acute and chronic social defeat are likely to be driven by associative learning, with the former group relating painful experiences with a specific individual and the latter group towards conspecifics that share the same features of their aggressor[62,242,243]. Experiments supporting the involvement of associative learning include those in which the effects of chronic social defeat can be successfully reversed, resulting in the normalisation of social behaviour. In such cases, the uncoupling of prior (painful) experiences with a stimulus (i.e., aggressor / particular features of their aggressor) can be achieved through extinction training in which some intruder-resident interactions are permitted through a mesh wall but not attacks[62].

The BLA is well documented for its role in fear conditioning and is described as a critical site for the plasticity underlying social defeat-induced changes in behaviour[244,245]. In rodents, the inactivation of the BLA by blocking protein synthesis, N-methyl-d-aspartic acid (NMDA) receptors, or 5-HT2a receptors before social defeat reduces avoidance behaviour 24 hours later[245–249]. Furthermore, inhibiting BLA immediately before the post-defeat social avoidance test also reduces avoidant behaviour[246]. Conversely, injection of TCB-2 (5-HT2a agonist) into the BLA increases the acquisition of conditioned social-defeat and anxiety-like behaviour[247]. Although the NAc is essential for driving approach behaviour towards conspecifics (*see* **Circuitry underlying approach**), it is also a region necessary for social avoidance. Considering the NAc is a significant area downstream of the BLA, unsurprisingly, pharmacological inhibition of the NAc 24 hours after defeat, like in the BLA, also reduces social avoidance[250]. Additionally, direct optogenetic activation of the BLA-NAc neurons increases social avoidance in undefeated mice[251].

Equivalent to the BLA is the medial dorsal telencephalon (Dm) in zebrafish, which is also implicated in fear and stress responses such as avoidance behaviour. Studies have shown that chronic social stress impairs neurogenesis and cell proliferation in the Dm and significantly in the Vv in subordinate male zebrafish[252]. Moreover, ablation of populations of neurons located in

subregions of the Dm (i.e., 120A-Dm neurons) causes reduced performance in Pavlovian fear conditioning[253]. Together, these results support BLA's/ Dm's role in modulating social defeat-induced behaviour at both the level of acquisition and expression.

Social isolation

Social isolation is a simple and effective technique to examine and tease apart the behavioural and neuronal mechanisms by which social interactions are regulated in many species, including our own. The adverse effects of social isolation have been documented in humans, rodents, domesticated ungulates and teleosts, such as rainbow trout, cichlids, angelfish and zebrafish[254–266]. Consistent findings among these studies are the reports of isolated individuals exhibiting avoidance-like behaviour toward social interaction and atypical social behaviour (i.e., anxiety-like behaviour) during social encounters.

To date, how social isolation affects the social brain circuitry, bringing about atypical behaviour, is poorly understood. Early findings suggest the involvement of zOT and AVT, which align with the role of the neuropeptides in modulating social approach and are supported by the avoidance of conspecifics observed in socially isolated individuals[201]. Furthermore, the involvement of zOT and AVT also suggest that social isolation may predominantly affect the POA and the HT, in which the neuropeptides are highly expressed, as well as other diverse brain areas receiving nonpeptidic projections from the POA.

Research also shows that social isolation may alter individuals' perception of conspecifics. For instance, Cacioppo et al., (2009) reported that loneliness (the perception of social isolation) in humans is associated with greater activation of the visual cortex in response to viewing negative social stimuli than negative non-social images (objects)[267]. Complementary to these findings, Bangee et al., 2018, reported that lonely individuals initially fixate more on socially threatening elements of social scenes than individuals with low levels of loneliness, who instead fixate on the positive aspects of the same visual cues[268].

Together these results suggest that social isolation likely works on the detection, approach and investigation circuitry to bring about atypical preference behaviour.

Differences between social isolation and loneliness

Social isolation and loneliness are conceptually distinct, describing various aspects of limited social interaction in humans and animals. Social isolation is the complete absence or insufficient contact with conspecifics and can be objectively defined based on quantitative measures of social network size or frequency of social contact with others[269]. Contrastingly, loneliness describes the

subjective perception of being alone, which originates from the discrepancy between an individual's preferred and actual social relationships based on a definition first suggested by Perlman and Peplau in 1981[270], and thus describes the emotional aspect of social isolation[271]. This definition underscores that social isolation and feeling lonely are not necessarily exclusive, meaning that it is possible to feel lonely without being socially isolated[272]. This thesis focuses on the impact of social isolation, and loneliness is referred to only in the context of contrasting behaviours of individuals towards conspecifics following periods of social isolation.

Health-related consequences of social isolation and loneliness

Social environments influence human and animal health, potentially altering the risk of disease and mortality[4,58,79,252,273–276]. In humans, low quality and low quantity of social relationships lead to a predicted ~50% increase in factors with an associated risk of mortality (e.g., smoking, obesity, and alcoholism), an effect size consistently observed across all age groups and sexes[277–282]. Contrastingly, living with and forming healthy, satisfying relationships with family members has been associated with preventing depression[283]. Social network sizes have been identified as protective against common mental health disorders[284].

In the average population, elderly individuals were identified as the most at risk of being socially isolated, with up to 50% of people aged 60 and one-third of the population expected to experience some level of loneliness in their life with health-related implications[285–288]. However, following the 2019-2021 coronavirus COVID-19 pandemic, social isolation has caused a significant increase in the number of individuals feeling lonely across all age groups[289,290]. In addition to the rising loneliness, global growth in anxiety and depression has also been observed over this same period[291,292]. Mounting evidence suggests that children and adolescents are disproportionally affected by isolation. Thus, the adverse consequences of social isolation over the recent pandemic will be prevalent for years to come[293,294]. Moreover, previous studies assessing the impact of early-life social isolation show concerning findings. Specifically, longitudinal studies on orphaned children from Romania have previously reported mild neurocognitive impairment, impulsivity, attention and social deficits, and behavioural abnormities such as motor-stereotypies, self-stimulatory behaviours, and indiscriminately friendly behaviour[295,296]. Furthermore, these findings emphasise the critical importance of social factors in health and wellbeing, especially during development, reinforcing the necessity of establishing new models to understand the mechanisms and find new treatments.

Social isolation can be self-perpetuating and exacerbate behavioural and emotional adjustment difficulties in individuals who already have underlying deficits in social processing. Autistic individuals, characterised by impaired reciprocal social interaction and repetitive

behaviour, respond to social interactions with stress, displaying anxiety-like behaviours and avoidance[297,298]. On the other side of the scale, individuals with Williams' Syndrome display hyper-sociability, often exhibiting an indiscriminate approach towards strangers[299]. The two contrasting behaviours represent how social dysfunction can result in different behavioural outcomes, ranging from social avoidance to inappropriate friendly behaviour with strangers. However, both these behaviours typically result in unsuccessful social interactions. The lack of social integration through avoidance or inappropriate social behaviours often results in a progressive withdrawal from relationships and social living, inducing a state of loneliness. This further worsens psychiatric symptoms and increases the risk of other diseases attributed to poor quality social relationships. Therefore, understanding the biology underlying the effects of social environments on health promises to provide new opportunities for effective interventions, which can aid in breaking the cycle of social isolation.

Social isolation in animal models

Similar to human findings, studies in laboratory animal models have shown that socially induced stress alone is sufficient to impact health and life span negatively. Such studies have reported direct effects on immune function and disease susceptibility, contributing to a shorter life span[276,252,273,300,301]. Furthermore, these studies have revealed that pervasive changes in response to social adversity are detectable at the molecular level[252]. For example, one hour of social isolation from pair mates is sufficient to elevate plasma cortisol levels in titi monkeys, whose partner preference is high[302]. Together these results echo those observed in humans, in which social adversity predicts increased mortality risk from almost all significant causes of death mentioned previously.

Beyond health implications, the impact of social isolation on behaviour has also been investigated in animal models. Chronic isolation lasting no less than two weeks in rodents leads to multiple behavioural changes. For instance, typical responses to prolonged social isolation in mice include aggression towards unfamiliar males, increased anxiety-like responses to threatening stimuli, and enhanced reactivity to footshock[303–306]. Similar observations are also made in rats following isolation commencing at postweaning with further annotations on decreased latency to emerge into unfamiliar open-fields and less time spent interacting with novel conspecifics[307]. As in mammals, in fish, the social environment is one of the most significant modulating factors of individual behaviour and can potentially enhance or restrict an individual's behavioural responses. For instance, it has been previously described that fish are more active and display increased exploratory behaviour when in the company of others[308–310]. Conversely, when isolated, fish are more persistent in their attention towards novel visual stimuli[310].

Behavioural anomalies have also been described in non-human primates following periods of social isolation. In rhesus monkeys, early-life social deprivation produces highly reactive individuals who, when faced with a range of stimuli, respond with a behavioural expression of fear and anxiety, accompanied by prolonged activation of the hypothalamic-pituitary-adrenal axis. Thus, laboratory animals provide a unique opportunity to investigate the causal links between social isolation, health and behaviour in a model where social interactions and settings are better controlled.

Zebrafish as an animal model for social behaviour

While early research described zebrafish behaviour as stereotyped and straightforward, particularly in their responses to pain[311], recent studies demonstrate that fish are capable of complex and context-dependent behaviours[312,313]. One of the most investigated is the social behaviour of adult zebrafish which includes shoaling and schooling[314]. As with many species, social behaviour in zebrafish is central for their survival representing essential strategies for the early detection of natural predators such as eels, enhanced foraging, and increased mating opportunities[315–318]. Native to India and Myanmar, zebrafish exhibit various shoaling behaviours, gathering in small shoals in slow-flowing waters and forming large tightly knit groups, hundreds in size, termed 'schools' in faster-flowing rivers[315,319,320]. It is worth pointing out that while shoaling and schooling may seem similar, there are distinct differences between the two behaviours[321,322]. Typically, shoaling refers to any group of fish that remains together for social reasons; that is shoaling is not limited to responding to external stimulus, and may exhibit, with little coordination between individuals. Schools are more synchronised forms of shoals, where individual fish move in the same direction in unison. In zebrafish, social group behaviours develop with age[322,323]. For instance, shoaling can be detected as early as 7 days post fertilisation (dpf)[110,323], with fish forming the tightest shoals by 79 dpf[322]. The development of conspecific preference accompanies this reduction in inter-individual distances, becoming robust around three weeks of age[91]. Once established, both behaviours persist throughout the fish's lifespan, showing minor alteration with changes in social environments[324].

Currently, two major approaches are used to investigate the variety of social behaviours exhibited by invertebrates and mammals, including humans. One focuses on the mechanisms, the other on the functions of a studied behaviour[323,325]. Laboratory animals such as zebrafish and rodents have already shown their potential in investigating the mechanisms of social behaviour, and several specialised protocols have been developed to facilitate the exploration of normal and abnormal social behaviours in these models[326–328].

Why are zebrafish becoming increasingly popular to study social behaviour and the underlying circuitry?

The power of the zebrafish behavioural tests must be compared to other developed rodent social models. For a long time, rodents have been the "go-to" model to study social deficits and associated behavioural disorders due to their small size and complex social phenotypes, which resemble human social behaviours and interactions[329]. However, the zebrafish animal model offers additional advantages. Zebrafish are small, inexpensive to house, fast-growing, reaching sexual maturity by two months of age, and capable of producing several hundred in size clutches. These features make the zebrafish a great model for high throughput social studies that rodents cannot match. Following this, zebrafish are diurnal[330], meaning that they can perform social behaviours under standard lighting conditions, exhibiting less sensitivity to environmental disturbances than their nocturnal rodent counterparts[327]. Moreover, it has also been shown that zebrafish show a higher degree of social cohesion than rats, indicating that fish models may be better suited to assess some social behaviours[327].

To fully address questions such as how environmental perturbations like social isolation give rise to atypical social behaviour, it is essential to investigate the underlying brain circuitry for alterations that affect the typical selection of appropriate motor programs for social behaviour. Advances in functional neuroimaging allow us to identify with increasing precision which brain regions correlate with a particular behavioural output. However, brain-wide visualization, permitted by electroencephalography (EEG) and functional magnetic resonance imaging (fMRI), does not even begin to reliably approach the cellular or synaptic spatial resolution of brain processing[331,332]. Contrariwise, electrophysiological or high-resolution imaging methods to record neural activity are difficult to extend beyond discrete social brain regions, as seen with human and rodent studies. To understand the neural basis of social behaviours, a challenging goal in neuroscience will be to bridge the gap between these distant levels i.e., to record and analyse the entire social brain with single neuron accuracy.

Zebrafish, sharing conserved neurochemistry and broad brain organisation with their mammalian counterparts, offer a means to bridge this gap and give the first insights into circuit dynamics, from the whole brain level to the molecular changes during conserved social behaviours. In this regard, the zebrafish model provides five key advantages: (i) compact brain size, (ii) conservation of the neuropeptide pool, (iii) linear organization of brain regions, (iv) structural accessibility of internal nuclei (no overlaying neocortex), and (v) optical clarity. The coupling of the optical clarity of the zebrafish brain[333–338] with its compact size, measuring about 400 × 800 × 250 µm3 (W × L × H) at larval stages[339], allows for the entire brain volume of the model to be captured at single-cell resolution by standard microscopy magnifications. This feature is

exploited in **Chapter 4** of this thesis. Central to understanding the circuitry-behaviour interplay in social behaviour, zebrafish possess homologous structures to human social brain areas, including the hypothalamus, preoptic area (POA), and amygdala (pallium and subpallium regions in zebrafish)[340–343]. The linear organisation of these and other major brain areas from the olfactory bulbs to the tip of the spinal cord[344] further facilitates brain-wide imaging. In addition, while the aforementioned amygdala and brain regions, such as the hippocampus and habenula, are difficult to scan in mammals due to their deep location beneath the neocortex, their position is inverted in zebrafish[345]. Specifically, while the anterior neural tube of mammals undergoes invagination during development leading to their deep location beneath the neocortex, the eversion process during fish development makes these behaviourally significant structures the most dorsal nuclei of the telencephalon[345] and therefore more accessible for high-resolution imaging.

As with all animal models, the zebrafish model is not without drawbacks. While the zebrafish model displays homology with 84% of genes associated with human diseases, the duplication of genes observed in the model can make genetic manipulation complex[346]. In addition, some brain areas in the zebrafish remain unknown or are less well defined, leading to difficulties interpreting results. With zebrafish only recently becoming a popular animal model, it is therefore also no surprise that little data is available characterising their behaviour in comparison to rodents[129]. However, with more sophisticated techniques to address the precision of genetic modification[347], and the continued development of dedicated brain atlases[348–350], the zebrafish models' limitations are slowly being addressed, ever-increasing its status as a tractable species to study brain behaviour relationships.

Social behaviour assays in zebrafish

The study of group forming behaviour in zebrafish is approached in two primary ways. The first and most frequently used approach is to record the shoaling behaviour of freely swimming fish. Parameters such as inter-individual distances and synchronisation can be extracted and analysed from these data-rich recordings[322,351–354]. In addition, social preference, which is the drive of individuals to be near others of the same species – conspecifics – has been widely studied in the model. Details of the tests used to quantify social preferences are described in **Chapter 2 (**see **Zebrafish social preference test)** of the work presented here. Briefly, this approach involves the presentation of a conspecific or a shoal of fish to a single individual to investigate the behavioural response[91,113,355,356]. While distances from social cues and the synchronisation can also be quantified using social preference tests, this method is better suited to studying the response of single fish towards social stimuli in greater detail. It facilitates investigating the underlying circuity

that governs social behaviour since the preference assay can be augmented to accommodate high-resolution imaging techniques, particularly in smaller fish.

Non-affiliative social interactions, which also form the repertoire of social behaviours, can also be measured in the zebrafish model. For example, in adult zebrafish, aggression and anti-predatory behaviours can be measured using a mirror or a simple predator exposure test. While the former quantifies fish aggression through biting and "charging" behaviour directed at its mirror image, the latter assesses anxiety and fear-related responses to live or virtual predators[129,352,357]. Interestingly, exposing zebrafish to alarm substances, chemicals released from epidermal skin cells upon skin damage, evokes darting-like motion and subsequent 'tightening' of shoaling fish without the accompaniment of visual cue[133], suggesting that social behaviour is not limited to optical sensory inputs alone.

Furthermore, social memory and learning can be assessed in the zebrafish model. Gerlach and others showed that larval zebrafish could distinguish between kin and non-kin when olfactory cues were provided at 6dpf[352]. Since then, further studies have revealed that zebrafish are not limited to discrimination through early imprinting but can readily distinguish between familiar and unfamiliar conspecifics in social preference tests, consistently spending more time near new/unknown fish[358]. Zebrafish learning starts to form reliably at around three weeks, reaching maximum performance at around six weeks[359]. Additionally, studies have shown that, like humans, adult zebrafish can learn from one another. Naïve zebrafish readily escaped from an approaching "trawl" net, opting to take routes learnt from a trained demonstrator fish, and the behaviour was reproducible across three generations of social learning[360].

Lastly, zebrafish social behaviour can also be modulated pharmacologically, as shown with the use of Buspirone in **Chapter 4** of this thesis. There is mounting evidence that neurochemical alterations can serve as a reliable biomarker of zebrafish state, e.g., changes in brain monoamine levels mediated by social interactions[361]. Studies have also shown that pharmacological modulation of these monoamine levels and other neuropeptides such as oxytocin, social behaviours can, in turn, be modulated[113,291,358]. Furthermore, several physiological biomarkers conventionally explored in stress research (e.g., brain c-Fos expression and whole-body cortisol levels) are strongly correlated with stress and anxiety-like behaviours in zebrafish. Additionally, these behaviours can also be modulated by pharmaceutical interventions[362,363]. The pharmacological accessibility of zebrafish makes it a powerful translational model to study the molecular mechanisms of social behaviour.

Aims of the thesis

The work described in this thesis uses juvenile zebrafish to study how the social brain circuit gives rise to a wide diversity of social preference behaviours and how environmental adversity, such as isolation, can lead to socially dysfunctional behaviour during development. These specific aims can be described in terms of four sequential steps:

1. Characterise social preference behaviour of juvenile zebrafish population, mainly focusing on two extreme and opposite social phenotypes, asocial and prosocial (**Chapter 2**).
2. Investigate the effects of early life chronic and acute isolation on behaviour, including but not limited to social preference (**Chapter 3**) of asocial and prosocial phenotypes.
3. Map the social brain network in the whole brain of juvenile zebrafish, comparing functional responses of individuals reared in social and isolated settings (**Chapter 4**).
4. Establish whether and how impaired social preference behaviour can be rescued through pharmacological manipulation (**Chapter 4**).

Chapter 2: Social Preference

Chapter 2 Summary

In this chapter, the stability and robustness of social preferences are assessed. Following this, social preference behaviour of socially reared three-week-old juvenile zebrafish is characterised together with eleven additional parameters, namely: proximity to conspecifics, the average location of fish, the total number of entries into predefined areas, total number of bouts, bout duration, total distance travelled, percentage of time spent freezing, percentage of time spent moving, absolute X motion and absolute Y motion and body orientation. The social preference index is also validated with subsequent use of these parameters. Finally, the behavioural responses of asocial and prosocial phenotypes towards conspecifics are comprehensively described using the parameters above.

2.1. Introduction

Defining social preference

The definition of social preference differs across various fields of study, and even within these fields, there is still no agreement on the term's precise meaning. Whilst economists loosely define social preference as "*a concern for the payoff allocated to other relevant reference agents in addition to the concern for one's own payoff*"[364], in behavioural biology, the term is often used to describe the inclination of individuals to be near others of the same species – conspecifics[365]. The difference in the definitions reflects the purpose of the term in the two fields. The former utilises it to explain the motivation behind a non-self-serving behaviour in humans, whilst the latter uses it to describe a behavioural outcome without any attempt to explain the rationale behind the action of an animal. Throughout this thesis, the behavioural biology definition of social preference is used.

Social preferences in zebrafish

Typically, conspecifics are a rewarding stimulus for zebrafish; however, not all conspecifics are equal. For instance, zebrafish are attracted to individuals who share similar colouration and patterning and with whom they were reared[324]. This preference remains stable into adulthood, despite changes in social environments in later life[324]. In addition, zebrafish prefer more active[366] and larger sizes[91,367] of shoals. Finally, social preferences can also be seen in the sexual context, where females usually prefer males with specific physical characteristics, such as larger size[368].

Zebrafish social preference test

To engage in social preference, zebrafish need to be able to identify conspecifics and initiate approach manoeuvres[369]. This fundamental preference is at the basis of all complex social behaviours, such as schooling in zebrafish[322,323,354], and it is innate for social species, including humans. The propensity of zebrafish to be near conspecifics can be assessed using social preference tests similar to those already used in rodent models[352,370].

Social preference assays usually begin with an acclimation phase, with the introduction of test fish into the test environment to allow fish the time to explore. The acclimation duration varies depending on the assay (usually 5-20 minutes)[91,371,372], and on the age of the fish (older fish

acclimate faster to a new environment)[373]. This phase is used to define an individual baseline for each fish to distinguish socialisation from novelty/exploratory behaviour. In addition, it controls for changes in behaviour not related to social preference, e.g., anxiety to a novel space, and allows the elimination of fish that show atypical behaviour (e.g., swimming only on one side of the arena or unhealthy). The acclimation phase is followed by the social/test phase, also varying between 5-20 minutes, where a social cue is presented to the single test fish. Both steps are usually recorded, and behavioural responses are analysed to determine social preference.

Depending on the research question and the study's experimental design, various social stimuli can be used, e.g., individual/multiple, live/pre-recorded, familiar/unfamiliar and size-matched/unmatched to the test fish[91,141,374,375]. The design of the chamber is influenced by the type of social stimulus to be used during testing. In this thesis, the social preference assay consists of two conspecifics, placed in one of two smaller chambers adjoined to a larger rectangular compartment, thus completing the C-shape design (**Figure 2.1 and 2.2**). Separation of the test fish from social stimuli is achieved via a transparent barrier made of clear glass or acrylic.

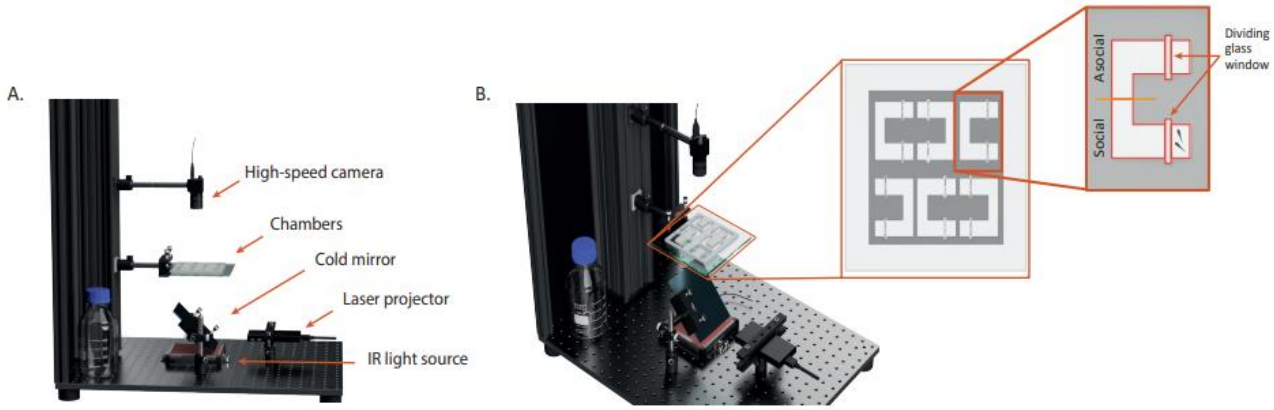


Figure 2.1: CAD drawings of arrangement generated on 360 Fusion. A. Side profile of the assay setup with labels. White light from a laser projector is delivered to the chambers from below through a cold mirror. IR light source provides homogeneous lighting for recording with a high-speed camera fitted with an IR filter. **B.** Angled view of the same design shown in **A.** The inset displays the arrangement of six chambers and shows the C-shaped design of a single chamber. Conspecifics are separated from the test fish by a glass window as depicted. The orange line depicts the division of the chamber into two equal regions: asocial, the side with no stimulus and social, with the conspecifics.

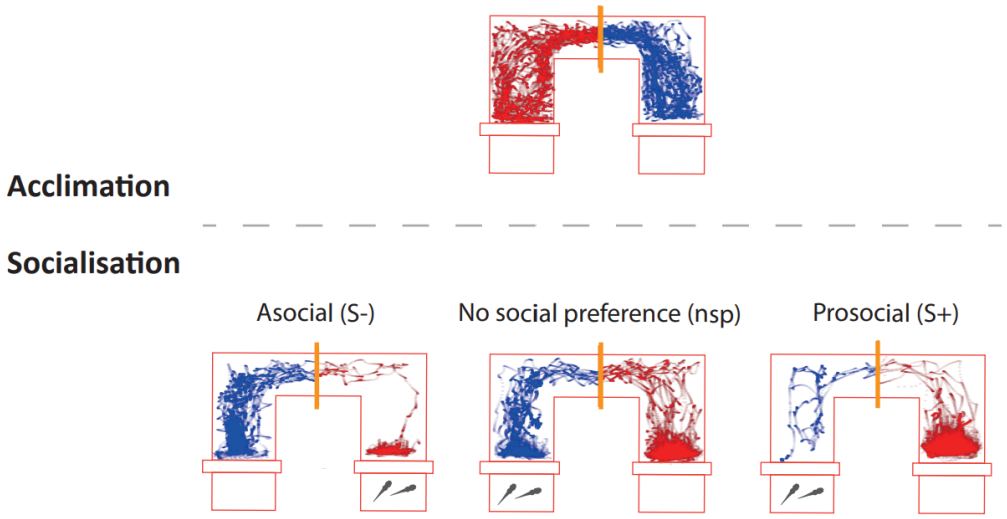


Figure 2.2: Example of fish tracking fish during acclimation and socialisation phases. Projected fish tracking examples given for each subpopulation as determined by social preference. Asocial (S-) fish spend more time in the asocial side of the chamber, no preference (NSP) fish display no preference between either side, and prosocial fish (S+) spend more time in the social side of the chamber. Blue and red indicate motion in the asocial and social side of the chamber, respectively.

Regardless of the chamber design employed, social preference is usually assessed by quantifying the time spent by the test fish near the social stimulus and expressed either as a percentage of total time or a score on an index scale ranging from -1 to 1[91,371,376]. Occasionally, the social preference chamber may be divided into zones reflecting their distance from social stimuli, with most proximal areas termed 'strong' and the farthest areas 'weak' serving a similar purpose to quantify social preference[372,377]. The work presented in this thesis uses an index scale to measure social preference, called the 'Visual Preference Index' (VPI), where scores are representative of the time fish spend near conspecifics (see **Material and Methods: Visual Preference Index** for greater detail, including formula).

Several behavioural parameters can be extracted from recordings to reveal more about individual experiences during testing. For example, fish entries and latency to enter predefined areas of a chamber, time spent swimming close to the surface of a tank, average inter- distances between individuals, average speeds, and time spent freezing may all be used to assess anxiety in response to novel environments and stimuli. Combining social preference scores with the mentioned behavioural parameters produces a powerful tool for evaluating the conspecific perception of socially deprived fish.

A quantitative measure of sociality

Several continuous indices have been developed to quantify various aspects of sociality, including reproductive skew[378,379], dominance hierarchies[374] and social complexity[380]; however, such indices only loosely measure social preference at the core of all social behaviours. One common approach to calculating social preference in zebrafish is to measure the percentage of time that test fish spend near conspecifics[91,374], such as the Visual Preference Index (VPI). Although extensively used, little is currently known about how such indices, created around one parameter, truly represents social preference and to what extent it can represent several other features of social behaviour. Therefore, investigating and validating such indexes is essential for their correct application and evaluation of individual preferences.

Stability of social preferences

It is known that initial behavioural responses of an individual to a sensory stimulus may not be reproduced in subsequent exposures[381,382]. For instance, male rats presented with an intruder male may initially attack but decide to ignore it on the next contact[383]. Thus, the same individual can often differ in behavioural progression and action selection despite the same sensory cues. The behavioural passage may also be relevant for social preference behaviours in the presence of conspecifics, particularly during development when preferences may not be established, and plasticity is described to be at its highest.

Longitudinal studies have shown that social preference in humans remains stable over extended periods in both adults[384] and children[385,386]. However, few studies have looked at changes in choice on shorter timescales (i.e., 24 hours) despite its importance in reproducing results and the potential impact on sample sizes. Thus, this thesis also investigates whether juvenile zebrafish show alterations in social preference over a testing day.

2.2. Results

A two-choice behavioural assay was used to assess social preference exhibited by 3-week-old juvenile zebrafish in response to the presentation of conspecifics - two age and size-matched conspecifics. This assay has been previously described in Dreosti et al. 2015 and consists of a C-shaped chamber where a test fish can swim freely (**Figure 2.1 and 2.2**)[91]. Here, test fish were introduced into the chamber and allowed to explore the environment for fifteen minutes. Following this acclimation phase, two conspecifics were randomly assigned to one of the small compartments adjacent to the main chamber and separated by a glass window (**Figure 2.1B**). During this phase, the test fish could see the conspecifics through these glass dividers, and actively choose their proximity to conspecifics within the chamber. After 15 minutes, fish were euthanised and fixed for further analysis.

Quantifying social preference behaviour in the average population

Animal species can be classified into a continuum of social categories that range from solitary, sub-social to social, based on the broad qualitative features of their social systems[387–389]. A wide range of quantitative measurements has been used to describe sociality and social preference in various species[387,388,390], including zebrafish[91,372,374].

Here, social preference - the drive to be with conspecifics – was measured similarly to Dreosti et al., 2015, with some modifications to how the social area of the chamber is defined (see **Materials and Methods - Behavioural test for social choice** for details). The resulting values were scored using a Visual Preference Index (VPI) ranging from 1 (fish spending all its time in the social area) to -1 (fish spending all its time in the asocial area) and used as a proxy for sociality in juvenile zebrafish.

During the acclimation phase, density graphs of VPI values revealed that test fish display no biases for any chamber areas. Evidence of this is the bell-shaped curve of the VPI distribution where equivalent numbers of fish are found around a central value of 0 in the population (**Figure 2.3A**; VPI < 0: n = 191, VPI > 0: n = 189, skewness = 0.012 and kurtosis = -0.81). During the socialisation phase juvenile zebrafish consistently showed a strong preference for social stimuli, in line with the previous studies[91,372]. Specifically, many fish showed VPI values close to 1 (reflecting a strong bias to remain almost entirely on the side with social cues), the net effect of which resulted in a shift of the median VPI value towards 1 in the socialisation phase (**Figure 2.3B**: skewness = -0.868, kurtosis = -0.253) (**Figure 2.3A and B**; Acclimation: Med = -0.00213; vs; Socialisation: Med = 0.55108; ,*Wilcoxon signed-rank test*, w = 12582, $p \leq 0.0001$, n = 380).

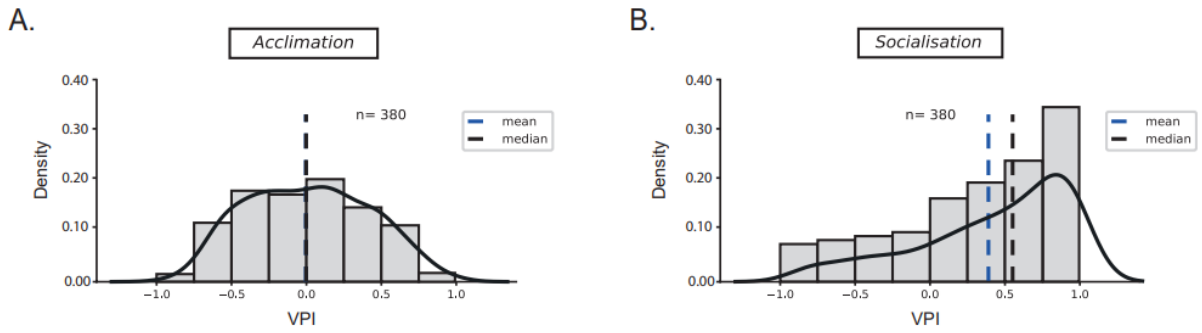


Figure 2.3: Distribution of social preference in the average juvenile zebrafish population. Visual Preference Index (VPI) used as a measure of sociality. A value of +1.0 indicates high social preference behaviour, and -1.0 indicates avoidance behaviour. The data represent VPI of fish. **A.** VPIs of fish are normally distributed during fifteen minutes of the acclimation period. Density is indicated by a continuous black line overlaying histogram. Skewness = 0.102 and kurtosis = -0.81. The blue dashed line indicates the mean, and the black dashed line indicates the median (0.0046 and -0.0021, respectively). **B.** VPI distribution of the same fish as in A. during fifteen minutes with exposure to conspecifics. The presence of conspecifics significantly alters VPI distribution, shifting the density to the right ($p \leq 0.05$). Skewness = -0.86 and kurtosis = -0.254. Mean and median, 0.390 and 0.55108, respectively. n = 380.

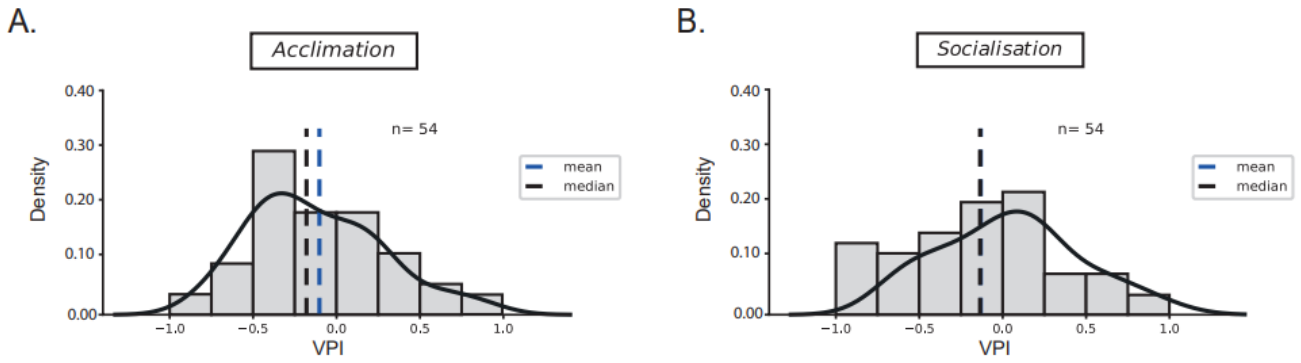


Figure 2.4: Juvenile zebrafish maintain exploratory behaviour in the absence of conspecific. A. Visual Preference Index (VPI) is normally distributed during acclimation**.** Density is indicated by a continuous black line overlaying histogram. Skewness = 0.548 and kurtosis = 0.234. The blue dashed line indicates the mean, and the black dashed line indicates the median (-0.1024 and -0.1798, respectively). **B.** VPI of the same fish as in **A.** during additional fifteen minutes in the assay without exposure to conspecifics. No significant change in VPI distribution is observed ($p > 0.05$). Skewness = 0.193 and kurtosis = 0.5532. Mean and median, -0.1313 and -0.13549, respectively. n = 54.

To understand whether this observed shift in VPI could be attributed to the presence of cues, the VPIs of fish subjected to the assay in the absence of conspecifics, NSC fish, were also analysed and compared first across assay phases (**Figure 2.4A** and **B**) then across conditions (**Figure 2.3B** vs. **Figure 2.4B**). Since NSC fish were never tested in the presence of conspecifics, 'asocial side' and 'social side' areas of the testing chamber were randomly assigned to determine acclimation and second phase VPIs, where the latter phase is equivalent to the socialisation phases in fish tested with conspecifics.

Initial comparison of VPI density graphs of NSC fish across acclimation and socialisation sessions revealed no notable change in the distribution of VPI values, with both acclimation and socialisation data presenting a near to normal spread (**Figure 2.4A** and **B**; Acclimation: *Med* = -0.17981, skewness = 0.564, kurtosis = 0.234; vs; Socialisation with no cues (NSC); *Med* = -0.13549, skewness = 0.193, kurtosis = 0.553;*Wilcoxon signed-rank test*, w = 737, p = 0.96223, n = 54). This result indicated that NSC fish maintain exploratory behaviour throughout the two experimental phases. Further comparison of VPIs across conditions showed that fish in the presence of conspecifics presented greater VPI values compared to NSC fish (**Figure 2.3B** vs **4B**; Controls: *Med* = 0.55108, *n* = 380; NSC: *Med = -0.13549, n* = 54; , *Mann-Whitney U test*, u = 4795, p ≤ 0.0001) indicating that the presence of social cues drives alterations in VPI scores.

To investigate the source of the observed shifts in VPI values across the experimental phases, all fish subjected to the social preference assay (tested with cues and without cues) were divided into three phenotypic groups, each representing different levels of sociality as determined by VPI scores acquired in the second experimental phase: a) asocial (S-) fish with VPIs equal to and below -0.5; b) no-social preference fish with -0.5 < VPI < 0.5; and c) prosocial (S+) fish with VPIs equal to and above 0.5.

The evaluation of VPI values of fish tested with social cues revealed that the proportion of asocial animals was not altered across experimental phases, staying on average around 12% of the total population (**Table 2.1**). However, no-social preference fish and prosocial fish groups did not follow this trend, exhibiting marked changes across phases. More precisely, the proportion of the no-social preference fish group showed a significant reduction (~36%), whilst the prosocial fish group exhibited a significant increase (~39%) in the proportion of animals assigned during socialisation (**Table 2.1**; Acclimation vs Socialisation: No-social preference (NSP): Students paired t-test, t = 7.15744, p = 0.00025; Prosocial (S+): Students paired t-test, t = 10.73887, p = 0.00025). These results suggest that the source of the shift observed in VPIs is predominantly through alterations in the proportion of no-social preference fish. The change in the number of prosocial fish increased almost correspondingly to the decrease observed in the no-social preference fish group, with the asocial fish contributing minimally. Hence, cues motivate test fish to make an active choice in the amount of time they spend in the two sides of the assay chamber.

A similar evaluation of NSC fish social groups proved that VPI values across experimental phases are robust on a population level (**Table 2.1**, No Social Cue (NSC)), providing further evidence that social preference is visually motivated.

In summary, social preference is visually mediated by the presence of conspecifics. A spectrum of social preferences can be readily observed within the average population of juvenile zebrafish, with a more significant proportion of fish displaying prosocial behaviour when given conspecifics.

Condition	Experimental phase	Proportion of asocial fish (S-)	Proportion of no-social preference fish (nsp)	Proportion of prosocial fish (S+)
No Social Cue	Acclimation	0.130	0.778	0.0923
	Socialisation	0.241	0.649	0.111
Social Cue	Acclimation	0.137	0.729	0.134
	Socialisation	0.105	0.368	0.526

Condition	Comparisons	Test statistic	p-value
No Social Cue	Acclimation (S-) vs Socialisation (S-)	1.36196	0.08973
	Acclimation (nsp) vs Socialisation (nsp)	0.79813	0.79824
	Acclimation (S+) vs Socialisation (S+)	0.29309	0.38554
Social Cue	Acclimation (S-) vs Socialisation (S-)	1.27073	0.10255
	Acclimation (nsp) vs Socialisation (nsp)	7.15744	0.00025 ***
	Acclimation (S+) vs Socialisation (S+)	10.73887	0.00025 ***

Table 2.1: The presence of conspecifics reveals the social preference of test fish. Two independent fish populations tested with differing socialisation phase conditions in the assay: fish with or without social cues, n = 380, n = 54, respectively. Students Paired T-test was used to compare the number of fish assigned to each social preference group as determined by VPI scores: asocial (S-, VPIs ≤ -0.5), no social preference (NSP, -0.5 < VPI < 0.5) and prosocial (S+, VPIs ≥ 0.5) across acclimation and socialisation phases for each testing condition. Conspecifics result in a significant increase in the proportion of fish assigned to no-social preference and prosocial groups. Asterisks indicate significance ($p \leq 0.05$).

Differences in sociality at two different time points in the day (Morning vs Afternoon)

Activity and resting periods are determined by the daily rhythms of light/dark cycles. Like humans, zebrafish show a diurnal activity pattern and sleep during the dark period; furthermore, alterations in visual sensitivity and locomotive activity have also been reported in zebrafish studies throughout a day[391–393]. Therefore, visual acuity towards conspecifics and locomotive activity

conducted at the start and end of the testing day was assessed to determine whether all behavioural data could be pooled together for more comprehensive behaviour analysis.

Social preference is unaffected over the testing day period

Comparisons of VPI for AM and PM sessions revealed morning social preference values to be distributed evenly during acclimation but tending more towards prosocial (VPI > 0.5) during socialisation (**Figure 2.5A and B**: Morning (AM); Acclimation: *Med* = -0.065; vs; Socialisation: *Med* = 0.570; *Mann-Whitney U test, u* = 686, *p* = ≤ 0.0001, *n* = 90, ; and Afternoon (PM): Acclimation: *Med* = -0.077; vs; Socialisation: *Med* = 0.593; *Mann-Whitney U test, u* = 7.3, p ≤ 0.0001, *n* = 90).

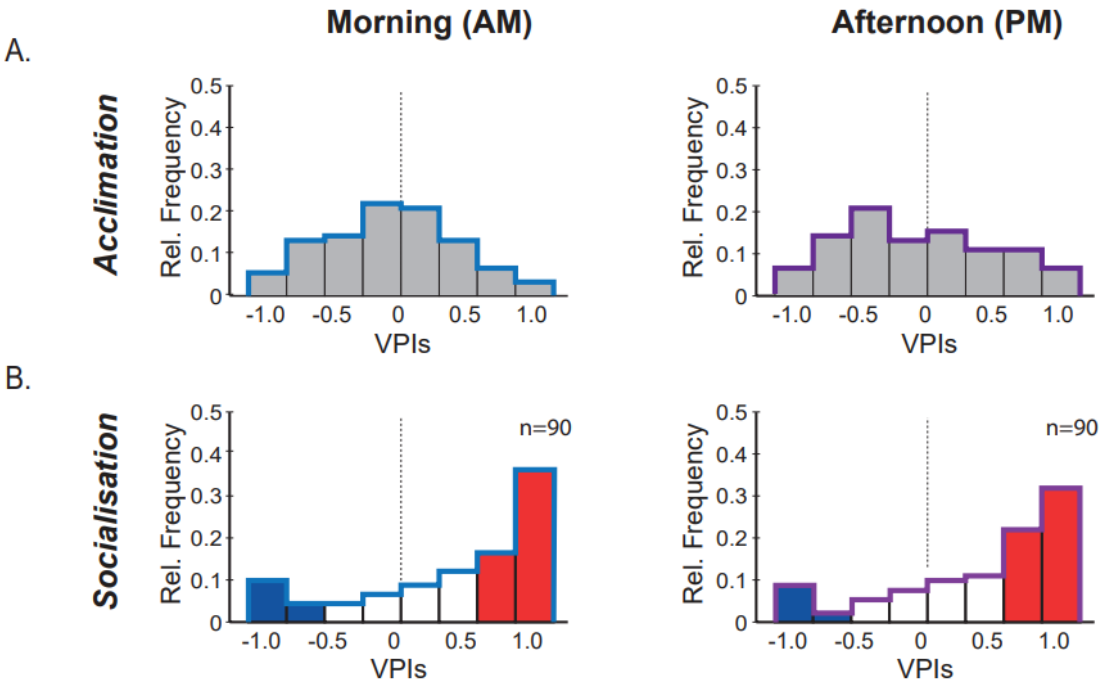


Figure 2.5: The social preference behaviour of fish tested in morning and afternoon sessions. A. Histogram displaying morning and afternoon VPIs of fish during acclimation. **B.** Histogram of VPIs in the presence of conspecifics. For visual clarity, blue bars highlight asocial fish (S-, VPIs ≤ -0.5), white no social preference fish (NSP, -0.5 < VPI < 0.5), and red bars highlight prosocial fish (S+, VPIs ≥ 0.5). n = 90 and n = 90. Note the increase in the relative frequency of VPI values above 0.5 in both morning and afternoon sessions during socialisation.

Temporal inspections of VPIs in one-minute intervals further supported this finding with similar trends observed for AM and PM sessions (Morning (AM); Acclimation vs Socialisation: two-

fifteen minutes, *n* = 90, p > 0.05, and Afternoon (PM); Acclimation vs Socialisation: one-fifteen minutes, *n* = 90, p > 0.05).

Further comparisons of median VPIs between AM and PM sessions during acclimation and socialisation showed no notable differences, suggesting that test fish habituate similarly in the chamber and to social cues (**Figure 2.5A**; Morning (AM): *Med* = -0.065, *n* = 90; vs; Afternoon (PM): *Med* = -0.077, *n* = 90; *Mann-Whitney U test, u* = 4042, *p* = 0.49144) (**Figure 2.5B**; Morning (AM): *Med* = 0.57, *n* = 90; vs; Afternoon (PM): *Med* = 0.59, *n* = 90; *Mann-Whitney U test, u* = 4026.5, *p* = 0.47375). Once again, temporal analysis of VPIs further supported these findings (**Figure 2.6**, Acclimation: Morning (AM), *n* = 90, vs, Afternoon (PM), *n* = 90; one-thirteen minutes and fifteen, p>0.05) (**Figure 2.6**, Socialisation: Morning (AM), *n* = 90, vs, Afternoon (PM); two-fifteen minutes; *n* = 90; *p* = >0.05).

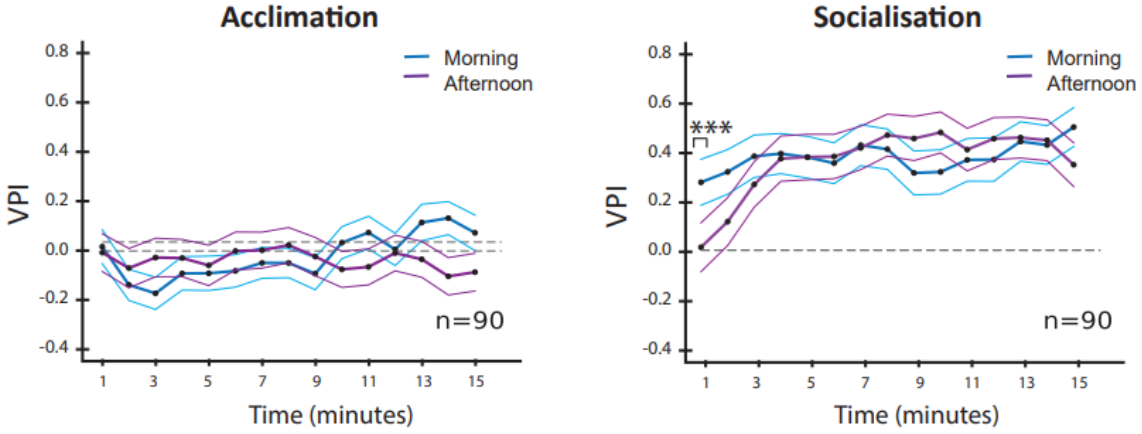


Figure 2.6: Temporal VPI scores of fish tested in morning or afternoon sessions. Acclimation and socialisation VPIs presented as one-minute bins. The thin lines indicate the standard error. Asterisks mark significant differences (p-value = 0.035, Mann-Whitney U-Test, n = 90).

The presence of conspecifics overcomes initial morning and afternoon differences in movement activity

Comparison of movement activity between AM and PM testing sessions revealed that juvenile fish tested in the mornings spent a more significant percentage of their time in the assay moving during acclimation than fish sampled in the afternoon (**Figure 2.7A**, Acclimation; AM: *Med* = 27.02%, *n* = 90; vs; PM: *Med* = 21.16%, *n* = 90; *Mann-Whitney U test, u* = 3056, *p* = 0.00224).

Further comparisons of movement activity between morning and afternoon sessions, in one-minute bins, revealed differences in percentage time fish spent moving were detectable across thirteen of the fifteen minutes comprising the acclimation period (**Figure 2.7B**, Acclimation; one-thirteen minutes, AM: *n* = 90; vs; PM: *n* = 90; $p \leq 0.05$). The comparability of movement activity supported the use of fifteen minutes period for acclimation.

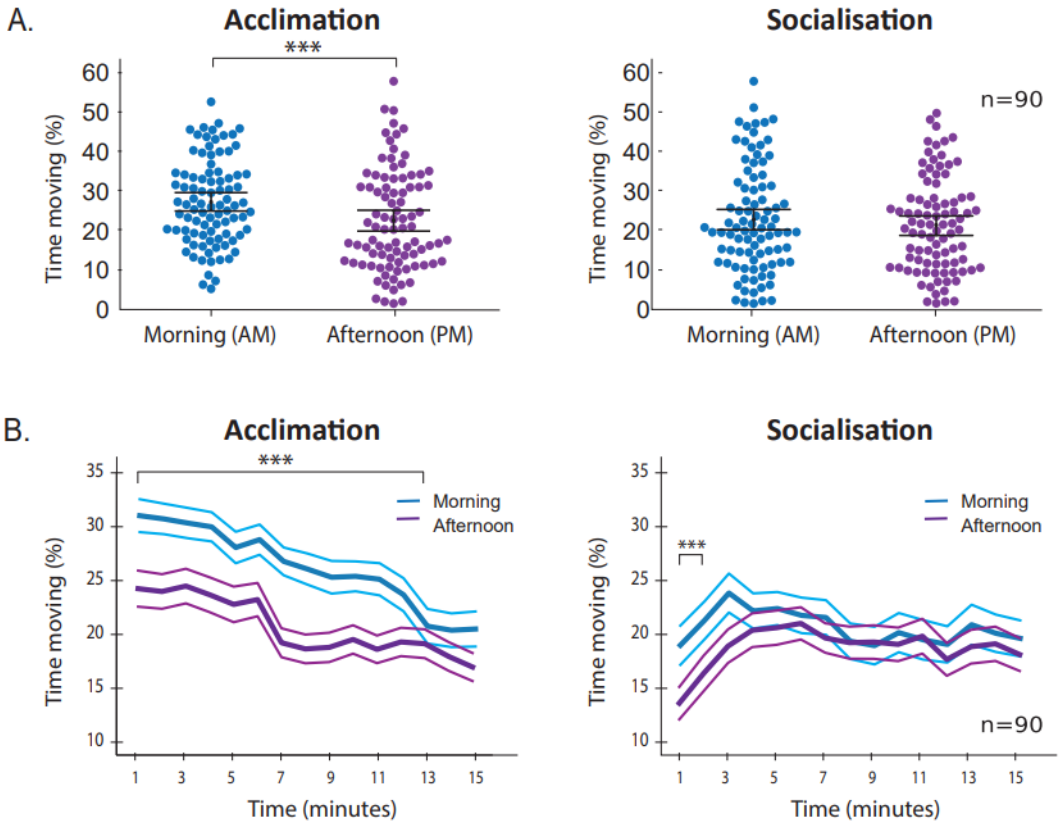


Figure 2.7: Test fish movement activity is comparable during social interaction despite initial differences in average baseline motion. A. Swarm plots comparing average activity levels of fish during acclimation and socialisation periods, expressed as a percentage time moving for each fifteen-minute condition (blue dots morning (AM), purple dots afternoon (PM), n = 90). Black lines indicate 95% confidence intervals. **B.** Percentage of time moving displayed as one-minute bins. Thin lines indicate standard error. Sample sizes shown on the right panels. Asterisks mark significant differences ($p \leq 0.05$) between AM and PM conditions.

In contrast, no considerable difference in time spent moving was detected between AM and PM sessions in the presence of conspecifics (**Figure 2.7A**, Socialisation; AM: *Med* = 20.03%, *n* = 90; vs; PM: *Med* = 20.79%, *n* = 90; *Mann-Whitney U test, u = 3871*, *p* = 0.30479), indicating that initial difference in the time AM and PM fish spent moving are overcome by the introduction of

conspecifics. Temporal comparison of movement activity between AM and PM sessions revealed an initial difference in the first three minutes of socialisation with no differences over the remaining twelve minutes (**Figure 2.7B**, Socialisation; one-three minutes, AM: *n* = 90; vs; PM: *n* = 90; $p \geq 0.05$; four-fifteen minutes, AM: *n* = 90; vs; PM: *n* = 90; $p \geq 0.05$). The short timeframe by which differences in movement activity become negligible indicates the presence of conspecifics as a potent stimulus.

Since no differences in VPI were observed across the early morning and late afternoon sessions and movement activity were comparable between fish tested in the early morning and late afternoon sessions in the presence of conspecifics, these results justified combining behavioural data obtained.

Stability of social preference in the same fish

Robustness of social preference in individual zebrafish has only been studied over long periods of development[394,395]. Yet it is possible preferences may change over shorter timescales such as a single day. Therefore, to test the robustness of social choice over a shorter course of time, for example, a single day, and thus the ability to accurately identifying asocial and prosocial phenotypes, VPI of fish exposure to the social preference assay once in the morning (AM_1st) and again in the afternoon (PM_2nd) were assessed.

Analysis was performed on VPIs in the morning or afternoon at the whole population level. These data showed no significant differences in acclimation or socialisation phases (**Figure 2.8A**, Acclimation: AM_1st: *Med* = 0.045; vs; PM_2nd: *Med* = 0.23; *Wilcoxon signed-rank test*, w = 1608, *p* = 0.80477, *n* = 81) (**Figure 2.8B**, Socialisation: AM_1st: *Med* = 0.68; vs; PM_2nd: *Med* = 0.60; *Wilcoxon signed-rank test*, w = 1420, *p* = 0.25849, *n* = 81).

To eliminate chance in the coupling of observed AM_1st and PM_2nd VPI values, and since socialisation values across morning and afternoon sessions did not follow the normal distribution, bootstrapping analysis was used to construct 10,000 pseudo-samples with 81 observations (see Methods for more details). Comparisons of the true and the pseudo-sample means revealed a significant difference (**Figure 2.9**: *True mean* = -0.1311, n = 81; vs; *pseudo mean* = 0.53842, *n* = 10,000; *p* = 0.00410, Two-sample T-test), indicating that the correlation between AM_1st and PM_2nd VPIs was not obtained by chance.

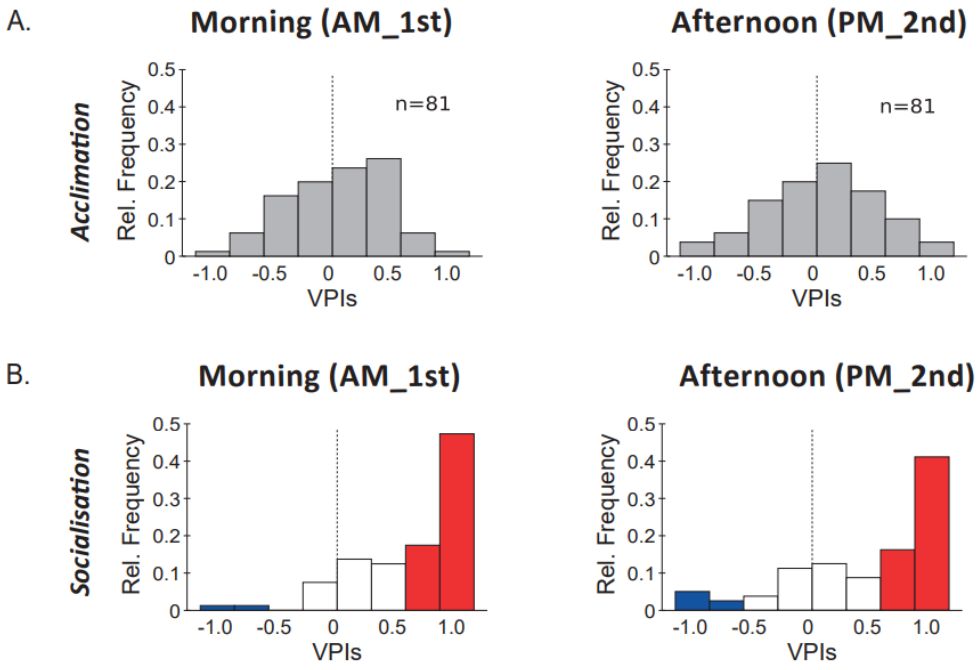


Figure 2.8: Social preference behaviour is robust to repeated testing. VPI Histograms of fish exposed to the social preference assay twice, once in the morning and again in the afternoon. **A.** Comparison of VPIs in the absence of conspecifics during morning and afternoon. **B.** Similar comparison as **A.** in the presence of conspecifics. For visual clarity, blue bars highlight asocial fish (S-, VPIs ≤ -0.5); white bars no social preference fish (NSP, -0.5 < VPI < 0.5); and red bars highlight prosocial fish (S+, VPIs ≥ 0.5). n = 81

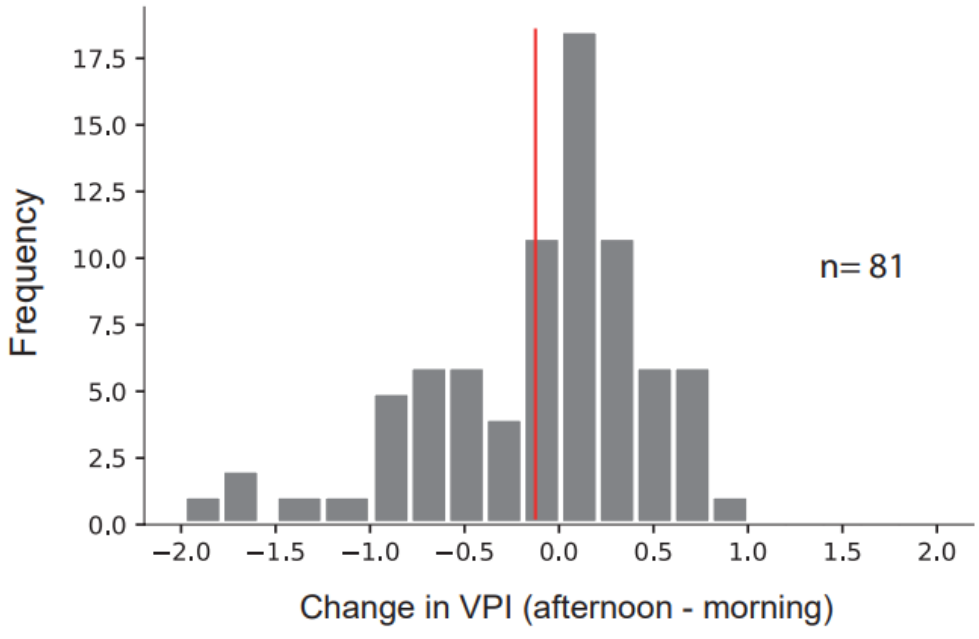


Figure 2.9: Morning and repeated afternoon VPIs are not coupled by chance. Bootstrapping analysis of the average change in VPI of fish with repeated exposure to the assay (morning and afternoon). The red line indicates the mean difference (-0.1311) of the true data of 81 fish, and grey bars show the average change in VPI of pseudo-dataset generated by 10,000 permutations of decoupling and recoupling the true dataset. The difference is calculated by subtracting morning VPI from afternoon VPI (PM_2nd - AM_1st). Fish with VPIs higher in morning sessions than afternoon are represented with negative values (PM_2nd < AM_1st), conversely fish with afternoon VPIs higher than morning sessions are illustrated with positive values (PM_2nd > AM_1st). A value of 0 indicates no differences across the two conditions (PM_2nd = AM_1st).

Since population-level differences in measurements of VPI were comparable, it was predicted that morning and afternoon should be positively correlated. A linear regression model was used to predict afternoon VPIs based on individual fish's morning values (**Figure 2.10**). Analysis of VPIs revealed, as expected, a significant correlation between VPIs ($F(1, 80) = -2992.17$, $p = 0.00912$), with an R^2 of 0.0823. Although a low R^2 value was identified, suggesting high variability in the data, the trend indicates that morning VPIs still provide information about expected afternoon VPI values even though the data points fall farther from the regression line. A closer analysis revealed that only 5% of test fish displayed extreme switching between VPI groups, and 40% of test fish showed no switching between social groups, with the remaining 55% of fish displaying more minor switching to and from non-social preference groups.

Together, these results demonstrate that strong preferences, such as asocial and prosocial phenotypes, both within the population and at the single fish level are robust enough to be accurately identified over a single testing day.

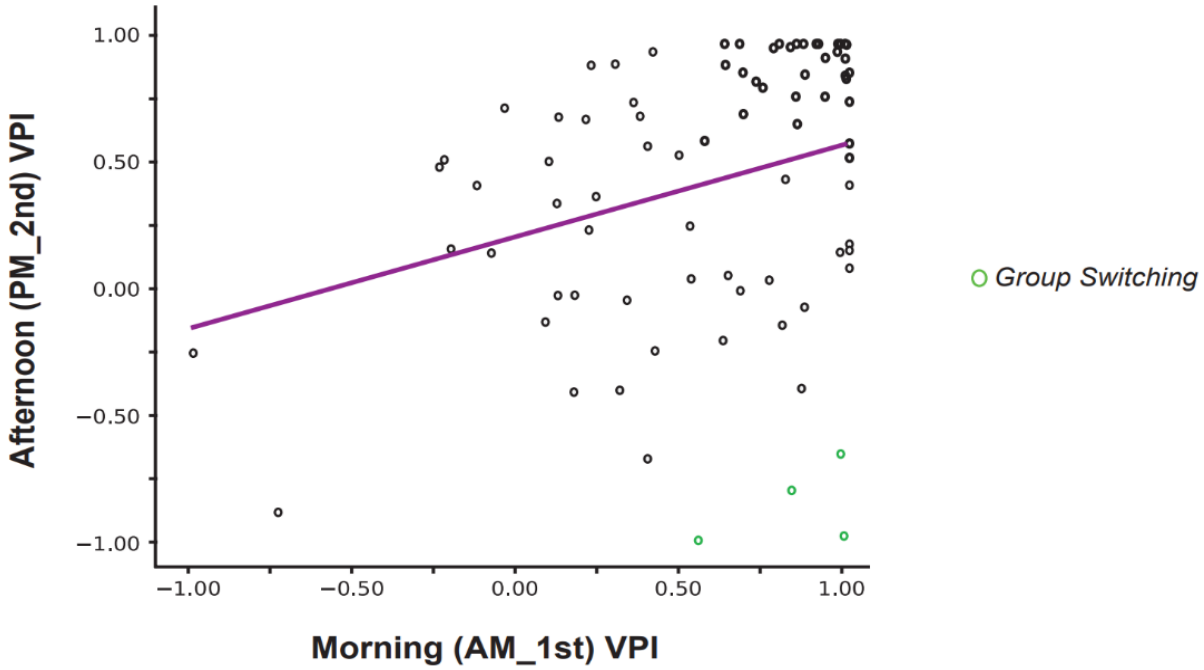


Figure 2.10: Morning and repeated afternoon VPIs are correlated. Scatterplot of VPIs across morning and afternoon sessions. Green circles high light fish that display significant changes in social preference behaviour between single (morning) and repeated (afternoon) measurements (i.e., asocial to prosocial or vice versa only). The regression line is shown in purple. n = 81.

Characterising juvenile fish social preference behaviour

After establishing that behavioural data collected from various time points during the day can be pooled and that single measurements are sufficient to identify asocial and prosocial phenotypes, the social behaviour of juvenile zebrafish was subsequently analysed.

A total of eleven parameters, previously used to describe behaviour dynamics both in rodents and fish[91,353,374,395–397], were selected to comprehensively characterise the social preference behaviour of 380 juvenile zebrafish (**Figure 2.11** and **2.12**). These included: proximity to conspecifics, the average location of fish, the total number of entries into predefined areas, the total number of bouts, bout duration, total distance travelled, percentage of time spent freezing, percentage of time spent moving, absolute X motion, absolute Y motion, and body orientations.

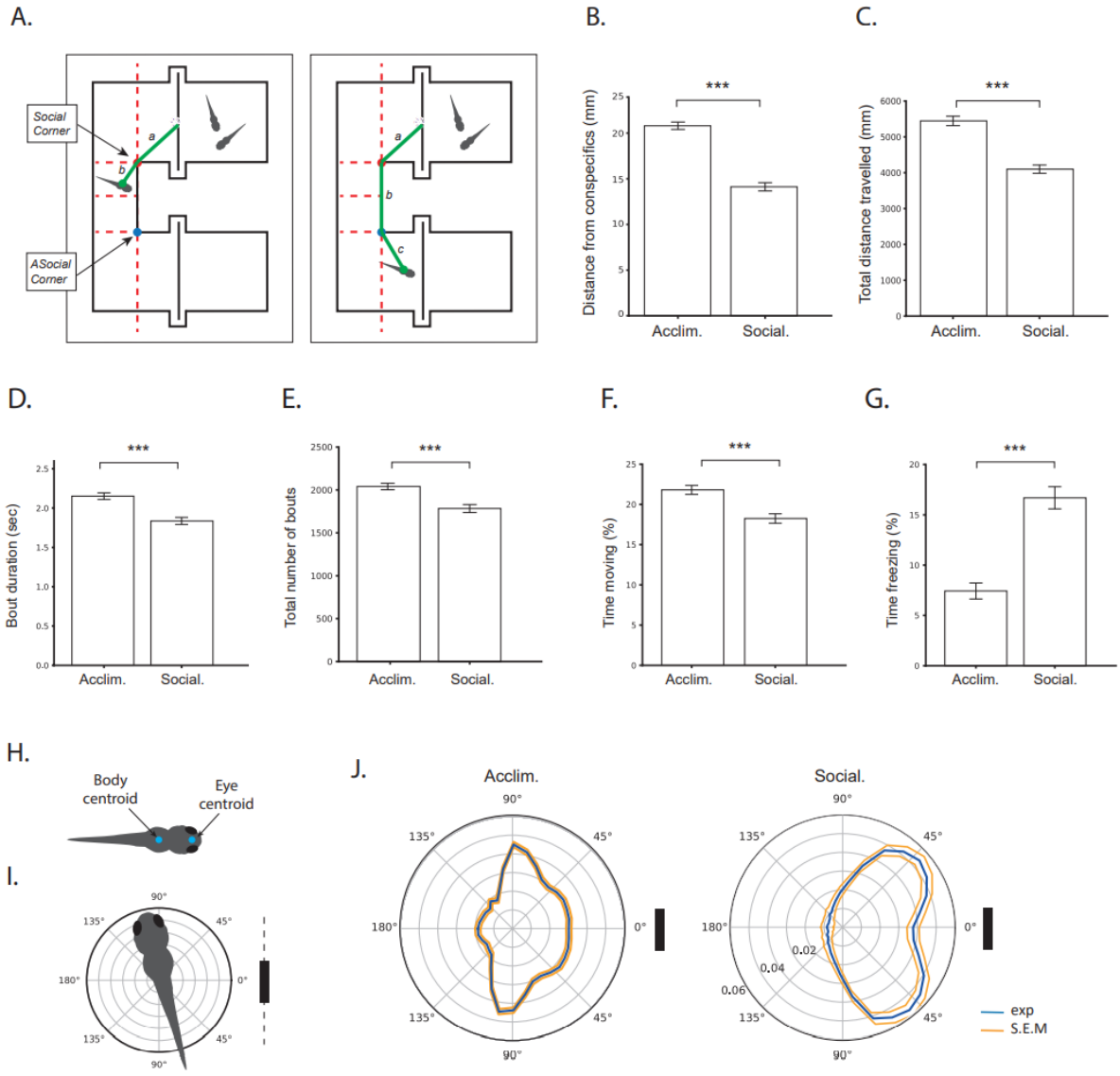


Figure 2.11: Fish alter behaviour across experimental phases. Comparison of measured metrics during acclimation and socialisation phases. **A.** Examples show how the distance of the test fish from the conspecifics is calculated for two locations. Distances from conspecifics are calculated as the shortest length to the closest inner corner with the distance to the middle point of the glass window separating conspecifics. B and C: During social cue exposure, fish spend more time near the conspecifics (B) and exhibit less explorative behaviour; hence a significant reduction in total distance travelled (C). **D to G.**: Fish display a significant reduction in bout duration (D), the total number of bouts (E) with a substantial decrease in motion (F) and an increase in pauses ((G) freezes > three seconds). Thin lines indicate standard error or the mean. H and **I.**: Schematic depicting how body orientations of the test fish are calculated relative to conspecifics. **J.** Polar histograms, averaged across all tested fish, of body orientations of the observer fish when within the social side of the chamber. Data for acclimation and socialisation are presented. Test fish interact with conspecifics at 45 degrees. Conspecifics are located at zero degrees, indicated as a black line. Numbers indicate the relative frequency, and orange lines standard error. Statistics performed by Wilcoxon signed-rank test comparing acclimation and socialisation phases. Asterisks indicate significance ($p \leq 0.05$). B to G: n = 380 and J: n = 366.

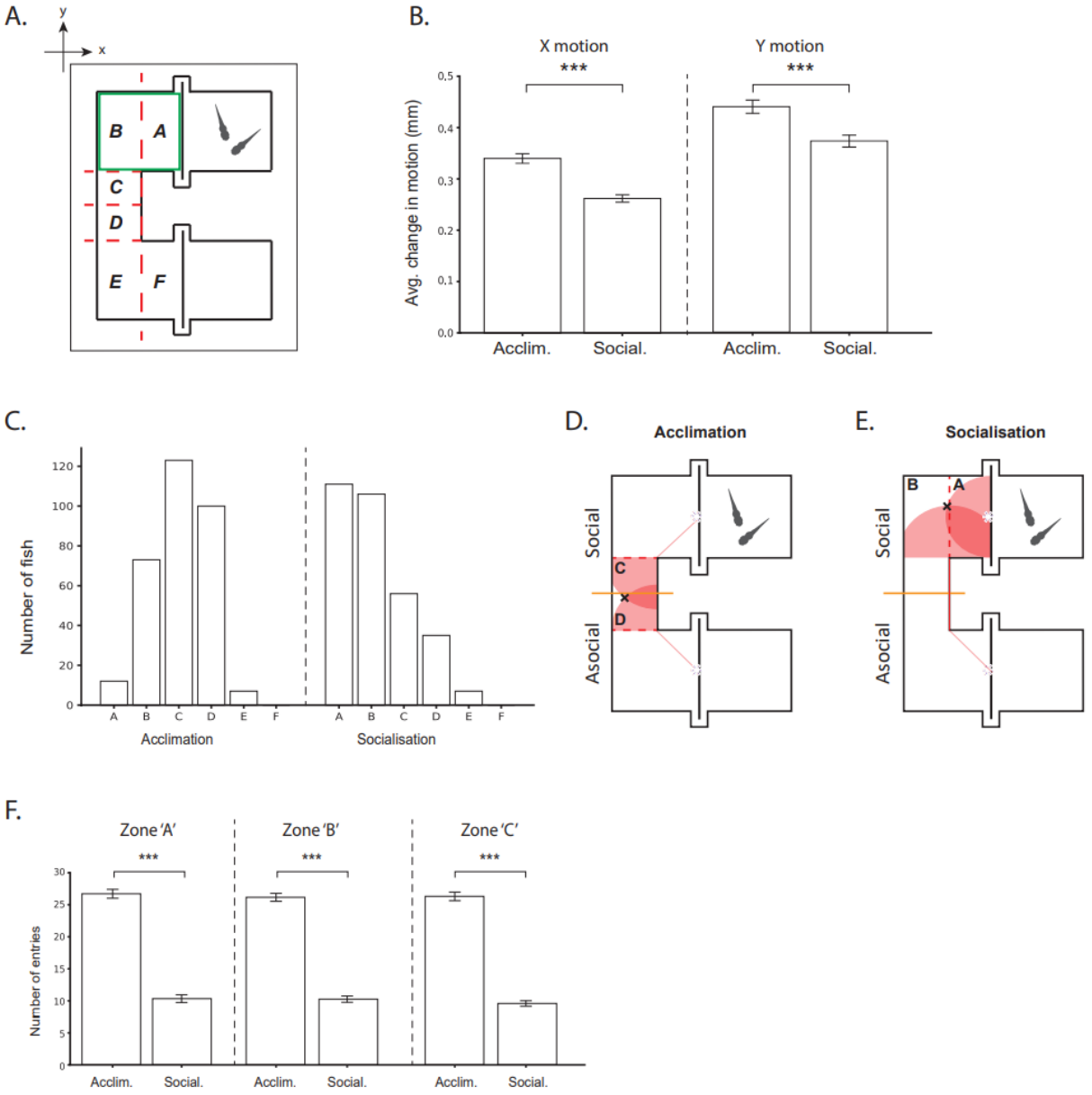


Figure 2.12: Juvenile fish navigate through the chamber differently in the presence of social cues. A.: Schematic of the division of the assay chamber into six regions. Zone 'AB' is indicated in green. **B.**: Total vertical and lateral movement of fish, X and Y motion, respectively, relative to the centre of the social window. **C.** Average position of test fish given as corresponding zones during acclimation and socialisation. **D. to E.**: Schematic displaying the average position of fish across all frames. Indicated are adjacent zones and the social and asocial sides of the chamber used for calculating social preference. **F.**: Total number of entries performed into zones A, B and C, which form the social side of the chamber. Red areas highlight distance from social or asocial windows. In the presence of conspecifics, juvenile zebrafish reduce exploratory behaviour resulting in a significant reduction in motion observed in zone 'AB' (B) and a decrease in entries into other zones (F). Fish display a preference to be near social cues (D to E). Thin lines indicate the standard error of the mean. Statistics performed by Wilcoxon signed-rank test comparing acclimation and socialisation phases. Asterisks indicate significance ($p \leq 0.05$). Sample sizes are as follows: A, and C to F: n = 380; and B: n = 366.

Fish proximity to the social window is increased in the presence of conspecifics

Test fish proximity to the social window that separated them from their siblings was assessed to investigate how conspecifics may be perceived by test fish, for example, frightening or rewarding (see **Material and Methods** for details).

Juvenile zebrafish displayed no bias to approach either of the two glass windows during acclimation and typically maintained a median (*Mdn*) distance of 20.84 mm (Acclimation, Social window: *Med* = 21.06 mm; Asocial window: *Med* = 20.62 mm; *Wilcoxon signed-rank test*, w = 24802, *p* = 0.47301, *n* = 380). In the presence of conspecifics, however, test fish significantly increased their proximity to the glass window, displaying a strong preference to be closer to conspecifics, in line with previous studies (**Figure 2.11B**; Acclimation, Social window: *Med* = 26.72 mm; Socialisation, Social window: *Med* = 13.11 mm; *Wilcoxon signed-rank test*, w = 7094, p ≤ 0.0001, *n* = 380).

To evaluate whether this behavioural change was specific to the addition of a social cue and not to the fatigue of fish, for instance, the behaviour of test fish was compared to fish tested in the absence of social cues (Non-Social Cues, NSC). Test fish in social cues exhibited shorter distances and closer proximity to the window that divided them from conspecifics (Socialisation with cues: *Med* = 13.11 mm, *n* = 380; NSC: *Med* = 21.95 mm, *n* = 54; *Mann-Whitney U test, u* = 647, p ≤ 0.0001). Furthermore, there was no difference between the two conditions during acclimation (p>0.91). Collectively, these results demonstrate that social cues are responsible for closer proximity during the second experimental phase.

Zebrafish navigation of the assay chamber

Like rats, zebrafish show thigmotactic or wall-hugging behaviour in response to anxiety. Thus, fish typically prefer edges with occasional entries to the centre of an open area[375,398,399] in novel environments. Furthermore, zebrafish display a strong attraction for conspecifics[91]. Bringing these details together, it was hypothesised that juvenile zebrafish would travel differently in the chamber across experimental phases and in response to the presence of social cues. To better understand how juvenile zebrafish move around the assay chamber, test fish positions and a total number of entries into predefined regions of the chamber were analysed.

Zebrafish show a place preference to areas nearest to conspecifics

The average position of each test fish (**Figure 2.12F**) was determined across all frames. The average zone where fish resided was obtained by cross-referencing test fish coordinates to one of six predefined zones in the chamber (**Figure 2.12A**). For a visual representation, the average

position within a zone was determined across the acclimation and socialisation phases of the assay.

During acclimation, test fish location analysis revealed that fish preferred to swim back and forth in zones C and D, the narrowest regions of the chamber (**Figure 2.12F**: Acclimation), positioning themselves on average within zone D (**Figure 2.12E**). With the addition of social cues, test fish increased their proximity to social cues and swum predominantly in zones A and B (**Figure 2.12F**: Socialisation). Importantly, this strong preference to be near social stimuli observed in fish tested with cues was absent in NSC fish.

Exploration versus social drive

The analysis of the total number of entries was conducted only on the social side of the chamber (comprised of three zones A, B and C) as the most considerable differences in the number of entries were anticipated in these regions in response to the presence of conspecifics.

Test fish displayed many entries into all social zones during acclimation (**Figure 2.12F**: Zones A, B and C). During the socialisation, test fish performed significantly fewer entries in all social assay zones (**Figure 2.12F**, Acclimation vs Socialisation; Zone A: *Wilcoxon signed-rank test*, w = 1421, $p \leq 0.0001$; Zone B: *Wilcoxon signed-rank test*, w = 1019, $p \leq 0.0001$; Zone C: *Wilcoxon signed-rank test*, w = 1038, $p \leq 0.0001$; $n = 380$). To fully appreciate whether changes in the number of entries were attributable to the viewing of cues, the phase differences across the two testing conditions were compared and this process repeated for each social zone. Results revealed that fish tested in the presence of cues significantly reduced the number of entries they performed into zones B and C compared to NSC fish, indicating that the preference to be with conspecifics drives the observed changes in behaviour in these zones (Differences [Socialisation – Acclimation]: Socialisation with cues: $n = 380$; vs; NSC: $n = 54$; Zone B:*Wilcoxon signed-rank test*, w = 1329.5, $p = 0.05031$; Zone C:*Wilcoxon signed-rank test*, w = 1094.5, $p = 0.005392$).

Although the comparison of entries into zone A across experimental phases between fish tested with and without cues were comparable (Differences [Socialisation – Acclimation]: Socialisation with cues: Med = -16 entries $n = 380$; vs; NSC: Med = -12 entries, $n = 54$; Zone A:*Wilcoxon signed-rank test*, w = 1766, $p = 0.30710$), this result coupled with the average positions of fish (Fish tested with cues: zone B, and NSC: zone C), suggests that the presence of conspecifics alters the movement of fish around the chamber. Fish presented with social cues tend to spend extended periods engaging with conspecifics by confining their actions to areas close to the social window.

Conspecifics motivate test fish to travel further distances

Increased locomotor activation measured as total distance travelled can indicate boldness[397,400], whilst decreased locomotion has been linked to anxiety-like states[397] and a response to a novel environment[401].

Analysis of total distance travelled across the two experimental phases revealed substantial alterations in locomotion (**Figure 2.11C**). During the acclimation phase, test fish typically travelled 5091.45 mm but displayed a marked reduction (30.7%) in total distance travelled in the company of conspecifics (**Figure 2.2C**; Acclimation: *Med* = 5091.45 mm; vs; Socialisation: *Med* = 3896.12 mm*; Wilcoxon signed-rank test*, w = 4894, p ≤ 0.0001, *n* = 380).

Surprisingly, the analysis of NSC fish also revealed a reduction in total distance travelled (NSC: Acclimation: *Med* = 6297.24 mm vs; Socialisation without cues: *Med* = 4707.14 mm;*Wilcoxon signed-rank test*, w = 805, p ≤ 0.0001, *n* = 54). However, this reduction was significantly more in NSC fish (Differences [Socialisation - Acclimation]: Fish with cues: *Med* = -1277.19 mm, n = 380; NSC: *Med* = -1473.25 mm, *n* = 54, *Mann-Whitney U test, u* = 8775, *p* = 0.042605), indicating that the presence of conspecifics altered motivated fish to stay active and thus cover greater total ditances.

Swim bout alterations

Juvenile zebrafish organise their swimming behaviour in sequences of discrete bouts that consist of tail oscillations that propel fish through the water. To assess whether test fish altered the kinetics of their bouts in response to conspecifics, the total number of swim bouts across each experimental phase was extracted and compared across phases and testing conditions.

Total number of swim bouts

Juvenile zebrafish showed a marked reduction in swimming bouts across experimental phases (**Figure 2.11E**: Acclimation: *Med* = 2078; vs; Socialisation: *Med* = 1693; *Wilcoxon signed-rank test*, w = 21999, p ≤ 0.0001, *n* = 380), which aligned with the decline in total distances travelled and time fish spent moving. A similar observation was also made in NSC fish (NSC; Acclimation: *Med* = 2186; vs; Socialisation without cues: *Med* = 1886; *Wilcoxon signed-rank test*, w = 273, p ≤ 0.0001, *n* = 54), indicating that marked changes in the total number of bouts are expected across experimental phases and that the presence of social cues has no significant impact on bout generation. This was further supported through comparing the behaviour of fish tested with and without social cues across experimental phases, which found changes in total bouts to be comparable between the two testing conditions (Differences [Socialisation -

Acclimation]: Fish with cues: *Med* = -263 bouts, *n* = 380; vs; NSC: *Med* = -305 bouts, *n* = 54; *Wilcoxon signed-rank test*, w = 9284, *p* = 0.12902).

Bout Duration

Analysis of bout duration across experimental phases in fish tested with cues showed that zebrafish exhibit significantly shorter bouts during the viewing of conspecifics (**Figure 2.11D**; Acclimation: Med = 2.18s; vs; Socialisation: Med = 1.81s; *Wilcoxon signed-rank test*, w = 19765, p ≤ 0.0001, n = 380). However, NSC fish show similar results during acclimation (NSC; Acclimation: Med = 2.40s; vs; Socialisation without cues: Med = 2.04s; *Wilcoxon signed-rank test*, w = 239, p ≤ 0.0001, n = 54) NSC and socialisation (Differences [Socialisation - Acclimation]: Fish with cues: Med = -0.34s, n = 380; vs; NSC: Med = -0.35s, n = 54; *Wilcoxon signed-rank test*, w = 9182, p = 0.10577), indicating that the presence of social cues also has little impact on swim bout durations exhibited by test fish.

Average swim bout displacement

The average displacement of swim bouts was measured across experimental phases. Bout displacement was calculated by dividing the distance test fish travelled by the number of bouts per experimental phase. This combinatory parameter allowed further investigation of swim alterations that may not be detected when looking at the total number of bouts and bout durations alone.

Initial comparisons of average bout displacement of both fish tested with and without cues revealed that fish travelled significantly shorter distances per swim bout across experimental phases (Acclimation: *Med* = 2.48 mm/bout vs; Socialisation: *Med* = 2.20 mm/bout; *Wilcoxon signed-rank test*, w = 11754, p ≤ 0.0001, *n* = 380). However, NSC fish also travelled notably shorter distances per swim bout across the experimental phases, suggesting that marked reductions in bout displacement are an expected consequence of prolonged exposure to the assay (NSC; Acclimation: *Med* = 2.67 mm/bout; vs; Socialisation without cues: *Med* = 2.42 mm/bout; *Wilcoxon signed-rank test*, w = 215, p ≤ 0.0001, *n* = 54). Further analysis comparing test fish and NSC fish verified that prolonged exposure, and not the presence of conspecifics, drives the changes in behaviour observed across experimental phases (Differences [Socialisation - Acclimation]: Fish with cues: *Med* = -034 mm/bout, *n* = 380; vs; NSC: *Med* = -0.21 mm/bout, *n* = 54; *Wilcoxon signed-rank test*, w = 9131, *p* = 0.09536).

The results from the total number of swim bouts, the durations and displacement per swim bout suggest that these parameters were ineffective in identifying social interaction in the behaviour of fish exposed to the social preference assay. However, these parameters indicate a change in the internal fish state across experimental phases.

Increased freezing behaviour is a hallmark of prolonged exposure to the chamber

When placed into a novel environment, zebrafish can exhibit anxiety-like behavioural responses. Similarly, changes in immediate surroundings, such as alteration in illumination, can also be anxiety-inducing since each unfamiliar environment can carry a potential danger[362,402–404]. Typically, anxiety can be measured through bottom-dwelling activity in open field tests[362,405–407], but it can also be estimated through freezing behaviour alterations in response to stressors and novel environments.

Analysis of NSC fish, subject to prolonged periods in the assay without cues, revealed that test fish exhibit a marked increase in the time spent freezing during the second phase of the experiment (Acclimation: *Med* = 0.17%; vs; NSC: *Med* = 7.42%; *Wilcoxon signed-rank test*, w = 97, p ≤ 0.0001, *n* = 54). Test fish exposed to social cues showed a substantial increase in the time spent freezing (**Figure 2.2G**; Acclimation: *Med* = 1.17%; vs; Socialisation: *Med* = 7.83%; *Wilcoxon signed-rank test*, w = 10658, p ≤ 0.0001, *n* = 380). Finally, comparisons between fish tested with and without social cues show no significant differences. (Differences [Socialisation - Acclimation]: Fish with cues: *Med* = 4.03%, *n* = 380; vs; NSC: *Med* = 4.27%, *n* = 54; *Wilcoxon signed-rank test*, w = 9337, *p* = 0.14235). This result demonstrates that an increase in freezing behaviour is an expected outcome of testing and thus not attributable to the presence of social cues.

Fish movement is increased in the presence of conspecifics

In zebrafish, increased locomotion in response to sudden sensory stimuli or novel environments has previously been reported. Like elevation in freezing behaviour, increased locomotion (hyperactivity) can also indicate anxiety[408–410], negatively impacting social preference[411]. Conversely, increased locomotion can also mean increased interest or excitement during social interactions; for example, zebrafish display increased velocity and distance travelled in their behaviour during mating, and dogs display increased motion upon seeing their owners'.

To further explore whether anxiety in response to a novel environment decreases during habituation to be later replaced by the natural tendency to explore, the percentage of time spent moving by fish across experimental phases was evaluated.

Analysis of movement activity during acclimation and socialisation aligned with earlier findings on the total distance travelled, with test fish showing a significantly smaller reduction in time spent moving across experimental phases compared to NSC fish and thus attributed to the presence of conspecifics (**Figure 2.11F**; Acclimation: *Med* = 21.03%; vs; Socialisation: *Med* = 17.50%;*Wilcoxon signed-rank test*, w = 20743, p ≤ 0.0001, *n* = 380) (Differences [Socialisation - Acclimation]: Fish with cues: *Med* = -4.47%, *n* = 380; vs; NSC: *Med* = -8.2%, *n* = 54;*Wilcoxon signed-rank test*, w = 6947, p ≤ 0.0001). Taking these results together, it can be concluded that the

presence of conspecifics in the assay has a motivating effect on fish movement behaviour resulting in fish keeping more active when viewing cues.

Lateral motion and social preference

To identify behavioural changes of the test fish when viewing social cues, the total change in X and Y motion in zone 'AB' areas (**Figure 2.12C**) was measured. The X and Y motion is calculated relative to the window; therefore, when close to conspecifics and relative to the social cue, a change in X motion indicates approach or retreat, whilst a change in Y suggested side movement (see **Figure 2.3A**).

Test fish exposed to social cues showed significant change in their X and Y motion across experimental phases (**Figure 2.3B**, X motion; Acclimation: *Med* = 0.32 mm; vs; Socialisation: *Med* = 0.23 mm; *Wilcoxon signed-rank test*, w = 6843, p ≤ 0.0001, *n* = 366, ; Y motion; Acclimation: *Med* = 0.39 mm vs Socialisation: *Med* = 0.33 mm, *Wilcoxon signed-rank test*, w = 13750, *n* = 366, p ≤ 0.0001).

NSC fish showed a comparable reduction in X motion (X motion differences [Socialisation - Acclimation]: Fish with cues: *Med* = -0.08 mm, *n* = 366; vs; NSC: *Med* = -0.09 mm, *n* = 54;*Wilcoxon signed-rank test*, w = 1545, *p* = 0.11963) but not in Y motion (Y motion differences [Socialisation - Acclimation]: Fish with cues: *Med* = -0.06 mm, *n* = 366; vs; NSC: *Med* = -0.13 mm, *n* = 54;*Wilcoxon signed-rank test*, w = 1234, *p* = 0.01685), suggesting Y movement behaviour was specific to social interaction.

Orientation towards conspecifics

Visual attention is an intrinsic part of social relationships, and emotional content, both rewarding or punishing, can modulate selective attention[412]. Since previous studies show that zebrafish perform a stereotyped orienting behaviour while viewing social cues, indicating social attention[110,111,120], body orientation was measured (**Figure 2.11H**) in 366 fish that entered zone A (see **Figure 2.12A**).

Mean polar body orientation histograms of the population and thus the directional focus of fish revealed differing distribution patterns across experimental phases (**Figure 2.11J**). During acclimation, test fish orientated themselves on average at 95° (**Figure 2.11J**, left panel; Right side: 90°, Rel. frequency = 0.046; Left side: 100°, Rel. frequency = 0.045), orienting themselves nearly perpendicular to the social window that divided them from conspecifics in the subsequent experimental phase. In contrast, test fish position themselves at +/-45° in the presence of conspecifics such that they could alternate social viewing with their left or right eye (**Figure 2.11J**, right panel; Right side: 40°, Rel. frequency = 0.057; Left side: 50°, Rel. frequency = 0.056). This is

in line with previous studies on zebrafish[91,120] and aligns with the notion of social cues being stimulating and rewarding in nature[109].

VPI as a suitable measure of social preference

It was predicted that fish with similar social drives would show other behavioural similarities, summarised well by VPI scores. To test this hypothesis and validate the use of VPI as an accurate measure to define sociality, dimension reduction through the T-distributed stochastic neighbour embedding (t-SNE) method was applied to the complete behavioural recording data of 322 fish.

Data from previously mentioned parameters (excluding test fish body orientations due to technical limitations) were included in the t-SNE analysis, and the resulting maps were labelled according to three social preference groups.

Asocial and prosocial phenotypes represent the two extremes of the social preference scale, with fish displaying avoidance or strong drive for social interaction assigned to asocial or prosocial groups, whilst non-social fish represented fish demonstrating no strong preference in either direction. Fish were assigned to each phenotype according to VPI scores (see section: **Quantifying social preference behaviour in the average population**).

Comparisons of t-SNE maps across experimental phases revealed marked differences in data organisation (**Figure 2.13**). Inspection of the acclimation t-SNE map revealed little to no apparent clustering corresponding to social preference (**Figure 2.13A**) with all three social groups (asocial, non-social and prosocial) mixing, indicating that it is not possible to precisely predict social preference based on prior exploratory behaviour of juvenile fish during acclimation. In contrast, during socialisation (**Figure 2.13B**), pronounced clusters corresponding to asocial and prosocial phenotypes were observed in line with expectations that social phenotypes share similar behavioural characteristics. Furthermore, the overlap between non-social phenotypes and asocial and prosocial groups also recapitulated the sociality lies on a spectrum. These two findings in the socialisation data validate VPI as an accurate quantitative measure of social preference and also show that, during socialisation, asocial and prosocial phenotypes are more distinguishable.

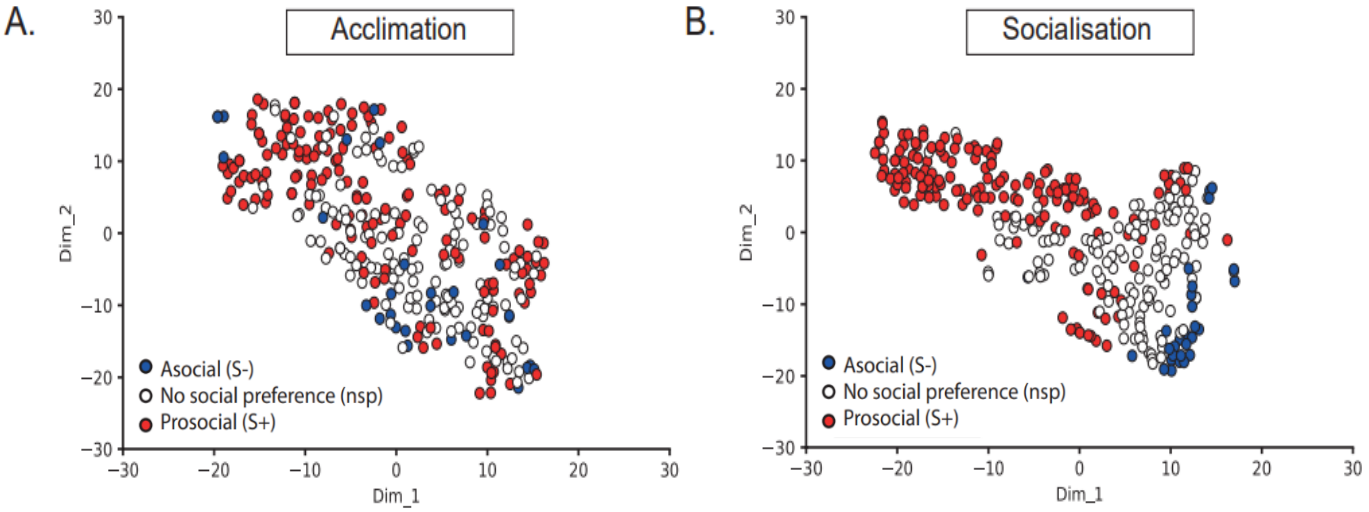


Figure 2.13: Fish with similar VPI scores display behavioural traits that correlate with sociality. A. and **B.**: Dimension reduction applied using t-SNE on behavioural data acquired during acclimation (A) and socialisation (B). The lack of clustering observed in A, and distinct clustering in B validates the use of VPI as an index of sociality in future experiments. Dim_1 and Dim_2 indicate the top two principal components defined by t-SNE on thirteen recorded behavioural metrics. Plot generated using the default parameters of the t-SNE library and coloured according to the social preference; (blue: asocial (S-, VPIs ≤ -0.5, grey: no social preference (NSP, -0.5 < VPIs < 0.5 and red: prosocial (S+, VPIs ≥ 0.5). n = 366.

Behavioural characteristics of asocial and prosocial phenotypes

In this section, using the behavioural parameters utilised to characterise the preference behaviour at the population level, the social preference behaviour of asocial and prosocial phenotypes is described. In addition, it is investigated whether the behavioural response of a given social phenotype is primarily responsible for the differentiation of the groups during socialisation as observed in the t-SNE results.

Prosocial fish increase their proximity to conspecifics

Zebrafish regulate their social encounters through alterations in inter-fish distances during social interactions (e. g. as shoaling) similarly to humans[372,377,413,414]. Therefore, it was expected that differences in asocial and prosocial fish responses to conspecifics would be readily observable in their proximity to the social window and, therefore, conspecifics.

As expected, asocial test fish significantly increased their distance from conspecifics window by a median value of 3.86 mm across experimental phases (Asocial (S-); Acclimation: *Med* = 24.94

mm; vs; Socialisation: *Med* = 28.80 mm; *Wilcoxon signed-rank test*, w = 12, p ≤ 0.0001, *n* = 39) in a response attributable to the presence of conspecifics as determined by further comparisons with NSC fish (Differences [Socialisation - Acclimation]; Asocial (S-): *Med* = -3.09 mm, *n* = 193; vs; NSC: *Med* = -0.01 mm, *n* = 54; *Mann-Whitney U test, u* = 102, *p* = 0.02661). In contrast, prosocial fish significantly reduced their distance by a median value of 11.39 mm (Prosocial (S+); Acclimation: *Med* = 19.42 mm; vs; Socialisation: *Med* = 7.81 mm; *Wilcoxon signed-rank test*, w = 23, p ≤ 0.0001, *n* = 193) in a response similarly attributable to the presence of conspecifics (Differences [Socialisation - Acclimation]; Prosocial (S+): *Med* = -11.21 mm, *n* = 193; vs; NSC: *Med* = -0.01 mm, *n* = 54; *Mann-Whitney U test, u* = 91, p ≤ 0.0001). Together these results indicate that asocial and prosocial phenotypes alter their behaviour in an opposing manner.

Comparison of the magnitudes of response asocial and prosocial fish exhibited, relative to NSC fish, revealed the reduction asocial fish showed in proximity to the social window to be significantly less than that observed in prosocial fish (Abs [Normalised]; Asocial (S-): *Med* = 2.09, *n* = 39; vs; Prosocial (S+): *Med* = 10.21, *n* = 193; *Mann-Whitney U test, u* = 1130, p ≤ 0.0001), therefore facilitating more the differentiation of the two groups during the second experimental phase.

Distances travelled by asocial and prosocial fish are distinct in the presence of conspecifics

Total distance travelled was also measured to characterise the different behaviour between asocial and prosocial fish.

Existing differences in distances travelled by asocial and prosocial during acclimation became more pronounced with the addition of conspecifics (Acclimation; Asocial (S-): *Med* = 4056.73 mm, *n* = 39; vs; Prosocial (S+): *Med* = 5831.96 mm, *n* = 193; *Mann-Whitney U test, u* = 1468, *p* = 0.00256) (**Figure 2.14B**; Socialisation, Asocial (S-): *Med* = 1629.00 mm**,** *n* = 39; vs; Prosocial (S+): *Med* = 4973.03 mm, *n* = 139; *Mann-Whitney U test, u* = 782, p ≤ 0.0001). Thus, asocial and prosocial fish are distinguishable by total distance travelled during acclimation and especially during socialisation.

Asocial fish exhibited approximately a 60% reduction in total distance travelled in the presence of social cues (Asocial; Acclimation: *Med* = 4056.73 mm; vs; Socialisation: *Med* = 1629.00 mm; *Wilcoxon signed-rank test*, w = 7, p ≤ 0.0001, n = 39), while prosocial fish had a much lower reduction (~15%, Prosocial; Acclimation: Med = 5831.96 mm; vs; Socialisation: *Med* = 4973.03 mm; *Wilcoxon signed-rank test*, w = 2042, p ≤ 0.0001, *n* = 39).

Comparison of asocial and prosocial responses to NSC fish revealed that the reduction in distance travelled by the two phenotypes were both attributable to the presence of conspecifics

(Differences [Socialisation - Acclimation]; Asocial (S-): *Med* = -1879.21 mm, *n* = 193; vs; NSC: *Med* = -1604.42 mm, *n* = 54; *Mann-Whitney U test, u* = 155, *p* = 0.35609) (Differences [Socialisation - Acclimation]; Prosocial (S+): *Med* = -1189.46 mm, *n* = 193; vs; NSC: *Med* = -1604.42, *n* = 54; *Mann-Whitney U test, u* = 91, *p* = 0.08281). Therefore, the magnitude of the responses could be directly compared, revealing that asocial fish responses were more significant than that of prosocial fish (Abs [Normalised]; Asocial (S-): *Med* = 1878.21 mm, *n* = 39; vs; Prosocial (S+): *Med* = 1396.37 mm, *n* = 193; *Mann-Whitney U test, u* = 1701, *p* = 0.02858) and contributed more to the two groups' differentiation during the socialisation phase.

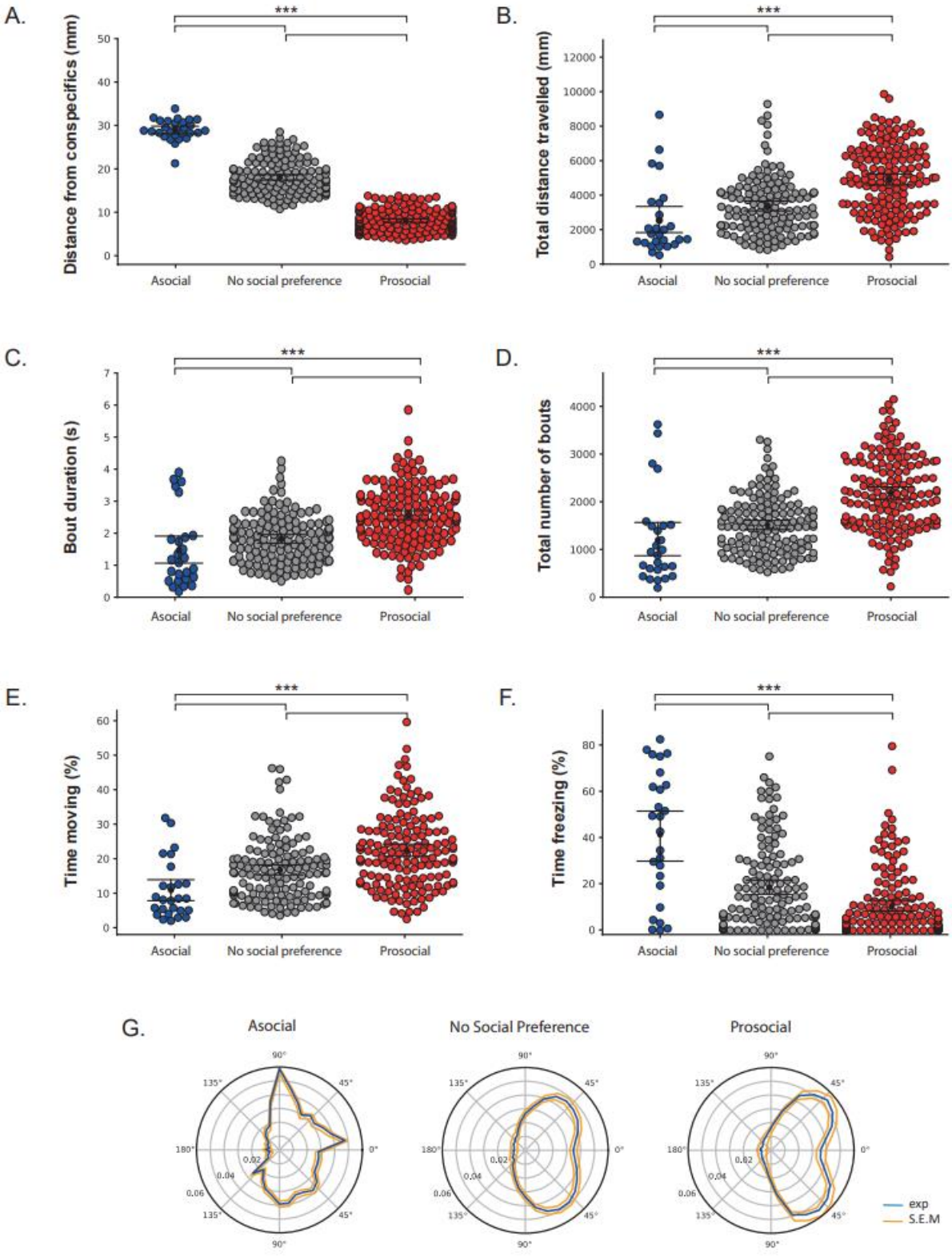

A.
Distance from conspecifics (mm)
***
Asocial
No social preference
Prosocial
B.
Total distance travelled (mm)
***
Asocial
No social preference
Prosocial
C.
Bout duration (s)
***
Asocial
No social preference
Prosocial
D.
Total number of bouts
***
Asocial
No social preference
Prosocial
E.
Time moving (%)
***
Asocial
No social preference
Prosocial
F.
Time freezing (%)
***
Asocial
No social preference
Prosocial
G.
Asocial
No Social Preference
Prosocial
exp
S.E.M

Figure 2.14: Social groups display different behaviours in the presence of conspecifics. Swarm plots of various metrics during the socialisation phase. Fish visual preference index values used as an indicator of social preference and grouping (blue; asocial (S-, VPIs ≤ -0.5), n = 39; grey: no social preference (NSP, -0.5 < VPIs < 0.5), n = 148; and red: prosocial (S+, VPIs ≥ 0.5, n = 193)). **A.** The average distance from the social window. **B.** The total distance travelled by test fish. **C.** Bout duration. **D.** Total number of bouts performed across each experimental phase. **E.** Average percentage time spent moving. **F.** Average percentage of time spent freezing **G.** Test fish body orientations relative to the midpoint of the social window when near conspecifics. Note how the no social preference fish group display intermediate responses to the presence of conspecifics compared to asocial and prosocial groups. Mean, and 95% confidence intervals are shown. Statistics performed by Mann Whitney U-test comparing subpopulations, ***p ≤ 0.05.

Swim bout alterations

Prosocial fish maintain their total number of bouts in the presence of conspecifics

Although bout analysis of control fish showed a reduction in total bouts with and without a social cue, it was possible that asocial and prosocial fish might have opposite behaviour that cancels one another when collectively analysed. The total number of bouts performed by asocial and prosocial phenotypes was analysed to test this.

The median of total number of swim bouts was reduced by 1111 bouts in asocial fish (Asocial; Acclimation: *Med* = 1888 bouts; vs; Socialisation: *Med* = 777 bouts; *Wilcoxon signed-rank test*, w = 30, p ≤ 0.0001, *n* = 39), and by 125 in prosocial fish (Prosocial; Acclimation: *Med* = 2225 swim bouts; vs; Socialisation: *Med* = 2100 bouts; *Wilcoxon signed-rank test*, w = 5548, *p* = 0.25209, *n* = 39).

As described before for total distance travelled by test fish, the difference in the number of bouts was more significant during the socialisation phase (**Figure 2.14**; Asocial (S-): *Med* = 1107 bouts, *n* = 39; vs; Prosocial (S+): *Med* = 2100 swim bouts, *n* = 139; *Mann-Whitney U test, u* = 761.5, p ≤ 0.0001), providing evidence that fish behaviour undergoes considerable change across experimental phases. However, only prosocial fish responses were found to be attributable to the presence of conspecifics since prosocial fish displayed a significantly smaller reduction in the total number of bouts when compared to NSC fish responses (Differences [Socialisation - Acclimation]; Prosocial (S+): *Med* = -56 bouts, *n* = 193; vs; NSC: *Med* = -613 bouts, *n* = 54; *Mann-Whitney U test, u* = 540, *p* = 0.00705), indicating that prosocial fish responses drive the differentiation between the two social groups during the socialisation phase, as also supported by analysis of the magnitudes of responses (Abs[Normalised]: Asocial (S-): *Med* = 962, *n* = 39; vs; Prosocial (S+): *Med* = 469, *n* = 193; *Mann-Whitney U test, u* = 1412, *p* = 0.00131).

Since differences in the total number of bouts could not previously be attributed to the presence of conspecifics at the population level, the results here show that behavioural differences may be masked by the net effect of asocial and prosocial behaviours cancelling one another out, therefore highlighting the importance of differentiating between social phenotypes.

Prosocial fish respond to conspecifics by maintaining bout duration

Evaluation of the bout durations during acclimation and socialisation phases showed that asocial fish reduced their bouts by 59% (Asocial; Acclimation: *Med* = 2.1s; vs; Socialisation: *Med* = 0.86s; *Wilcoxon signed-rank test*, w = 25, p ≤ 0.0001, *n* = 39). Prosocial fish showed a smaller reduction (9%) in their bout duration across experimental phases (Prosocial; Acclimation: *Med* = 2.32s; vs; Socialisation: *Med* = 2.11s; *Wilcoxon signed-rank test*, w = 4925, *p* = 0.025277, *n* = 193). As asocial and prosocial bout durations were comparable during acclimation, social phenotypes were only distinguishable during socialisation, with prosocial fish exhibiting significantly longer bouts than their asocial counterparts (**Figure 2.7C**; Asocial (S-): *Med* = 0.86s, *n* = 39; vs; Prosocial (S+): *Med* = 2.11s, *n* = 193; *Mann-Whitney U test, u* = 903, p ≤ 0.0001). Only the reduction in bout duration displayed by prosocial fish was attributable to the presence of conspecifics when compared to NSC responses (Differences [Socialisation - Acclimation]; Prosocial (S+): *Med* = -0.16s, *n* = 193; vs; NSC: *Med* = -0.83s, *n* = 54; *Mann-Whitney U test, u* = 465, *p* = 0.00177).

In line with the above findings, the comparison of response magnitudes of social groups exhibited across experimental phases revealed that prosocial fish responses primarily mediated the differentiation of the two groups during the viewing of conspecifics (Abs [Normalised]; Asocial (S-): *Med* = -0.10, *n* = 39; vs; Prosocial (S+): *Med* = -0.53, *n* = 193; *Mann-Whitney U test, u* = 1347, *p* = 0.00056).

Freezing behaviour is increased in asocial fish

Next, the freezing behaviour in asocial and prosocial fish phenotypes was compared. Results revealed that the prolonged periods of inactivity observed in asocial fish were significantly greater than those observed in prosocial fish (Acclimation; Asocial (S-): *Med* = 3.83%%, *n* = 39; vs; Prosocial (S+): *Med* = 0.56%, *n* = 193; *Mann-Whitney U test, u* = 11336.5, *p* = 0.00139) which suggested underlying differences in individual dispositions such as 'shy' and 'bold' personality traits between the two phenotypes. Shyness is often characterised by low explorative, thus increasing freezing behaviour, and bold individuals are explorative and more significant risk-takers[415–417]. Shy and bold personality traits can be applied to the observed behaviour in the experimental phases with the asocial and prosocial fish. Where freezing behaviour is observed, the quality of shyness may be attributed and, inversely, boldness to high activity fish during acclimation. If correct, with this explanation, it should be possible to predict the two phenotype responses to cues during the

second experimental phase. Thus, it was expected that asocial fish would display significantly more freezing behaviours, shying away and freezing from their conspecifics in the social chamber, whilst their bolder prosocial counterparts exhibited social interest accompanied by shorter inactivity. The similar comparison of asocial and prosocial test fish responses during socialisation revealed, as predicted, asocial fish spent 45.86 more time freezing than their prosocial counterparts (**Figure 2.14F**; Asocial (S-): *Med* = 49.79%, *n* = 39; vs; Prosocial (S+): *Med* = 3.93%, *n* = 139; *Mann-Whitney U test, u* = 766.5, p ≤ 0.0001) in alignment with previous t-SNE results that asocial and prosocial groups are more distinct in the presence of cues.

Across experimental phases the presence of conspecifics increased the prolonged periods of freezing asocial fish exhibited were comparable to NSC fish responses (Differences [Socialisation - Acclimation]; Asocial (S-): *Med* = 29.15%, *n* = 193; vs; NSC: *Med* = 5.31%, *n* = 54; *Mann-Whitney U test, u* = 107, *p* = 0.03708) whilst prosocial fish exhibited a decreased response when also compared to NSC fish (Differences [Socialisation - Acclimation]; Prosocial (S+): *Med* = 1.74%, *n* = 193; vs; NSC: *Med* = 5.31%, *n* = 54; *Mann-Whitney U test, u* = 91, *p* = 0.01911). Thus, conspecifics had opposing effects on asocial and prosocial fish behaviour.

A comparison of response magnitudes of social groups exhibited across experimental phases revealed that asocial fish reactions primarily mediated the differentiation of the two groups during the viewing of social cues with asocial fish responses determined to be approximately twelve times greater than prosocial responses (Abs[Normalised]; Asocial (S-): *Med* = 28.15, *n* = 39; vs; Prosocial (S+): *Med* = 2.33, *n* = 193; *Mann-Whitney U test, u* = 943, p ≤ 0.0001).

Prosocial fish display motivated behaviour in the company of conspecifics

To test whether the two social groups were distinguishable pre- and post-social cue exposure, the percentage time fish spent moving was compared for each experimental phase in asocial and prosocial fish. Analysis of acclimation behaviour showed that the two groups were distinct prior to viewing conspecifics (Acclimation; Asocial (S-): *Med* = 18.30%, *n* = 39; vs; Prosocial (S+): *Med* = 22.40%, *n* = 193; *Mann-Whitney U test, u* = 1726, *p* = 0.03544). However, this separation was more apparent in the presence of social cues aligning to previous t-SNE results, with prosocial fish spending significantly greater time moving than asocial counterparts (**Figure 2.7F**; Asocial (S-): *Med* = 7.32%, *n* = 39; vs; Prosocial (S+): *Med* = 20.29%, *n* = 139; *Mann-Whitney U test, u* = 776, p ≤ 0.0001).

Across experimental phases, only prosocial fish showed alteration in movement activity that was not explainable by prolonged assay exposure (after comparing to NSC fish responses: Differences [Socialisation - Acclimation]; Prosocial (S+): *Med* = -2.37%, *n* = 193; vs; NSC: *Med* = -7.95%, *n* = 54; *Mann-Whitney U test, u* = 508, *p* = 0.00398), indicating the presence of conspecifics

to motivate movement activity in prosocial phenotypes solely. Further analysis comparing absolute magnitudes of asocial and prosocial fish responses to NSC fish revealed that prosocial fish responses to conspecifics substantially facilitate the differentiation of the two groups during the second experimental phase.

X and Y motion while viewing conspecifics

Measurements of X-motion of asocial and prosocial fish were indistinguishable during acclimation and socialisation, as were overall responses to conspecifics, indicating this parameter was ineffective in identifying social or avoidance behaviour.

Lateral (Y) motion facilitates the differentiation of asocial and prosocial fish groups during the viewing of conspecifics

Inspection of the behaviour of all fish, across experimental phases, previously unmasked lateral movement unique to the socialisation phase suggesting that alterations in Y-motion were motivated by social drive. Therefore, it was hypothesised that prosocial fish with a strong preference for conspecifics would readily exhibit this unique behaviour exclusively in the presence of conspecifics.

Results revealed that the two social phenotypes were indistinguishable through comparisons in lateral motion during the acclimation phases with asocial and prosocial fish displaying similar movement in the Y direction (Acclimation; Asocial (S-): *Med* = 35 mm, *n* = 28; vs; Prosocial (S+): *Med* = 0.46 mm, *n* = 193; *Mann-Whitney U test, u* = 1949, *p* = 0.17055). However, the two social phenotypes became distinct during the socialisation phases, with prosocial fish exhibiting more significant movement in the Y-direction than asocial fish as predicted (**Figure 2.15A**; Asocial (S-): *Med* = 0.28 mm, *n* = 28; vs; Prosocial (S+): *Med* = 0.39 mm, *n* = 157; *Mann-Whitney U test, u* = 1586, *p* = 0.00957).

Across experimental phases, only prosocial fish responses were attributable to the presence of conspecifics, with prosocial fish showing significantly less reduction in Y-motion than fish tested without social cues (Differences [Socialisation - Acclimation]; Prosocial (S+): *Med* = -0.04 mm, *n* = 157; vs; NSC: *Med* = -0.13 mm, *n* = 54; *Mann-Whitney U test, u* = 738, *p* = 0.0111).

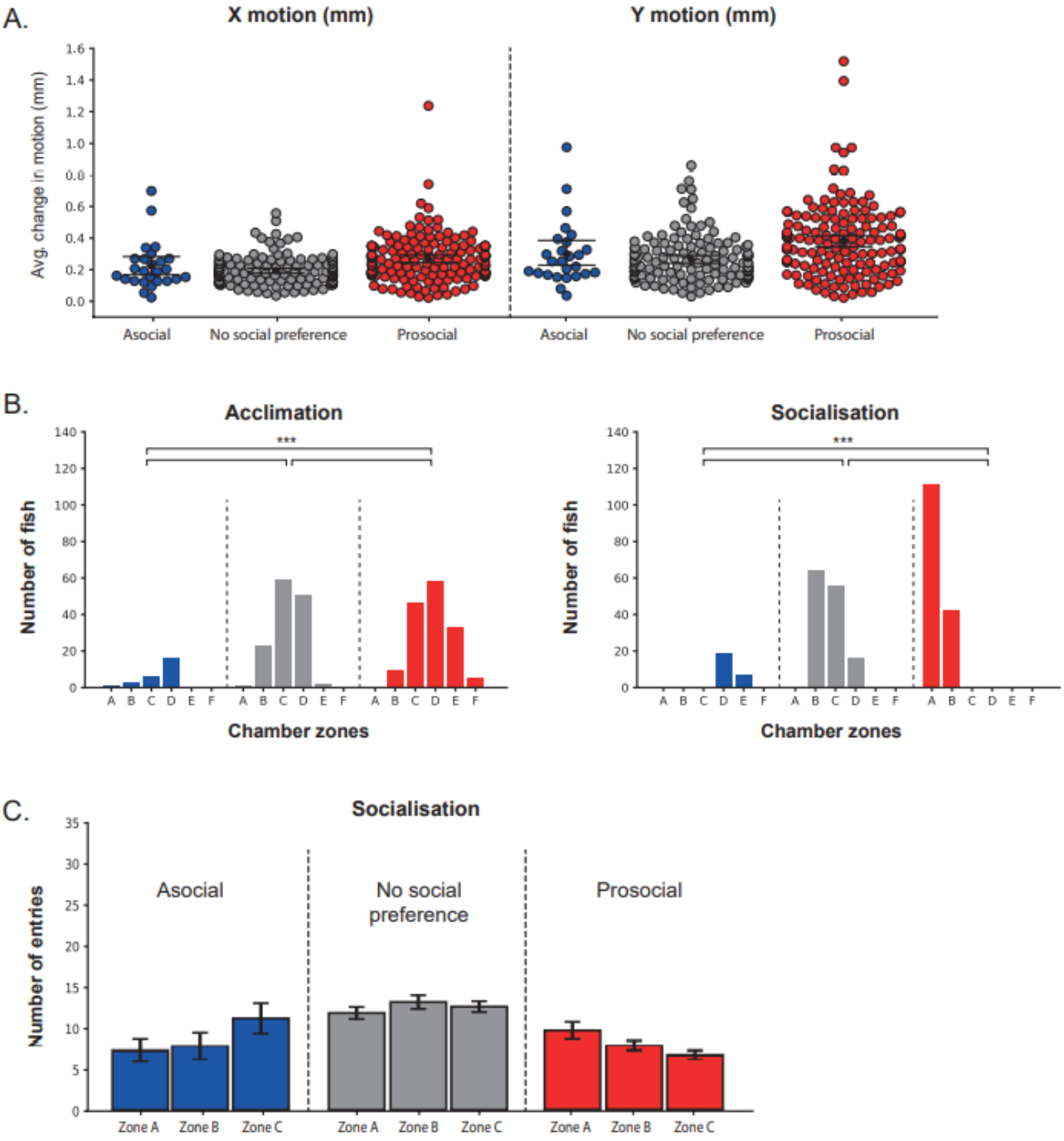

A.
X motion (mm)
Y motion (mm)
Avg. change in motion (mm)
Asocial
No social preference
Prosocial
B.
Acclimation
Socialisation
Number of fish
Chamber zones
***
C.
Socialisation
Asocial
No social preference
Prosocial
Number of entries
Zone A
Zone B
Zone C

Figure 2.15: Navigation of fish social groups. Fish visual preference index values used as an indicator of social preference and grouping (blue; asocial (S-, VPIs ≤ -0.5); grey: no social preference (NSP, -0.5 < VPIs < 0.5); and red: prosocial (S+, VPIs ≥ 0.5). **A.** Swarm plots of x and y motion of test fish during socialisation. Asocial and prosocial fish exhibit similar movement in X and Y direction when near conspecifics. **B.** Average position of test fish given as corresponding zones for acclimation and socialisation phases. Zone A is the closest possible zone to conspecifics, while zone F is the furthest. **C.** Bar graph displaying the average number of entries into zones A, B and C by test fish during socialisation. Mean and standard error are shown. Statistics performed by Mann Whitney U-test comparing social groups, ***p ≤ 0.05. Sample sizes are as follows: A: S-, n = 28; NSP, n = 181; and S+, n = 157; and B and C: S-, n = 39; NSP, n = 148; and S+, n = 193.

Comparison of absolutes of normalised responses in Y-motion showed that asocial and prosocial alterations in Y-motion were similar in magnitude relative to NSC fish alterations (Abs [Normalised]; Asocial (S-): *Med* = 1.13, *n* = 28; vs; Prosocial (S+): *Med* = 1.10, *n* = 157; *Mann-Whitney U test, u* = 1831, *p* = 0.08105). Thus, the lack of response asocial displayed and altered response exhibited by prosocial fish contributed equally to the clear separation of the two phenotypes during cue viewing.

Prosocial fish display interest in conspecifics

Initial analysis of acclimation viewing angles revealed that asocial fish displayed an average angle of 95°, orienting their bodies nearly perpendicularly to the social chamber (i.e. the section occupied by conspecifics during the second experimental phase) (Asocial; left side: mode = 100°; right side: mode = 90°) similar to that of prosocial and NSC fish (Prosocial; left side: mode = 100°; right side: mode = 90° and NSC; left side: mode = 100°; right side: mode = 80°). However, the similarity of both asocial and prosocial groups with NSC fish behaviour did not extend to the socialisation phase. The average viewing angle displayed by asocial fish near the social window mainly remained unaltered (95°) with the addition of conspecifics, indicating no social interest (**Figure 2.14G:** Asocial; left side: mode = 90°; right side: mode = 90°). These results were comparable to NSC fish over the same experimental phase (NSC; left side: mode = 100°; right side: mode = 80°). In contrast, prosocial fish orientated their bodies, reducing their viewing angle to 45°, directing their focus towards their conspecifics in the social chamber, suggesting high interest. (**Figure 2.8G**: Prosocial; left side: mode = 50°; right side: mode = 40°).

These results show that asocial and prosocial responses are distinct and demonstrate that prosocial fish behaviour differentiates the two social groups during viewing cues.

2.3. Discussion

This chapter reports significant findings. First, the stability and robustness of social preference in juvenile zebrafish, an increasingly attractive model for behaviour and systems neuroscience, is described. Second, social preference behaviour results represent the first comprehensive report in the juvenile zebrafish to date. Third, the suitability of VPI as an indicator of social preference is validated. Last, social behavioural of asocial and prosocial fish in response to conspecifics are systemically characterised for the first time.

Stability and robustness of social preference

This chapter reports that social preference behaviours, determined by VPI scores, remain stable over the testing day (**Figures 2.5 and 2.6**). Furthermore, other important aspects of fish behavioural responses to conspecifics, such as time spent moving, also remain unaltered over the same period (**Figure 2.7**). Together, these findings indicate no circadian element or ongoing developmental contribution to social preference or that any developmental effect is on a longer (multiday) timescale. Thus, experiments conducted anytime within the testing day can be pooled together.

Differences in behavioural progression and action selection can often be shown by the same individual over a short time, despite the presentation of the same sensory cue. Only a few studies have investigated such a phenomenon with regards to social preference behaviour. Therefore, this chapter reports that juvenile zebrafish exhibit robust social preference when repeatedly tested with conspecifics on the same day. Only a negligible number of fish (~5%) alter their preference towards conspecifics (**Figure 2.10**). This finding will have implications for determining sample sizes in future studies as it assures that a single measurement is sufficient to accurately represent juvenile zebrafish's social preference.

The next step following these lines of investigation would be to establish whether the social preference is robust over longer periods. A parallel study was designed to this end which involved testing the social preference of group-housed juvenile zebrafish over consecutive days. Google Cloud Vision AI and IdTracker were among the two approaches utilised to identify individual zebrafish. Unfortunately, the experiment was unsuccessful due to software limitations, lighting issues, and the fish's rapid growth. Since this, however, further improvements have been made in the software and lighting problems can be resolved, thus warranting repeating this experiment. Such an experiment would allow for a better understanding of how individual social preferences arise within a given population and answer questions such as “do extreme differences in social

preference towards conspecifics change/develop with age?". Results from this line of enquiry would be significant since, in humans, adolescence has been proposed as a period of hypersensitivity to the social environment[418] and a crucial period for the onset of mental health problems[419]. Thus, these results would also open new lines of investigation into whether a critical/sensitive period exists for the development of social preference in zebrafish.

Population-level characterisation of social preference behaviour

Comparisons between fish tested with and without conspecifics allowed the identification and characterisation of social preference behaviour. In the presence of conspecifics, the average juvenile zebrafish readily approach two conspecifics located on one end of the chamber. Following initial viewing, juvenile fish confine their movement to regions proximal to conspecifics, only occasionally making brief visits to other areas. Therefore, subsequent movement activity predominately occurs in the social half of the assay chamber. The place preference of juvenile fish is mirrored in proceeding socialisation phase VPI scores. In the presence of conspecifics, 21 days old fish maintain a more significant proportion of movement activity (i.e., percentage time spent moving) initially exhibited in the acclimation phase of the assay. Unsurprisingly, the maintenance of movement activity is reflected in the total distances travelled by fish during the socialisation phase. Juvenile fish orient themselves at +/- 45° degrees relative to conspecifics and minor reduction and, therefore, Y-motion maintenance when near conspecifics. The insignificant decrease in Y motion quantifies the swimming behaviour of juvenile fish when following the length of the dividing glass window - a side to side motion relative to conspecifics. Angles below 90 degrees and body orientation and maintenance of Y motion are unique characteristics of zebrafish social interaction, with no such behaviours observed during acclimation or in NSC fish.

Seven of the eleven behavioural parameters extracted (i.e. proximity to conspecifics, the average location of fish, the total number of entries into predefined areas, total distance travelled, percentage of time spent moving, absolute Y motion, and body orientations) showed social preference behaviour-specific changes. As in humans, a broad spectrum of social preferences has been found in the zebrafish population[91]. Subsequent evaluation of parameters determined that the proportion of prosocial fish in the measured population zebrafish was the cause of this curious observation in all but one parameter (X motion), highlighting the importance distinguishing phenotypes. Absolute change in X motion represented forward and backward movement performed by test fish near the conspecifics. The similarities in the X motion observed between fish tested with and without conspecifics are explained when multiple behavioural parameters are considered and compared. For instance, fish tested with conspecifics displayed strong place preference to areas near siblings. A significant increase in Y motion was seen as fish often

swimming pressed against the dividing glass window, consequently reducing X-directed movement.

In contrast, the reduction in X-motion observed in NSC directly resulted from reduced entries into zone AB during the second experimental phase. Therefore, to distinguish between the two behaviours in future experiments, the X motion parameter could be improved by normalising values by the time fish spend in zone AB from which this parameter is extracted. The smaller resulting values from this improved measurement would represent responses attributable to conspecifics and the larger values representing prolonged exposure to the assay.

Of note, fish tested with and without conspecifics exhibited a significant reduction in all parameters accompanied by an equally substantial increase in freezing behaviour across experimental phases, strongly suggesting a change in the internal state, for example, increased fatigue or reduced interest in surroundings with the diminishing novelty of the chamber over time.

While an increase in freezing behaviour and decrease in movement activity typically indicate elevated stress or anxiety states, the context in which these parameters are measured is essential for correct interpretation. The habituation behaviour of rodents and zebrafish to novel environments may also be similarly described using these two parameters[409,420]. For example, when placed into an unfamiliar environment, zebrafish initially explore a chamber in a heightened state of anxiety (displayed as hyperactivity), followed by a comparatively more casual investigation of the same environment resembling hypo-locomotion, which can be reflected as increased freezing behaviour. Therefore, habituation may be incorrectly interpreted as increased stress and anxiety levels. Although the changes in freezing and movement activity are most likely to be habituation to the novel environment, since zebrafish tested with rewarding conspecifics also display this behaviour, this can be confirmed via quantifying stress by measuring cortisol levels. In humans, stress responses heavily rely on the hypothalamic-pituitary-adrenocortical axis (HPA). Similarly, in zebrafish, the primary hormone released following the activation of the HPA is cortisol[301] and can be measured using one of many techniques and protocols currently available, e.g., ELISA[421]. Thus, to assess if behaviour change is due to exploration or stress/anxiety, cortisol levels could be evaluated from samples of water taken from the assay chamber in a non-invasive manner post acclimation and again following the socialisation phase. Subsequently, cortisol measurements could be compared between fish tested with and without conspecifics, with higher cortisol concentrations indicating rising stress/ anxiety levels in response to the novel environment. In contrast, lower levels suggest habituation to the assay chamber.

VPI as a suitable measure of social preference

In this chapter, dimension reduction, through the T-distributed stochastic neighbour embedding (t-SNE method), was utilised to explore and visualise the high-dimensional behavioural data of juvenile zebrafish and ultimately validate the visual preference index (VPI) for measuring social preference (**Figure 2.13**). Although used extensively, previously reported social preference indices have not been validated. Typically, VPI is calculated using test fish proximity to conspecifics or time spent near these social cues; however, it was currently unknown how well they represent the other features of social behaviour.

This chapter reports that juvenile zebrafish with similar VPI scores share similar characteristics in their social preference behaviour. Crucially, during acclimation, asocial and prosocial phenotypes show significant differences in behavioural parameters, i.e., total bouts performed, total distance travelled, proximity to a social window, percentage time spent moving and freezing, average position in chamber and number of entries into zone A, becoming more distinguishable in the presence of conspecifics. Lastly, non-social fish phenotypes lay between both asocial and prosocial phenotypes, agreeing that social preference is a spectrum.

Characterising the behaviour of asocial and prosocial phenotypes

In the final section of this chapter, the social preference behaviour of fish that display aversion and strong attraction towards conspecifics is characterised in-depth for the first time (**Figures 2.11 and 2.12**). The two social groups (asocial and prosocial) identified through VPI scores represent the two opposing ends of the social scale. In this research, the proportion of asocial and prosocial fish found in the 3-week-old juvenile zebrafish population match those reported by Dreosti and co-workers[91]. Since 2015, when the two social groups were first quantified in juvenile zebrafish[91], no studies have investigated how they differ in detail in their social preference behaviour. The distinct acclimation behaviours and reactions to conspecifics that asocial and prosocial groups exhibit are summarised in the following statements. Upon placement into the assay chamber, asocial fish prefer the chamber's narrowest region, performing fewer entries to the comparably more open-field areas. Asocial fish display more periodic bouts, travel shorter distances, spend less time moving and show significantly more anxiety-like responses. In the presence of conspecifics, asocial fish respond by further reducing the total distance they travel, considerably increasing the time spent inactive in the chamber, and overall display avoidance like behaviour, indicated by the significantly increased distances from social cues.

Conversely, prosocial fish explore more of the assay chamber, displaying substantially greater overall locomotion and freezing for a shorter time than asocial fish. Although asocial and prosocial

fish phenotypes seem to be distinguishable before viewing social cues, this is made difficult with the presence of non-social fish seen in the average population, which shows overlap in behaviour with both asocial and prosocial phenotypes (see t-SNE). Therefore, separation of asocial and prosocial phenotypes is not possible without socialisation data.

Following the introduction of conspecifics, prosocial fish exhibit great interest in social cues, which they readily approach, displaying place preference for areas nearest to conspecifics to and orientating their bodies at a -/+45° angles. Like other fish, zebrafish eyes frequently protrude from their head, providing almost a 360° view of their surroundings in all directions (laterally and vertically)[422]. Notably, the positional anatomy of the eye on the zebrafish head means that angles below 90° (perpendicular to the eyes) are the most optimal visual acuity[422]. Therefore, the body orientations of prosocial fish reported align with this information and recapitulate previous studies showing that juvenile fish orient their body at 45° angles concerning the position of conspecifics during social interaction[91].

Prosocial fish display significantly longer bout durations, increased number of bouts, total distances travelled, movement activity, and reduced time spent freezing than their asocial counterparts. The maintenance in the previously mentioned parameters prosocial fish exhibit across the experimental phases suggests that conspecifics have a positive and stimulating influence on zebrafish, in line with previous studies reporting social stimuli rewarding[103,369,396,412,423–425].

Interestingly, before viewing cues, the asocial and prosocial phenotypes displayed behaviour resembling shy and bold personality traits characterised by low exploration and increased freezing behaviour, or greater exploration and decreased freezing, respectively[415,417,426]. Boldness and shyness have been previously described as coping styles to environmental stressors, with shy animals responding to stress with higher activation of the hypothalamic-pituitary-adrenal axis, leading to higher post-stress levels glucocorticoids than bolder individuals[427]. This physiological stress response is also recapitulated in shy and bold fish, with shy individuals displaying higher activation of the hypothalamic-pituitary-interrenal axis (the teleostean homologue of the mammalian adrenal axis), subsequently leading to higher post-stress levels of cortisol[428]. Although much interest exists around the interplay between personalities and social interactions in other species, little is currently understood about how personalities may drive individual social preference in zebrafish since few studies report the personality traits before testing social preference.

Jolles and co-workers (2014), using the three-spined stickleback fish, showed that social interaction of fish occurs at further distances when one of the pairs of fish are bold, indicating that bold fish are not as socially attractive conspecifics. The same study also reported that social

attraction was positively correlated with better coordination between fish pairs[426]. Yet here in this chapter, it is said that asocial zebrafish that displayed introverted tendencies maintain longer viewing distances from conspecifics than their prosocial counterparts. The reason for such inconsistency in fish distances between interacting pairs reported in Jolles et al., with the results reported in this thesis, is currently unknown but could be due to differences in experimental design and characterisation of bold and shy personalities and species. Jolles and co-workers allowed stickleback fish to acclimate to their environment over three days and characterised boldness using the proportion of fish spent out of cover. In this chapter, juvenile zebrafish were allowed to acclimate for 15 minutes with no available shelter/cover.

Furthermore, zebrafish were not categorised as bold by quantifying a particular behavioural measure and instead assessed relatively between asocial and prosocial phenotypes based on overall locomotive activity. Boldness and shyness in isolated fish can be investigated using assays established in the zebrafish model[426,429,430]. The link between social phenotypes and personality traits may be assessed using these in conjunction with the social preference assay, either preceding or proceeding. Experiments using personality traits to characterize differences in social individualities could be very informative in understanding and even predicting how asocial and prosocial phenotypes may respond to social environmental factors.

Conclusion

This is the first systematic study characterising the social preference behaviour of juvenile zebrafish using multiple behavioural parameters. A wide range of individual social preferences exists in the zebrafish population, and the associated behaviours are well summarised by the robust and stable visual preference index score. Most importantly, critical behavioural differences in asocial and prosocial phenotypes exist, possibly reflecting the differences in how conspecifics are perceived. It remains a mystery how the underlying social circuitry in the brain gives rise to the variety of individual social preferences found in each population. One way to uncover this mystery is by identifying social phenotypes and coupling these results with high-resolution imaging techniques to investigate differences in the function of the social brain circuitry. This exact approach is described in **Chapter 4** of this thesis.

A greater understanding of how asocial and prosocial tendencies develop promises to provide prevention and early intervention strategies to tackle atypical social preferences associated with neuropsychiatric disorders and possibly modify psychopathological trajectories. Furthermore, these approaches are likely to be more efficacious than treatment once asocial tendencies are fully established[431].

Overall, the second chapter provides essential information on expected behaviours in the juvenile wildtype zebrafish at both the population and individual level, which could be used to subsequently identify atypical behaviour following periods of social adversity faced in the environment.

Chapter 3: Social Isolation

Chapter 3 Outline

In this chapter, the effects of isolation on behaviour are assessed, with results in the previous chapter serving as baseline references to determine whether fish responses to social cues are altered by isolation. Specifically, using previously mentioned parameters: proximity to conspecifics, the average location of fish, the total number of entries into predefined areas, the total number of bouts, bout duration, total distance travelled, percentage of time spent freezing, percentage of time spent moving, absolute X motion, absolute Y motion, and body orientations, analysis of isolated fish behaviour is presented with detailed comparisons with socially reared fish test with and without conspecifics.

3.1. Introduction

The increasing popularity of social isolation studies

A simple PubMed search for "social isolation" returns 11,727 reports in the last five years. An overwhelming amount is based on human studies producing 8,304 hits, with rats and mice returning 57% of all non-human research. Unsurprisingly, the tiny and highly social zebrafish has seen a 200% increase in publications in the same timeframe totalling 18 research articles, 5 of which coincide with the COVID-19 pandemic, thus reflecting its rise in popularity as a model to study the effects of social isolation. It is worth highlighting that, although the quantity of human studies prevails over animal studies, the contribution of animal models to our current understanding of the implications of social isolation is invaluable. The importance of such models becomes most apparent when considering that controlled research on severe social isolation in humans is complex and rare due to ethical implications.

Social isolation in zebrafish

Of the few studies conducted in zebrafish, undesired social isolation has returned conflicting results regarding social preference, locomotion and anxiety-related behaviours. For instance, in the adult zebrafish, in which most isolation studies have been carried out, a period of 24 hours of social isolation is reported to decrease whole-brain levels of serotonin and dopamine without impacting locomotor activity or social response stimuli[432]. Continuous social deprivation lasting between 3-5 days has been associated with disrupted swimming activity of shoals resembling dopaminergic neurotoxin administration[433]. Ninety days of prolonged social isolation has been described to decrease anxiety-like behaviours such as thigmotaxis and serotonin levels without alterations to locomotion[375]. Of the limited number of studies assessing the impact of social isolation during development, impaired differentiation between kin and non-kin[434], decreased preference for differently pigmented shoal-mates[315], decreased shoaling[395], no consequences on shoaling[435], increase in an anxiogenic-like responses[352,365], as well as hyperactivity with reduced anxiety-like responses[395] have been reported.

A common feature of the above studies is that the impact of social deprivation (conditions without visual, tactile or olfactory access to conspecifics) on general behaviour is assessed without any reference to social preferences. In the few studies where the social behaviour of isolated fish is reported, sociality is evaluated through the analysis of shoaling behaviours and individual preferences are never reported. While shoaling behaviour in fish is classified as social behaviour,

the complex dynamics of shoaling often reflect the overall preferences and traits of participating members rather than individuals.

Furthermore, environmental effects, including social experiences, on social behaviour may be amplified or reduced in shoals' conduct depending on the experimental design. For example, one can imagine a scenario where social isolation may alter preferred proximity to conspecifics[436] and a shoal comprised of such fish would reflect all the atypical preferences of members, resulting in loosely formed shoals[395]. However, when a single isolated fish is placed in a group of socially reared individuals, the effect of isolation may be undetected/masked. This masking issue is particularly problematic when considering that the impact of social isolation may be different in individuals preferring solitude compared to social fish. Therefore, investigating the effects of developmental social deprivation in a manner where single zebrafish are independently assessed is crucial to enhancing our understanding of how isolation alters social behaviour, particularly on a level that also reflects the diversity of individual preferences in our species.

3.2. Results

Isolation alters social preference

Two isolation models were employed to investigate the effects of social deprivation on social preference behaviour during development (**Figure 3.1**). Complete isolation (Full Isolation (Fi)), spanning twenty-one days from fertilisation, reproduced chronic isolation conditions like those previously described in humans and other mammalian species[306,437–439]. In contrast, partial isolation of 48 hours and 24 hours (Pi48 and Pi24, respectively) applied before testing replicated two conditions of social isolation previously described in vertebrates, including songbirds[395,440]. Isolation conditions were maintained until testing to capture first responses to conspecifics.

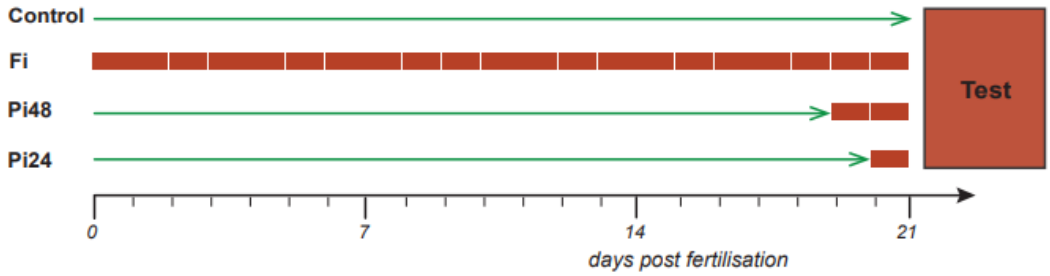


Figure 3.1: Schematic representation of the experimental timeline for various rearing conditions. All fish are collected as embryos within 1 hour of fertilisation and tested at 21 days post fertilisation. Green arrows depict periods of grouped housing, and red boxes periods of isolation in 24-hour blocks. Animals reared in full isolation from the point of fertilisation until testing are represented as F**I.** Partial isolation of 48hrs or 24hrs applied before testing are represented by Pi48 and Pi24, respectively. All control fish are reared in social groups up until testing.

Specially designed rearing tanks constructed from opaque acrylic facilitated the isolation of focal fish during development (**Figure 3.2**). Matte surfaces of the internal walls promoted the perception of isolation by eliminating reflections throughout rearing; this ensured stress and social behaviour observed following isolation were not attributed to stress responses from viewing themselves[441,442]. Live diets provided to group-housed controls were maintained across all isolates to eliminate the novelty of moving stimuli during rearing.

As in **Chapter 2**, Social preference was quantified by calculating the visual preference index (VPI) of fish that underwent the social isolation protocols described above. Results showed that the distribution of VPI scores across all conditions (Fi, Pi48 and Pi24) were similar during the acclimation phase, indicating that no differences exists between rearing conditions before the

viewing of conspecifics (**Figure 3.3**, Acclimation: Control (C): *Med* = -0.002, *n* = 380, vs; Full isolation (Fi): *Med* = 0.03, n = 47, *Mann-Whitney U test,* u = 8373, p = 0.24280; Partial isolation 48hrs (Pi48): *Med* = 0.04, n = 157, *Mann-Whitney U test,* u = 8373, p = 0.09263; and Partial isolation 24hrs (Pi24): *Med* = 0.004, n = 71, *Mann-Whitney U test,* u = 13332, p = 0.43792).

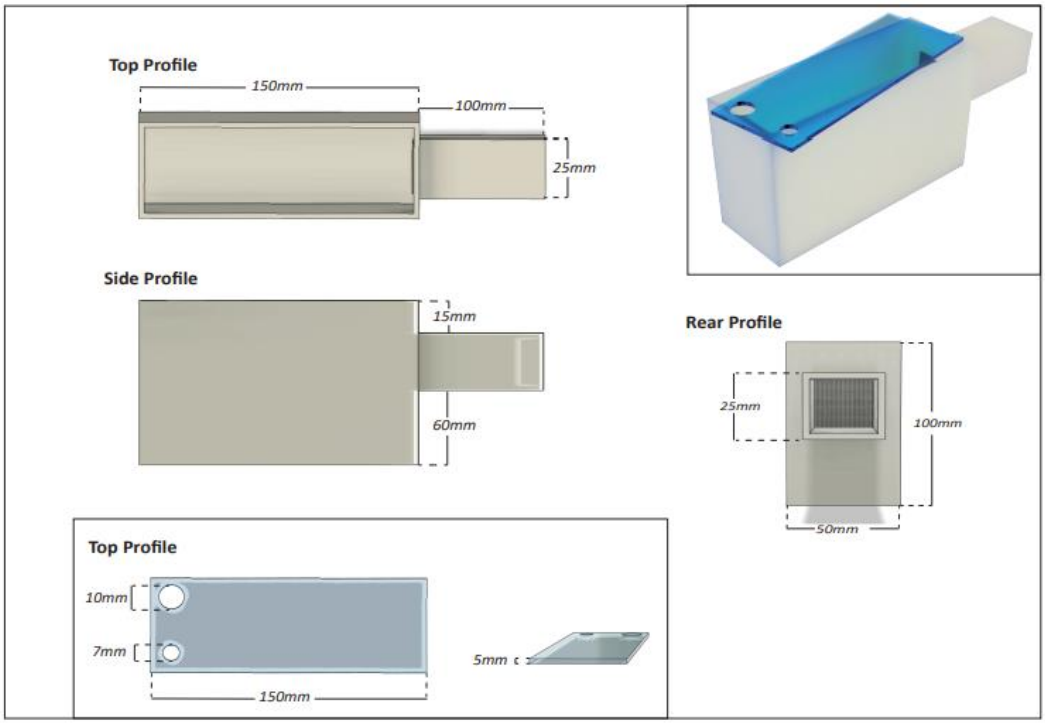


Figure 3.2: Isometric drawing of isolation tanks used for social deprivation. Body and lid constructed from acrylic. The opaque white walls prevent fish from viewing conspecifics in adjacent tanks, and matt surfaces inhibit reflections. A translucent lid allows illumination from the surrounding environment. Back compartment facilitates directed outflow of water.

Social isolation profoundly affected the social preference behaviour of juvenile zebrafish reared in all isolation conditions (**Figure 3.3**). Full social isolation (Fi) caused a significant decrease in average social preference scores relative to socially reared sibling controls (**Figure 3.3**, Socialisation; Controls (C): *Med* = 0.55, *n* = 380; vs; Full isolation (Fi): *Med* = -0.21, *n* = 47; *Mann-Whitney U test, u* = 4753, p ≤ 0.0001). Similar results were also observed in partially isolated fish conditions; however, these were to a lesser extent than full isolation, with 24 hours of social deprivation having the lowest impact of the two partial conditions on the distribution of VPIs, although still significant (**Figure 3.3**, Socialisation; Controls (C): *Med* = 0.55, *n* = 380; vs; Partial isolation 48hrs (Pi48): *Med* = -0.002, *n* = 157; *Mann-Whitney U test, u* = 20921, p ≤ 0.0001, and Partial isolation 24hrs (Pi24): *Med* = 0.03208, *n* = 71; *Mann-Whitney U test, u* = 9317.5, p ≤ 0.0001).

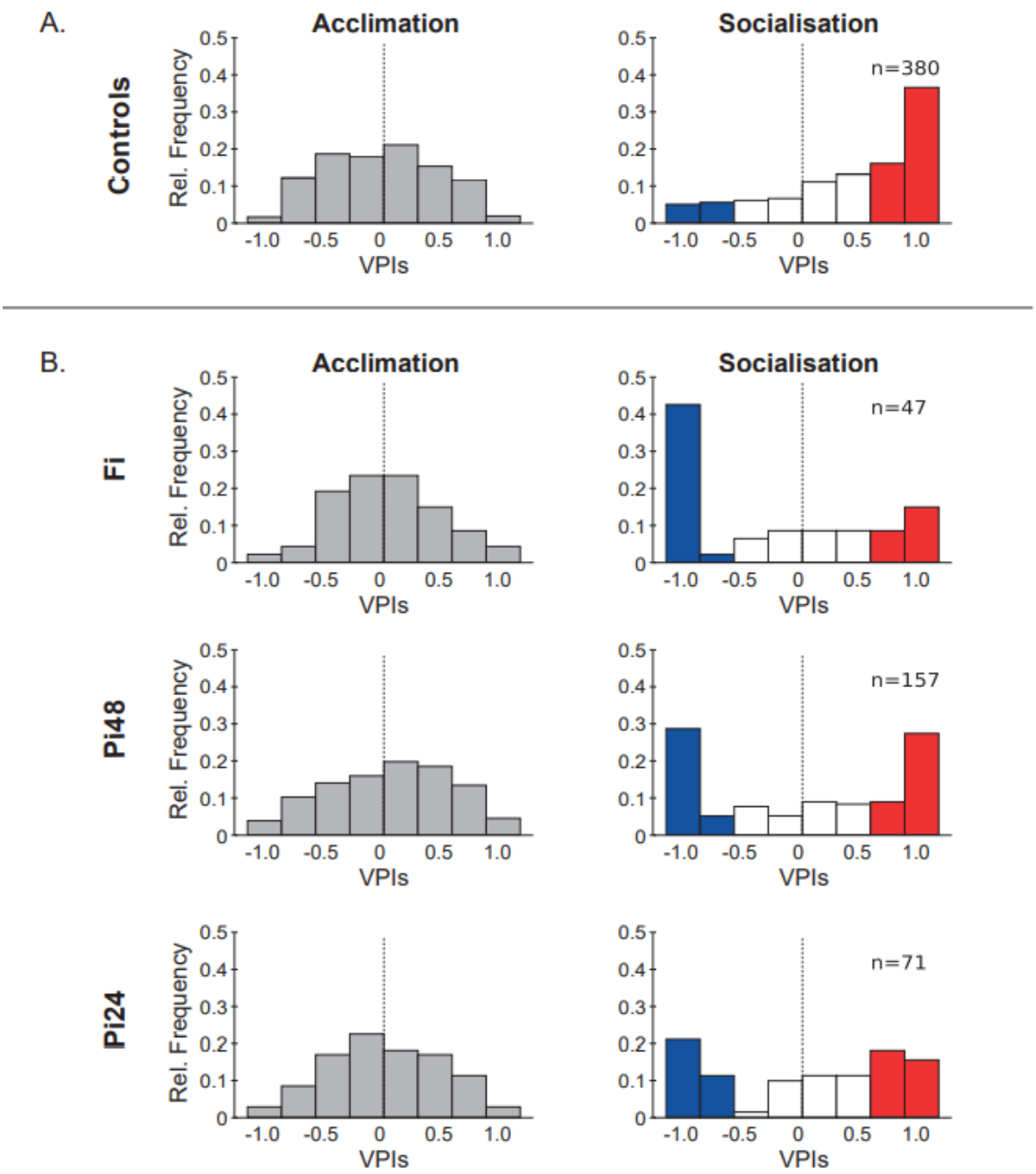


Figure 3.3: Isolation alters social preference behaviour. Left to Right: Acclimation and socialisation phases of the behavioural assay. **A.** Histogram of VPIs for control animals reared in social groups without isolation. **B.** Histogram of VPIs of fish subjected to social isolation during development. Three isolation conditions tested: Full (Fi) 21 days isolation; partial 48hrs (Pi48), and partial 24hrs (Pi24), 48hrs and 24hrs hours isolation prior to testing respectively. For visual clarity, blue bars highlight asocial fish (S-, VPIs ≤ -0.5), white no social preference fish (NSP, -0.5 < VPI < 0.5), and red bars highlight prosocial fish (S+, VPIs ≥ 0.5). Sample sizes are indicated. Note the changes in the frequency of asocial and prosocial fish groups reared in isolation conditions.

The correlation between isolation duration and impact on social preferences scores was further reflected in the subsequent calculations of effect sizes. This was done by comparing the standardised mean differences between controls and isolation conditions (expressed as Cohen's *d*). Specifically, full isolation was determined to have the most apparent effect size at d = 0.93, followed by partial isolation of 48 hours and partial isolation of 24 hours, calculated at d = 0.59, and d = 0.57, respectively.

Since the VPI distributions indicated the number of fish assigned to asocial, no-social preference and prosocial groups was altered by social deprivation, the proportions of fish found in each social group were compared across rearing conditions (**Table 3.1**). Statistical analysis revealed that isolation significantly increased the quantity of fish assigned to the asocial phenotype group whilst considerably reducing the number of prosocial fish in the population (see **Table 3.1**, $p<0.05$). The comparatively more minor, yet still significant, change observed in the no-social preference group (48 hours of partial isolation) hints that social deprivation shifts preferences instead of substituting one strong preference for another, i.e. prosocial to asocial, suggesting both prosocial and no-social preference phenotypes susceptible to the influence of social deprivation (**Table 3.1**, No-social preference: Controls (C): $\hat{p}$ = 0.387, n = 380; vs; Partial isolation 48hrs (Pi48): $\hat{p}$ = 0.293, n = 157; Z = 2.1, p = 0.0389, Two-sample Z test).

Rearing condition	Proportion of asocial fish (S-)	Proportion of no-social preference fish (nsp)	Proportion of prosocial fish (S+)
Control (C)	0.103	0.387	0.508
Full isolation (Fi)	0.447	0.319	0.234
Partial 48hrs isolation (Pi48)	0.344	0.293	0.363
Partial 24hrs isolation (Pi24)	0.324	0.338	0.338

Comparisons of rearing condition and proportion of sample populations		z-score	p-value
Controls (C) vs. Full isolation (Fi)	Asocial (S-) vs. Asocial (S-)	6.4	< 0.0001 ***
	No-social preference (nsp) vs. No-social preference (nsp)	0.9	0.3648
	Prosocial (S+) vs. Prosocial (S+)	3.5	0.0004 ***
Controls (C) vs. Partial 48hrs isolation (Pi48)	Asocial (S-) vs Asocial (S-)	6.7	< 0.0001 ***
	No-social preference (nsp) vs. No-social preference (nsp)	2.1	0.0389 ***
	Prosocial (S+) vs. Prosocial (S+)	3.1	0.0022 ***
Controls (C) vs. Partial 24hrs isolation (Pi24)	Asocial (S-) vs Asocial (S-)	5.0	< 0.0001 ***
	No-social preference (nsp) vs. No-social preference (nsp)	0.8	0.4348
	Prosocial (S+) vs. Prosocial (S+)	2.6	0.0085 ***

Table 3.1: Isolation alters the proportion of asocial and prosocial fish in a population. The Two-Sample Z-test was used to compare the number of fish assigned to each social preference group as determined by VPI scores: asocial (S-, VPIs ≤ -0.5), nonsocial (NSP, -0.5 < VPI < 0.5) and prosocial (S+, VPIs ≥ 0.5) in the presence of conspecifics. Isolation results in a significant increase in the proportion of asocial fish accompanied by a substantial decrease in prosocial animals. Asterisks indicate significance ($p \leq 0.05$).

Temporal stability of isolated fish VPIs

To investigate whether socially deprived fish recovered social preference, the stability of average VPIs of isolated and control fish were examined temporally in one-minute bins (**Figure 3.4**). The stability of VPI scores for each rearing condition was determined using three different approaches. The first approach compared temporal VPIs across rearing conditions; the second searched for differences between start and end VPIs; the third evaluated each binned VPI with preceding values, for example, the second minute vs the first minute. The latter two approaches did not involve direct comparisons between rearing conditions, hence were carried out independently.

During the acclimation phase, VPI remained stable (**Figure 3.4,** Acclimation; Control (C) vs isolation, first to the fifteenth minute: $p>0.05$), and no indication of inherent bias for any part of the chamber was observed in any rearing condition (**Figure 3.4,** Acclimation; Control (C) and isolation, first vs the fifteenth minute: $p>0.05$) (**Figure 3.4**, Acclimation; Control (C) and Isolated fish (Fi, Pi48 and Pi24), first-fifteen minutes: $p>0.05$).

In contrast, all isolated fish displayed a significant reduction in VPI compared to socially reared fish (**Figure 3.4**, Socialisation; Control (C) vs Isolated fish, one-fifteen minutes: $p<0.05$), consistent with previous data (see preceding section: **Isolation alters social preference**). Additional analysis comparing the average difference in binned VPIs between controls and isolated rearing conditions (see **Materials and Methods: Temporal VPI** for formula) further supported this finding, also revealing that full isolation has the most profound effect on social preference (Average difference of binned VPI: Full isolation (Fi): $\mu = 0.16$ > Partial isolation 48hrs (Pi48): $\mu = 0.09$ > Partial isolation 24hrs (Pi24): $\mu = 0.06$).

Comparisons of start and end VPIs per rearing condition revealed no apparent differences in all but one rearing condition, namely Pi48, which displayed significant changes between the first and last minute of their socialisation VPI values, suggesting some recovery with continued exposure

to social cues (**Figure 3.4**, Socialisation; Partial isolation 48hrs (Pi48): one vs fifteen minutes: p<0.05).

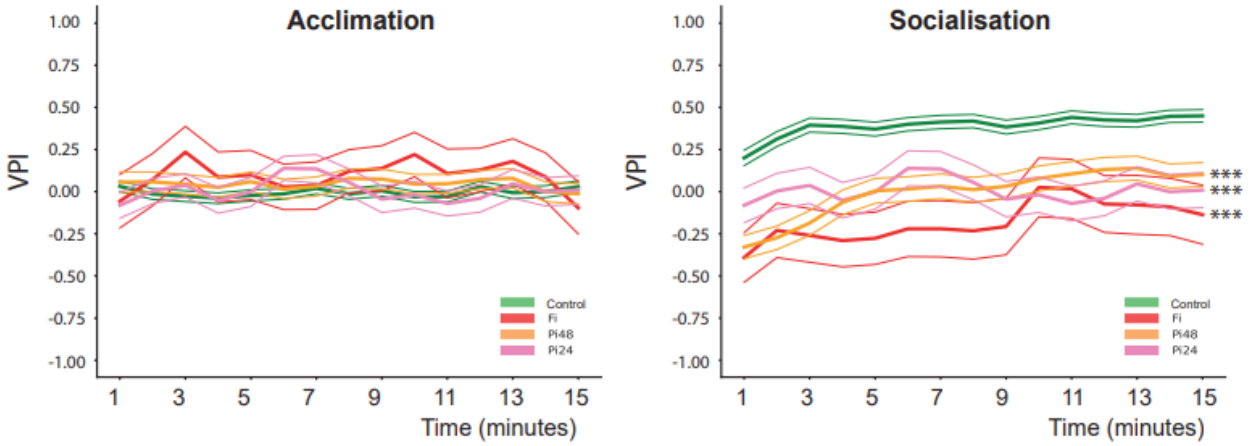


Figure 3.4: Social preferences of isolated fish are distinct from socially reared controls. The VPI shown in one-minute intervals for acclimation and socialisation phases. Thin lines indicate standard error. Left panel: Isolated fish display comparable VPIs to controls (light green line, n = 380) reared in social groups. Right panel: In the presence of conspecifics, isolated animals exhibit a significant reduction of VPI values over the fifteen-minute phase. Sample sizes are as follows full isolation (Fi, red line) n = 47; partial 48hrs isolation (Pi48, orange line), n = 157; and partial 24hrs isolation (Pi24, pink), n = 71. Statistics performed by Mann Whitney U-test comparing each isolation condition to control, ***p ≤ 0.05.

Following this finding, the stability of Pi48 fish VPIs was analysed to investigate the time scale over which VPI scores changed significantly. Since it was already established that Pi48 VPIs increase during social viewing of conspecifics as described above, the rolling window of time used for comparisons was expanded to two minutes and applied, i.e., fourth minutes vs second, fifth minute vs third etc. The first indication of significant differences was identified between the first and third minutes and the second and fourth minutes of testing, revealing that recovery of Pi48 fish was on a two-minute rolling window (**Figure 3.4**, Socialisation; Partial isolation 48hrs (Pi48): one vs three minutes: $p = 0.03756$, *Mann-Whitney U test,* $u = 10940$, $n = 47$; two vs four minutes, $p = 0.03223$, *Mann-Whitney U test,* $u = 10893.5$, $n = 47$).

This comprehensive approach was also applied to controls with no significant difference ($p > 0.05$) detected between VPI scores regardless of the rolling window size. Application to full isolation showed no significance regardless of windows size applied ($p > 0.05$), whilst partial isolation of 24 hours showed a significant difference with a rolling window size of nine minutes (**Figure 3.4**, Socialisation; Partial isolation 24hrs (Pi24): four vs thirteen minutes: $p = 0.04640$,

Mann-Whitney U test, u = 2119, *n* = 71). Therefore, controls were the most stable, followed by full, partial isolation of 24 hours, and last, partial isolation of 48 hours showed recovery.

Characterising the behaviour of isolated fish

Once established that isolation increases the number of asocial fish in a population, with full isolation having the most significant impact, it was hypothesised that these changes would also be reflected in their general behaviour. It was predicted that isolated fish behaviour would display a similar level of locomotion and bout kinematics to asocial fish, with the magnitude of isolation impacting the extent of kinematic changes.

Movement activity in isolated fish

Although several studies have investigated the impact of social isolation on movement activity, conflicting results have been reported ranging from hyperactivity, reduced movement and no effects on locomotion[443,395,444,432,433]. Thus, to assess whether the presence of conspecifics equally motivated isolated fish to move, the average percentage of time test fish spent moving was compared across the various rearing conditions for each experimental phase (**Figure 3.5**).

Isolation reduces the time test fish spend moving

The comparison between controls and isolated fish movement activity during socialisation revealed a similar trend (**Figure 3.5**): isolated fish spent significantly less time moving during acclimation and socialisation with social cues than controls (**Figure 3.5A and B** and **Table 3.2**). Across experimental phases, only the minor reduction in movement activity exhibited by fish isolated for 48 hours was significant compared to socially reared controls, indicating that Pi48 responses to social cues were altered, with fish seemingly more interested and motivated to move in the presence of social cues (**Figure 3.5A** and **B**, Differences [Socialisation - Acclimation]; Controls: *Med* = -3.76%, *n* = 380; vs; Partial isolation 48hrs (Pi48): *Med* = -2.01%, *n* = 157; *Mann-Whitney U test, u* = 25348, *p* = 0.00307).

Analysis of the percentage of time isolated fish spent moving supports earlier findings that isolated fish spend significantly less time moving than socially reared controls during acclimation and socialisation (**Figure 3.6**). Furthermore, it was revealed that Pi48 fish movement behaviour was dynamic, becoming indistinguishable from controls by eleven minutes into the socialisation phase of the assay (**Figure 3.6**, Socialisation; Controls (C) vs Partial isolation 48hrs (Pi48): one to ten minutes: $p<0.05$ and eleven to fifteen: $p>0.05$).

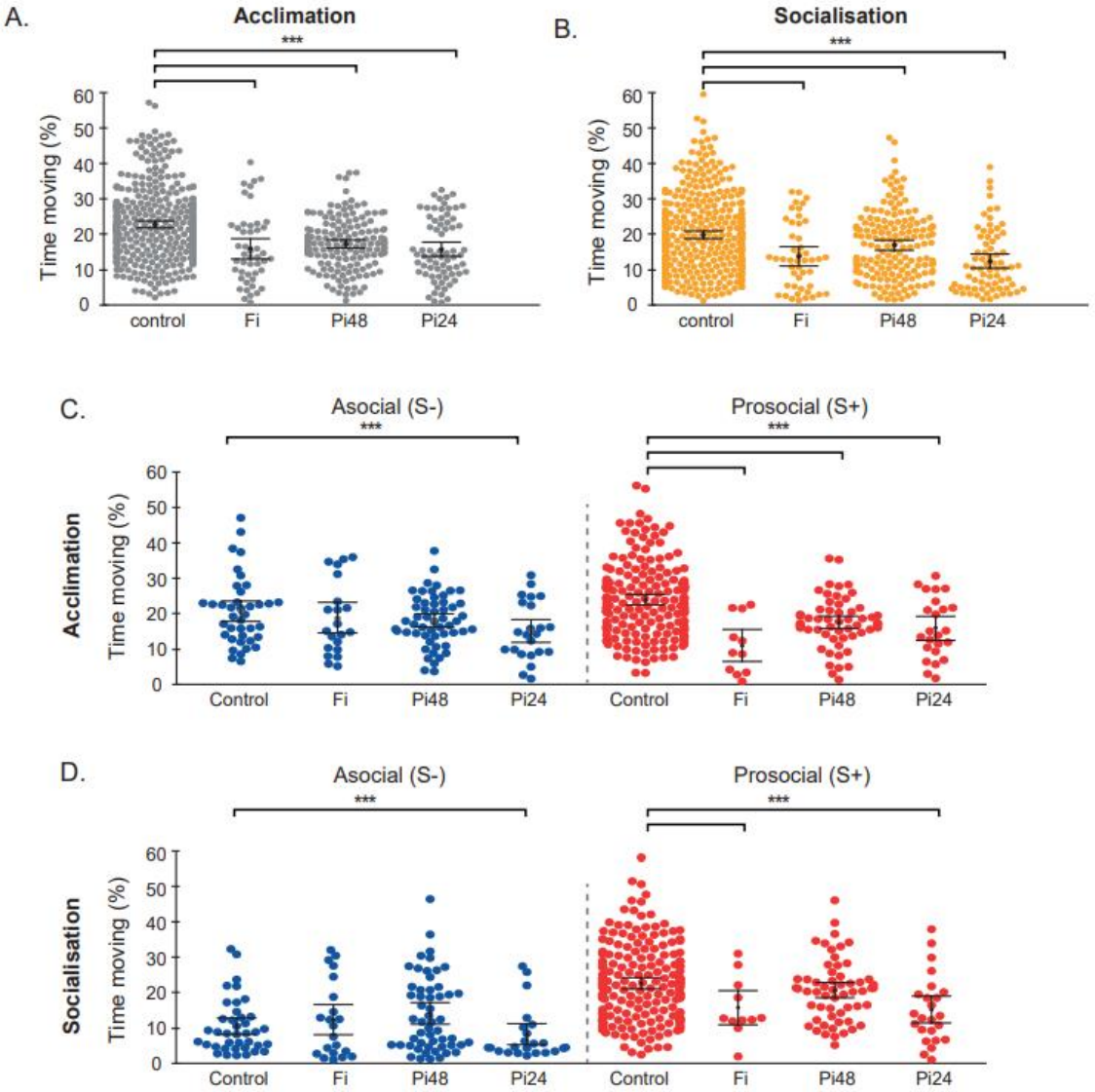


Figure 3.5: Isolation alters swimming activity in juvenile zebrafish. A. Swarm plots comparing fish activity levels during acclimation, expressed as percentage time moving (control, n = 380; Fi, n = 47; Pi48, n = 157 and Pi24, n = 71). Mean and 95% confidence intervals are shown. **B.** Comparisons of time spent moving of test fish in the presence of conspecifics for each rearing condition. **C.** Swarm plots comparing the activity levels of asocial (S-) and prosocial (S+) fish for the acclimation phase of each rearing condition: asocial (control n = 39; Fi, n = 21; Pi48, n = 53 and Pi24, n = 23) and prosocial (control n = 193; Fi, n = 11; Pi48, n = 57 and Pi24, n = 24). **D.** Comparison of the activity levels of asocial (S-) and prosocial (S+) fish groups during socialisation. Statistics performed by Mann Whitney U-test comparing each isolation condition to controls, ***$p \leq 0.05$.

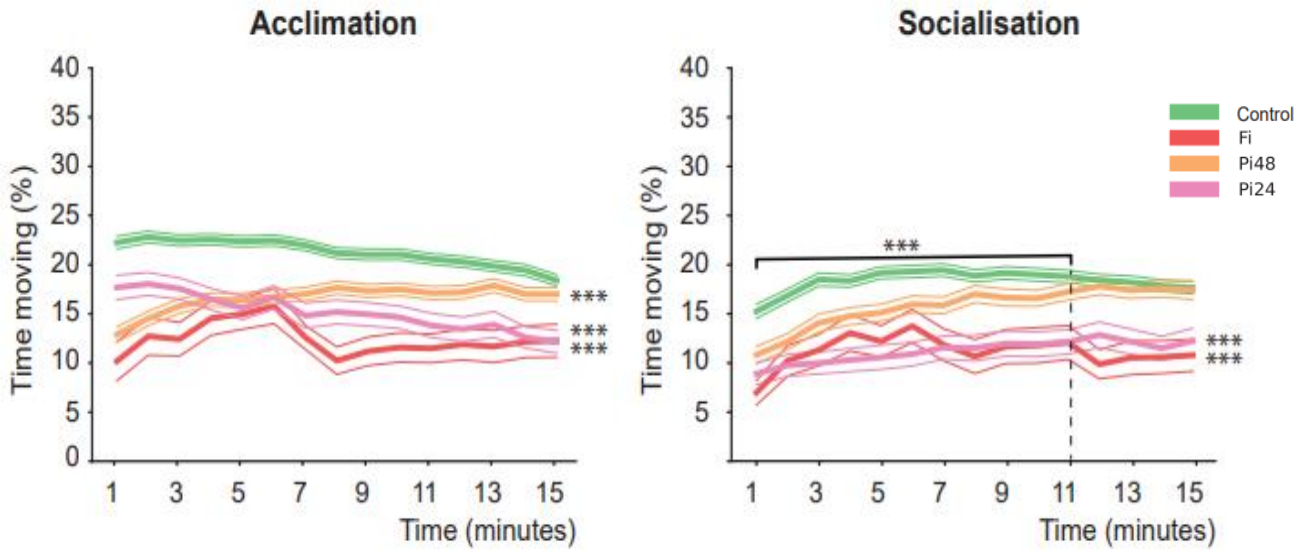


Figure 3.6: Line graph showing swimming activity of fish through time. The average percentage of time moving calculated in one-minute bins for fish reared in various conditions. Thin lines indicate standard error. Left panel: Isolated fish displayed a significant reduction in the average time moving throughout acclimation relative to fish reared under normal conditions (control, green line, n = 380). Right panel: The presence of conspecifics significantly reduces the average time spent moving in fully (Fi, red line, n = 47) and 24hrs partially isolated fish (Pi24, pink line, n = 71) over the fifteen-minute phase. Fish isolated for 48hrs (Pi48, orange line, n = 157) show significantly less movement activity until the eleventh minute into testing, shown as a dashed Line. Statistics performed by Mann Whitney U-test comparing each isolation condition to controls, ***p ≤ 0.05.

	Acclimation	Socialisation
Condition	**Mean (%)**	**Mean (%)**
Control (C)	21.90	18.69
Full isolation (Fi)	15.31	12.56
Partial 48hrs isolation (Pi48)	16.57	15.77
Partial 24hrs isolation (Pi24)	15.06	11.22

Comparisons		**W-statistic**	**p-value**
Acclimation	Control (C) vs Full isolation (Fi)	5507	≤ 0.0001 ***
	Control (C) vs Partial 48hrs isolation (Pi48)	20298	≤ 0.0001 ***
	Control (C) vs Partial 24hrs isolation (Pi24)	8321	≤ 0.0001 ***
Socialisation	Control (C) vs Full isolation (Fi)	6028	≤ 0.0001 ***
	Control (C) vs Partial 48hrs isolation (Pi48)	25530	≤ 0.0001 ***
	Control (C) vs Partial 24hrs isolation (Pi24)	7724	≤ 0.0001 ***

Comparisons		**W-statistic**	**p-value**
Control (C):	Acclimation vs. Socialisation	20743	≤ 0.0001 ***
Full isolation (Fi):	Acclimation vs. Socialisation	417	0.11980
Partial 48hrs isolation (Pi48):	Acclimation vs. Socialisation	4990	≤ 0.0001 ***
Partial 24hrs isolation (Pi24):	Acclimation vs. Socialisation	557	≤ 0.0001 ***

Table 3.2: Isolation alters movement activity. Top panel: Mean of average percentage time spent moving across the various rearing conditions shown. Middle panel: W-statistic and p-values following analysis of top panel. Statistics performed by *Mann-Whitney U test* comparing each rearing condition per experimental phase. Bottom panel: W-statistic and p-values following analysis of top panel. Statistics performed by Wilcoxon Signed-Ranked Test comparing acclimation and socialisation phases for each rearing condition. Asterisks indicate significance ($p \leq 0.0001$).

Movement activity of asocial and prosocial phenotypes

During acclimation, asocial fish from the Pi24 rearing condition showed significant reductions in time spent moving, while all other rearing conditions did not (**Figure 3.5C:** Asocial (S-) and **Table 3.3**: Acclimation, Asocial (S-)). In contrast, prosocial comparisons showed that all isolated fish exhibited a significant decrease in the time spent moving compared to socially reared fish, suggesting that prosocial fish are more susceptible to the influences of isolation (**Figure 3.5C**: Prosocial (S+) and **Table 3.3:** Acclimation, Prosocial (S+)).

During socialisation, similar results were obtained comparing the movement activity of controls and isolated fish as observed with acclimation behaviour which also included the significant difference between asocial controls and asocial Pi24 phenotypes (**Figure 3.5D**: Asocial (S-) and **Table 3.3:** Socialisation, Asocial (S-)). The only exception to the continued trend between acclimation and socialisation phases of social phenotypes was the percentage of time prosocial Pi48 spent moving since previous significance during acclimation was found not significant during the socialisation phase (**Figure 3.5**D: Prosocial (S+) and **Table 3.3:** Socialisation, Prosocial (S+)).

Interestingly, only asocial Pi48 fish displayed a significantly altered response to the presence of social cues in the adjacent chamber by retaining more of their movement activity than fish with prolonged exposure to the assay without conspecifics (Differences [Socialisation - Acclimation]; NSC: *Med* = -7.72%, *n* = 54; vs; Asocial Partial Isolation 48hrs (Pi48(S+)): *Med* = -4.486%, *n* = 53; *Mann-Whitney U test, u* = 1039, *p* = 0.00506;). This finding suggests that isolation of 48 hours may heighten fish receptiveness/sensitivity to conspecifics and thus interest in conspecifics, a phenomenon reported in humans[267,445,446].

The extension of these comparisons to prosocial phenotypes revealed varying results. Control and Pi24 fish showed a significant reduction in their movement activity not attributable to prolonged assay exposure when compared to NSC fish (Differences [Socialisation - Acclimation]; NSC: *Med* = -7.7%, *n* = 54; vs; Prosocial Control (C(S+)): *Med* = -1.88%, *n* = 193; *Mann-Whitney U test, u* = 2907, $p \leq 0.0001$; and Prosocial Partial Isolation 24hrs (Pi24(S+)): *Med* = -0.72%, *n* = 24; *Mann-Whitney U test, u* = 337, *p* = 0.00387;), whereas both prosocial Fi and Pi48 fish showed a significant increase in movement activity attributable to the presence of social cues (Differences

[Socialisation - Acclimation]; NSC: *Med* = -7.7%, *n* = 54; vs; Prosocial Full Isolation (Fi(S+)): *Med* = 4.16%, *n* = 11; *Mann-Whitney U test, u* = 38, p ≤ 0.0001; and Prosocial Partial Isolation 48hrs (Pi48(S+)): *Med* = 1.97%, *n* = 57; *Mann-Whitney U test, u* = 450, p ≤ 0.0001), suggesting that full and partial isolation of 48 hours modifies prosocial fish responses towards conspecifics.

A.

	Subpopulation	Condition	Mean (%)
Acclimation	Asocial	Control (C)	19.43
		Full isolation (Fi):	17.50
		Partial 48hrs isolation (Pi48):	16.97
		Partial 24hrs isolation (Pi24):	14.01
	Prosocial	Control (C)	23.34
		Full isolation (Fi):	10.48
		Partial 48hrs isolation (Pi48):	17.05
		Partial 24hrs isolation (Pi24):	15.52

	Subpopulation	Condition	Mean (%)
Socialisation	Asocial	Control (C)	9.37
		Full isolation (Fi):	11.21
		Partial 48hrs isolation (Pi48):	12.96
		Partial 24hrs isolation (Pi24):	6.95
	Prosocial	Control (C)	22.32
		Full isolation (Fi):	15.41
		Partial 48hrs isolation (Pi48):	20.38
		Partial 24hrs isolation (Pi24):	14.77

B.

		Comparisons	U-statistic	p-value
Acclimation	Asocial (S-)	Control (C) vs Full isolation (Fi)	348	0.17222
		Control (C) vs Partial 48hrs isolation (Pi48)	942	0.19481
		Control (C) vs Partial 24hrs isolation (Pi24)	312	0.02375 ***
	Prosocial (S+)	Control (C) vs Full isolation (Fi)	342	≤ 0.0001 ***
		Control (C) vs Partial 48hrs isolation (Pi48)	3454	≤ 0.0001 ***
		Control (C) vs Partial 24hrs isolation (Pi24)	1307	0.00025 ***

		Comparisons	U-statistic	p-value
Socialisation	Asocial (S-)	Control (C) vs Full isolation (Fi)	406	0.48146
		Control (C) vs Partial 48hrs isolation (Pi48)	882	0.09218
		Control (C) vs Partial 24hrs isolation (Pi24)	329	0.04145 ***
	Prosocial (S+)	Control (C) vs Full isolation (Fi)	684	0.02387 ***
		Control (C) vs Partial 48hrs isolation (Pi48)	4973	0.13596
		Control (C) vs Partial 24hrs isolation (Pi24)	1375	0.00059 ***

Table 3.3: Isolation alters the movement activity of social groups differently. **A.** Mean of average percentage time spent moving for asocial and prosocial fish across the various rearing conditions. **B.** U-statistic and p-values following analysis of top panel. Only asocial fish isolated for 24 hours exhibit significantly altered movement, whilst all prosocial fish across all isolated conditions exhibit a significant reduction in activity compared to controls. Statistics performed by Mann Whitney U-test comparing isolation conditions to controls. Asterisks indicate significance ($p \leq 0.05$).

Freezing behaviour in isolated fish

Previously in **Chapter 2**, it was established that an increased freezing behaviour, the average percentage of time spent in continuous periods (>3 sec) without motion, across experimental phases, was not attributable to the presence conspecifics. Instead, it was determined to be a general response to prolonged exposure to the assay without social cues.

Several studies have reported that isolation induces stress and anxiety-like behaviours in zebrafish and rodents[303,447–449]. Although freezing is often considered a hallmark of anxiety-like behaviour observed in many species and reported in zebrafish exposed to stressors[395,450], including periods of social isolation[353,362], reduction in freezing following social deprivation has also been reported in studies[451]. Therefore, to investigate if isolation periods cause freezing behaviour changes, test fish quiescence periods were quantified and compared to controls similar to the movement activity parameter in the previous sections.

Isolation increases freezing behaviour

Inspection of raw behavioural data revealed that isolated fish behaved qualitatively differently, exhibiting prolonged periods of quiescence (freezing) during both acclimation and socialisation phases, compared to socially reared conspecifics (Video: https://static-movie-usa.glencoesoftware.com/mp4/10.7554/394/7196c34494e888aa49d8865541ae269805e1201c/elife-55863-video1.mp4).

This observation was further confirmed by statistical analysis of freezing behaviour between rearing conditions and experimental phases (**Figure 3.7** and **Table 3.4**). In detail, comparisons of freezing behaviour during acclimation revealed that socially deprived fish exhibited significantly more freezing behaviour than their control conspecifics (**Figure 3.7A** and **Table 3.4**), suggesting that social deprivation could induce anxiety. Fully isolated fish showed significantly more freezing than partially isolated fish, which was an expected find considering the more significant effect size of complete isolation. Interestingly, a comparison of the freezing behaviours of partially isolated fish revealed Pi24 fish spent substantially more time freezing than Pi48 fish, suggesting that a

shorter 24-hour period of isolation had a more significant impact on fish movement than 48 hours of isolation (**Figure 3.7A**; Partial isolation 48hrs (Pi48): *Med* = 3.93%, *n* = 157; vs; Partial isolation 24hrs (Pi48): *Med* = 11.03%, *n* = 71; *Mann-Whitney U test, u* = 4517.5, *p* = 0.01075).

Both controls and isolated fish significantly increased their freezing time (**Table 3.4**). Interestingly, Fi fish spent the greatest period freezing, followed by Pi24, Pi48 and controls fish (**Figure 3.7B**). The acclimation and socialisation data were similar in magnitude, suggesting that the differences between controls and isolated fish during socialisation were linked to pre-existing differences observed before viewing conspecifics. This indication was later confirmed by cross comparing the change in freezing behaviour across phases between rearing conditions, where no significance was detected ($p > 0.05$).

To investigate whether the increase in freezing behaviour across phases in isolated fish could be attributable to prolonged exposure to the assay (as established in controls), Fi, Pi48 and Pi24 fish reactions were compared to NSC fish behaviour.

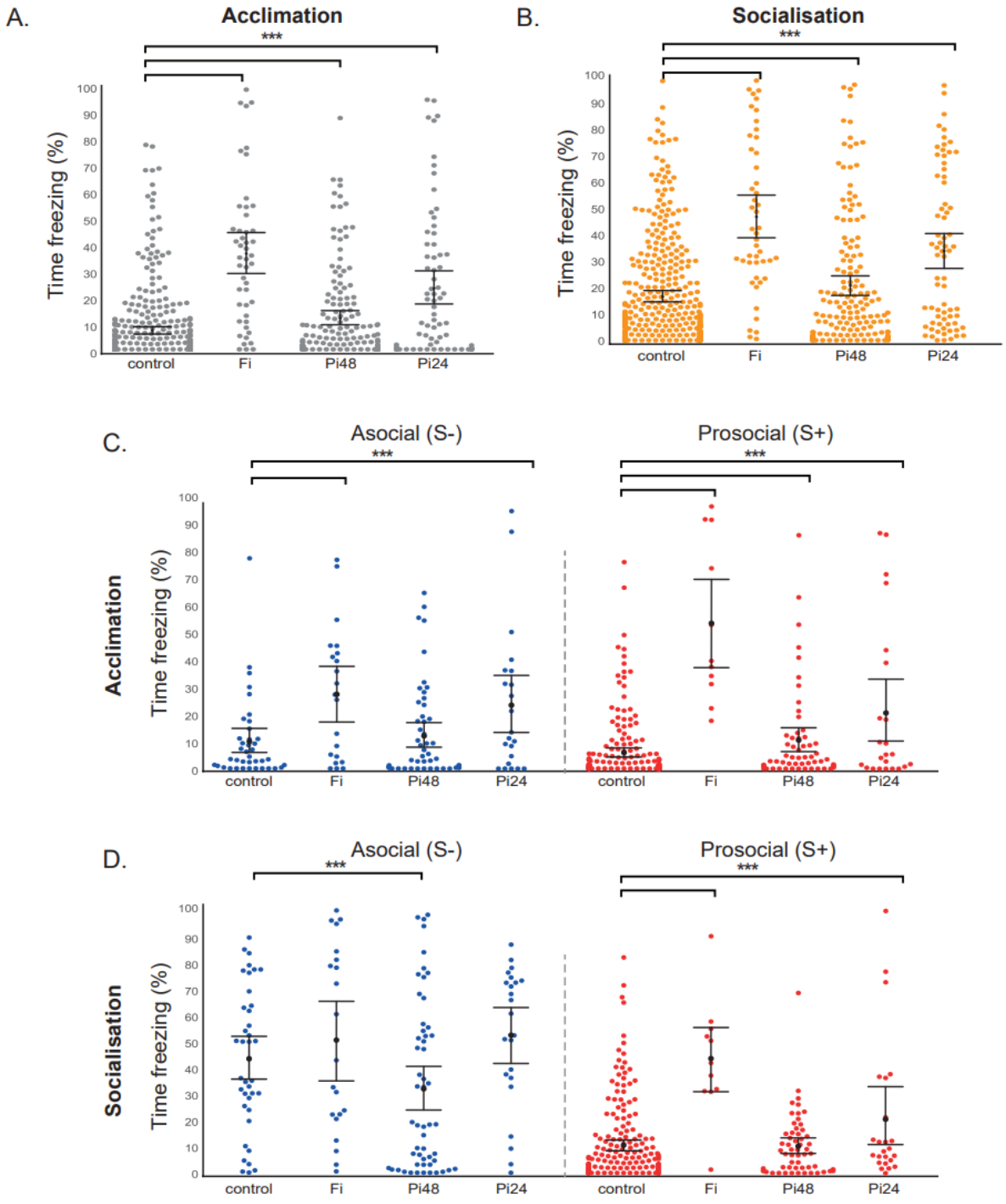


Figure 3.7: Isolation increases freezing behaviour in juvenile zebrafish. A. Swarm plots comparing freezing durations longer than three seconds during acclimation, expressed as percentage time freezing (control, n = 380; Fi, n = 47; Pi48, n = 157 and Pi24, n = 71). Mean and 95% confidence intervals are shown. **B.** Comparisons of time spent freezing by test fish in the presence of conspecifics for each rearing condition. **C.** Swarm plots comparing the time spent freezing of asocial (S-) and prosocial (S+) fish for the acclimation phase of each rearing condition: asocial (control, n = 39; Fi, n = 21; Pi48, n = 53 and Pi24, n = 23) and prosocial (control, n = 193; Fi, n = 11; Pi48, n = 57 and Pi24, n = 24). **D.** Comparison of the freezing durations of asocial (S-) and prosocial (S+) fish groups during socialisation. Statistics performed by Mann Whitney U-test comparing each isolation condition to controls, ***$p \leq 0.05$.

These comparisons revealed that socially deprived fish altered the time they spent freezing across experimental phases comparably to NSC fish ($p > 0.05$), showing that isolated fish responses were linked to the presence of social cues. Since a similar response was also observed in controls, this also suggested that socially deprived fish exhibiting greater anxiety during acclimation respond to conspecifics like controls (**Figure 3.7A and D**, and **Table 3.4**).

As with the percentage time moving, freezing behaviour was also inspected temporally (**Figure 3.8**). Analysis of the time fish spent freezing in one-minute bins revealed that freezing

	Acclimation	Socialisation
Condition	Mean (%)	Mean (%)
Control (C)	7.08	16.72
Fi isolation (Fi)	36.01	47.30
Partial 48hrs isolation (Pi48)	11.70	21.02
Partial 24hrs isolation (Pi24)	22.92	34.21

Comparisons		W-statistic	p-value
Control (C):	Acclimation vs. Socialisation	10658	≤ 0.0001 ***
Full isolation (Fi):	Acclimation vs. Socialisation	346	0.02106 ***
Partial 48hrs isolation (Pi48):	Acclimation vs. Socialisation	3234	≤ 0.0001 ***
Partial 24hrs isolation (Pi24):	Acclimation vs. Socialisation	548	≤ 0.0001 ***

Comparisons		W-statistic	p-value
Acclimation	Control (C) vs Full isolation (Fi)	2579	≤ 0.0001 ***
	Control (C) vs Partial 48hrs isolation (Pi48)	23322	≤ 0.0001 ***
	Control (C) vs Partial 24hrs isolation (Pi24)	8776	≤ 0.0001 ***
Socialisation	Control (C) vs Full isolation (Fi)	3267	≤ 0.0001 ***
	Control (C) vs Partial 48hrs isolation (Pi48)	26728	0.02887 ***
	Control (C) vs Partial 24hrs isolation (Pi24)	8266	≤ 0.0001 ***

Table 3.4: Isolation increases freezing behaviour. Top panel: Mean of average percentage time spent freezing across the various rearing conditions shown. Bottom panel: W-statistic and p-values following analysis of top panel. Statistics performed by Wilcoxon Signed-Ranked Test comparing acclimation and socialisation phases for each rearing condition. Asterisks indicate significance ($p \leq 0.05$).

behaviour profiles were unique to each rearing condition. In-depth, controls fish exhibited stable freezing responses, which showed minor alteration between the start and end of the acclimation phase. Fi and Pi48 fish displayed a gradual reduction in time spent freezing with the progression of the first fifteen-minute phase Pi48 fish freezing behaviour was indistinguishable from controls fish by the ninth minute of testing, suggesting that a shorter period of 48 hours isolation is more

easily reversed than complete social deprivation (**Figure 3.8**, Acclimation; Control (C): *n* = 380; vs; Partial isolation 48hrs (Pi48): *n* = 157; one to ten minutes: p>0.05, eleven to fifteen minute: p<0.05). In contrast to the decrease Fi and Pi48 exhibited, Pi24 fish gradually increased the time they spent freezing, suggesting a gradual increase in anxiety.

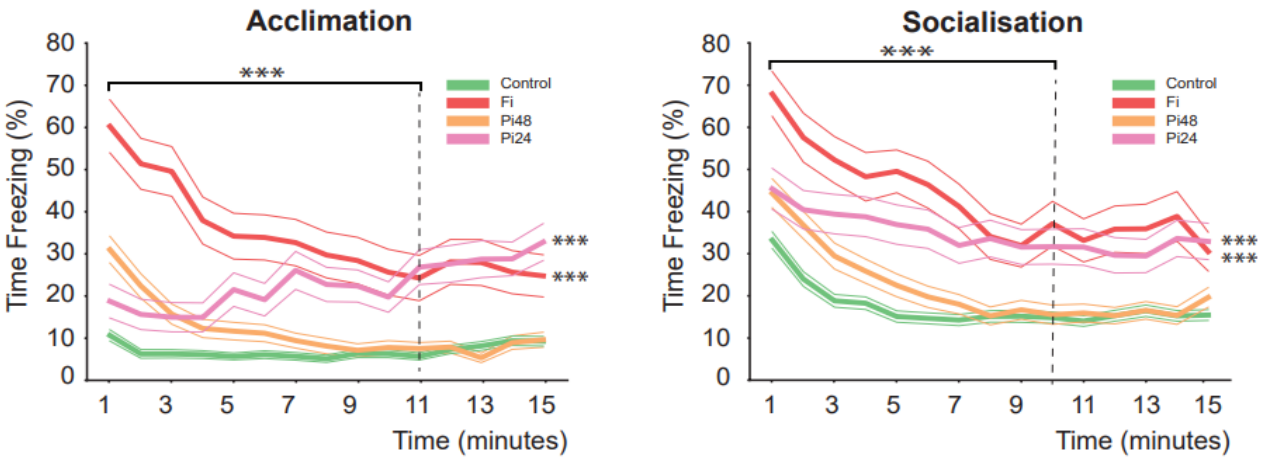


Figure 3.8: The freezing behaviour of isolated fish is distinct from socially reared controls. The average percentage of time freezing calculated in one-minute bins for fish reared in various conditions. Thin lines indicate standard error. Left panel: Isolated fish display an increase in the average time spent freezing throughout acclimation relative to fish reared under normal conditions (controls, green line, n = 380). This increase is consistently significantly throughout acclimation for fully (Fi, red line, n = 47) and 24hrs partially isolated fish (Pi24, pink line, n = 71). Fish isolated for 48hrs (Pi48, orange line, n = 157) become comparable to controls eleven minutes into the initial phase of the assay, shown as a dashed line. Right panel: The presence of conspecifics significantly increases the aver- age time spent freezing in Fi and Pi24 fish during socialisation. Similar to acclimation, Pi48 fish freezing becomes comparable to controls after ten minutes, shown as a dashed line. Statistics performed by Mann Whitney U-test comparing each isolation condition to controls, ***p ≤ 0.05.

In the presence of conspecifics Fi and Pi48 fish continued to show a reduction in freezing behaviour with Pi48 fish once more becoming indistinguishable from controls by an earlier time of seven minutes (**Figure 3.8**, Acclimation; Control (C): *n* = 380; vs; Partial isolation 48hrs (Pi48): *n* = 157; one to nine: p>0.05, ten to fifteen minutes: p<0.05). However, as the decreased freezing of Pi48 fish during socialisation was on a similar time scale as observed during acclimation, reducing time spent freezing may be associated with habituation.

The freezing activity of isolated asocial and prosocial phenotypes

Since periods of separation from conspecifics may be differently perceived by asocial and prosocial phenotypes, and the impact on these phenotypes may be masked by population-level analysis, freezing behaviour of isolated asocial and prosocial isolated fish were independently evaluated.

Comparison of freezing behaviours of asocial fish during acclimation revealed that the effects of isolation could be detected in asocial Fi and Pi24 populations, with both spending significantly more time freezing than control fish (**Figure 3.7C**: Asocial and **Table 3.5**). In the presence of social cues all asocial fish including those reared in socially deprived conditions, showed a significant increase in the time they spend freezing, becoming indistinguishable from control fish in the presence of conspecifics (**Figure 3.7C** and **D** Asocial, Acclimation vs Socialisation; Control (C(S-)): *n* = 39,*Wilcoxon signed-rank test*, w = 213, p ≤ 0.0001; Asocial Full isolation (Fi(S-)): *n* = 21,*Wilcoxon signed-rank test*, w = 136, *p* = 0.01728; Partial isolation 48hrs(Pi48(S-)): *n* = 53,*Wilcoxon signed-rank test*, w = 880, *p* = 0.00019; Partial isolation 24hrs(Pi24(S-)): *n* = 23,*Wilcoxon signed-rank test*, w = 109.5, *p* = 0.00034;). Across experimental phases only Fi and Pi48 fish changed their behaviour in the presence of conspecifics, increasing time spent freezing significantly more than that observed in NSC fish, unlike Pi24 and socially reared controls (Differences [Socialisation - Acclimation]; NSC: *Med* = 4.27%, *n* = 54; vs; Asocial Control (C(S-)): *Med* = 30.08%, *n* = 39; *Mann-Whitney U test, u* = 517, p ≤ 0.0001; Asocial Full Isolation (Fi(S-)): *Med* = 23.78%, *n* = 21; *Mann-Whitney U test, u* = 442, *p* = 0.0.07090; Asocial Partial Isolation 48hrs (Pi48(S-)): *Med* = 20.01%, *n* = 53; *Mann-Whitney U test, u* = 1223.5, *p* = 0.07524; and Asocial Partial Isolation 24hrs (Pi24(S-)): *Med* = 32.58%, *n* = 23; *Mann-Whitney U test, u* = 323.5, *p* = 0.00047).

All prosocial fish reared in isolated conditions spent significantly more time freezing during acclimation and socialisation (**Table 3.4**). Although all prosocial fish displayed a significant reduction in time spent moving across experimental phases compared to NSC fish (Differences [Socialisation - Acclimation]; NSC: *Med* = 4.27%, *n* = 54; vs; Prosocial Full Isolation (Fi(S+)): *Med* = -9.44%, *n* = 11; *Mann-Whitney U test, u* = 154, *p* = 0.00633; Prosocial Partial Isolation 48hrs (Pi48(S+)): *Med* = 0%, *n* = 57; *Mann-Whitney U test, u* = 916.5, *p* = 0.00012; Prosocial Partial Isolation 24hrs (Pi24(S+)): *Med* = 2.40%, *n* = 24; *Mann-Whitney U test, u* = 471.5, *p* = 0.02836;), only the reduced freezing of prosocial Fi and Pi48 fish showed in response to conspecifics were significantly lower than socially reared conditions and, therefore, transformed by social deprivation (Differences [Socialisation - Acclimation]; Prosocial Control (C(S+)): *Med* = -11.49%, *n* = 193; vs; Prosocial Full Isolation (Fi(S+)): *Med* = -9.44%, *n* = 11; *Mann-Whitney U test, u* = 688154, *p* = 0.02502; Prosocial Partial Isolation 48hrs (Pi48(S+)): *Med* = 0%, *n* = 57; *Mann-Whitney U test, u* = 4559.5, *p* = 0.02489; Prosocial Partial Isolation 24hrs (Pi24(S+)): *Med* = 2.40%, *n* = 24; *Mann-Whitney U test, u* = 2136.5, *p* = 0.26849;).

Together, these results suggest that only more extended periods of isolation, i.e., full and partial isolation of 48 hours, alter fish freezing activity, counterintuitively reducing them.

A.

	Subpopulation	Condition	Mean (%)
Acclimation	Asocial	Control (C)	9.74
		Full isolation (Fi):	26.93
		Partial 48hrs isolation (Pi48):	11.83
		Partial 24hrs isolation (Pi24):	23.00
	Prosocial	Control (C)	5.91
		Full isolation (Fi):	54.12
		Partial 48hrs isolation (Pi48):	10.63
		Partial 24hrs isolation (Pi24):	20.80

	Subpopulation	Condition	Mean (%)
Socialisation	Asocial	Control (C)	43.22
		Full isolation (Fi):	50.30
		Partial 48hrs isolation (Pi48):	31.84
		Partial 24hrs isolation (Pi24):	52.24
	Prosocial	Control (C)	10.27
		Full isolation (Fi):	42.63
		Partial 48hrs isolation (Pi48):	9.99
		Partial 24hrs isolation (Pi24):	20.05

B.

		Comparisons	U-statistic	p-value
Acclimation	Asocial (S-)	Control (C) vs Full isolation (Fi)	239	0.00411 ***
		Control (C) vs Partial 48hrs isolation (Pi48)	1050	0.49221
		Control (C) vs Partial 24hrs isolation (Pi24)	305	0.01815 ***
	Prosocial (S+)	Control (C) vs Full isolation (Fi)	75	≤ 0.0001 ***
		Control (C) vs Partial 48hrs isolation (Pi48)	3951	0.00048 ***
		Control (C) vs Partial 24hrs isolation (Pi24)	1484.5	0.00164 ***

		Comparisons	U-statistic	p-value
Socialisation	Asocial (S-)	Control (C) vs Full isolation (Fi)	365	0.24764
		Control (C) vs Partial 48hrs isolation (Pi48)	791.5	0.02106 ***
		Control (C) vs Partial 24hrs isolation (Pi24)	351.5	0.07983
	Prosocial (S+)	Control (C) vs Full isolation (Fi)	240	≤ 0.0001 ***
		Control (C) vs Partial 48hrs isolation (Pi48)	4971	0.13448
		Control (C) vs Partial 24hrs isolation (Pi24)	1549	0.00405 ***

Table 3.5: Isolation alters freezing behaviour in asocial and prosocial fish. **A.** Mean of average percentage time spent moving for asocial and prosocial fish across the various rearing conditions. **B.** U-statistic and p-values following analysis of top panel. Statistics performed by Mann Whitney U-test comparing isolation conditions to controls. Asterisks indicate significance ($p \leq 0.05$).

Isolation alters distances travelled

Comparison of distances fish travelled during acclimation revealed that all isolated fish covered significantly shorter distances than the controls (**Figure 3.9A**). Fully isolated fish showed the most significant difference (**Figure 3.9A**, Acclimation; Control (C): *Med* = 5091.44 mm, *n* = 380; vs; Full isolation (Fi): *Med* = 3363.85 mm, *n* = 47; *Mann-Whitney U test, u* = 4062, $p \leq 0.0001$;). Pi24 fish, which travelled 1764 mm less than controls (**Figure 3.9A**, Acclimation; Control (C): *Med* = 5091.44 mm, *n* = 380; vs; Partial isolation 24hrs (Pi24): *Med* = 4915.04 mm, *n* = 157, *Mann-Whitney U test, u* = 27063, *p* = 0.04536). Pi48 travelled 3828.11 mm (**Figure 3.9A**, Acclimation; Control (C): *Med* = 5091.44 mm, *n* = 380; vs; Partial isolation 48hrs (Pi48): *Med* = 3828.11 mm, *n* = 71, *Mann-Whitney U test, u* = 8944, $p \leq 0.0001$;). The travelling of Pi48 was unexpected since it was assumed that 48 hours of isolation would have a more considerable impact on the behaviour of fish than a shorter period of 24 hours based on earlier findings on VPI and effect sizes. However, these results align with previous findings, showing that Pi48 spent more time moving and less freezing.

In the presence of social cues, all fish, regardless of rearing conditions, displayed a significant reduction in the total distance covered (**Figure 3.9A**: Socialisation). To test whether distances travelled by isolated fish were attributable to the viewing of conspecifics, differences in behaviour across experimental phases were compared to NSC fish responses. Analysis revealed that similar to controls, all isolated fish showed a significant reduction in total distances covered during socialisation, outside the boundaries explainable by prolonged exposure to the assay without social cues (**Figure 3.9A**, Differences [Socialisation - Acclimation]; NSC: *Med* = -1473.25 mm, *n* = 54; vs; Control (C): *Med* = -1277.19 mm, *n* = 380, *Mann-Whitney U test, u* = 8775, *p* = 0.04261; Full isolation (Fi): *Med* = -429.98 mm, *n* = 47, *Mann-Whitney U test, u* = 840, *p* = 0.001764; Partial isolation 48hrs (Pi48): *Med* = -664.24 mm, *n* = 157, *Mann-Whitney U test, u* = 2789, $p \leq 0.0001$; and Partial isolation 24hrs (Pi24): *Med* = -974.52 mm, *n* = 71, *Mann-Whitney U test, u* = 1564, *p* = 0.03947;).

The analysis of distances of isolated fish revealed that Pi24 fish travelled similar lengths compared to controls ($p<0.05$), suggesting that 24 hours of isolation does not alter fish responses to social cues. In contrast, Fi and Pi48 fish showed a significantly reduction in the total distance

travelled compared to controls, suggesting that longer periods of isolation impact fish responses to conspecifics (**Figure 3.9A**, Differences [Socialisation - Acclimation]; Controls(C): *Med* = -1277.18 mm, *n* = 380; vs; Full isolation (Fi): *Med* = -429.97 mm, *n* = 47, *Mann-Whitney U test, u* = 7109, *p* = 0.01128; and Partial isolation 48hrs (Pi48): *Med* = -664.24 mm, *n* = 157, *Mann-Whitney U test, u* = 24034, *p* = 0.00020). Together, these results confirm earlier indications that Pi48 responses towards conspecifics are unique in terms of distance travelled and suggest that Fi responses are too atypical of those observed in controls. Thus, isolation beyond 24 hours impacts fish responses towards conspecifics.

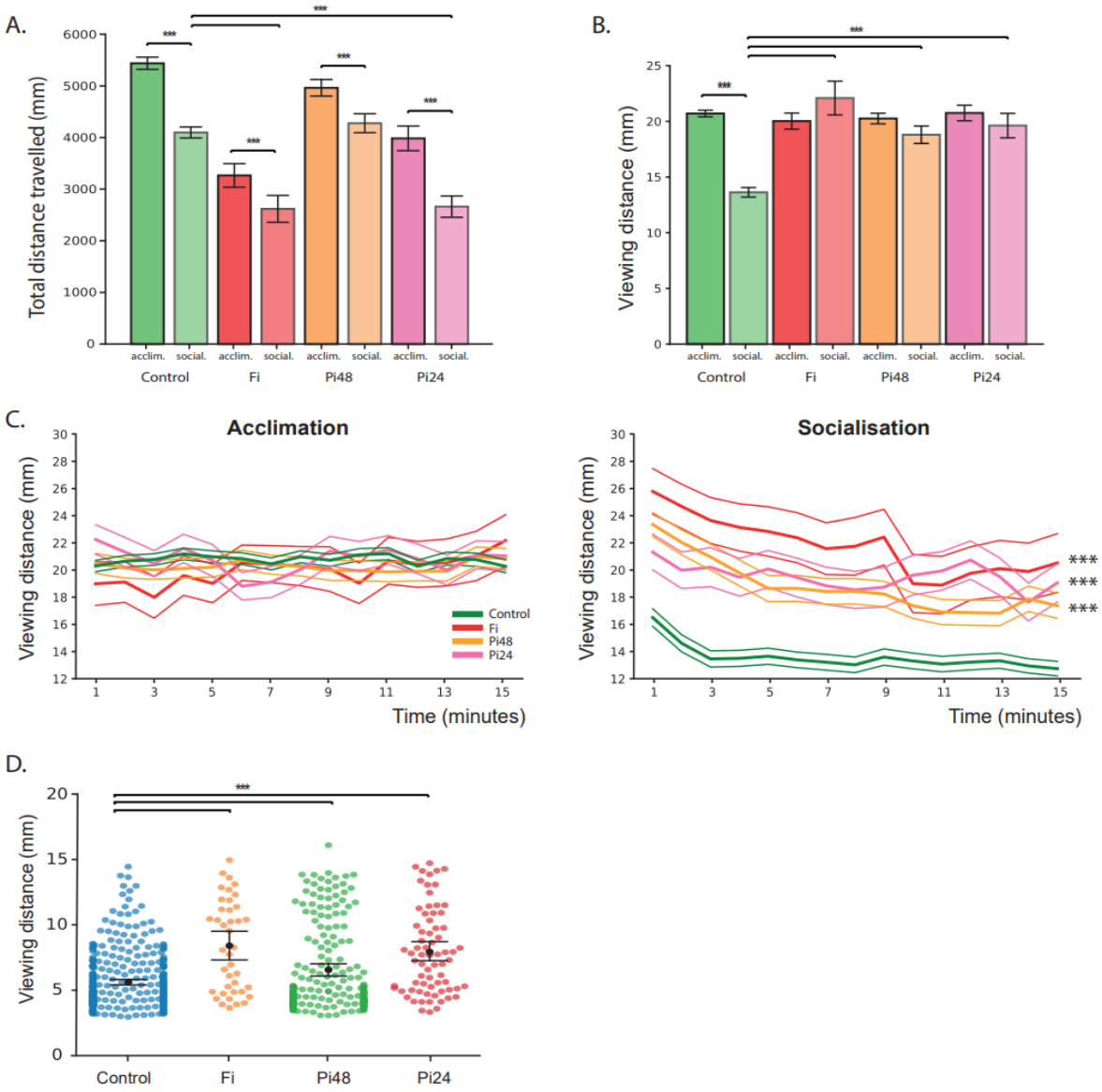


Figure 3.9: Isolation alters fish locomotion and viewing distances. **A.** Total distances travelled by test fish during acclimation and socialisation. Isolated fish travel shorter distances in the chamber during both phases of the assay. **B.** Bar graph of the average viewing distance of test fish in the presence of conspecifics. Isolated fish significantly alter their proximity to the dividing glass window in the presence of social cues. **C.** Line graph showing average focal fish distances from conspecifics displayed as one-minute bins. All isolated fish distances are comparable to controls during acclimation, while in the presence of cues, isolated fish show a significant preference to be further away from social cues. **D.** Swarm plot of the average test fish distance to conspecifics when in zone 'AB'. Isolation increases test fish distances to conspecifics. Mean and standard errors are shown. Statistics performed by Wilcoxon signed-rank test for comparing acclimation to socialisation phases and Mann Whitney U-test comparing each isolation condition to controls, ***$p \leq 0.05$. Sample sizes are as follows: A, B and C: Control, n = 380; Fi, n = 47; Pi48, n = 157; Pi24, n = 71; and D: Control, n = 366; Fi, n = 40; Pi48, n = 157; Pi24, n = 71.

Isolated fish view conspecifics from greater distances

During acclimation, all fish reared in isolated conditions viewed conspecifics from a similar distance from the social window as socially reared controls (**Figure 3.9B**; Acclimation: Control vs Full isolation (Fi), Partial isolation 48hrs (Pi48) and Partial isolation 24hrs (Pi24), $p>0.05$).

In the presence of conspecifics, isolated fish from Fi, Pi48 and Pi24 conditions did not show the same reduction in distance from the social windows as socially reared controls, instead opting for viewing distances significantly further back (**Figure 3.9B**: Socialisation; Control (C): *Med* = -1277.19 mm, *n* = 380, *vs;* Full isolation (Fi): *Med* = -429.98 mm, *n* = 47, *Mann-Whitney U test, u* = 840, *p* = 0.001764; Partial isolation 48hrs (Pi48): *Med* = -664.24 mm, *n* = 157, *Mann-Whitney U test, u* = 2789, $p \leq 0.0001$; and Partial isolation 24hrs (Pi24): *Med* = -974.52 mm, *n* = 71, *Mann-Whitney U test, u* = 1564, *p* = 0.03947;). As close physical distance (proximity) to a stimulus can readily trigger fear/anxiety, this hints that isolated fish may be fearful/anxious about conspecifics[452], possibly through alterations in the interpersonal space preferences[436].

Across experimental phases, all socially deprived fish displayed isolation-induced behaviour transformation in terms of proximity to the social window with the presence of conspecifics. Fi fish showed an active avoidance of conspecifics across experimental phases by significantly increasing their distance from the social window compared to NSC fish (Differences [Socialisation - Acclimation]; NSC: Med = -0.86 mm, n = 54; vs; Med = 4.60 mm, n = 47; *Mann-Whitney U test*, u = 900, p = 0.04519) In contrast, alteration in the proximity of Pi48 and Pi24 fish across experimental phases were attributable with prolonged assay exposure dissimilar to the behaviour observed in socially reared controls; therefore, suggesting short periods of social isolation alters fish interest towards conspecifics on the population level.

Since all the above analysis also included non-visual contact regions, to get a better resolution of fish responses when viewing social cues, distance analysis was applied to the portion of the social preference chamber where fish can readily view social stimuli (zone 'AB') (**Figure 3.9D**). These results further corroborated earlier findings that isolated fish adopt farther distances from the social window than socially reared control fish.

Pi48 fish reduce their viewing distances over time

Test fish distances were temporally inspected in one-minute bins to investigate whether isolated fish altered their viewing proximity to conspecifics throughout experimental phases (**Figure 3.9C**). Evaluation of the acclimation behaviour revealed that socially deprived fish were indistinguishable before viewing cues (**Figure 3.9C**: Acclimation). However, in line with earlier findings, the presence of conspecifics altered this similarity with isolated fish keeping significantly greater distances away from the social window throughout the entirety of the socialisation phase (**Figure 3.9**, Socialisation: Control (C) vs Full isolation (Fi), Partial isolation 48hrs (Pi48), Partial isolation 24hrs (Pi24); one-fifteen minute: $p<0.05$).

Regardless of rearing conditions, all fish reduced their proximity to social cues with the progression of the socialisation phase. However, evaluation of differences in viewing distances at the start and end of socialisation between the various rearing conditions with NSC responses found that only Pi48 reduction in viewing distances was attributable to the presence of social cues (Differences [15 minute – 1 minute]: NSC: *Med* = -2.15 mm, $n = 54$; vs; Partial isolation 48hrs (Pi48): *Med* = -1.84 mm, $n = 157$, *Mann-Whitney U test,* $u = 4061$, $p = 0.040366$). This finding indicates that only fish isolated for 48 hours showed a reduction in proximity responding to the presence of conspecifics. This important finding may have otherwise been missed without temporal inspection of isolated fish's viewing distances.

Isolated fish increase Y motion similar to controls

To evaluate the social interaction behaviour in isolated fish, total X motion was divided by Y motion in zone 'AB'. The resulting ratio plotted against average conspecific viewing distances allowed investigation into whether fish altered their behaviour with proximity to conspecifics (**Figure 3.10**). Motion scores larger than one highlighted fish with greater X motion readily approaching and retreating from the social stimuli in a 'back' and 'forward' motion. A total of 268 isolated fish motions were extracted (Fi; 40, Pi48; 157, and Pi24; 71).

Analysis revealed that juvenile fish, regardless of rearing condition, exhibited greater changes in lateral Y motion, moving 'side-to-side' relative to the social window, during both phases of the assay (**Figure 3.10**: Acclimation and Socialisation). Closer inspection of the average position of

controls fish movement across phases (**Figure 3.10A**: Acclimation) revealed substantial differences in how fish moved around the area nearest the social window. During acclimation controls, fish remained an average of 8 mm away from the social window, which placed fish in the middle of zone 'AB'. Comparing this to movement during socialisation controls fish displayed greater lateral movement with closer proximity to social cues (**Figure 3.10A**: Acclimation: *Med* = 0.09; vs; Socialisation: *Med* = 0.13; *Wilcoxon signed-rank test*, w = 6270, p ≤ 0.0001, *n* = 366).

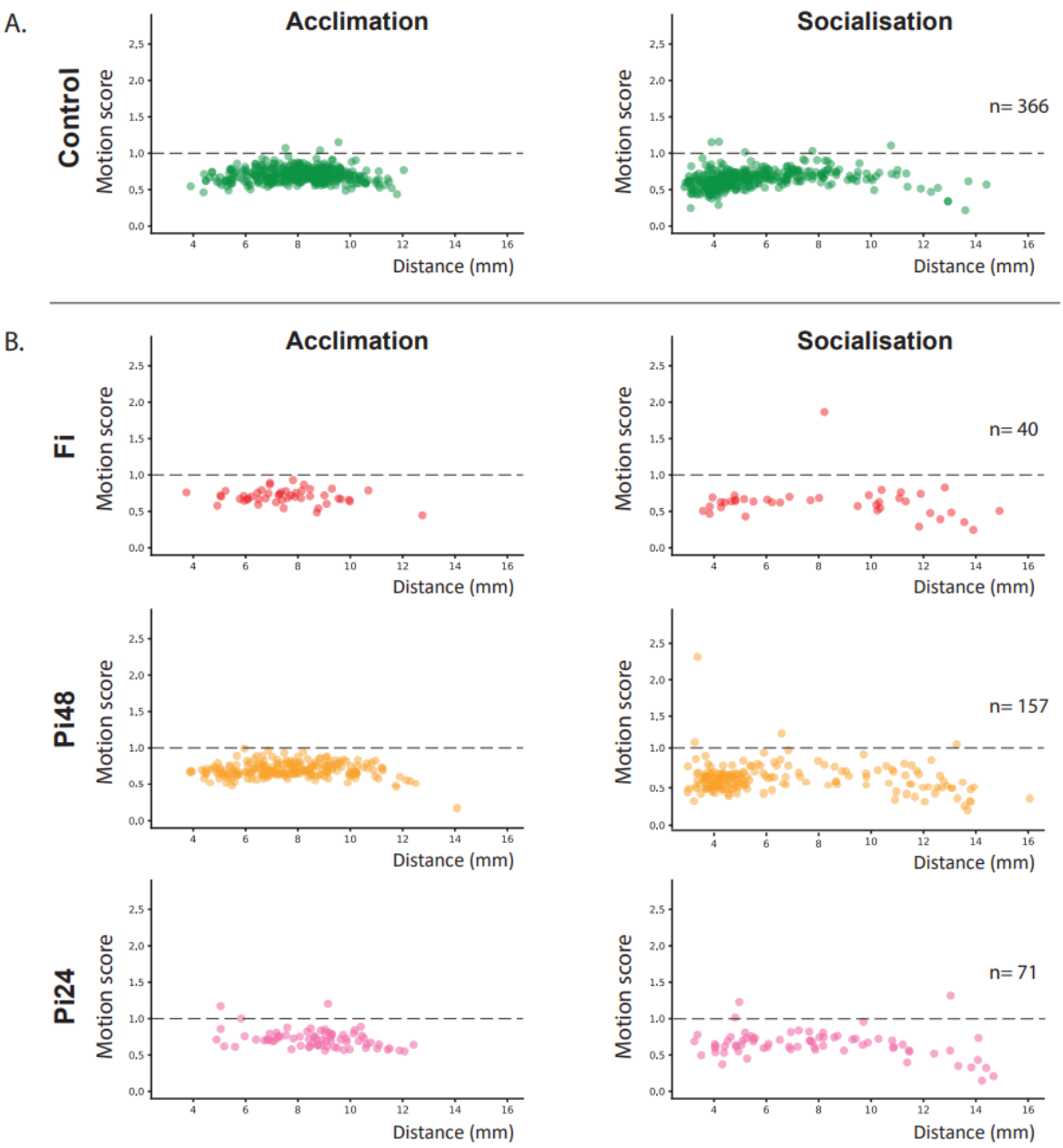


Figure 3.10: Isolation alters fish motion dynamics. Left to Right: Motion scores (change in x motion divided by change in y motion (x/y)) of focal fish plotted against their average distance in zone 'AB' for acclimation and socialisation phases. The dashed lines indicate equal movement in x (forward and back) and y-direction (side to side) relative to conspecifics. A. Motion data for control fish reared in social groups. Control fish alter their motion with proximity to conspecifics. B. Similar to A, motion data of isolated fish. Three types of isolation conditions are shown: full 21 days isolation (Fi), partial 48hrs (Pi48), and 24hrs (Pi24) isolation applied to fish 48hrs and 24hrs before testing, respectively. No significant difference in motion scores was detected across conditions but note curvature of data. Fish with closest and furthest distances from conspecifics display more noticeable changes in y-direction similar to that observed in controls. Sample sizes indicated on the right panels.

Across experimental phases, evaluation of isolated fish behaviour revealed that only Pi48 and Pi24 fish altered their Y motion significantly in the presence of social cues (**Figure 3.10B**: Acclimation to Socialisation; Partial isolation 48hrs (Pi48): *Wilcoxon signed-rank test*, w = 5073, p ≤ 0.0001, *n* = 157; and Partial isolation 24hrs (Pi24): *Wilcoxon signed-rank test*, w = 813, *p* = 0.01834, *n* = 71). However, further comparisons between Pi48 and Pi24 with NSC fish behaviour revealed that only Pi48 fish were lateral motion and the approach to social cues attributable to the presence of conspecifics (Differences [Socialisation - Acclimation]; NSC *Med* = 0.002, *n* = 54 vs; Partial isolation 48hrs (Pi48): *Med* = 0.02, *n* = 157; *Wilcoxon signed-rank test*, w = 3098, *p* = 0.00047). Thus, only Pi48 fish were motivated to approach the social window to interact with social cues indicating that 48 hours of isolation does not abolish the social drive to interact unlike full and 24 hours of isolation.

Isolated and socially reared fish swim bouts are comparable

Comparison of bout durations revealed that isolated fish exhibited significantly shorter bouts than socially reared controls during acclimation (**Figure 3.11A**; Acclimation: Controls: Med = 2.16s, n = 380; vs; Full isolation (Fi): Med = 1.55s, n = 47, *Mann-Whitney U test*, u = 6306, p = 0.00051; Partial isolation 48hrs (Pi48): Med = 2.07s, n = 157, *Mann-Whitney U test*, u = 26491.5, p = 0.02062; Partial isolation 24hrs (Pi24): Med = 1.78s, n = 71; *Mann-Whitney U test*, u = 9515, p ≤ 0.0001). During socialisation, all fish reared in isolated conditions showed a reduction in bout duration (**Figure 3.11A**; Socialisation) which was comparable to socially reared controls tested with conspecifics and NSC fish.

Analysis of the total number of fish swim bouts performed by fish reared in social isolation revealed that while some differences were found in acclimation behaviour of isolated fish with Fi and Pi24 displaying significantly few bouts compared to socially reared controls (**Figure 3.11A**: Acclimation: Controls: Med = 2077.5, n = 380; vs; Full isolation (Fi): Med = 1338 bouts, n = 47,

Mann-Whitney U test, u = 3720, p ≤ 0.0001; Partial isolation 48hrs (Pi48): Med = 2089 bouts, n = 157, *Mann-Whitney U test*, u = 29670.5, p = 0.46128; Partial isolation 24hrs (Pi24): Med = 1626 bouts, n = 71; *Mann-Whitney U test*, u = 9171.5, p ≤ 0.0001). The further reduction of bouts observed in all isolated fish during socialisation was not attributable to conspecifics compared to NSC fish. It was found to be comparable to socially reared controls. Therefore, no new responses were detected in the total number of bouts of fish reared in Fi, Pi48 and Pi24 conditions.

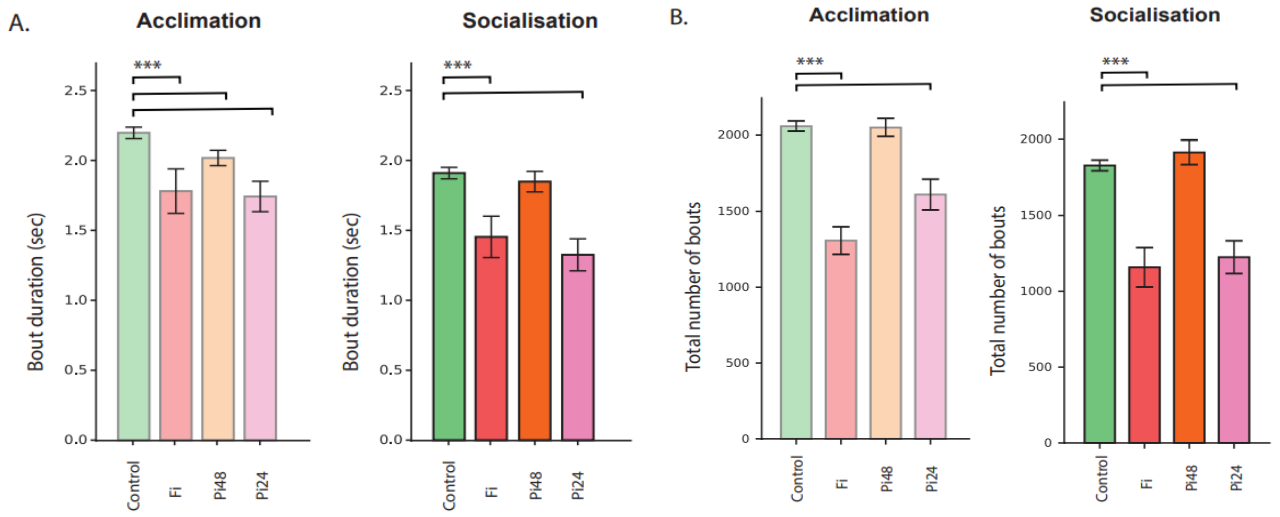


Figure 3.11: Fish bout kinematics are altered by isolation. A. Barplot of bout durations of fish reared under various conditions. **B.** Barplot of the average total number of bouts performed by fish. Left to Right: Acclimation and socialisation phases of the behavioural assay. Bars indicate SEM. Three isolation conditions tested: Full (Fi) 21 days isolation; partial 48hrs (Pi48), and partial 24hrs (Pi24), 48hrs and 24hrs hours isolation before testing, respectively. Control fish were never isolated. Sample sizes are as follows: Control, n = 380; Fi, n = 47; Pi48, n = 157; and Pi24, n = 71.

Viewing angles in isolated fish

Since isolated fish showed increased asocial behaviour, it was hypothesised that body orientation would be similar to the asocial fish already described (**Figure 2.14G**). To test this hypothesis, the body orientation, thus the average viewing angle, of isolated fish were extracted from all entries into zone A (**Figure 2.12A**) performed by fish during both acclimation and socialisation phases and subsequently compared. The number of isolated fish which entered zone A were as follows: Fi; *n* = 40, Pi48; *n* = 157, and Pi24; *n* = 71.

Fi and Pi48 fish show greater interest to conspecifics

During acclimation, the body orientation of isolated fish was comparable to socially reared control fish (**Figure 3.12**: Acclimation), similarly as described in **Chapter 2**. In the presence of social cues, all isolated fish, on average, orientated towards conspecifics (**Figure 3.12**: Socialisation). Pi24 fish showed a decrease in their viewing angle in the presence of social cues, which suggested an interest towards conspecifics in the social chamber; however, this angle was greater than the viewing angle of controls fish reared in normal conditions, averaging at 65° (**Figure 3.12B**, Full isolation (Fi): Left side: 80°, Right side: 50°), reflecting the number of asocial fish in the population. Contrastingly, fully and 48 hours isolated fish displayed body orientations in the presence of social cues (**Figure 3.12**) similar to controls. While fully isolated fish orientated themselves at an average angle of 40° (**Figure 3.12B**, Full isolation (Fi): Left side: 30°, Right side: 50°) and Pi48 fish orientated themselves at 35° (**Figure 3.12B**, Partial isolation 48hrs (Pi48): Left side: 40°, Right side: 30°), both of which were lower than the 45° viewing angle of controls when viewing social cues against predictions and suggesting greater interest towards social cues than controls.

These results indicate that social isolation beyond 24 hours has opposing effects on behaviour, particularly viewing angles.

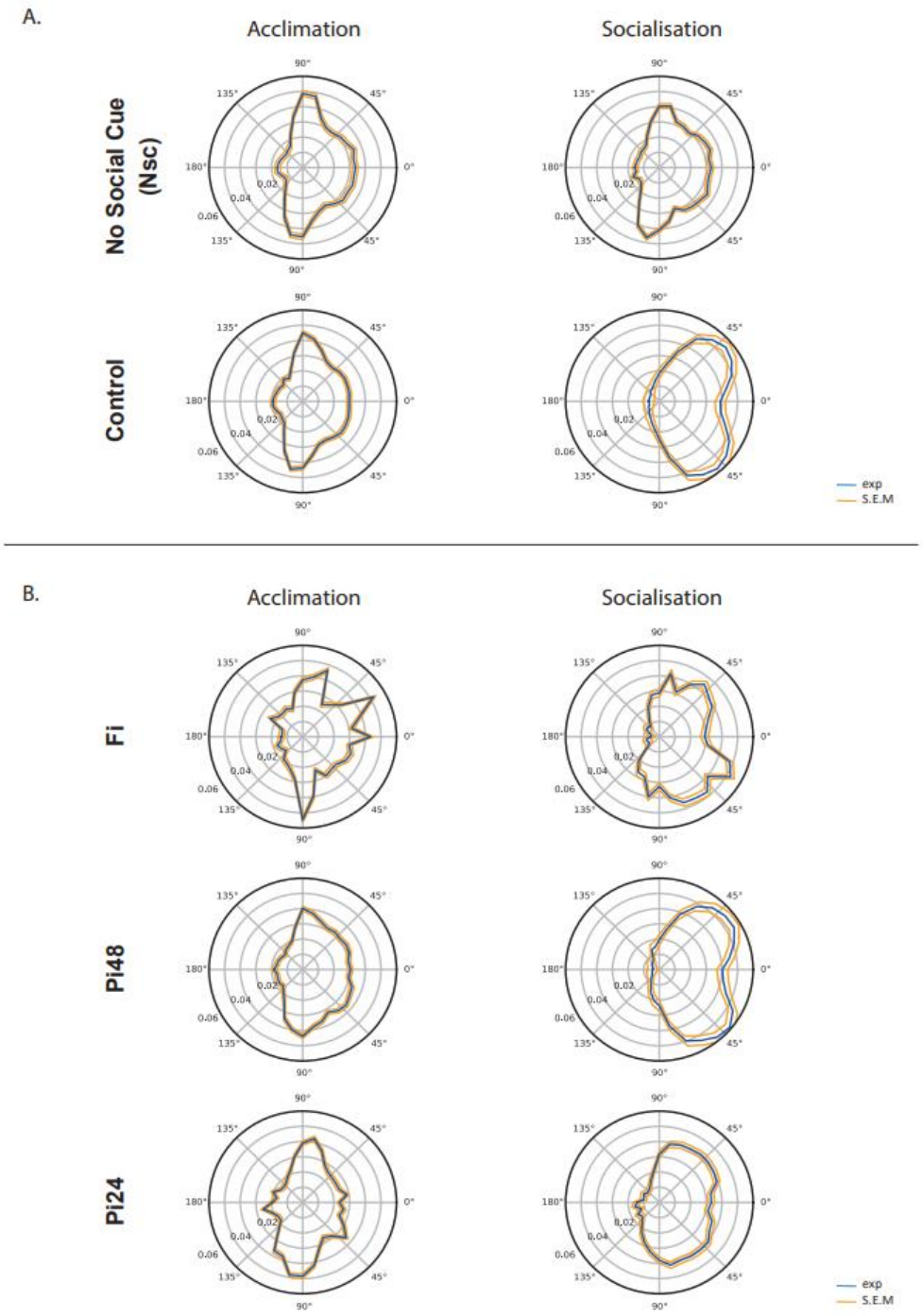


Figure 3.12: Isolation alters fish interest towards conspecifics. Polar histograms of body orientations of test fish during acclimation and socialisation presented for different rearing conditions. **A.** Test fish reared in social groups and tested with social cues (for convenience controls, same as Figure 2.11J are shown) and without (No Social Cue) are shown and serve as positive and negative controls. **B.** Isolated fish body orientations. Complete isolation of 21 days (Fi) drastically alters the viewing angle of test fish, and 24hrs of isolation applied before testing (Pi24) reduces the relative frequency of angles. Conspecifics are located at zero degrees. Numbers indicate the relative frequency. The blue and orange lines indicate mean and confidence intervals (95%). Sample sizes are as follows: Control, n = 366; Fi, n = 40; Pi48, n = 157; Pi24, n = 71.

Viewing angles of asocial and prosocial phenotypes are altered by isolation

One theory that may explain why fully and Pi48 fish exhibited reduced angles in the presence of social cues is that extended periods of isolation alter fish's visual sensitivity, making them more perceptive towards social cues. To test this theory and investigate whether asocial and prosocial fish in these populations were affected, fish reared in isolated conditions were divided according to social preferences and their viewing angles of these asocial and prosocial populations compared to their equivalents raised under normal conditions.

Comparisons of asocial fish viewing angles revealed that isolation resulted in asocial fish with greater interest in social cues. Asocial Fi, Pi48 and Pi24 fish exhibited average viewing angles of 85°, 75° and 55°, respectively (Asocial Full isolation (Fi(S-)): Left side: 90°, Right side: 80°) (Asocial Partial isolation (Pi48(S-)): Left side: 80°, Right side: 70°) (Asocial Partial isolation (Pi24(S-)): Left side: 50°, Right side: 60°), all lower than their controls equivalent which typically maintained 90° (**Figure 3.12A**: Asocial; Left side: 90°, Right side: 90°). These results indicate that shorter periods of isolation on asocial fish have more confounding effects on viewing angles, perhaps reflecting fish interest in conspecifics.

A similar comparison with prosocial groups revealed that full and 24 hours of isolation did not produce prosocial fish with greater interest towards conspecifics and instead increased the viewing angles of these fish to 60° and 55°, respectively (Prosocial Full isolation (Fi (S+)): Left side: 70°, Right side: 50°) (Prosocial Partial isolation (Pi24(S+)): Left side: 50°, Right side: 60°). However, this was not the case for 48 hours of isolation which resulted in prosocial fish reducing their viewing angles to an average angle of 35° (Prosocial Partial isolation 48hrs (Pi48(S+)): Left side: 40°, Right side: 30°) in the presence of social cues, suggesting that 48 hours is long enough to heighten prosocial fish interest towards conspecifics.

The above findings on body orientations suggest both asocial and prosocial groups are susceptible to isolation. Furthermore, these results hint that isolated asocial fish are different from asocial fish reared under normal conditions. Further experiments are needed to clarify why such differences are observed between asocial and prosocial fish from isolated and socially reared conditions (see **Chapter 4**).

3.3. Discussion

It is well established that social environment, including social deprivation, especially during early stages of development, can have lasting effects on behaviour and cognitive brain function in humans[295,453,454] and rodents[455–458]. Previous reports suggest that social isolation alters zebrafish behaviour, stress and anxiety levels, and immune responses. However, most of these results are predominately reported in adult zebrafish[375,]; therefore, little is currently known about the consequences of early developmental social deprivation on behaviour. Of the few studies available, conflicting results are reported, with some studies describing developmental isolation to have no impact on behaviour while others reported significant effects.

This chapter reported that developmental social isolation, that is, isolation from conspecifics in early life, significantly impacts the distribution of individual social preferences found in a population. Specifically, full isolation spanning 21 days and partial isolation of 48 and 24 hours before behavioural testing leads to a significant increase in the proportion of asocial fish in the population (**Figure 3.3** and **Table 3.1**). This result aligns with studies reporting aversive behaviour, a characteristic of the asocial phenotype, following periods of isolation[436,395]. For the first time, the effect sizes of full and partial isolation are quantified, showing that full isolation has the greatest effect size on visual preference index scores, which represent social preferences, followed by 48 hours and then 24 hours of social deprivation.

Previous studies in many species have shown that social isolation alters locomotor activity in both magnitude and direction[459,460]. Such results highlight the importance of thoroughly distinguishing general locomotion and social-specific effects when assessing the impact of social isolation on social behaviour. In this chapter, the comparison of several behavioural parameters between isolated and socially reared fish tested either with (controls) or without conspecifics (NSC) assessed the effect of social isolation on general locomotion (exposure to the assay chamber). For the first time, it is reported that full and partial isolation on the behaviour of juvenile zebrafish during development has different effects on locomotion and social preference behaviours. This chapter shows that the impact on these two measures depends on the duration of social isolation, with full and 48 hours isolation impacting both general locomotion and social preference and 24 hours affecting only the former measure (**Figures 3.3– 3.12** and **Tables 3.1–3.5**).

To date, it is not fully understood how developmental isolation alters the underlying social circuity, which gives rise to social dysfunction. In this chapter, the first step into this investigation begins with comparing asocial and prosocial behaviours of juvenile zebrafish reared in various social conditions. For the first time, it is shown that full and partial isolation of 48 hours produces

asocial and prosocial fish with atypical acclimation behaviour and responses to conspecifics not observed in socially reared controls.

Effect of full isolation on general behaviour

Full isolation significantly alters fish locomotion during acclimation. It was found that isolated fish reduce time spent moving, distance travelled, the total number of bouts and shorter bout duration during acclimation. Importantly, Fi social phenotypes display varied locomotion in the absence of conspecifics with differences in time spent freezing observed between asocial and prosocial fish compared to their socially reared counterparts. The analysis presented in this thesis is more detailed than previously published papers reporting on chronically isolated rodents and zebrafish models[432,436,458,461]. The work show in this chapter of this thesis is the first to divide asocial and prosocial phenotypes before subsequent analysis of parameters such as time spent moving. Secondly, sample sizes used here are more extensive than those previously described in social isolation experiments, where cohorts typically consist of no more than 20 animals[395,432,461]. These two factors may explain conflicting findings on the effects of chronic social isolation on fish locomotion. Previous studies potentially reported cohorts comprised mainly of non-social and prosocial individuals that do not correctly reflect the increased proportion of asocial phenotypes associated with social isolation. This emphasises the importance of ascertaining the social preference of individuals in social isolation experiments, as presented here in this thesis, thus making the findings in this chapter significant.

Following the publication of the results in this chapter (in early 2020)[113], Groneberg and co-workers (in late 2020) similarly reported reduced locomotion in larval zebrafish following long periods of isolation[436]. Interestingly, however, they attributed the reduced movement to feeding interactions, noticing in a pilot experiment that isolated juvenile fish grew faster when fed and displayed increased locomotion than group reared fish. This is an unlikely possible cause of this thesis's results since isolated zebrafish in this chapter showed similar growth rates to conspecifics raised in social conditions allowing size matching. A more likely scenario is that social isolation alters zebrafish perception of novel surroundings, consequently seen in their behaviour.

For many species, including our own, developmental isolation elevates anxiety[436,449,458]. Although it is not possible to differentiate fear from anxiety, especially since brain areas associated with these states show comparable responses to threats[462], freezing behaviour in a social context is commonly associated with anxiety[362,397,405,463]. A significant increase in the percentage of time spent freezing was observed in fish following three weeks of full isolation. This result is in line with the findings that long periods of isolation increase anxiety-like behaviour in zebrafish. For example, Collymore et al., 2015 showed that adult fish isolated for 21 days increased bottom-

dwelling – another behavioural measure of anxiety[464]. The next step in this line of investigation would be to confirm anxiety levels by evaluating whole-body cortisol levels since elevated cortisol concentrations indicate elevated anxiety/fear.

Although several protocols exist for quantifying cortisol in zebrafish, this may not be so straightforward. Previous studies on developmental isolation have reported that six months of social deprivation do not affect cortisol levels[465]. Interestingly, in an unpredictable chronic stress study, Rambo et al., 2016 reported that male zebrafish contain high whole-body cortisol levels. No differences in cortisol levels were found in stressed females, highlighting the relevance of studying the physiological parameters separately in both sexes[466]. Since the sex of fish used to assess cortisol levels in fish following periods of social isolation is often not disclosed, insignificant results on the impact of isolation may be explained by the high number of female fish in samples. Unfortunately, at three weeks old, the physical features typically used to identify zebrafish sexes, such as the ovipositor, are not readily visible in the juvenile zebrafish. Therefore, this requires either rearing fish to 28 days or sexual maturity (approximately ten weeks[467]), when sex determination can be conclusively clarified, or qPCR quantification for sex-related genes. The former of these two options may come with possible implications relating to prolonged social isolation exceeding beyond the 21 days utilised in this thesis. For example, Shams and co-workers reported contradictory findings to the results presented here, reporting changes in shoal cohesion and anxiety[432] but no differences in general locomotion in zebrafish chronically isolated for 180 days. Unfortunately, since individual fish' social preference was not investigated by Shams et al., it is challenging to make concluding comparisons between the two studies. Recently, King et al., 2020 reported that the expression of genes such as cyp17a1, cyp19a1a, vtg1, igf3 and dmrt1 might be used to determine the sex of fish younger than 28 days, describing the first four of these genes to be highly expressed in females and the last one highly expressed in male zebrafish[465]. Coupling this approach to sexing with whole-body cortisol levels should reveal whether isolation results in an elevation of anxiety levels.

Effect of partial isolation on general behaviour

Like full isolation, significant behavioural changes are also found in the general locomotor behaviour of partially isolated fish. For example, developmental isolation of 48 and 24 hours results in reduced locomotion, i.e., the time spent moving and total distance travelled in a manner resembling those observed in full isolation. The only behavioural difference noted between fish isolated for 48 and 24 hours is the total swim bouts. Pi48 fish exhibits a comparable total number of bouts to socially reared conspecifics, while Pi24 fish is similar to fully isolated fish. This result indicates that different durations of social isolation may have differing effects on behaviour.

The effects of acute/partial isolation are rarely studied in zebrafish. Currently, no literature is available specifically on the impact of 48 and 24 hours early-life social isolation on social behaviour. The shortest duration of developmental social isolation applied to zebrafish is reported in work by Zellner and co-workers[468] and previously mentioned Shams and co-workers[395], researching the effects of five and seven days of isolation, respectively. The outcomes in this chapter are consistent with hypoactivity described by Zellner et al., 2011 in fish isolated from 0 to 5 dpf and subsequently tested at 6 dpf[468]. The results presented here with those described by Zellner and co-workers suggest that social isolation encountered during early development impacts the general locomotor behaviour of fish.

Work by Gerlach and co-workers previously identified a 24 hour window at 6 dpf critical for olfactory the imprinting process for kin recognition by socially isolating fish larvae with effects seen at adulthood[434]. Although social preference is predominately visually driven, likely, other senses are also involved, as recognition is a crucial requirement for social interactions. Therefore, more than one critical window for social preference may exists during development. Further research along these lines of investigation will reveal much about the timing of such periods during development. It is yet to be established whether a critical period during development exists for general locomotion, specifically whether periods of social isolation are an influencing factor. A pilot study was conducted in parallel with the work presented in this thesis, where fish were allowed to re-socialise following seven days periods of social isolation, hyperactivity similar to that described in Shams et al., 2017[432] was observed in fish socially deprived between 0-7 dpf, 8-14 dpf and 0-14 dpf. However, fish showed reduced locomotion when socially deprived at 15-21 dpf without re-socialisation before testing, similar to the isolation conditions used in this thesis. These results indicate that a critical period for the effect of developmental social isolation on general locomotion may not exist and instead is influenced by the recency of adverse events -as is social isolation explaining why social isolation studies report conflicting findings on locomotion. It is worth noting that it is also possible that the critical window extends beyond 21 days, probably up to 1 month, which is when fish start to establish shoaling behaviour and have been shown to recognise members of their species -conspecifics. Thus, further studies using more prolonged periods of social isolation applied during development will reveal whether a critical window exists in the zebrafish model.

48 and 24 hours of isolation increased the percentage time freezing exhibited by fish before viewing conspecifics, indicating elevated anxiety. These results align with the work described on zebrafish by Shams and co-workers (2018), which investigated the impact of 24 hours social isolation in adults. As reported in this chapter, Shams et al., 2018 also described acute social isolation induces the elevation of anxiety-like behaviours using behavioural parameters such as reduced turn angle and reduced fish proximity to the bottom of a tank[395].

Further evidence that the degree of impact from developmental isolation is dependent on duration is visible when the movement and freezing activities that asocial and prosocial fish exhibit are evaluated separately. For instance, asocial fish separated for 48 hours from conspecifics display comparable percentage moving and freezing to socially reared controls, while 24 hours of social isolation produces asocial fish with significantly less movement activity and similar freezing behaviour to control fish. However, this difference between asocial 48- and 24-hours isolated fish does not extend to prosocial fish groups, indicating that partial isolation, regardless of duration, similarly affects the general locomotion of prosocial phenotypes. These results may explain why some studies fail to identify any impact on movement activity and anxiety-like behaviours and even describe anxiolytic effects[375,469] of short periods of isolation.

Effect of isolation on responses to conspecifics

Isolation experiments primarily measure social behaviour with little or no analysis of other behaviours such as locomotion. Where the effect of isolation on social interaction has been considered in zebrafish, assessment of these have been predominantly considered using shoaling behaviour[395,433], with only one study reporting the mean distance of isolated fish to conspecifics[432]. However, the dynamics of shoaling is complex and often reflects the overall preferences and traits of participating members rather than individual preferences. Currently, no data is available on the impact of early life social isolation on the social preference of single fish; therefore, the findings in this chapter are described for the first time.

Fully isolated fish display avoidance behaviour, actively increasing their distances from social stimuli and reducing overall travelled distance. This finding is in line with results reported in previously mentioned Shams et al., 2017, where adult fish chronically isolated displayed increased inter-individual distances in shoals[395]. This chapter also shows that fish isolated for 24 hours behave similarly to socially reared fish in response to conspecifics, with no relative differences observed in duration, total bouts, distances travelled, and percentage of time spent moving and freezing. In line with isolation studies on adult fish[432], these findings indicate that an acute period of social isolation 24 hours before testing does not alter the fish's ability to interact with conspecifics at the population level. Conversely, full developmental social deprivation of three weeks or 48 hours produces juvenile zebrafish with atypical conspecific responses. Zebrafish partially separated for 48 hours show no active avoidance or attraction towards conspecifics than socially reared controls. However, Pi48 exhibits more significant maintenance of bout duration, the total bouts, distance travelled, and movement activity when in the presence of conspecifics than controls. This result suggests that 48 hours of social isolation may lead to heightened sensitivity towards social stimuli, perhaps creating a sense of longing for social interaction similar

to the effects of acute isolation observed in humans, whereby the brain responses of individuals socially deprived for 10 hours towards social cues resemble individuals viewing food cues following a fasting period[470]. This result indicates zebrafish as a suitable model to assess loneliness for the first time, opening new investigation lines to understand how it manifests in a model whose whole brain is visually accessible with high-resolution imaging techniques, unlike pre-existing human and rodent models with physically larger brains.

Finally, this chapter shows that full and partial isolation of 48 but not 24 hours produces asocial and prosocial phenotypes with differing responses to conspecifics. Asocial Pi48 fish display significantly greater movement activity maintenance than socially reared controls and, like fully isolated asocial phenotype, show abolished freezing behaviour attributable to conspecifics. The exact reason for differences between social phenotypes of various isolation conditions remains unclear but may be linked to personality traits. In the previous chapter, differences in acclimation behaviour between asocial and prosocial phenotypes reared in social conditions indicated a link between shy and bold characteristics associated with the two social phenotypes, respectively. As in many species, boldness in fish is associated with the active exploration of a novel environment, whilst retreating and freezing responses indicate shyness[471–473]. Periods of social isolation exceeding 24 hours may result in bold fish, which would have been otherwise prosocial, becoming asocial while also maintaining its tendency to display less anxiety-like behaviour in response to novelty, in line with being bold. Hence the atypical percentage of time spent freezing in isolated asocial phenotypes. Retrospectively, an extension of the analysis of asocial fish responses towards conspecifics to include other measured parameters, i.e., proximity to social window mentioned in the previous chapter (i.e., proximity to social window and the total distance travelled by fish), would provide crucial information to this extent. In contrast, prosocial fully or partially 48 hours isolated fish show similar reactions to the presence of conspecifics, displaying a significant increase and decrease in movement and freezing activity, respectively, instead of the expected general maintenance of movement and freezing activity observed in controls.

These findings once again highlight the importance of ascertaining the social preference phenotype of individuals in social isolation experiments, crucial in obtaining a clearer understanding of the effects of social deprivation in future studies.

Conclusion

In conclusion, periods of social isolation profoundly affect juvenile zebrafish, influencing both behavioural responses to a novel chamber and the presence of conspecifics, with asocial and prosocial phenotypes affected differently. Variations observed across isolated rearing conditions in these measures directly result from the duration of isolations applied during early development.

Together, these results show that understanding how developmental social isolation gives rise to social dysfunction requires precise experimental controls and detailed behavioural analysis at the level of social phenotypes.

Combining the behavioural findings described in this chapter with high-resolution imaging techniques, it will be possible to investigate how the functioning social circuity gives rise to atypical social behaviour. This exact approach is described in **Chapter 4** of this thesis.

Chapter 4: The Underlying Social Circuits

Chapter 4 Summary

In this chapter, the functional brain responses to conspecifics of asocial and prosocial juvenile fish reared in various social conditions are mapped using high-resolution two-photon imaging. Whole mount in *situ* hybridisation is used to confirm serotonin and dopamine cell populations in social brain nodes identified from brain activity maps. Finally, since isolated fish exhibited increased anxiety-like responses, it is investigated whether the reduced social preference of isolated fish can be improved by administering an anxiolytic (buspirone).

4.1. Introduction

Social behaviour between pairs or larger groups involves active detection and response to cues from multiple sensory modalities and is instantly shaped by participants' dynamic and mutual feedback[13]. Yet studies have shown that visual stimulation is sufficient to facilitate social interactions[91]. Regardless, social interactions are complex. The 'social behaviour network' model describes how the brain processes social information to make operational social behavioural decisions. In its original application in mammals, the model represents a network of six nodes (brain areas) that respond distinctly to various social stimuli, forming different patterns that modulate behavioural outputs[202]. The six nodes are comprised of five regions that lie in the forebrain and include: the amygdala, lateral septum, POA, hypothalamus (both ventromedial and anterior); and one midbrain area, which has in itself several other structures, mainly the periaqueductal/ central grey and various regions of the tegmentum[202]. Since its initial suggestion, the model has been extended to a broader range of species, including birds and teleosts[474–478]. Crucial to understanding the social brain circuitry in the zebrafish model and its translatability to humans, the six nodes that control aggression, social recognition, responses to social stressors, and various forms of communication behaviours in mammals have also been mapped in the zebrafish brain[202,319,474,475,479,480]. Brain areas that process social information have been identified in humans using fMRI studies[341]. However, imaging at single-cell resolution is not achievable using fMRI. Since the social network nodes are also distributed throughout the brain, including many deep, optically inaccessible areas, high-resolution imaging of the whole brain is difficult in humans and rodents. For this reason, the small size and translucent zebrafish provide a unique opportunity to investigate the whole-brain network underlying social preference with single-cell resolution.

Social interactions are rewarding in nature with growing evidence that the pattern of activity of these nodes in response to a social stimulus is influenced by the mesolimbic reward system, an organisation of several structures the basolateral amygdala, ventral tegmental area and ventral palladium[477,481].

Several neurotransmitters such as dopamine and serotonin have been associated with social behaviour. The juvenile zebrafish presents itself as an ideal model for high-throughput pharmacological screening of drugs with its high fecundity and ease of drug administration, thus a great candidate to explore this association. For example, in zebrafish, pharmacological manipulation of the dopamine system through a D1 receptor antagonist, such as SCH23390, leads to reduced social preference[482]. Moreover, elevated dopamine levels have also been reported following exposure to conspecifics in several zebrafish studies[483–485]. Interestingly, alterations in dopamine signalling have been recently implied in neuropsychiatric disorders associated with

abnormal social functions such as autism[486]. Therefore, interventions with pharmacological agents may offer a potential avenue to improve social interaction in individuals with social recognition dysfunction[487–489].

The neurotransmitter serotonin has also been shown to modulate the social behaviour network. Its effect on the social behaviour network is neither beneficial nor detrimental but depends on the environmental context in which it appears[490]. The 'for-better-and-for-worse' concept has long been used to describe the conflicting results across several studies on serotonin[424,491–494]. The thought is that increased levels of serotonin increase sensitivity to environmental stimuli, which may itself encourage or discourage different social interactions. For example, if an association with a stimulus is positive, i.e., food, increased serotonin levels may further drive interaction with the stimulus. Similarly, if negative, this association may also be amplified. Modulating serotonin, i.e., decreasing levels may also increase the exposure duration to a stimulus, thus creating a new threshold, particularly for negative stimuli.

As mentioned in the previous chapters, periods of social isolation have adverse effects on mental health conditions, including depression, stress and anxiety[495]. Medications such as fluoxetine and buspirone are often prescribed in humans who suffer from social anxiety. These pharmacological agents exert their effect by controlling serotonin levels through serotonin receptors[496–498]. In zebrafish, both fluoxetine[499] and buspirone[113,500,501] eliminates anxiety-like behaviours, such as freezing and decreasing aggressive behaviour[499] via either increasing[502,503] or reducing serotonin levels in the brain, respectively[501,504]. Therefore, there remains scope to investigate the effects of social isolation and possible treatments to alleviate the effects with buspirone in this chapter.

4.2. Results

Zebrafish brain activity in response to conspecifics

The differences in behavioural responses observed between naturally occurring asocial and prosocial phenotypes in socially reared conditions (**Chapter 2**) suggest differential brain activation, which can be investigated in zebrafish. Furthermore, isolated fish phenotypes (asocial and prosocial) showed behavioural differences from their socially reared counterparts (**Chapter 3**), suggesting isolation-induced changes in brain activation. Therefore, whole-brain two-photon imaging of c-Fos expression, an immediate early gene whose expression is associated with increased neural activity[505], was performed following the social preference assay, to detect for changes in brain activities.

Dissected brains were imaged from ventral to dorsal with the ventral surface up closest to the objective to achieve clear views of the ventral brain structures previously implicated in the social brain network (**Figure 4.1A**). Comparisons were made between the average activity map for each rearing/sociality condition, with similarly raised conspecifics in the absence of any social cues (No Social Cue, NSC). The resulting 'Normalised' difference stacks [e.g. (S+ - NSC)/ NSC] facilitated the identification of any changes in neural activity associated with exposure to a visual social cue (**Figure 4.1A**, also see **Methods and Materials** for more details).

Distinct c-Fos expression in socially reared asocial and prosocial fish

Although several brain areas showed activation or inhibition following social cue exposure, subsequent analysis primarily focused on two regions previously described as social brain areas[506], namely the caudal hypothalamus and preoptic area (POA), where differences between social groups were most striking (**Figure 4.1B**: asocial (C (S-)) and prosocial (C (S+))). The caudal hypothalamus of fish reared in social groups displayed differential activation between asocial phenotypes and their prosocial equivalents (C (S-) and C (S+), respectively). More specifically, the dorsal subregion of the caudal hypothalamus along with the adjacent ventral sub-region showed significantly reduced activity in asocial controls (**Figure 4.1B** and D; vHc: C (S-) vs C (NSC), $p = 0.003$, $n = 5$, Mann Whitney U test), whilst being significantly activated in prosocial controls (**Figure 4.1B** and D; dHc: C (S+) vs C (NSC), $p = 0.007$, $n = 5$, Mann Whitney U test).

The caudal hypothalamus is known to express high levels of serotonin and dopamine[507,508]. Since both serotonergic and dopaminergic pathways have been previously described to modulate the social behaviour network, the expression of Serotonin Transporter (*SERTB/ 5-HTT/ SLC6A4B*) and *TH1, TH2*, and Dopamine Active Transporter (*DAT/ SLC6A3*)[508,509] were characterised. Whole-

mount in *situ* hybridisation staining showed overlap with c-Fos activation in the caudal hypothalamus (**Figure 4.1C**), suggesting the two pathways modulate social preference behaviour through acting on the caudal hypothalamus.

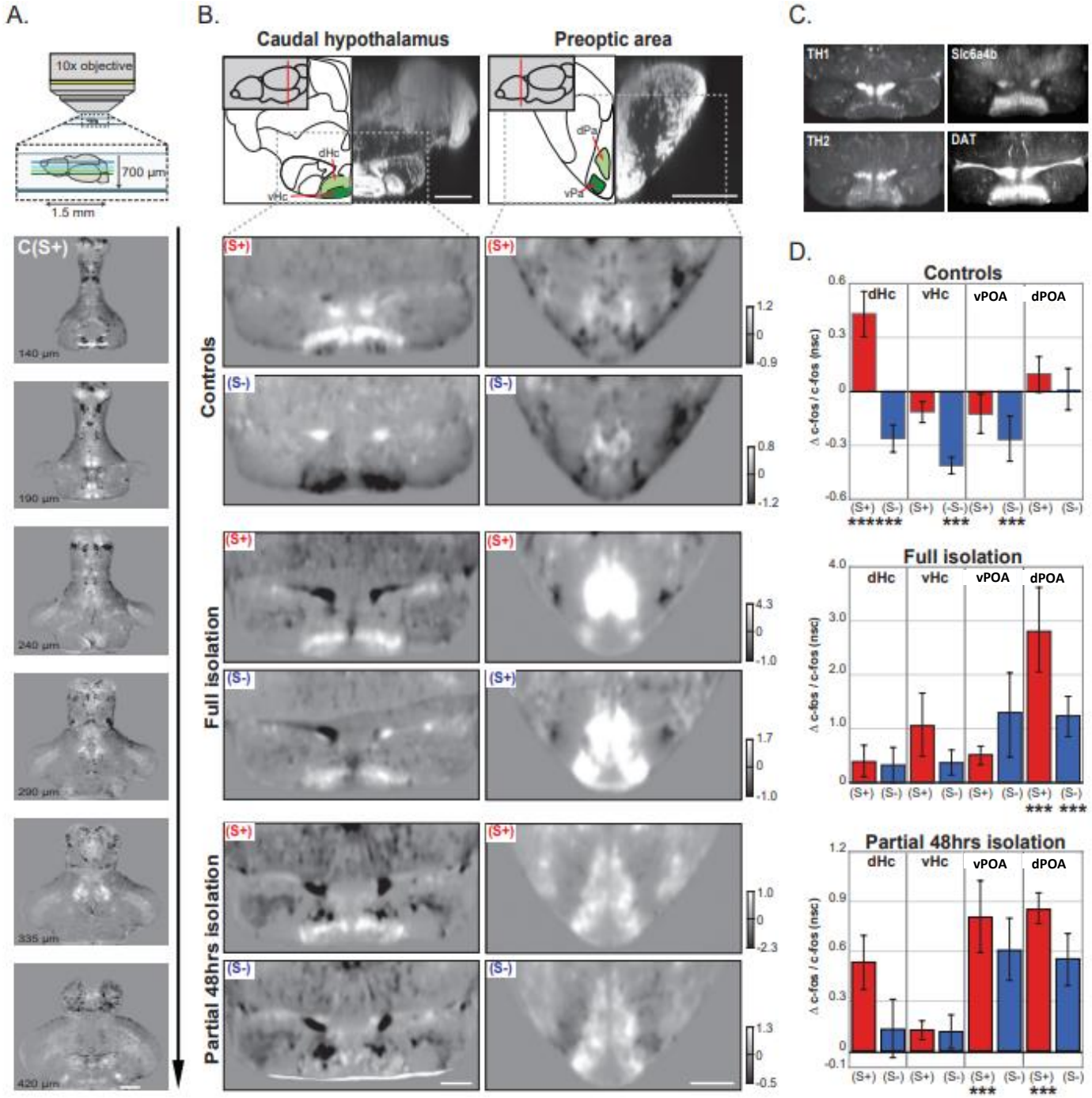


Figure 4.1. Functional maps of the social brain in normal and isolated fish. A. Schematic of the custom-built two-photon microscope used for acquiring whole-brain volumes of dorsal-down mounted fish brains (top panel). Horizontal sections of pro-social control fish (C(S-)) responses at increasing imaging depth (lower panels). Images are average differences between (C(S+)) and siblings not presented with a social cue. Positive values (white) indicate increased c-Fos expression in socially preferring fish, while negative values (black) indicate decreased expression. Scale bar is 200µm. The intensity scale bar is shown in B, C(S+) row. B. Region analysis of two different brain areas that have been implicated in social behaviour: caudal hypothalamus and preoptic fish A schematic of the anatomical regions and corresponding DAPI staining is shown (top panel) with two subregions highlighted in green. Images showing changes in c-Fos activation in these areas for prosocial (S+) and asocial (S-) controls, fully isolated, and partially isolated for 48 hours fish are shown. Images are horizontal sections of the average difference between each test group and their corresponding sibling group not presented with a social cue. Scale bar is 100µm. Intensity scale bar is shown for each group. C. Average image of TH1, TH2, SLC6A4B, and DAT expression in the same section of the caudal hypothalamus as B (n = 3 each). Scale bar is 100µm. D. Summary graphs showing the change in c-Fos activation for four different brain areas calculated by using the average difference images shown in B and using 3D masks (a single plane of each area of the masks is shown in green in B). Positive values indicate increases in c-Fos expression; asterisks mark significant changes ($p < 0.05$) relative to No Social Cue siblings. dHc = dorsal caudal hypothalamus, vHc = ventral caudal hypothalamus; vPOA = ventrolateral preoptic area, dPOA = dorsal preoptic area.

Evaluation of the POA of asocial and prosocial revealed similar trends in activation patterns with an in the dorsal preoptic area (dPOA) accompanied by a decrease in the ventral preoptic area (vPOA). Only asocial controls fish presented a significant reduction in the ventral region compared to NSC (**Figure 4.1B** and **D**; vPOA: Control (S-) vs No social cue (NSC), p = 0.003, n = 3, Mann Whitney U test).

Taking the caudal hypothalamus and preoptic area data together, the unique pattern of activation and inhibition observed in asocial and prosocial phenotypes indicates these two brain areas could be crucial in regulating social preference.

Modified c-Fos expression in socially isolated fish

To investigate whether differences in behaviour underlie activity changes in aforementioned brain-areas following the presentation of social cues, c-Fos expression of social phenotypes reared in social (control) and isolated conditions (Fi, Pi48 and Pi24) were also compared.

C-Fos functional maps in asocial, fully isolated fish (Fi (S-)) revealed an entirely different activity profile compared to those in socially reared, asocial fish (**Figure 4.1B** and **D**: Fi (S-) vs C (S-)). The ventral sub-region of the caudal hypothalamus (vHc) of fully isolated (Fi (S-)) fish showed strong activation in response to the social assay, while the POA was strongly activated in both the dorsal (dPOA) and the ventral (vPOA) regions, but significantly only in the dorsal (**Figure 4.1B** and **D**, Fi (S-) vs Fi (NSC): dPOA, Mann Whitney U test; p = 0.006; vPOA, n = 5, p = 0.07).

Interestingly, prosocial fully isolated fish (Fi (S+)) fish (**Figure 4.1B**: Fi (S+)), which exhibited an increase of freezing and reduced motility compared to control fish when viewing conspecifics, presented a similar activation to prosocial controls (C (S+)) in the caudal hypothalamus, specifically in the dorsal area (**Figure 4.1D**) accompanied by an increased expression of activity in the dPOA. The dPOA of fish (Fi) revealed a substantial increase in activity absent in the socially reared fish (C (S)). The presence of social cues activated the preoptic area differently in prosocial (Fi (S+)) and asocial isolated fish (Fi (S-)) with the presence of conspecifics strongly activating only the dPOA in the fully isolated prosocial fish group (**Figure 4.1B** and **D**, Fi (S+) vs Fi: vPOA, Mann Whitney U test: n = 5, p = 0.04; dPOA, Mann Whitney U test: n = 5, p = 0.002).

Asocial and prosocial fish exposed to a brief isolation period of only 48 hours prior to testing, showed similar functional activity changes to fully isolated fish, albeit to a lesser extent ((**Figure 4.1B** and **D**: Pi48 (S-) vs Pi48 (NSC); dHc, Mann Whitney U test: p = 0.18; vHc, Mann Whitney U test: p = 0.28; vPOA, Mann Whitney U test: p = 0.04; dPOA, Mann Whitney U test: p = 0.04) and (**Figure 4.1B** and **D**: Pi48 (S+) vs Pi48 (NSC): dHc, Mann Whitney U test: p = 0.17; vHc, Mann Whitney U test: p = 0.05; vPOA, Mann Whitney U test: p = 0.007; dPOA, Mann Whitney U test: p = 0.006)). These results indicate that even short-term social isolation (i.e., 48 hours) is sufficient to alter neural responses during the viewing of social cues and may explain the changes in behavioural phenotype described previously in **Chapter 3**.

Together, these results show that social isolation causes abnormal neural responses when viewing social cues and suggests that the impact of social deprivation on the functional circuitry of preference behaviour may be cumulative.

Why social isolation promotes social aversion instead of increasing the drive for social interaction

In evolution, loneliness/isolation has been posited to serve adaptive ends by motivating connection and reconnection with others to ensure safety and survival[446,510]. Yet, juvenile zebrafish subject to social isolation, both long-term and partial (48 or 24 hours), displayed asocial preference behaviour, suggesting that loneliness (modelled here as the undesired isolation of juvenile zebrafish) perpetuates itself. Further analysis of brain map activities was carried out to understand why social isolation promotes social aversion instead of a predictably increased drive for social interactions.

An important clue was found in the pattern of brain activity changes unique to isolated fish before exposure to social cues. Direct comparison of the normalised c-Fos functional brain maps of isolated and control fish that were not exposed to social cues during the assay (**Figure 4.2A**)

revealed significant increases in two areas; the first associated with visual processing, the optic tectum (OT)[511], and the second involved in stress responses, the posterior tuberal nucleus (PTN)[512].

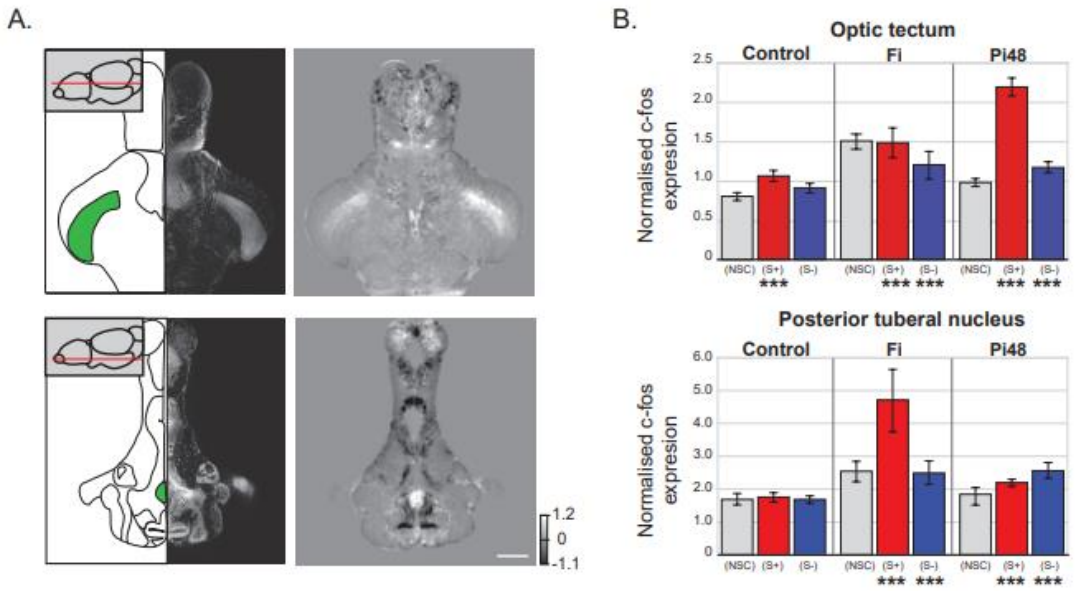


Figure 4.2: Changes in baseline brain activity following isolation. A. Images of two areas that show c-Fos activation in fully isolated fish independent of social stimuli (optic tectum and posterior tuberal nucleus (PTN)). Schematics of the horizontal sections and corresponding DAPI image are shown in the left panels. One plane of the 3D mask regions used for subsequent analysis is indicated (green). Images of Fully isolated fish c-fos neuronal activity, calculated as average differences between fully isolated (Fi) fish and normally raised fish tested with no social cues are shown in the right panels. Scale bar 200 µm. **B.** Summary graphs showing the normalised c-Fos expression in the optic tectum (OT) and posterior tuberal nucleus (PTN) 3D masks for each experimental condition: No Social Cue (NSC), prosocial (S-) and asocial (S-) for all the controls (C), fully isolated (Fi), and partially isolated for 48 hours (Pi48) fish. Asterisks mark significant changes ($p \leq 0.05$) of isolated fish relative to NSC.

The presence of social cues resulted in a significant increase of neuronal activity in the OT of socially reared prosocial controls (**Figure 4.2B**, OT: C (S+) vs C (NSC), p = 0.004, Mann Whitney U test). However, analysis of the neuronal activity in fully isolated fish revealed an increased baseline activity in the absence of social cues compared to their control counterparts (**Figure 4.2B**, OT: Fi (NSC) vs C (NSC), Mann Whitney U test: p = 0.0004), suggesting that isolation heightens visual sensitivity, as previously reported in humans[267,445,446]. The observed significant increase in sensitivity of fully isolated fish not presented with social cues was also observed in partially isolated fish, albeit to a lesser extent (**Figure 4.2B**, OT: Pi48 (NSC) vs C (NSC), p = 0.03, Mann Whitney U test). Furthermore, a much more significant increase in tectal activity was observed in partially isolated prosocial fish (Pi48 (S+)) compared to socially reared controls following interaction with social cues, further indicating visual sensitisation had occurred (**Figure 4.2B**, OT: Pi48 (S+) vs C (S+), p = 0.0002, Mann Whitney U test). Additionally, an increase in tectal activity

was also present in both fully and partially isolated asocial fish (**Figure 4.2**B, OT: Fi (S-) vs C (S-), Mann Whitney U test: , *n* = 5, *p* = 0.048; Pi48 (S-) vs C (S-), Mann Whitney U test: *n* = 5 *p* = 0.005), even though these fish largely avoided the area in the chamber visually accessible to conspecifics.

Isolation related activity was also observed to increase in the Posterior Tuberal Nucleus (PTN), a brain region associated with stress responses, including in the zebrafish[512,513]. Analysis of c-Fos in the PTN also revealed that complete isolation caused a significant increase in PTN activity in the absence of social cues (**Figure 4.2B**, PTN: Fi (NSC) vs C (NSC), Mann Whitney U test: *n* = 5, *p* = 0.015) and both asocial and prosocial fish exposed to social cues (**Figure 4.2B**, PTN; Fi (S+) vs C (S+): Mann Whitney U test, *n* = 5, *p* = 0.003; Fi (S-) vs C (S-): Mann Whitney U test: *n* = 5, *p* = 0.016). Partial isolation of 48 hours slightly increased the PTN activity in the absence of social cues (**Figure 4.2B**, PTN: Pi48 (NSC) vs C (NSC), *p* = 0.29, Mann Whitney U test), but significantly in prosocial and asocial fish in the presence of social cues ((**Figure 4.2B**, PTN: Pi48 (S+) vs C (S+), Mann Whitney U test: *n* = 5, *p* = 0.018) and (**Figure 4.2B**, PTN: Pi48 (S-) vs C (S-), Mann Whitney U test: *n* = 5, *p* = 0.0005)).

These results suggest that changes occur in the visual pathway of socially isolated fish, like that shown in humans, and in stress/anxiety-related brain areas, also demonstrated in humans. Furthermore, both findings support the behavioural data described in **Chapter 3**.

Buspirone treatment rescues behavioural phenotype induced by isolation.

Since changes in c-Fos activity were detected in the caudal hypothalamus, an area modulated by serotonin, and with the knowledge that serotonin targeting drugs are often prescribed to treat social anxiety in humans, pharmacological manipulation of the social brain circuitry was conducted to establish whether the impaired preference in isolated fish could also be rescued. Given the increased activity in the optic tectum and posterior tuberal nucleus, it was hypothesised that isolation initially heightens sensitivity to social stimuli, with 48 hours of isolation creating a longing for conspecifics in socially deprived fish. However, when isolation is prolonged, this heightened sensitivity could increase stress and anxiety levels during social viewing, which leads to an aversion to social stimuli. To test the hypothesis that reducing anxiety could reverse the aversive behaviour observed in isolated fish, controls and Pi48 fish were acutely treated with an anxiolytic - buspirone.

The selection of 48 hours partial isolation fish for these experiments was motivated by the intermediate behavioural and functional phenotype of partial isolation relative to normal-rearing and complete isolation, which allowed for easier detection of both positive and negative impacts of treatment on sociality. The choice of buspirone, an agonist of the auto-inhibitory 5-HT_{1A}

receptor, was aided by the changes in activity observed in the caudal hypothalamus of isolated fish and further supported by the fact that the caudal hypothalamus and the POA strongly express Htr1ab receptors, one of the two orthologues of the 5-HT_{1A} receptor[514].

Since all previous experiments on the effects of buspirone on zebrafish have been conducted on either adults or larval stages[404,463,500,501,515,516], juvenile controls fish reared in social groups were treated with different concentrations of buspirone to establish the effects of acute exposure to the anxiolytic. Treatment consisted of a ten-minute exposure to either 30 µM or 50 µM of buspirone which was previously determined to be effective doses based on previously conducted pilot studies, coincidently compared to previously reported amounts in adult zebrafish[404].

When treated with 30 µM of buspirone, socially reared control fish showed a significant increase in social preference relative to untreated conspecifics (**Figure 4.3A**, Controls treated (30 µM); C (no drug): *Med* = 0.797, *n* = 52; vs; C (30 µM): *Med* = 0.551, *Mann-Whitney U test: n* = 380; *u* = 7959, *p* = 0.01). Interestingly, a 10% of asocial fish remained as in the normal population (**Table 4.1**; C (no drug): asocial (S-) $\hat{p}$ = 0.103; vs; C (buspirone): asocial (S-) $\hat{p}$ = 0.096; *z* = 0.2, *p* = 0.876, two-sample Z-test). Comparisons between social groups across treatment conditions (treated vs untreated) revealed a sizeable decrease in the proportion of no-social preference (NSP) fish (13.7%) within the treated population (**Table 4.1**; C (no drug): No-Social Preference (NSP) $\hat{p}$ = 0.387; vs; C (buspirone): No-Social Preference (NSP) $\hat{p}$ = 0.250; two-sample Z-test*: z* = 1.9, *p* = 0.055). A marked increase in the proportion of prosocial fish (14.6%) was also detected in treated conditions compared to untreated controls (**Table 4.1**; C (no drug): prosocial (S+) $\hat{p}$ = 0.508; vs; C (buspirone): prosocial (S+) $\hat{p}$ = 0.654; two-sample Z-test*: z* = 2.1, *p* = 0.048). These results, taken together, suggest that buspirone increases social preference at the population level by reducing serotonin signalling and, therefore, sensitivity to stress in no-social preference and prosocial fish groups.

Acute administration of 30 µM buspirone on socially reared fish significantly reduced percentage time spent moving before viewing conspecifics (**Figure 4.4**, Acclimation: controls vs control+ buspirone (30 µM): first minute to the fifteenth minute; Mann Whitney U test: $p \leq 0.05$). This reduction in movement activity was replicated during socialisation from the first minute of the second phase of the preference assay (Socialisation: controls vs control+ buspirone (30 µM): first minute to the fifteenth minute; $p<0.05$, Mann Whitney U test).

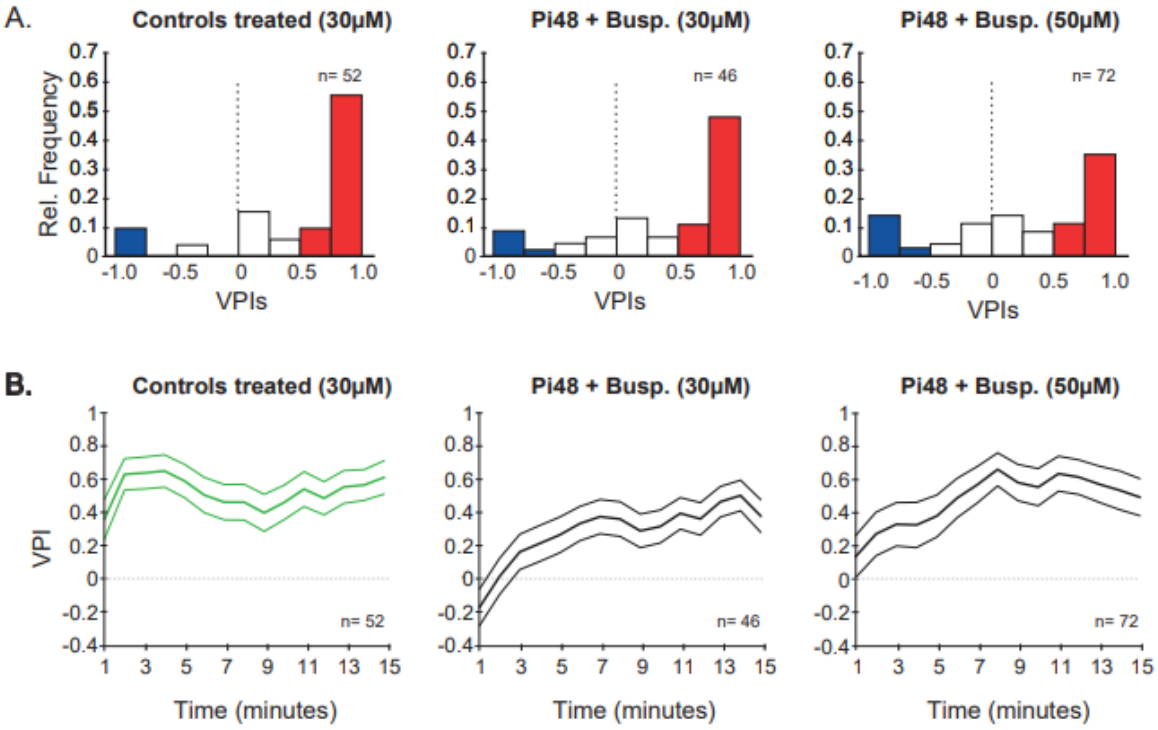


Figure 4.3: Buspirone increases social preference in fish. A. Histograms of VPIs during the socialisation phase in controls fish treated with 30µM of buspirone, in partially isolated (Pi48) fish treated with 30µM and 50µM of buspirone. For visual clarity, blue bars asocial fish (S-, VPIs ≤ -0.5), white no social preference fish (NSP, -0.5 < VPI < 0.5) and red bars highlight prosocial fish (S+, VPIs ≥ 0.5). **B.** Temporal VPI values calculated every minute for controls treated with 30µM of buspirone, Pi48 fish treated with 30µM and 50µM of buspirone. Thin lines indicate standard error. Note how buspirone rescues social preference within the first few minutes.

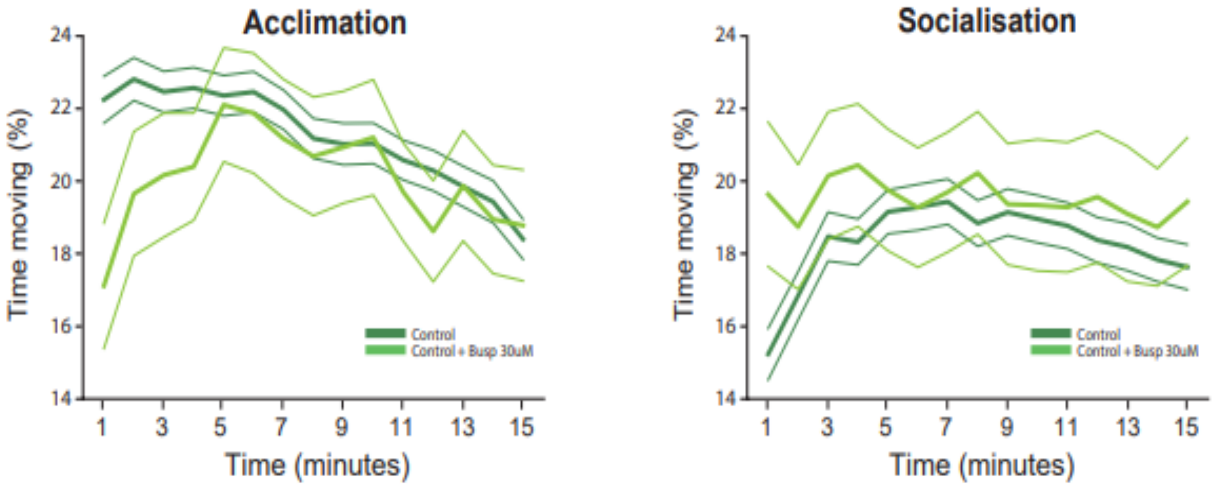


Figure 4.4: Percentage of time spent moving in buspirone treated control fish during acclimation and socialisation. Temporal VPI values calculated every minute for socially reared fish untreated (dark green) and treated with 30µM of buspirone (light green). Thin lines indicate standard error.

Fish partially isolated for 48 hours were treated with 30 µM or 50 µM (*n* = 46 and *n* = 72, respectively) of buspirone (**Figure 4.3** and **4.5**). Remarkably, the acute drug treatment was sufficient in both concentrations to reverse the asocial phenotype caused by isolation (**Figure 4.3A**; Pi48: *Med* = 0.09, *n* = 157; vs; Pi48 +buspirone (30 µM and 50 µM combined): *Med* = 0.54, *n* = 118; *Mann-Whitney U test, u* = 6620, $p \leq 0.0001$, Mann Whitney U test). Closer inspection looking at the proportion ($\hat{p}$) of fish assigned to the three social groups, asocial, no-social preference and prosocial, showed that fish numbers were comparable between untreated socially reared controls and treated isolated conditions, thus confirming that buspirone reversed the effects of isolation (**Table 4.2**). Comparisons of proportions across treated and untreated isolated conditions (Pi48+buspirone and Pi48, respectively) revealed a substantial reduction in the number of asocial animals (20%) that contributed to the significant increase in the number of prosocial fish (14.5%) (**Table 4.2**; Pi48: asocial (S-) $\hat{p}$ = 0.344; vs; Pi48+ buspirone: asocial (S-) $\hat{p}$ = 0.144; z = 3.8, p = 0.002, two-sample Z-test) (**Table 4.2**; Pi48: prosocial (S+) $\hat{p}$ = 0.363; vs; Pi48+buspirone: prosocial (S+) $\hat{p}$ = 0.508; z = 3.8, p = 0.0161, two-sample Z-test). Furthermore, while treatment with buspirone increased the proportion of no-social preference fish (5.4%) in partially isolated fish relative to their untreated equivalents, this was insignificant (**Table 4.2**). These results support earlier findings that anxiety is the driving factor that governs socially isolated fish's aversive behaviour since socially reared no-social preferences are expected to be in a lower state of social anxiety.

	Proportion of asocial (S-)	Proportion of no-social preference (nsp)	Proportion of prosocial (S+)	Total number of fish
Controls	0.103	0.387	0.508	380
Controls +Buspirone (30µM)	0.096	0.25	0.654	52

Comparison	Comparisons of proportion of sample populations	z-score	p-value
Controls (C) vs. Controls +Buspirone (30µM)	Asocial (S-) vs. Asocial (S-)	0.2	0.876
	No social preference (nsp) vs. No social preference (nsp)	1.9	0.055
	Prosocial (S+) vs. Prosocial (S+)	2.0	0.048 ***

Table 4.1 Buspirone increases the proportions of prosocial fish. Top panel: Table of proportions of asocial (S-, VPIs ≤ -0.5), No Social Preference (NSP, -0.5 < VPI < 0.5) and prosocial (S+, VPIs ≥ 0.5) fish found in normally reared untreated and treated fish populations. Sample sizes provided in the last column. Treatment is 10 minutes of 30µM of buspirone. Bottom panel: Results from the Two-Sample Z-test was used to compare the number of fish assigned to each social preference group in the presence of conspecifics. Asterisks indicate significance (p ≤ 0.05).

	Proportion of asocial (S-)	Proportion of no social preference (nsp)	Proportion of prosocial (S+)	Total number of fish
Controls	0.103	0.387	0.508	380
Pi48	0.344	0.293	0.363	157
Pi48 +Buspirone (30µM and 50µM)	0.144	0.347	0.508	118

Comparison	Comparisons of proportion of sample populations	z-score	p-value
Controls vs. Pi48 +Buspirone (30µM and 50µM)	Asocial (S-) vs. Asocial (S-)	1.2	0.2186
	No social preference (nsp) vs. No social preference (nsp)	0.8	0.4336
	Prosocial (S+) vs. Prosocial (S+)	0	1
Pi48 vs. Pi48 +Buspirone (30µM and 50µM)	Asocial (S-) vs. Asocial (S-)	3.8	0.0002 ***
	No social preference (nsp) vs. No social preference (nsp)	0.7	0.4965
	Prosocial (S+) vs. Prosocial (S+)	2.4	0.0161 ***

Table 4.2: Buspirone rescues the proportion of asocial and prosocial groups in isolated fish. Top panel: Table of proportions of asocial (S-, VPIs ≤ -0.5), No Social Preference (NSP, -0.5 < VPI < 0.5) and prosocial (S+, VPIs ≥ 0.5) fish found in normally reared untreated and treated and isolated treated fish populations. Sample sizes provided in the last column. Treatment is 10 minutes of 30µM or 50µM buspirone. Bottom panel: Results from the Two-Sample Z-test was used to compare the number of fish assigned to each social preference group in the presence of conspecifics. Asterisks indicate significance (p ≤ 0.05).

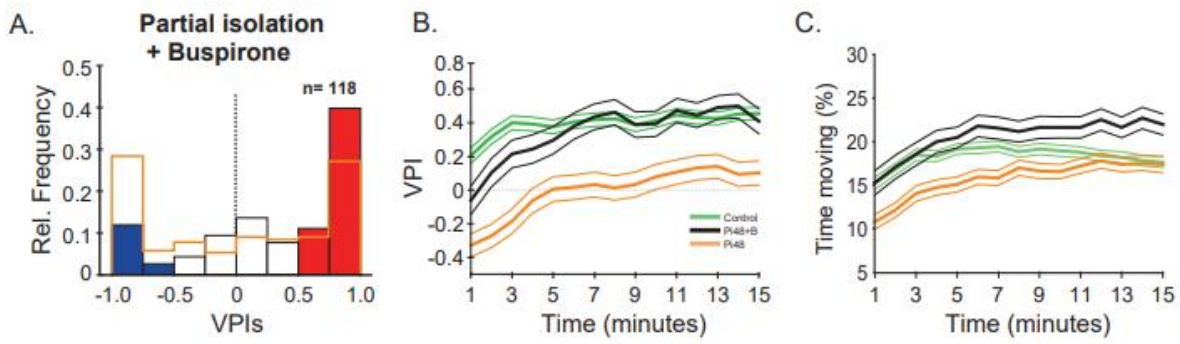


Figure 4.5: Buspirone rescues social preference in isolated fish. A. Histograms of VPIs during the social cue period is partially isolated (Pi48) fish treated with 30µM or 50µM of buspirone combined. Orange line displays VPI of partially isolated without treatment. For visual clarity, blue bars highlight asocial fish (S-, VPIs ≤ -0.5), white no social preference fish (NSP, -0.5 < VPI < 0.5) and red bars highlight prosocial fish (S+, VPIs ≥ 0.5). B. Temporal VPI values calculated every minute for controls (green line, n = 380), Pi48 (orange line, n = 157), and Pi48 treated with buspirone (black line, n = 118). Note how buspirone rescues social preference already within the first five minutes. C. Percentage of time moving calculated in one-minute bins for the same fish as B, thin lines indicate standard error.

To investigate the time course for the rescue of the asocial aversive phenotype, VPIs computed in one-minute bins during social cue viewing were compared (**Figure 4.3B**). Partially isolated fish treated with buspirone (Pi48+ buspirone), while initially asocial, displayed rapid recovery to normal social preference behaviour within the first five minutes of exposure to social cues (**Figure 4.3B**; C: *n* = 380; vs; Pi48+Buspirone (30 µM and 50 µM combined); first minute: *Med* = 0.64, *Med* = -0.06, *Mann-Whitney U test, u* = 18543, *p* = 0.017160; fourth minute: *Med* = 0.89, *Med* = 0.24, *Mann-Whitney U test, u* = 20809, *p* = 0.38046). In contrast, the VPI's of untreated isolated fish (Pi48) remained significantly lower than controls throughout the entire session (**Figure 4.3B**; C: *n* = 380; vs; Pi48 (Buspirone): Mann Whitney U test: *n* = 157; *p* ≤ 0.05)

Similar temporal comparisons of the proportion of time moving (**Figure 4.3C**) revealed that fish motility generally increased quickly over the first five minutes of social viewing. Notably, the activity of isolated fish treated with buspirone was already at the level of the controls at the start of the social viewing session (**Figure 4.3B**; C: *n* = 380; vs; Pi48 (Buspirone): *n* = 157; first minute: *Med* = 11.84%, *Med* = 10.41%, *Mann-Whitney U test, u* = 21758.5, *p* = 0.31), suggesting that the recovery of regular movement activity, through reduced anxiety, precedes the recovery of normal social preference.

Discussion

In this chapter, using high-resolution imaging, it is determined for the first time that there are key differences between socially reared asocial and prosocial zebrafish brain activities. It is demonstrated that social and prosocial zebrafish reared in social isolation have different functional responses to conspecifics than those in the socially reared population. The functional changes caused by social deprivation are consistent with increased anxiety resulting from hyper-sensitisation to conspecifics, similar to the effects of isolation on humans[517]. The final experiment presents that the adverse impact of social isolation in zebrafish can be reversed using an existing anxiolytic drug that acts on the monoaminergic system.

Functional brain activity

The 'social behaviour network' model describes a network of brain areas that respond distinctly to various social stimuli, collectively forming different patterns that modulate behavioural outputs[202]. In the first two experiments, in line with this model, differential brain activity is reported in two of the several brain regions that comprise the social behaviour network, namely the hypothalamus and preoptic areas (**Figure 4.1**). Furthermore, brain areas associated with visual processing and stress responses (optic tectum and posterior tuberal nucleus, respectively) are differently activated between asocial and prosocial zebrafish phenotypes within the same and across the different social rearing conditions.

Hypothalamus

In zebrafish, the hypothalamus is in the diencephalon, with the rostral regions positioned below the thalamus and the caudal areas below the mesencephalic tegmentum[518–521]. The hypothalamus is particularly sensitive to interpretations of threats and stressors by the brain[517]. Through its interactions with the pituitary and adrenal glands, which together make up the hypothalamic-pituitary-adrenocortical (HPA) in mammals or the hypothalamic-pituitary-interrenal (HPI) axis in fish, the hypothalamus acts as an interface between the autonomic and endocrine systems, regulating a broad spectrum of physiological and behavioural processes, including but not limited to light-seeking and motor behaviours[522,523], feeding[524,525] and sleep cycles[526] as well as social behaviour[521].

The caudal hypothalamus has been described to express serotonin, dopamine, glutamate, and histamine highly. The segregation into distinct dorsal and ventral areas of the caudal hypothalamus was previously described with markers targeting these pathways, such as Tyrosine Hydroxylase 1 and 2 (*TH1* and *TH2*) enzymes involved in the synthesis of dopamine[527].

In this present chapter, riboprobes targeting the serotonergic and dopaminergic systems were selected since changes in serotonin and dopamine levels have been widely documented in

response to social interaction[528], viewing social cues[423], and social isolation[244,375,395]. Whilst the serotoninergic system has been linked to stress and arousal[529], the dopamine circuitry has been shown to regulate the reward system underlying social behaviour[530].

In line with previous findings, whole-mount in *situ* hybridisation staining shows a potential overlap of TH1, TH2, and dopamine and serotonin transporters, DAT and SLC6A4B with c-Fos activation in the caudal hypothalamus (**Figure 4.1C**)[509,531]. Since the caudal hypothalamus expresses both dopamine and serotonin, and the brain activity inferred by c-Fos staining demonstrates a unique pattern of activation/ inhibition that is distinct for prosocial and asocial phenotypes, these results suggest that this area could be crucial in regulating social preference.

Several studies have shown that social isolation in mice leads to dysregulation of the HPA axis resulting in the increased release of cortisol[532,533] with similar effects, also observed in socially deprived rats[370,534]. Results in this chapter are in line with these findings with fully and 48 hours, isolated fish display atypical activation in the caudal hypothalamus, compared to socially reared controls fish, inferring distorted HPA axis activation. Furthermore, the different activation of social phenotypes between fully and partially isolated fish are consistent with behavioural variations reported in **Chapter 3,** e.g., fish proximity to a social window and freezing responses**.** Thus**,** supporting the earlier hypothesis that periods of full (21 days) and partial (48 and 24 hours) isolation may exert differing behavioural changes through dissimilarly altered activation of the social circuitry, particularly through the caudal hypothalamus.

Aside from the impact of dysregulation of the HPA axis, research in mice and rats have shown that social isolation has multiple effects on the serotonergic and dopaminergic systems propagating their disruption[375,395,432,486,533]. Studies that have investigated the neurochemical manifestations of social isolation using high-performance liquid chromatography have described significant changes in whole-brain serotonin and dopamine levels (as well as their respective metabolites, 5-hydroxyindoleacetic acid (5HIAA), and 3,4-dihydroxyphenylacetic acid (DOPAC), respectively). However, published evidence is conflicting with studies reporting either increase, decrease or no changes in levels in many models, including the adult zebrafish[375,395,432,486,533].

The analysis of whole-brain homogenate has deterred any reference to neuroanatomy when citing changes in transmitter and metabolite concentrations[375,395,535,536]. Thus, using the tiny optically transparent brains of juvenile zebrafish reared in different social settings, it would be advantageous to classify the neuroanatomical and neurochemical character of areas of disparate activation by generating a high-resolution three-dimensional spatial representation of serotonergic and dopaminergic networks. Based on the differences in brain activity between asocial and prosocial fish reported here, extending such a study to include the separation of the two social phenotypes may explain why conflicting serotonin and dopamine levels have been

previously described. Furthermore, it would be of interest to continue expanding the breadth of tested probes, presented in this chapter, to include other neuromodulators signally through the metabotropic receptors, such as noradrenaline, histamine, and hypocretin indicated in higher-order psychological processes, including perception (categorisation of stimuli)[537,538] to recapitulate the full complement of c-Fos expression elicited by viewing of conspecifics[539] which are not fully captured by the serotonergic and dopaminergic systems alone.

Preoptic area

Located in the most anterior part of the hypothalamus[540,541], the teleost preoptic area (POA) has been described as analogous to the mammalian preoptic area in terms of neurochemistry and hodology receiving fibres from and projecting to diverse brain areas[506,542–544], for example, the habenula[545]. Like the hypothalamus, the POA has been previously detailed to play an essential role in regulating social behaviours such as courtship, aggression and in some fish species, parental care through the expression of several neuropeptides such as oxytocin, vasotocin and arginine vasopressin[483,546]. It was recently shown that oxytocin does not seem to be responsible for social interaction, where oxytocin receptor mutants do not exhibit alterations in social preference but instead display reduced social recognition. In rats, the activation of oxytocin neurons during social interaction is believed to generate social reward[547]. Furthermore, oxytocin injections into the brain of zebrafish do not have any effect on social interaction and shoaling behaviour[548]. The neuropeptide vasotocin has been shown to reduce interaction exclusively[548] without impacting shoaling behaviour. Additionally, oxytocin has also been shown to be involved in aggression[173] and stress by stimulating the release of cortisol.

In this chapter, the reported activation of the preoptic area during social behaviour is consistent with previous literature in several species following social interaction[134,506,549]. Interestingly, activation in the preoptic area following social interaction in isolated fish is significantly higher than in socially reared fish. Conflicting information has been reported on the activity of the preoptic area in animals following periods of social isolation. For example, Pousa and co-workers (2019) described that isolation of 24 hours significantly lowered neuronal activity and reduced vasotocin positive cells in the anterior preoptic area of weakly pulse-type electric fish (Brachyhypopomus gauderio)[549]. In contrast, Stowe and co-workers (2005) found increased neuronal activity in the medial preoptic area of prairie voles following only an acute isolation period of 24 hours and not two weeks[550]. Although differences between reported results may seem like discrepancies, it is likely due to variations in how the preoptic area is subdivided. In this present chapter, the preoptic area is divided dorsally, and ventrally whilst Pousa et al. instead separated the same region anteriorly and posteriorly (preopticus ventricularis anterior and posterior, respectively)[549]. Contrastingly, Stowe et al. analysed the entire preoptic brain area of isolated prairie voles[550]. These differences highlight that within a single brain structure, several

patterns of activation may be present, and these are potentially missed when these structures are analysed as a whole. Since the functional imaging of juvenile zebrafish in this chapter is completed on whole brains, it would be possible simple to extend the analysis to the preoptic subregions described by Pousa et al. and other studies to facilitate more direct comparisons.

Interestingly, Pousa and co-workers also reported no differences in the activation of vasotocin expressing cells between isolated and socially kept fish but found a clear association between the number of vasotocin-positive cells and courtship behaviour[549]. Therefore, a follow-up experiment investigating the phenotype of preoptic c-Fos expressing cells presented in this thesis could reveal whether the underlying difference in the number of vasotocin expressing cells are responsible for the atypical social behaviour readily observed in isolated fish.

In rats, the neuropeptide neurotensin has been linked to the reward system, with neurons in the medial preoptic area generating social reward via activation of the dopaminergic producing neurons of the ventral tegmental area[551,552]. Therefore, the differing activation of the preoptic area in isolated zebrafish may reflect an altered perception of conspecifics in terms of reward. Thus, follow-up experiments to identify the cell phenotype are warranted, including techniques such as double fluorescent in situ staining and immunohistochemistry or optogenetic approaches. Furthermore, the expression pattern of the neuropeptides and their respective receptors, in conjunction with c-Fos activity, would provide valuable information on the mechanism by which isolation leads to dysfunctional social behaviour, highlighting again the benefit of expanding the breadth of tested probes to beyond the serotonin and dopamine pathways, for example, vasotocin, neurotensin as well as previously mentioned neuropeptides oxytocin and arginine vasopressin.

Why is social deprivation self-perpetuating?

The optic tectum (superior colliculus in mammals) is a highly laminated structure located in the midbrain with a central role in visuomotor transformation in all vertebrates[370,553–556]. Aside from the retinal afferents, nonretinal tectal afferents have been described anatomically in many adult fish and include examples such as the hypothalamus and the raphe[557–561]. The capacity of the hypothalamus to regulate several behaviours has been previously mentioned. The serotonergic neurons in the raphe nucleus, also affected by hypothalamic neurons, have been shown to work with the visual information to arbitrate the classification of visual stimuli as either appetitive or predatory based[562].

Depending on the species, visual stimuli can generate specific motor responses. For instance, the overhead presentation of a visual stimulus increasing rapidly in size (a so-called looming stimulus) in mice evokes a flight response similar to that triggered by an approaching predator. In

contrast, a slower-moving visual stimulus resembling a cruising predator induces a freezing reaction[563]. Contrastingly, in zebrafish, slowly moving dots similar in size to the microorganisms they feed on (i.e., paramecium)[564] while looming stimuli induce escape swimming in larval zebrafish[562].

Across species, both flight and fright responses are thought to be controlled by the optic tectum. Specifically, through direct and indirect inputs from other brain areas, it is believed that the optic tectum selects between one of these two visually triggered motor actions, which are subsequently conveyed through downstream reticulospinal circuits[565]. Therefore, the differences in optic tectum activity observed between socially reared or isolated fish may reflect an altered perception of conspecifics, supported by behavioural data in **Chapter 3.**

The increased optic tectum activity in isolated fish this chapter aligns with functional magnetic resonance imaging research on humans[267]. Cacioppo et al. reported that loneliness (the perception of social isolation) in humans is associated with greater activation of the visual cortex in response to viewing negative social stimuli than negative non-social images (objects)[267]. Complementary to these findings, Bangee and co-workers (2018) reported that lonely individuals initially fixate more on socially threatening elements of social scenes than individuals with low levels of loneliness, who instead fixate on the positive aspects of the same visual cues[268]. In both these human studies, this hypervigilance was ascribed to increased emphasis on self-preservation in the face of social-environmental adversity[120,267], which perhaps increases the likelihood of survival[517]. For example, hypervigilance may aid an individual to identify and solicit social-mediated resources such as food, shelter, and reproductive opportunities alongside preparing them to detect and defend against any potential assault in their environment. This idea can also be extended to other species, including teleosts. For instance, fish on the outer edges of schools face a greater risk of predation due to the ease they may be isolated from the group and attacked by predatory fish[566]. In such cases, social behaviour is preferred, with fish actively co-ordinating their swimming to stay near conspecifics, particularly within the centre of the school[566], as the alternative action poses a danger to an individual's immediate health. Once separated, it is in the isolated fish's interest to stay vigilant to potential assault until a time by which the typical social environment is restored, i.e., due to the risk of predation. Although the overall function of hypervigilance may be to increase the likelihood of short-term survival, it is logical that long-term increased sensitivity carries costs, especially when the perception of isolation becomes chronic, i.e., the active avoidance behaviour in fully isolated fish (see **Chapter 3**).

Furthermore, research in Drosophila has shown that chronic but not acute social isolation reduces total sleep incrementally[567]. Many studies have reported the detrimental effects of shorted/interrupted sleep on neurobehavioral performance, particularly during critical developmental windows[568]. In this present chapter, the more significant impact of full isolation at

both the functional and behavioural level than partial isolation of 48 hours supports the notion that social isolation has a cumulative effect. While the behavioural responses of fish isolated for 24 hours also support this suggestion, further functional studies on such fish would give more validation[267].

Acute and chronic stress can precipitate depression and pathological anxiety disorders, both previously implicated with social isolation[288,289,292,517,569]. Studies have shown that the zebrafish posterior tuberal nucleus (PTN), a region near the caudal hypothalamus, is involved in stress responses. For example, Ziv and co-workers (2013) reported that adult zebrafish acutely stressed through confinement in a narrow glass tube for ten minutes showed increased corticotropin-releasing hormones in the PTN involved in promoting the release of cortisol. Furthermore, Choi and co-workers (2018) reported that Sam2-expressing cells in the PTN regulate anxiety-like behaviour in zebrafish[570]. Therefore, the increased PTN activity of isolated fish reported in the analysis presented here suggests that both prolonged and short incidents of social isolation (full and 48 hours, respectively) result in altered stress responses, and this is reflected by the increased anxiety-like behaviour observed in both fully and partially isolated fish before viewing conspecifics as reported in **Chapter 3**.

Buspirone rescues social preference

The last experiment demonstrated that single acute administration of buspirone effectively rescues social preference in young fish challenged with social deprivation of 48 hours before testing.

Initially designed to treat psychosis in mental disorders such as schizophrenia, buspirone is used to treat generalised and social anxiety disorders due to its efficacy as an anxiolytic. Although it has been successfully administered in humans for over two decades[497,571–573], the exact mechanisms by which buspirone reduces social anxiety are not yet fully understood[496]. Several studies have shown that it has a high affinity for the 5-HT_{1A} receptor and lower affinities for dopamine D_2 and 5-HT_2 receptors[553,574–576]. Over the last decade, there has been growing evidence indicating that buspirone's anxiolytic action is derived from its effects at the 5-HT_{1A} autoreceptors on the presynapses of serotonergic neurons, acting as a partial agonist to reduce circulating 5-HT (serotonin) levels in the braiin[501,504].

Buspirone has been shown to reduce anxiety in humans, and in many mammalian models[404,577,578] enhance social interaction[579,580] and reduce social phobia[497,581]. Bencan and co-workers (2009) first showed that buspirone significantly reduces the novel tank diving response in zebrafish without its sedative effects[404]. Several studies have described the anxiolytic effects of

buspiron[501,579,582] and its ability to enhance sociability in zebrafish- prosocial effects[404,500,579,580,582,583]. Yet, to date, buspirones' ability to counter the impact of social isolation has not been investigated[113].

The anxiolytic effects of buspirone have been reported in zebrafish. Where individual fish behaviours have been studied, a significant reduction in bottom-dwelling in adults and no effects on locomotion have been reported at dose concentrations of 3 mg/L or 5 mg/L (7.11 µM and 11.85 µM, respectively)[583,584]. However, aside from anxiolytic effects, a concentration of 50 µM in larvae has also been associated with a significant reduction in locomotion[585]. Yet, in this present chapter, concentrations of 30 µM and 50 µM do not reduce movement activity but increase in isolated juvenile zebrafish. The difference between previously mentioned locomotive findings and those reported here are likely to be down to two reasons: 1) variation in how locomotion is measured and 2) contextual differences in types of anxiety.

Previous studies have quantified locomotion through measurements such as velocity, whilst locomotion in this chapter was defined by the percentage of time fish spent moving. However, since total distances were also extracted from behavioural data, test fish velocities may also be calculated retrospectively, thus directly comparing results reported here with previous publications. To explain why different types of anxiety may result in differences in the locomotive effect of buspirone, rodent models (in which the anxiolytic effects have been described following social disruption/ isolation)[574,586] and human data are best used. For example, in a chapter by Haller and co-workers (2004), where female rats underwent alternating periods of social isolation and crowded housing resulting in an unstable social environment, buspirone was reported to exert anxiolytic effects. Interestingly, the social instability that increased the apparent anxiolytic effect of buspirone (i.e., reduction in agonistic interactions such as aggressive grooming, chasing, fighting and biting) also attenuated the locomotor suppressive effects of the drug[586]. Similar results have been reported in male rats and mice following separation from conspecifics[574], with periods of social isolation appearing to enhance the anxiolytic efficacy of serotonergic agents[587–589], including buspirone[590]. These studies suggest that buspirone reduces the anxiety induced by social environmental factors without affecting basal anxiety levels. Human data further supports this idea. Rather than decreasing anxiety, buspirone instead causes sleepiness, drowsiness, and other side effects such as nervousness and even anxiety in healthy humans[591–593].

Similar results are reported in the present chapter. Whilst the acute administration of buspirone on fish isolated for 48 hours results in a significant increase in movement activity, the equal treatment of socially reared juvenile zebrafish results in a substantial reduction in this same parameter compared to untreated equivalents. The findings add to the growing evidence that social context is an essential determinant of drug action. Further evidence is provided by the

differences in the balance of social phenotypes found in the treated 48 hours isolated fish population but not treated socially reared controls.

The findings in this chapter indicate that VPI scores of partially isolated fish are restored within the first minute of social interaction. Furthermore, increase in percentage time moving in treated isolated fish are detectable within the first five minutes of socialisation. Notably, the movement activity of socially deprived fish treated with buspirone is already at the level of controls from the start of the socialisation phase. These results are in line with previous reports on the rapid behavioural effect of buspirone, where the impact on zebrafish height in a tank test following ten minutes of buspirone treatment was detectable from the first minute of exposure[501]. Compared to untreated isolated fish, the relative movement of buspirone treated Pi48 fish within the first minute suggests that recovery of regular movement activity precedes the recovery of normal preference. It is beyond the scope of work presented here to fully establish whether it is buspirone's direct action on the serotonin pathway or whether its anxiolytic effect allows isolated fish facilitates their more prolonged exposure to conspecifics that drives the recovery of social behaviour. However, buspirone's impact on the recovery rate of social preference indicates that it may work by lowering anxiety, perhaps at the caudal hypothalamic and preoptic area, allowing circuit plasticity to down-regulate the hypersensitivity to social stimuli acquired during the isolation period.

Given the promising effect of buspirone on partial social isolation, investigating its efficacy in chronically isolated juvenile fish would be the next logical step. Buspirone is typically prescribed beyond single use in humans when treating generalised anxiety disorder. Behavioural results in the previous chapter, coupled with the different functional brain activities between the two isolated fish conditions presented here, indicate an accumulative effect of social isolation on preference behaviour, similar to the impact of chronic and acute isolation on short- and long-term social recognition memory already reported in rats[594]. Therefore, it is likely that settings with prolonged social deprivation may require continuous administration to reach therapeutic levels. Consequently, it would be beneficial to investigate buspirones' effectiveness in treating social isolation under chronic administration regimens.

Since behaviour correlates to brain activity and buspirone rescues the social preference of juvenile zebrafish socially deprived of conspecifics for 48 hours, a direct follow-up experiment would be to image the functional brain activity of isolated social phenotypes following buspirone treatment, after which active serotoninergic cells may be identified using in *situ* hybridisation techniques. Currently, no study has reported a correlation between social behavioural changes following buspirone treatment, and the neuroanatomical modification of the serotonergic system in isolated fish, as suggested above. The work of Varga and co-workers (2020) have come closest, reporting increased serotonin in the forebrain of isolated larvae[595]. However, they too have fallen

short of extending their investigation to the level of asocial and prosocial phenotypes; therefore, the extent to which the brain can be rescued from the effects of isolation (i.e., 48 hours) remained undocumented. Such studies would provide both the groundwork for assessing the effectiveness of future treatments and insight into the susceptibility of socially deprived individuals to future incidents of isolation.

Conclusion

Evidence derived from the zebrafish model strongly suggests that social isolation can alter both stress responses and the perception of conspecifics following prolonged and short isolation. Based on the observations in the four brain areas (the caudal hypothalamus, preoptic area, optic tectum and the PTN), it is concluded that isolation heightens sensitivity to social stimuli. When prolonged, this heightened sensitivity towards social cues results in increased stress and anxiety levels during social viewing, leading to subsequent aversion to conspecifics. This view is further supported by the subsequent recovery of social preference behaviour in isolated juvenile zebrafish treated with an anxiolytic, buspirone.

Together, these results provide an understanding of the impact of social isolation on the neural circuitry at the functional level, which gives rise to atypical social preference behaviour, and novel insights into the role of serotonin signalling in the impaired phenotype observed in socially isolated zebrafish. Although the brain regions in this chapter are modulated by serotonin or dopamine, it is unlikely that the functional and behavioural abnormalities detected in socially isolated zebrafish are solely explainable by changes in these two systems. Therefore, a systematic approach will be required to disentangle the intricate relationships between neurotransmitters, modulatory peptides, and their consequences on social circuitry and behaviour.

Finally, the juvenile zebrafish with its tiny translucent brain presents a powerful tool for studying the impact of isolation on brain function and exploring different strategies for reducing or even reversing its adverse effects through manipulating involved pathways.

Chapter 5: General discussion

Summary of main findings

The overall aim of this thesis was to study how the social brain circuitry gives rise to a diversity of social preference behaviours and the mechanism by which social-environmental adversity (social isolation) during development causes dysfunctional behaviour. The juvenile zebrafish, an excellent laboratory model to study social preference both behaviourally and functionally, is used due to its extensive repertoire of social behaviours combined with its small brain size, amenable to high-resolution imaging techniques. Below, the principal findings are briefly summarised in order as reported in each chapter.

1. Socially reared zebrafish visual preference index scores are stable over the testing day and robust against repeated testing. This suggests that a single VPI measurement is sufficient to represent a given fish's sociality accurately.

2. In fish, social preference behaviour is distinguishable from general locomotion through several parameters, which lends itself to a detailed analysis of how social interactions are perceived. These parameters include test fish proximity to conspecifics, the average location of fish in the chamber, the total number of entries into predefined areas, total distance travelled, percentage of time spent freezing, percentage of time spent moving, absolute Y motion, and body orientations.

3. The social preference behaviour of juvenile zebrafish can be summarised using VPI scores which recapitulates several of the analysed parameters, thus providing a single measure by which sociality can be assessed.

4. Asocial and prosocial phenotypes, identifiable by VPI scores, are behaviourally distinct and show differences during acclimation. In addition, these differences become more pronounced following the placement of conspecifics into one of two adjoined and visually accessible smaller chambers. These points suggest that a) existing differences may underly the variations in fish responses towards conspecifics, and b) the viewing of conspecifics is perceived dissimilarly between the two social phenotypes.

5. Fish responses in terms of distances from the social window (and thus conspecifics) varied across isolated conditions. Fi fish actively maintained greater distances, whilst Pi48 fish increased their proximity over time in the presence of conspecifics. Changes in Pi24 fish proximity to the social window were not attributable to the presence of conspecifics. This result indicates different durations of isolation have differing effects on social preference behaviour.

6. Only Pi48 fish responses to conspecifics show dynamic change, i.e., proximity to the social window, freezing and movement activity. Furthermore, asocial and prosocial Pi48 responses are higher than their socially reared counterparts. This suggests that 48 hours of social isolation may lead to a heightened sensitivity towards social stimuli, perhaps creating a sense of longing for social interaction, whilst prolonged isolation is self-perpetuating through aversion.

7. Whole-brain functional maps of asocial and prosocial phenotypes within the socially reared population are distinct, supporting behavioural data indicating that social phenotypes perceive conspecifics differently.

8. The functional maps of isolated social phenotypes are distinct from social phenotypes found in the average population. In addition, these isolation-induced activity changes reveal profound disruption of neural activity in brain areas linked to social behaviour, social cue processing, and anxiety/stress, proposing possible mechanisms by which isolation gives rise to atypical social behaviour.

9. Acute administration of buspirone, an anxiolytic, rescues the social preference in juvenile zebrafish challenged with social deprivation by acutely reducing serotonin levels. This confirms the crucial role of serotonin signalling in the impaired social preference phenotype observed in isolated zebrafish and suggests the involvement of anxiety.

Measuring loneliness

Social relationships are intricately tied to individuals' health, and a lack of social connection has an adverse impact on health and well-being[596,597]. In an extensive systematic review involving a meta-analysis of 148 studies examining social relationships and mortality risk, Holt-Lunstad et al., 2010, documented those older individuals with weaker social relationships had a 50% increased likelihood of mortality than those with stronger social relationships[598]. Furthermore, subsequent research by this group found that social isolation, loneliness, and living alone increased the risk for mortality more than obesity alone[599], even though the increased risk of obesity has also been associated with social isolation[278,598]. Loneliness is also associated with a higher risk for depression and other mental health problems[238,283,291,292,597,598], a constellation of socioemotional states and adaptation disorders, such as overconsumption of alcohol, fear or negative evaluation, increased anger, dysmorphia and loss of self-esteem[600], as well as perturbed sleep[601–603] and impaired cognition[604–607]. Although loneliness has been associated with several adverse health and well-being outcomes, there is currently little understanding of how feelings of

loneliness manifest as adverse health and well-being consequences, including behavioural issues, thus, warranting further research.

Currently, two main approaches exist for measuring loneliness in humans: single-item and multi-item scales[608,609]. These measures differ in terms of their methodological approach and theoretical positions covering the nature of loneliness; therefore, each offers unique advantages and disadvantages[610].

Cognitive theories of loneliness, based upon personal assumptions, such as the discrepancy between desired and available relationships[270] which give rise to maladaptive patterns of cognitive processing to generate feelings of loneliness[611–613], underpin the self-rating scale approaches to measuring loneliness. In this viewpoint, loneliness is considered a state that can be induced and manipulated hence the interest in social isolation and intervention studies[614–616], respectively.

Single-item measures are simple to use and, as suggested by the name, are single direct questions asked to measure loneliness, e.g., "how often are you lonely?"[617]. Such questions require respondents to rate their levels of loneliness from 'never' to 'always' with some intermediate gradations of response[618]. Hence, this approach presumes that loneliness is a unidimensional concept and that the main difference between lonely individuals is in the intensity of the experience. Although the simple scale of the single-item approach has been used in many studies[619–622], it is better adapted to measuring loneliness in adults without severe cognitive impairment, (age 50 or older)[623], even though it does not elicit information about the amount, nature, value, or meaning of loneliness, nor about its causes or consequences. The simplicity of the single-item approach, which serves as an advantage, also serves as a disadvantage since the single question presumes a common understanding of the concept of loneliness by all involved (i.e., questioners, clinicians and responding patients) and, therefore, can be highly culturally specific[624]. More fundamentally, loneliness may be seen as a stigmatising concept[625,626], further compromising the status of an individual in society; therefore, older respondents may choose not to define themselves as lonely and thus downshift their loneliness rating[627].

To circumnavigate the issue of the stigma surrounding self-identifying as 'lonely', a variety of multi-item scales that avoid the term have been developed to indirectly measure loneliness. Included in this category are two of the most widely used measures of loneliness, the Revised UCLA Loneliness Scale (R-UCLA)[628,629] and the De Jong Gierveld Loneliness Scale[630].

The R-UCLA, a revised version of the original 1980 loneliness scale initially developed and validated among college students at the University of California Los Angeles (UCLA)[631], is one of the most widespread scales of loneliness, used extensively in studies conducted in the United States[632–635]. The R-UCLA is a unidimensional, 20-item self-report scale that asks respondents to rate how often they feel certain emotions that implicitly capture the sense of loneliness (such as,

"How often do you feel left out?" and "How often do you feel you lack companionship?") on a 4-point Likert-type scale ranging from 1 (*never*) to 4 (*often*)[608]. Scores are calculated as the sum of all the items, and the loneliest respondents score closer to 100. Of the several shortened versions of the R-UCLA with proven good psychometric properties and, therefore, used in social surveys, the shortest version comprises only three items rated on a 3-point scale, resulting in 9 as the maximum possible score[608].

On the other hand, the De Jong Gierveld Loneliness Scale is an 11-item, two-dimensional scale more prevalent in European research projects on loneliness (e.g., Netherlands[636–638]). The formulation of this scale reflects the proposition that loneliness can be perceived as a multidimensional phenomenon comprising two distinct dimensions; a deprivation component that relates to the loss of an intimate attachment (social loneliness, e.g., divorce or the death of a partner) and a range of emotional aspects of loneliness, such as sadness, guilt, frustration, anxiety, and desperation[627,630]. Statements such as “I can call on my friends whenever I need them” pertain to the social loneliness component, whilst statements such as “I often feel rejected” relate to the emotional aspects of loneliness. 6 of the 11 items, such as “I can call on my friends whenever I need them” pertain to the social loneliness component, whilst the remaining 5 items relate to the emotional aspects of loneliness (e.g., “I often feel rejected”). Response categories for each item range from 1 to 5, resulting in a maximum possible score of 55 on the De Jong Gierveld Loneliness Scale[630]. The main difference between the two indirect loneliness scales lies in the conceptualisation of loneliness as either a unidimensional or a multidimensional construct, respectively[639].

As mentioned earlier, both single- and multi-item approaches are used extensively in loneliness research, albeit not without problems. An issue shared by both approaches is the subjective nature of reporting loneliness. Both require that the experiencing individual be aware of the true nature of their aversive affective state of loneliness at their cores. This issue is further compounded by the fact that multiple factors, such as health, age, gender, and an individual's prior social experience, can influence the likelihood of individuals experiencing loneliness and the extent to which they feel lonely[640,641]. For example, studies have shown that younger men living in individualistic cultures are most vulnerable to loneliness when age, gender and cultural backgrounds are considered[641]. In addition, gender differences in the likelihood of individuals reporting loneliness have also been documented, with more women reporting feeling lonely to their general health practitioners (GP) than men[642]. Hence, current studies on loneliness must use complex analyses and various controls requiring large sample sizes to overcome such issues. Therefore, it would be advantageous in the field of social psychology and behavioural sciences to have an alternative approach by which loneliness can be objectively assessed.

However, how and what could be used to obtain such a measure remains to be ascertained. A possible solution to this question comes from revisiting the adverse effects on health and well-being associated with loneliness. Since the discrepancy between desired and available relationships is believed to give rise to maladaptive patterns of cognitive processing, generating feelings of loneliness with adverse effects on health and well-being, the negative outcomes may serve as objective measures of loneliness. Specifically, sleep disruption and cognitive function offer two possible measurements by which loneliness (on both a physical and mental level) can be objectively assessed in lonely individuals following periods of social isolation.

The link between cognition and loneliness

Cognition is the ability to comprehend and encompasses the mental action or process (both conscious and unconscious) by which knowledge is acquired, retained, and used, e.g., perception, learning, memory, and thinking[643]. It is believed that most neuropsychiatric disorders such as schizophrenia, depression, and anxiety are cognitive at their core[644]. Research suggests that social isolation and low quality or disrupted interpersonal relations, which lead to feelings of loneliness, increase the risk of an individual developing the named neuropsychiatric disorders[644,645].

Studies have shown that negative emotions substantially influence cognitive processes, including perception[646,647], attention[646], learning memory[648], reasoning[649], and problem-solving[650]. Therefore, it is no surprise that loneliness, a negative emotional state that arises following a discrepancy between an individual's desired and actual levels of social interactions[651], correlates with poorer performances in cognitive tasks[615,652]. In a mass human study which involved 342 participants ranging from 18 to 72 years of age and spanning multiple ethnic backgrounds, the effects of short-term social isolation on the various components of cognition were assessed[615]. Participants were required to complete a plethora of online tasks to measure mood, attention, memory, decision-making, time-estimation and learning over 5 time points spanning 13 weeks. Throughout the study, conditions of social isolation gradually declined from the first week, becoming minimal by week 13. Negative mood was measured using 10 items from the 1992 40-item profile of mood state scale[653], with two items taken from each of the five subscales-confusion, tension, depression, fatigue, and anger. A flanker test[654] measured selective attention in which participants had to press left or right keys per the direction of a centre target image faced, flanked by nontarget distractor stimuli. A Digital Symbol Substitution Test (DSST, adapted from[655]) measured working memory in which participants were required to determine whether a symbol-digit pair matched any of those on a lookup table. The DSST has been described as sensitive to changes in cognitive functioning in patients with major depressive disorder[656]. A gambling task (adapted from[657]) measured decision-making in which participants were tasked to maximize profit on a loan of play money by deciding from which four virtual decks they turned over cards.

Unbeknownst to each participant, each deck varied in the value and frequency of profits and penalties. A time perception task (adapted from[658]) measured time estimation in which participants were assessed on their ability to estimate when a certain amount of time (500, 1,000, 1,500, or 4,000 ms) had passed. Previous research has shown that emotional variations due to social isolation may influence the morning perceptions of time in humans[658]. Lastly, a symbol-learning task (adapted from[659]) measured learning ability, in which participants were required to learn mandarin characters, and after a short mathematical distractor task, match the symbols with their meaning. Results showed that self-reported negative mood ratings significantly improved with the ease of social isolation. Selective attention was at its lowest at five weeks of isolation, corresponding with an increase in a negative mood. However, with the ease of social isolation, flanker task performance generally increased. Similarly, participants showed a general improvement over time on DSST and gambling tasks, with the greatest improvements seen in weeks 5-9 with the easing conditions of social isolation. Symbol learning consistently showed improvement throughout the study. Finally, in the time-perception task, as isolation conditions eased, participants went from significant underestimation to significant overestimation of time[615]. Cravo et al., 2022, reported similar findings on social isolation and the perception of time, documenting that at the beginning of the COVID-19 pandemic, when feelings of loneliness were the high, time was perceived as expanded and decreased across the weeks with ease of social isolation[606]. Hence, feelings of loneliness correspond with the distortion of time awareness.

Like short-term, chronic periods of social isolation also adversely affect cognitive function, and these adverse effects remain detectable over long periods[607,660]. For example, Chen et al., 2021, reported that social isolation was correlated with the accelerated decline of cognitive function and neuropsychiatric symptoms in patients with neurodevelopmental diseases, at even six months after isolation[607]. Specifically, over a 1-year longitudinal study, 177 patients with Mild Cognitive Impairment (MCI), Alzheimer's Disease (AD) or Dementia with Lewy Bodies (DLB) were evaluated for cognitive function and physical activity at two-time points: the first at baseline (before the COVID-19 pandemic) and the last at 1-year follow-up. Self-reporting questionnaires assessed patients' social contact levels and physical activity. Global cognition was evaluated using a Mini-Mental Status Examination (MMSE) in conjunction with the Montreal Cognitive Assessment (MOCA). The Neuropsychiatric Inventory (NPI) measured the frequency and degree of Neuropsychiatric Symptoms (NPS), and the Hamilton depression rating (HAMD) assessed depression levels. Lastly, the Activities of Daily Living (ADL) scale and the Epworth Sleepiness Scale (ESS) were used to evaluate patients' capacity to care for themselves independently and daytime sleepiness, respectively. Results showed that AD and DLB patients who experienced social isolation for about four months had significantly declined MMSE and MOCA scores, indicating a substantial reduction in general cognitive function, detectable at the end of the study.

Furthermore, at the 1-year follow-up, AD patients also showed a marked increase in NPI, ADL, HAMD and ESS scores, indicating increased NPS, ADL, depression, and daytime sleepiness (due to sleep disturbances), respectively[607]. Lara et al., 2019, reported similar findings on the adverse effects of social isolation on cognition, explicitly reporting that both loneliness and social isolation continued to be associated with decreased cognitive ability over a 3-year follow-up period[660]. Continuing, Chen also showed variations in degrees of deterioration of cognitive function between patients with different neurodegenerative diseases. Specifically, DLB patients showed a greater decline in general cognitive ability than AD patients even when the expected yearly decline of each condition was factored in. Moreover, MMSE scores in patients with DLB dropped more than twice as fast as those with AD throughout the study, indicating that DLB patients showed a more rapid decline in cognition than AD patients during periods of social isolation and, therefore, greater susceptibility to the adverse effects of loneliness.

Loneliness is often described as a negative experience unique to everyone, highlighting that personal variables are relevant for understanding the differences in distress between individuals. Three of these personal variables are health status (as previously mentioned), age[641], and personality[661]. Barreto et al., 2021, reported that feelings of loneliness decreased with age and, in conjunction with gender and culture, could be used to predict the frequency of loneliness[641]. Losada-Baltar et al., 2021, also reported that age was a predictor of differences in distress, with older people reporting less psychological distress, e.g., loneliness[662]. Wang et al., 2018, reported that neuroticism was associated with a higher likelihood of feeling lonely, whereas conscientiousness was associated with a decreased risk of loneliness[661]. Together these studies emphasise the importance of controlling for variables in future studies, including work using animal models such as zebrafish. Furthermore, when most of the personal variables are accounted for in an experimental design, e.g., health, age, and gender, the resulting data may provide valuable insights into the contribution of other variables, such as personality traits, which can better help our understanding on how social isolation impacts preference.

Considering the association between social isolation and cognitive function, as exemplified by the previously mentioned studies, there seems to be enough evidence to support that disturbed cognitive function may be used as an objective measure of loneliness in socially isolated individuals. Furthermore, research in this line of investigation may hold important implications for how we prevent and even treat the adverse impact of loneliness.

Sleep as a readout of loneliness

Feeling safe is essential for sleeping soundly in humans and animals, particularly in high-risk predation environments[663,664]. If an individual does not feel secure in their environment this results in a heightened vigilance for threats in their surroundings due to an increased feeling of

vulnerability. When the sense of safety is not restored it is rational to think that an individual's heightened vigilance continues into the night and manifests as disrupted sleep. Similarly, studies have shown that perceived social isolation (i.e., loneliness) also heightens sensitivity to negative social stimuli/ threats[604,665]. Thus, it is possible that the absence of a secure social environment, leading to a sense of loneliness, can too impact sleep and may be expressed as restlessness or more fragmented sleep.

Cacioppo et al., 2002, tested this theory by investigating the sleep efficiency of 64 participants as measured by objective Nightcap recordings and the Pittsburgh Sleep Quality Inventory[666,667]. Participants were grouped according to scores from the R-UCLA scale[609], administered by questionnaire prior to sleep assessment. The cohort consisted of participants with test scores from upper, lower, or middle quintiles. The upper and lower quintiles represented the two extreme levels of loneliness; individuals scoring in the upper quintile represented the lonely group, and those from the lower quintile were the nonlonely group. Results showed that loneliness was related to more micro-awakenings and less restful sleep (e.g., higher reports of daytime fatigue), with lonely individuals spending more time awake after sleep onset than nonlonely individuals. The middle group did not differ from either group on the same measure. Furthermore, Cacioppo reported that these results could not be explained by differences in sleep duration, depressive symptomatology, or other risk factors, meaning that loneliness alone could predict sleep efficiency[668].

Although Cacioppo's work links increased loneliness to reduced restful sleep, it does not address whether the brains of lonely individuals remain vigilant during sleep or whether individuals who exhibit less restful sleep are more predisposed to become lonely. In 2010, these questions were studied by Hawley and colleagues, who asked 229 participants to complete end-of-day diaries on three consecutive days[602]. Diary questions probed sleep duration, daytime dysfunction (i.e., low energy, fatigue, and sleepiness), physical symptoms, and feelings of depression experienced that day. Participants were also asked six items from the same UCLA loneliness scale also utilised by Cacioppo et al., 2002, to assess feelings of social isolation; thus, loneliness was also probed with the end-of-day dairy. Detailed analysis of the complex data via cross-lagged panel models and relevant controls (including but not limited to race/ethnicity, sleep duration, and depressive symptomatology) revealed that daily variations in loneliness predicted feelings of daytime dysfunction the next day. In contrast, daytime dysfunction was not a good predictor of subsequent loneliness. Furthermore, daytime dysfunction was independent of sleep duration, indicating that individuals that felt more socially isolated, compared to non-isolated, found the same amount of sleep less salubrious[602].

Researchers on loneliness and poor sleep have used adults of a wide range of ages. For instance, Kurina et al., 2011, conducted a cross-sectional study to test whether loneliness is

associated with sleep fragmentation or sleep duration in 95 members of a traditional, communal, agrarian society, with ages ranging from 19 - 84 years old[603]. Participants were required to wear an actigraph on the wrist for one week to measure objective sleep properties, namely: fragmentation and sleep duration, and self-reports were used to measure loneliness, depression, anxiety, stress, and subjective aspects of sleep. Results showed that loneliness was a significant predictor of sleep fragmentation. Higher loneliness scores were associated with significantly higher levels of sleep fragmentation (with each unit increase in the UCLA loneliness scale resulting in an approximately 8% increase in sleep fragmentation) even after controlling for covariates such as age, sex, body mass index, risk of sleep apnea and negative affect (i.e., depression and anxiety)[603].

Taking the current findings on loneliness and sleep, sleep quality, at least as indexed by sleep fragmentation, might be a good candidate to evaluate loneliness in individuals, particularly when questionnaires are redundant when using animal models.

Future directions

Linking cognition and social isolation in the zebrafish model

In the zebrafish model, it is possible to explore the effects of social isolation on cognition observed in humans by assessing zebrafish performances on a task set within a maze-like surrounding (typically shaped as a 'T')[669–672]. Specifically, learning and cognitive phenotypes can be modelled in zebrafish using tasks which assess choice discrimination[673], associative learning[674–676], spatial learning[674,676], and memory retention[677].

Like mammalian models, studies on zebrafish have shown that chronic stress (e.g., overcrowding, temperature changes, restraint, and handling) increases anxiety and impairs cognitive function when applied continuously or repeatedly[677–680]. Although the adverse effect of social isolation on cognition has been studied in the zebrafish model, it has been in conjunction with other stressors[677] and or the context of social learning[681,682]. For instance, Piato et al., 2011, reported that social isolation, when used as part of a protocol in conjunction with other stressors, such as handling, increased the expression of corticotrophin-releasing factor and anxiety levels whilst also impairing cognitive function[677]. Gleason et al., 1977, demonstrated that zebrafish learn avoidance responses to electric shocks faster when taught together with conspecifics compared to singly taught individuals[681]. Furthermore, Lindeyer et al., 2010, showed that groups of naïve zebrafish could learn escape routes in response to an approaching trawl from a demonstrator fish with prior experience. Furthermore, newly learned zebrafish could act as demonstrators to new naïve fish, thus simulating three generations of social learning[360]. Currently, the zebrafish model

lacks information about the direct link between isolation and cognition without the impact of additional stressors and social learning. However, cognition has been studied in other social fish species subjected to conditions of social isolation. For example, Brandão et al., 2015, reported that isolation impairs cognition in cichlid fish (*Cichlasoma paranaense*)[605]. In detail, a T-maze was used to assess the learning ability of cichlid fish isolated for 10 days. Fish had to learn to associate a green or yellow visual landmark and, or the position (left or right) of one of two chambers with accessible food. Learning was assumed only when fish found food in nine out of ten trials. Results revealed that whilst socially isolated and non-isolated cichlids struggled to meet the learning criterion, socially isolated fish were significantly less likely to learn the task (3 out of 15 fish) than non-isolated (7 out of 14 fish), thus providing evidence that isolation impairs cognition[605].

Similar to chronic stress, acute stressors can also disrupt memory in zebrafish. For example, Gaikwad et al., 2011, showed that acute stress in the form of an alarm pheromone or exposure to a natural sympatric predator such as an Indian leaf fish (*Nandus nandus*) adversely impacts spatial and cued memory. In detail, in the cued memory task, zebrafish were required to associate a red card placed at different arms of the chamber with a food reward (target arm). In the spatial memory protocol, fish needed to correlate the spatial location of the food reward using external cues of the experimental room. Following 20 days of trials which included habituation to the chamber and the food bait reward, fish were exposed to one of the two stressors (alarm pheromone or predator) for 6 minutes and then tested on cued and spatial memory. Results showed that single exposure to acute inescapable stress markedly reduced the number of correct arm entries and time fish spent in the target arm, suggesting that spatial and cued memory was impaired.

Taking the fact that acute social isolation is a well-reported stressor in adult zebrafish[683,684] with the previous example, it is likely that social isolation, even when acutely administered, is capable of impacting memory like other acutely experienced stressors. A simple approach to test this theory would be to use Gaikwad's experimental design, replacing the chemical stressor with acute social isolation followed up by the social preference assay. Relevant controls would include but not be limited to non-isolated zebrafish, used to establish the effect size of social isolation, and fish socially isolated but not tested in the T-maze, used to probe the extent of which preference behaviour is impacted by exposure to the maze task. Since zebrafish do not exhibit significant conditioning until 3 weeks of age[685] (coinciding with when robust social preference behaviour is also exhibited), the point in time when fish are subjected to the preference assay would need to be postponed. Delaying the time point of social preference testing to when socially isolated fish are 41 dpf would facilitate the required time to train and assess the memory of fish reared in social isolation before testing social preference. With the application of both assays in a sequential manner as described above, it would be possible to explore any linkages between

loneliness (mandated social isolation), preference behaviour and memory, which is not achievable when using only one of the two assays to investigate the impact of social isolation. Furthermore, this new combined approach would further build on the current work presented in this thesis, thus benefitting from tried-and-tested codes used for data extraction and analysis.

Lastly, although not as attractive to study as cued response and spatial memory, habituation to novelty, including new environments, is also a cognitive phenotype in zebrafish. Studies have shown that anxiogenic drugs such as pentylenetetrazole and caffeine negatively affect habituation[403], whilst anxiolytics, such as ethanol, nicotine, buspirone and fluoxetine, have a positive effect[686,687]. Of the mentioned anxiolytics, the most relevant to the work presented in this thesis is the reported effects of buspirone (utilised in **Chapter 4**) and fluoxetine since it too exerts an anxiolytic effect through the serotonin pathway like buspirone. In a study by Costa de Melo et al., 2019, zebrafish were initially treated with either an anxiogenic agent such as caffeine or with one of three depressors: 1% ethanol, unpredictable chronic stress, or social isolation. Following this, fish were post-treated with either hydroethanolic extract (HELAp), buspirone or fluoxetine. Anxiety and depression in fish were assessed using a light-dark or a novel tank diving test, respectively. Thus, the experimental design was set as follows: Light-dark test; naive fish, caffeine, caffeine co-treated with buspirone or HELAp, and novel tank diving test; naive fish, alcohol, alcohol co-treated with fluoxetine or HELAp, light stress co-treated with fluoxetine or HELAp, social isolation, and social isolation co-treated with fluoxetine or HELAp. Results showed that compared to caffeine-dosed fish, zebrafish co-treated with caffeine and buspirone showed a significant reduction in the time spent in the white compartment, latency entering the dark areas of the chamber, and the time spent freezing. Compared to caffeine-dosed fish, zebrafish co-treated with caffeine and buspirone also showed a substantial increase in the number of toggles between the light and dark areas. In the novel tank diving test, the treatment of socially isolated fish with fluoxetine resulted in a significant increase in the time spent at the top of a novel chamber and the number of crossed quadrants compared to socially isolated fish. Socially isolated fish post-treated with fluoxetine also exhibited a considerable reduction in erratic swimming and time spent freezing compared to untreated equivalents. Furthermore, immersion with fluoxetine in the novel tank diving test significantly increased the distance travelled by socially isolated fish, thus restoring locomotive activity. In summary, Costa de Melo demonstrated that the anxiety-like behaviour of fish dosed with caffeine and depressive symptomology of socially isolated fish in response to novel environments were reverted by buspirone and fluoxetine, respectively.

Chapter 3 showed that socially isolated fish exhibited atypical acclimation behaviour compared to socially reared fish, in alignment with Costa de Melo's findings. Specifically, Fi, Pi48, and Pi24 fish across the first fifteen minutes of being introduced to the novel chamber showed a significant decrease in the time spent moving, total distance travelled, and a marked increase in

the time spent freezing compared to socially reared controls. Furthermore, buspirone treatment in **Chapter 4** reduced the time socially isolated Pi48 fish were frozen during the acclimation phase and restored the average VPI score within the first minute of the socialisation phase. Given that habituation to a novel environment is considered a cognitive phenotype, the atypical acclimation behaviour of socially isolated fish may be interpreted as a marker of underlying disturbances in cognitive function, which would also be supported by the different brain activities of Fi and Pi48 fish (see **Chapter 4)**. Therefore, to further assess responses to a novel environment, it would be advantageous to subject the Fi, Pi48 and Pi24 fish (with and without buspirone treatment) to the novel diving test used by Costa de Melo. This line of investigation could provide supporting evidence on the cognitive functional ability of Fi, Pi48 and Pi24 fish obtained when using the T-maze task and the social preference test consecutively.

The prospect of sleep in zebrafish social isolation studies

Although experimental manipulations have been widely applied to studying the control of sleep and wakefulness in animal models, how normal sleep is perturbed by social isolation remains largely unexplored, with only two studies reported to date. Despite the few available studies, early emerging data has already begun to show that animal social isolation is similarly associated with reduced quality and quantity of sleep like in humans. For instance, adult male (C57BL/6J) mice that were continuously socially isolated for five weeks, compared with pair-housed mice, showed a marked reduction in electroencephalography (EEG) delta power in non-rapid eye movement (NREM) sleep during baseline conditions. Furthermore, compared with pair-housed mice, the socially isolated group also showed a blunted homeostatic sleep response to 8 hours of sleep deprivation. Specifically, although socially paired and isolated mice both exhibited significant increases in EEG delta power during the initial 6 hours of NREM sleep following sleep deprivation, this rise was not persistent throughout the dark period in socially isolated mice, indicating a reduction in sleep depth and quality compared with matched pair-housed mice[688]. In drosophila, chronic but not acute social isolation results in sleep loss and increased feeding. Specifically, flies chronically socially isolated for 5 or 7 days, compared with 1 or 3 days, displayed alterations in sleep architecture primarily during the daytime and especially during the initial hours following lights on. Chronically socially isolated flies showed a significant reduction in total daily sleep, daytime sleep and sleep between the start and the 4 hours after lights-on in a light-dark cycle. Furthermore, compared to acutely socially isolated flies, the socially isolated group showed altered expression of metabolic genes (such as Limostatin and Drosulfakinin, involved in insulin suppression and satiety, respectively) and exhibited increased total food consumption[601], an adaptation to provide the energy needed during insufficient sleep[689].

Despite zebrafish being a great organism to investigate the effects of social isolation on preference behaviour and widely regarded as an advantageous model for studying human sleep[690], there is lacking information about the impact of social isolation on the sleep patterns of zebrafish. Current sleep studies in zebrafish focus on the effects of depression and anxiety on sleep and associated treatments[691,692]; however, they fall short of connecting social isolation (a significant risk factor for depression and anxiety in humans[693–695]) and perturbations in sleep.

Chapter 4 of this thesis reported that the increased optic tectum activity in isolated zebrafish aligned with functional resonance imaging research in humans, in which greater activation of the visual cortex was reported in individuals viewing negative social stimuli and that this may be ascribed to hypervigilance. Sleep studies in which hypervigilance may be investigated as a behavioural output, i.e., a reduction in sleep quality and quantity coupled with increased feeding, would provide an alternative method to verify and understand better the impact of social isolation on the perception of visual social stimuli, without the need for invasive techniques. Such experiments could involve monitoring fully and partially socially isolated zebrafish from 4 dpf, when swim bouts are both complex and robust[696,697] since sleep duration is quantified as a continuous period without movement (typically 1 minute)[691,697], leading up to the social preference test at 21 dpf.

An additional area of investigation using the experimental design outlined above would be to address whether perturbations of sleep, caused by social isolation can be used as a predictor of atypical social preference behaviour. To date, there has been little success in identifying behavioural markers helpful in predicting social preference in humans and animals. Although human studies have shown sleep not to be a good predictor of self-reported loneliness, it may still serve as a good predictor when used as a parameter to report on levels of loneliness experienced by socially isolated fish.

Furthermore, it is likely that any changes in sleep due to hypervigilance, such as fragmentation, may also account for differences in preference behaviour in socially isolated zebrafish (as reported in **Chapter 3**). Specifically, changes in sleep may provide insights into personality traits and the social experiences of each fish before testing when considering the following three points: 1) boldness positively correlates to dominance and is not merely a consequence of social dominance[430], 2) acute and chronic social defeat has been associated with avoidance behaviour of conspecifics[62,230], and 3) numerous studies have established linkages between shyness and psychological difficulties, such as a tendency to exhibit fear and wariness in social situations, coupled with hypervigilance [430,698].

Lastly, several studies in social psychology suggest that compromised sleep quality and quantity may be a key factor by which persistent loneliness may be linked to adverse health

conditions in humans[603,699]. Therefore, research on the connection between sleep and loneliness is warranted. Using the zebrafish model, which offers many advantages, such as high-throughput in vivo compound screening to assess drug efficacy and toxicity, it would also be possible to follow up on any findings on loneliness and associated health conditions with potential treatments.

Functional mapping of social circuitry

Several studies, including the work presented in this thesis, have identified some of the critical regions of the brain that are linked to social behaviour. Yet, these studies only begin to touch the surface of the work to be done, with many areas still waiting to be identified and studied. Furthermore, upstream and downstream targets of brain regions crucial for social behaviour, such as the hypothalamus and preoptic area, have not been fully characterised. Investigation into these targets would allow us to develop our understanding beyond brain regions to social circuit connectivity.

Lastly, an area of investigation that is currently lacking in research regarding the social circuitry on which social behaviour is based in the role of non-neuronal cells in the construction and regulation of the circuit. Astrocytes have been identified as having a functional role in some non-social behaviour[700,701]; thus, it is also possible that astrocytes may also have an active role in regulating the social behaviour circuitry networks.

Understanding the functional units in circuits

Specifically, investigating the functional units in circuits brain regions, specific projections, cell types are areas of study shown to be functionally relevant for the social circuitry. A key area open to further future investigation is the composition of these cell types, including the heterogeneous projection patterns of specific cells. Significant improvements have been made in understanding the compositions of cells in the brain areas associated with social behaviour. This development is due to the advancements in single-cell RNA sequencing alongside multiplexed fluorescent in situ hybridization. The brain regions investigated using these improved techniques are the hypothalamus and the amygdala, including their activation states[702–704].

Other techniques that also include the successful growing use of adeno-associated viruses or novel vesicular stomatitis viruses in zebrafish, which facilitate the identification of projection patterns of individual neurons or synapse-specific projections of brain regions, will prove valuable in the advancement of our knowledge in the projection composition of cells that comprise the social circuit[705–708]. With the growing popularity of single-cell approaches to analyse the nervous system, a fundamental question concerning the definition of a cell type has arisen. Several key

components to the characterisation of neuronal subtype, including molecular signature, morphology, and connectivity, has been proposed[704].

The use of in vivo imaging allows the study of activity patterns of animals during their social encounters at a population level. Previously imaging the activity of deep brain areas has been proven difficult in freely behaving animals, but it is now possible with advances in miniature fluorescent microscopy[709]. Rodent social behaviour studies have now begun to use this approach to analyse the activity of circuits during social interaction. They have revealed that social cues and subsequent behavioural responses are represented at the individual neuron and the population level[710,711]. In zebrafish, monitoring whole-brain neural activity in freely swimming larval zebrafish is now possible with advances in volume imaging techniques and 3D tracking[712,713]. These systems make it possible to investigate a wide range of behaviour previously partially or entirely inaccessible to neural imaging. Although further advances are required to move from looking at 7dpf to 21 dpf when social behaviour is established, studies continuing down this line of investigation will significantly contribute to our understanding of the encoding of social behaviours.

Social interaction as a coupled dynamic system

Previously, the process involved in studying the interactions between two or more individuals has proven challenging due to the complexity of the exchanges. These challenges are further complicated by variability in interpretation and how behavioural data is scored across studies across different research groups. These differences can result in incorrect conclusions when studying the same neural circuits causing the observed behaviours. Thus, methodology and equipment capable of minimising the aforementioned issues are much needed to address these common challenges frequently experienced by behavioural neuroscience as a whole[714]. Such approaches were employed in work presented in this thesis, where automated tracking and advanced programming scripts quantify aspects of social behaviours. However, many improvements and features of social interactions are still yet to be explored, i.e., coordinated movement between two or more social organisms.

In humans, coordination or synchronicity of movement underlies many social activities, for example, dancing and even walking and talking with friends. Such synchronised behaviour can be intentional, i.e., when dancing, or unintentional, such as two people sitting side-by-side in rocking chairs[715]. Interestingly, studies have shown that disruption to the coordination of behaviours may serve as important biomarkers of neurodevelopmental disorders, i.e., autism, where the loss of synchronised eye-blinking[716] and contagious yawning[717] are observed. In zebrafish, the coupling motion of one fish to that of another is an essential prerequisite for the coordinated behaviour that predominates in groups of schooling fish[322] making the zebrafish a suitable model to

investigate coupled dynamic systems, which may help better understand the nature of social impairment in autistic patients.

Concluding remarks

In this thesis, several novel and exciting findings have been reported. The approaches outlined in this thesis provide a framework to investigate social behaviour and the underlying social brain circuitry. Furthermore, the results serve as a foundation to address many unanswered questions, such as how are emotional components of social behaviour encoded within the brain? How do social cues generate social reward? Are social and non-social reward stimuli encoded similarly in the brain, given that social interactions are also rewarding in nature? How are social behavioural decisions regulated in the brain? Is it a social brain network, and if so, is it dedicated?

A critical goal of studying social preference behaviour and the impact of developmental social isolation in the zebrafish model system, specifically at the juvenile stage, is to identify mechanisms that can be applied to our understanding of human social interactions and related disorders. Humans are highly social in that we are uniquely advanced in our degree of social communication, including interactions with other species, making our social behaviours remarkably plastic[718,719]. The disruption of typical functional brain circuitry and subsequent social behaviour has been associated with many neurodevelopmental disorders, i.e., autism[720] and schizophrenia[721], and is being actively studied in zebrafish models[722] (for reviews see[723–725]). There is growing evidence in the zebrafish model suggesting that the disruption of social behaviours in neurodevelopmental disorders may occur at various levels, from social preference recognition to auditory perception[726–728]. Therefore, furthering our knowledge of social preference, the fundamental basis of all more complex social behaviours, within zebrafish would not only help us develop effective strategies to combat social isolation and loneliness, significant with the current pandemic, but also contribute to our understanding of the environmental basis of many neurodevelopmental disorders. Results from these investigation lines will significantly improve the quality of lives of millions by effects on mental and physical well-being on a global scale.

Chapter 6: Methods

Animals and Housing

Zebrafish (Danio rerio) of the ABxTüpLF strain of wild-type fish were used for all the experimental procedures listed in this thesis. Paired males and females were allowed to spawn in breeding tanks (Tecniplast), producing clutches sizes of about 200 embryos. The following day, all embryos were surface disinfected and then maintained in Petri dishes containing system water at an approximate density of 50 embryos per 90ml. After reaching 4dpf, fish larvae (controls) were transferred into permanent holding tanks (approximately 30 fish per 3.5-litre system rack tank, Tecniplast). Tanks were connected to a central recirculating filtration system, and the water was maintained at 28 °C. The recirculating design of the system ensured fish were exposed to olfactory cues during development. All fish were entrained on a 14h:10h light-dark cycle with room lights switched on at 0900 hours. Fish were fed twice a day from 4dpf with a dry food diet from Skretting (Gemma, particle size 100-200) and saltwater rotifer (Branchionus Plicatilis) until 10dpf; after which brine shrimp (Artemia salina) is incorporated into the diet. From 15dpf, the diet consisted of dry food (Gemma, particle size 200-300) and brine shrimp.

For social deprivation studies, zebrafish were separated from conspecifics at the point of fertilisation, or 9:30 am hours on predetermined days following schedules selected explicitly to address the question of interest. For all instances where fish were isolated from 0dpf, singularly housed fish were maintained in 30 mL Petri dishes with white electrical tape covering the outer wall surfaces to prevent fish interactions across adjacent plates. After reaching 4dpf, individual fish larvae were transferred into the custom-built isolation chambers (**Figure 3.1**) constructed from matt opaque white acrylic (Moonlight white 1T41 frost, cast Perspex acrylic sheet, thickness 3 mm) with translucent blue lids (Arctic Blue 7T77, cast Perspex acrylic sheet thicknesses: 3 mm with 75% light transmission) for continued social deprivation under the selected rearing condition. In contrast, when isolation commenced beyond 7dpf, fish reared in social groups were randomly selected and individually placed in isolation tanks for the duration required. Where fish were scheduled for socialisation following social deprivation, previously isolated fish raised in identical conditions were grouped at 9:30 am on the day to form new social groups at densities comparable to socially reared controls. It is worth noting that live diets provided to group-housed controls were maintained across all isolates to eliminate the novelty of moving stimuli during rearing.

The local ethical committee (AWERB Bloomsbury Campus UCL) and the United Kingdom Home Office approved all husbandry procedures and experiments. Project Licence: PAE2ECA7E and Personal Licence: 70/7321

Behavioural test for social preference

Apparatus and setup

A custom-built behavioural setup (**Figure 2.1**), assembled and mounted using structural framing (Misumi, Germany) with photonic components (Thorlabs) as described in Dreosti et al., 2005, was used to record juvenile fish behaviour.

Juvenile fish were allowed to swim freely in 5 mm thick laser-cut arenas constructed from white opaque acrylic. The setup design facilitated the recording of six fish simultaneously. Each testing arena consisted of two sizeable conspecific viewing chambers (15 mm square) adjoined by a 6 mm passage (**Figure 2.1**). Glass windows inserted between viewing and conspecific sections (also 15 mm square) separated focal fish from conspecifics, thus, completing the C-shaped multi-partition design of the chamber.

Fish were illuminated with visible white light from below and filmed from above with a high-speed monochrome camera (FLIR, Cat. #Flea®3 FL3-U3-32S2M-CS) fitted with a varifocal lens (Fujinon, Cat. #YV2.8x2.8SAS-A2) and a 700 nm long-pass filter (Hoya, JP, Cat. #R70) to block the passage of all visible light. The transmission of white light (sourced from a laser projector (Microvision, ShowX+, USA)) to the testing arenas required the use of a cold mirror (Edmund Optics, Cat. #64-452) fixed to the breadboard base using Thorlabs components and angled at 45°. Two main methods were employed to obtain light homogeneity in the setup. The first method involved using a 100 *100 mm infrared LED-based diffusive backlight at 880 nm wavelength (Edmund Optics, Cat. #66-844) placed between the cold mirror and projector. The selection of an 880 nm wavelength was made based on the properties of the zebrafish visual system and determined to be outside the detectable spectrum of zebrafish[729–731]. The second method involved using a gel diffuser paper (LEE Filters, 216 White Diffusion) applied on the transparent base of the assay, which created softer lighting by scattering beams and thus prevented the formation of bright spots or dark shadows on the arena. Combining both methods facilitated the fish's accurate behavioural tracking and motion analysis. All experiments were conducted in a light-tight enclosure which shielded experimental fish from external fluctuations in illumination.

Assay procedure

All behavioural experiments were typically carried out between 10 am (after feeding) and 6 pm, when the most active fish were. Three-week-old zebrafish were transferred from housing tanks (system rack tanks or isolation tanks) and placed into either 90 mL Petri dishes at a density of 30 per dish or individually into 30 mL containers- identical to that used in the early stages of isolation, depending on the housing conditions of the fish on the day of experiments. All dishes

were placed in a box and transported to the designated animal room for testing. Once relocated, all fish were given ten minutes of quiescence in the same quiet corner of the room, away from moving shadows and vibrations.

The design of the custom-built assay chambers meant that a total of six fish could be accommodated in the behavioural setup per individual recording session (see **Figure 2.1**). Each behavioural chamber was filled with approximately 5 mL of system water (from the same facility where the fish were reared) collected on the experiment day.

Individual focal fish were positioned into the middle of the C-shaped assay chambers using a 10 mL pipette with the tip shorted to accommodate the size of the fish. Following a thirty-second delay between the placement of the sixth experimental fish into the assay chamber and the initiation of the recording, fish were subject to a fifteen-minute monitored acclimation phase. Previous studies demonstrated that the novelty of a testing arena could be aversive to zebrafish for the first three minutes of exposure[732,733]. Therefore, the fifteen minutes provided was considered sufficient to establish baseline activity that could be dissociated from the effect of novelty. The acclimation phase was swiftly followed by a second fifteen-minute phase where conspecifics (two fish of the same age and similar size) were presented to experimental fish in one of two conspecifics chambers selected pseudo-randomly (**Figure 2.1**).

Immediately after completing the thirty-minute (total) testing period, focal fish were euthanised by ethyl 3-aminobenzoate methanesulfonate overdose and stored in sweetened ice-cold fixative (4% PFA + 4% sucrose). Thus, unless stated otherwise, all fish were used only once. All samples were stored at 4 °C for future analysis.

Tracking system

Focal fish in six individual arenas were simultaneously tracked at 100 frames per second using custom-written workflows in Bonsai (version 2.3.0.), an open-source data stream processing framework[91]. The compressed movies recorded were cropped, background subtracted, and fish were identified by applying a threshold filter. Each focal fish's centroid was found using the “find contours” and “find largest particle” algorithm using Bonsai.

Behaviour analysis

Images were analysed using custom-written computer vision scripts in Python based on OpenCV (https://www.dreo-sci.com/resources/). The test fish's centroid, position, orientation, and per frame motions were identified and stored in a CSV file. All videos were saved with H.264 compression for subsequent offline analysis and are available upon request. The source code can be downloaded at http://www.dreo-sci.com/resources/.

Visual Preference Index (VPI)

The test chamber was horizontally divided by an arbitrary line to give two areas of equal size. The resulting asocial and social sides were more extensive than that described in Dreosti et al. 2015 which further divided the two areas[91]; thus, a more conservative approach to measuring social preference was used in this thesis.

Visual preference index values (VPIs) were calculated by first subtracting the total number of frames the focal fish spends in the testing arena closest to the viewing chamber, occupied by conspecifics (social side), with the total number of frames spent in the opposite side - absent of social cues (asocial side). The resulting number is then divided by the total number of frames in the socialisation period (about 90,000). The formula is:

$$VPI = \frac{\left(Social\ side_{(frames)} - Asocial\ side_{(frames)}\right)}{\left(Social\ side_{(frames)} + Asocial\ side_{(frames)}\right)}$$

Acclimation VPI scores were calculated using the locations of conspecifics during the socialisation phase to determine the social and non-social sides of the chamber. VPI values ranged from -1 to 1, reflecting strong avoidance (asocial) to conspecifics when the value was closer to -1, and strong social preference (prosocial) to conspecifics when nearer to 1. Following this, tested fish, except for fish tested with No Social Cues NSC, were assigned to one of three subpopulations determined by their test phase VPI values: asocial (S-) fish where VPIs below -0.5, no social preference (NSP) fish with -0.5 < VPI <0.5, prosocial (S+) fish with VPI above 0.5.

The VPI of each NSC fish was determined by randomly assigning the social side of the chamber before calculating VPI scores using the formula above.

Temporal VPI

The average difference in binned VPIs between controls and isolated rearing conditions using the following formula:

$$\mu = \sum_{T=1}^{15} \frac{ABS(mean(Control_T)) - ABS(mean(Isolated_T))}{15}$$

where μ is the temporal change in fish responses; T = time in minutes, ABS(mean($Control_T$)) and ABS(mean($Isolated_T$)) are absolute differences in mean responses of control and socially isolated fish at a given time.

Determining magnitudes of asocial and prosocial fish responses

Absolutes of normalised values were used to determine magnitudes of changes. First, the normalisation of measured differences across experimental phases was achieved by subtracting asocial or prosocial behavioural changes during acclimation from socialisation data ($[Socialisation - Acclimation]$). Following this, the average change across phases observed in NSC fish was removed from the resulting values and finally divided by the average change across phases observed in NSC fish, e.g., $[\Delta Asocial - mean(\Delta NSC)/(mean(\Delta NSC)]$.

Subsequently, the absolutes of normalised values were calculated with the following equations:

for **Asocial**:

$$magnitude\ of\ change\ = \left[\frac{ABS(\Delta Asocial) - ABS\big(mean(\Delta NSC)\big)}{ABS\big(mean(\Delta NSC)\big)}\right]$$

for **Prosocial**:

$$magntidute\ of\ change\ = \left[\frac{ABS(\Delta Prosocial) - ABS\big(mean(\Delta NSC)\big)}{ABS\big(mean(\Delta NSC)\big)}\right]$$

Where ABS (Δ Asocial) and ABS (Δ Prosocial) = differences in asocial and prosocial responses across experimental phases ([Socialisation – Acclimation]) and ABS(Δ mean(NSC)) = absolute differences in NSC fish mean responses also across experimental phases.

Average viewing distance

The average viewing distance of experimental fish in the assay chamber was calculated by dividing it into six areas and assigning each with letters A-F reflecting their proximity to the conspecifics, with area A closest to, and area farthest from the conspecifics (**Figure 2.12A**).

When fish positions were in areas A-D, viewing distances were calculated as the shortest distance to the social corner within the chamber (**Figure 2.12A**) and the shortest distance to the midpoint of the dividing glass window that separated the conspecifics. When fish entered areas E or F, viewing distances were calculated by summing the shortest distance to the asocial corner of the chamber with the length to the social corner, followed by the shortest distance to the glass window as previously described.

The above step was repeated across all frames and then averaged by dividing the total number of frames. Finally, the constant 'k' was calculated by dividing the chamber height measured in pixels with the length of the chamber measured in millimetres (42 mm) and applied to the resulting figure above to obtain the average viewing distance in millimetres.

The maximum possible distance from conspecifics in zone 'AB' was governed by the chamber dimensions and determined to be 17.46 mm [$\mathrm{Dist}_{max} = \sqrt{(1.6mm^2 + 0.7mm^2)}$].

Total distance travelled

To calculate the total distance travelled by each fish, the X and Y coordinates were determined across all frames. Changes in X position were calculated as differences in x-coordinates between frames [current frame – previous frame]. The resulting figure was then divided by the chamber width measured in pixels, followed by multiplying by the width of the chamber in millimetres (16 mm). Similarly, changes in Y positions were also calculated by dividing the difference in y-coordinates between two frames with the pixel length of the chamber and the resulting value multiplied by the entire length of the chamber (42 mm). Once figures were generated, trigonometry was applied to calculate distances across frames. This process was repeated for all frames comprising each assay phase, and the total travelled distance was determined as the sum of all lengths across frames.

The determination of total distance covered by fish took into account an estimation of the distance covered when fish could not be tracked by considering it proportional to the distance travelled from the last known position of the fish to when subsequently detected. Furthermore, calculations for total distance travelled excluded changes in lengths of 0.5 mm or less across frames deemed drifting.

Time spent moving

The percentage of time moving was calculated by counting each frame with detectable changes in the fish image relative to the previous frame (i.e., motion) and dividing the resulting figure by the total number of frames.

Time spent freezing

The time test fish spent freezing was defined as the complete cessation of movement (except for gill and eyes)[362,734–736] by fish when in the social side of the assay chamber. Similar to time moving, the percentage of time freezing was calculated by dividing the sum of all frames without motion (freezes > 3 seconds) by the total number of social frames and subsequently expressed as a percentage.

Fish location in the assay chamber

Similarly, the average location of test fish in the assay chamber was determined using the X and Y coordinates of the fish per frame and calculating the mean throughout the fifteen-minute

experimental phase. The resulting X and Y were subsequently cross-referenced to one of the chamber's six zones (**Figure 2.12A**), giving the average zone where each fish resided.

Number of entries into predefined areas

The division of the chamber into six areas (**Figure 2.12A**), as mentioned previously, was utilised to calculate the number of fish entries into various regions of the chamber. Fish positions across frames were determined, and the corresponding locations were stored as a sequence with a length equal to the total number of frames of the recorded testing phase. The series of areas for each fish were then analysed for changes, comparing each new location in the arrangement to the previous. Counts (the number of entries) were assigned to the current location rather than the last in line with the fish's motion, i.e., a transition from area A to B was recorded against area B. This method of counting prevented the duplication of data across areas. The total number of exits validated the total number of entries performed by test fish in predefined chamber areas since; entries = ±1 exist for equivalent regions.

Body orientation of fish

The orientation of each focal fish was inferred by its body position relative to the stimulus. For each test fish, body orientation was calculated using eye and body positions when in zone 'AB' of the assay chamber (see **Figure 2.12A**). Two separate thresholds were utilised to identify the fish's body (comprised of shades of dark grey) and the eye (darkest 10% of pixels). Once identified, centroids for the body and eyes were determined, resulting in two single values for each region (**Figure 2.2H**). The position of these two regions was used to simulate a line which could then be used to calculate the vector angle (body angle) of fish from the vertical line of the dividing glass window (**Figure 2.2I**).

Bout duration and total bouts

The total number of bouts performed by experimental fish were extracted from motion signal data, comprised of the speed and angle of the test fish from all frames. Bout initiation - motion greater than 0.03ms, peak motion, and bout termination -motion lower than 0.01 ms were computed along with the number of instances crossing these thresholds tallied per fish for each experimental phase.

Bout durations were subsequently calculated by dividing the number of bouts by the number of seconds where fish were in motion using the mentioned thresholds.

X and Y motion while viewing conspecifics

X and Y motion of fish were calculated from all frames when fish were in zone 'AB', the area where experimental fish could view conspecifics. Absolute differences in x-coordinates were computed across frames when the above criteria were met, and the resulting figures were summed to produce an X motion value measured in pixels. This X motion was then converted into millimetres via multiplying by the constant 'k' described and used to calculate average viewing distances. Y motion was calculated using the same procedure by utilising the y-coordinates and converted to millimetres.

Statistical analysis

As the distributions for acclimation and versus socialisation phases (absence/ presence of social cues) of the assay were not normally distributed, and the n values varied between testing conditions, the same non-parametric statistical test was applied to all comparisons made within a given rearing condition: a Wilcoxon signed-ranked test of paired samples. Further, the Wilcoxon signed-ranked test was also preferred to compare all repeated measurements, given that the population of animals was identical. However, the application of this test did not extend to evaluating the proportions of animals assigned to asocial (S-), non-social (NSC), and prosocial (S+) subpopulations, where instead a student's-paired T-test was favoured.

A Mann Whitney U test was employed when comparisons were made across rearing conditions or time points, except when comparing the proportions of fish assigned to subpopulations. For this, a Two-Sample Z-test was preferred. All statistical significance was defined as $p \leq 0.05$. Statistical analysis was performed using custom processing routines written in Python using the SciPy stats package (SciPy).

Differences in sociality at two different time points in the day (morning vs afternoon)

Morning (AM) sessions were defined as between 10 am and 12 pm and afternoon (PM) sessions between 4 pm and 6 pm. Of the 380 focal fish (which were subject to the behavioural assay once) as described in **Chapter 2**, 180 juvenile fish were pseudo-randomly selected, meeting the time criteria for testing as above (90 per session).

The VPI distribution and temporal VPI values of fish were compared across conditions for both acclimation and socialisation phases of the assay. Similar comparisons were made across groups for both assay phases to assess whether potential differences in AM and PM VPI values

could be attributed to fatigue which may be observed as a reduction in the time fish spend moving between AM and PM acclimation phases.

Stability of social preference over time on the same fish

Three-week-old juvenile zebrafish were subject to the behavioural assay twice, once in the morning and again in the afternoon. Like the above section, Morning (AM_1st) and Afternoon (PM_2nd) sessions are defined according to time of recording; with the first VPI value obtained between the hours of 10 am and 12 pm and a repeated measurement obtained between 4 pm and 6 pm, thus ensuring a minimum of 4 hours resting period between testing sessions (See: Differences in sociality at two different time points in the day, page 133). 81 fish were subject to the behavioural assay and tested over four separate experimental days. The assay's acclimation and socialisation phases were analysed using a to compare population-level differences in VPI.

To investigate a possible relationship between single and repeated VPI measurements on an individual level, linear regression analysis was used to evaluate "AM_1st" and "PM_2nd" VPI values of singular juvenile fish. Furthermore, differences between morning and afternoon were calculated by subtracting the first recorded VPI value from the repeated VPI measurement (PM_2nd – AM_1st). Numerous permutations were employed to eliminate chance on the observed data to create a series of pseudo-data sets with corresponding absolute means. The combined result of these pseudo means of VPI was compared to the real absolute mean. Permutations were carried out as follows; observed VPI values from 81 fish were uncoupled and grouped according to morning and afternoon sessions and subsequently shuffled, resulting in two independently shuffled datasets with 81 values each. The two shuffled datasets were then recoupled to create a single pseudo-data set per permutation. This process was repeated 10,000 times, with the absolute mean calculated.

Buspirone hydrochloride treatment

Buspirone hydrochloride was kindly supplied by the laboratory of Jason Rihel (University College London). A 100 µM stock solution was prepared by dissolving buspirone hydrochloride (CAS:33386-02, Sigma-Aldrich Cat. #B7148, MW: 421.96) in H_2O (milli-Q water purification system) and subsequently stored at 4 °C. The desired working concentrations of 30 µM or 50 µM of buspirone hydrochloride were prepared by diluting the stock solution with fish water.

Juvenile zebrafish were immersed in 6 mL of either fish water (control fish) or one of the two concentrations of buspirone hydrochloride for ten minutes - based on previous studies reporting behavioural changes in locomotion without effects of sedation (Bencan, Sledge, and Levin 2009;

Gebauer et al. 2011). Before testing, fish were washed three times by transferring fish into new dishes with fresh fish water to eliminate drug transfer into the assay. A total of 118 partially isolated fish (Pi48, separated 48 hours before testing) were treated across both concentrations of buspirone hydrochloride (30 µM; *n* = 48 and 50 µM; *n* = 72) and compared to two controls groups; partially isolated fish (Pi48, *n* = 157) and socially raised fish, controls (C, *n* = 380).

Dissection

In accordance with the HomeOffice regulations, juvenile zebrafish of the appropriate age and size were Schedule 1 killed via ethyl 3-aminobenzoate methanesulfonate overdose (>5% concentration, Sigma). Fish confirmed deceased by the onset of rigour Mortis were immediately transferred into sweetened ice-cold fixative (4% PFA + 4% sucrose) and refrigerated until dissection. The addition of sucrose facilitated aided dissection where the eyes and cranial skin of fixed fish were removed, exposing the full extent of the brain to the rostral spinal cord while the body was left intact. Dissected fish were then dehydrated in increasing methanol concentrations in phosphate buffer saline solution with 0.1% Tween (PBSTw) (25%, 50%, 75%, and 100%), with five minutes allotted per step, and stored at -20 °C overnight. All dissections were performed on in-house prepared Sylgard plates (Sylgard 184, Dow Corning, Cat. #2065622).

cDNA library preparation

Total RNA (tRNA) was extracted from six snap-frozen juvenile zebrafish in 1 mL of Trizol (Invitrogen, Cat. #15596026). The tissue was homogenised with a micro-pestle, and by using a 30-gauge needle, the samples were incubated at room temperature for five minutes. For RNA extraction, 200 µL of chloroform was added, and the samples were incubated at room temperature for three minutes, followed by centrifugation (12,000 g) for fifteen minutes at 4 °C. The aqueous phase was transferred into a clean tube, and a ten-minute incubation precipitated RNA at room temperature with the addition of 500 µL of ice-cold isopropanol to the sample. After a repeated fifteen minutes of centrifugation at 4 °C, the pellet was washed in 75% ethanol, dried, resuspended in RNase free water (100 µL) and stored at -80 °C.

cDNA synthesis was carried out using an Invitrogen Superscript II Reverse transcriptase kit (SS-II RT kit, Thermo Fisher, Cat. #18064-022). 200 ng of random primers (Invitrogen, Cat. #48190011), 10 mm dNTP mix (Promega, Cat. #U1511), and 2.5 ng of total RNA was mixed in MQ water to make up a final volume of 12 µL, incubated at 65 °C for five minutes and chilled on ice for the equivalent time. 4 µL of 5X First-Strand Buffer (SS-II RT kit), 2 µL of 0.1M DTT (SS-II RT kit) and 1 µL RNaseOUT (Invitrogen, Cat. #10777019) were added to the reaction, and the samples

were incubated at 25 °C for two minutes. After adding 1 µL of SuperScript II Reverse transcriptase, cDNA synthesis was carried out in the thermocycler using the following programme: 25 °C for ten minutes, 42 °C for fifty minutes, 70 °C for fifteen minutes and chilled down to 4 °C to end the reaction. RNA was removed from the prepared cDNA using RNSase treatment for 1 hours at 37 °C before subsequent cleaning using DNA purification columns (Qiagen, # Cat. #28104) following the manufactures guidance. The resulting cDNA was stored in -20 °C for future use.

Antisense mRNA probe generation

Plasmids for dopaminergic markers (dopamine transporter (*DAT*), tyrosine hydroxylase 1 (*TH1*), tyrosine hydroxylase 2 (*TH2*) as described in Filippi et al., 2010[508], were kindly supplied by the laboratory of Prof. Wolfgang Driever (University of Freiburg), while the marker for the 5-HT transporter gene (SLC6A4B) as described in Norton et al., 2008[514] was provided by the laboratory of Dr William Norton (University of Leicester).

A polymerase chain reaction was used to construct the c-Fos probe from the plasmid curtesy of Ricardo N. Silva (forward primer: 5'-*CCGATACACTGCAAGCTGAA*-3' and reverse primer: 5'-*ATTGCAGGGCTATGGAAGTG*-3').

Plasmid construction

The sequence for the c-Fos antisense probes was amplified from the cDNA library prepared from juvenile zebrafish. Amplicons were generated by standard PCR reaction, performed in a final volume of 25 µL. Briefly, 1 µL of cDNA was mixed with 0.5 µL of forward and reverse primers (10 µM), 18 µL of PCR mix (made inhouse), 0.1 µL Taq DNA Polymerase (New England BioLabs, Cat. #M0267), 2.5 µL Betaine solution (5M, Sigma-Aldrich, Cat. #B0300-fVL) and 2.5 µL of nuclease-free water. The thermo-cycling profile consisted of a denaturation temperature of 94 °C and an annealing temperature of 58 °C. The elongation period was determined as "1 minute per 1000 base pairs" as per the manufacturers' guidance thus a period of 90 seconds was used. The produced fragments from the reaction above were cloned into the pCRII-TOPO vector (Thermo Fischer Scientific, Cat. #K460001) by mixing equal volumes of salt solution, TOPO vector and fresh PCR product (1 µL) with 3 µL of nuclease-free water following the manufacturer's instructions. Following a final five-minute incubation at room temperature, the plasmid was ready for cloning.

Bacterial transformation

Frozen One Shot chemically competent Escherichia coli cells (Thermo Fisher, Cat. #C404010) were thawed on ice in 50 µL lots. For every 25 µL of competent cells used, 1 µL of resuspended plasmid was directly added. DNA was mixed into cells by gentle tapping. Vials containing DNA and cells were incubated on ice for 15 minutes, heat-shocked in a 42 °C water bath for 30 seconds,

then returned to the ice for 2 minutes. To each vial, 250 µL of room temperature super optimal broth with catabolite repression (SOC medium, Thermo Fisher, Cat. #15544034) was added. Each vial was placed in a 37 °C shaking incubator for at least 30 minutes up to 1 hour at 225 rpm. 150 µL of the 276 µL prepared mix was aseptically spread onto selective agar plates with ampicillin and incubated overnight at 37 °C.

Midiprep

The next day, isolated colonies were selected (deliberately avoiding satellite colonies) with a 100 µL pipette tip. The entire pipette tip was added into a large (approximately five times the volume of contained liquid) flask containing 100 mL of a 1:1000 dilution of selection agent stock (ampicillin, 50 µg/ µL) in sterile Lysogeny broth (20 g/L, Sigma-Aldrich, Cat. #L3022). Flasks were covered and placed into a shaking incubator overnight at 37 °C. The broth was centrifuged at 11,000 rpm for ten minutes at 4 °C and the supernatant decanted. According to the provided instructions, plasmid DNA (pDNA) was purified from the resultant bacterial pellet using a Plasmid Midi Kit (QIAGEN, Cat. #12143). The final pDNA product was resuspended in 100 µL of nuclease-free water, and yield was determined using a spectrophotometer (Thermo Fisher, Cat. #ND-2000) and accompanying NanoDrop software.

Sanger sequencing (Source BioScience) was employed to confirm the construct and its orientation of the insertion, particularly important for generating the c-Fos antisense probe as the plasmid contained two promoters.

Plasmid linearization

A total reaction volume of 100 µL was aseptically prepared, containing 15 µg of pDNA (from midiprep), 10 µL of 10X MULTI-CORE restriction buffer (Cat.R9991, Promega), 50 units of restriction enzyme (Promega), and nuclease-free water as needed. The necessary restriction enzymes were SLC6A4B, ApaI; DAT, XbaI; TH1, XhoI; and TH2, NotI. The reaction was incubated for the prescribed time (depending on DNA concentration and units/ µL of enzyme) at 37 °C.

In vitro transcription

Digoxigenin antisense RNA probes were generated by in *vitro* transcription. A total reaction volume of 11 µL was aseptically prepared with the following constituents: 1µg of linearized template pDNA, 4 µL 5X transcription buffer, 2 µL 0.1M DTT, 2 µL Digoxigenin RNA labelling mix (Sigma, Cat. #11207733910), 1ul RNAse inhibitor, 1 µL RNA polymerase. The resulting mixture was incubated for 2 hours on a heat block (Cat.BLO1314, Grant) set at 37 °C to obtain the maximum yield. T7 RNA polymerase transcribes *SLC6A4B* and *TH2* probes, while T7 and T3 were used for *DAT* and *TH1* probes.

The generated mRNA probes were treated with 1 µL Turbo DNAse (Thermo Fisher, Cat. #AM2238) and purified using RNeasy Mini Kit columns (QIAGEN, Cat. #74104) as per provided instructions before being eluted with 30 µL nuclease-free water. All undiluted mRNA probes were stored at -20 °C until use.

Whole-mount fluorescent in *situ* hybridisation

Samples previously dehydrated and stored at -20 °C were returned to room temperature and rehydrated in decreasing methanol concentrations in PBSTw (100, 75, 50, 25% and two rounds of 100% PBSTw), with five minutes allotted per step. Following rehydration, samples were permeabilised with 0.02mg/ mL proteinase K (PK, Cat.03115887001, Sigma) for twenty minutes at room temperature and then bleached for the equivalent time in bleaching solution (250 µL formamide, 125 µL 20X saline sodium citrate (SSC), 3.6 mL water and 1 mL H_2O_2), also at room temperature. Fish were post-fixed with 4% PFA (w/v) in PBS for twenty minutes to maintain the integrity of samples, washed in PBSTw a total of four times for five minutes each, and incubated for a minimum of three hours at 65 °C (in a QB digital block heater, Grant) in standard hybridisation solution containing 50% formamide. Antisense mRNA probes generated previously were diluted in a hybridisation buffer containing 5% by volume dextran sulphate (Sigma, Cat. #42867) at a 2ng/ µL, and the samples were incubated in the resulting probe solution overnight at 65 °C.

The following day, diluted riboprobes were aseptically rescued and stored at -20 °C for repeated use. Samples were successively washed at 65 °C through a graded series of hybridisation solutions and 2X SSC (100, 75, 50, and 25%) for ten minutes, followed by a thirty-minute wash in 2X SSC and two times thirty-minute washes in 0.2X SSC. At room temperature, samples were further washed with PBSTw for ten minutes before blocking with 10% normal goat serum (NGS, Sigma, Cat. #G6767) in PBStw for 2-3hours with gentle shaking temperature.

Fluorescent staining protocol

DIG-labelled probes were detected by overnight incubation with anti-Digoxigenin-POD Fab fragments (1:500) (Roche, Cat. #11207733910) for Tyramide-based fluorescent in *situ* hybridisation (horseradish peroxidase-conjugated antibody). Concurrent staining with 4',6-diamidino-2-phenylindole (DAPI, 20 mg/mL, Sigma, Cat. #D9564) – a fluorescent nuclear stain that binds to A-T-rich regions in DNA, was achieved by adding 1:500 dilution into the overnight incubation solution of probes.

The following day, samples were washed in PBSTw four times for a minimum of 15 minutes per wash. A commercial Tyramide Signal Amplification (TSA) kit (Perkin Elmer, Inc., Cat. #NEL744001KT) along with Cyanine 3 (Cy3) prepared with 60 µL of dimethyl sulphoxide (DMSO,

Sigma, Cat. #D2650), was used to detect fluorescent expression profiles. All samples were incubated in TSA solution in darkness for one hour, followed by five-minute rinses in PBSTw at room temperature before being left to wash for at least 48 hours, gently shaking, in freshly made PBSTw at 4 °C. Samples were then transferred into increasing concentrations of glycerol diluted in PBSTw (20:80, 40:60, 60:40, 80:20), ensuring with each step that samples sunk to the bottom of the vials before proceeding with the next concentration. Finally, samples were stored in the final solution at four °C for two-photon imaging.

Mounting of samples for imaging by two-photon microscopy

Samples were mounted in 2% low melting-point agarose prepared with 80% glycerol (Thermo Fisher, Cat. #16500500) prepared in PBSTw by placing them within a transparent glass ring. Two glass coverslips were used to seal the ring with silicone grease (RS Components UK, Cat. #494124). As ventral brain regions were of primary interest, whole brains were mounted such that imaging was performed from ventral to dorsal (**Figure 4.1**A).

Imaging and registration

A custom-built two-photon microscope (INSS) was used for image acquisition of whole-brain in *situs*. Both DAPI and Cy3 Images were collected with a 10X objective (Olympus, W Plan-Apochromat 10x/0.5 M27 75 mm) using a 'Chameleon' titanium–sapphire laser tuned to 1030 nm (Coherent Inc, Santa Clara, CA, US) and controlled using custom-written software in LabView. In situ images were registered using ANTs (Advanced Normalisation Tools) version 2.1.0 running on the UCL Legion compute cluster. Images were downsampled to 512*512, and parameters were slightly modified from 404 fixed registration as below:

antsRegistration -d 3 -float 1 -o [Registered_Image_, Registered_Image _warped.nii.gz] -interpolation WelchWindowedSinc -use-histogram-matching 0 -r [reference_Image, Registered_Image,1] -t rigid[0.1] -m MI[reference_Image, Registered_Image _0.nii,1,32,Regular,0.25] -c [1000x500x250x100,1e-8,10] -shrink-factors 12x8x4x2 -s 4x3x2x1 -t Affine[0.1] -m MI[reference_Image, Registered_Image,1,32,Regular,0.25] -c [1000x500x250x100,1e-8,10] -shrink-factors 12x8x4x2 -s 4x3x2x1 -t SyN[0.1,6,0] -m CC[reference_Image, Registered_Image _0.nii,1,2] -c [1000x500x500x250x100,1e-7,10] -shrink-factors 12x8x4x2x1 -s 4x3x2x1x0

antsApplyTransforms -d 3 -v 0 -float -n WelchWindowedSinc -i Registered_Image _1.nii -r reference_Image -o Registered_Image _warped_red.nii.gz -t Registered_Image _1Warp.nii.gz -t Registered_Image _0GenericAffine.mat

Intensity normalisation

The registered image stacks were normalised to adjust for intensity variations between imaging sessions caused by various sources (staining efficiency, laser power fluctuations, light detector sensitivity, etc.). Normalisation was accomplished by computing an intensity histogram for each fish brain's volume (with 10000 discrete intensity bins spanning the range −4000.0 to 70000.0) for all 512*512*273 voxels. The minimum value bin (with at least 100 voxels) was used as the bias offset and subtracted from all voxel values. The mode value, minus the bias, provided a robust estimate of the background/baseline fluorescence and was thus used to normalise voxel values for the entire volume. Therefore, after normalisation, an intensity value of 1 reflected the background level while two indicated fluorescence level that is twice the background, and so on. Histogram normalisation was performed for each fish's brain volume before any region or voxel-based analysis.

Figure 4.1B and 4.2A: Reconstruction of cross-section images were obtained by using Fiji 'Volume viewer' plugin. Schematics of cross- and horizontal-section were obtained by using the 'Neuroanatomy of the zebrafish brain'.

Figure 4.1D: Percentages of c-Fos activation were calculated for each of the six areas highlighted in Figure 4.1B and 4.2A, using custom-written Python functions, as stated below. A 3D mask for each area was generated by using the 'Segmentation Editor' plugin Fiji (*https://imagej.net/Segmentation_Editor*). C-Fos percentage values for each condition (C (S+), C (S-), Fi (S-), Pi48 (S-)) were obtained by subtracting and then dividing each c-Fos average value of the mask by the basal c-Fos average value calculated in controls fish No Social Cu1.

References

1. Elsabbagh, M. *et al.* Global Prevalence of Autism and Other Pervasive Developmental Disorders. *Autism Research* **5**, 160–179 (2012).

2. Charlson, F. J. *et al.* Global epidemiology and burden of schizophrenia: Findings from the global burden of disease study 2016. *Schizophr Bull* **44**, 1195–1203 (2018).

3. Rubenstein, D. R. & Abbot, P. *Comparative social evolution*. *Comparative Social Evolution* (2017). doi:10.1017/9781107338319.

4. Wilson, E. O. Some central problems of sociobiology. *Social Science Information* **14**, 5–18 (1975).

5. Lösel, F. *International Encyclopedia of the Social & Behavioral Sciences*. *International Encyclopedia of the Social & Behavioral Sciences* (Elsevier, 2015).

6. Faris, E. The Primary Group: Essence and Accident. *American Journal of Sociology* **38**, 41–50 (1932).

7. Riehl, C. Evolutionary routes to non-kin cooperative breeding in birds. *Proceedings of the Royal Society B: Biological Sciences* vol. 280 Preprint at https://doi.org/10.1098/rspb.2013.2245 (2013).

8. Walters, J. R., Doerr, P. D. & Carter, J. H. Delayed dispersal and reproduction as a life-history tactic in cooperative breeders: fitness calculations from red-cockaded woodpeckers. *American Naturalist* **139**, 623–643 (1992).

9. Taylor, L. A., Wittemyer, G., Lambert, B., Douglas-Hamilton, I. & Vollrath, F. Movement behaviour after birth demonstrates precocial abilities of African savannah elephant, Loxodonta africana, calves. *Anim Behav* **187**, 331–353 (2022).

10. Rubenstein, D. I. & Rubenstein, D. R. Social Behavior. in *Encyclopedia of Biodiversity* 571–579 (Elsevier, 2013). doi:10.1016/B978-0-12-384719-5.00126-X.

11. Turillazzi, S. & Francescato, E. Patrolling behaviour and related secretory structures in the males of some Stenogastrine wasps (Hymenoptera, Vespidae). *Insectes Soc* **37**, 146–157 (1990).

12. Beam, L. & Turillazzi, S. Aerial patrolling and the stripes-display in males of Parischnogaster mellyi (Hymenoptera Stenogastrinae). *Ethol Ecol Evol* **6**, 43–46 (1994).

13. Chen, P. & Hong, W. Neural Circuit Mechanisms of Social Behavior. *Neuron* vol. 98 16–30 Preprint at https://doi.org/10.1016/j.neuron.2018.02.026 (2018).

14. Gammie, S. C. Stress and Social Behavior. *Encyclopedia of Behavioral Neuroscience* 334–341 (2010) doi:10.1016/B978-0-08-045396-5.00237-2.

15. Martin, E. & Hine, R. Oxford Dictionary of Biology (Oxford Paperback Reference). *A Dictionary of Biology* (2008).

16. Noë, R. Cooperation experiments: coordination through communication versus acting apart together. *Anim Behav* **71**, 1–18 (2006).

17. Sachs, J. L., Mueller, U. G., Wilcox, T. P. & Bull, J. J. The evolution of cooperation. *Q Rev Biol* **79**, 135–160 (2004).

18. Gardner, A., Griffin, A. S. & West, S. A. Theory of Cooperation. *eLS* (2009) doi:10.1002/9780470015902.A0021910.

19. Bshary, R., Zuberbühler, K. & van Schaik, C. P. Why mutual helping in most natural systems is neither conflict-free nor based on maximal conflict. *Philosophical Transactions of the Royal Society B: Biological Sciences* **371**, 20150091 (2016).

20. Brosnan, S. F., Salwiczek, L. & Bshary, R. The interplay of cognition and cooperation. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**, 2699–2710 (2010).

21. Dale, R., Marshall-Pescini, S. & Range, F. What matters for cooperation? The importance of social relationship over cognition. *Sci Rep* **10**, 11778 (2020).

22. Oliveira, R. F. & Bshary, R. Expanding the concept of social behavior to interspecific interactions. *Ethology* **127**, 758–773 (2021).

23. Schlupp, I. The Evolutionary Ecology of Gynogenesis. *Annu Rev Ecol Evol Syst* **36**, 399–417 (2005).

24. Soares, M. C. The Neurobiology of Mutualistic Behavior: The Cleanerfish Swims into the Spotlight. *Front Behav Neurosci* **11**, (2017).

25. Grutter, A. S. Relationship between cleaning rates and ectoparasite loads in coral reef fishes. *Mar Ecol Prog Ser* **118**, 51–58 (1995).

26. Isabelle M. Cote. Evolution and ecology of cleaning symbioses in the sea. in *Oceanography and marine biology: an annual review.* vol. 38 311–355 (Taylor Francis, London (UK), 2000).

27. Bronstein, J. L. Our Current Understanding of Mutualism. *Q Rev Biol* **69**, 31–51 (1994).

28. de MAZANCOURT, C., LOREAU, M. & DIECKMANN, U. Understanding mutualism when there is adaptation to the partner. *Journal of Ecology* **93**, 305–314 (2005).

29. Krebs, C. J. *Ecology: the experimental analysis of distribution and abundance*. (Harper & Row, 1972).

30. Bender, E. A., Case, T. J. & Gilpin, M. E. Perturbation Experiments in Community Ecology: Theory and Practice. *Ecology* **65**, 1–13 (1984).

31. Schoener, T. W. Field Experiments on Interspecific Competition. *Am Nat* **122**, 240–285 (1983).

32. Faeth, S. H. & Sullivan, T. J. Mutualistic Asexual Endophytes in a Native Grass Are Usually Parasitic. *Am Nat* **161**, 310–325 (2003).

33. Bruno, J. F., Stachowicz, J. J. & Bertness, M. D. Inclusion of facilitation into ecological theory. *Trends Ecol Evol* **18**, 119–125 (2003).

34. Wright, A. J., Wardle, D. A., Callaway, R. & Gaxiola, A. The Overlooked Role of Facilitation in Biodiversity Experiments. *Trends Ecol Evol* **32**, 383–390 (2017).

35. Fontaine, C. *et al.* The ecological and evolutionary implications of merging different types of networks. *Ecol Lett* **14**, 1170–1181 (2011).

36. Ollerton, J. Pollinator Diversity: Distribution, Ecological Function, and Conservation. *Annu Rev Ecol Evol Syst* **48**, 353–376 (2017).

37. Wilkinson, G. S. Food sharing in vampire bats. *Sci Am* **262**, 76–82 (1990).

38. Carter, G. G. & Wilkinson, G. S. Food sharing in vampire bats: reciprocal help predicts donations more than relatedness or harassment. *Proc Biol Sci* **280**, (2013).

39. Shim, E., Chapman, G. B., Townsend, J. P. & Galvani, A. P. The influence of altruism on influenza vaccination decisions. *J R Soc Interface* **9**, 2234 (2012).

40. Post, S. G. Altuism, happiness, and health: it's good to be good. *Int J Behav Med* **12**, 66–77 (2005).

41. Summers, K., Weigt, L., Boag, P. & Herpetologica, E. B. The evolution of female parental care in poison frogs of the genus Dendrobates: evidence from mitochondrial DNA sequences. *Herpetologica* **55**, 254–270 (1999).

42. Lynn, S. E. Endocrine and neuroendocrine regulation of fathering behavior in birds. *Horm Behav* **77**, 237–248 (2016).

43. Dulac, C., O'Connell, L. A. & Wu, Z. Neural control of maternal and paternal behaviors. *Science (1979)* **345**, 765–770 (2014).

44. Fernandez-Duque, E., Valeggia, C. R. & Mendoza, S. P. The Biology of Paternal Care in Human and Nonhuman Primates. *http://dx.doi.org/10.1146/annurev-anthro-091908-164334* **38**, 115–130 (2009).

45. Hrdy, S. B. Variable postpartum responsiveness among humans and other primates with 'cooperative breeding': A comparative and evolutionary perspective. *Horm Behav* **77**, 272–283 (2016).

46. Ainsworth, M. S. & Bowlby, J. An ethological approach to personality development. *American Psychologist* **46**, 333–341 (1991).

47. Trickett, P. K. & McBride-Chang, C. The Developmental Impact of Different Forms of Child Abuse and Neglect. *Developmental Review* **15**, 311–337 (1995).

48. Ochi, M. & Fujiwara, T. Association Between Parental Social Interaction and Behavior Problems in Offspring: a Population-Based Study in Japan. *Int J Behav Med* **23**, 447–457 (2016).

49. Miranda, M., Morici, J. F., Zanoni, M. B. & Bekinschtein, P. Brain-Derived Neurotrophic Factor: A Key Molecule for Memory in the Healthy and the Pathological Brain. *Front Cell Neurosci* **13**, 363 (2019).

50. Lu, B., Nagappan, G. & Lu, Y. BDNF and synaptic plasticity, cognitive function, and dysfunction. *Handb Exp Pharmacol* **220**, 223–250 (2014).

51. Franchini, L. *et al.* Linking NMDA Receptor Synaptic Retention to Synaptic Plasticity and Cognition. *iScience* **19**, 927 (2019).

52. Curley, J. P. & Champagne, F. A. Influence of Maternal Care on the Developing Brain: Mechanisms, Temporal Dynamics and Sensitive Periods. *Front Neuroendocrinol* **40**, 52 (2016).

53. Bredy, T. W., Humpartzoomian, R. A., Cain, D. P. & Meaney, M. J. Partial reversal of the effect of maternal care on cognitive function through environmental enrichment. *Neuroscience* **118**, 571–576 (2003).

54. Liu, D., Diorio, J., Day, J. C., Francis, D. D. & Meaney, M. J. Maternal care, hippocampal synaptogenesis and cognitive development in rats. *Nature Neuroscience 2000 3:8* **3**, 799–806 (2000).

55. Unternaehrer, E. *et al.* Childhood maternal care is associated with DNA methylation of the genes for brain-derived neurotrophic factor (BDNF) and oxytocin receptor (OXTR) in peripheral blood cells in adult men and women. *Stress* **18**, 451–461 (2015).

56. Meaney, M. J. Maternal Care, Gene Expression, and the Transmission of Individual Differences in Stress Reactivity Across Generations. *http://dx.doi.org/10.1146/annurev.neuro.24.1.1161* **24**, 1161–1192 (2003).

57. Garcia, M., Charlton, B. D., Wyman, M. T., Fitch, W. T. & Reby, D. Do red deer stags (Cervus elaphus) use roar fundamental frequency (F0) to assess rivals? *PLoS One* **8**, e83946 (2013).

58. Freeman, L. C., Freeman, S. C. & Romney, A. K. The implications of social structure for dominance hierarchies in red deer, Cervus elaphus L. *Anim Behav* **44**, 239–245 (1992).

59. Lazaro-Perea, C., de Arruda, M. F. & Snowdon, C. T. Grooming as a reward? Social function of grooming between females in cooperatively breeding marmosets. *Anim Behav* **67**, 627–636 (2004).

60. Golden, S. A., Covington, H. E., Berton, O. & Russo, S. J. A standardized protocol for repeated social defeat stress in mice. *Nat Protoc* **6**, 1183–91 (2011).

61. Butler, M. C. & Gettinger, M. Learned Aggression in Humans. in *Encyclopedia of the Sciences of Learning* 1737–1740 (Springer US, 2012). doi:10.1007/978-1-4419-1428-6_547.

62. Ayash, S., Schmitt, U. & Müller, M. B. Chronic social defeat-induced social avoidance as a proxy of stress resilience in mice involves conditioned learning. *J Psychiatr Res* **120**, 64–71 (2020).

63. West, S. A., Griffin, A. S. & Gardner, A. Social semantics: altruism, cooperation, mutualism, strong reciprocity and group selection. *J Evol Biol* **20**, 415–432 (2007).

64. Bourke, A. F. G. Hamilton's rule and the causes of social evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences* **369**, (2014).

65. van Veelen, M. Can hamilton's rule be violated? *Elife* **7**, (2018).

66. Hamilton, W. D. The genetical evolution of social behaviour. I. *J Theor Biol* **7**, 1–16 (1964).

67. Alexander, R. D. & Tinkle, D. W. Natural selection and social behavior : recent research and new theory. 532 (1981).

68. Scantlebury, M., Clutton-Brock, T. H. & Speakman, J. R. Energetics of cooperative breeding in meerkats Suricata Suricatta. *Int Congr Ser* **1275**, 367–374 (2004).

69. Clutton-Brock, T. H. *et al.* Individual contributions to babysitting in a cooperative mongoose, Suricata suricatta. *Proceedings of the Royal Society B: Biological Sciences* **267**, 301 (2000).

70. Skinner, B. F. (Burrhus F. *Science and human behavior*. (Macmillan, 1953).

71. Sidowski, J. B., Wyckoff, L. B. & Tabory, L. The influence of reinforcement and punishment in a minimal social situation. *J Abnorm Psychol* **52**, 115–119 (1956).

72. Sidowski, J. B. Reward and punishment in a minimal social situation. *J Exp Psychol* **54**, 318–326 (1957).

73. Kelley, H. H., Thibaut, J. W., Radloff, R. & Mundy, D. The development of cooperation in the 'minimal social situation'. *Psychological Monographs: General and Applied* **76**, 1–19 (1962).

74. Hake, D. F. & Vukelich, R. A classification and review of cooperation procedures. *J Exp Anal Behav* **18**, 333 (1972).

75. Fehr, E., Fischbacher, U. & Gächter, S. Strong reciprocity, human cooperation, and the enforcement of social norms. *Human Nature 2002 13:1* **13**, 1–25 (2002).

76. el Mouden, C., West, S. A. & Gardner, A. The enforcement of cooperation by policing. *Evolution* **64**, 2139–2152 (2010).

77. Guala, F. Reciprocity: Weak or strong? What punishment experiments do (and do not) demonstrate. *Behavioral and Brain Sciences* **35**, 1–15 (2012).

78. Hamilton, W. D. Selfish and Spiteful Behaviour in an Evolutionary Model. *Nature 1970 228:5277* **228**, 1218–1220 (1970).

79. Hamilton, W. D. The genetical evolution of social behaviour. I. *J Theor Biol* **7**, 1–16 (1964).

80. Hamilton, W. Innate social aptitudes of man: an approach from evolutionary genetics. (1975).

81. Eberhard, M. J. W. The Evolution of Social Behavior by Kin Selection. *Q Rev Biol* **50**, 1–33 (1975).

82. Tinbergen, N. On the Analysis of Social Organization Among Vertebrates, with Special Reference to Birds. *American Midland Naturalist* **21**, 210 (1939).

83. Rodrigues, A. M. M. Viscous Population. *Encyclopedia of Evolutionary Psychological Science* 8419–8424 (2021) doi:10.1007/978-3-319-19650-3_3092.

84. Smith, J. M. Group Selection and Kin Selection. *Nature 1964 201:4924* **201**, 1145–1147 (1964).

85. Dawkins, R. & Davis, N. *The selfish gene*. (Madcat Library, 2017).

86. Jansen, V. A. A. & van Baalen, M. Altruism through beard chromodynamics. *Nature* **440**, 663–666 (2006).

87. Gardner, A. The greenbeard effect. *Current Biology* **29**, R430–R431 (2019).

88. Wei, D., Talwar, V. & Lin, D. Neural circuits of social behaviors: Innate yet flexible. *Neuron* **109**, 1600–1620 (2021).

89. Johnston, R. E. Chemical Communication in Rodents: from Pheromones to Individual Recognition. *J Mammal* **84**, 1141–1162 (2003).

90. Rymer, T. L. The Role of Olfactory Genes in the Expression of Rodent Paternal Care Behavior. *Genes (Basel)* **11**, (2020).

91. Dreosti, E., Lopes, G., Kampff, A. R. & Wilson, S. W. Development of social behavior in young zebrafish. *Front Neural Circuits* **9**, 39 (2015).

92. Aubin, T., Jouventin, P. & Hildebrand, C. Penguins Use the Two-Voice System to Recognize Each Other. *Proc Biol Sci* **267**, 1081–1087 (2000).

93. Swaney, W. T., Curley, J. P., Champagne, F. A. & Keverne, E. B. The paternally expressed gene Peg3 regulates sexual experience-dependent preferences for estrous odors. *Behavioral neuroscience* **122**, 963–973 (2008).

94. Brennan, P. A. & Keverne, E. B. Something in the air? New insights into mammalian pheromones. *Curr Biol* **14**, (2004).

95. Lai, S. C., Vasilieva, N. Y. & Johnston, R. E. Odors providing sexual information in Djungarian hamsters: evidence for an across-odor code. *Horm Behav* **30**, 26–36 (1996).

96. Payne, A. P. The attractiveness of Harderian gland smears to sexually naive and experienced male golden hamsters. *Anim Behav* **27**, 897–904 (1979).

97. Kurnikova, A., Moore, J. D., Liao, S.-M., Deschênes, M. & Kleinfeld, D. Coordination of Orofacial Motor Actions into Exploratory Behavior by Rat. *Current Biology* **27**, 688–696 (2017).

98. Spinello, C., Yang, Y., Macrì, S. & Porfiri, M. Zebrafish adjust their behavior in response to an interactive robotic predator. *Frontiers Robotics AI* **6**, 38 (2019).

99. Renee L. Rosier & Tracy Langkilde. Behavior Under Risk: How Animals Avoid Becoming Dinner. *Nature Education Knowledge* **2**, 8 (2011).

100. Oliveira, T. A. *et al.* Stress responses to conspecific visual cues of predation risk in zebrafish. *PeerJ* **2017**, (2017).

101. Mulholland, M. M. *et al.* Are conspecific social videos rewarding to chimpanzees (Pan troglodytes)? A test of the social motivation theory. *PLoS One* **16**, (2021).

102. Fleming, A. S., Korsmit, M. & Deller, M. Rat pups are potent reinforcers to the maternal animal: Effects of experience, parity, hormones, and dopamine function. *Psychobiology* **22**, 44–53 (1994).

103. Trezza, V., Campolongo, P. & Vanderschuren, L. J. M. J. Evaluating the rewarding nature of social interactions in laboratory animals. *Dev Cogn Neurosci* **1**, 444–458 (2011).

104. Thompson, B., Leonard, K. C. & Brudzynski, S. M. Amphetamine-induced 50 kHz calls from rat nucleus accumbens: a quantitative mapping study and acoustic analysis. *Behavioural brain research* **168**, 64–73 (2006).

105. Burgdorf, J., Panksepp, J., Brudzynski, S. M., Kroes, R. & Moskal, J. R. Breeding for 50-kHz positive affective vocalization in rats. *Behav Genet* **35**, 67–72 (2005).

106. Palagi, E. *et al.* Rough-and-tumble play as a window on animal communication. *Biol Rev Camb Philos Soc* **91**, 311–327 (2016).

107. Knutson, B., Burgdorf, J. & Panksepp, J. Ultrasonic vocalizations as indices of affective states in rats. *Psychol Bull* **128**, 961–977 (2002).

108. Wöhr, M. & Schwarting, R. K. W. Affective communication in rodents: ultrasonic vocalizations as a tool for research on emotion and motivation. *Cell Tissue Res* **354**, 81–97 (2013).

109. Atkinson, M. A., Simpson, A. A. & Cole, G. G. Visual attention and action: How cueing, direct mapping, and social interactions drive orienting. *Psychonomic Bulletin and Review* vol. 25 1585–1605 Preprint at https://doi.org/10.3758/s13423-017-1354-0 (2018).

110. Hinz, R. C. & de Polavieja, G. G. Ontogeny of collective behavior reveals a simple attraction rule. *Proc Natl Acad Sci U S A* **114**, 2295–2300 (2017).

111. Stednitz, S. J. *et al.* Forebrain Control of Behaviorally Driven Social Orienting in Zebrafish. *Current Biology* **28**, 2445-2451.e3 (2018).

112. Pita, D. & Fernández-Juricic, E. Zebrafish Neighbor Distance Changes Relative to Conspecific Size, Position in the Water Column, and the Horizon: A Video-Playback Experiment. *Front Ecol Evol* **8**, 499 (2021).

113. Tunbak, H., Vazquez-Prada, M., Ryan, T., Kampff, A. R. & Dreosti, E. Whole-brain mapping of socially isolated zebrafish reveals that lonely fish are not loners. *Elife* **9**, (2020).

114. Kepecs, A., Uchida, N. & Mainen, Z. F. The Sniff as a Unit of Olfactory Processing. *Chem Senses* **31**, 167–179 (2006).

115. Charpentier, M. J. E., Crawford, J. C., Boulet, M. & Drea, C. M. Message 'scent': lemurs detect the genetic relatedness and quality of conspecifics via olfactory cues. *Anim Behav* **80**, 101–108 (2010).

116. Laska, M., Bautista, R. M. R., Höfelmann, D., Sterlemann, V. & Salazar, L. T. H. Olfactory sensitivity for putrefaction-associated thiols and indols in three species of non-human primate. *Journal of Experimental Biology* **210**, 4169–4178 (2007).

117. Jänig, S., Weiß, B. M. & Widdig, A. Comparing the sniffing behavior of great apes. *Am J Primatol* **80**, e22872 (2018).

118. Liberles, S. D. Mammalian pheromones. *Annu Rev Physiol* **76**, 151–75 (2014).

119. Wesson, D. W. Sniffing Behavior Communicates Social Hierarchy. *Current Biology* **23**, 575–580 (2013).

120. Abril-De-Abreu, R., Cruz, J. & Oliveira, R. F. Social Eavesdropping in Zebrafish: Tuning of Attention to Social Interactions. *Sci Rep* **5**, 12678 (2015).

121. Kim, D. G. *et al.* Social Interaction Test in Home Cage as a Novel and Ethological Measure of Social Behavior in Mice. *Exp Neurobiol* **28**, 247–260 (2019).

122. Strasser, S. & Dixon, A. K. Effects of visual and acoustic deprivation on agonistic behaviour of the albino mouse (M. musculus L.). *Physiol Behav* **36**, 773–778 (1986).

123. Smotherman, W. P., Bell, R. W., Starzec, J., Elias, J. & Zachman, T. A. Maternal responses to infant vocalizations and olfactory cues in rats and mice. *Behav Biol* **12**, 55–66 (1974).

124. Ryan, B. C., Young, N. B., Moy, S. S. & Crawley, J. N. Olfactory cues are sufficient to elicit social approach behaviors but not social transmission of food preference in C57BL/6J mice. *Behavioural brain research* **193**, 235–242 (2008).

125. Spehr, M. *et al.* Signaling in the Chemosensory Systems. *Cellular and Molecular Life Sciences CMLS 2006 63:13* **63**, 1476–1484 (2006).

126. Feldman, J. L. & Kam, K. Facing the challenge of mammalian neural microcircuits: taking a few breaths may help. *J Physiol* **593**, 3–23 (2015).

127. Nummela, S. & Thewissen J.G.M. *Sensory Evolution on the Threshold: Adaptations in Secondarily Aquatic Vertebrates*. *Sensory evolution on the threshold: adaptations in secondarily aquatic vertebrates* (University of California Press, 2008).

128. Hinz, C. *et al.* Olfactory imprinting is triggered by MHC peptide ligands. *Sci Rep* **3**, 2800 (2013).

129. Gerlai, R. Zebrafish antipredatory responses: A future for translational research? *Behavioural Brain Research* **207**, 223–231 (2010).

130. Speedie, N. & Gerlai, R. Alarm substance induced behavioral responses in zebrafish (Danio rerio). *Behavioural Brain Research* **188**, 168–177 (2008).

131. Pfeiffer, W. The Distribution of Fright Reaction and Alarm Substance Cells in Fishes. *Copeia* 653 (1977) doi:10.2307/1443164.

132. Pfeiffer, W. Die Schreckreaktion der Fische und Kaulquappen. *Naturwissenschaften* **53**, 565–570 (1966).

133. Waldman, B. Quantitative and Developmental Analyses of the Alarm Reaction in the Zebra Danio, Brachydanio rerio. *Copeia* **1982**, 1 (1982).

134. Korsching, S. Aquatic Olfaction. in *Chemosensory Transduction* 81–100 (Elsevier, 2016). doi:10.1016/B978-0-12-801694-7.00005-6.

135. Ahuja, G. *et al.* Zebrafish crypt neurons project to a single, identified mediodorsal glomerulus. *Scientific Reports 2013 3:1* **3**, 1–9 (2013).

136. Mueller, T., Dong, Z., Berberoglu, M. A. & Guo, S. The dorsal pallium in zebrafish, Danio rerio (Cyprinidae, Teleostei). *Brain Res* **1381**, 95–105 (2011).

137. Geng, Y. & Peterson, R. T. The zebrafish subcortical social brain as a model for studying social behavior disorders. *DMM Disease Models and Mechanisms* **12**, (2019).

138. Kermen, F., Franco, L. M., Wyatt, C. & Yaksi, E. Neural circuits mediating olfactory-driven behavior in fish. *Front Neural Circuits* **7**, (2013).

139. deCarvalho, T. N., Akitake, C. M., Thisse, C., Thisse, B. & Halpern, M. E. Aversive cues fail to activate fos expression in the asymmetric olfactory-habenula pathway of zebrafish. *Front Neural Circuits* **7**, 98 (2013).

140. Korsching, S. I. Taste and Smell in Zebrafish. in *The Senses: A Comprehensive Reference* 466–492 (Elsevier, 2020). doi:10.1016/B978-0-12-809324-5.24155-2.

141. Gerlai, R. Animated images in the analysis of zebrafish behavior. *Curr Zool* **63**, 35–44 (2017).

142. Gerlai, R. Social behavior of zebrafish: From synthetic images to biological mechanisms of shoaling. *J Neurosci Methods* **234**, 59–65 (2014).

143. Kappel, J. M. *et al.* Visual recognition of social signals by a tectothalamic neural circuit. *Nature* **608**, 146–152 (2022).

144. MacLean, E. L. *et al.* The evolution of self-control. *Proceedings of the National Academy of Sciences* **111**, E2140–E2148 (2014).

145. Santacà, M., Busatta, M., Lucon-Xiccato, T. & Bisazza, A. Sensory differences mediate species variation in detour task performance. *Anim Behav* **155**, 153–162 (2019).

146. Santacà, M., Dadda, M. & Bisazza, A. The role of visual and olfactory cues in social decisions of guppies and zebrafish. *Anim Behav* **180**, 209–217 (2021).

147. Blaiss, C. A. & Janak, P. H. The nucleus accumbens core and shell are critical for the expression, but not the consolidation, of Pavlovian conditioned approach. *Behavioural Brain Research* **200**, 22–32 (2009).

148. Floresco, S. B. The Nucleus Accumbens: An Interface Between Cognition, Emotion, and Action. *Annu Rev Psychol* **66**, 25–52 (2015).

149. Floresco, S. B. The Nucleus Accumbens: An Interface Between Cognition, Emotion, and Action. *Annu Rev Psychol* **66**, 25–52 (2015).

150. Rogers-Carter, M. M., Djerdjaj, A., Gribbons, K. B., Varela, J. A. & Christianson, J. P. Insular Cortex Projections to Nucleus Accumbens Core Mediate Social Approach to Stressed Juvenile Rats. *The Journal of Neuroscience* **39**, 8717–8729 (2019).

151. Williams, A. v. *et al.* Social approach and social vigilance are differentially regulated by oxytocin receptors in the nucleus accumbens. *Neuropsychopharmacology* **45**, 1423–1430 (2020).

152. Yu, C. J., Zhang, S. W. & Tai, F. D. Effects of nucleus accumbens oxytocin and its antagonist on social approach behavior. *Behavioural Pharmacology* **27**, 672–680 (2016).

153. Walsh, J. J. *et al.* 5-HT release in nucleus accumbens rescues social deficits in mouse autism model. *Nature* **560**, 589 (2018).

154. Scribner, J. L. *et al.* A neuronal signature for monogamous reunion. *Proceedings of the National Academy of Sciences* **117**, 11076–11084 (2020).

155. Tye, K. M. *et al.* Dopamine neurons modulate neural encoding and expression of depression-related behaviour. *Nature* **493**, 537–541 (2013).

156. Mirenowicz, J. & Schultz, W. Preferential activation of midbrain dopamine neurons by appetitive rather than aversive stimuli. *Nature* **379**, 449–451 (1996).

157. Kalivas, P. W. & Nakamura, M. Neural systems for behavioral activation and reward. *Curr Opin Neurobiol* **9**, 223–227 (1999).

158. Chaudhury, D. *et al.* Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons. *Nature* **493**, 532–536 (2013).

159. Budygin, E. A. *et al.* Aversive stimulus differentially triggers subsecond dopamine release in reward regions. *Neuroscience* **201**, 331–337 (2012).

160. Gunaydin, L. A. *et al.* Natural Neural Projection Dynamics Underlying Social Behavior. *Cell* **157**, 1535–1551 (2014).

161. Brischoux, F., Chakraborty, S., Brierley, D. I. & Ungless, M. A. Phasic excitation of dopamine neurons in ventral VTA by noxious stimuli. *Proc Natl Acad Sci U S A* **106**, 4894–4899 (2009).

162. Li, Z. *et al.* Cell-Type-Specific Afferent Innervation of the Nucleus Accumbens Core and Shell. *Front Neuroanat* **12**, (2018).

163. Bekkers, J. M. & Suzuki, N. Neurons and circuits for odor processing in the piriform cortex. *Trends Neurosci* **36**, 429–438 (2013).

164. Yadon, C. A. & Wilson, D. A. The role of metabotropic glutamate receptors and cortical adaptation in habituation of odor-guided behavior. *Learning & Memory* **12**, 601–605 (2005).

165. Parsana, A. J., Li, N. & Brown, T. H. Positive and Negative Ultrasonic Social Signals Elicit Opposing Firing Patterns in Rat Amygdala. *Behavioural brain research* **226**, 77 (2012).

166. Willuhn, I. *et al.* Phasic Dopamine Release in the Nucleus Accumbens in Response to Pro-Social 50 kHz Ultrasonic Vocalizations in Rats. *Journal of Neuroscience* **34**, 10616–10623 (2014).

167. Sadananda, M., Wöhr, M. & Schwarting, R. K. W. Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain. *Neurosci Lett* **435**, 17–23 (2008).

168. Hintiryan, H. *et al.* Connectivity characterization of the mouse basolateral amygdalar complex. *Nat Commun* **12**, 2859 (2021).

169. Dieterich, A. *et al.* Activation of Basolateral Amygdala to Nucleus Accumbens Projection Neurons Attenuates Chronic Corticosterone-Induced Behavioral Deficits in Male Mice. *Front Behav Neurosci* **15**, (2021).

170. Schönfeld, L.-M., Zech, M.-P., Schäble, S., Wöhr, M. & Kalenscher, T. Lesions of the rat basolateral amygdala reduce the behavioral response to ultrasonic vocalizations. *Behavioural Brain Research* **378**, 112274 (2020).

171. Ocampo Daza, D., Bergqvist, C. A. & Larhammar, D. The Evolution of Oxytocin and Vasotocin Receptor Genes in Jawed Vertebrates: A Clear Case for Gene Duplications Through Ancestral Whole-Genome Duplications. *Front Endocrinol (Lausanne)* **12**, (2022).

172. Elphick, M. R., Mirabeau, O. & Larhammar, D. Evolution of neuropeptide signalling systems. *Journal of Experimental Biology* **221**, (2018).

173. Teles, M. C., Gozdowska, M., Kalamarz-Kubiak, H., Kulczykowska, E. & Oliveira, R. F. Agonistic interactions elicit rapid changes in brain nonapeptide levels in zebrafish. *Horm Behav* **84**, 57–63 (2016).

174. Urano, A. & Ando, H. Diversity of the hypothalamo-neurohypophysial system and its hormonal genes. *Gen Comp Endocrinol* **170**, 41–56 (2011).

175. Saito, D., Komatsuda, M. & Urano, A. Functional organization of preoptic vasotocin and isotocin neurons in the brain of rainbow trout: central and neurohypophysial projections of single neurons. *Neuroscience* **124**, 973–984 (2004).

176. Akinrinade, I. *et al.* Oxytocin regulation of social transmission of fear in zebrafish reveals its evolutionary conserved role in emotional contagion. *bioRxiv* 2021.10.06.463413 (2021) doi:doi.org/10.1101/2021.10.06.463413;

177. Syed, A. S., Sansone, A., Hassenklöver, T., Manzini, I. & Korsching, S. I. Coordinated shift of olfactory amino acid responses and V2R expression to an amphibian water nose during metamorphosis. *Cellular and Molecular Life Sciences* **74**, 1711–1719 (2017).

178. Kelliher, K. R. The combined role of the main olfactory and vomeronasal systems in social communication in mammals. *Horm Behav* **52**, 561–570 (2007).

179. Gerlach, G. & Wullimann, M. F. Neural pathways of olfactory kin imprinting and kin recognition in zebrafish. *Cell and Tissue Research 2021 383:1* **383**, 273–287 (2021).

180. He, J. *et al.* Distinct signals conveyed by pheromone concentrations to the mouse vomeronasal organ. *J Neurosci* **30**, 7473–7483 (2010).

181. Pankevich, D. E., Cherry, J. A. & Baum, M. J. Effect of vomeronasal organ removal from male mice on their preference for and neural Fos responses to female urinary odors. *Behavioral neuroscience* **120**, 925–936 (2006).

182. Stowers, L., Holy, T. E., Meister, M., Dulac, C. & Koentges, G. Loss of Sex Discrimination and Male-Male Aggression in Mice Deficient for TRP2. *Science (1979)* **295**, 1493–1500 (2002).

183. Kimchi, T., Xu, J. & Dulac, C. A functional circuit underlying male sexual behaviour in the female mouse brain. *Nature* **448**, 1009–1014 (2007).

184. Chamero, P. *et al.* G protein Gαo is essential for vomeronasal function and aggressive behavior in mice. *Proceedings of the National Academy of Sciences* **108**, 12898–12903 (2011).

185. Montani, G. *et al.* Aggressive behaviour and physiological responses to pheromones are strongly impaired in mice deficient for the olfactory G-protein γ-subunit Gγ8. *J Physiol* **591**, 3949–3962 (2013).

186. Overath, P., Sturm, T. & Rammensee, H.-G. Of volatiles and peptides: in search for MHC-dependent olfactory signals in social communication. *Cellular and Molecular Life Sciences* **71**, 2429–2442 (2014).

187. Leinders-Zufall, T. *et al.* MHC Class I Peptides as Chemosensory Signals in the Vomeronasal Organ. *Science (1979)* **306**, 1033–1037 (2004).

188. Leinders-Zufall, T. *et al.* A Family of Nonclassical Class I MHC Genes Contributes to Ultrasensitive Chemodetection by Mouse Vomeronasal Sensory Neurons. *Journal of Neuroscience* **34**, 5121–5133 (2014).

189. Bruce, H. M. An Exteroceptive Block to Pregnancy in the Mouse. *Nature 1959 184:4680* **184**, 105–105 (1959).

190. Biechl, D., Tietje, K., Gerlach, G. & Wullimann, M. F. Crypt cells are involved in kin recognition in larval zebrafish. *Sci Rep* **6**, 24590 (2016).

191. Mann, K. D., Turnell, E. R., Atema, J. & Gerlach, G. Kin Recognition in Juvenile Zebrafish (Danio rerio) Based on Olfactory Cues. *https://doi.org/10.2307/1543264* **205**, 224–225 (2016).

192. Gerlach, G. & Lysiak, N. Kin recognition and inbreeding avoidance in zebrafish, Danio rerio, is based on phenotype matching. *Anim Behav* **71**, 1371–1377 (2006).

193. Gerlach, G., Hodgins-Davis, A., Avolio, C. & Schunter, C. Kin recognition in zebrafish: a 24-hour window for olfactory imprinting. *Proceedings of the Royal Society B: Biological Sciences* **275**, 2165–2170 (2008).

194. Gerlach, G. *et al.* Behavioural and neuronal basis of olfactory imprinting and kin recognition in larval fish. *J Exp Biol* **222**, (2019).

195. Kress, S., Biechl, D. & Wullimann, M. F. Combinatorial analysis of calcium-binding proteins in larval and adult zebrafish primary olfactory system identifies differential olfactory bulb glomerular projection fields. *Brain Struct Funct* **220**, 1951–1970 (2015).

196. Ferrando, S. *et al.* Observations of crypt neuron-like cells in the olfactory epithelium of a cartilaginous fish. *Neurosci Lett* **403**, 280–282 (2006).

197. Miyasaka, N. *et al.* From the Olfactory Bulb to Higher Brain Centers: Genetic Visualization of Secondary Olfactory Pathways in Zebrafish. *Journal of Neuroscience* **29**, 4756–4767 (2009).

198. Nunes, A. R. *et al.* Perceptual mechanisms of social affiliation in zebrafish. *Sci Rep* **10**, 1–14 (2020).

199. Jetti, S. K., Vendrell-Llopis, N. & Yaksi, E. Spontaneous Activity Governs Olfactory Representations in Spatially Organized Habenular Microcircuits. *Current Biology* **24**, 434–439 (2014).

200. Dreosti, E., Vendrell Llopis, N., Carl, M., Yaksi, E. & Wilson, S. W. Left-Right Asymmetry Is Required for the Habenulae to Respond to Both Visual and Olfactory Stimuli. *Current Biology* **24**, 440–445 (2014).

201. Wee, C. L. *et al.* Social isolation modulates appetite and avoidance behavior via a common oxytocinergic circuit in larval zebrafish. *Nature Communications 2022 13:1* **13**, 1–17 (2022).

202. NEWMAN, S. W. The Medial Extended Amygdala in Male Reproductive Behavior A Node in the Mammalian Social Behavior Network. *Ann N Y Acad Sci* **877**, 242–257 (1999).

203. Cheek, J. M. & Buss, A. H. Shyness and sociability. *J Pers Soc Psychol* **41**, 330–339 (1981).

204. Wells, A. A cognitive model of social phobia. in *Social Phobia: Diagnosis, Assessment and Treatment* (1995).

205. Baumeister, R. F. & Leary, M. R. The Need to Belong: Desire for Interpersonal Attachments as a Fundamental Human Motivation. *Interpersonal Development* 57–89 (2018) doi:10.4324/9781351153683-3/NEED-BELONG-DESIRE-INTERPERSONAL-ATTACHMENTS-FUNDAMENTAL-HUMAN-MOTIVATION-ROY-BAUMEISTER-MARK-LEARY.

206. Krach, S., Paulus, F. M., Bodden, M. & Kircher, T. The rewarding nature of social interactions. *Front Behav Neurosci* **4**, (2010).

207. Dunn, E. W., Aknin, L. B. & Norton, M. I. Spending money on others promotes happiness. *Science (1979)* **319**, 1687–1688 (2008).

208. Kuroda, T. Reinforcement of approaching conspecifics in zebrafish (Danio Rerio) using a real-time 3D tracking system. *Revista Mexicana de Analisis de la Conducta* **45**, 359–381 (2019).

209. Engeszer, R. E., Wang, G., Ryan, M. J. & Parichy, D. M. Sex-specific perceptual spaces for a vertebrate basal social aggregative behavior. *Proc Natl Acad Sci U S A* **105**, 929–933 (2008).

210. Sawangjit, A., Kelemen, E., Born, J. & Inostroza, M. Sleep enhances recognition memory for conspecifics as bound into spatial context. *Front Behav Neurosci* **11**, (2017).

211. Reeve, H. K. The Evolution of Conspecific Acceptance Thresholds. *Am Nat* **133**, 407–435 (1989).

212. Suarez, A. v., Scharf, H. M., Reeve, H. K. & Hauber, M. E. Signal detection, acceptance thresholds and the evolution of animal recognition systems. *Philosophical Transactions of the Royal Society B: Biological Sciences* **375**, 20190464 (2020).

213. Anneser, L. *et al.* The neuropeptide Pth2 modulates social behavior and anxiety in zebrafish. *iScience* **25**, 103868 (2022).

214. Hyde, J. S. & Sawyer, T. F. Estrous cycle fluctuations in aggressiveness of house mice. *Horm Behav* **9**, 290–295 (1977).

215. Denson, T. F., O'Dean, S. M., Blake, K. R. & Beames, J. R. Aggression in women: Behavior, brain and hormones. *Front Behav Neurosci* **12**, 81 (2018).

216. Aguiar, A. S., Speck, A. E., Amaral, I. M., Canas, P. M. & Cunha, R. A. The exercise sex gap and the impact of the estrous cycle on exercise performance in mice. *Sci Rep* **8**, (2018).

217. Meziane, H., Ouagazzal, A.-M., Aubert, L., Wietrzych, M. & Krezel, W. Estrous cycle effects on behavior of C57BL/6J and BALB/cByJ female mice: implications for phenotyping strategies. *Genes Brain Behav* **6**, 192–200 (2007).

218. Mhaouty-Kodja, S., Naulé, L. & Capela, D. Sexual Behavior: From Hormonal Regulation to Endocrine Disruption. *Neuroendocrinology* **107**, 400–416 (2018).

219. Erskine, M. S. Solicitation behavior in the estrous female rat: A review. *Horm Behav* **23**, 473–502 (1989).

220. Diamond, L. M., Dickenson, J. A. & Blair, K. L. Menstrual Cycle Changes in Daily Sexual Motivation and Behavior Among Sexually Diverse Cisgender Women. *Arch Sex Behav* **51**, 577–588 (2022).

221. Cafazzo, S., Bonanni, R., Valsecchi, P. & Natoli, E. Social Variables Affecting Mate Preferences, Copulation and Reproductive Outcome in a Pack of Free-Ranging Dogs. *PLoS One* **9**, e98594 (2014).

222. Hong, K. & Choi, Y. Role of estrogen and RAS signaling in repeated implantation failure. *BMB Rep* **51**, 225–229 (2018).

223. Dey, S. *et al.* Cyclic Regulation of Sensory Perception by a Female Hormone Alters Behavior. *Cell* **161**, 1334–1344 (2015).

224. Almey, A., Milner, T. A. & Brake, W. G. Estrogen receptors in the central nervous system and their implication for dopamine-dependent cognition in females. *Horm Behav* **74**, 125 (2015).

225. Proaño, S. B., Morris, H. J., Kunz, L. M., Dorris, D. M. & Meitzen, J. Estrous cycle-induced sex differences in medium spiny neuron excitatory synaptic transmission and intrinsic excitability in adult rat nucleus accumbens core. *J Neurophysiol* **120**, 1356–1373 (2018).

226. Calipari, E. S. *et al.* Dopaminergic dynamics underlying sex-specific cocaine reward. *Nat Commun* **8**, 13877 (2017).

227. E. Yoest, K., A. Cummings, J. & B. Becker, J. Estradiol, Dopamine and Motivation. *Cent Nerv Syst Agents Med Chem* **14**, 83–89 (2015).

228. Tritsch, N. X. & Sabatini, B. L. Dopaminergic Modulation of Synaptic Transmission in Cortex and Striatum. *Neuron* **76**, 33–50 (2012).

229. McHenry, J. A. *et al.* Hormonal gain control of a medial preoptic area social reward circuit. *Nat Neurosci* **20**, 449 (2017).

230. Qi, C. C. *et al.* Interaction of basolateral amygdala, ventral hippocampus and medial prefrontal cortex regulates the consolidation and extinction of social fear. *Behavioral and Brain Functions* **14**, 1–13 (2018).

231. Razzoli, M., Carboni, L., Andreoli, M., Ballottari, A. & Arban, R. Different susceptibility to social defeat stress of BalbC and C57BL6/J mice. *Behavioural Brain Research* **216**, 100–108 (2011).

232. Huang, C.-H., Kuo, M.-T. & Lai, W.-S. Characterization of behavioural responses in different test contexts after a single social defeat in male golden hamsters (Mesocricetus auratus). *Behavioural Processes* **86**, 94–101 (2011).

233. Lai, W.-S., Ramiro, L.-L. R., Yu, H. A. & Johnston, R. E. Recognition of familiar individuals in golden hamsters: a new method and functional neuroanatomy. *Soc Neuroscience* (2005) doi:10.1523/JNEUROSCI.2124-05.2005.

234. Lukas, M. *et al.* The neuropeptide oxytocin facilitates pro-social behavior and prevents social avoidance in rats and mice. *Neuropsychopharmacology* **36**, 2159–2168 (2011).

235. Hollis, F. & Kabbaj, M. Social Defeat as an Animal Model for Depression. *ILAR J* **55**, 221–232 (2014).

236. van Doeselaar, L. *et al.* Chronic social defeat stress in female mice leads to sex-specific behavioral and neuroendocrine effects. *Stress* **24**, 168–180 (2021).

237. Rygula, R. *et al.* Anhedonia and motivational deficits in rats: Impact of chronic social stress. *Behavioural Brain Research* **162**, 127–134 (2005).

238. Kudryavtseva, N. N., Bakshtanovskaya, I. v. & Koryakina, L. A. Social model of depression in mice of C57BL/6J strain. *Pharmacol Biochem Behav* **38**, 315–320 (1991).

239. Toth, I. & Neumann, I. D. Animal models of social avoidance and social fear. *Cell Tissue Res* **354**, 107–118 (2013).

240. Meerlo, P., Overkamp, G. J. F., Daan, S., van den Hoofdakker, R. H. & Koolhaas, J. M. Changes in Behaviour and Body Weight Following a Single or Double Social Defeat in Rats. *Stress* **1**, 21–32 (1996).

241. Ruis, M. A. W. *et al.* Housing familiar male wildtype rats together reduces the long-term adverse behavioural and physiological effects of social defeat. *Psychoneuroendocrinology* **24**, 285–300 (1999).

242. Koban, L., Kusko, D. & Wager, T. D. Generalization of learned pain modulation depends on explicit learning. *Acta Psychol (Amst)* **184**, 75 (2018).

243. Diaz, V. & Lin, D. Neural circuits for coping with social defeat. *Curr Opin Neurobiol* **60**, 99–107 (2020).

244. Huang, J. Y. *et al.* Huang et al. Respond to "Multigenerational Social Determinants of Health". *Am J Epidemiol* **182**, 583–584 (2015).

245. Day, D. E., Cooper, M. A., Markham, C. M. & Huhman, K. L. NR2B subunit of the NMDA receptor in the basolateral amygdala is necessary for the acquisition of conditioned defeat in Syrian hamsters. *Behavioural Brain Research* **217**, 55–59 (2011).

246. Jasnow, A. M. & Huhman, K. L. Activation of GABAA receptors in the amygdala blocks the acquisition and expression of conditioned defeat in Syrian hamsters. *Brain Res* **920**, 142–150 (2001).

247. Clinard, C. T., Bader, L. R., Sullivan, M. A. & Cooper, M. A. Activation of 5-HT2a receptors in the basolateral amygdala promotes defeat-induced anxiety and the acquisition of conditioned defeat in Syrian hamsters. *Neuropharmacology* **90**, 102–112 (2015).

248. Markham, C. M. & Huhman, K. L. Is the medial amygdala part of the neural circuit modulating conditioned defeat in Syrian hamsters? *Learning & Memory* **15**, 6–12 (2008).

249. Markham, C. M., Taylor, S. L. & Huhman, K. L. Role of amygdala and hippocampus in the neural circuit subserving conditioned defeat in Syrian hamsters. *Learning & Memory* **17**, 109–116 (2010).

250. Luckett, C., Norvelle, A. & Huhman, K. The role of the nucleus accumbens in the acquisition and expression of conditioned defeat. *Behavioural Brain Research* **227**, 208–214 (2012).

251. Folkes, O. M. *et al.* An endocannabinoid-regulated basolateral amygdala-nucleus accumbens circuit modulates sociability. *J Clin Invest* **130**, 1728–1742 (2020).

252. Tea, J., Alderman, S. L. & Gilmour, K. M. Social stress increases plasma cortisol and reduces forebrain cell proliferation in subordinate male zebrafish (Danio rerio). *Journal of Experimental Biology* **222**, (2019).

253. Lal, P. *et al.* Identification of a neuronal population in the telencephalon essential for fear conditioning in zebrafish. *BMC Biol* **16**, 45 (2018).

254. Sørensen, C., Nilsson, G. E., Summers, C. H. & Øverli, Ø. Social stress reduces forebrain cell proliferation in rainbow trout (Oncorhynchus mykiss). *Behavioural Brain Research* **227**, 311–318 (2012).

255. Galhardo, L. & Oliveira, R. F. The effects of social isolation on steroid hormone levels are modulated by previous social status and context in a cichlid fish. *Horm Behav* **65**, 1–5 (2014).

256. EARLEY, R. *et al.* Social interactions tune aggression and stress responsiveness in a territorial cichlid fish (Archocentrus nigrofasciatus). *Physiol Behav* **88**, 353–363 (2006).

257. Gómez-Laplaza, L. M. & Morgan, E. Effects of short-term isolation on the locomotor activity of the angelfish (Pterophyllum scalare). *J Comp Psychol* **105**, 366–375 (1991).

258. Guesdon, V. *et al.* Behavioral and endocrine evaluation of the stressfulness of single-pen housing compared to group-housing and social isolation conditions. *Physiol Behav* **147**, 63–70 (2015).

259. Wei, X. Y., Yang, J. Y., Dong, Y. X. & Wu, C. F. Anxiolytic-like effects of oleamide in group-housed and socially isolated mice. *Prog Neuropsychopharmacol Biol Psychiatry* **31**, 1189–1195 (2007).

260. Mucignat-Caretta, C. *et al.* Age and isolation influence steroids release and chemical signaling in male mice. *Steroids* **83**, 10–16 (2014).

261. Aguiar, A. S. *et al.* Effects of exercise on mitochondrial function, neuroplasticity and anxio-depressive behavior of mice. *Neuroscience* **271**, 56–63 (2014).

262. Lapiz, M. D. S. *et al.* Influence of Postweaning Social Isolation in the Rat on Brain Development, Conditioned Behavior, and Neurotransmission. *Neuroscience and Behavioral Physiology 2003 33:1* **33**, 13–29 (2003).

263. Fone, K. C. F. & Porkess, M. V. Behavioural and neurochemical effects of post-weaning social isolation in rodents—Relevance to developmental neuropsychiatric disorders. *Neurosci Biobehav Rev* **32**, 1087–1102 (2008).

264. FERDMAN, N., MURMU, R., BOCK, J., BRAUN, K. & LESHEM, M. Weaning age, social isolation, and gender, interact to determine adult explorative and social behavior, and dendritic and spine morphology in prefrontal cortex of rats. *Behavioural Brain Research* **180**, 174–182 (2007).

265. Hawkley, L. C. & Cacioppo, J. T. Loneliness and pathways to disease. *Brain Behav Immun* **17**, 98–105 (2003).

266. Cacioppo, J. T., Hawkley, L. C., Norman, G. J. & Berntson, G. G. Social isolation. *Ann N Y Acad Sci* **1231**, 17–22 (2011).

267. Cacioppo, J. T., Norris, C. J., Decety, J., Monteleone, G. & Nusbaum, H. In the eye of the beholder: Individual differences in perceived social isolation predict regional brain activation to social stimuli. *J Cogn Neurosci* **21**, 83–92 (2009).

268. Bangee, M. & Qualter, P. Examining the visual processing patterns of lonely adults. *Scand J Psychol* **59**, 351–359 (2018).

269. Wenger, G. C., Davies, R., Shahtahmasebi, S. & Scott, A. Social Isolation and Loneliness in Old Age: Review and Model Refinement. *Ageing Soc* **16**, 333–358 (1996).

270. Perlman, D. & Peplau, L. A. Toward a Social Psychology of Loneliness. in *Personal Relationships in Disorder* (eds. Duck S. & Gilmour R.) vol. 3 31–56 (Academic Press, 1981).

271. Cornwell, E. Y. & Waite, L. J. Social disconnectedness, perceived isolation, and health among older adults. *J Health Soc Behav* **50**, 31–48 (2009).

272. National Academies of Sciences and Medicine, E. *Social Isolation and Loneliness in Older Adults*. (National Academies Press, 2020). doi:10.17226/25663.

273. Filby, A. L., Paull, G. C., Bartlett, E. J., van Look, K. J. W. & Tyler, C. R. Physiological and health consequences of social status in zebrafish (Danio rerio). *Physiol Behav* **101**, 576–587 (2010).

274. Snyder-Mackler, N. *et al.* Social determinants of health and survival in humans and other animals. *Science* vol. 368 Preprint at https://doi.org/10.1126/science.aax9553 (2020).

275. Porcelli, S. *et al.* Social brain, social dysfunction and social withdrawal. *Neuroscience and Biobehavioral Reviews* vol. 97 10–33 Preprint at https://doi.org/10.1016/j.neubiorev.2018.09.012 (2019).

276. Razzoli, M. *et al.* Social stress shortens lifespan in mice. *Aging Cell* **17**, (2018).

277. Seid, A. K. Social interactions, trust and risky alcohol consumption. *Health Econ Rev* **6**, 1–9 (2016).

278. Pachucki, M. C. & Goodman, E. Social Relationships and Obesity: Benefits of Incorporating a Lifecourse Perspective. *Current obesity reports* vol. 4 217–223 Preprint at https://doi.org/10.1007/s13679-015-0145-z (2015).

279. Holman, C. D. J., English, D. R., Milne, E. & Winter, M. G. Meta-analysis of alcohol and all-cause mortality: A validation of NHMRC recommendations. *Medical Journal of Australia* **164**, 141–145 (1996).

280. Shavelle, R. M., Paculdo, D. R., Strauss, D. J. & Kush, S. J. Smoking habit and mortality: a meta-analysis. *J Insur Med* **40**, 170–178 (2008).

281. Katzmarzyk, P. T., Janssen, I. & Ardern, C. I. Physical inactivity, excess adiposity and premature mortality. *Obesity Reviews* vol. 4 257–290 Preprint at https://doi.org/10.1046/j.1467-789X.2003.00120.x (2003).

282. Holt-Lunstad, J., Smith, T. B. & Layton, J. B. Social relationships and mortality risk: A meta-analytic review. *PLoS Medicine* vol. 7 e1000316 Preprint at https://doi.org/10.1371/journal.pmed.1000316 (2010).

283. Han, S. & Lee, H. S. Social capital and depression: Does household context matter? *Asia Pac J Public Health* **27**, NP2008–NP2018 (2015).

284. Alegría, M., NeMoyer, A., Falgàs Bagué, I., Wang, Y. & Alvarez, K. Social Determinants of Mental Health: Where We Are and Where We Need to Go. *Current Psychiatry Reports* vol. 20 95 Preprint at https://doi.org/10.1007/s11920-018-0969-9 (2018).

285. Victor, C. R., Scambler, S. J., Bowling, A. & Bond, J. The prevalence of, and risk factors for, loneliness in later life: A survey of older people in Great Britain. *Ageing Soc* **25**, 357–375 (2005).

286. Grenade, L. & Boldy, D. Social isolation and loneliness among older people: Issues and future challenges in community and residential settings. *Australian Health Review* **32**, 468–478 (2008).

287. Ibrahim, R., Abolfathi Momtaz, Y. & Hamid, T. A. Social isolation in older Malaysians: Prevalence and risk factors. *Psychogeriatrics* **13**, 71–79 (2013).

288. Leigh-Hunt, N. *et al.* An overview of systematic reviews on the public health consequences of social isolation and loneliness. *Public Health* vol. 152 157–171 Preprint at https://doi.org/10.1016/j.puhe.2017.07.035 (2017).

289. Hwang, T. J., Rabheru, K., Peisah, C., Reichman, W. & Ikeda, M. Loneliness and social isolation during the COVID-19 pandemic. *International Psychogeriatrics* vol. 32 1217–1220 Preprint at https://doi.org/10.1017/S1041610220000988 (2020).

290. Jeste, D. v., Lee, E. E. & Cacioppo, S. Battling the Modern Behavioral Epidemic of Loneliness: Suggestions for Research and Interventions. *JAMA Psychiatry* vol. 77 553–554 Preprint at https://doi.org/10.1001/jamapsychiatry.2020.0027 (2020).

291. Castaldelli-Maia, J. M., Marziali, M. E., Lu, Z. & Martins, S. S. Investigating the effect of national government physical distancing measures on depression and anxiety during the COVID-19 pandemic through meta-analysis and meta-regression. *Psychological Medicine* vol. 51 881–893 Preprint at https://doi.org/10.1017/S0033291721000933 (2021).

292. Porter, C. *et al.* Impact of the COVID-19 pandemic on anxiety and depression symptoms of young people in the global south: Evidence from a four-country cohort study. *BMJ Open* **11**, e049653 (2021).

293. Saladino, V., Algeri, D. & Auriemma, V. The Psychological and Social Impact of Covid-19: New Perspectives of Well-Being. *Front Psychol* **11**, 2550 (2020).

294. Nicola, M. *et al.* The socio-economic implications of the coronavirus pandemic (COVID-19): A review. *International Journal of Surgery* vol. 78 185–193 Preprint at https://doi.org/10.1016/j.ijsu.2020.04.018 (2020).

295. Chugani, H. T. *et al.* Local brain functional activity following early deprivation: A study of postinstitutionalized Romanian orphans. *Neuroimage* **14**, 1290–1301 (2001).

296. Chisholm, K., Carter, M. C., Ames, E. W. & Morison, S. J. Attachment security and indiscriminately friendly behavior in children adopted from Romanian orphanages. *Dev Psychopathol* **7**, 283–294 (1995).

297. Bellini, S. Social Skill Deficits and Anxiety in High-Functioning Adolescents With Autism Spectrum Disorders. *Focus Autism Other Dev Disabl* **19**, 78–86 (2004).

298. Spain, D. *et al.* Social anxiety in adult males with autism spectrum disorders. *Res Autism Spectr Disord* **32**, 13–23 (2016).

299. Capitão, L. *et al.* Williams syndrome hypersociability: A neuropsychological study of the amygdala and prefrontal cortex hypotheses. *Res Dev Disabil* **32**, 1169–1179 (2011).

300. Evans, J. R., Torres-Pérez, J. v., Petrazzini, M. E. M., Riley, R. & Brennan, C. H. Stress reactivity elicits a tissue-specific reduction in telomere length in aging zebrafish (Danio rerio). *Scientific Reports 2021 11:1* **11**, 1–11 (2021).

301. Menezes, F. P., Padilha de Sousa, I. & Luchiari, A. C. Early Mistreatment Contributes to Social Behavior Disorders in Zebrafish. *Front Behav Neurosci* **14**, 179 (2020).

302. Mendoza, S. P. & Mason, W. A. Contrasting responses to intruders and to involuntary separation by monogamous and polygynous New World monkeys. *Physiol Behav* **38**, 795–801 (1986).

303. Matsumoto, K., Pinna, G., Puia, G., Guidotti, A. & Costa, E. Social isolation stress-induced aggression in mice: A model to study the pharmacology of neurosteroidogenesis. *Stress* vol. 8 85–93 Preprint at https://doi.org/10.1080/10253890500159022 (2005).

304. Zelikowsky, M. *et al.* The Neuropeptide Tac2 Controls a Distributed Brain State Induced by Chronic Social Isolation Stress. *Cell* **173**, 1265-1279.e19 (2018).

305. Valzelli, L. The 'isolation syndrome' in mice. *Psychopharmacologia* **31**, 305–320 (1973).

306. Ieraci, A., Mallei, A. & Popoli, M. Social Isolation Stress Induces Anxious-Depressive-Like Behavior and Alterations of Neuroplasticity-Related Genes in Adult Male Mice. *Neural Plast* **2016**, (2016).

307. Wallace, D. L. *et al.* CREB regulation of nucleus accumbens excitability mediates social isolation-induced behavioral deficits. *Nat Neurosci* **12**, 200–209 (2009).

308. Jolles, J. W. *et al.* The role of previous social experience on risk-taking and leadership in three-spined sticklebacks. *Behavioral Ecology* **25**, 1395–1401 (2014).

309. Webster, M. M., Ward, A. J. W. & Hart, P. J. B. Boldness is influenced by social context in threespine sticklebacks (Gasterosteus aculeatus). *Behaviour* **144**, 351–371 (2007).

310. GÓmez-Laplaza, L. M. & Morgan, E. Towards an isolation syndrome for the angelfish, Pterophyllum scalare. *J Fish Biol* **29**, 179–187 (1986).

311. Rose, J. D. The Neurobehavioral Nature of Fishes and the Question of Awareness and Pain. *Reviews in Fisheries Science* vol. 10 1–38 Preprint at https://doi.org/10.1080/20026491051668 (2002).

312. Agetsuma, M. *et al.* The habenula is crucial for experience-dependent modification of fear responses in zebrafish. *Nat Neurosci* **13**, 1354–1356 (2010).

313. Ahmed, O., Seguin, D. & Gerlai, R. An automated predator avoidance task in zebrafish. *Behavioural Brain Research* **216**, 166–171 (2011).

314. Orger, M. B. & de Polavieja, G. G. Zebrafish Behavior: Opportunities and Challenges. *Annu Rev Neurosci* **40**, 125–147 (2017).

315. Engeszer, R. E., Patterson, L. B., Rao, A. A. & Parichy, D. M. Zebrafish in the wild: A review of natural history and new notes from the field. *Zebrafish* vol. 4 21–40 Preprint at https://doi.org/10.1089/zeb.2006.9997 (2007).

316. Krausz, R. R. Living in Groups. *Transactional Analysis Journal* **43**, 366–374 (2013).

317. Harpaz, R. & Schneidman, E. Social interactions drive efficient foraging and income equality in groups of fish. *Elife* **9**, 1–46 (2020).

318. Morrell, L. J. & James, R. Mechanisms for aggregation in animals: Rule success depends on ecological variables. *Behavioral Ecology* **19**, 193–201 (2008).

319. Fontana, B. D. *et al.* Using zebrafish (Danio rerio) models to understand the critical role of social interactions in mental health and wellbeing. *Progress in Neurobiology* 101993 Preprint at https://doi.org/10.1016/j.pneurobio.2021.101993 (2021).

320. Suriyampola, P. S. *et al.* Zebrafish Social Behavior in the Wild. *Zebrafish* **13**, 1–8 (2016).

321. Pitcher, T. J. Heuristic definitions of fish shoaling behaviour. *Anim Behav* **31**, 611–613 (1983).

322. Miller, N. & Gerlai, R. From Schooling to Shoaling: Patterns of Collective Motion in Zebrafish (Danio rerio). *PLoS One* **7**, e48865 (2012).

323. Buske, C. & Gerlai, R. Shoaling develops with age in Zebrafish (Danio rerio). *Prog Neuropsychopharmacol Biol Psychiatry* **35**, 1409–1415 (2011).

324. Engeszer, R. E., da Barbiano, L. A., Ryan, M. J. & Parichy, D. M. Timing and plasticity of shoaling behaviour in the zebrafish, Danio rerio. *Anim Behav* **74**, 1269–1275 (2007).

325. Székely, T., Moore, A. J. & Komdeur, J. *Social behaviour: Genes, ecology and evolution*. *Social Behaviour: Genes, Ecology and Evolution* (Cambridge University Press, 2010). doi:10.1017/CBO9780511781360.

326. Taborsky, M. *et al.* Taxon matters: Promoting integrative studies of social behavior: NESCent Working Group on Integrative Models of Vertebrate Sociality: Evolution, Mechanisms, and Emergent Properties. *Trends in Neurosciences* vol. 38 189–191 Preprint at https://doi.org/10.1016/j.tins.2015.01.004 (2015).

327. Saverino, C. & Gerlai, R. The social zebrafish: Behavioral responses to conspecific, heterospecific, and computer animated fish. *Behavioural Brain Research* **191**, 77–87 (2008).

328. Silverman, J. L., Yang, M., Lord, C. & Crawley, J. N. Behavioural phenotyping assays for mouse models of autism. *Nature Reviews Neuroscience* vol. 11 490–502 Preprint at https://doi.org/10.1038/nrn2851 (2010).

329. Hånell, A. & Marklund, N. Structured evaluation of rodent behavioral tests used in drug discovery research. *Front Behav Neurosci* **8**, 252 (2014).

330. Menger, G. J., Koke, J. R. & Cahill, G. M. Diurnal and circadian retinomotor movements in zebrafish. *Vis Neurosci* **22**, 203–209 (2005).

331. Lenkov, D. N., Volnova, A. B., Pope, A. R. D. & Tsytsarev, V. Advantages and limitations of brain imaging methods in the research of absence epilepsy in humans and animal models. *J Neurosci Methods* **212**, 195–202 (2013).

332. THAI, N. Disconnected brains: What is the role of fMRI in connectivity research? *International Journal of Psychophysiology* **73**, 27–32 (2009).

333. Takacs, C. A., Krivak, T. C. & Napolitano, P. G. Insulinoma in pregnancy: a case report and review of the literature. *Obstet Gynecol Surv* **57**, 229–235 (2002).

334. Charonis, A. S. *et al.* Laminin Alterations After In Vitro Nonenzymatic Glycosylation. *Diabetes* **39**, 807–814 (1990).

335. Appelbaum, L. *et al.* Sleep–wake regulation and hypocretin–melatonin interaction in zebrafish. *Proceedings of the National Academy of Sciences* **106**, 21942–21947 (2009).

336. Berman, J. R., Skariah, G., Maro, G. S., Mignot, E. & Mourrain, P. Characterization of two melanin-concentrating hormone genes in zebrafish reveals evolutionary and physiological links with the mammalian MCH system. *Journal of Comparative Neurology* **517**, 695–710 (2009).

337. Friedrich, R. W., Jacobson, G. A. & Zhu, P. Circuit Neuroscience in Zebrafish. *Current Biology* **20**, R371–R381 (2010).

338. Engert, F. & Wilson, S. Zebrafish neurobiology: From development to circuit function and behaviour. *Dev Neurobiol* **72**, 215–217 (2012).

339. Bruzzone, M. *et al.* Whole brain functional recordings at cellular resolution in zebrafish larvae with 3D scanning multiphoton microscopy. *Sci Rep* **11**, 11048 (2021).

340. Adolphs, R. The social brain: Neural basis of social knowledge. *Annual Review of Psychology* vol. 60 693–716 Preprint at https://doi.org/10.1146/annurev.psych.60.110707.163514 (2009).

341. Blakemore, S. J. The social brain in adolescence. *Nature Reviews Neuroscience* vol. 9 267–277 Preprint at https://doi.org/10.1038/nrn2353 (2008).

342. Frith, C. D. The social brain? in *Philosophical Transactions of the Royal Society B: Biological Sciences* vol. 362 671–678 (The Royal SocietyLondon, 2007).

343. Heap, L., Goh, C. C., Kassahn, K. S. & Scott, E. K. Cerebellar output in zebrafish: An analysis of spatial patterns and topography in eurydendroid cell projections. *Front Neural Circuits* (2013) doi:10.3389/fncir.2013.00053.

344. Maeyama, K. & Nakayasu, H. Postembryonic Neurogenesis in Zebrafish (Danio rerio) Brain: Presence of Two Different Systems. *Zoolog Sci* **17**, 959–966 (2000).

345. Folgueira, M. *et al.* Morphogenesis underlying the development of the everted teleost telencephalon. *Neural Dev* **7**, 212 (2012).

346. Howe, K. *et al.* Corrigendum: The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **505**, 248–248 (2014).

347. Kroll, F. *et al.* A simple and effective f0 knockout method for rapid screening of behaviour and other complex phenotypes. *Elife* **10**, 1–34 (2021).

348. Kunst, M. *et al.* A Cellular-Resolution Atlas of the Larval Zebrafish Brain. *Neuron* **103**, 21-38.e5 (2019).

349. Wullimann, M. F., Rupp, B. & Reichert, H. *Neuroanatomy of the Zebrafish Brain*. *Neuroanatomy of the Zebrafish Brain* (Birkhäuser Basel, 1996). doi:10.1007/978-3-0348-8979-7.

350. Randlett, O. *et al.* Whole-brain activity mapping onto a zebrafish brain atlas. *Nat Methods* **12**, 1039–1046 (2015).

351. Hamilton, T. J., Krook, J., Szaszkiewicz, J. & Burggren, W. Shoaling, boldness, anxiety-like behavior and locomotion in zebrafish (Danio rerio) are altered by acute benzo[a]pyrene exposure. *Science of the Total Environment* **774**, 145702 (2021).

352. Pham, M. *et al.* Assessing Social Behavior Phenotypes in Adult Zebrafish: Shoaling, Social Preference, and Mirror Biting Tests. in vol. 66 231–246 (Humana Press, Totowa, NJ, 2012).

353. Shams, S., Seguin, D., Facciol, A., Chatterjee, D. & Gerlai, R. Effect of social isolation on anxiety-related behaviors, cortisol, and monoamines in adult zebrafish. *Behavioral Neuroscience* **131**, 492–504 (2017).

354. Gerlai, R. Animated images in the analysis of zebrafish behavior. *Curr Zool* **63**, 35–44 (2017).

355. Landin, J. *et al.* Oxytocin Receptors Regulate Social Preference in Zebrafish. *Sci Rep* **10**, 1–12 (2020).

356. Aslanzadeh, M. *et al.* The Body Size of Stimulus Conspecifics Affects Social Preference in a Binary Choice Task in Wild-Type, but Not in dyrk1aa Mutant, Zebrafish. *Zebrafish* **16**, 262–267 (2019).

357. Audira, G. *et al.* A versatile setup for measuring multiple behavior endpoints in zebrafish. *Inventions* **3**, 75 (2018).

358. Ribeiro, D. *et al.* Oxytocin receptor signalling modulates novelty recognition but not social preference in zebrafish. *J Neuroendocrinol* **32**, e12834 (2020).

359. Valente, A., Huang, K. H., Portugues, R. & Engert, F. Ontogeny of classical and operant learning behaviors in zebrafish. *Learning and Memory* **19**, 170–177 (2012).

360. Lindeyer, C. M. & Reader, S. M. Social learning of escape routes in zebrafish and the stability of behavioural traditions. *Anim Behav* **79**, 827–834 (2010).

361. Teles, M. C., Dahlbom, S. J., Winberg, S. & Oliveira, R. F. Social modulation of brain monoamine levels in zebrafish. *Behavioural Brain Research* **253**, 17–24 (2013).

362. Egan, R. J. *et al.* Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research* **205**, 38–44 (2009).

363. Lau, J. Y. *et al.* Distinct neural signatures of threat learning in adolescents and adults. *Proc Natl Acad Sci U S A* **108**, 4500–4505 (2011).

364. Carpenter, J. Social Preferences. in *The New Palgrave Dictionary of Economics* 1–5 (Palgrave Macmillan, London, 2008). doi:10.1057/978-1-349-95121-5_1974-1.

365. Liu, X. *et al.* Social preference deficits in juvenile zebrafish induced by early chronic exposure to sodium valproate. *Front Behav Neurosci* **10**, 201 (2016).

366. Pritchard, V. L., Lawrence, J., Butlin, R. K. & Krause, J. Shoal choice in zebrafish, Danio rerio: The influence of shoal size and activity. *Anim Behav* **62**, 1085–1088 (2001).

367. Ruhl, N. & McRobert, S. P. The effect of sex and shoal size on shoaling behaviour in Danio rerio. *J Fish Biol* **67**, 1318–1326 (2005).

368. Pyron, M. Female preferences and male-male interactions in zebrafish (Danio rerio). *Can J Zool* **81**, 122–125 (2003).

369. Fareri, D. S. & Delgado, M. R. Social rewards and social networks in the human brain. *Neuroscientist* vol. 20 387–402 Preprint at https://doi.org/10.1177/1073858414521869 (2014).

370. Holson, R. R., Scallet, A. C., Ali, S. F. & Turner, B. B. 'Isolation stress' revisited: Isolation-rearing effects depend on animal care methods. *Physiol Behav* **49**, 1107–1118 (1991).

371. Stednitz, S. J. & Washbourne, P. Rapid Progressive Social Development of Zebrafish. *Zebrafish* **17**, 11–17 (2020).

372. Landin, J. *et al.* Oxytocin Receptors Regulate Social Preference in Zebrafish. *Sci Rep* **10**, 1–12 (2020).

373. Woodward, M. A., Winder, L. A. & Watt, P. J. Enrichment Increases Aggression in Zebrafish. *Fishes 2019, Vol. 4, Page 22* **4**, 22 (2019).

374. Norton, W. H. J., Manceau, L. & Reichmann, F. The visually mediated social preference test: A novel technique to measure social behavior and behavioral disturbances in Zebrafish. in *Methods in Molecular Biology* vol. 2011 121–132 (Humana Press Inc., 2019).

375. Shams, S., Chatterjee, D. & Gerlai, R. Chronic social isolation affects thigmotaxis and whole-brain serotonin levels in adult zebrafish. *Behavioural Brain Research* **292**, 283–287 (2015).

376. Norton, W. H. J., Manceau, L. & Reichmann, F. The visually mediated social preference test: A novel technique to measure social behavior and behavioral disturbances in Zebrafish. in *Methods in Molecular Biology* vol. 2011 121–132 (Humana, New York, NY, 2019).

377. Aslanzadeh, M. *et al.* The Body Size of Stimulus Conspecifics Affects Social Preference in a Binary Choice Task in Wild-Type, but Not in dyrk1aa Mutant, Zebrafish. *Zebrafish* **16**, 262–267 (2019).

378. Nonacs, P. Measuring and using skew in the study of social behavior and evolution. *American Naturalist* **156**, 577–589 (2000).

379. Sherman, P. W., Lacey, E. A., Reeve, H. K. & Keller, L. Forum: The eusociality continuum. *Behavioral Ecology* **6**, 102–108 (1995).

380. Blumstein, D. T. & Armitage, K. B. Life history consequences of social complexity: A comparative study of ground-dwelling sciurids. *Behavioral Ecology* **9**, 8–19 (1998).

381. Hubert, J., Booms, E., Witbaard, R. & Slabbekoorn, H. Responsiveness and habituation to repeated sound exposures and pulse trains in blue mussels. *J Exp Mar Biol Ecol* **547**, 151668 (2022).

382. Daalmans, J. Human Behavior in Hazardous Situations. *Human Behavior in Hazardous Situations* (2013) doi:10.1016/C2012-0-02612-9.

383. Lore, R. & Flannelly, K. Rat Societies. *Sci Am* **236**, 106–118 (1977).

384. Carlsson, F., Johansson-Stenman, O. & Nam, P. K. Social preferences are stable over long periods of time. *J Public Econ* **117**, 104–114 (2014).

385. Lansford, J. E., Killeya-Jones, L. A., Miller, S. & Costanzo, P. R. Early Adolescents' Social Standing in Peer Groups: Behavioral Correlates of Stability and Change. *J Youth Adolesc* **38**, 1084 (2009).

386. Maassen, G. H. & Verschueren, K. A Two-Dimensional Ratings-Based Procedure for Sociometric Status Determination as an Alternative to the Asher and Dodge System. *Merrill Palmer Q* **51**, 192–212 (2005).

387. Reiczigel, J., Lang, Z., Rózsa, L. & Tóthmérész, B. Measures of sociality: two different views of group size. *Animal Behaviour* vol. 75 715–721 Preprint at https://doi.org/10.1016/j.anbehav.2007.05.020 (2008).

388. Avilés, L. & Harwood, G. A Quantitative Index of Sociality and Its Application to Group-Living Spiders and Other Social Organisms. *Ethology* **118**, 1219–1229 (2012).

389. Ward, A. & Webster, M. *Sociality: The behaviour of group-living animals*. *Sociality: The Behaviour of Group-Living Animals* (Springer International Publishing, 2016). doi:10.1007/978-3-319-28585-6.

390. Hayes, R. A. Analyzing Animal Societies: Quantitative Methods for Vertebrate Social Analysis. *Austral Ecol* **36**, e23–e23 (2011).

391. Li, L. & Dowling, J. E. Zebrafish visual sensitivity is regulated by a circadian clock. *Vis Neurosci* **15**, 851–857 (1998).

392. López-Olmeda, J. F., Madrid, J. A. & Sánchez-Vázquez, F. J. Light and temperature cycles as zeitgebers of zebrafish (Danio rerio) circadian activity rhythms. *Chronobiol Int* **23**, 537–550 (2006).

393. Kopp, R., Legler, J. & Legradi, J. Alterations in locomotor activity of feeding zebrafish larvae as a consequence of exposure to different environmental factors. *Environmental Science and Pollution Research* **25**, 4085–4093 (2018).

394. Wircer, E. *et al.* Homeodomain protein otp affects developmental neuropeptide switching in oxytocin neurons associated with a long-term effect on social behavior. *Elife* **6**, (2017).

395. Shams, S., Amlani, S., Buske, C., Chatterjee, D. & Gerlai, R. Developmental social isolation affects adult behavior, social interaction, and dopamine metabolite levels in zebrafish. *Dev Psychobiol* **60**, 43–56 (2018).

396. Netser, S. *et al.* Distinct dynamics of social motivation drive differential social behavior in laboratory rat and mouse strains. *Nat Commun* **11**, 1–19 (2020).

397. Angiulli, E. *et al.* Increase in environmental temperature affects exploratory behaviour, anxiety and social preference in Danio rerio. *Sci Rep* **10**, 1–12 (2020).

398. Hamilton, T. J. *et al.* Establishing zebrafish as a model to study the anxiolytic effects of scopolamine. *Sci Rep* **7**, 1–9 (2017).

399. Blaser, R. E. & Rosemberg, D. B. Measures of anxiety in zebrafish (Danio rerio): Dissociation of black/white preference and novel tank test. *PLoS One* **7**, 36931 (2012).

400. Tran, S. & Gerlai, R. Individual differences in activity levels in zebrafish (Danio rerio). *Behavioural Brain Research* **257**, 224–229 (2013).

401. Sackerman, J. *et al.* Zebrafish Behavior in Novel Environments: Effects of Acute Exposure to Anxiolytic Compounds and Choice of Danio rerio Line. *International journal of comparative psychology / ISCP ; sponsored by the International Society for Comparative Psychology and the University of Calabria* **23**, 43 (2010).

402. Tran, S., Facciol, A. & Gerlai, R. Alcohol-induced behavioral changes in zebrafish: The role of dopamine D2-like receptors. *Psychopharmacology (Berl)* **233**, 2119–2128 (2016).

403. Wong, K. *et al.* Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behavioural Brain Research* **208**, 450–457 (2010).

404. Bencan, Z., Sledge, D. & Levin, E. D. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. *Pharmacol Biochem Behav* **94**, 75–80 (2009).

405. Cachat, J. *et al.* Three-dimensional neurophenotyping of adult zebrafish behavior. *PLoS One* **6**, 17597 (2011).

406. Levin, E. D., Bencan, Z. & Cerutti, D. T. Anxiolytic effects of nicotine in zebrafish. *Physiol Behav* **90**, 54–58 (2007).

407. Blaser, R. & Gerlai, R. Behavioral phenotyping in zebrafish: Comparison of three behavioral quantification methods. in *Behavior Research Methods* vol. 38 456–469 (Springer New York LLC, 2006).

408. Mejias, R. *et al.* Increased novelty-induced locomotion, sensitivity to amphetamine, and extracellular dopamine in striatum of Zdhhc15-deficient mice. *Transl Psychiatry* **11**, 65 (2021).

409. Langova, V., Vales, K., Horka, P. & Horacek, J. The Role of Zebrafish and Laboratory Rodents in Schizophrenia Research. *Frontiers in Psychiatry* vol. 11 Preprint at https://doi.org/10.3389/fpsyt.2020.00703 (2020).

410. Strekalova, T., Spanagel, R., Dolgov, O. & Bartsch, D. Stress-induced hyperlocomotion as a confounding factor in anxiety and depression models in mice. *Behavioural Pharmacology* **16**, 171–180 (2005).

411. Lo, S. C., Scearce-Levie, K. & Sheng, M. Characterization of Social Behaviors in caspase-3 deficient mice. *Sci Rep* **6**, 1–9 (2016).

412. Rochais, C. *et al.* Visual attention, an indicator of human-animal relationships? A study of domestic horses (Equus caballus). *Front Psychol* **5**, 108 (2014).

413. Schaefer, I. C. *et al.* The side-by-side exploratory test: A simple automated protocol for the evaluation of adult zebrafish behavior simultaneously with social interaction. *Behavioural Pharmacology* **26**, 691–696 (2015).

414. Kroczek, L. O. H., Pfaller, M., Lange, B., Müller, M. & Mühlberger, A. Interpersonal Distance During Real-Time Social Interaction: Insights From Subjective Experience, Behavior, and Physiology. *Front Psychiatry* **11**, 561 (2020).

415. Oswald, M. E., Singer, M. & Robison, B. D. The Quantitative Genetic Architecture of the Bold-Shy Continuum in Zebrafish, Danio rerio. *PLoS One* **8**, 68828 (2013).

416. Mustafa, A., Roman, E. & Winberg, S. Boldness in Male and Female Zebrafish (Danio rerio) Is Dependent on Strain and Test. *Front Behav Neurosci* **13**, (2019).

417. Sih, A., Bell, A. M., Johnson, J. C. & Ziemba, R. E. Behavioral syndromes: An integrative overview. *Quarterly Review of Biology* vol. 79 241–277 Preprint at https://doi.org/10.1086/422893 (2004).

418. Crone, E. A. & Dahl, R. E. Understanding adolescence as a period of social-affective engagement and goal flexibility. *Nat Rev Neurosci* **13**, 636–650 (2012).

419. RC, K. *et al.* Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry* **62**, 593–602 (2005).

420. Ennaceur, A., Michalikova, S. & Chazot, P. L. Models of anxiety: Responses of rats to novelty in an open space and an enclosed space. *Behavioural Brain Research* **171**, 26–49 (2006).

421. Yeh, C. M., Glöck, M. & Ryu, S. An optimized whole-body cortisol quantification method for assessing stress levels in larval zebrafish. *PLoS One* **8**, 79406 (2013).

422. Wilby, D., Temple, S. E. & Gagnon, Y. L. Losing focus: how lens position and viewing angle affect the function of multifocal lenses in fishes. *JOSA A, Vol. 33, Issue 9, pp. 1901-1909* **33**, 1901–1909 (2016).

423. Saif, M., Chatterjee, D., Buske, C. & Gerlai, R. Sight of conspecific images induces changes in neurochemistry in zebrafish. *Behavioural Brain Research* **243**, 294–299 (2013).

424. Homberg, J. R. *et al.* Adaptations in pre- and postsynaptic 5-HT1A receptor function and cocaine supersensitivity in serotonin transporter knockout rats. *Psychopharmacology (Berl)* **200**, 367–380 (2008).

425. Al-Imari, L. & Gerlai, R. Sight of conspecifics as reward in associative learning in zebrafish (Danio rerio). *Behavioural Brain Research* **189**, 216–219 (2008).

426. Mustafa, A., Roman, E. & Winberg, S. Boldness in Male and Female Zebrafish (Danio rerio) Is Dependent on Strain and Test. *Front Behav Neurosci* **13**, (2019).

427. Koolhaas, J. M. *et al.* Coping styles in animals: Current status in behavior and stress-physiology. *Neurosci Biobehav Rev* **23**, 925–935 (1999).

428. Samaras, A. *et al.* Repeatability of cortisol stress response in the European sea bass (Dicentrarchus labrax) and transcription differences between individuals with divergent responses. *Sci Rep* **6**, 1–11 (2016).

429. Way, G. P., Southwell, M. & McRobert, S. P. Boldness, Aggression, and Shoaling Assays for Zebrafish Behavioral Syndromes. *J Vis Exp* **2016**, 54049 (2016).

430. Dahlbom, S. J., Lagman, D., Lundstedt-Enkel, K., Sundström, L. F. & Winberg, S. Boldness predicts social status in zebrafish (Danio rerio). *PLoS One* **6**, (2011).

431. Marmot, M., Friel, S., Bell, R., Houweling, T. A. & Taylor, S. Closing the gap in a generation: health equity through action on the social determinants of health. *The Lancet* **372**, 1661–1669 (2008).

432. Shams, S., Seguin, D., Facciol, A., Chatterjee, D. & Gerlai, R. Effect of social isolation on anxiety-related behaviors, cortisol, and monoamines in adult zebrafish. *Behav Neurosci* **131**, 492–504 (2017).

433. Saszik, S. M. & Smith, C. M. The impact of stress on social behavior in adult zebrafish (Danio rerio). *Behavioural Pharmacology* **29**, 53–59 (2018).

434. Gerlach, G., Hodgins-Davis, A., Avolio, C. & Schunter, C. Kin recognition in zebrafish: A 24-hour window for olfactory imprinting. *Proceedings of the Royal Society B: Biological Sciences* **275**, 2165–2170 (2008).

435. Miller, N. Y. & Gerlai, R. Shoaling in zebrafish: What we don't know. *Rev Neurosci* **22**, 17–25 (2011).

436. Groneberg, A. H. *et al.* Early-Life Social Experience Shapes Social Avoidance Reactions in Larval Zebrafish. *Current Biology* **30**, 4009-4021.e4 (2020).

437. Weber, J. *et al.* Neurophysiological, neuropsychological, and cognitive effects of 30 days of isolation. *Exp Brain Res* **237**, 1563–1573 (2019).

438. Cruz, F. C. *et al.* Adolescent vulnerability to cardiovascular consequences of chronic social stress: Immediate and long-term effects of social isolation during adolescence. *Dev Neurobiol* **76**, 34–46 (2016).

439. Zlatković, J. & Filipović, D. Chronic social isolation induces NF-κB activation and upregulation of iNOS protein expression in rat prefrontal cortex. *Neurochem Int* **63**, 172–179 (2013).

440. George, J. M. *et al.* Acute social isolation alters neurogenomic state in songbird forebrain. *Proc Natl Acad Sci U S A* **117**, 23311–23316 (2020).

441. Kay, R. & Hall, C. The use of a mirror reduces isolation stress in horses being transported by trailer. *Appl Anim Behav Sci* **116**, 237–243 (2009).

442. Parrott, R. F., Houpt, K. A. & Misson, B. H. Modification of the responses of sheep to isolation stress by the use of mirror panels. *Appl Anim Behav Sci* **19**, 331–338 (1988).

443. Begni, V. *et al.* Social isolation in rats: Effects on animal welfare and molecular markers for neuroplasticity. *PLoS One* **15**, e0240439 (2020).

444. Clemenza, K. *et al.* Social isolation is closely linked to a marked reduction in physical activity in male mice. *J Neurosci Res* **99**, 1099–1107 (2021).

445. Cacioppo, S., Grippo, A. J., London, S., Goossens, L. & Cacioppo, J. T. Loneliness: Clinical Import and Interventions. *Perspectives on Psychological Science* **10**, 238–249 (2015).

446. Cacioppo, J. T. *et al.* Loneliness within a nomological net: An evolutionary perspective. *J Res Pers* **40**, 1054–1085 (2006).

447. Ieraci, A., Mallei, A. & Popoli, M. Social Isolation Stress Induces Anxious-Depressive-Like Behavior and Alterations of Neuroplasticity-Related Genes in Adult Male Mice. *Neural Plast* **2016**, (2016).

448. Golla, A., Østby, H. & Kermen, F. Chronic unpredictable stress induces anxiety-like behaviors in young zebrafish. *Sci Rep* **10**, 1–10 (2020).

449. Forsatkar, M. N., Safari, O. & Boiti, C. Effects of social isolation on growth, stress response, and immunity of zebrafish. *Acta Ethol* **20**, 255–261 (2017).

450. Giacomini, A. C. V. V. *et al.* My stress, our stress: Blunted cortisol response to stress in isolated housed zebrafish. *Physiol Behav* **139**, 182–187 (2015).

451. Yusufishaq, S. & Rosenkranz, J. A. Post-weaning Social Isolation Impairs Observational Fear Conditioning. *Behavioural brain research* **242**, 142 (2013).

452. Adolphs, R. The biology of fear. *Current Biology* vol. 23 R79 Preprint at https://doi.org/10.1016/j.cub.2012.11.055 (2013).

453. Kaler, S. R. & Freeman, B. J. Analysis of Environmental Deprivation: Cognitive and Social Development in Romanian Orphans. *Journal of Child Psychology and Psychiatry* **35**, 769–781 (1994).

454. Kumsta, R. *et al.* 5HTT genotype moderates the influence of early institutional deprivation on emotional problems in adolescence: Evidence from the English and Romanian Adoptee (ERA) study. *J Child Psychol Psychiatry* **51**, 755–762 (2010).

455. Lukkes, J. L., Mokin, M. v., Scholl, J. L. & Forster, G. L. Adult rats exposed to early-life social isolation exhibit increased anxiety and conditioned fear behavior, and altered hormonal stress responses. *Horm Behav* **55**, 248–256 (2009).

456. Amiri, S. *et al.* Protective effects of gabapentin against the seizure susceptibility and comorbid behavioral abnormalities in the early socially isolated mice. *Eur J Pharmacol* **797**, 106–114 (2017).

457. Yasuda, H. *et al.* Artificially reared mice exhibit anxiety-like behavior in adulthood. *Exp Anim* **65**, 267–274 (2016).

458. Lapiz, M. D. S., Mateo, Y., Parker, T. & Marsden, C. Effects of noradrenaline depletion in the brain on response to novelty in isolation-reared rats. *Psychopharmacology (Berl)* **152**, 312–320 (2000).

459. Wilkinson, L. S. *et al.* Social isolation in the rat produces developmentally specific deficits in prepulse inhibition of the acoustic startle response without disrupting latent inhibition. *Neuropsychopharmacology* **10**, 61–72 (1994).

460. Dalrymple-Alford, J. C. & Benton, D. Activity differences of individually and group-housed male and female rats. *Anim Learn Behav* **9**, 50–55 (1981).

461. Levine, J. B. *et al.* Isolation rearing impairs wound healing and is associated with increased locomotion and decreased immediate early gene expression in the medial prefrontal cortex of juvenile rats. *Neuroscience* **151**, 589–603 (2008).

462. Hur, J. *et al.* Anxiety and the neurobiology of temporally uncertain threat anticipation. *Journal of Neuroscience* **40**, 7949–7964 (2020).

463. Maximino, C. *et al.* Fingerprinting of psychoactive drugs in zebrafish anxiety-like behaviors. *PLoS One* **9**, 103943 (2014).

464. Collymore, C., Tolwani, R. J. & Rasmussen, S. The behavioral effects of single housing and environmental enrichment on adult zebrafish (Danio rerio). *Journal of the American Association for Laboratory Animal Science* **54**, 280–285 (2015).

465. Lindsey, B. W. & Tropepe, V. Changes in the social environment induce neurogenic plasticity predominantly in niches residing in sensory structures of the zebrafish brain independently of cortisol levels. *Dev Neurobiol* **74**, 1053–1077 (2014).

466. Rambo, C. L. *et al.* Gender differences in aggression and cortisol levels in zebrafish subjected to unpredictable chronic stress. *Physiol Behav* **171**, 50–54 (2017).

467. Westerfield, M. *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 5th Edition*. (University of Oregon Press, Eugene, 2007).

468. Zellner, D., Padnos, B., Hunter, D. L., MacPhail, R. C. & Padilla, S. Rearing conditions differentially affect the locomotor behavior of larval zebrafish, but not their response to valproate-induced developmental neurotoxicity. *Neurotoxicol Teratol* **33**, 674–679 (2011).

469. Parker, M. O., Millington, M. E., Combe, F. J. & Brennan, C. H. Housing conditions differentially affect physiological and behavioural stress responses of zebrafish, as well as the response to anxiolytics. *PLoS One* **7**, e34992 (2012).

470. Tomova, L. *et al.* Acute social isolation evokes midbrain craving responses similar to hunger. *Nat Neurosci* **23**, 1597–1605 (2020).

471. Budaev, S. v., Zworykin, D. D. & Mochek, A. D. Consistency of individual differences in behaviour of the lion-headed cichlid, Steatocranus casuarius. *Behavioural Processes* **48**, 49–55 (1999).

472. Sloan Wilson, D., Clark, A. B., Coleman, K. & Dearstyne, T. Shyness and boldness in humans and other animals. *Trends Ecol Evol* **9**, 442–446 (1994).

473. Wilson, D. S., Coleman, K., Clark, A. B. & Biederman, L. Shy-bold continuum in pumpkinseed sunfish (Lepomis gibbosus): an ecological study of a psychological trait. *J Comp Psychol* **107**, 250–260 (1993).

474. Geng, Y. & Peterson, R. T. The zebrafish subcortical social brain as a model for studying social behavior disorders. *DMM Disease Models and Mechanisms* vol. 12 Preprint at https://doi.org/10.1242/dmm.039446 (2019).

475. Oliveira, R. F. Mind the fish: Zebrafish as a model in cognitive social neuroscience. *Frontiers in Neural Circuits* vol. 7 131 Preprint at https://doi.org/10.3389/fncir.2013.00131 (2013).

476. de Polavieja, G. G. & Orger, M. B. Social Behavior: A Neural Circuit for Social Behavior in Zebrafish. *Current Biology* vol. 28 R828–R830 Preprint at https://doi.org/10.1016/j.cub.2018.06.065 (2018).

477. O'Connell, L. A. & Hofmann, H. A. Evolution of a vertebrate social decision-making network. *Science (1979)* **336**, 1154–1157 (2012).

478. Goodson, J. L. The vertebrate social behavior network: Evolutionary themes and variations. *Horm Behav* **48**, 11–22 (2005).

479. Facciol, A. & Gerlai, R. Zebrafish Shoaling, Its Behavioral and Neurobiological Mechanisms, and Its Alteration by Embryonic Alcohol Exposure: A Review. *Frontiers in Behavioral Neuroscience* vol. 14 174 Preprint at https://doi.org/10.3389/fnbeh.2020.572175 (2020).

480. Brandl, H. B., Farine, D. R., Funghi, C., Schuett, W. & Griffith, S. C. Early-life social environment predicts social network position in wild zebra finches. *Proceedings of the Royal Society B: Biological Sciences* **286**, (2019).

481. O'Connell, L. A. & Hofmann, H. A. Genes, hormones, and circuits: An integrative approach to study the evolution of social behavior. *Frontiers in Neuroendocrinology* vol. 32 320–335 Preprint at https://doi.org/10.1016/j.yfrne.2010.12.004 (2011).

482. Schalbroeck, R. *et al.* Striatal dopamine synthesis capacity in autism spectrum disorder and its relation with social defeat: an [18F]-FDOPA PET/CT study. *Transl Psychiatry* **11**, 1–10 (2021).

483. Heinrichs, M., von Dawans, B. & Domes, G. Oxytocin, vasopressin, and human social behavior. *Frontiers in Neuroendocrinology* Preprint at https://doi.org/10.1016/j.yfrne.2009.05.005 (2009).

484. Engeszer, R. E., Ryan, M. J. & Parichy, D. M. Learned social preference in zebrafish. *Current Biology* **14**, 881–884 (2004).

485. Fernandes, Y. & Gerlai, R. Long-term behavioral changes in response to early developmental exposure to ethanol in Zebrafish. *Alcohol Clin Exp Res* **33**, 601–609 (2009).

486. Scerbina, T., Chatterjee, D. & Gerlai, R. Dopamine receptor antagonism disrupts social preference in zebrafish: A strain comparison study. *Amino Acids* **43**, 2059–2072 (2012).

487. Beherec, L. *et al.* Retrospective review of clozapine in the treatment of patients with autism spectrum disorder and severe disruptive behaviors. *J Clin Psychopharmacol* **31**, 341–344 (2011).

488. Marcus, R. N. *et al.* Safety and tolerability of aripiprazole for irritability in pediatric patients with autistic disorder: A 52-week, open-label, multicenter study. *Journal of Clinical Psychiatry* **72**, 1270–1276 (2011).

489. LeClerc, S. & Easley, D. Pharmacological therapies for autism spectrum disorder: A review. *P and T* **40**, 389–397 (2015).

490. Kiser, D., SteemerS, B., Branchi, I. & Homberg, J. R. The reciprocal interaction between serotonin and social behaviour. *Neuroscience and Biobehavioral Reviews* vol. 36 786–798 Preprint at https://doi.org/10.1016/j.neubiorev.2011.12.009 (2012).

491. Ellis, B. J., Boyce, W. T., Belsky, J., Bakermans-Kranenburg, M. J. & van Ijzendoorn, M. H. Differential susceptibility to the environment: An evolutionary- neurodevelopmental theory. *Dev Psychopathol* **23**, 7–28 (2011).

492. Way, B. M. & Taylor, S. E. Social influences on health: Is serotonin a critical mediator? *Psychosomatic Medicine* vol. 72 107–112 Preprint at https://doi.org/10.1097/PSY.0b013e3181ce6a7d (2010).

493. Cools, R., Nakamura, K. & Daw, N. D. Serotonin and dopamine: Unifying affective, activational, and decision functions. *Neuropsychopharmacology* vol. 36 98–113 Preprint at https://doi.org/10.1038/npp.2010.121 (2011).

494. Cools, R., Roberts, A. C. & Robbins, T. W. Serotoninergic regulation of emotional and behavioural control processes. *Trends in Cognitive Sciences* vol. 12 31–40 Preprint at https://doi.org/10.1016/j.tics.2007.10.011 (2008).

495. Torales, J., O'Higgins, M., Castaldelli-Maia, J. M. & Ventriglio, A. The outbreak of COVID-19 coronavirus and its impact on global mental health. *International Journal of Social Psychiatry* vol. 66 317–320 Preprint at https://doi.org/10.1177/0020764020915212 (2020).

496. Vardanyan, R. S. & Hruby, V. J. Anxiolytics (Tranquilizers). in *Synthesis of Essential Drugs* 69–82 (Elsevier, 2006). doi:10.1016/b978-044452166-8/50005-4.

497. Schneier, F. R. *et al.* Buspirone in social phobia. *J Clin Psychopharmacol* **13**, 251–256 (1993).

498. Wong, R. Y., Oxendine, S. E. & Godwin, J. Behavioral and neurogenomic transcriptome changes in wild-derived zebrafish with fluoxetine treatment. *BMC Genomics* **14**, 1–13 (2013).

499. Theodoridi, A., Tsalafouta, A. & Pavlidis, M. Acute exposure to fluoxetine alters aggressive behavior of zebrafish and expression of genes involved in serotonergic system regulation. *Front Neurosci* **11**, 223 (2017).

500. Maaswinkel, H., Zhu, L. & Weng, W. The immediate and the delayed effects of buspirone on zebrafish (Danio rerio) in an open field test: A 3-D approach. *Behavioural Brain Research* **234**, 365–374 (2012).

501. Gebauer, D. L. *et al.* Effects of anxiolytics in zebrafish: Similarities and differences between benzodiazepines, buspirone and ethanol. *Pharmacol Biochem Behav* **99**, 480–486 (2011).

502. Vaswani, M., Linda, F. K. & Ramesh, S. Role of selective serotonin reuptake inhibitors in psychiatric disorders: a comprehensive review. *Prog Neuropsychopharmacol Biol Psychiatry* **27**, 85–102 (2003).

503. Theodoridi, A., Tsalafouta, A. & Pavlidis, M. Acute exposure to fluoxetine alters aggressive behavior of zebrafish and expression of genes involved in serotonergic system regulation. *Front Neurosci* **11**, 223 (2017).

504. Frick, A. *et al.* Serotonin synthesis and reuptake in social anxiety disorder a positron emission tomography study. *JAMA Psychiatry* **72**, 794–802 (2015).

505. Herrera, D. G. & Robertson, H. A. Activation of c-fos in the brain. *Progress in Neurobiology* vol. 50 83–107 Preprint at https://doi.org/10.1016/S0301-0082(96)00021-4 (1996).

506. O'Connell, L. A. & Hofmann, H. A. The Vertebrate mesolimbic reward system and social behavior network: A comparative synthesis. *Journal of Comparative Neurology* vol. 519 3599–3639 Preprint at https://doi.org/10.1002/cne.22735 (2011).

507. Kaslin, J. & Panula, P. Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (Danio rerio). *Journal of Comparative Neurology* (2001) doi:10.1002/cne.1390.

508. Filippi, A., Mahler, J., Schweitzer, J. & Driever, W. Expression of the paralogous tyrosine hydroxylase encoding genes th1 and th2 reveals the full complement of dopaminergic and noradrenergic neurons in zebrafish larval and juvenile brain. *J Comp Neurol* **518**, 423 (2010).

509. Lillesaar, C. The serotonergic system in fish. *Journal of Chemical Neuroanatomy* vol. 41 294–308 Preprint at https://doi.org/10.1016/j.jchemneu.2011.05.009 (2011).

510. Hawkley, L. C. & Capitanio, J. P. Perceived social isolation, evolutionary fitness and health outcomes: A lifespan approach. *Philosophical Transactions of the Royal Society B: Biological Sciences* vol. 370 Preprint at https://doi.org/10.1098/rstb.2014.0114 (2015).

511. McDowell, A. L., Dixon, L. J., Houchins, J. D. & Bilotta, J. Visual processing of the zebrafish optic tectum before and after optic nerve damage. *Vis Neurosci* **21**, 97–106 (2004).

512. Ziv, Y. *et al.* Long-term dynamics of CA1 hippocampal place codes. *Nat Neurosci* **16**, 264–266 (2013).

513. Wee, C. L. *et al.* Zebrafish oxytocin neurons drive nocifensive behavior via brainstem premotor targets. *Nat Neurosci* **22**, 1477–1492 (2019).

514. Norton, W. H. J., Folchert, A. & Bally-Cuif, L. Comparative analysis of serotonin receptor (HTR1A/HTR1B families) and transporter (slc6a4a/b) gene expression in the zebrafish brain. *Journal of Comparative Neurology* (2008) doi:10.1002/cne.21831.

515. Peeters, B. W. M. M., Moeskops, M. & Veenvliet, A. R. J. Color Preference in Danio rerio: Effects of Age and Anxiolytic Treatments. *Zebrafish* **13**, 330–334 (2016).

516. Chin, J. S. R., Albert, L. T., Loomis, C. L., Keene, A. C. & Duboué, E. R. Behavioral approaches to studying innate stress in Zebrafish. *Journal of Visualized Experiments* **2019**, e59092 (2019).

517. Cacioppo, J. T., Cacioppo, S., Capitanio, J. P. & Cole, S. W. The neuroendocrinology of social isolation. *Annu Rev Psychol* **66**, 733–767 (2015).

518. Vaz, R., Hofmeister, W. & Lindstrand, A. Zebrafish models of neurodevelopmental disorders: Limitations and benefits of current tools and techniques. *International Journal of Molecular Sciences* vol. 20 Preprint at https://doi.org/10.3390/ijms20061296 (2019).

519. Wullimann, M. F., Rupp, B. & Reichert, H. The brain of the zebrafish Danio rerio: a neuroanatomical atlas. in *Neuroanatomy of the Zebrafish Brain* 19–87 (Birkhäuser Basel, 1996). doi:10.1007/978-3-0348-8979-7_5.

520. Bassi, I. *et al.* The zebrafish: An emerging animal model for investigating the hypothalamic regulation of reproduction. *Minerva Endocrinologica* vol. 41 250–265 Preprint at https://www.minervamedica.it/en/journals/minerva-endocrinology/article.php?cod=R07Y2016N02A0250 (2016).

521. Machluf, Y., Gutnick, A. & Levkowitz, G. Development of the zebrafish hypothalamus. *Ann N Y Acad Sci* **1220**, 93–105 (2011).

522. McPherson, A. D. *et al.* Motor Behavior Mediated by Continuously Generated Dopaminergic Neurons in the Zebrafish Hypothalamus Recovers after Cell Ablation. *Current Biology* **26**, 263–269 (2016).

523. Fernandes, A. M. *et al.* Deep brain photoreceptors control light-seeking behavior in zebrafish larvae. *Current Biology* **22**, 2042–2047 (2012).

524. Yokobori, E. *et al.* Stimulatory effect of intracerebroventricular administration of orexin A on food intake in the zebrafish, Danio rerio. *Peptides (N.Y.)* **32**, 1357–1362 (2011).

525. Yokobori, E. *et al.* Neuropeptide Y Stimulates Food Intake in the Zebrafish, Danio rerio. *J Neuroendocrinol* **24**, 766–773 (2012).

526. Chiu, C. N. & Prober, D. A. Regulation of zebrafish sleep and arousal states: Current and prospective approaches. *Front Neural Circuits* **7**, (2013).

527. Yamamoto, M. *et al.* Mib-Jag1-Notch signalling regulates patterning and structural roles of the notochord by controlling cell-fate decisions. *Development* **137**, 2527–2537 (2010).

528. Scerbina, T., Chatterjee, D. & Gerlai, R. Dopamine receptor antagonism disrupts social preference in zebrafish: A strain comparison study. *Amino Acids* **43**, 2059–2072 (2012).

529. Backström, T. & Winberg, S. Serotonin coordinates responses to social stress-What we can learn from fish. *Frontiers in Neuroscience* Preprint at https://doi.org/10.3389/fnins.2017.00595 (2017).

530. Teles, R., Teles, F., Frias-Lopez, J., Paster, B. & Haffajee, A. Lessons learned and unlearned in periodontal microbiology. *Periodontol 2000* **62**, 95–162 (2013).

531. Filippi, M. *et al.* The brain functional networks associated to human and animal suffering differ among omnivores, vegetarians and vegans. *PLoS One* **5**, 10847 (2010).

532. Ehlert, U., Gaab, J. & Heinrichs, M. Psychoneuroendocrinological contributions to the etiology of depression, posttraumatic stress disorder, and stress-related bodily disorders: The role of the hypothalamus-pituitary-adrenal axis. *Biol Psychol* **57**, 141–152 (2001).

533. Tan, O., Martin, L. J. & Bowen, M. T. Divergent pathways mediate 5-HT1A receptor agonist effects on close social interaction, grooming and aggressive behaviour in mice: Exploring the involvement of the oxytocin and vasopressin systems. *Journal of Psychopharmacology* **34**, 795–805 (2020).

534. Mumtaz, F., Khan, M. I., Zubair, M. & Dehpour, A. R. Neurobiology and consequences of social isolation stress in animal model—A comprehensive review. *Biomedicine and Pharmacotherapy* vol. 105 1205–1222 Preprint at https://doi.org/10.1016/j.biopha.2018.05.086 (2018).

535. Thomas, J., Khanam, R. & Vohora, D. A validated HPLC-UV method and optimization of sample preparation technique for norepinephrine and serotonin in mouse brain. *http://dx.doi.org/10.3109/13880209.2014.991837* **53**, 1539–1544 (2015).

536. Chatterjee, D. & Gerlai, R. High precision liquid chromatography analysis of dopaminergic and serotoninergic responses to acute alcohol exposure in zebrafish. *Behavioural brain research* **200**, 208 (2009).

537. Terbeck, S., Savulescu, J., Chesterman, L. P. & Cowen, P. J. Noradrenaline effects on social behaviour, intergroup relations, and moral decisions. *Neuroscience and Biobehavioral Reviews* vol. 66 54–60 Preprint at https://doi.org/10.1016/j.neubiorev.2016.03.031 (2016).

538. Pizza, F., Magnani, M., Indrio, C. & Plazzi, G. The hypocretin system and psychiatric disorders. *Curr Psychiatry Rep* **16**, 1–10 (2014).

539. Panula, P. *et al.* The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases. *Neurobiology of Disease* vol. 40 46–57 Preprint at https://doi.org/10.1016/j.nbd.2010.05.010 (2010).

540. Folgueira, M., Anadón, R. & Yáñez, J. Experimental study of the connections of the telencephalon in the rainbow trout (Oncorhynchus mykiss). II: Dorsal area and preoptic region. *Journal of Comparative Neurology* **480**, 204–233 (2004).

541. Tsuneoka, Y. & Funato, H. Cellular Composition of the Preoptic Area Regulating Sleep, Parental, and Sexual Behavior. *Frontiers in Neuroscience* vol. 15 327 Preprint at https://doi.org/10.3389/fnins.2021.649159 (2021).

542. Carreño Gutiérrez, H. *et al.* Endothelin neurotransmitter signalling controls zebrafish social behaviour. *Sci Rep* **9**, 1–17 (2019).

543. Herget, U., Gutierrez-Triana, J. A., Thula, O. S., Knerr, B. & Ryu, S. Single-cell reconstruction of oxytocinergic neurons reveals separate hypophysiotropic and encephalotropic subtypes in larval zebrafish. *eNeuro* **4**, 278–294 (2017).

544. Larson, E. T., O'Malley, D. M. & Melloni, R. H. Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. *Behavioural Brain Research* **167**, 94–102 (2006).

545. Turner, K. J. *et al.* Afferent Connectivity of the Zebrafish Habenulae. *Front Neural Circuits* **10**, (2016).

546. Herget, U. & Ryu, S. Coexpression analysis of nine neuropeptides in the neurosecretory preoptic area of larval zebrafish. *Front Neuroanat* **9**, 1–11 (2015).

547. Dölen, G., Darvishzadeh, A., Huang, K. W. & Malenka, R. C. Social reward requires coordinated activity of nucleus accumbens oxytocin and serotonin. *Nature* **501**, 179–184 (2013).

548. Langen, E. M. A., Lindeyer, C. M., Reader, S. M. & Swaney, W. T. Nonapeptide influences on social behaviour: Effects of vasotocin and isotocin on shoaling and interaction in zebrafish. *Behaviour* **152**, 897–915 (2015).

549. Pouso, P., Cabana, Á., Goodson, J. L. & Silva, A. Preoptic Area Activation and Vasotocin Involvement in the Reproductive Behavior of a Weakly Pulse-Type Electric Fish, Brachyhypopomus gauderio. *Front Integr Neurosci* **13**, 37 (2019).

550. Stowe, J. R., Liu, Y., Curtis, J. T., Freeman, M. E. & Wang, Z. Species differences in anxiety-related responses in male prairie and meadow voles: The effects of social isolation. *Physiol Behav* **86**, 369–378 (2005).

551. McHenry, J. A. *et al.* Hormonal gain control of a medial preoptic area social reward circuit. *Nat Neurosci* **20**, 449–458 (2017).

552. Caba, M., Melo, A. I., Fleming, A. & Meza, E. Maternal care activates the ventral tegmental area but not dopaminergic cells in the rat. *J Neuroendocrinol* **31**, (2019).

553. Pecknold, J., Luthe, L., Munjack, D. & Alexander, P. A double-blind, placebo-controlled, multicenter study: With alprazolam and extended-release alprazolam in: The treatment of panic disorder. *J Clin Psychopharmacol* **14**, 314–321 (1994).

554. Aswar, U., Shende, H. & Aswar, M. Buspirone, a 5-HT1A agonist attenuates social isolation-induced behavior deficits in rats: a comparative study with fluoxetine. *Behavioural Pharmacology* **33**, 309–321 (2022).

555. Heap, L. A. *et al.* Hypothalamic projections to the optic tectum in larval zebrafish. *Front Neuroanat* **11**, 135 (2018).

556. Wang, Y. C., Bianciardi, M., Chanes, L. & Satpute, A. B. Ultra High Field fMRI of Human Superior Colliculi Activity during Affective Visual Processing. *Sci Rep* **10**, 1–7 (2020).

557. de Arriba, M. D. C. & Pombal, M. A. Afferent connections of the optic tectum in lampreys: An experimental study. *Brain Behav Evol* **69**, 37–68 (2006).

558. Muto, A. *et al.* Activation of the hypothalamic feeding centre upon visual prey detection. *Nat Commun* **8**, 1–10 (2017).

559. Zupanc, G. K. H. & Sîrbulescu, R. F. Adult neurogenesis and neuronal regeneration in the central nervous system of teleost fish. *European Journal of Neuroscience* **34**, 917–929 (2011).

560. Fiebig, E., Ebbesson, S. O. E. & Meyer, D. L. Afferent connections of the optic tectum in the piranha (Serrasalmus nattereri). *Cell Tissue Res* **231**, 55–72 (1983).

561. Orger, M. B. The Cellular Organization of Zebrafish Visuomotor Circuits. *Current Biology* vol. 26 R377–R385 Preprint at https://doi.org/10.1016/j.cub.2016.03.054 (2016).

562. Filosa, A., Barker, A. J., Dal Maschio, M. & Baier, H. Feeding State Modulates Behavioral Choice and Processing of Prey Stimuli in the Zebrafish Tectum. *Neuron* **90**, 596–608 (2016).

563. Yilmaz, M. & Meister, M. Rapid innate defensive responses of mice to looming visual stimuli. *Current Biology* **23**, 2011–2015 (2013).

564. Bianco, I. H., Kampff, A. R. & Engert, F. Prey capture behavior evoked by simple visual stimuli in larval zebrafish. *Front Syst Neurosci* **0**, 101 (2011).

565. Suzuki, D. G., Pérez-Fernández, J., Wibble, T., Kardamakis, A. A. & Grillner, S. The role of the optic tectum for visually evoked orienting and evasive movements. *Proc Natl Acad Sci U S A* **116**, 15272–15281 (2019).

566. Ioannou, C. C., Guttal, V. & Couzin, I. D. Predatory fish select for coordinated collective motion in virtual prey. *Science (1979)* **337**, 1212–1215 (2012).

567. Li, W. *et al.* Chronic Social Isolation Signals Starvation in the Drosophila Brain and Reduces Sleep. *Nature 2021* (under review) (2021) doi:10.1038/s41586-021-03837-0.

568. Zhao, Z., Zhao, X. & Veasey, S. C. Neural consequences of chronic short sleep: Reversible or lasting? *Frontiers in Neurology* vol. 8 1 Preprint at https://doi.org/10.3389/fneur.2017.00235 (2017).

569. Begni, V. *et al.* Social isolation in rats: Effects on animal welfare and molecular markers for neuroplasticity. *PLoS One* **15**, e0240439 (2020).

570. Choi, J. H. *et al.* Targeted knockout of a chemokine-like gene increases anxiety and fear responses. *Proc Natl Acad Sci U S A* **115**, E1041–E1050 (2018).

571. Clark, D. B. & Agras, W. S. The assessment and treatment of performance anxiety in musicians. in *American Journal of Psychiatry* vol. 148 598–605 (1991).

572. Gammans, R. E. *et al.* Use of buspirone in patients with generalized anxiety disorder and coexisting depressive symptoms: A meta-analysis of eight randomized, controlled studies. *Neuropsychobiology* **25**, 193–201 (1992).

573. Rabatin, J. & Keltz, L. B. Generalized anxiety and panic disorder. *West J Med* **176**, 164–168 (2002).

574. Majercsik, E. *et al.* The effect of social factors on the anxiolytic efficacy of buspirone in male rats, male mice, and men. in *Progress in Neuro-Psychopharmacology and Biological Psychiatry* vol. 27 1187–1199 (Elsevier, 2003).

575. McMillen, B. A. & McDonald, C. C. Selective effects of buspirone and molindone on dopamine metabolism and function in the striatum and frontal cortex of the rat. *Neuropharmacology* **22**, 273–278 (1983).

576. Loane, C. & Politis, M. Buspirone: What is it all about? *Brain Research* vol. 1461 111–118 Preprint at https://doi.org/10.1016/j.brainres.2012.04.032 (2012).

577. Patel, S. & Hillard, C. J. Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: Further evidence for an anxiolytic role for endogenous cannabinoid signaling. *Journal of Pharmacology and Experimental Therapeutics* **318**, 304–311 (2006).

578. Lalonde, R. & Strazielle, C. Relations between open-field, elevated plus-maze, and emergence tests in C57BL/6J and BALB/c mice injected with GABA- and 5HT-anxiolytic agents: ORIGINAL ARTICLE. *Fundam Clin Pharmacol* **24**, 365–376 (2010).

579. File, S. E. & Seth, P. A review of 25 years of the social interaction test. *European Journal of Pharmacology* vol. 463 35–53 Preprint at https://doi.org/10.1016/S0014-2999(03)01273-1 (2003).

580. Gould, G. G. *et al.* Density and function of central serotonin (5-HT) transporters, 5-HT 1A and 5-HT2A receptors, and effects of their targeting on BTBR T+tf/J mouse social behavior. *J Neurochem* **116**, 291–303 (2011).

581. van Vliet, I. M., den Boer, J. A., Westenberg, H. G. M. & Ho Pian, K. L. Clinical effects of buspirone in social phobia: A double-blind placebo- controlled study. *Journal of Clinical Psychiatry* **58**, 164–168 (1997).

582. Barba-Escobedo, P. A. & Gould, G. G. Visual social preferences of lone zebrafish in a novel environment: Strain and anxiolytic effects. *Genes Brain Behav* **11**, 366–373 (2012).

583. Maaswinkel, H., Le, X., He, L., Zhu, L. & Weng, W. Dissociating the effects of habituation, black walls, buspirone and ethanol on anxiety-like behavioral responses in shoaling zebrafish. A 3D approach to social behavior. *Pharmacol Biochem Behav* **108**, 16–27 (2013).

584. Gebauer, D. L. *et al.* Effects of anxiolytics in zebrafish: Similarities and differences between benzodiazepines, buspirone and ethanol. *Pharmacol Biochem Behav* **99**, 480–486 (2011).

585. Varga, Z. K. *et al.* The swimming plus-maze test: a novel high-throughput model for assessment of anxiety-related behaviour in larval and juvenile zebrafish (Danio rerio). *Sci Rep* **8**, (2018).

586. Haller, J., Baranyi, J., Bakos, N. & Halász, J. Social instability in female rats: Effects on anxiety and buspirone efficacy. *Psychopharmacology (Berl)* **174**, 197–202 (2004).

587. Wright, I. K., Ismail, H., Upton, N. & Marsden, C. A. Effect of isolation rearing on 5-HT agonist-induced responses in the rat. *Psychopharmacology (Berl)* **105**, 259–263 (1991).

588. Fone, K. C. F., Shalders, K., Fox, Z. D., Arthur, R. & Marsden, C. A. Increased 5-HT2C receptor responsiveness occurs on rearing rats in social isolation. *Psychopharmacology (Berl)* **123**, 346–352 (1996).

589. Motta, V., Maisonnette, S., Morato, S., Castrechini, P. & Brandão, M. L. Effects of blockade of 5-HT2 receptors and activation of 5-HT1A receptors on the exploratory activity of rats in the elevated plus-maze. *Psychopharmacology (Berl)* **107**, 135–139 (1992).

590. Haller, J., Halász, J. & Makara, G. B. Housing conditions and the anxiolytic efficacy of buspirone: The relationship between main and side effects. *Behavioural Pharmacology* **11**, 403–412 (2000).

591. Rush, C. R., Critchfield, T. S., Troisi, J. R. & Griffiths, R. R. DISCRIMINATIVE STIMULUS EFFECTS OF DIAZEPAM AND BUSPIRONE IN NORMAL VOLUNTEERS. *J Exp Anal Behav* **63**, 277–294 (1995).

592. Murasaki, M. *et al.* Phase I study of a new antianxiety drug, buspirone. *Prog Neuropsychopharmacol Biol Psychiatry* **13**, 137–144 (1989).

593. Bond, A. J. & Lader, M. H. Comparative effects of diazepam and buspirone on subjective feelings, psychological tests and the EEG. *Int Pharmacopsychiatry* **16**, 212–220 (1981).

594. Shahar-Gold, H., Gur, R. & Wagner, S. Rapid and Reversible Impairments of Short- and Long-Term Social Recognition Memory Are Caused by Acute Isolation of Adult Rats via Distinct Mechanisms. *PLoS One* **8**, (2013).

595. Varga, Z. K. *et al.* Conserved Serotonergic Background of Experience-Dependent Behavioral Responsiveness in Zebrafish (Danio rerio). *Journal of Neuroscience* **40**, 4551–4564 (2020).

596. House, J. S., Landis, K. R. & Umberson, D. Social Relationships and Health. *Science (1979)* **241**, 540–545 (1988).

597. Hagerty, B. M. & Williams, &NA; A. The Effects of Sense of Belonging, Social Support, Conflict, and Loneliness on Depression. *Nurs Res* **48**, 215–219 (1999).

598. Holt-Lunstad, J., Smith, T. B. & Layton, J. B. Social Relationships and Mortality Risk: A Meta-analytic Review. *PLoS Med* **7**, e1000316 (2010).

599. Holt-Lunstad, J., Smith, T. B., Baker, M., Harris, T. & Stephenson, D. Loneliness and Social Isolation as Risk Factors for Mortality. *Perspectives on Psychological Science* **10**, 227–237 (2015).

600. Jones, W. H., Hobbs, S. A. & Hockenbury, D. Loneliness and social skill deficits. *J Pers Soc Psychol* **42**, 682–689 (1982).

601. Li, W. *et al.* Chronic social isolation signals starvation and reduces sleep in Drosophila. *Nature* **597**, 239–244 (2021).

602. Hawkley, L. C., Preacher, K. J. & Cacioppo, J. T. Loneliness impairs daytime functioning but not sleep duration. *Health Psychology* **29**, 124–129 (2010).

603. Kurina, L. M. *et al.* Loneliness Is Associated with Sleep Fragmentation in a Communal Society. *Sleep* **34**, 1519–1526 (2011).

604. Cacioppo, J. T. & Hawkley, L. C. Perceived social isolation and cognition. *Trends Cogn Sci* **13**, 447–454 (2009).

605. Brandão, M. L., Braithwaite, V. A. & Gonçalves-de-Freitas, E. Isolation impairs cognition in a social fish. *Appl Anim Behav Sci* **171**, 204–210 (2015).

606. Cravo, A. M. *et al.* Time experience during social distancing: A longitudinal study during the first months of COVID-19 pandemic in Brazil. *Sci Adv* **8**, 7205 (2022).

607. Chen, Z. C. *et al.* The Impact of the COVID-19 Pandemic and Lockdown on Mild Cognitive Impairment, Alzheimer's Disease and Dementia With Lewy Bodies in China: A 1-Year Follow-Up Study. *Front Psychiatry* **12**, (2021).

608. Hughes, M. E., Waite, L. J., Hawkley, L. C. & Cacioppo, J. T. A Short Scale for Measuring Loneliness in Large Surveys. *Res Aging* **26**, 655–672 (2004).

609. Russell, D., Peplau, L. A. & Cutrona, C. E. The revised UCLA Loneliness Scale: Concurrent and discriminant validity evidence. *J Pers Soc Psychol* **39**, 472–480 (1980).

610. Shiovitz-Ezra, S. & Ayalon, L. Use of Direct Versus Indirect Approaches to Measure Loneliness in Later Life. *Res Aging* **34**, 572–591 (2012).

611. Qualter, P. *et al.* Loneliness across the life span. *Perspect Psychol Sci* **10**, 250–264 (2015).

612. Byrne, C., Salas, C. E., Coetzer, R. & Ramsey, R. Understanding Loneliness in Brain Injury: Linking the Reaffiliation Motive Model of Loneliness With a Model of Executive Impairment. *Front Integr Neurosci* **16**, 66 (2022).

613. Koç, H., Assist, R. & Arslan, C. The Mediating Role of Loneliness in the Relationship Between Maladaptive Thinking Styles and Emotional Expressivity. *Psycho-Educational Research Reviews* **11**, 93–107 (2022).

614. Tomova, L. *et al.* Acute social isolation evokes midbrain craving responses similar to hunger. *Nat Neurosci* **23**, 1597–1605 (2020).

615. Ingram, J., Hand, C. J. & Maciejewski, G. Social isolation during COVID-19 lockdown impairs cognitive function. *Appl Cogn Psychol* **35**, 935–947 (2021).

616. Matthews, G. A. *et al.* Dorsal Raphe Dopamine Neurons Represent the Experience of Social Isolation. *Cell* **164**, 617–631 (2016).

617. Kotwal, A. A. *et al.* A single question assessment of loneliness in older adults during the COVID -19 pandemic: A nationally-representative study. *J Am Geriatr Soc* **70**, 1342–1345 (2022).

618. Victor, C., Scambler, S., Bond, J. & Bowling, A. Loneliness in later life: Preliminary findings from the Growing Older project. *Qual Ageing Older Adults* **3**, 34–41 (2002).

619. Thurston, R. C. & Kubzansky, L. D. Women, Loneliness, and Incident Coronary Heart Disease. *Psychosom Med* **71**, 836–842 (2009).

620. Shiovitz-Ezra, S. & Ayalon, L. Situational versus chronic loneliness as risk factors for all-cause mortality. *Int Psychogeriatr* **22**, 455–462 (2010).

621. Savikko, N., Routasalo, P., Tilvis, R. S., Strandberg, T. E. & Pitkälä, K. H. Predictors and subjective causes of loneliness in an aged population. *Arch Gerontol Geriatr* **41**, 223–233 (2005).

622. Routasalo, P. E., Savikko, N., Tilvis, R. S., Strandberg, T. E. & Pitkälä, K. H. Social Contacts and Their Relationship to Loneliness among Aged People – A Population-Based Study. *Gerontology* **52**, 181–187 (2006).

623. Fees, B. S., Martin, P. & Poon, L. W. A Model of Loneliness in Older Adults. *J Gerontol B Psychol Sci Soc Sci* **54B**, P231–P239 (1999).

624. Jylhä, M. Old Age and Loneliness: Cross-sectional and Longitudinal Analyses in the Tampere Longitudinal Study on Aging. *Can J Aging* **23**, 157–168 (2004).

625. Lau, S. & Gruen, G. E. The Social Stigma of Loneliness: Effect of Target Person's and Perceiver's Sex: *http://dx.doi.org/10.1177/0146167292182009* **18**, 182–189 (2016).

626. Kerr, N. A. & Stanley, T. B. Revisiting the social stigma of loneliness. *Pers Individ Dif* **171**, 110482 (2021).

627. Victor, C., Grenade, L. & Boldy, D. Measuring loneliness in later life: a comparison of differing measures. *Rev Clin Gerontol* **15**, 63–70 (2005).

628. Russell, D., Peplau, L. A. & Cutrona, C. E. The revised UCLA Loneliness Scale: Concurrent and discriminant validity evidence. *J Pers Soc Psychol* **39**, 472–480 (1980).

629. Russell, D. W. UCLA Loneliness Scale (Version 3): Reliability, Validity, and Factor Structure. *J Pers Assess* **66**, 20–40 (1996).

630. de Jong-Gierveld, J. & Kamphuls, F. The Development of a Rasch-Type Loneliness Scale. *Appl Psychol Meas* **9**, 289–299 (1985).

631. Deckx, L., van den Akker, M. & Buntinx, F. Risk factors for loneliness in patients with cancer: A systematic literature review and meta-analysis. *European Journal of Oncology Nursing* **18**, 466–477 (2014).

632. VanderWeele, T. J., Hawkley, L. C., Thisted, R. A. & Cacioppo, J. T. A marginal structural model analysis for loneliness: Implications for intervention trials and clinical practice. *J Consult Clin Psychol* **79**, 225–235 (2011).

633. Steptoe, A., Owen, N., Kunz-Ebrecht, S. R. & Brydon, L. Loneliness and neuroendocrine, cardiovascular, and inflammatory stress responses in middle-aged men and women. *Psychoneuroendocrinology* **29**, 593–611 (2004).

634. Hawkley, L. C., Masi, C. M., Berry, J. D. & Cacioppo, J. T. Loneliness is a unique predictor of age-related differences in systolic blood pressure. *Psychol Aging* **21**, 152–164 (2006).

635. Cacioppo, J. T., Hawkley, L. C. & Thisted, R. A. Perceived social isolation makes me sad: 5-year cross-lagged analyses of loneliness and depressive symptomatology in the Chicago Health, Aging, and Social Relations Study. *Psychol Aging* **25**, 453–463 (2010).

636. de Jong Gierveld, J. & van Tilburg, T. The De Jong Gierveld short scales for emotional and social loneliness: tested on data from 7 countries in the UN generations and gender surveys. *Eur J Ageing* **7**, 121–130 (2010).

637. van Baarsen, B., Snijders, T. A. B., Smit, J. H. & van Duijn, M. A. J. Lonely but Not Alone: Emotional Isolation and Social Isolation as Two Distinct Dimensions of Loneliness in Older People. *Educ Psychol Meas* **61**, 119–135 (2001).

638. Tijhuis, M. Changes in and factors related to loneliness in older men. The Zutphen Elderly Study. *Age Ageing* **28**, 491–495 (1999).

639. Marangoni, C. & Ickes, W. Loneliness: A Theoretical Review with Implications for Measurement. *J Soc Pers Relat* **6**, 93–128 (1989).

640. Steptoe, A., Shankar, A., Demakakos, P. & Wardle, J. Social isolation, loneliness, and all-cause mortality in older men and women. *Proc Natl Acad Sci U S A* **110**, 5797–5801 (2013).

641. Barreto, M. *et al.* Loneliness around the world: Age, gender, and cultural differences in loneliness. *Pers Individ Dif* **169**, 110066 (2021).

642. Burns, A., Leavey, G., Ward, M. & O'Sullivan, R. The impact of loneliness on healthcare use in older people: evidence from a nationally representative cohort. *J Public Health (Bangkok)* **30**, 675–684 (2022).

643. Khera, T. & Rangasamy, V. Cognition and Pain: A Review. *Front Psychol* **12**, 1819 (2021).

644. Robbins, T. W. Cognition: The Ultimate Brain Function. *Neuropsychopharmacology* **36**, 1 (2011).

645. Eglit, G. M. L., Palmer, B. W., Martin, A. S., Tu, X. & Jeste, D. v. Loneliness in schizophrenia: Construct clarification, measurement, and clinical relevance. *PLoS One* **13**, e0194021 (2018).

646. Vuilleumier, P. How brains beware: neural mechanisms of emotional attention. *Trends Cogn Sci* **9**, 585–594 (2005).

647. Schupp, H. T. *et al.* Selective Visual Attention to Emotion. *Journal of Neuroscience* **27**, 1082–1089 (2007).

648. Um, E. "Rachel", Plass, J. L., Hayward, E. O. & Homer, B. D. Emotional design in multimedia learning. *J Educ Psychol* **104**, 485–498 (2012).

649. Jung, N., Wranke, C., Hamburger, K. & Knauff, M. How emotions affect logical reasoning: evidence from experiments with mood-manipulated participants, spider phobics, and people with exam anxiety. *Front Psychol* **5**, 570 (2014).

650. Isen, A. M., Daubman, K. A. & Nowicki, G. P. Positive affect facilitates creative problem solving. *J Pers Soc Psychol* **52**, 1122–1131 (1987).

651. Peplau, L. A. Perspective on loneliness. in *Lonelinss: a sourcebook of current theory, research and therapy.* (1982).

652. Manca, R. *et al.* The Impact of Social Isolation Due to COVID-19 on Symptom Progression in People With Dementia: Findings of the SOLITUDE Study. *Front Psychiatry* **13**, 867 (2022).

653. Grove, J. R. & Harry Prapavessis. Preliminary evidence for the reliability and validity of an abbreviated profile of mood states. *Int J Sport Psychol* (1992).

654. Wylie, S. A., Ridderinkhof, K. R., Eckerle, M. K. & Manning, C. A. Inefficient response inhibition in individuals with mild cognitive impairment. *Neuropsychologia* **45**, 1408–1419 (2007).

655. Chatterjee, D., Gavas, R. D., Chakravarty, K., Sinha, A. & Lahiri, U. Evaluating Age-Related Variations of Gaze Behavior for a Novel Digitized-Digit Symbol Substitution Test. *J Eye Mov Res* **12**, 1–15 (2019).

656. Jaeger, J. Digit Symbol Substitution Test. *J Clin Psychopharmacol* **38**, 513–519 (2018).

657. Bechara, A., Damasio, A. R., Damasio, H. & Anderson, S. W. Insensitivity to future consequences following damage to human prefrontal cortex. *Cognition* **50**, 7–15 (1994).

658. Tortello, C. *et al.* Subjective time estimation in Antarctica: The impact of extreme environments and isolation on a time production task. *Neurosci Lett* **725**, 134893 (2020).

659. Yang, C., Potts, R. & Shanks, D. R. The forward testing effect on self-regulated study time allocation and metamemory monitoring. *J Exp Psychol Appl* **23**, 263–277 (2017).

660. Lara, E. *et al.* Are loneliness and social isolation associated with cognitive decline? *Int J Geriatr Psychiatry* **34**, 1613–1622 (2019).

661. Wang, B. & Dong, X. The Association Between Personality and Loneliness: Findings From a Community-Dwelling Chinese Aging Population. *Gerontol Geriatr Med* **4**, 233372141877818 (2018).

662. Losada-Baltar, A. *et al.* Longitudinal Correlates of Loneliness and Psychological Distress During the Lockdown Situation due to COVID-19. Effects of Age and Self-Perceptions of Aging. *The Journals of Gerontology: Series B* **77**, 652–660 (2022).

663. Umlauf, M. G., Bolland, A. C., Bolland, K. A., Tomek, S. & Bolland, J. M. The effects of age, gender, hopelessness, and exposure to violence on sleep disorder symptoms and daytime sleepiness among adolescents in impoverished neighborhoods. *J Youth Adolesc* **44**, 518–542 (2015).

664. Lima, S. L., Rattenborg, N. C., Lesku, J. A. & Amlaner, C. J. Sleeping under the risk of predation. *Anim Behav* **70**, 723–736 (2005).

665. Norris, C. J. & Wu, E. Accentuate the positive, eliminate the negative: Reducing ambivalence through instructed emotion regulation. *Emotion* **21**, 499–512 (2021).

666. Buysse, D. J., Reynolds, C. F., Monk, T. H., Berman, S. R. & Kupfer, D. J. The Pittsburgh sleep quality index: A new instrument for psychiatric practice and research. *Psychiatry Res* **28**, 193–213 (1989).

667. AJILORE, O., STICKGOLD, R., RITTENHOUSE, C. D. & HOBSON, J. A. Nightcap: Laboratory and home-based evaluation of a portable sleep monitor. *Psychophysiology* **32**, 92–98 (1995).

668. Cacioppo, J. T. *et al.* Do Lonely Days Invade the Nights? Potential Social Modulation of Sleep Efficiency. *Psychol Sci* **13**, 384–387 (2002).

669. Darland, T. & Dowling, J. E. Behavioral screening for cocaine sensitivity in mutagenized zebrafish. *Proceedings of the National Academy of Sciences* **98**, 11691–11696 (2001).

670. Swain, H. A., Sigstad, C. & Scalzo, F. M. Effects of dizocilpine (MK-801) on circling behavior, swimming activity, and place preference in zebrafish (Danio rerio). *Neurotoxicol Teratol* **26**, 725–729 (2004).

671. Braida, D. *et al.* Role of neuronal nicotinic acetylcholine receptors (nAChRs) on learning and memory in zebrafish. *Psychopharmacology (Berl)* **231**, 1975–1985 (2014).

672. Ngoc Hieu, B. T. *et al.* Development of a Modified Three-Day T-maze Protocol for Evaluating Learning and Memory Capacity of Adult Zebrafish. *Int J Mol Sci* **21**, 1464 (2020).

673. Bilotta, J., Risner, M. L., Davis, E. C. & Haggbloom, S. J. Assessing Appetitive Choice Discrimination Learning in Zebrafish. *Zebrafish* **2**, 259–268 (2005).

674. Sison, M. & Gerlai, R. Associative learning in zebrafish (Danio rerio) in the plus maze. *Behavioural Brain Research* **207**, 99–104 (2010).

675. Gerlai, R. Associative Learning in Zebrafish (Danio rerio). in *Methods in Cell Biology* vol. 101 249–270 (Academic Press, 2011).

676. Karnik, I. & Gerlai, R. Can zebrafish learn spatial tasks? An empirical analysis of place and single CS–US associative learning. *Behavioural Brain Research* **233**, 415–421 (2012).

677. Piato, Â. L. *et al.* Unpredictable chronic stress model in zebrafish (Danio rerio): Behavioral and physiological responses. *Prog Neuropsychopharmacol Biol Psychiatry* **35**, 561–567 (2011).

678. Babkiewicz, E., Surga, K., Gliwicz, Z. M. & Maszczyk, P. The effect of temperature on the spatial learning rate of zebrafish (Danio rerio). *Ethology* **127**, 632–642 (2021).

679. Pradhan, L. K., Sahoo, P. K., Chauhan, N. R. & Das, S. K. Temporal exposure to chronic unpredictable stress induces precocious neurobehavioral deficits by distorting neuromorphology and glutathione biosynthesis in zebrafish brain. *Behavioural Brain Research* **418**, 113672 (2022).

680. Aparna, S. & Patri, M. Benzo[a]pyrene exposure and overcrowding stress impacts anxiety-like behavior and impairs learning and memory in adult zebrafish, Danio rerio. *Environ Toxicol* **36**, 352–361 (2021).

681. Gleason, P. E., Weber, P. G. & Weber, S. P. Effect of group size on avoidance learning in zebra fish,Brachydanio rerio (Pisces: Cyprinidae). *Anim Learn Behav* **5**, 213–216 (1977).

682. Lindeyer, C. M. & Reader, S. M. Social learning of escape routes in zebrafish and the stability of behavioural traditions. *Anim Behav* **79**, 827–834 (2010).

683. Marchetto, L. *et al.* Auditory environmental enrichment prevents anxiety-like behavior, but not cortisol responses, evoked by 24-h social isolation in zebrafish. *Behavioural Brain Research* **404**, 113169 (2021).

684. Kalueff, A. v., Echevarria, D. J. & Stewart, A. M. Gaining translational momentum: More zebrafish models for neuroscience research. *Prog Neuropsychopharmacol Biol Psychiatry* **55**, 1–6 (2014).

685. Roberts, A. C., Bill, B. R. & Glanzman, D. L. Learning and memory in zebrafish larvae. *Front Neural Circuits* **7**, (2013).

686. Levin, E. D. Zebrafish assessment of cognitive improvement and anxiolysis: filling the gap between in vitro and rodent models for drug development. *revneuro* **22**, 75–84 (2011).

687. Wong, K. *et al.* Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behavioural Brain Research* **208**, 450–457 (2010).

688. Kaushal, N., Nair, D., Gozal, D. & Ramesh, V. Socially isolated mice exhibit a blunted homeostatic sleep response to acute sleep deprivation compared to socially paired mice. *Brain Res* **1454**, 65–79 (2012).

689. Markwald, R. R. *et al.* Impact of insufficient sleep on total daily energy expenditure, food intake, and weight gain. *Proceedings of the National Academy of Sciences* **110**, 5695–5700 (2013).

690. Sorribes, A. The ontogeny of sleep–wake cycles in zebrafish: a comparison to humans. *Front Neural Circuits* **7**, (2013).

691. Singh, C., Rihel, J. & Prober, D. A. Neuropeptide Y Regulates Sleep by Modulating Noradrenergic Signaling. *Current Biology* **27**, 3796-3811.e5 (2017).

692. Lachowicz, J. *et al.* Zebrafish as an Animal Model for Testing Agents with Antidepressant Potential. *Life 2021, Vol. 11, Page 792* **11**, 792 (2021).

693. National Academies of Sciences, E. and M. *et al. Social Isolation and Loneliness in Older Adults*. *Social Isolation and Loneliness in Older Adults: Opportunities for the Health Care System.* (National Academies Press, 2020). doi:10.17226/25663.

694. Cacioppo, J. T., Hughes, M. E., Waite, L. J., Hawkley, L. C. & Thisted, R. A. Loneliness as a specific risk factor for depressive symptoms: Cross-sectional and longitudinal analyses. *Psychol Aging* **21**, 140–151 (2006).

695. Cacioppo, J. T. & Cacioppo, S. Loneliness in the Modern Age: An Evolutionary Theory of Loneliness (ETL). *Adv Exp Soc Psychol* **58**, 127–197 (2018).

696. Buss, R. R. & Drapeau, P. Synaptic Drive to Motoneurons During Fictive Swimming in the Developing Zebrafish. *J Neurophysiol* **86**, 197–210 (2001).

697. Rihel, J. & Schier, A. F. Behavioral screening for neuroactive drugs in zebrafish. *Dev Neurobiol* **72**, 373–385 (2012).

698. Bowker, J. C., Santo, J. B. & Adams, R. E. A Dynamic Examination of the Associations between Shyness, Psychological Difficulties, and Stressful Life Events during Early Adolescence. *J Abnorm Child Psychol* **47**, 1183–1195 (2019).

699. Cacioppo, J. T. *et al.* Loneliness and Health: Potential Mechanisms. *Psychosom Med* **64**, 407–417 (2002).

700. Suzuki, A. *et al.* Astrocyte-neuron lactate transport is required for long-term memory formation. *Cell* **144**, 810–823 (2011).

701. Martin-Fernandez, M. *et al.* Synapse-specific astrocyte gating of amygdala-related behavior. *Nat Neurosci* **20**, 1540–1548 (2017).

702. Romanov, R. A. *et al.* Molecular interrogation of hypothalamic organization reveals distinct dopamine neuronal subtypes. *Nature Neuroscience 2016 20:2* **20**, 176–188 (2016).

703. Wu, Y. E., Pan, L., Zuo, Y., Li, X. & Hong, W. Detecting Activated Cell Populations Using Single-Cell RNA-Seq. *Neuron* **96**, 313-329.e6 (2017).

704. Zeng, H. & Sanes, J. R. Neuronal cell-type classification: challenges, opportunities and the path forward. *Nature Reviews Neuroscience 2017 18:9* **18**, 530–546 (2017).

705. Tervo, D. G. R. *et al.* A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons. *Neuron* **92**, 372–382 (2016).

706. Kebschull, J. M. *et al.* High-Throughput Mapping of Single-Neuron Projections by Sequencing of Barcoded RNA. *Neuron* **91**, 975–987 (2016).

707. Callaway, E. M. & Luo, L. Monosynaptic Circuit Tracing with Glycoprotein-Deleted Rabies Viruses. *The Journal of Neuroscience* **35**, 8979 (2015).

708. Ma, M., Kler, S. & Pan, Y. A. Structural Neural Connectivity Analysis in Zebrafish With Restricted Anterograde Transneuronal Viral Labeling and Quantitative Brain Mapping. *Front Neural Circuits* **13**, (2020).

709. Grewe, B. F. *et al.* Neural ensemble dynamics underlying a long-term associative memory. *Nature* **543**, 670–675 (2017).

710. Li, Y. *et al.* Neuronal Representation of Social Information in the Medial Amygdala of Awake Behaving Mice. *Cell* **171**, 1176-1190.e17 (2017).

711. Remedios, R. *et al.* Social behaviour shapes hypothalamic neural ensemble representations of conspecific sex. *Nature 2017 550:7676* **550**, 388–392 (2017).

712. Kim, D. H. *et al.* Pan-neuronal calcium imaging with cellular resolution in freely swimming zebrafish. *Nature Methods 2017 14:11* **14**, 1107–1114 (2017).

713. Cong, L. *et al.* Rapid whole brain imaging of neural activity in freely behaving larval zebrafish (Danio rerio). *Elife* **6**, (2017).

714. Anderson, D. J. & Perona, P. Toward a science of computational ethology. *Neuron* vol. 84 18–31 Preprint at https://doi.org/10.1016/j.neuron.2014.09.005 (2014).

715. Richardson, M. J., Marsh, K. L., Isenhower, R. W., Goodman, J. R. L. & Schmidt, R. C. Rocking together: Dynamics of intentional and unintentional interpersonal coordination. *Hum Mov Sci* **26**, 867–891 (2007).

716. Sears, L. L., Finn, P. R. & Steinmetz, J. E. Abnormal classical eye-blink conditioning in autism. *Journal of Autism and Developmental Disorders 1994 24:6* **24**, 737–751 (1994).

717. Senju, A. *et al.* Absence of contagious yawning in children with autism spectrum disorder. *Biol Lett* **3**, 706–708 (2007).

718. Ebstein, R. P., Israel, S., Chew, S. H., Zhong, S. & Knafo, A. Genetics of Human Social Behavior. *Neuron* vol. 65 831–844 Preprint at https://doi.org/10.1016/j.neuron.2010.02.020 (2010).

719. Young, S. N. The neurobiology of human social behaviour: An important but neglected topic. *Journal of Psychiatry and Neuroscience* vol. 33 391–392 Preprint at /pmc/articles/PMC2527715/ (2008).

720. Misra, V. The social brain network and autism. *Annals of Neurosciences* vol. 21 69–73 Preprint at https://doi.org/10.5214/ans.0972.7531.210208 (2014).

721. Burns, J. The social brain hypothesis of schizophrenia. *Psychiatr Danub* **18**, 225–229 (2006).

722. Lee, S. *et al.* Plausibility of the zebrafish embryos/larvae as an alternative animal model for autism: A comparison study of transcriptome changes. *PLoS One* **13**, e0203543 (2018).

723. Sakai, C., Ijaz, S. & Hoffman, E. J. Zebrafish Models of Neurodevelopmental Disorders: Past, Present, and Future. *Frontiers in Molecular Neuroscience* vol. 11 294 Preprint at https://doi.org/10.3389/fnmol.2018.00294 (2018).

724. Langova, V., Vales, K., Horka, P. & Horacek, J. The Role of Zebrafish and Laboratory Rodents in Schizophrenia Research. *Frontiers in Psychiatry* vol. 11 703 Preprint at https://doi.org/10.3389/fpsyt.2020.00703 (2020).

725. Campbell, P. D. & Granato, M. Zebrafish as a tool to study schizophrenia-associated copy number variants. *DMM Disease Models and Mechanisms* vol. 13 Preprint at https://doi.org/10.1242/dmm.043877 (2020).

726. Constantin, L. *et al.* Altered brain-wide auditory networks in a zebrafish model of fragile X syndrome. *BMC Biol* **18**, 1–17 (2020).

727. Durand, C. M. *et al.* Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nat Genet* **39**, 25–27 (2007).

728. Liu, C. X. *et al.* CRISPR/Cas9-induced shank3b mutant zebrafish display autism-like behaviors. *Mol Autism* **9**, (2018).

729. Krauss, A. & Neumeyer, C. Wavelength dependence of the optomotor response in zebrafish (Danio rerio). *Vision Res* **43**, 1275–1284 (2003).

730. Risner, M. L., Lemerise, E., Vukmanic, E. v. & Moore, A. Behavioral spectral sensitivity of the zebrafish (Danio rerio). *Vision Res* **46**, 2625–2635 (2006).

731. Nava, S. S., An, S. & Hamil, T. Visual detection of UV cues by adult zebrafish (Danio rerio). *J Vis* **11**, 1–5 (2011).

732. Wong, R. Y., French, J. & Russ, J. B. Differences in stress reactivity between zebrafish with alternative stress coping styles. *R Soc Open Sci* **6**, (2019).

733. Fulcher, N., Tran, S., Shams, S., Chatterjee, D. & Gerlai, R. Neurochemical and Behavioral Responses to Unpredictable Chronic Mild Stress Following Developmental Isolation: The Zebrafish as a Model for Major Depression. *Zebrafish* **14**, 23–34 (2017).

734. Cachat, J. M. *et al.* Video-Aided Analysis of Zebrafish Locomotion and Anxiety-Related Behavioral Responses. *Neuromethods* **51**, 1–14 (2011).

735. Maximino, C. *et al.* Parametric analyses of anxiety in zebrafish scototaxis. *Behavioural brain research* **210**, 1–7 (2010).

736. Blaser, R. E., Chadwick, L. & McGinnis, G. C. Behavioral measures of anxiety in zebrafish (Danio rerio). *Behavioural brain research* **208**, 56–62 (2010).

737. Marquart, G. D. *et al.* High-precision registration between zebrafish brain atlases using symmetric diffeomorphic normalization. *Gigascience* **6**, (2017).

Appendix

Title: Whole-brain mapping of socially isolated zebrafish reveals that lonely fish are not loners

Journal: eLife

Year: 2020

Authors: Hande Tunbak [1], Mireya Vazquez-Prada [1], Thomas Michael Ryan [1], Adam Raymond Kampff [2], Elena Dreosti [1]

[1] The Wolfson Institute for Biomedical Research, University Street, University College London, London, United Kingdom

[2] Sainsbury Wellcome Centre, Howland Street, University College London, London, United Kingdom

DOI: 10.7554/eLife.55863

SHORT REPORT

Whole-brain mapping of socially isolated zebrafish reveals that lonely fish are not loners

Hande Tunbak[1], Mireya Vazquez-Prada[1], Thomas Michael Ryan[1], Adam Raymond Kampff[2], Elena Dreosti[1]*

[1]The Wolfson Institute for Biomedical Research, University Street, University College London, London, United Kingdom; [2]Sainsbury Wellcome Centre, Howland Street, University College London, London, United Kingdom

Abstract The zebrafish was used to assess the impact of social isolation on behaviour and brain function. As in humans and other social species, early social deprivation reduced social preference in juvenile zebrafish. Whole-brain functional maps of anti-social isolated (lonely) fish were distinct from anti-social (loner) fish found in the normal population. These isolation-induced activity changes revealed profound disruption of neural activity in brain areas linked to social behaviour, social cue processing, and anxiety/stress. Several of the affected regions are modulated by serotonin, and we found that social preference in isolated fish could be rescued by acutely reducing serotonin levels.

Introduction

Social preference behaviour, the drive for individuals to identify and approach members of their own species (***Rogers-Carter et al., 2018***; ***Winslow, 2003***), is a fundamental component of all social behaviour. We previously found that most zebrafish develop a strong social preference by 2–3 weeks of age (***Dreosti et al., 2015***), yet we also found a small number (~10%) of 'loner' fish that were averse to social cues. A similar diversity of individual social preferences has been found in many species, including humans (***Sloan Wilson et al., 1994***). Loneliness, undesired isolation from social interaction, has been linked to a reduction in social preference (***Engeszer et al., 2004***; ***Shams et al., 2018***). We therefore asked whether the socially-averse *loner* fish found in the normal population would show a similar behavioural phenotype and neuronal activity to socially-averse *lonely* fish raised in isolation. To answer this question, we compared the behavioural and functional responses of isolated fish to controls during viewing of conspecifics. This comparison found that isolation induces patterns of brain activity that are not present in the normal population. We then asked if we could rescue the aversive behaviour of isolated fish. Since some of the highly activated areas in isolated fish are serotoninergic, we used Buspirone, a 5HT1A receptor agonist. These findings will have important implications for how we understand and treat the impact of social isolation.

Prolonged periods of social isolation are particularly detrimental to humans during early development. However, even brief periods of social isolation have been shown to impact mental and physical health. We therefore tested two models of social isolation, Full (fish raised completely without social interaction) and Partial (fish isolated for 48 hr prior to behavioural testing). Each experiment comprised two sessions, 15 min of acclimation to the chamber followed by 15 min of exposure to two size matched sibling fish that were not isolated. To quantify social preference, we calculated a visual preference index (VPI) that compares the amount of time fish spend in the chamber nearest the conspecifics versus the opposite chamber where they are visually isolated from social cues (see Materials and methods). Full social isolation (Fi) caused a significant decrease in social preference relative to normally raised sibling controls (C) (***Figure 1A***, left and middle panel: C vs Fi, p=8.3e ^{8},

***For correspondence:** e.dreosti@ucl.ac.uk

Competing interests: The authors declare that no competing interests exist.

Funding: See page 11

Received: 10 February 2020
Accepted: 23 April 2020
Published: 05 May 2020

Reviewing editor: Peggy Mason, University of Chicago, United States

eLife digest Socialising is good for people's mental health and wellbeing. The connections and relationships that we form can make us more resilient and healthier. Researchers also know that prolonged periods of social isolation, and feeling lonely, can be detrimental to our health, especially in early childhood. The paradox is that loneliness often results in an even lower desire for social contact, leading to further isolation. But not everyone craves social contact. Some people prefer to be alone and feel more comfortable avoiding social interaction.

Zebrafish display the same social preferences. This, along with their transparent brains, makes them a useful model to study the links between social behaviour and brain activity. Like humans, zebrafish are social animals, with most fish taking a strong liking to social interactions by the time they are a few weeks old. A small number of 'loner' fish, however, prefer to avoid interacting with their siblings or tank mates. And so, if loneliness quells the desire for more social contact, the question becomes, does isolation turn otherwise social fish into loners?

Here, Tunbak et al. use zebrafish to study how social isolation changes brain activity and behaviour. Social fish were isolated from others in the tank for a few days. These so-called 'lonely fish' were then allowed back in contact with the other fish. This revealed that, after isolation, previously social fish did avoid interacting with others.

With this experimental set-up, Tunbak et al. also compared the brains of lonely and loner fish. When fish that prefer social interaction were deprived of social contact, they had increased activity in areas of the brain related to stress and anxiety. These lonely fish became anxious and very sensitive to stimuli; and their brain activity suggested that social interaction became overwhelming rather than rewarding. Positively, the lonely fish quickly recovered their normal, social behaviour when given a drug that reduces anxiety.

This work provides a glimpse into how human behaviour could be affected by lengthy periods in isolation. These results suggest that humans could feel anxious upon returning to normal life after spending a long time alone. Moreover, the findings show the impact that social interaction and isolation can have on the young, developing brain.

Mann-Whitney). Specifically, there was an increase in the number of individuals that had a large negative VPI. We therefore decided to divide the fish into three sociality groups: a) anti-social (-S) fish with VPIs below −0.5; b) pro-social (+S) fish with VPIs above +0.5; c) non-social fish with −0.5 < VPI < +0.5. Fish that underwent Partial isolation (Pi), exhibited an intermediate, yet highly significant, change in social preference (***Figure 1A***, right panel: C vs Pi, p=2.5e ^{8}, Mann-Whitney).

As previously reported (***Zellner et al., 2011***), we found that fish raised in isolation were significantly less active than their normally raised siblings during the acclimation period (***Figure 1B***: C vs Fi, p=9.0e ^{6}; C vs Pi, p=2.8e-^{9} Mann-Whitney) and during the social viewing session (***Figure 1—figure supplement 1A***: left C vs Fi, p=0.0001; C vs Pi, p=0.004 Mann-Whitney). We then divided fish into groups based on their social preference. Interestingly, anti-social fully and partially isolated fish showed very similar movement activity compared to anti-social controls during the acclimation (***Figure 1C*** left: C (-S) vs Fi (-S), p=0.17 Mann-Whitney; C (-S) vs Pi (-S) p=0.23 Mann-Whitney) and during the social viewing session (***Figure 1—figure supplement 1B*** left: C (-S) vs Fi (-S), p=0.48 Mann-Whitney; C (-S) vs Pi (-S) p=0.10 Mann-Whitney). The pro-social isolated fish, which also exhibited a reduction in activity relative to controls during the acclimation session (***Figure 1C*** right: C (-S) vs Fi (-S), p=8.0e ^{5} Mann-Whitney; C (-S) vs Pi (-S) p=1.0e ^{7} Mann-Whitney), instead showed similar activity relative to controls during social viewing (***Figure 1—figure supplement 1B*** right: C (-S) vs Fi (-S), p=0.02 Mann-Whitney; C (-S) vs Pi (-S) p=0.14 Mann-Whitney). In addition, we noticed that all isolated fish behaved qualitatively differently, exhibiting prolonged periods of quiescence (freezing) even when observing conspecifics (***Figure 1D*** and ***Video 1***).

Freezing is a hallmark of anxiety-like behaviour observed in many species, and reported in zebrafish exposed to stressors (***Giacomini et al., 2015***; ***Shams et al., 2018***), including periods of social isolation (***Egan et al., 2009***; ***Shams et al., 2017***). In order to quantify freezing behaviour, we measured the percentage of time spent in continuous periods (>3 s) without motion (***Figure 1—figure supplement 1C-D***). We found that both fully and partially isolated fish exhibited significantly more

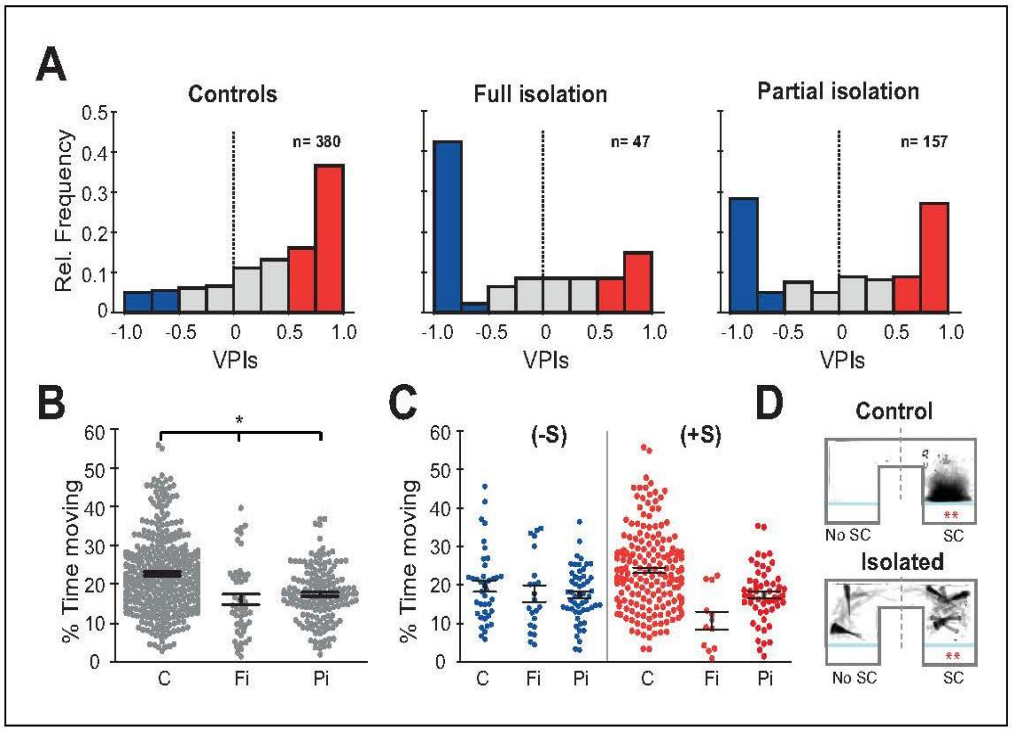


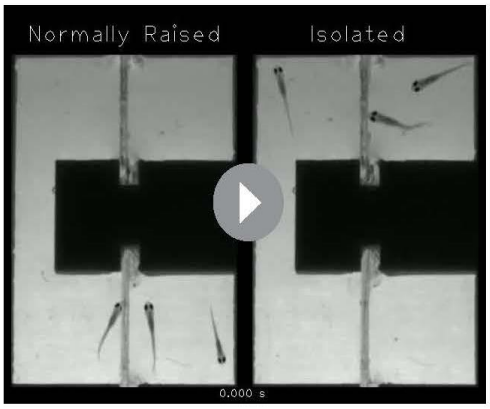
Figure 1. Isolation alters social preference behavior and swimming activity. (**A**) Histograms of all the VPIs during the social cue period across different conditions: controls (C, left), full isolation (Fi, middle), and partial isolation (Pi, right). For visual clarity, red bars highlight strong pro-social fish (+S, VPIs > 0.5), blue bars anti-social fish(-S, VPIs < -0.5), and gray non-social fish (ns, -0.5 < VPI < +0.5). (**B**) Swarm plots comparing the activity levels of fish during the acclimation period expressed as percent time moving (C, n=380; Fi, n=47; Pi, n=157). Mean and standard errors are shown. (**C**) Swarm plots comparing the activity levels of anti-social (left) and social (fish) fish during visual social cue exposure for each rearing condition (C (-S), n=39; Fi (-S), n=21; Pi (-S), n=53) or (C (+S), n=193; Fi, n=11; Pi (+S), n=57). (**D**) Time projection through the video of a pro-social control, C(+S), and a fully isolated, Fi (+S), fish during social cue exposure. The dashed lines mark the division between the social cue side (SC) and the side without social cues (No SC) that was used to calculate VPI.

The online version of this article includes the following figure supplement(s) for figure 1:

Figure supplement 1. Isolation alters social preference behaviour and swimming activity.

freezing than controls during the acclimation period (***Figure 1—figure supplement 1C*** left: C vs Fi, p=3.4e ^{16} Mann-Whitney; C vs Pi, p=2.8e ^{5} Mann-Whitney), and that this increase relative to controls persisted for fully isolated fish during social viewing, but was reduced in partially isolated fish, perhaps representing some recovery during the 15 min of social interaction (***Figure 1—figure supplement 1D*** left: C vs Fi, p=6.3e ^{13} Mann-Whitney; C vs Pi, p=0.03 Mann-Whitney). When we compared freezing behaviour of groups with similar social preference, we found, as expected, that anti-social fish exhibited increased freezing during social viewing regardless of rearing condition. However, pro-social fully isolated fish also showed increased freezing during social viewing, suggesting that they were not engaged in typical social interaction, but rather remained immobile on the side with the conspecifics (***Figure 1—figure supplement 1D*** right).

The behavioural similarities between anti-social isolated (*lonely*) and anti-social control (*loner*) fish led us to hypothesize that isolation might simply predispose fish to the same anti-social state found in the normal population. If this is the case, neural activity of anti-social isolated and anti-social control fish should be similar when presented with social cues. To test this hypothesis, we performed whole-brain two-photon imaging of *c-fos* expression, an immediate early gene whose expression is associated with increased neural activity (***Herrera and Robertson, 1996***), in juvenile brains following testing in the social preference assay. Dissected brains were imaged with the dorsal surface down (bottom-up) to achieve clear views of the ventral brain structures that have been previously implicated in the social brain network (***Figure 2A***, also see Materials and methods). Volumes of 1.5 mm x 1.5 mm x 700 µm, with a voxel size of 1 × 1×3 µm, were acquired from 135 zebrafish brains across all experimental groups and registered to a reference brain (***Marquart et al., 2017***). These *c-fos*



Video 1. Example of a control and a fully isolated +S fish video during social cue presentation. Two minutes of behaviour is shown in 20 s (6x playback acceleration). The control fish shows a strong social preference for the social cue and has a stereotypical social phenotype (left). The test fish spends most of its time watching the social cue with a 45-degree angle and synchronizing its bout motion with the other two conspecifics. The fully isolated fish spends long periods of time as well on the side of the conspecifics. Its behaviour, however, its characterized by long pauses while watching the conspecifics (right).
https://elifesciences.org/articles/55863#video1

whole-brain functional maps were first normalised to a background intensity level (see Materials and methods) and then used to compare the neural activity patterns of different test groups. We compared the average activity map for each rearing/sociality condition with the average map acquired from similarly raised sibling fish that were placed in the behavioural assay for 30 min without any social cues (nsc, no social-cue). The resulting normalised difference stacks (e.g. (+S - nsc)/nsc) allowed us to identify changes in neural activity associated with exposure to a visual social cue (***Figure 2A***).

Several brain areas showed strong activation or inhibition in normally raised fish upon social cue exposure. We focused on areas that have been reported as social brain areas (***O'Connell and Hofmann, 2011***) and show differences between our experimental groups (***Figure 2B***: C (+S and -S)). The caudal hypothalamus was differentially activated in pro- vs. anti-social control fish. A dorsal sub-region was significantly activated in pro-social controls (***Figure 2B and D***: dHc - C (+S) vs C (nsc), p=0.007, Mann-Whitney), whereas it was inhibited, along with the adjacent ventral sub-region, in anti-social controls (***Figure 2B and D***: vHc - C (-S) vs C (nsc), p=0.003, Mann-Whitney). The caudal hypothalamus is known to express high levels of serotonin and dopamine, as well as glutamate and histamine (***Filippi et al., 2010***; ***Kaslin and Panula, 2001***). Furthermore, a segregation into distinct dorsal and ventral areas of the caudal hypothalamus has already been shown for some of these markers, such as tyrosine hydroxylase 1 and 2, (Th1 and Th2) (***Yamamoto et al., 2010***) and we confirmed these previous results with immunostaining (***Figure 2C*** left), as well as for the dopamine and serotonin transporters, DAT and slc6a4b (***Figure 2C*** right) (***Filippi et al., 2010***; ***Lillesaar, 2011***). Changes in serotonin and dopamine levels have been widely documented in response to social interaction (***Scerbina et al., 2012***), viewing social cues (***Saif et al., 2013***), and social isolation (***Huang et al., 2015***; ***Shams et al., 2018***; ***Shams et al., 2015***). While the serotoninergic system has been linked to stress and arousal (***Backström and Winberg, 2017***), the dopamine circuitry has been shown to regulate the reward system underlying social behaviour (***Teles et al., 2013***). Since the caudal hypothalamus expresses both of these neurotransmitters, and our data demonstrate a pattern of activation/inhibition that is distinct for pro- and anti-social fish, then this area could be crucial in regulating social preference.

The second social brain area we investigated was the preoptic area. Our data showed a similar activation pattern for anti-social and pro-social fish characterised by a small increase in the dorsal preoptic area (dPa) and a small decrease in the ventral preoptic area (vPa). However, only anti-social control fish showed a significant change in the ventral area (***Figure 2B and D***: C (-S) vs C (nsc), vPa p=0.003, Mann-Whitney). The activation of the preoptic area during social behaviour is consistent with previous literature in a number of species (***O'Connell and Hofmann, 2011***). This area has been shown to express several neuropeptides involved in social behaviour such as arginine/vasotocin and oxytocin (***Heinrichs et al., 2009***; ***Herget and Ryu, 2015***). It was recently shown that oxytocin does not seem to be responsible for social interaction (***Ribeiro et al., 2019***) as mutants for oxytocin receptors shows no alteration in social preference, but rather reduced social recognition. Furthermore, injections of oxytocin do not have any effect on shoaling and interaction (***Langen et al., 2015***). The neuropeptide vasotocin, instead, has been shown to have a specific effect on reducing social interaction (***Langen et al., 2015***) and not shoaling behaviour. This neuropeptide has also been shown to be involved in aggression (***Teles et al., 2016***) and stress by stimulating cortisol release.

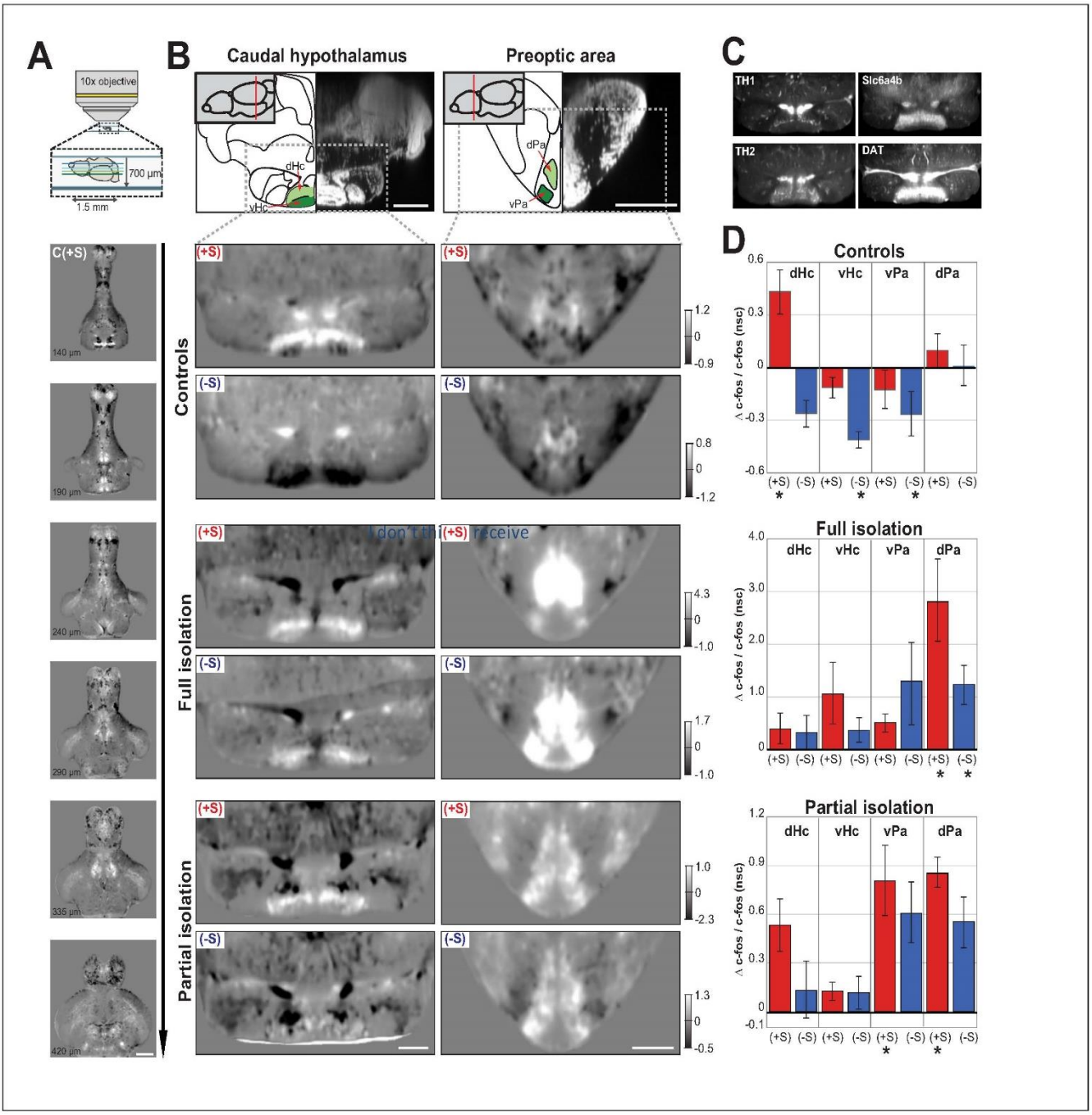


Figure 2. Functional maps of the social brain in normal and isolated fish. (**A**) Schematic of the custom-built two-photon microscope used for acquiring whole-brain volumes of dorsal-down mounted fish brains (top panel). Horizontal sections of pro-social control fish (C(+S)) responses at increasing imaging depth (lower panels). Images are average differences between (C(+S)) and siblings not presented with a social cue. Positive values (white) indicate increased cFos expression in socially preferring fish, while negative values (black) indicate decreased expression. Scale bar is 200µm. The intensity scale bar is shown in **B**, C(+S) row. (**B**) Region analysis of two different brain areas that have been implicated in social behavior: caudal hypothalamus and preoptic area. A schematic of the anatomical regions and corresponding DAPI staining is shown (top panel) with two sub-regions highlighted in green. Images showing changes in cFos activation in these areas for pro- (+S) and anti-social (-S) controls, fully isolated, and partially isolated fish are shown. Images are horizontal sections of the average difference between each test group and their corresponding sibling group not presented with a social cue. Scale bar is 100µm. Intensity scale bar is shown for each group. (**C**) Average image of TH1, TH2, Slc6a4b, and DAT expression in the same section of the caudal hypothalamus as 2B (n=3 each). Scale bar is 100µm. (**D**) Summary graphs showing the change in cFos activation for four different brain areas calculated by using the average difference images shown in (**B**) and using 3D masks (a single plane of each area of the masks is shown in green in **B**). Positive values indicate increases in cFos expression; asterisks mark significant changes relative to no social cue siblings. D=dorsal and V=ventral caudal hypothalamus; Pa=ventrolateral preoptic area, PM=dorsal preoptic area.

We then compared the brain activity maps of anti- and pro-social control fish with fully and partially isolated fish. As described previously, anti-social control (*loner*) fish showed a behavioural phenotype very similar to anti-social isolated (*lonely*) fish. Therefore, we investigated whether their brain activity maps were also similar following the presentation of a social cue. Contrary to our hypothesis, *c-fos* functional maps of anti-social fully isolated fish (***Figure 2B***: Fi (-S)) revealed a completely different activity profile than their anti-social sibling controls (***Figure 2B***: C (-S)). The ventral sub-region of the caudal hypothalamus (vHc) of Fi (-S) fish was not inactivated, while the preoptic area was strongly activated in both the dorsal (dPa) and the ventral (vPa) regions, but significantly only in the dorsal (***Figure 2B and D***: Fi (-S) vs Fi (nsc), p=0.006 dPa; p=0.07 vPa, Mann-Whitney). Furthermore, the pro-social fully isolated fish (***Figure 2B***: Fi (+S)), who exhibited an increase of freezes and reduced motility compared to control fish when viewing conspecifics, showed a similar activation to pro-social controls in the caudal hypothalamus, but increased activity in the dorsal preoptic area. Interestingly, the preoptic area was activated differently in pro-social and anti-social isolated fish, with only the dorsal preoptic area strongly activated in the pro-social group (***Figure 2B and D***: Fi (+S) vs Fi, p=0.04 vPa, p=0.002 dPa, Man-Whitney). These data suggest that long social isolation causes abnormal neural responses during viewing of social cues.

Furthermore, anti- and pro-social fish exposed to a brief isolation for only 48 hr prior to testing, showed similar functional activity changes to fully isolated fish, albeit less strong (***Figure 2B and D***: Pi (-S) vs Pi (nsc), p=0.18 dHc; p=0.28 vHc; p=0.04 vPa; p=0.04 dPa, Mann-Whitney; ***Figure 2B and D***: Pi (+S) vs Pi (nsc), p=0.17 dHc; p=0.05 vHc; p=0.007 vPa; p=0.006 dPa). Together with the behavioural data, this finding supports the idea that short term isolation is enough to induce brain activity changes similar to those observed following complete isolation, and strikingly different than those observed in anti-social controls.

We were next interested in understanding why social isolation promotes social aversion instead of increasing the drive for social interaction. An important clue was found in the pattern of brain activity changes that were unique to isolated fish. When we directly compared the normalised *c-fos* functional brain maps of isolated and control fish that were not exposed to social cues during the assay (***Figure 3A***), we found a significant increase in two interesting areas; one associated with visual

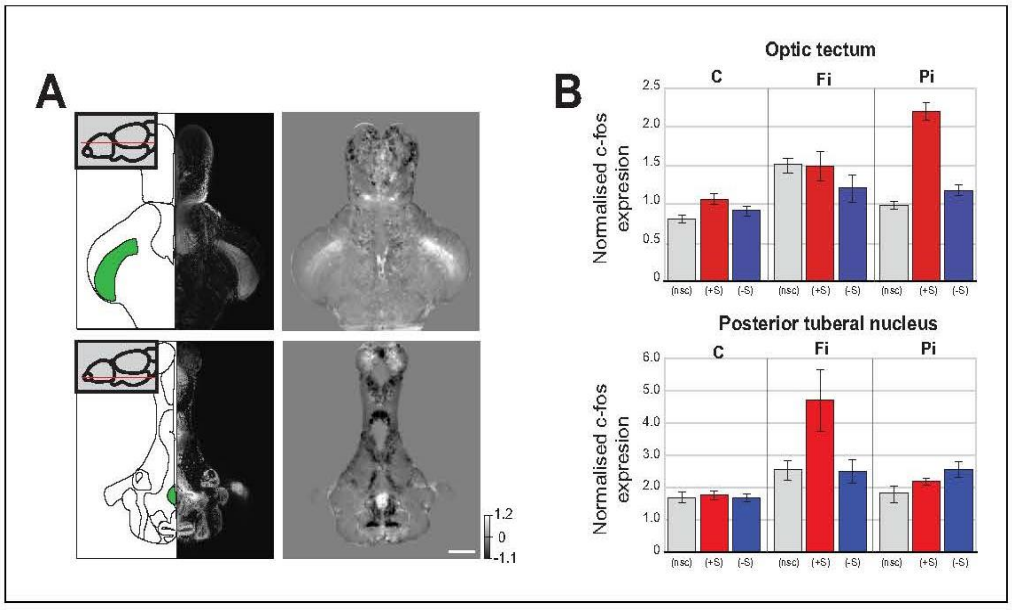


Figure 3. Changes in baseline brain activity following isolation. (**A**) Images of two areas that show strong c-fos activation in fully isolated fish independent of social stimuli (optic tectum and posterior tuberal nucleus (PTN)). Schematics of the horizontal sections and corresponding DAPI image are shown in the left panels. One plane of the 3D mask regions used for subsequent analysis is indicated (green). Images of Fully isolated fish c-fos neuronal activity, calculated as average differences between fully isolated (Fi) fish and normally raised fish without social cues (nsc) are shown in the right panels. Scale bar 200 µm. (**B**) Summary graphs showing the normalised c-fos expression in the optic tectum and PTN 3D masks for each experimental condition: non social cue (nsc), pro-social (+S) and anti-social (-S) for all the controls (C), fully isolated (Fi), and partially isolated (Pi) fish.

processing, the optic tectum, [**McDowell et al., 2004**]), and one involved in stress responses, the posterior tuberal nucleus (**Ziv et al., 2013**).

In pro-social control fish, viewing social cues resulted in a significant increase of neuronal activity in the optic tectum (**Figure 3B** top: C (+S) vs C (nsc), p=0.004 Mann-Whitney). However, in fully isolated fish, there was already increased neuronal activity in the optic tectum in the absence of social cues (**Figure 3B** top: Fi (nsc) vs C (nsc), p=0.0004, Mann-Whitney), suggesting that isolation increases visual sensitivity, as previously reported in humans (**Cacioppo et al., 2015**). This increased sensitivity of fully isolated fish not presented with social cues was weaker in partially isolated fish (**Figure 3B** top: Pi (nsc) vs C (nsc), p=0.03, Mann-Whitney). However, a much larger increase in tectal activity was observed when pro-social partially isolated fish viewed conspecifics, revealing that some visual sensitization had occurred (**Figure 3B** top: Pi (+S) vs C (+S), p=0.0002, Mann-Whitney). In addition, increased tectal activity was also present in both fully and partially isolated anti-social fish (**Figure 3B** top: Fi (-S) vs C (-S), p=0.048; Pi (-S) vs C (-S), p=0.005, Mann-Whitney), even though these fish largely avoided the chamber with visual access to conspecifics.

We also observed isolation-related activity increases in the posterior tuberal nucleus, an area associated with stress responses in zebrafish (**Wee et al., 2019**; **Ziv et al., 2013**). Full isolation caused a significant increase in posterior tuberal nucleus activity in the absence of social cues (**Figure 3B** bottom: Fi (nsc) vs C (nsc), p=0.015, Mann-Whitney) and in both anti-social and pro-social fish exposed to social cues (**Figure 3B** bottom: Fi (+S) vs C (+S), p=0.003; Fi (-S) vs C (-S), p=0.016, Mann-Whitney). Following partial isolation, posterior tuberal nucleus activity was not increased in the absence of social cues (**Figure 3B** bottom: Pi (nsc) vs C (nsc), p=0.29, Mann-Whitney), only slightly in pro-social fish (**Figure 3B** bottom: Pi (+S) vs C (+S), p=0.018), but significantly so in anti-social fish (**Figure 3B** bottom: Pi (-S) vs C (-S), p=0.0005).

Given these results from the optic tectum and posterior tuberal nucleus, we propose that isolation initially heightens sensitivity to social stimuli. However, when prolonged, this heightened sensitivity results in an increase of stress and anxiety levels during social viewing that leads to an aversion for social stimuli.

To test our hypothesis that reducing anxiety could reverse the anti-social behaviour observed in isolated zebrafish, we acutely treated control and partially isolated fish with Buspirone, an agonist of the auto-inhibitory $5HT_{1A}$ receptor. The choice of isolation duration was motivated by the intermediate behavioural and functional phenotype of partial isolation relative to normal-rearing and full isolation, which would allow us to more easily detect both positive and negative impacts of treatment on sociality. The choice of Buspirone was supported by the changes in activity observed in the caudal hypothalamus of isolated fish, and by the fact that the caudal hypothalamus and the preoptic area strongly express Htr1ab receptors, one of the two orthologues of the $5HT_{1A}$ receptor (**Norton et al., 2008**). Buspirone has been shown to reduce anxiety in humans, mice, and zebrafish (**Bencan et al., 2009**; **Lalonde and Strazielle, 2010**; **Lau et al., 2011**; **Patel and Hillard, 2006**). While it is not fully understood how Buspirone reduces anxiety, it has been shown to enhance social interaction of rats (**File and Seth, 2003**; **Gould et al., 2011**), sociability of zebrafish (**Barba-Escobedo and Gould, 2012**), and reduce social phobia in humans (**Schneier et al., 1993**; **van Vliet et al., 1997**). Its ability to counter the effects of social isolation in zebrafish has not been investigated.

We first tested the effects of acute exposure to Buspirone in control fish, and, as expected, we observed a small significant increase in social preference relative to untreated controls, however, a population of ~10% anti-social fish remained (**Figure 4—figure supplement 1**; C (no drug) vs C (30 µM), p=0.01, Mann-Whitney). We then treated partially isolated fish with 30 µM and 50 µM (**Figure 4—figure supplement 1**, n = 46, n = 72 fish) of Buspirone. Remarkably, the acute drug treatment was sufficient in both concentrations to reverse the anti-social phenotype caused by isolation (**Figure 4A**; Pi vs Pi (Buspirone 30 µM and 50 µM combined), p=2.56 e-05, Mann-Whitney).

When we then compared the time course of this phenotype reversal by computing the VPIs for each minute throughout the 15 min of the behavioural experiment (**Figure 4B**). We found that the isolated fish treated with Buspirone, while initially anti-social, would rapidly recover normal social preference behaviour within the first 5 min of exposure to social cues (**Figure 4B**: C vs Pi (Buspirone), p=0.016, first minute; p=0.37, fourth minute, Mann-Whitney). In contrast, the VPIs of untreated isolated fish remained significantly lower than controls throughout the entire session. We next compared the time course of movement activity (**Figure 4C**), and found that it generally increased quickly throughout the first 5 min of the social viewing session. Notably, the activity of isolated fish

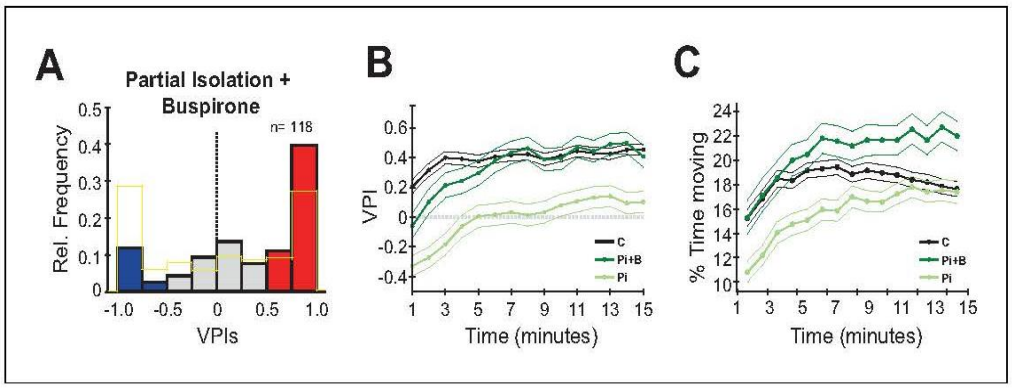


Figure 4. Buspirone rescues social preference in isolated fish. (**A**) Histogram of VPIs during the social cue period in partially isolated (Pi) fish treated with 30 µM and 50 µM of Buspirone (combined). For visual clarity, the bars are colored as in ***Figure 1***. (**B**) VPI values calculated in one-minute time bins for controls (C, black line, n=380), partial isolated (Pi, blue line, n=157), and Pi treated with Buspirone (Pi+B, green line, n=118). Note how Buspirone treated fish recover normal social preference within the first 5 minutes. (**C**) Percentage of time moving calculated in one-minute bins for the same fish as B, thin lines indicate standard error.

The online version of this article includes the following figure supplement(s) for figure 4:

Figure supplement 1. Buspirone rescues social preference in isolated fish.

treated with Buspirone was already at the level of controls from the start of the social viewing session (***Figure 4B***: C vs Pi (Buspirone), p=0.31, first minute, Mann-Whitney), which suggests that the recovery of normal movement activity, possibly as a result of reduced anxiety, precedes the recovery of normal social preference. Therefore, Buspirone's impact on the rate of recovery of social preference indicates that it may do so by reducing anxiety, perhaps at the level of the preoptic and/or caudal hypothalamic area, allowing circuit plasticity to down-regulate the hypersensitivity to social stimuli acquired during the isolation period.

In summary, our results demonstrate that *lonely* fish, which have been isolated from social cues and show anti-social behaviour, have a completely different functional response to social stimuli than *loner* fish, anti-social fish found in the normal population. In addition, the functional changes caused by social deprivation are consistent with an increase in anxiety resulting from hyper-sensitization to social stimuli, similar to the effects of isolation on humans. We could reverse isolation's effects in zebrafish with an existing anxiolytic drug that acts on the monoaminergic system. Zebrafish will thus provide a powerful new tool for studying the impact of loneliness (isolation) on brain function and exploring different strategies for reducing, or even reversing, its harm.

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Antibody	Anti- digoxigenin-POD, sheep, polyclonal Fab fragments	Sigma-Aldrich, Rouche	Roche, Cat# 11207733910, RRID:AB_514500	1:3000
Sequence-based reagent	cFos _F	This paper	PCR primers	CCGATACACTGCAAGCTGAA
Sequence-based reagent	cFos_R	This paper	PCR primers	ATTGCAGGGCTATGGAAGTG
Peptide, recombinant protein	Proteinase K	Sigma-Aldrich	Cat# P6556-10MG	2 mg/ml
Commercial assay	TSA Plus Cyanine three system	Sigma-Aldrich, Perkin Elmer	Cat# NEL74401KT	Dilution 1:50

Continued on next page

Continued

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Chemical compound, drug	Buspirone hydrochloride	Sigma-Aldrich	Cat# B7148-1G	30 uM and 50 uM
Software, algorithm	Anaconda, Spyder	Anaconda (https://www.anaconda.com/)	Spyder, RRID:SCR_017585	Version 4.0.1
Software	ImageJ	NIH (http://imagej.nih.gov/ij/)	RRID:SCR_003070	
Software	ANTs- Advanced Normalisation Tools	http://stnava.github.io/ANTs/	RRID:SCR_004757	Version 2.1.0
Other	DAPI staining	Sigma-Aldrich	Cat# D9564-10MG	1 mg/ml
Other	Slc6a4b RNA probe	***Norton et al., 2008***		
Other	DAT RNA probe	***Filippi et al., 2010***		
Other	Th1 RNA Probe	***Filippi et al., 2010***		
Other	Th2 RNA probe	***Filippi et al., 2010***		

Animals

AB strain zebrafish maintenance and breeding was performed at 28.5C with a 14 hr:10 hr light-dark cycle. Isolated fish were housed in custom chambers (length = 15 cm, width = 5 cm, height = 10 cm) made of opaque white acrylic with translucent lids, either from fertilization (full isolation) or for 48 hr prior to the behavioural experiment (partial isolation). All experiments were performed according to protocols approved by local ethical committee (AWERB Bloomsbury Campus UCL) and the UK Home Office.

Behavioural assay and analysis

Experimental details and image acquisition were performed as described previously (***Dreosti et al., 2015***). Fish were positioned in custom-built behavioural arenas (***Figure 1D***) made of white acrylic, and illuminated with visible light using a laser light projector (Microvision, ShowwX+, US). The videography system comprised a high-speed camera (Flea3, PointGrey, CA), an infrared light (Advanced Illumination, US, 880 nm), an IR filter (R70, Hoya, JP), and a vari-focal lens (Fujinon, JP). Experiments were recorded using custom written workflows in Bonsai (***Langen et al., 2015***). Test fish were positioned in the main C-shape compartment of the arena by pipetting, and left for 15 min to acclimate. A social cue, two fish of same age and similar size, was then introduced into one of the two adjacent chambers randomly. Test fish could see the social cue through a glass window. Each fish was run only once in the behavioural assay.

Images were analysed using custom written computer vision scripts in Python based on OpenCV (https://www.dreo-sci.com/resources/). Each frame was cropped, background subtracted, and thresholded. The centroid, position, orientation, and per frame motions of the test fish were identified, and stored in a CSV file. All videos have been saved with H.264 compression for subsequent offline analysis, and are available upon request.The source code can be downloaded at http://www.dreo-sci.com/resources/.

The visual preference index (VPI) was calculated by subtracting the number of frames in which the fish was located on the side of the arena nearest the social stimulus (Social cue (SC) side in ***Figure 1B***) by the number of frames located on the opposite side of the arena (nsc (No SC) side). This difference was then divided by the total number of frames recorded [VPI = (SC – No SC)/Total frames]. The percent time moving was calculated by counting each frame with detectable changes in the fish image relative to the previous frame (i.e. motion), and dividing by the total number of frames. The percent time freezing was calculated by detecting contiguous sequences without motion longer than 3 s, counting all frames that are part of such sequences, and dividing by the total number of frames.

Whole mount in situ hybridisation

Fluorescent in situ hybridizations using digoxigenin-labelled *c-fos* were performed on dissected juvenile zebrafish with few modification to the original method (**Brend and Holley, 2009**). After overnight fixation in 4% PFA, protein K treatment (2 mg/ml 20 min of incubation), inactivation of endogenous peroxidase with H_2O_2 (22% v/v for 30 min at room temperature), additional fixation (30 min at room temperature) and 3 hr of incubation with the hybridisation buffer, fish were incubated with the *c-fos* probe (courtesy from Ricardo N. Silva (Forward CCGATACACTGCAAGCTGAA and Reverse ATTGCAGGGCTATGGAAGTG), or with dopamine transporter (DAT), tyrosine hydroxylase 1 (Th1), tyrosine hydroxylase (Th2) (**Filippi et al., 2010**), or the 5-HT transporter, solute carrier family 6 member 4b (Slc6a4b) probes (**Norton et al., 2008**). *C-fos*, *DAT* and *Slc6a4b* probes were detected with anti-Digoxigenin-POD, Fab fragments (Roche, 1:3000) and TSA Plus Cyanine 3 System (Perkin Elmer, 1:50). Nuclear staining was obtained using DAPI (Sigma-Aldrich, 1: 500). Fish were then mounted for imaging in low melting point agarose (2.5% Agarose, 0.8% glycerol, PBS-Tween) and imaged.

Imaging and registration

A custom built two-photon microscope (INSS) was used for image acquisition of whole-brain in situs. Both DAPI and Cy3 Images were collected with a 10x objective (Olympus, W Plan-Apochromat 10x/0.5 M27 75 mm) using a 'Chameleon' titanium–sapphire laser tuned to 1030 nm (Coherent Inc, Santa Clara, CA, US) and controlled using custom written software in LabView. Registration of in-situ images was performed using ANTs (Advanced Normalisation Tools) version 2.1.0 running on the UCL Legion compute cluster. Images were down-sampled to 512*512 and parameters were slightly modified from **Marquart et al. (2017)** fixed registration:

```
antsRegistration -d 3 –float 1 -o [Registered_Image_, Registered_Image _warped.nii.gz] –interpolation WelchWindowedSinc –use-histogram-matching 0 r [reference_Image, Registered_Image,1] -t rigid[0.1] -m MI[reference_Image, Registered_Image _0.nii,1,32,Regular,0.25] -c [1000 × 500×250 × 100,1e-8,10] –shrink-factors 12 × 8×4 × 2 s 4 × 3×2 × 1 t Affine[0.1] -m MI[reference_Image, Registered_Image,1,32,Regular,0.25] -c [1000 × 500×250 × 100,1e-8,10] –shrink-factors 12 × 8×4 × 2 s 4 × 3×2 × 1 t SyN[0.1,6,0] -m CC[reference_Image, Registered_Image _0.nii,1,2] -c [1000 × 500×500x250 × 100,1e-7,10] –shrink-factors 12 × 8×4x2 × 1 s 4 × 3×2x1 × 0
antsApplyTransforms -d 3 v 0 –float -n WelchWindowedSinc -i Registered_Image _1.nii -r reference_Image -o Registered_Image _warped_red.nii.gz -t Registered_Image _1Warp.nii.gz -t Registered_Image _0GenericAffine.mat
```

Intensity normalisation

The registered image stacks were then normalised to adjust for intensity variations between imaging sessions caused by a variety of sources (staining efficiency, laser power fluctuations, light detector sensitivity, etc.). Normalisation was accomplished by computing an intensity histogram for each fish brain's volume (with 10000 discrete intensity bins spanning the range −4000.0 to 70000.0) for all 512*512*273 voxels. The minimum value bin (with at least 100 voxels) was used as the bias offset, and subtracted from all voxel values. The mode value, minus the bias, provided a robust estimate of the background/baseline fluorescence and was thus used to normalise voxel values for the entire volume. Therefore, after normalisation, an intensity value of 1 reflected the background level while two indicates fluorescence level that is twice the background, and so on. Histogram normalisation was performed for each individual fish's brain volume prior to any region or voxel-based analysis.

Figures 2B and ***3A*** Reconstruction of cross section images were obtained by using the Fiji 'Volume viewer' plugin. Schematics of cross- and horizontal-section were obtained by using the 'Neuroanatomy of the zebrafish brain'.

Figures 2D Percentages of *c-fos* activation were calculated for each of the six different areas highlighted in ***Figures 2B*** and ***3A***, using custom written Python functions, in the following way. A 3D mask for each area was generated by using the 'Segmentation Editor' plugin Fiji (https://imagej.net/

Segmentation_Editor). *C-fos* percentage values for each condition (C (+S), C (-S), Fi (-S), Pi (-S)) were obtained by subtracting and then dividing each *c-fos* average value of the mask by the basal *c-fos* average value calculated in control fish No Social Cue.

Statistics

Statistical analysis was performed using Python scipy stats libraries. Since VPI, percent time moving/freezing, and *c-fos* activity distributions were generally not normally distributed, we used the non-parametric Mann-Whitney U-test of independent samples for hypothesis testing throughout the manuscript.

Drug treatment

Juvenile fish were treated with 30 µM or 50 µM Buspirone (Buspirone HCl, Sigma) for 10 min prior the experiment. After washing, fish were run through the behavioural assay. Each fish was used only once.

Data availability

All the images, video, protocols, analysis scripts, and data that support the findings of this study are available from this website (http://www.dreo-sci.com/resources/), or our GitHub repository (https://github.com/Dreosti-Lab/Lonely_Fish_2020; ***Dreosti, 2020***; copy archived at https://github.com/elifesciences-publications/Lonely_Fish_2020), or from the corresponding author upon request.

Acknowledgements

The authors acknowledge Steve Wilson for providing lab resources. Ricardo Neto Silva for the *c-fos* probe. Jason Rihel for some of the reagents. Wolfgang Driver for the dopaminergic probes and William Norton for the serotoninergic probes. UCL fish facility team for fish care/husbandry. This work was supported by the Wellcome Trust Grant Ref 202465/Z/16/Z.

Additional information

Funding

Funder	Grant reference number	Author
Wellcome	202465/Z/16/Z	Elena Dreosti
Gatsby Charitable Foundation	090843/F/09/Z	Adam Raymond Kampff

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions

Hande Tunbak, Conceptualization, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing - review and editing; Mireya Vazquez-Prada, Data curation; Thomas Michael Ryan, Software, Writing - review and editing; Adam Raymond Kampff, Software, Formal analysis, Visualization, Writing - review and editing; Elena Dreosti, Conceptualization, Resources, Data curation, Software, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Methodology, Writing - original draft, Project administration, Writing - review and editing

Author ORCIDs

Hande Tunbak https://orcid.org/0000-0003-3180-1401
Mireya Vazquez-Prada https://orcid.org/0000-0001-7964-7576
Thomas Michael Ryan https://orcid.org/0000-0001-9469-4135
Adam Raymond Kampff https://orcid.org/0000-0003-3079-019X
Elena Dreosti https://orcid.org/0000-0002-6738-7057

Ethics

Animal experimentation: All experiments were performed according to protocols approved by local ethical committee (AWERB Bloomsbury Campus UCL) and the UK Home Office. PAE2ECA7E.

Decision letter and Author response

Decision letter https://doi.org/10.7554/eLife.55863.sa1
Author response https://doi.org/10.7554/eLife.55863.sa2

Additional files

Supplementary files

- Transparent reporting form

Data availability

All the images, video, protocols, analysis scripts, and data that support the findings of this study are available on our website (www.dreo-sci.com/resources) and GitHub repository (https://github/Dreosti-Lab/Social_Zebrafish), or from the corresponding author upon request.

References

Backström T, Winberg S. 2017. Serotonin coordinates responses to social Stress-What we can learn from fish. *Frontiers in Neuroscience* **11**:595. DOI: https://doi.org/10.3389/fnins.2017.00595, PMID: 29163002

Barba-Escobedo PA, Gould GG. 2012. Visual social preferences of lone zebrafish in a novel environment: strain and anxiolytic effects. *Genes, Brain and Behavior* **11**:366–373. DOI: https://doi.org/10.1111/j.1601-183X.2012.00770.x

Bencan Z, Sledge D, Levin ED. 2009. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. *Pharmacology Biochemistry and Behavior* **94**:75–80. DOI: https://doi.org/10.1016/j.pbb.2009.07.009

Brend T, Holley SA. 2009. Zebrafish whole mount High-Resolution double fluorescent in situ hybridization. *Journal of Visualized Experiments* **25**:29. DOI: https://doi.org/10.3791/1229

Cacioppo JT, Cacioppo S, Capitanio JP, Cole SW. 2015. The neuroendocrinology of social isolation. *Annual Review of Psychology* **66**:733–767. DOI: https://doi.org/10.1146/annurev-psych-010814-015240, PMID: 25148851

Dreosti E, Lopes G, Kampff AR, Wilson SW. 2015. Development of social behavior in young zebrafish. *Frontiers in Neural Circuits* **9**:39. DOI: https://doi.org/10.3389/fncir.2015.00039, PMID: 26347614

Dreosti E. 2020. Analysis code used in Tunbak et al. 2020. *GitHub*. d80c2f4. https://github.com/Dreosti-Lab/Lonely_Fish_2020

Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, Kalueff AV. 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research* **205**:38–44. DOI: https://doi.org/10.1016/j.bbr.2009.06.022, PMID: 19540270

Engeszer RE, Ryan MJ, Parichy DM. 2004. Learned social preference in zebrafish. *Current Biology* **14**:881–884. DOI: https://doi.org/10.1016/j.cub.2004.04.042, PMID: 15186744

File SE, Seth P. 2003. A review of 25 years of the social interaction test. *European Journal of Pharmacology* **463**:35–53. DOI: https://doi.org/10.1016/S0014-2999(03)01273-1, PMID: 12600701

Filippi A, Mahler J, Schweitzer J, Driever W. 2010. Expression of the paralogous tyrosine hydroxylase encoding genes *th1* and *th2* reveals the full complement of dopaminergic and noradrenergic neurons in zebrafish larval and juvenile brain. *The Journal of Comparative Neurology* **518**:423–438. DOI: https://doi.org/10.1002/cne.22213, PMID: 20017209

Giacomini AC, de Abreu MS, Koakoski G, Idalêncio R, Kalichak F, Oliveira TA, da Rosa JG, Gusso D, Piato AL, Barcellos LJ. 2015. My stress, our stress: blunted cortisol response to stress in isolated housed zebrafish. *Physiology & Behavior* **139**:182–187. DOI: https://doi.org/10.1016/j.physbeh.2014.11.035, PMID: 25449397

Gould GG, Hensler JG, Burke TF, Benno RH, Onaivi ES, Daws LC. 2011. Density and function of central serotonin (5-HT) transporters, 5-HT1A and 5-HT2A receptors, and effects of their targeting on BTBR T+tf/J mouse social behavior. *Journal of Neurochemistry* **116**:291–303. DOI: https://doi.org/10.1111/j.1471-4159.2010.07104.x, PMID: 21070242

Heinrichs M, von Dawans B, Domes G. 2009. Oxytocin, vasopressin, and human social behavior. *Frontiers in Neuroendocrinology* **30**:548–557 . DOI: https://doi.org/10.1016/j.yfrne.2009.05.005

Herget U, Ryu S. 2015. Coexpression analysis of nine neuropeptides in the neurosecretory preoptic area of larval zebrafish. *Frontiers in Neuroanatomy* **9**:2. DOI: https://doi.org/10.3389/fnana.2015.00002, PMID: 25729355

Herrera DG, Robertson HA. 1996. Activation of c-fos in the brain. *Progress in Neurobiology* **50**:83–107. DOI: https://doi.org/10.1016/S0301-0082(96)00021-4, PMID: 8971979

Huang C-C, Lu R-B, Yen C-H, Yeh Y-W, Chou H-W, Kuo S-C, Chen C-Y, Chang C-C, Chang H-A, Ho P-S, Liang C-S, Cheng S, Shih M-C, Huang S-Y. 2015. Dopamine transporter gene may be associated with bipolar disorder and its personality traits. *European Archives of Psychiatry and Clinical Neuroscience* **265**:281–290 . DOI: https://doi.org/10.1007/s00406-014-0570-0

Kaslin J, Panula P. 2001. Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (*Danio rerio*). *The Journal of Comparative Neurology* **440**:342–377. DOI: https://doi.org/10.1002/cne.1390, PMID: 11745628

Lalonde R, Strazielle C. 2010. Relations between open-field, elevated plus-maze, and emergence tests in C57BL/6J and BALB/c mice injected with GABA- and 5HT-anxiolytic agents. *Fundamental & Clinical Pharmacology* **24**:365–376. DOI: https://doi.org/10.1111/j.1472-8206.2009.00772.x, PMID: 19735300

Langen EMA, Lindeyer CM, Reader SM, Swaney WT. 2015. Nonapeptide influences on social behaviour: effects of vasotocin and isotocin on shoaling and interaction in zebrafish. *Behaviour* **152**:897–915. DOI: https://doi.org/10.1163/1568539X-00003261

Lau BY, Mathur P, Gould GG, Guo S. 2011. Identification of a brain center whose activity discriminates a choice behavior in zebrafish. *PNAS* **108**:2581–2586. DOI: https://doi.org/10.1073/pnas.1018275108, PMID: 21262817

Lillesaar C. 2011. The serotonergic system in fish. *Journal of Chemical Neuroanatomy* **41**:294–308. DOI: https://doi.org/10.1016/j.jchemneu.2011.05.009, PMID: 21635948

Marquart GD, Tabor KM, Horstick EJ, Brown M, Geoca AK, Polys NF, Nogare DD, Burgess HA. 2017. High-precision registration between zebrafish brain atlases using symmetric diffeomorphic normalization. *GigaScience* **6**:1–15. DOI: https://doi.org/10.1093/gigascience/gix056, PMID: 28873968

McDowell AL, Dixon LJ, Houchins JD, Bilotta J. 2004. Visual processing of the zebrafish optic tectum before and after optic nerve damage. *Visual Neuroscience* **21**:97–106. DOI: https://doi.org/10.1017/S0952523804043019, PMID: 15259561

Norton WH, Folchert A, Bally-Cuif L. 2008. Comparative analysis of serotonin receptor (HTR1A/HTR1B families) and transporter (*slc6a4a/b*) gene expression in the zebrafish brain. *The Journal of Comparative Neurology* **511**:521–542. DOI: https://doi.org/10.1002/cne.21831, PMID: 18839395

O'Connell LA, Hofmann HA. 2011. The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *The Journal of Comparative Neurology* **519**:3599–3639. DOI: https://doi.org/10.1002/cne.22735, PMID: 21800319

Patel S, Hillard CJ. 2006. Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: further evidence for an anxiolytic role for endogenous cannabinoid signaling. *Journal of Pharmacology and Experimental Therapeutics* **318**:304–311. DOI: https://doi.org/10.1124/jpet.106.101287, PMID: 16569753

Ribeiro J, Davids K, Araújo D, Guilherme J, Silva P, Garganta J. 2019. Exploiting Bi-Directional Self-Organizing tendencies in team sports: the role of the game model and tactical principles of play. *Frontiers in Psychology* **10**:213. DOI: https://doi.org/10.3389/fpsyg.2019.02213

Rogers-Carter MM, Varela JA, Gribbons KB, Pierce AF, McGoey MT, Ritchey M, Christianson JP. 2018. Insular cortex mediates approach and avoidance responses to social affective stimuli. *Nature Neuroscience* **21**:404–414. DOI: https://doi.org/10.1038/s41593-018-0071-y, PMID: 29379116

Saif M, Chatterjee D, Buske C, Gerlai R. 2013. Sight of conspecific images induces changes in neurochemistry in zebrafish. *Behavioural Brain Research* **243**:294–299. DOI: https://doi.org/10.1016/j.bbr.2013.01.020, PMID: 23357085

Scerbina T, Chatterjee D, Gerlai R. 2012. Dopamine receptor antagonism disrupts social preference in zebrafish: a strain comparison study. *Amino Acids* **43**:2059–2072 . DOI: https://doi.org/10.1007/s00726-012-1284-0

Schneier FR, Saoud JB, Campeas R, Fallon BA, Hollander E, Coplan J, Liebowitz MR. 1993. Buspirone in social phobia. *Journal of Clinical Psychopharmacology* **13**:251???256–256. DOI: https://doi.org/10.1097/00004714-199308000-00004

Shams S, Chatterjee D, Gerlai R. 2015. Chronic social isolation affects thigmotaxis and whole-brain serotonin levels in adult zebrafish. *Behavioural Brain Research* **292**:283–287. DOI: https://doi.org/10.1016/j.bbr.2015.05.061, PMID: 26119237

Shams S, Seguin D, Facciol A, Chatterjee D, Gerlai R. 2017. Effect of social isolation on anxiety-related behaviors, cortisol, and monoamines in adult zebrafish. *Behavioral Neuroscience* **131**:492–504. DOI: https://doi.org/10.1037/bne0000220, PMID: 29189020

Shams S, Amlani S, Buske C, Chatterjee D, Gerlai R. 2018. Developmental social isolation affects adult behavior, social interaction, and dopamine metabolite levels in zebrafish. *Developmental Psychobiology* **60**:43–56. DOI: https://doi.org/10.1002/dev.21581, PMID: 29091281

Sloan Wilson D, Clark AB, Coleman K, Dearstyne T. 1994. Shyness and boldness in humans and other animals. *Trends in Ecology & Evolution* **9**:442–446. DOI: https://doi.org/10.1016/0169-5347(94)90134-1, PMID: 21236920

Teles MC, Dahlbom SJ, Winberg S, Oliveira RF. 2013. Social modulation of brain monoamine levels in zebrafish. *Behavioural Brain Research* **253**:17–24. DOI: https://doi.org/10.1016/j.bbr.2013.07.012, PMID: 23850359

Teles MC, Gozdowska M, Kalamarz-Kubiak H, Kulczykowska E, Oliveira RF. 2016. Agonistic interactions elicit rapid changes in brain nonapeptide levels in zebrafish. *Hormones and Behavior* **84**:57–63. DOI: https://doi.org/10.1016/j.yhbeh.2016.05.020, PMID: 27235811

van Vliet IM, den Boer JA, Westenberg HG, Pian KL. 1997. Clinical effects of buspirone in social phobia: a double-blind placebo-controlled study. *The Journal of Clinical Psychiatry* **58**:164–168. DOI: https://doi.org/10.4088/jcp.v58n0405, PMID: 9164427

Wee CL, Nikitchenko M, Wang WC, Luks-Morgan SJ, Song E, Gagnon JA, Randlett O, Bianco IH, Lacoste AMB, Glushenkova E, Barrios JP, Schier AF, Kunes S, Engert F, Douglass AD. 2019. Zebrafish oxytocin neurons drive nocifensive behavior via brainstem premotor targets. *Nature Neuroscience* **22**:1477–1492. DOI: https://doi.org/10.1038/s41593-019-0452-x, PMID: 31358991

Winslow JT. 2003. Mouse social recognition and preference. *Current Protocols in Neuroscience* **22**:8.16.1–8.16.8. DOI: https://doi.org/10.1002/0471142301.ns0816s22

Yamamoto K, Ruuskanen JO, Wullimann MF, Vernier P. 2010. Two tyrosine hydroxylase genes in vertebrates new dopaminergic territories revealed in the zebrafish brain. *Molecular and Cellular Neurosciences* **43**:394–402. DOI: https://doi.org/10.1016/j.mcn.2010.01.006, PMID: 20123022

Zellner D, Padnos B, Hunter DL, MacPhail RC, Padilla S. 2011. Rearing conditions differentially affect the locomotor behavior of larval zebrafish, but not their response to valproate-induced developmental neurotoxicity. *Neurotoxicology and Teratology* **33**:674–679. DOI: https://doi.org/10.1016/j.ntt.2011.06.007

Ziv L, Muto A, Schoonheim PJ, Meijsing SH, Strasser D, Ingraham HA, Schaaf MJ, Yamamoto KR, Baier H. 2013. An affective disorder in zebrafish with mutation of the glucocorticoid receptor. *Molecular Psychiatry* **18**:681–691. DOI: https://doi.org/10.1038/mp.2012.64, PMID: 22641177