### Developmental Influences on Social Competence and Neuroplasticity: The Impact of Early Social Complexity in a Cooperatively Breeding Fish



Inaugural dissertation of the Faculty of Science, University of Bern

presented by

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from France

Supervisor of the doctoral thesis:

Professor Barbara Taborsky

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# General Introduction



The study of sociality is a fundamental field in biology. Across nature, we are witnessing many instances of animals living and interacting together in different ways. One common characteristic of sociality is group living. Group living is widespread among taxa (Biedermann & Taborsky, 2011; Bilde & Lubin, 2011; Cant, 2012; Koenig & Dickinson, 2016), highlighting its ecological and evolutionary significance. In invertebrates and vertebrates, group living systems have evolved as a strategy to enhance survival, resource acquisition, and cooperation (Clutton-Brock, 2006; Creel & Creel, 1995; Ewald, 1987; Riehl, 2011; Salomon & Lubin, 2007). Group size is a particularly important component for group-living animals. For example, in the cooperatively breeding *N. pulcher*, large groups are more stable, less vulnerable to extinction and have more reproductive success than smaller groups (Heg et al., 2005). In meerkats (Suricata suricatta), large groups contribute to better survival of adults when the predation pressure is high (Clutton-Brock et al., 1999). In cooperatively breeding magpies (Gymnorhina tibicen), larger groups were found to increase cognitive performance across different cognitive tests as well as promote the spread of information and innovation within the group, suggesting that living in larger groups promotes cognitive development (Ashton et al., 2018, 2019). To understand sociality, it is important to study how it evolved and how its social systems, such as cooperative breeders, are maintained.

Group size is not the only important factor to consider, groups can vary in both size and complexity in nature. A group composed of different size classes, sexes or social roles (Groenewoud et al., 2016) is assumed to be more complex compared to a group where the individuals are arranged in a homogeneous cohort, such as fish schools (Krause & Ruxton, 2002). The effects of current group size on behavioural traits, life history and fitness have been investigated in many cooperatively breeding vertebrates (Balshine et al., 2001; Clutton-Brock, 2006; Clutton-Brock et al., 1999; Heg et al., 2005; Koenig & Dickinson, 2016). Previous studies on meerkats, for example, have shown that group size can directly impact the survival and reproductive success of individuals (Clutton-Brock, 2006; Clutton-Brock et al., 1999, 2001). Cooperatively breeding vertebrates, as opposed to eusocial insects, are presumed to specialize into breeders and helpers during later life stages (English et al., 2015). Previous work on the cooperatively breeding cichlid *N. pulcher* using extreme early-life group size variation suggested that the early social environment triggered life-long differences in social trajectories, with individuals socially deprived in early life developing a breeder phenotype whereas individuals raised with adults developed a more philopatric phenotype (Fischer et al., 2017). However, social deprivation is not a situation that would exist in nature as young left without adults to defend them would not survive. The long-term influence of natural group composition has not been studied so far. In particular, very little is known about how the variation of group size and complexity experienced by offspring early in life shapes later-life social and life history trajectories.

Social behaviour plays a pivotal role in shaping the survival, reproduction, and group dynamics of organisms across diverse taxa. There are many examples of social interaction in nature, agonistic or not, and most of them are unavoidable to a wide range of species, especially for group living systems. Within social groups, individuals must navigate complex social dynamics, communicate effectively, and exhibit appropriate social behaviours to maintain group cohesion and individual fitness. The acquisition of social competence, defined as the ability to adaptively adjust behaviour to the prevailing social context, is therefore crucial for successful social interactions (Taborsky & Oliveira, 2012). Social competence plays a central role in maintaining sociality, particularly among highly social species, where critical activities rely extensively on social interactions (Taborsky & Oliveira, 2012). Social performance in each social interaction has been suggested to add up small fitness benefits over time and over the many thousands of interactions social species have during life (Taborsky, 2021). To grant long-term, substantive fitness benefits, social competence should be repeatable at the individual level, an aspect so far ignored in the concept of social competence (Oliveira, 2009; Taborsky & Oliveira, 2012). Consequently, the potential for selection to favour increased social competence may be determined through the extent to which social competence is consistent within individuals and varies consistently among individuals. Social competence assumes diverse forms and manifests through a spectrum of behaviours. In the context of within-group dominance hierarchies, submission acts as a potent signal, effectively reducing the toll of agonistic interactions by mitigating injury risks, eviction probabilities, and the energetic expenditure in conflicts (Camerlink et al., 2019; Lehner et al., 2011; Reddon et al., 2021). Beyond performance in social exchanges, the early environment exerts its influence across multiple dimensions of social and life history trajectories. Instances include the tendency to provide alloparental brood care in cooperative breeders (Fischer et al., 2017), predisposition to dispersal (Fischer et al., 2017; Zepeda et al., 2021), and investment in reproduction (Antunes & Taborsky, 2020; Fleming et al., 1997; Lindström, 1999; Pigeon et al., 2017). As illustrated by prior research (Taborsky & Oliveira, 2012), grasping the mechanisms of social competence acquisition remains pivotal for understanding the maintenance of social systems.

One way of acquiring social competence is through developmental plasticity. This refers to the ability of organisms to modify their phenotype in response to environmental conditions encountered during their development. This phenomenon is prevalent throughout the natural world and significantly shapes our understanding of phenotypic diversity and adaptation (Taborsky, 2017; Uller, 2008; West-Eberhard, 2003). Developmental plasticity occurs, in particular, in changing but predictable environments where individuals benefit from opportunities to gather information on their current and future environment and shape their phenotype accordingly. Here lies a critical link between an organism's early social environment and the process of shaping social competence.

Developmental plasticity can be costly in case of a mismatch between the early

environment and the future environment. One strategy to mitigate the risks associated with developing a phenotype ill-suited for later conditions is the extension or presence of multiple sensitive periods to environmental cues, allowing individuals more opportunities to gather information about their environment. Sensitive periods are times during ontogeny in which an individual's phenotype is particularly sensitive to changes (Fawcett & Frankenhuis, 2015). Typically, individuals are most sensitive to external influences during early development (Champagne & Curley, 2005). Nonetheless, experiencing environmental change during ontogeny may lead to prolonged or multiple sensitive periods (Panchanathan & Frankenhuis, 2016). For instance, some mammals and birds exhibit a second sensitive period during adolescence, coinciding with sexual and social maturation (Ruploh et al., 2013; Sachser et al., 2013, 2018). In rats, behavioural deficiencies as well as gene expression patterns from individuals raised with low-quality maternal care could be reversed later in life by providing them with a socially enriched environment (Champagne & Meaney, 2007). The existence of a second or extended sensitive period enables individuals to better align their phenotype with the later-life environment (Sachser et al., 2018). Animals experiencing highly variable yet predictable social environments should benefit from a second sensitive period to acquire social competence. In group-living species like cooperative breeders, dispersal is typically a period where individuals can experience changing environments. Yet, it is unclear if a second sensitive period for the acquisition of adequate social behaviour is also found in cooperative breeding societies prior dispersal decision.

Expanding upon the concept of developmental plasticity, it is imperative to examine the influence of the early social environment on the acquisition of social competence. In various vertebrate species, evidence consistently points to the early-life social environment as a key determinant of subsequent social behaviour and competence (Arnold & Taborsky, 2010; D'Andrea et al., 2007; Kempes et al., 2008; Taborsky, 2017). For instance, studies reveal that mice (Mus musculus) reared in communal nests exhibit heightened allogrooming and allosniffing tendencies toward cage mates later in life compared to those raised in singlemother nests (D'Andrea et al., 2007) and display quicker adoption of hierarchy roles (Branchi et al., 2006). Jumping spiders (Marpissa muscosa) show better learning ability and more appropriate social behaviours when reared in an enriched social environment (Liedtke & Schneider, 2017). In *Neolamprologus pulcher*, a cooperatively breeding cichlid fish, individuals raised in socially deprived environments display lower social competence than fish raised in larger more complex groups of conspecifics (Arnold & Taborsky, 2010; Fischer et al., 2015, 2017; Nyman et al., 2017; Taborsky et al., 2012). The variability of the early social environment stems from factors such as group size, composition, and types of interactions between group members.

Developmental plasticity, through the early social environment, seems to be one main mechanism behind the acquisition of social competence, likely governed by neurological processes at the brain level. Understanding how individuals acquire social competence is a fundamental research topic in evolutionary biology. It can unravel the complexities of social interactions and their adaptive significance. To broaden our understanding we need to investigate the process of acquisition of social competence at different levels, diving into a more mechanistic approach by focusing on brain circuitry and how developmental plasticity and the early social environment influences neurological processes.

The social decision-making network (SDMN) is a well-conserved network of interconnected brain nodes responsible for processing and integrating social information and controlling social behaviour expression (O'Connell & Hofmann, 2011, 2012). On the more mechanistic side, social competence is thought to be accomplished by rewiring or by biochemically switching between nodes of the SDMN, facilitated by neuromodulators like neuropeptides, monoamines, or hormones, responding to current social information (Cardoso et al., 2015). Structural and functional changes in the brain are mediated by neuroplasticity processes, particularly through the influence of neurotrophins, which play a crucial role in mediating the rewiring of the SDMN and shaping social competence. Restructuration of neural networks in response to novel experiences or alterations in behaviour or environment is done through neuroplasticity (Kleim & Jones, 2008). Notably, neurotrophins have been proposed as key mediators of short- and long-term neuroplasticity, thereby enabling environmental experiences to shape brain structure and function (Branchi et al., 2004).

Neurotrophins are of key interest in the study of early social-environmental effects on social competence. BDNF, a well-preserved neurotrophin across vertebrates (Lucini et al., 2018), is a good candidate for in-depth examination of the long-term behavioural and neurological consequences of early social experiences. BDNF engages with two receptors, p75NTR and TrkB (Purves et al., 2004), each contributing distinct functions to neuroplasticity and acting as a significant regulator of excitatory synapse plasticity (Leal et al., 2015). There are two pathways to synaptic plasticity: BDNF/TrkB (Long Term Potentiation, long-lasting synaptic enhancement and synapse strength) and BDNF/p75NTR (Long Term Depression, synapse elimination) (Sakuragi et al., 2013). Experiments manipulating the early social environment in rodents have demonstrated both short and long-term impacts on brain neurotrophin expression (Branchi et al., 2006; Cirulli et al., 2003; Liu et al., 2000). In cooperatively breeding fish, social environment and social challenge impacted the expression of the neurotrophin bdnf in the hypothalamus (Nyman et al., 2017). While we understand the impact of manipulations of the early social environment on social competence in cooperative breeders, the relationship between variations in social competence and neuroplasticity markers, such as neurotrophins, remains unclear.

#### **Thesis aim**

The work of this thesis aimed to provide a comprehensive perspective on the interplay between the early social environment and the acquisition of social competence. I investigated the underlying mechanisms of acquisition of social competence using the cooperatively breeding fish *Neolamprologus pulcher* as a model species, through different questions.

In the first chapter, I aimed to answer three questions: (i) How does early-life experimental variation in a natural range of group sizes and complexity influence the acquisition of social competence? (ii) Do *N. pulcher* behave adequately and consistently in repeated tests of a particular behavioural trait and whether repeatability of a social trait depends on early-life group size and complexity? And (iii) how does early social complexity shape the expression of important life-history traits such as helping behaviour, exploration, and dispersal? I predicted that individuals raised in larger, socially more complex groups would exhibit enhanced social competence compared to those from smaller groups (Fischer et al., 2015). Additionally, fish from larger groups were expected to consistently display appropriate responses to repeated social challenges, in contrast to fish from smaller groups. Previous experiments on N. pulcher demonstrated that early-life behavioural competence influenced adult strategies, with fish raised alongside adults exhibiting higher social competence and philopatry yet reduced helping behaviour compared to socially deprived fish (Fischer et al., 2017). Building on these findings, I further predicted that individuals raised in larger, complex groups would exhibit reduced helping behaviour and a preference for group living when provided the chance to disperse for independent breeding.

In the second chapter, I aimed to decipher when during the lifespan social competence was acquired and whether this acquisition could be reversed by exposure to different social experiences later in life. Arnold & Taborsky (2010) argued that, in *N. pulcher*, the differences in social competence resulting from exposure to different early social environments are due to differences in the opportunity to learn appropriate social behaviour. Following this argument, I now asked whether when provided with additional opportunities fish could change their pattern of acquisition of social competence. I investigated how changing and predictable environments influence the acquisition of social competence, particularly in the context of cooperative societies and dispersal decisions. By manipulating the social environment as individuals transition through life stages, I aimed to shed light on the presence or absence of extended or multiple sensitive windows in *N. pulcher*. I predicted that fish raised in socially enriched groups in early life would always display a higher social competence regardless of later life social experience. I further predicted that fish reared without adults but later exposed to a socially enriched environment will display a higher social competence than fish deprived of adults early and that these fish will stay

either with siblings or alone during the second experience phase.

For my third chapter, I focused on the underlying neural mechanisms of social competence acquisition. By probing into neuroplasticity and neurotrophins, notably brain-derived neurotrophic factor (BDNF), I aimed to elucidate how the early social environment impacts brain plasticity gene expression. I measured gene expression related to neurotrophins and their receptors within specific brain regions involved in the expression of social behaviour. I sought to uncover the underlying mechanisms behind the differences in social competence demonstrated in my first experiment. I predicted that increased social competence is associated with specific neuroplasticity patterns, suggesting that complex early social environments promote the upregulation of synaptic plasticity mechanisms within the SDMN. Prior fish studies demonstrated the correlation between social phenotype and distinct bdnf expression patterns in different nodes of the SDMN involved in the expression of social behaviour (Nyman et al., 2017; Teles et al., 2016). Drawing from these results, I predicted that early social complexity influenced bdnf expression in the SDMN. Consequently, I expected that higher complexity would align with the elevation of synaptic plasticity pathways in the regions of the brain I measured.

With this approach, I enhanced our understanding of the role of development in the acquisition of social competence in *Neolamprologus pulcher* and the underlying mechanisms of this acquisition. By enhancing our understanding of the connections between the early social environment, neural mechanisms, and sensitive windows during development, my research provided insights into the complex interplay between sociality, behavioural development, and the capacity to adapt within dynamic environments.

#### **Study species**

The East African cichlid *Neolamprologus pulcher* has proven to be a highly suitable model species in the study of the effects of the early social environment on social competence (Antunes & Taborsky, 2020; Arnold & Taborsky, 2010; Fischer et al., 2017; Nyman et al., 2017; Taborsky et al., 2012; M. Taborsky, 2016). *N. pulcher* is a cooperatively breeding cichlid endemic to Lake Tanganyika in Africa (M. Taborsky, 2016). Individuals live in groups that can vary in size and composition. Typically groups are composed of a dominant breeding pair and a variable number of subordinates ranging from 1 to 30 per group (Groenewoud et al., 2016; M. Taborsky, 2016). These fish breed cooperatively, in groups organized in linear size-based hierarchies (Dey et al., 2013). Subordinate helpers can be related or unrelated to the breeders (Dierkes et al., 2005). Subordinate fish perform a range of helping behaviours towards the dominants, such as direct brood care by cleaning or defending eggs against predators, territory maintenance by cleaning shelter spaces, and territory defence against space competitors or predators (Balshine et al., 2001; Bruintjes & Taborsky, 2011; M. Taborsky, 1984, 2016; M. Taborsky & Limberger, 1981). As cooperative breeders, they are involved in many different social interactions

between group members every day and therefore should benefit from the early acquisition of social competence (Taborsky et al., 2012).

#### **Chapter Overview**

Chapter 1: "Early social complexity influences social behaviour but not social trajectories in a cooperatively-breeding cichlid fish."

The first chapter of this thesis investigates the influence of the early social environment on social competence and life-history traits in the cooperatively breeding cichlid species *Neolamprologus pulcher*. I investigated how group size and social complexity during the first two months of life affect social competence and life-history traits in *N. pulcher* between the ages of four and twelve months. I performed a set of behavioural tests to measure social competence and three life history traits (exploration, helping and dispersal). I demonstrated that, as predicted, fish raised in larger more complex groups exhibit higher social competence. Specifically, by exhibiting increased submission in response to aggression from dominant conspecifics, earlier submission, and greater flexibility in the expression of submissive behaviour compared to those raised in small groups. These findings emphasize the significance of early-life social complexity in the development of social abilities in this species. Moreover, contrary to my predictions my results indicate that early social complexity does not appear to influence aggressive and exploration behaviour, nor does it influence the propensities for dispersal and helping behaviour. By investigating these dynamics, this chapter contributes to a better understanding of the impact of early social experiences on the development of social competence and associated life-history traits in cooperatively breeding cichlids. This study is in revision in Royal Society Open Science.

## *Chapter 2: "Social competence is influenced by early but not late-life social experience in a cooperatively breeding fish."*

This chapter aims to investigate whether a second or extended sensitive period exists for the acquisition of social competence. The study raises questions about whether individuals retain plasticity to adjust their social competence later in life when the predictability of the early social environment is manipulated. Juveniles *N. pulcher* were raised for two months with or without adults and subsequently assessed for social competence. Fish were then assigned to different social contexts, such as being housed alone, with siblings, or with a new group, to examine the impact of subsequent social experiences on social competence. I demonstrated that only the early social environment triggered long-term behavioural differences. The findings from this chapter contribute to our understanding of the factors shaping social competence and the interplay between sociality and social competence. By investigating the potential sensitive periods and plasticity in social competence acquisition, my study enhances our knowledge of how individuals adapt to changing and predictable environments in cooperative breeders like *N. pulcher*. I suggest that limited opportunities for the acquisition of social competence later in life may drive the preference for philopatry, reinforcing the interplay between sociality and social competence. This study has been submitted to *Animal Behaviour*.

### Chapter 3: "Social complexity during early development has long-term effects on neuroplasticity in the social decision-making network.."

This chapter investigates the underlying neural mechanisms and plasticity associated with the acquisition of social competence. Specifically, it explores the differential expression of neurotrophins and their receptors in key brain regions involved in social behaviour. I studied the expression of brain-derived neurotrophic factor (bdnf) and its two receptors p75 and TrkB in four nodes of the social decision-making network (SDMN) of *N. pulcher* raised in large or small groups in early life. I selected one region in the telencephalon, two in the hypothalamus and one in the preoptic area. I showed that early social environment induces long-term differences in the expression of bdnf, and its receptors in three specific nodes of the SDMN (specifically DL, aTn and POA). These findings underscore the significance of social complexity during early development and its potential impact on the expression of neurotrophins and their receptors in specific brain regions. This chapter enhances our understanding of the molecular mechanisms underlying the effects of the early social environment on brain structure and function, contributing to the broader knowledge of how neuroplasticity and neurotrophins mediate the acquisition of social competence.

# Appendix 1: "Fish can infer relations between colour cues in a non-social learning task".

Transitive inference (TI) describes the ability to infer relationships between stimuli that have never been seen together before (Guez & Audley, 2013; Vasconcelos, 2008). Social cichlids can use TI in a social setting where observers assess dominance status after witnessing contests between different dyads of conspecifics (Grosenick et al., 2007). If cognitive processes are domain-general, animals should use abilities evolved in a social context also in a non-social context (Varela et al., 2020). Therefore, if TI is domain-general in fish, social fish should also be able to use TI in non-social tasks. Here we tested whether the cooperatively breeding cichlid *Neolamprologus pulcher* can infer transitive relationships between artificial stimuli in a non-social context. We used an associative learning paradigm where the fish received a food reward when correctly solving a colour discrimination task. Eleven of twelve subjects chose the predicted outcome for TI in the first test trial and five subjects performed with 100% accuracy in six successive test trials. We found no evidence that the fish solved the TI task by value transfer. My findings show that fish also use TI in non-social tasks with artificial stimuli, thus generalizing past results reported in a social context and hinting toward a domain-general cognitive mechanism.

This study has been conducted conjointly with a student as a bachelor thesis project and published in *Biology Letters* in 2022.

## Appendix 2: "Do rearing group-size and social rank influence the affective state of a cooperatively-breeding cichlid fish?"

The affective state of animals, i.e. their mood and emotions, are altered by stressful (negative) or enriching (positive) experiences. In turn, the affective state influences decision making, thereby helping animals to cope with environmental challenges and opportunities. Particularly in the social domain, it is largely unknown how social experiences modulate the affective state. Here, we performed a judgment bias test to study the effects of rearing group-size and experimentally assigned current rank on the affective state of the cooperatively-breeding cichlid fish Neolamprologus pulcher. To assess affective state, we developed and validated a judgment bias test for this species. Fish learned to discriminate between a positive and a negative stimulus as shown by different latencies to approach the stimulus. Furthermore, the fish showed the response curves expected in judgement bias tests. They showed an intermediate latency to approach an ambiguous stimulus, which significantly differed from the latencies to approach the positive and the negative stimulus. Unexpectedly, there were no significant effects of rearing group size and current social rank on the affective state of N. pulcher, despite known effects of these two social parameters on behaviours and physiology of this species. This study has been conducted conjointly with a student as a bachelor thesis project and is in preparation to be submitted to Ethology.

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# **Chapter 1**

Early social complexity influences social behaviour but not social trajectories in a cooperativelybreeding cichlid fish.

In revision in Royal society Open Science



# Early social complexity influences social behaviour but not social trajectories in a cooperatively-breeding cichlid fish.

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#### <u>Abstract</u>

Social competence - defined as the ability to optimise the social behaviour to prevailing social information – as well as a suite of life history traits, can be influenced by the early social environment in cooperatively breeding vertebrates. The size of social groups influences the current behavioural phenotype of these species, but whether group size during early life induces behavioural phenotypes and life history is yet unknown. Here we compared how being reared in large vs. small groups for the first two months of life affects social behaviour and life-history traits in the cooperatively-breeding cichlid *Neolamprologus pulcher* between the age of four and twelve months. As we had predicted, fish raised in larger, more complex groups showed higher social competence: they showed submission earlier and exhibited more flexibility in the expression of submissive behaviour compared to fish raised in small groups. This emphasizes the importance of early-life social complexity for the development of social competence. In contrast, there was no evidence that early social complexity affects aggressive and exploration behaviour, and the propensities to disperse or to show helping behaviour.

#### **Introduction**

For group-living species the early life is especially important to develop the appropriate social skills to navigate their social environment. Social competence is based on behavioural flexibility [1,2] and denotes the ability to optimise the social behaviour depending on available social information [1,3]. Such adjustments come with small fitness benefits that add up with each social interaction [4]. In a range of vertebrates, the social environment experienced during early life shapes later social behaviour and social competence [5–7]. For instance, mice reared in communal nests show a higher level of allogrooming and allosniffing towards cage mates later in life, when compared to mice reared in singlemother nests [6] and take a shorter time to adopt their respective roles in a hierarchy [8]. Social competence is likely to have substantial fitness consequences, in particular for highly social species, in which most of the activities necessary for survival (e.g., foraging, predator defence) and mating require social interactions [1,4]. As an example of social competence in a mating context, male sage grouse Centrocercus urophasianus have higher mating success when they adjust their courting effort to the presence of females [9]. In the context of within-group dominance hierarchies, submission is a powerful signal to lower the cost of agonistic interaction between group members and therefore reduce chances of injury and eviction as well as the energetic cost of fighting [10–12]. In cooperatively breeding societies submission has also been found to be displayed as pre-emptive appeasement in order to avoid punishment by dominants [13]. Beyond the performance in social interactions, the early environment has been demonstrated also to influence other aspects of social and life history trajectories throughout life, like the propensity to provide alloparental brood care in cooperative breeders ('helping') [14], the propensity to disperse [14,15] and investment in reproduction [16–19].

Group size and composition are important determinants of the social environment. Beyond group size, a more diverse composition of group members contributes to an enhanced complexity of the social environment [20]. A stable group of cooperative breeders composed of breeders and helpers with different size classes, sex or social roles [21] can be assumed to allow for more numerous and diverse interactions and thus to represent higher social complexity than a uniform group of individuals such as often seen in fish schools or gnu herds, for instance [22]. The effects of current group size on behavioural traits, life history and fitness have been investigated in many cooperatively breeding vertebrates [23–27]. Group size has been shown to promote survival and reproductive success in cooperative meerkats, for example [24,25,28]. However, nearly nothing is known about how the variation of group size and complexity experienced by offspring early in life shapes later-life social and life history trajectories. Cooperatively breeding vertebrates, as opposed to eusocial insects, are assumed to specialize into breeders and helpers only during later life stages [29]. Thus far, there has been a single study experimentally varying early-life group size in a cooperatively breeding vertebrate. This experiment, done in

our study species *Neolamprologus pulcher*, reported that manipulating early-life group size and complexity affected the expression of social behaviour shortly after the social experience phase [30]. Several other experiments in this fish species varied the early social environment, but all of them compared behavioural and life history traits between a small cooperatively-breeding group and a socially deprived group of siblings without breeders and helpers, a situation not found in nature [5,14,31]. In contrast, *N. pulcher* show a highly variable range of group sizes in the wild, with breeders always present and the number of subordinates ranging from 1 to 25 per group [20]. Therefore, here we asked, how early-life experimental variation in a natural range of group sizes and complexity affects social competence, helping behaviour as well as an important life-history decision, the propensity to leave a group and disperse for independent breeding, in the cooperatively breeding vertebrate *N. pulcher*.

Small fitness benefits accrued by a better social performance in each social interaction have been suggested to add up over time and over the many thousands of interactions social species have during life [4]. Social competence may yield only a small fitness benefit in any given social interaction. However, with repeated social interactions, these benefits should add up for individuals that repeatedly behave appropriately in a specific context. Thus, to yield long term, substantive fitness benefits, social competence should itself be repeatable at the individual level, an aspect thus far ignored in the concept of social competence [1,2]. Thus, the extent to which social competence is consistent within individuals and varies consistently among individuals may determine the potential for selection to favour increased social competence. Therefore, here we investigated whether *N. pulcher* behave adequately and consistently in repeated tests of a particular behavioural trait and whether repeatability of a social trait depends on early-life group size and complexity.

To answer these questions, we manipulated rearing groups, so they differed in size and social complexity. We then tested individuals differing in early social experience for a series of behavioural and life history traits at different points of their lives. In brief, for the first 60 days of life, we reared fish in large groups, composed of a dominant breeder pair and eight subordinates of different sizes and sexes, or in small groups comprising only a breeder pair and one small helper ("experience phase"). In *N. pulcher*, the diversity of helper sizes reflecting different ranks in the social hierarchy contributes to a higher diversity of interactions and thus enhanced social complexity [20,21]. To test the influence of early-life social complexity on social competence and behavioural trajectories, we exposed the experimental fish to a suite of behavioural tests that have been developed in our laboratory [14,30,32], based on an in-depth knowledge of *N. pulcher* have a linear-sizedbased hierarchy to design social tests, where the appropriate social behaviour is known.

We predicted that individuals raised in larger, socially more complex groups will have more social competence than individuals raised in small groups [30]. This implies

that fish from large groups should show a steeper increase in their response to social information, in our case a steeper increase of submission relative to received aggression, indicating higher levels of flexibility. Moreover, fish raised in large groups should also display appropriate behaviours more consistently in response to repeated exposure to a social challenge than small group-raised fish. Previous developmental experiments on *N. pulcher* reared with or without older group members showed that the behavioural competence acquired in early life influenced adult life-history strategies, with fish raised with adults showing higher social competence and higher philopatry as adults, but instead showed less helping behaviour, compared to fish raised in a socially deprived environment [14]. Based on this earlier study, we therefore predicted that individuals raised in a larger, more complex group will show less help and will choose to stay in a group when given the opportunity to disperse for independent breeding.

#### **Methods**

#### Model species

The East African Cichlid *N. pulcher* has proven to be a highly suitable model species in the study of the effects of the early social environment on social behaviour and lifehistory trajectories [5,14,19,20,31,33]. These fish breed cooperatively, with groups being organized in linear size-based hierarchies [34]. In nature, social groups are typically composed of a dominant breeding pair and subordinate helpers of various sizes and sexes [20,21]. Subordinate helpers can be related or unrelated to the breeders [35]. Helping behaviour includes alloparental care, territory defence and territory maintenance [20,36]. As cooperative breeders, they are involved in many different social interactions between group members every day and therefore should benefit from the early acquisition of social competence [31].

#### *Rearing treatments*

We used a laboratory-bred *N. pulcher* population as the parental generation for the experimental fish, which was derived from wild-caught fish from Kasakalawe Point, Mpulungu, Zambia. We reared juvenile *N. pulcher* in the lab in two different early social environments: (i) small groups were composed of three adult individuals, two breeders [5.0-7.0 cm standard length (SL)] and one small helper (1.5-2.5 cm SL); and (ii) large groups were composed of ten adult individuals, two breeders (5.0-7.0 cm SL), four large helpers (two males, two females, 4.0-4.7 cm), and two medium-sized helpers (2.6-3.5 cm) and two small helpers of unknown sex (1.5-2.5cm). Breeders and helpers were unrelated. Reproduction was sometimes shared among females in the large groups so the clutches used for this experiment may have been laid by either the dominant female or one of the large female helpers. The juveniles stayed for 60 days after they were free swimming (i.e., day 0) in the group tanks for the 'social experience phase' (Figure 1).

At day 60, the 21 broods were transferred to 50-L tanks, where they were kept only among their siblings for another 60 days ('neutral phase'). We discarded all clutches with less than four young at day 60. Discarded clutches were placed in aggregation tanks where they grew to become later part of our stock population. We kept sibling groups of up to 10 individuals in 50-L tanks (on average 8 individuals per tank). The remaining siblings were placed together in a 200-L aggregation tank and raised for future use in other studies; they were not used in this experiment anymore. The neutral phase is important to ensure that any behavioural differences between treatments measured in the later behavioural tests are not a direct effect of the different conditions during the early-experience phase, but reflect longer-term, developmentally plastic effects.

#### Experimental phase 1

At the age of 120 days, we started the first set of tests. We selected two focal fish (average size  $2.2 \pm 0.19$  cm SL) per sibling group and marked them with a unique elastomer colour tag [37]. We excluded the biggest and the smallest fish, thereby excluding the fish at both ends of the size-based hierarchy to avoid differences in behaviour merely due to their extreme rank. Further, fish below 1.9 cm standard length (SL) were too small to be individually marked and were therefore also not used. Among the remaining fish, we selected the two focal fish randomly from each sibling group.

We exposed the fish to two social challenges, a 'hierarchy test' and an 'aggression test'. Both were repeated three times per fish, with hierarchy tests taking place on days 120, 127 and 150 after free swimming (Figure 1). Aggression tests were carried out on the day after each hierarchy test (i.e., on days 121, 128, 151). We tested 20 fish from the large-group treatment and 20 fish from the small-group treatment. Due to some technical difficulties and naturally occurring mortality, some individuals could not undergo all three tests for each behavioural trait. Fourty fish underwent the first test, 37 the second and 26 all three tests. The sample size was always balanced across treatments. In experimental phase 1, the fish were not yet sexually mature, so we did not consider their sex in the statistical analysis.

#### *Hierarchy test*

For measuring submission, we let our focal fish interact with a larger dominant conspecific, a situation where the appropriate behaviour is to show submission in order to retain access to a territory and its resources. We introduced a focal fish in a 20-L tank equipped with a shelter as centre of a territory and allowed it to acclimatise and claim the shelter overnight [30]. The following day we added an 'intruder' in the 20-L tank, which was 4-5 mm bigger than the focal fish (about 25% larger). We used a different intruder for each trial. The size of the intruder was chosen to assure its dominance over the focal fish. We immediately recorded all aggressive and submissive displays by the focal fish and intruder for the next 20 min. We distinguished three categories of aggressive displays: fin

spreads, restrained aggression (i.e. without physical contact) and overt aggression (i.e. with physical contact) [38]. We recorded the duration spent by the focal fish performing submissive displays, all aggressive behaviours by the intruder towards the focal fish, and the latency of the focal fish to show submission for the first time. It is good to note that the fish did not interact non-stop during our trials, as fish would sometimes swim alone in the tank or hide in the shelter. Four hours after the release of the intruder, we observed both fish for 10 min to establish the acceptance status of the focal fish by the larger intruder. 'Fully accepted' focal individuals had access to the shelter or at least to its close vicinity of the shelter (<1 body length) as well as everywhere else in the tank, and the dominant did not show aggression towards the focal fish. "Accepted" focal fish could swim everywhere in the tank except in the close vicinity of the shelter closely. The dominant was only aggressive when the focal fish tried to access the shelter. "Evicted" focal fish were restricted to less than a third of the tank that was the furthest away from the shelter and they received aggression by the dominant when being close to the dominant. In two cases, the eviction of the intruder by the focal fish happened. No fish were injured in the hierarchy test. All behavioural recordings of this and the below behavioural tests were done with a Sony Handycam HDR-PJ260 and coded with the software BORIS [39].

#### Aggression test

To compare aggression between treatments, we exposed our focal fish to videos of a smaller conspecific. In this situation, competent fish should be aggressive to assert dominance and keep ownership of the territory over a small conspecific. We varied the level of aggressive behaviour of the small conspecific displayed in the video to see if aggression was adjusted to these different situations. We presented videos of aggressive displays by conspecifics we had previously recorded through a one-way mirror such that aggressive behaviours appear to be directed at the focal fish. Presenting these videos to the focal fish allowed us to standardize the intensity of the received aggression by a virtual intruder perceived by the focal fish. Video presentations to test for aggressive tendencies of focal fish were done in the same experimental tanks that were used for the hierarchy tests. We presented a video on a Samsung A5 screen, positioned vertically, of a smaller conspecific (about 1cm on the screen) to the focal fish, which either displayed 'fast approaches' (strong aggression), 'head-down' display (low aggression) or swam calmly (no aggression) [38]. As video presentations are constrained to present restrained aggression (i.e. aggression with no physical contact), we classified the presented aggression as 'strong' or 'low' aggression. A video presentation lasted 10 min. On each test day, one presentation of each of the three aggressive levels was shown to a focal fish with a 1-h gap in between each presentation. Videos of the three aggressive levels were shown in a balanced order. We recorded all aggressive displays performed by the focal fish toward the videos. In this test focal fish are larger than the fish on the video and they already own a territory. Therefore, the appropriate (i.e., socially competent) behaviour to display in response to the videos

is aggression towards the smaller, virtual intruder, and aggression of the focal fish should increase with the increased aggression levels shown by the virtual intruder in the videos.

#### Experimental phase 2

At 1 year of age (±2 weeks) we tested the focal fish for their propensities to show exploration, helping behaviour and dispersal, over a 3-week testing period (Figure 1). We waited until the fish were 1 year old, as older fish engage more readily in defence behaviour [40] and fish only start to disperse after sexual maturity [41]. All tests were done in a 1000-L tank partitioned into different areas, depending on the task. We chose to test exploration propensity as an attempt to validate its use as a proxy for dispersal. We tested helping behaviour and dispersal because these traits were previously described as being part of a suite of life-history traits and social behaviours that was influenced by the early social environment [14]. At 1 year of age, 27 individuals of the 40 individuals tested in the first experimental phase were still alive and were tested in 'Experimental phase 2'. The fish were now sexually mature (size between 3.5 and 5.0 cm SL) so that we could determine their sex and include it in the analysis.

#### Exploration test

The focal fish was introduced in a 100L compartment of a 1000L experimental tank ('safe area'), which contained one flowerpot half as a shelter. On the first day, we let the focal fish acclimatise for 20 min in this compartment, before lifting a mesh divider between the safe area, where the fish was acclimatized, and an 'exploration zone'. The exploration zone was a 600-L compartment equipped with ten large shelters equally spaced forming three lines across the tank bottom and a filter [32]. The shelters were placed with the opening facing the front of the tank. The focal fish was recorded for 25 minutes, and we counted the number of visited shelters in the exploration zone. On the second day, we repeated the same test twice, with 5 hours break in between. Between the first and the second day, the fish stayed in the safe area overnight without physical or visual access to the exploration zone. Therefore, no acclimation time was necessary before the tests on day 2.

#### Helping test

We created a territory suitable for a group of fish within the 1000L tank. The territory was 115L composed of five large (11 cm x 6 cm [L x H]), three medium (10 cm x 6 cm [L x H]) and two small-sized shelters (8 cm x 4 cm [Lx H]). We gave the focal fish 20 min to acclimatise to the new territory. Then we introduced a pair of dominant breeder fish where the dominant female was chosen to be at least 0.5 cm SL bigger than the focal fish and the dominant male at least 0.5 cm SL larger that the dominant female. We set up a neighbouring group of five adult *N. pulcher* of various sizes in a compartment next to the territory, separated by a transparent divider. The purpose of the neighbouring group was to simulate a more natural environment to raise the probability that the dominant accepts

the focal fish as a helper (see 11).

During the second week, we performed three helping tests, each separated by 24 hours (Figure 1). We first placed an opaque partition between the neighbouring group and the group consisting of the pair and the focal fish, so that the neighbouring group did not distract the group during the helping test. Next, we presented an egg predator *Telmatochromis vittatus* inside a transparent plastic cylinder (Figure 1). All used egg predators were between 4.0 and 5.0 cm SL. To prepare the presentations, we first surrounded the transparent cylinder with the egg predator in it by an opaque cylinder and allowed for 10 min for both the egg predator and the pair and focal fish to acclimatize to the set-up. Then we lifted the opaque cylinder and immediately recorded the interaction of the pair and focal fish with the egg predator in its transparent tube for 15 min. Interactions seen in this test consisted of aggressive behaviours by the *N. pulcher* pair and focal fish towards the tube with the egg predator [42].

#### Dispersal test

For the dispersal test, we followed the method of Fischer et al. (2017), which we summarize here briefly. The dispersal test was done in 1000-L tanks of 2.6m length. At the onset of the dispersal test, the focal fish was still subordinate to the pair that had been introduced in the helping test (see above). We chose a potential mate from the focal fish that we placed in compartment 80 cm away from the territory of the group consisting of the dominant pair and the focal fish (group territory). Thus, between the group territory and the compartment of the mate there was an empty 80-cm zone containing only sand, which the focal fish had to cross to disperse to the compartment containing the potential mate. If the potential mate was a female, we chose it to be 0.5-1.0 cm SL smaller than the focal fish, and if it was a male, it was larger by that size difference than the focal fish. All fish were allowed to habituate to the set-up for 14 days, with two dividers in place separating the three compartments; an opaque divider between group territory and empty zone, and a transparent divider between empty zone on potential mate's territory. After this habituation period, we removed the divider between the group territory and the empty zone and we lifted the transparent divider between the empty zone and the potential mate's territory by 2 cm. To access the potential mate's territory, the focal fish thus had to cross the empty zone and then to swim through the 2-cm slit between tank bottom and transparent divider. The focal fish was allowed 7 days to decide to either stay as subordinate with the pair or become a breeder in the potential mate's territory. On day 21, we recorded the position of the focal fish; if the focal fish was in the mate's territory, we considered it to have dispersed.



Figure 1: Timeline of the experiment

#### Statistical analysis

We analysed behavioural data collected from the behavioural tests using linear mixed effect models fitted in R 4.1.2 [43] using packages "glmmTmb" [44], and "LmerTest" [45]. All models include early social treatment as a fixed effect and fish identity and family identity as random effects to account for repeated measures on individuals. Other fixed effects included are described below for each model. We present inference on fixed effects based on type III analysis of variance using Satterthwaite's method. Post hoc tests for pairwise comparison between significant factors were run using the package "emmeans" [46].

#### Social competence

In the hierarchy test we measured, and then modelled, four response variables; (1) the total duration of submissive displays performed by the focal fish, (2) the focal fish's latency to show the first submission, (3) the submission rate per received aggression and (4) the acceptance status of the focal fish at the end of each test. (1) In the model with the duration of submission, the response variable was log-transformed to achieve normality of the residuals. We investigated the effect of the interaction between received aggression by the intruder and rearing treatment as well as the effect of the test number (i.e., whether it was the 1st, 2nd or 3rd test). (2) Latency to the first submission was log-transformed to achieve normality of residuals. We investigated the effect of received aggression during the test, rearing treatment, and the effect of the test number. (3) The submission rate per received aggression was Box-Cox transformed to achieve normality of residuals distribution. We investigated the effect of the interaction of rearing treatment and category of received aggression as well as test number on the rate of performed submission per received aggression. (4) Acceptance status was a binary variable (accepted or not), so we used a generalised linear mixed model fitting a binomial distribution. We excluded the two focal fish from this analysis which evicted the intruder, as it was a rare event. Model assumptions were checked via visual inspection of residuals from Tukey-Anscombe plots and quantile-quantile (Q-Q) plots.

In the aggression test, we recorded the number of total aggressive behaviour performed by the focal against the videos and analysed this using a generalised linear mixed model (GLMM) with negative binomial error distribution as the data were overdispersed. Test number, level of received aggression and rearing treatment were included as fixed effects, with focal fish identity as a random effect.

We estimated the repeatability (R) of submission and aggression using the function "rpt" of the package "rptR" [47]. We estimated the adjusted repeatability of submission duration and the number of aggressive behaviours for all fish and separated by treatment. Submission durations were log-transformed to follow a normal distribution. The repeatability of total aggressive behaviour was estimated with data being square root transformed.

#### Social trajectory traits

For each of the exploration, helping, and dispersal tests we scored and analysed a single response variable only. In the exploration test, we modelled the total number of visited pots using a negative binomial GLMM with fixed effects of test number, sex, rearing treatment and focal identity as a random effect. The same GLMM structure was then used to model the total number of aggressive behaviours against the egg predator from the helping test. We used negative binomial distribution as the data were overdispersed in both cases. Finally, we analysed dispersal as a binary variable: fish either dispersed or not. Since there was only a single observation per focal fish and there was no detectable effect of family identity on the variance we used a GLM with binary distribution and fixed effects of rearing treatment, sex, size, exploration propensity and prospection on dispersal.

#### <u>Results</u>

#### Social Competence

#### **Hierarchy Test**

There was a significant interactive effect between the early social experience and the duration of received aggression on the duration of submission performed (Figure 2a, LMM, p=0.034, Table 1a). Fish reared in large groups showed more submission per received aggression than fish reared in small groups (Table 1a, Figure 2a), thus displaying a more flexible response than fish reared in small groups. Also, test number affected submission duration. The fish showed more submission in the second test compared to the third one (Figure 2a, Table 1a, LMM, p=0.012). The early social environment did not affect the likelihood of being accepted in the intruder fish territory (GLMM, df=1,  $\chi^2$ =0.029, p=0.866).

Table 1: Results of the LMMs fitting the effects on submission in the hierarchy test. (a) Effects of the test number (1,2,3), size and rearing treatment, received aggression and their interaction on the duration of submission. The duration of submission were log-transformed to achieve normality of model residuals (n=100 observations, n=40 individuals, n=21 family groups). (b) Effects of rearing treatment, category of received aggression [i.e. fin spread (score of 1), restrained aggression (2) or overt aggression (3) performed by the dominant individual that triggered submission events by the subordinate individual] and their interaction, and test number and size on the ratio of the duration of submission per duration of received aggression in each category. Duration of submission was boxcox-transformed to achieve normality of model residuals (n=300 observations, n=40 individuals, , n=21 family groups). (c) Effects of the test number (1,2, or 3), rearing treatment and received aggression on the latency to first submission (log-transformed) (n=91 observations, n=40 individuals). The post hoc results are pairwise comparisons between (d) the rearing treatments and test numbers from model (a) and (e) between rearing treatments compared within each category of received aggression and test number from the model (b). The duration of submission was boxcox-transformed to achieve normality of model residuals; the interpretation of estimates has to take account of this data transformation, which caused the estimate signs to reverse in direction. NumDF: Numerator degrees of freedom. DenDF: Denominator degrees of freedom. Significant *p*-values are highlighted in bold.

Model	Factor level	Estimate	SE	NumDF	DenDF	F value	p-value
(a) Time spent performing submission (total)							
Rearing treatment x Received aggression		52.809	22.931	1	79.955	5.304	0.024
Rearing treatment	large	0.966	0.428	1	19.569	5.096	0.036
Received aggression		6.661	7.205		80.12	8.402	0.005
Test number				2	86.704	4.263	0.017
Sizo	test 2 test 3	0.273 -1.032	0.315 0.465	1	40.020	0.270	0 500
(h) Time s	nent nerformi	0.155 ina suhmis	0.941 sion after r	1 receivina	49.059 aaaressia	0.279 n	0.599
Rearing treatment x Category of received aggression	large x restrained aggression	-0.124	0.601	2	255.16	5.099	0.007
	large x overt aggression	-0.191	0.061				
Rearing treatment	large	0.004	0.06	1 2	18.88 255.16	4.243 112.793	0.053 <b>&lt;0.001</b>
Category of received aggression	restrained aggression	-0.328	0.043				
	overt aggression	-0.305	0.043				
Test number				2	228.105	7.167	<0.001
	test 2 test 3	-0.052 0.109	0.029 0.047				
Size (c) Latency to the first	5	-0.065	0.107	1	53.315	0.36	0.551
Rearing treatment	large	-0.632	0.282	1	17.276	5.018	0.039
Received aggression		0.216	6.026	1	72.663	0.001	0.971
Test number				2	60.476	0.5	0.609
	test 2 test 3	0.051 0.387	0.258 0.391				
Size		-0.267	0.715	1	29.438	0.14	0.711



Figure 2: (a) Scatterplot representing the log of the total duration of submission per second compared to the total duration of received aggression per second during the three hierarchy tests. Blue: data points for fish reared in large groups (n=20); red: data points for fish reared in small groups (n=21). (b) Interquartile range plot representing the duration of submission for each received aggression category in the hierarchy test. The duration of submission was divided by the duration of received aggression (i.e. submission per received aggression) in each category and the log of this ratio is shown. "Low" represents the submission performed after the dominant showed fin spread behaviour; "Medium" represent the submission performed after the dominant showed restrained aggression (aggression without physical contact); and "High" represents submission performed after the dominant showed overt aggression (aggression with physical contact). Blue: data points for fish reared in large groups (n=20); red: data points for fish reared in small groups (n=21). Medians and first and third quartiles are shown. Dots are the individual data points.

Overall, submission duration across the three observations in the hierarchy test was repeatable (repeatability adjusted for test number, R = 0.213, CI = [0,0.447, p = 0.023). When estimated separately by early-life treatments submission was only significantly repeatable in fish raised in large groups (large group: R = 0.279, CI = [0,0.606], p= 0.049; small group: R = 0.050, CI = [0,0.377], p= 0.357). Based on the wide, and strongly overlapping confidence intervals, we note however that R does not itself differ significantly between the two treatment groups.

When calculating the relative duration of submission shown in response to different categories of received aggression reflecting increasing aggressive intensity (low: fin

spread; intermediate: threat display; high: overt aggression), there was also a significant interaction between the rearing treatment and the category of received aggression triggering the submissive event (Figure 2b, Table 1b, LMM, p=0.007). Post-hoc analyses showed that the fish reared in large groups have a steeper response when the intensity of received aggression increases compared to fish raised in small groups (Figure 2b, Table 1b, pairwise comparisons: medium p=0.030, high p<0.001). This means that fish reared in large groups exhibited relatively more submission with increasing levels of received aggression. There was again an effect of the test number on submission duration per category of received aggression. Fish showed less submission per category of received aggression in the second test compared to the third test (Figure 2b, Table 1b, LMM p<0.001). Finally, fish from the large-group treatment also showed a shorter latency to show the first display of submission than fish from the small-group treatment (Figure 3, Table 1c).



Figure 3: Interquartile range plot representing the latency to the first submission in the hierarchy test. Medians and first and third quartiles are shown. Dots are individual data points. Blue: data points for fish reared in large groups (n=20); red: data points for fish reared in small groups (n=21).

#### Aggression test

On average focal fish increased aggression to more aggressive stimuli (Table 2, GLMM p<0.001). There was no main effect of early-life treatment on the aggressiveness of the focal fish (Table 2, GLMM p=0.280), nor was there any support for an interaction between treatment and stimulus aggression level. This means that large and small group focal fish do not differ in how they adjust their own aggression according to the level of aggression by the stimulus fish in the video. Fish reduced their aggression frequency across tests (Table 2, GLMM p<0.001). Aggressive behaviour was repeatable across all observations (R = 0.388, SE=0.073, CI = [0.231,0.519], p<0.001). Estimates of repeatability for aggressive behaviour were almost identical in the subsets of data fish raised in different early-life

environments (large groups: R=0.383, SE=0.107, CI=[0.154,0.568], p<0.001; small groups: R=0.398, SE=0.110, CI=[0.170,0.582], p<0.001)

Table 2: A GLMM assuming a negative binomial distribution, to test for the effects of rearing treatment, test number and level of received aggression in the video (low, medium, high) on aggression in the aggression test (n= 508 trials, n= 38 individuals).

Fixed effects	Factor level	Estimate	SE	df	χ²	p-value
Rearing treatment	small	0.746	0.865	1	1.051	0.305
Received aggression		1.361	0.124	2	105.288	<0.001
Test Number				2	32.525	<0.001
	test 2	0.189	0.344			
	test 3	-2.784	0.425			

#### Social Trajectory traits

#### **Exploration test**

The early-life treatment did not affect explorative behaviour; fish showed more exploration in the second test (Table 3, GLMM p=0.036). Males were more explorative than females (Figure 4a, GLMM, p=0.005, Table 3), and smaller fish were more explorative than larger fish (Figure 5, GLMM, p=0.030).

Table 3: A GLMM assuming negative binomial distribution, to test for the effects of rearing treatment, test number, sex and size on the total number of visited pots in the exploration task (n= 63 trials, N= 21 individuals). The post hoc results are pairwise comparisons between test number and Sex.

Fixed effects	Factor level	Estimate	SE	df	χ²	p-value
<b>Rearing Treat</b>	ısmall	0.034	0.039	1	0.008	0.93
Test number				2	6.618	0.037
	test 2	1.264	0.477			
	test 3	0.905	0.48			
Sex	male	1.772	0.594	1	7.761	0.005
Size		-1.855	0.842	1	4.604	0.032
Comparisons			Estimate	SE	df	p-value
Test number	r					
Test 1 -Test	2		-1.264	0.477	54	0.028
Test 1 - Test	3		-0.905	0.48	54	0.153
Test 2 - Test	3		0.359	0.444	54	0.699
Sex						
Female - Ma	le		-1.77	0.594	54	0.004


Figure 4: Interquartile range plot representing the exploration score (total number of pots visited) by the fish during the exploration task between (a) rearing group size and sex and (b) disperser status and sex. In blue fish raised in large groups (n=14), in red fish raised in small groups (n=13), in black non-dispersers and in grey dispersers. Medians and first and third quartiles are shown. Small dots are individual data points.



Figure 5: Plot representing the exploration score (total number of pots visited) in the exploration task for fish of different sizes (standard length). In black the regression lines and individual scores for females and in grey for males.

## Helping test

There was no effect of the early-life treatment on the amount of helping behaviour (GLMM, p=0.265, Table 4). Females showed more helping behaviour than males (Figure 6, GLMM, p=0.009, Table 4).

Table 4: A GLMM assuming negative binomial distribution, on the effects of rearing treatment, test number and sex on the total number of helping behaviour performed by the focal individual in the helping task. (n= 74 trials, n=27 individuals).



Figure 6: Interquartile range plot representing the total number of aggressions performed by the fish towards an egg predator intruder during the helping task. Medians and first and third quartiles are shown. Dots are individual data points. Blue: data points for fish reared in large groups (n=14); red: data points for fish reared in small groups (n=13).

#### **Dispersal test**

The early-life treatment did not influence the dispersal behaviour of the fish (GLMM, p=0.334, Table 5a), nor did it influence the time the fish spent prospecting at the edge of the dominant's territory (GLMM, p=0.786, Table 5b). Time spent prospecting before the dispersal decision did not predict dispersal (GLMM, p=0.085, Table 5a). However, the exploration score from the exploration test predicted the level of dispersal; fish showing a higher exploration score were less likely to disperse (GLMM, p=0.014, Table 5a, Figure 4b). Males were more likely to disperse than females (GLMM, p=0.019, Table 5a). We had 9 fish dispersing out of 27 in the experiment, from these 9 fish one was a female, the others were all males.

Table 5: (a) A GLMM assuming binomial distribution, to test for the effects of rearing treatment, sex and exploitation score (total number of pots visited in the exploration task) on dispersal (binary variable 0 or 1) (n=21 trials, n=21 individuals). (b) LMM to test for effects of rearing treatment, sex and size on prospecting behaviour, described as the time spent near the door dividing the dominant's territory from the potential mate's territory (n=44 trials, n=24 individuals).

Fixed effects	Factor level	Estimate	SE	df		χ²	p-value
<u>(a) Dispersal</u>							
Rearing treatment	small	2.635	3.188	1	0.969		0.324
Sex	male	13.534	9.312	1	12.735		0.0004
Exploration Score		-0.645	0.473	1	7.705		0.005
Prospecting		0.022	0.041	1	0.341		0.559
Fixed effect	Factor level	Estimate	SE	NumDF	DenDF	F value	p-value
(b) Prospecting	<u>1</u>						
Rearing treatment	small	-0.273	0.653	1	10.519	0.175	0.685

# **Discussion**

Our study investigated the effect of the early social group size and complexity on social competence and social trajectories. Firstly, our results demonstrate the importance of a larger early social group size, which in our study species is related to greater social complexity, for the development of better social competence. Fish which experienced large groups early in life containing a mix of adults and juveniles of different sexes, sizes and social ranks, showed a steeper increase of submission relative to increasing received aggression. Fish raised in large groups increased their submission relative to increasing received aggression both in frequency and intensity, and they adopted the appropriate, subordinate behaviour faster in a social contest with a larger conspecific. In other words, fish raised in large groups showed more appropriate and more flexible behavioural responses when interacting with a dominant fish. However, there was no influence of the early social environment on aggressive behaviour toward videos of smaller conspecifics. The focal fish showed consistent aggressive behaviour towards the videos, and they increased their aggression when the video showed a more aggressive individual. This shows that the fish recognized the behaviours on the videos, and it further suggests that our fish from both early social treatments showed appropriate responses when competing against a smaller conspecific. There was also no evidence for the effects of the early social environment on explorative, helping and dispersal behaviour. Taken together, these results suggest that varying early social group size and complexity has long-term effects on submissive behaviour, but does not induce divergence in social and life history trajectories, opposite to what has been reported previously in fish raised in natural vs socially deprived social conditions [14].

The ability to flexibly adjust one's level of appropriate behaviour towards the behaviour of social partners during interactions is one indicator of social competence in animals [1]. In *N. pulcher*, an expression of higher social competence is to show a steeper response curve of submissive behaviour in response to the received aggression by a dominant fish [31]. In this species, the hierarchy is linear and size-based [34]. Thus, submission is the appropriate behaviour to show when interacting with a larger conspecific, which in

*N. pulcher* is typically dominant over smaller individuals because of its size advantage. Generally, submission helps avoid escalated fights and injuries [12] and enables the formation of a stable dominance hierarchy [48]. In *N. pulcher*, submissive behaviour is a very important mechanism to regulate aggression in the group; it can even be expressed to achieve pre-emptive appeasement in order to avoid punishment by dominants in large and small groups [13,49,50].

An alternative possibility for the observed response difference between fish raised in small or large groups might be that they differed in some other aspect, for instance size, growth or body condition. However, in our study fish did not differ in size between social treatments (LMM, df = 1, estimate = -0.013, p = 0.87; measured at 4 months of age). Moreover, previously *N. pulcher* raised in small or large groups did not differ in specific growth rate [30], and fish raised with or without adults did not differ in their body condition [5]. Therefore, it seems unlikely that the observed differences in responding to received aggression can be explained by divergence in size, growth or body condition induced by their early environment and we believe that the experimentally induced group size effect most likely reflects a difference in social competence.

To gain the accumulated benefits of social competence during the multitude of interactions group-living animals have every day, individuals need to consistently be able to adjust their responses appropriately to the current situation [1]. Here we found fish raised in large groups showed repeatable submission behaviour across the three observations, but also responded appropriately (on average) to varying levels of aggression received. Our current data are not directly informative for the repeatability (or consistency) of this plastic response by individuals to aggression received but we propose that this is an important question that has thus far been neglected in the study of social competence. Interestingly, our estimate of repeatability for submissive behaviour was lower (and non-significant) for fish raised in small groups, suggesting that early life experience can influence the structure of among-individual differences. However, we also acknowledge that the repeatability estimates have wide confidence intervals and did not differ significantly between treatments, so a more targeted experimental design may be required to formally investigate this.

Previous findings suggest that the early social environment triggers a life-long divergence of social and life history trajectories in *N. pulcher* [14,19] with a divergence between two social phenotypes, one being socially competent, philopatric but with low helping propensity and reproductive investment, and a second type with the opposite profile. When measuring a suite of social and non-social behavioural and life history traits, we found long-lasting effects on the propensity to show submission in our study, but not on other social and non-social traits. Thus, there was no evidence of an overarching behavioural phenotype induced by the early social environment. This may be explained by the major difference between previous and our studies. While we contrasted large and

small natural group sizes, previous *N. pulcher* studies compared group-living vs. socially deprived (brood mates only) rearing settings [14,19]. A recent long-term field study on *N. pulcher* found that group size did not influence the likelihood of dispersal [41], suggesting that the rearing conditions of the previous studies (socially deprived vs. natural social groups) may have represented a stronger contrast of the social environments thus affecting more traits than the comparison of two different, natural group sizes as done in our study.

Only a few fish in the dispersal test dispersed to a neighbouring compartment containing a potential mate for independent breeding. These were mostly males. A previous study investigating social trajectories in N. pulcher used three-year old fish, but due to the advanced age aggression was high in males, and group integration followed by dispersal could only be investigated in females [14]. Therefore, here we used younger fish of 1 year of age. While with this approach, we were able to study dispersal in both sexes, the younger age may also explain the generally low dispersal propensity in our experiment. Nevertheless, males were the more explorative sex and had a higher propensity to disperse than females, which is in line with natural dispersal patterns in this species [41] and findings from the laboratory [32]. It is also the general pattern in mammals and other fish species [51–53]. For example, like in *N. pulcher*, in meerkats (*Suricata suricatta*) females display higher levels of help compared to males and show higher levels of philopatry [54]. Moreover, in our study males showed lower levels of help than females, thus, validating previous findings [32]. Sex is an important determinant of life history strategies in vertebrates [32,54]. However, there is still behavioural variation within sex that can be explained by early-life experience. In coyotes Canis latrans, for instance, individuals of both sexes differ in their dispersal decisions dependent on their environment in early life [15].

We had expected that explorative behaviour would be positively related to dispersal propensities as this link has been widely reported in other species [55,56]. Against our expectations, more explorative individuals actually dispersed less. Exploration is susceptible to vary with time. For instance, in root voles, Microtus oeconomus, behavioural differences, including exploration differences, between disperser and resident are only temporary [57]. Prospecting before a dispersal decision did not impact whether a fish dispersed or not, so it could be that our measure of exploration was disconnected from the dispersal decision. In addition, exploration can also be the result of different environmental pressures. In European hares, Lepus europaeus, exploration is likely triggered by predation rather than by dispersal [58]. In line with these findings, it is possible that exploring novel shelters adjacent to the home territory (like in the exploration task) and dispersing to settle with a mate in a new territory (like in the dispersal task) represent two entirely different and unrelated ecological contexts for *N. pulcher*.

Animals living in social groups engage in many social interactions with conspecifics every day. For group-living individuals, being able to competently behave in each of the

many social interactions occurring in social groups will bring higher fitness benefits. Such benefits could be for example avoiding injuries or saving energy expenditures during contests [10]. Here we demonstrate that larger groups produce more socially competent offspring. Individuals with the appropriate social skills are more likely and faster to find their appropriate social role in a hierarchy [8] and more likely to be accepted and stay in social groups [31]. If social competence is transmitted environmentally or genetically across generations [4], this can contribute to increased group sizes and therefore to the enhanced production of socially competent individuals. This can generate a positive feedback loop based on individual-to-society feedback [59] enhancing sociality and promoting the formation of even larger groups of individuals [4]. Living in large groups can yield fitness benefits such as increased survival [25,27], better territory quality [23] and increased reproductive success for breeders [23,24,26,60]. Living in larger groups can also promote cognitive development as shown in cooperatively breeding Australian magpies (*Gymnorhina tibicen*). Individuals born and living in larger groups performed better in a series of cognitive tasks and benefitted from the facilitated transmission of information [61,62], and task performance was positively related to indicators of reproductive success [61], suggesting potential fitness benefits of group living via the acquisition of better cognitive skills.

In conclusion, our findings support the large body of literature showing that developmental plasticity has a key role in the expression of social behaviour and more specifically in the expression of social competence. We showed that increasing early social group size and complexity enhance social competence in a cooperative breeder. We stressed that social competence is likely to benefit an individual through cumulated fitness benefits over numerous social interactions, therefore consistency of social competence is crucial to render this social ability beneficial. However, we did not find differences in other social traits that resulted from developmental plasticity, suggesting that group size and complexity do not shape life-long life-history trajectories in *N. pulcher*. Taken together our results highlight the importance of the early social environment in shaping social competence.

#### **Ethics statement**

The study procedures followed the Swiss Animal Welfare laws and were approved by the Veterinary Office of the Kanton Bern, licence number BE93/18. No animals were harmed during the behavioural tests.

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# **Chapter 2**

Social competence is influenced by early but not late-life social experience in a cooperatively breeding fish.

In revision in Animal Behaviour



# Social competence is influenced by early but not late-life social experience in a cooperatively breeding fish.

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# **Abstract**

Social species rely on acquiring appropriate social skills to navigate their social environment and avoid eviction. Developmental plasticity allows individuals to develop the necessary phenotype by receiving cues from their social and non-social environment. However, committing to a specific phenotype early in life may prove maladaptive in case of mismatch between the early and future environment. To address potential mismatches, multiple or extended sensitive periods provide opportunities for individuals to gather the necessary information in changing and predictable environments. Cooperative breeders, like the highly social cichlid Neolamprologus pulcher, typically experience changing environments especially in the case of dispersal. While we know that the early development is critical for the acquisition of social competence, it remains uncertain if a second or extended sensitive period exists around the time of dispersal decisions in cooperative breeders. Here we manipulated the early and late life social environment of *N*. *pulcher* and asked whether individuals retain plasticity to adjust their social competence around sexual maturation. Juveniles were raised for two months with or without adults and tested for social competence. Shortly before reaching sexual maturity, they were randomly assigned to be housed alone, with siblings, or with a social group for an additional two months before undergoing further social competence testing. Aggressive and submissive behaviours were assessed in two social contexts. Fish raised with a large group exhibited greater social competence compared to those raised only among siblings, regardless of subsequent social experiences. This suggests that either early life cues reliably predict future environments or that constraints on late life plasticity exist.

# **Highlights**

- We study the acquisition of social competence in cooperatively breeding fish.
- We investigate whether sensitive periods exist early and late in life.
- Early development is critical in acquiring social competence in *N. pulcher*.
- Later development does not influence the social competence in *N. pulcher*.

# **Introduction**

Animals are bound to interact with their environment, which can pose many challenges such as acquiring mates, finding food patches, or interacting with conspecifics and group members. For social species, it is especially important to acquire the appropriate social skills to interact with group members or to avoid being evicted from a group (Taborsky & Oliveira, 2012). Group living individuals, therefore, benefit from early acquisition of social competence – i.e. the ability to adapt one's social behaviour to the prevailing social environment (Taborsky & Oliveira, 2012). One way to prepare for such challenges is through developmental plasticity, that is, the ability of a genotype to produce different phenotypes depending on the environmental conditions (Uller, 2008). Developing individuals can use cues from their social and non-social environment to develop a phenotype that is adapted to the environment. Developmental plasticity often has non-reversible effects leading to long-term consequences on the phenotype (Piersma & Drent, 2003).

Early-life environmental conditions often influence social behaviour and social competence. In mice for instance, individuals reared in communal nests show a higher level of allogrooming and allosniffing towards cage mates compared to individuals reared in single mother nests (D'Andrea et al., 2007). Jumping spiders (*Marpissa muscosa*) show better learning ability and more appropriate social behaviours when reared in an enriched social environment (Liedtke & Schneider, 2017). In *Neolamprologus pulcher*, a cooperatively breeding cichlid fish, the early social environment is critical in shaping social competence (Arnold & Taborsky, 2010; Fischer et al., 2015, 2017; Nyman et al., 2017; Taborsky et al., 2012). Yet, deciding on a particular phenotype already early life might be maladaptive in a scenario in which the early-life environment does not match the environment individuals will experience later in life (Piersma & Drent, 2003).

One way to mediate costs of developing a mismatching phenotype early in life that does not perform well in later conditions is to have extended or multiple sensitive periods. This allows more opportunities for individuals to gather information about the environment. Sensitive periods are intervals during ontogeny in which an individual's phenotype is particularly sensitive to changes (Fawcett & Frankenhuis, 2015). Typically, individuals are most sensitive to external influences during early development (Champagne & Curley, 2005). However, experiencing environmental change during the course of ontogeny may lead to prolonged or multiple sensitive periods (Panchanathan & Frankenhuis, 2016). Some mammals and birds, for instance, exhibit a second sensitive period during adolescence, i.e. the transitional period during sexual and social maturation, allowing individuals to better match their phenotype to the later-life environment (Ruploh et al., 2013; Sachser et al., 2013, 2018). For example, in rats, behavioural deficiencies as well as gene expression patterns from individuals raised with low-quality maternal care could be reversed later in life by providing them with a socially enriched environment (Champagne & Meaney, 2007).

Theory predicts that developmental plasticity occurs particularly in variable but predictable environments; thus when cue availability is variable but informative individuals should need extended opportunities to gather the necessary information in order to adjust their phenotypes to the envisaged conditions (Fawcett & Frankenhuis, 2015). Social species living in such environments might therefore benefit from a second sensitive period to be able to adjust their social behaviour in case the environment changes. One important environmental change to occur in social species is to disperse away from the natal territory to find opportunities for independent reproduction. Typically, natal dispersal occurs during adolescence. Yet, it is unclear if a second sensitive period exists during adolescence, where social behaviour and social competence can be adjusted to environmental change.

In cooperative breeders, group size can be highly variable depending on environmental conditions. For instance, in *Neolamprologus pulcher*, a highly social cichlid fish endemic from Lake Tanganyika, harsher environmental conditions lead to increased acceptance of immigrants as subordinate group members, promoting larger group sizes (Zöttl et al., 2013). Within a population, social groups in this species vary in size and composition (Groenewoud et al. 2016). They are typically composed of a dominant breeding pair and a greatly varying number of 1-30 subordinate helpers (Taborsky, 2016). Group size is autocorrelated across years and thus predictable as long as individuals stay in the same group (Heg et al., 2005). However, when *N. pulcher* disperse they do so by joining a new group, and these groups may differ in size (Jungwirth et al., 2023). This should favour the existence of a second sensitive period to accommodate potential mismatches between an individual's current environment and its early life conditions.

Previous studies have shown that group size and composition are important determinants of social competence in *N. pulcher*, where juveniles exposed to the presence of more adults early in life behave more socially competent in various social contexts (Arnold & Taborsky, 2010; Fischer et al., 2015; Taborsky et al., 2012). Arnold & Taborsky (2010) argued that, in *N. pulcher*, the differences in social competence resulting from exposure to different environments are due to enhanced opportunities to engage in social interactions in larger groups. When investigating the existence of an early sensitive period for the development of social competence in *N. pulcher*, results suggested continuous plastic behavioural adjustments during the first two months of life (Fischer et al., 2015). However, whether this sensitive period may be extended beyond that period or whether there may be a second sensitive period during later life, is unknown. Here we manipulated the variability of the social environment between life stages to test whether *N. pulcher* retain some developmental plasticity into adolescence to adjust their social competence to their current group size and composition. In particular, we asked if individuals developing poor social competence early in life are able to improve this ability when experiencing

socially enriched conditions later in life.

To investigate the possibility of recovery of social competence later during ontogeny, we exposed fish to two environmental experiences at two life stages. We first reared fish with or without adult group members, which is known to induce high or low social competence, respectively, in *N. pulcher* (Arnold & Taborsky, 2010; Fischer et al., 2017; Nyman et al., 2017; Taborsky et al., 2012). After keeping all fish under equal social conditions for two months (first 'neutral phase'), we did a first set of tests to evaluate differences in the appropriate use of submissive and aggressive behaviours. During a second experience phase later in life, shortly before sexual maturity, we randomly assigned three fish of each early social treatment group to a later-life social environment for 60 days: with their siblings, in social isolation, or in a new social group consisting of a breeder pair and a focal fish. After the second experience phase, we kept the fish again under equal conditions (second neutral phase) for two months before performing a second test of social competence. We predicted that fish raised in socially enriched groups in early life would always display better social competence regardless of later life social experience. We further predicted that fish reared without adults but are later exposed to a socially enriched environment will display better social competence than fish deprived of adults early and stay either with siblings or alone during the second experience phase.

# <u>Methods</u>

#### Model species

The East African Cichlid *N. pulcher* has proven to be a suited model species for the study of the effects of the early social environment on social competence (Antunes & Taborsky, 2020; Arnold & Taborsky, 2010; Fischer et al., 2017; Nyman et al., 2017; Taborsky et al., 2012; Taborsky, 2016). *N. pulcher* are cooperative breeders, with groups being organized in linear size-based hierarchies (Dey et al., 2013). Their social groups are typically composed of a dominant breeding pair and subordinate helpers of various sizes and sexes (Groenewoud et al., 2016; Taborsky, 2016), related or not to the breeders (Dierkes et al., 2005). As cooperative breeders, their frequent engagement in a wide range of social interactions among group members every day implies that they stand to gain benefits from acquiring social competence at an early stage (Taborsky et al., 2012). *N. pulcher* are able to disperse when sexually mature around the size of 3.5-4 cm, corresponding to the age of about 9 months (Taborsky & Limberger, 1981), but sometimes stay at the natal territory for extended periods (Jungwirth et al. 2023). Dispersers joining new groups should benefit from a second sensitive period to acquiring the social skills needed in their new environment.

#### Early social experience

We used a laboratory-bred N. pulcher population derived from wild-caught fish from

Kasakalawe Point, Mpulungu, Zambia. We reared 22 broods of juvenile *N. pulcher* in the lab from 11 large family groups, each housed in a 400L tank and composed of ten *N. pulcher*: two breeders (5.0-7.0 cm Standard Length, SL), four large helpers (two males, two females, 4.0-4.7 cm SL), and two medium-sized helpers (2.6-3.5 cm SL) and two small helpers of unknown sex (1.5-2.5 cm SL). Breeders and helpers were unrelated. From each family group, we collected two broods: one grew up in the presence of the adults in the family group home tank (referred to as G+), and one was collected at the egg stage immediately when spotted in the tank and was reared without a large group, only among siblings, in a 50L tank (referred to as G-). The order of collection of the broods was balanced across treatments: half of the G- broods were collected before the G+ and the other half was collected in the reverse order. Broods collected from the same family differed on average by 40 ± 33 S.E. days in age. During rearing the juveniles from both early social treatments were provided a pile of rocks to hide in, a 5cm layer of sand at the bottom of their tank and a biological filter. In addition, in the large group tanks one half of a clay flowerpot per adult was present as shelter, which formed the core of the group's territory. To collect the subjects for the G- treatment, eggs were collected by removing the flowerpot where they were attached to and placing it with water and air supply in a small box until hatching. After hatching, the larvae were placed in a 50L tank with a pile of stones to hide in. G+ fish received 60 days of social experience with their family group. Then all juveniles of a family group were collected and placed in 50L-tanks among their siblings. Similarly, after 60 days of experiencing only the presence of their siblings, G- fish were also moved to a new 50L-tank, to standardize the experience of being moved between tanks in both treatments after the end of the early-life experience phase. We ensured to keep a similar density of fish in the 50L-tanks of G+ and G- groups containing siblings produced by the same family group. Both G+ and G- fish were kept with their siblings only for another 60 days. This 'neutral phase' of equal conditions served to ensure that any behavioural differences between early life treatments measured in the subsequent behavioural tests are not a direct effect of the different conditions during the early-experience phase, but instead reflect longer-term effects.

#### Behavioural tests

Before the first behavioural test, we selected three focal fish per sibling group and marked them with a unique elastomer colour tag (Jungwirth et al., 2019). To prevent behavioural differences solely based on extreme rank, we excluded the largest, i.e. most dominant fish and the smallest, i.e. most subordinate, fish from each rearing group tank from our experiments. Additionally, fish below 1.9 cm standard length (SL) were not individually marked due to their small size and were consequently not included in our study. From the remaining fish, we randomly selected our focal fish from each sibling group. The same focal fish were used throughout all behavioural tests of the entire experiment.

#### **Submission**

At the age of 120 days after free swimming ( $\pm$  15 days) and 260 days ( $\pm$  11 days) we exposed the three marked focal fish of each brood to a larger conspecific in a hierarchy formation test (focal fish in test 1: 2.4  $\pm$  0.29 cm SL; focal fish in test 2: 3.4  $\pm$  0.41 cm SL). This test aimed to investigate their propensity to exhibit submissive behaviour in response to the received aggression by a dominant fish. Given the linear size-based hierarchy observed in *N. pulcher* (Dey et al., 2013), where the appropriate response of smaller fish towards aggression received by larger fish is to show submission, this test has been used previously as a measure social competence in this species (Fischer et al., 2015).

Hierarchy tests were performed in 20L tanks equipped with half of a clay flowerpot serving as shelter and centre of the focal fish's territory. In *N. pulcher* shelter owners are the dominants in a territory. We first gave the focal fish 24h to acclimate to the new environment and to settle in the provided territory. The same day, a competitor, which was 4-5mm larger than the focal fish, was chosen from one of the stock tanks of our laboratory. The competitor was placed together with a smaller fish (4-5mm smaller), also taken from stock tanks, in an empty 20L tank equipped with half of a clay flowerpot. This step allowed the competitor to assume a dominant role over a smaller fish prior to the test, as we wanted to ensure that the competitor behaved as a dominant fish when doing the hierarchy test the next day.

The next day we introduced the now-dominant competitor into the focal fish's tank. Aggressive and submissive displays by both the focal fish and competitor were immediately recorded for a duration of 20 minutes. Behavioural recordings for this experiment were captured using a Sony Handycam HDR-PJ260 and analysed by OL using the BORIS software (Friard & Gamba, 2016). The observer was blind to the treatment during the video analysis. The number of submissive displays by the focal fish, aggressive behaviours exhibited by the competitor towards the focal fish, and the time it took for the focal fish to display submission for the first time were measured. We then calculated the number of submissive behaviours from the focal fish per received aggression from the competitor as our measure of social competence as the variable to be used in statistical models.

Four hours after the introduction of the competitor, a 10-minute observation period was conducted to determine the acceptance status of the focal fish by the larger competitor. Focal fish that were "fully accepted" had access to the shelter or its close vicinity (within one body length) and could freely move throughout the tank, without receiving aggression from the dominant fish. "Accepted" focal fish were allowed to swim freely in the tank, except in the close vicinity of the shelter, where the dominant fish would display aggression if approached. "Evicted" focal fish were confined to a specific area of the tank farthest from the shelter, and they received aggression from the dominant fish when in close proximity. Since these were live observations, the observer could not be blind to the treatments. It is

important to note that no injuries occurred during the hierarchy test. In a very few cases the focal fish was able to evict the competitor, in which case we considered them as being dominant over the larger competitor. These fish were excluded from further analysis (n=5 fish). The competitor was removed after the test and returned to its home tank; the focal fish stayed in the tank for one day until the next behavioural test.

#### Aggression

Two days after the hierarchy test, we performed a mirror test to measure the aggressive behaviour of focal fish (Balzarini et al., 2014). The focal fish had stayed in the experimental tank between the hierarchy test and the aggression test, and the aggression test was performed in the same tank as the previous hierarchy test. We introduced a mirror (15\*15cm) in the tank just next to the fish shelter. We immediately recorded the interaction of the fish with the mirror for 10 minutes using a video camera Sony Handycam HDR-PJ260. Later Lauriane Bégué analysed all aggressive behaviours toward the mirror on the video recordings with the BORIS software (Friard & Gamba, 2016). The observer was blind to the treatments.

#### Late social experience

At the age of 140 days (± 11 days), the focal fish were assigned to one of three late-life social treatments such that each of the three marked fish of a family of origin entered a different treatment. (1) The first third of the fish was kept alone for 60 days in a 20L tank. The first day we placed the fish in the tank, we added 100 ml of its home tank water to reduce stress (social deprivation treatment). (2) Another third of the focal fish were put back in their home tank with their siblings (control treatment). The focal fish was separated from their siblings by a clear, perforated partition. Thus, these fish were physically, but not socially isolated from their siblings, and this set-up avoided any injuries of focal fish. (3) The last third of the fish was transferred to a 400L tank in which it was assigned to be a small helper in a group of 10 unfamiliar and unrelated fish (social enhancement treatment; the group composition was similar to the G+ treatment during the early social experience). The second social experience phase lasted 60 days. Afterwards, we moved each of the focal fish back with their siblings to a new 50L tank. Focal fish were again physically isolated from their siblings to avoid injuries, but had access to visual and olfactory cues to the siblings. We kept the focal fish in these new tanks for another 60 days (second neutral phase), after which we performed another hierarchy and another mirror test in the same way as described above.

#### Statistical Analysis

We analysed behavioural data collected from the behavioural tests using linear mixed effect models fitted in R 4.1.2 (R Core Team, 2020) using packages "glmmTmb" (Brooks et al., 2017), "LmerTest" (Kuznetsova et al., 2017) as well as "ggplot2" (Wickham, 2011)

for plots. All models include fixed effect of body size and family ID as a random factor. Models included other specific covariates as described below. Post hoc tests for pairwise comparison between significant factors were run using the package "emmeans" (Lenth, 2018).

#### Submission measure

We used the ratio of submission per received aggression as the response variable in our linear mixed models. We fitted a first model on the data collected after the early social experience including a fixed effect of the early social experience (G+ vs. G-). We fitted a second model with the data collected after the late social experience including fixed effects of the early social experience and the late social experience. For the second model we used a boxcox transformation to achieve normality of the residuals.

Two further models tested for effects on the outcome of the contest, that is, the acceptance state of the focal fish 4 h after the hierarchy test. These data were binary (0 = not accepted, 1 = accepted) so we fitted generalised linear mixed models assuming a binomial distribution. The first model testing on effects of the early social experience included early social experience and the ratio of submission per received aggression as fixed factors. The second model with the data collected after the late social experience included early social experience, late social experience, and the ratio of submission per received aggression as fixed aggression as fixed effects.

#### Aggression measure

We used the count of all aggressive behaviours towards the mirror as the response variable in our models. We used generalised linear mixed models following a negative binomial distribution and we analysed separately the data collected after the early and late social experience in the same way as when analysing submission per received aggression (see above). Accordingly, in a first model we analysed the data collected after the early social experience including a fixed effect of early social experience. And, we fitted a second model with the data collected after the late social experience including fixed effects of early social experience and late social experience.

#### Ethical note

All procedures followed the Swiss Animal Welfare laws and were approved by the Veterinary Office of the Kanton Bern, licence number BE93/18. After the hierarchy test, during the four hours where the two fish stayed together in order to record the final outcome of the interaction, we frequently checked the fish to ensure they were not inflicting any injuries on each other. None of the fish were injured during the behavioural tests. Between behavioural tests experimental fish were kept in isolation nets within their home tanks, where they had visual and olfactory cues from their siblings but were shielded from any physical aggression. All tanks involved in this experiment were regularly checked for

aggression and potential evictions of fish from the groups. Any evicted fish were isolated and reintegrated in the groups as soon as possible.

# <u>Results</u>

# Effect of early social experience

The early social experience significantly influenced the level of submission per received aggression displayed by the fish in the hierarchy test (Figure 1a; Table 1a; LMM, p= 0.0003), as well as the level of aggression displayed against a mirror (Figure 2a; Table 3a; GLMM, p= 0.005). Fish raised with a large group (G+) showed higher levels of submission per received aggression compared to fish raised only among siblings (G-) (Figure 1a; Table 1c; pairwise comparison, p=0.0003) and higher levels of aggression towards the mirror (Figure 2a; Table 3c; pairwise comparison, p=0.007). In addition, G+ fish were accepted more often by the dominant fish (i.e. the larger competitor, which took over the territory) 4 h after the hierarchy test (Table 2a; GLMM p=0.01; Table 2c; pairwise comparison, p=0.004).

Table 1: Results of the LMM on the ratio between number of submission and number of received aggression in the hierarchy test for (a) the first measure after the early social experience (n=65 observations) and (b) the second measure after the later social experience (n=61 observations, boxcox transformation). The post hoc results are pairwise comparisons between the different early experiences in the (c) first measure and (d) second measure.

Model	df	F value	p-value
(a) First measure			
Early Social Experience	1	15.4	0.0003
Size (b) Second measure	1	0.351	0.56
Early Social Experience	1	4.07	0.049
Late Social Experience	2	0.14	0.87
Size	1	3.71	0.06
Comparisons	Estimate ± SE	T ratio	p-value
(c) First measure			
G+ > G-	-0.562 ± 0.14	-3.92	0.0003
(d) Second measure			
G+ > G-	0.116 ± 0.06	2.01	0.041



Figure 1: Interquartile range plot representing the ratio of number of submission per received aggression in the (a) first measure between the G+ (raised with a large group) and G- (raised only among same age siblings) fish, and (b) in the second measure between the G+ and G- fish that went through the late social experience of either social deprivation in blue, social enhancement in black and stayed with their siblings in red. Dots represent the median and bottom and top whiskers represent the first and third quartile respectively.

#### Effects of early and late social experience

When analysing the amount of submission performed in the test after the late social experience, again the early social experience significantly influenced the amount of submission per received aggression (Figure 1b; Table 1b; LMM, p= 0.05). G+ fish showed higher levels of submission per received aggression compared to G- fish (Figure 1b; Table 1d; pairwise comparison, p=0.05). However, the early social experience did not influence the level of aggression performed against a mirror later in life (Figure 2b; Table 3b; GLMM, p= 0.24) nor did it influence the acceptance status of the fish by the dominant after the hierarchy test (Table 2b; GLMM p=0.25).



Figure 2: : Interquartile range plot representing the ratio of number of aggression towards the mirror in the (a) first measure between the G+ (raised with a large group) and G- (raised among same age siblings) fish, and (b) in the second measure between the G+ and G- fish that went through the late social experience of either social deprivation in blue, social enhancement in black and stayed with their siblings in red. Dots represent the median and bottom and top whiskers represent the first and third quartile respectively.

The second social experience did not influence any of the behavioural measures. It did not affect the amount of submission per received aggression performed by the fish in the hierarchy test (Figure 1b; Table 1b; LMM, p= 0.87), nor did it influence the amount of aggression displayed towards the mirror (Figure 2b; Table 3b; GLMM, p= 0.51). Bigger fish showed more aggression towards the mirror than smaller fish in the second test (Table 3b: GLMM, p= 0.01). The acceptance status was not influenced by the late social experience (Table 2b; GLMM p=0.42). However, fish that performed more submission per received aggression were accepted more often compared to fish that submitted less (Figure 3; Table 2b; GLMM p=0.05).

Table 2: Results of the GLMM (binomial distribution) on the acceptance status (1= accepted, 0= not accepted) in the hierarchy test for (a) the first measure after the early social experience (n=63 observations) and (b) the second measure after the later social experience (n=55 observations). Fish that achieved the dominant status were excluded from the analysis (n=5 fish). Post hoc results are pairwise comparisons between the different early experiences in the (c) first measure.

Model	df	LRT	p-value
(a) Outcome of the fight - First measure			
Early Social Experience	1	5.01	0.025
Ratio of Submission per received aggression	1	0.02	0.87
Size	1	3.37	0.07
(b) Outcome of the fight - Second measure			
Early Social Experience	1	1.35	0.25
Late Social Experience	2	1.73	0.42
Ratio of Submission per received aggression	1	3.99	0.045
Size	1	0.23	0.63
Comparisons	Estimate ± SE	T ratio	p-value
(c) Outcome of the fight - First measure			
G+ > G-	-1.52 ± 0.72	-2.11	0.039

Table 3: Results of the GLMM (negative binomial distribution) on the counts of aggression in the mirror test for (a) the first measure after the early social experience (n=64 observations) and (b) the second measure after the later social experience (n=59 observations). The post hoc results are pairwise comparisons between the different early experiences in the (c) first measure.

Model	df	LRT	p-value		
(a) Counts of aggression - First measure					
Early Social Experience	1	7.8	0.005		
Size	1	0.84	0.36		
(b) Counts of aggression - Second measure					
Early Social Experience	1	1.37	0.24		
Late Social Experience	2	1.35	0.51		
Size	1	6.14	0.01		
Comparisons	Estimate ± SE	T ratio	p-value		
(c) Counts of submission per received aggression - First measure					
G+ > G-	0.50 ± 0.18	2.82	0.007		



Figure 3: Interquartile range plot representing the ratio of number of submissions per received aggression in the second measure between accepted and non-accepted fish. Dots represent the median and bottom and top whiskers represent the first and third quartile respectively.

#### **Discussion**

Early life is a key developmental stage that critically influences the development of social competence (D'Andrea et al., 2007; Fischer et al., 2015; Kempes et al., 2008). An effect of early life on the acquisition of social competence was demonstrated previously in N. pulcher (Arnold & Taborsky, 2010; Fischer et al., 2015, 2017; Nyman et al., 2017; Taborsky et al., 2012). Yet, the social behaviours developed during this period may not always be beneficial when the early-life environment differs from the later life environment (Piersma & Drent, 2003), thus yielding a cost to loss of plasticity after earlylife. Theory predicts that animals living in highly variable but predictable environments should benefit from several or longer sensitive periods to overcome this cost (Fawcett & Frankenhuis, 2015; Fischer et al., 2014; Panchanathan & Frankenhuis, 2016). We had predicted that in a cooperatively breeding fish that under natural conditions lives in a variable but predictable social environment the early-life social experience will shape its social behaviour and social competence. We further had predicted that exposure to a changing social environment during late development should lead to late-life plasticity of social competence. Contrary to our second prediction, our results suggest that the period when *N. pulcher* are sensitive to social cues when developing social competence is restricted to early life and exposure to contrasting social environments later on does not induce an increase or decrease of social competence.

After the first social experience, individuals raised with a group of adults showed higher levels of submission per received aggression, which is the appropriate response of a subordinate to avoid eviction by a dominant from a territory. Additionally, the focal fish were more often accepted in the territory when interacting with a larger dominant conspecific. Individuals raised in G+ conditions also showed higher levels of aggression towards a mirror; however, this difference was not retained, or detected, in our second measure. Taken together these results show that individuals exposed in early life to a large group had overall higher social competence compared to their siblings raised among themselves. The differences in levels of submission towards a larger dominant conspecific between fish experiencing a socially enriched versus a deprived environment in early life were retained after the second social experience regardless of the nature of the second experience. This shows that the first, but not the second, social experience triggered longlasting differences in social competence.

In *N. pulcher*, fish live in groups that can vary in size and composition (Groenewoud et al., 2016; Taborsky, 2016). Fish typically experience variability in the composition of groups around dispersal (Jungwirth et al., 2015). In our experiment, fish were subjected to different treatments that simulated group composition variability that may be encountered around sexual maturity of the fish. Some individuals were placed in new groups, mimicking the experience of joining unfamiliar social groups, while others were isolated, as if dispersing alone. Additionally, the control group remained among their siblings, representing a scenario of philopatry. We predicted that induction of a change of the social environment would lead individuals to retain plasticity to acquire the appropriate social skills to navigate their environment. We found this was not the case, possibly because the social cues gathered in early life are in this species highly informative and reliable. This hypothesis is supported by a recent field study showing that in *N. pulcher*, only the few fish that gained dominance after dispersal experienced a change of group size afterwards, whereas fish dispersing and retaining their social ranks did not experience a significant change in group size (Jungwirth et al., 2023).

Alternatively, the costs of retaining plasticity into late life may be too high constraining later changes of social phenotype (Panchanathan & Frankenhuis, 2016; Utz et al., 2014). These costs can be energetic, or genes involved in plasticity could have negative pleiotropic effects, limiting the period to shape the phenotype to the early development (DeWitt et al., 1998; Fawcett & Frankenhuis, 2015). Additionally, maintaining the machinery for high plasticity can be costly and therefore plasticity is expected to decrease with age (Fawcett & Frankenhuis, 2015; Panchanathan & Frankenhuis, 2016). In drosophila, for instance, maintenance of plasticity through high learning ability over lifetime resulted in lower survival under competition in selection lines (Mery & Kawecki, 2003). Theoretical models also showed that the optimal peak of plasticity lies in the early life and decreases through development (English et al., 2016; Fawcett & Frankenhuis, 2015; B. Fischer et al., 2014).

During the second aggression test, i.e. after the late social experience, larger fish were more aggressive towards their mirror image reflecting a same size conspecific. Body size is generally an important factor mediating aggression behaviour. In many species, larger individuals show higher levels of aggression compared to smaller ones (Herrel et al., 2009; Näslund & Johnsson, 2016; Nowbahari et al., 1999). The fact that we did not see this effect in the first test after the early social experience could be explained by the fact that younger fish might not perceive same sized conspecifics as threatening as older fish do. Older (and larger) fish are starting to compete with other group members for rank (Taborsky, 2016) and they are also more likely to be unrelated to each other (Dierkes et al., 2005). Therefore, a same sized individual can pose more of a threat and increase motivation to show aggression to assert dominance. Age and size differences in aggressive behaviour might also explain why the effect of the early social environment in expression of aggression was not recovered during the second aggression test around sexual maturity.

Finally, during the second hierarchy test, neither early nor late social experience affected the acceptance status of a fish by the dominant individual. However, we found that the amount of submission shown in response to aggression predicted whether a fish would be accepted in the territory or not. This is in line with the results after the first behavioural test. There we did not find a direct effect of submission on acceptance, but fish raised with a large group displayed more submission per received aggression and were more often accepted in the territory. These results highlight the importance of social competence for promoting integration in groups and retain benefits of staying at a safe territory even after losing it to a more dominant conspecific. In group living species, individuals often gain substantial fitness benefits in staying with a group sharing a territory (Clutton-Brock, 2006; Koenig & Dickinson, 2016; Taborsky, 2016). Access to a territory can yield access to shelters from predators, support of the group for predator defences and foraging, for example (Clutton-Brock, 2006; Koenig & Dickinson, 2016; Taborsky, 2016). As in our experimental test, individuals were too small to claim the territory for themselves, they had to adopt the subordinate position to keep benefiting from accessing the shelter.

Our results show that, contrary to some mammals and birds (Ruploh et al., 2013; Sachser et al., 2013, 2018), in social cichlids sensitivity to environmental cues appears to be restricted to the early development. This demonstrates variation across social vertebrates in the mechanisms of acquisition of social phenotypes, with implications for their evolution (Hofmann et al., 2014; Taborsky, 2021). Here we showcase an example of a cooperative breeder where early, but not later, life critically shapes social competence. Thus far, it has been shown that in cooperative breeders, maternal effects can be critical in shaping the social phenotype of offspring (Russell & Lummaa, 2009). Moreover, in eusocial invertebrates an early and irreversible determination of social phenotype is common and often goes along with physiological specialisation (English et al., 2015).

Cooperative societies all have in common that individuals delay dispersal so stay and help in their natal group (Koenig & Dickinson, 2016). While the delay in dispersal could be used to gather information on outside options and potentially to adjust the phenotype to the envisaged life after dispersal, in some species only some individuals disperse (Bergmüller et al., 2005; Jungwirth et al., 2023; Suh et al., 2022). By remaining in familiar surroundings, more socially competent individuals can capitalize on the social knowledge they acquired, allowing them to navigate the social challenges within their group more effectively. As a result, these individuals may exhibit a preference for philopatry as it provides them with a higher likelihood of survival and may ultimately increase their fitness if they can inherit the territory (Taborsky, 2021).

In conclusion, our study demonstrates that the acquisition of social competence in cooperatively breeding fish is predominantly influenced by early life experiences. We found no evidence of a second nor of an extended plasticity window later in development. This highlights the importance of gathering social information during the early developmental period to acquire the appropriate social skills for the future. A preference for philopatry in cooperative breeders might be driven by limited opportunities for gathering social information later in life, reinforcing a feedback loop between sociality and social competence. Further research is needed to understand the timing of sensitive periods and the acquisition of social competence in other social species.

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# **Author contribution statement**

Océane La Loggia: Conceptualisation, Methodology, Formal Analysis, Investigation, Data Curation, Writing -Original Draft, Writing – Review and Editing, Visualisation. Barbara Taborsky: Conceptualisation, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

## Data availability statement

Data is available on Figshare https://figshare.com/s/712bd8b62f5320cb2534 (La Loggia & Taborsky, n.d.).

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# **Chapter 3**

Social complexity during early development has long-term effects on neuroplasticity in the social decision-making network.



# Social complexity during early development has long-term effects on neuroplasticity in the social decision-making network.

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# Abstract

In social species the early social environment is especially important to develop appropriate social behaviours during conspecific interactions. However, the underlying neuronal mechanisms responsible for the acquisition of appropriate social behaviours, aka social competence, are largely unknown. One key candidate to influence social competence is neuroplasticity, which functions to restructure neural networks in response to novel experiences, or alterations in behaviour or environment. One important mediator of this restructuring is the neurotrophin BDNF, which is well conserved among vertebrates. Here we studied a highly social fish, *Neolamprologus pulcher*, in which the impact of early social experience on social competence has been previously shown. We investigated the relationship between early social experience and neuroplasticity, by analysing the relative expression of the bdnf gene and its receptors (p75NTR and TrkB) within the Social Decision-Making Network. In fish raised in larger groups, *bdnf* and *TrkB* were both upregulated in the anterior tuberal nucleus of, while in the lateral part of the dorsal telencephalon TrkB was downregulated and *bdnf* was upregulated. In the preoptic area (POA) all three genes were upregulated in fish raised in large groups, suggesting that early social experiences might lead to changes of the neuronal connectivity in the POA. Our results highlight the importance of the early social experience in programming the constitutive expression of neuroplasticity markers and the potential long-term effects on neuronal branching. We suggest that the effects of early social experience on social competence might be due to the long-term changes on neuroplasticity.

# **Introduction**

During early ontogeny, the experienced social environment can lead to pervasive changes of phenotypic traits, including social behaviour (reviewed in Kasumovic & Brooks, 2011; B. Taborsky, 2017), dispersal decisions (S. Fischer et al., 2017; Gustafsson & Sutherland, 1988) and reproductive investment (Antunes & Taborsky, 2020; Naguib et al., 2006). Individuals which are socially deprived during development typically fail to develop the appropriate social skills later in life as shown in social spiders (Liedtke & Schneider, 2017), fish (Arnold & Taborsky, 2010; S. Fischer et al., 2015; B. Taborsky et al., 2012), mice (Branchi et al., 2006; D'Andrea et al., 2007), and apes (Kempes et al., 2008). Highly social species, such as cooperative breeders, typically engage in a diversity of interactions and form complex groups where individuals adopt different social roles and ranks in a mixed group of kin and non-kin (Clutton-Brock, 2006; Groenewoud et al., 2016a; Koenig & Dickinson, 2016; M. Taborsky, 2016). Consequently, they should particularly benefit from early acquisition of social competence, defined as the ability to optimally adjust one's social behaviour to the prevailing social information, hence reducing potential costs associated to extended philopatry (B. Taborsky, 2021; B. Taborsky & Oliveira, 2012).

During early life the Social Decision-Making Network (SDMN) is developing and different neural mechanisms, such as the monoaminergic and stress axis, are being adjusted (Antunes et al., 2021). The SDMN is a network of interconnected brain nodes responsible for processing and integrating social information and regulating the expression of social behaviour. The SDMN is well preserved across vertebrates (O'Connell & Hofmann, 2011, 2012). Early life experience impacts brain circuitry mediating defensive behaviour in rodents by, for example, modulating dendritic complexity and length in several brain regions of the SDMN such as the hippocampus or the medial prefrontal cortex (Chocyk et al., 2013; Murthy & Gould, 2020; Soztutar et al., 2016). Social competence is thought to be accomplished by rewiring or by biochemically switching nodes (i.e through diffuse action of neuromodulators like neuropeptides, monoamines or hormones) of the SDMN involved in the expression of social behaviour in response to the current social information (Cardoso et al., 2015). Neuroplasticity, which involves structural and functional changes in the brain, particularly through the influence of neurotrophins (Huang & Reichardt, 2001), plays a crucial role in mediating the rewiring of the SDMN and shaping social behaviour (Branchi et al., 2004).

Neuroplasticity functions to restructure neural networks in response to novel experiences or alterations in behaviour or environment (Kleim & Jones, 2008). Neurotrophins are secreted proteins that will act on cell-surface receptors from target cells, promoting survival and maturation of neurons (Bhattacharyya & Svendsen, 2003; Huang & Reichardt, 2001). Their activity mediates neuroplasticity in the short and long term, thereby facilitating effects of environmental experience on brain structure and

function (Branchi et al., 2004). Therefore, neurotrophins are of key interest in the study of early social environmental effects on social competence. An important neurotrophin involved in the development of social behaviour and social competence of communally breeding mice is the brain-derived neurotrophic factor (BDNF) (Branchi et al., 2006), which is well conserved among vertebrates (Lucini et al., 2018). Thus, the BDNF pathways involving BDNF itself and its two receptors are good candidates for investigating the long-term behavioural and neurological effects of early social experience in highly social vertebrates.

BDNF binds to the receptors p75NTR and TrkB (Huang & Reichardt, 2001; Purves et al., 2004), which have different functions in neuroplasticity. BDNF is known to be a major regulator of plasticity at the excitatory synapses (Leal et al., 2015). There are two pathways for synaptic plasticity: BDNF/TrkB (Long Term Potentiation, long-lasting synaptic enhancement and synapse strength, i.e the efficiency of the communication between two neurons at a synapse); and BDNF/p75NTR (Long Term Depression, synapse elimination) (Sakuragi et al., 2013). TrkB has a higher affinity for BDNF than p75NTR (Klein et al., 1991; Rodriguez-Tebar et al., 1990). The receptor p75NTR has three main neuroplasticity functions: cell cycle arrest, cell death and neurite growth (Purves et al., 2004). In mice downregulation of *p75NTR* in the hippocampus results in improved cognitive function (Maejima et al., 2018) and in autistic humans, *p75NTR* was correlated with impaired social cognition in a theory of mind test (Segura et al., 2015). The receptor *TrkB* has four functions all related to synaptic plasticity: cell survival, neurite outgrowth, neuronal differentiation, and activity-dependent plasticity (Purves et al., 2004). *TrkB* plays a significant role in both social behaviour and stress vulnerability in mice (Razzoli et al., 2011). For instance, after repeated social defeat, mice expressing a truncated *TrkB* variant (leading to a decrease in BDNF signalling) exhibited more consistent social avoidance behaviours than their wild-type counterparts (Razzoli et al., 2011). In mice, *TrkB* knockout mutants performed poorly in complex and stressful learning tasks suggesting that the TrkB receptor also has an important role in cognition (Minichiello et al., 1999).

Experiments manipulating the early social environment in rodents have shown a short and long-term impact on brain neurotrophin expression, with an enriched social environment triggering higher expression (Branchi et al., 2006; Cirulli et al., 2003; D. Liu et al., 2000). In the cooperatively breeding fish *Neolamprologus pulcher*, early social deprivation and a social challenge interactively influenced the expression of the *bdnf* gene in the hypothalamus, where fish reared in social deprivation showed a downregulation of *bdnf* when not being challenged and an upregulation of *bdnf* when they were socially challenged by a dominant individual (Nyman et al., 2017). However, it is yet unknown (i) how natural variation of the early social environment impacts markers of neuroplasticity such as neurotrophins and their receptors and (ii) how differences in social competence are accompanied by differences in neuroplasticity.

The East African Cichlid *N. pulcher* has emerged as a prominent model species for investigating the influence of the early social environment on neuroplasticity and social behaviour (Antunes & Taborsky, 2020; Arnold & Taborsky, 2010; S. Fischer et al., 2017; Nyman et al., 2017; B. Taborsky et al., 2012; M. Taborsky, 2016). These cooperative breeders form linear size-based hierarchies within groups, with social structures consisting of a dominant breeding pair and up to 25 subordinate helpers of various sizes and sexes (Dey et al., 2013; Groenewoud et al., 2016b; M. Taborsky, 2016). As cooperative breeders these fish engage in numerous daily social interactions among group members, indicating the benefits of acquiring social competence early on (B. Taborsky et al., 2012).

We investigated the relationship between early social experience and neuroplasticity in *N. pulcher* by analysing the relative brain gene expression of *bdnf* and its receptors (*p75NTR* and *TrkB*) within the SDMN. We performed brain microdissection of four brain nuclei of the SDMN all involved in the modulation of social behaviour (O'Connell & Hofmann, 2011). We selected one region in the telencephalon (DL: lateral zone of the dorsal telencephalic area, putative mammalian homologue: hippocampus), two in the hypothalamus (aTn: anterior tuberal nucleus, putative mammalian homologue: ventromedial hypothalamus VMH; TPp: posterior tuberculum, putative mammalian homologue: preoptic area plus paraventricular nucleus of the hypothalamus).

In *N. pulcher* early-life environment affects the expression of social behaviour; fish raised in larger, more complex social groups show more social competence, which means they can more flexibly adjust their social behaviour to social information than fish raised in smaller, less complex groups (La Loggia et al., in revision). We hypothesize that the ability to flexibly adjust one's social behaviour is linked to neuroplasticity and therefore more complex early social environments should favour upregulation of synaptic plasticity mechanisms in the SDMN. Previous fish studies have shown that the social phenotype is linked to different *bdnf* expression patterns in the lateral zone of the dorsal telencephalic area (DL) (Teles et al., 2016) and in the hypothalamus (Nyman et al., 2017). Based on these studies, we expect that early social complexity influences *bdnf* expression patterns in the DL, aTn and TPp. The POA, aTn and TPp are all involved in the expression of social behaviour (O'Connell & Hofmann, 2011) across vertebrates, therefore we expect to see different gene expression patterns to be related to early social complexity, with higher complexity being linked to the upregulation of synaptic plasticity pathways.

#### <u>Methods</u>

#### Rearing and housing conditions

We reared the fish in two early social conditions: large groups comprising ten adults (two breeders and eight unrelated subordinates), and small groups consisting of three
adults (two breeders and one unrelated subordinate) (see details in La Loggia et al., in revision). In order to control for density of individuals within tanks we housed large groups in 300L-tanks and small groups in 100L-tanks. Fish were reared in their respective groups for 60 days after free swimming, before being housed in sibling groups in 20L tanks (same social conditions) during a 'neutral' phase of 60 days. At the age of 4 months the fish were tagged with coloured Visible Implant Elastomer (VIE) tags (Northwest Marine Technology Inc.) in a scale pouch in the dorsal region to track individuals (Jungwirth et al., 2019). Behavioural tests were conducted between the ages of 4 months and 1 year to evaluate the effects of the early social environment on social behaviour (submission and aggression) and life history traits (exploration, helping and dispersal) (for detailed methods of these tests see La Loggia et al. in revision). After all behavioural tests were performed, i.e. when the fish reached 1 year of age, they were housed in sibling pairs in 50L tanks, separated from each other by clear, perforated partitions facilitating visual and olfactory communication. In total, we sampled 22 fish, eleven from large group-reared fish and eleven from small group-reared fish. As not all the siblings survived until an age of 1 year due to natural mortality, among the sampled fish there was a subset of individuals (three from each early-life treatment) that had been housed in 200L tanks alongside conspecifics from the same early life treatment.

### Candidate region of the SDMN

We investigated four nodes of the SDMN: (i) The anterior tuberal nucleus (aTn) is involved in aggression, reproduction and parental care in mammals (Félix & Oliveira, 2021; Lee et al., 2014; Y. Liu et al., 2019; McClellan et al., 2006; O'Connell & Hofmann, 2011; Olivier, 1977). In female plainfin midshipman fish Porichthys notatus, signs of neural activity were greater in the aTn when exposed to noises from conspecifics than ambient noise or heterospecific noises, suggesting that the aTn plays a role in the social behaviour network in fish (Mohr et al., 2018). (ii) The lateral zone of the dorsal telencephalic area (DL) is known to be involved in spatial learning via the storage of repeated experiences (Félix & Oliveira, 2021; O'Connell & Hofmann, 2011). In mammals, BDNF has been found to influence learning and memory in the hippocampus through its effects on long term potentiation (LTP) and long-term depression (LTD) (Egan et al., 2003; Kovalchuk et al., 2002; Park & Poo, 2013). In zebrafish, BDNF involvement in the DL has been suggested to improve the ability to recognise dominant conspecifics (Teles et al., 2016). (iii) The preoptic area (POA) is involved in regulating sexual behaviour, aggression, and parental care in teleosts (Félix & Oliveira, 2021; O'Connell & Hofmann, 2011). (vi) The posterior tuberculum (TPp) is involved in reward-related behaviour like motivation and in reproductive social behaviours such as parental care (Félix & Oliveira, 2021; O'Connell & Hofmann, 2011; Trutti et al., 2019).

# Candidate genes

We measured expression of the three candidate genes *bdnf*, *TrkB* and *P75NTR*. For measuring *bdnf* expression we used primers from (Nyman et al., 2017). For measuring expression of the tyrosine kinase receptor B (*Trkb*), primers developed based on the N. brichardi mRNA sequence were used (NCBI database ID number XM\_006787332.1, forward: GCTGGAACCACGATCCTCTG; reverse: GGGTCAGGTACACATTCTTGG, amplicon size 99), and for *P75NTR* (nerve growth factor receptor b in teleosts, referred to as *p75NTR* here), primers developed based on the N. brichardi mRNA sequence (NCBI database ID number XM\_006807519.1, forward: GAATCAGGCACAAACAGTCGTCAAC; reverse: CTAAACAGCAGCTTCTCCACTTTCTC, amplicon size 93) were used. The expression of *18s* was quantified as a house-keeping gene (Antunes et al., 2021; Nyman et al., 2017); we used primers from (B. Taborsky et al., 2013). Primers for *TrkB* and *P75NTR* were designed using primer-Blast (NCBI). The newly designed primers were tested prior to their utilisation. After performing a PCR (polymerase chain reaction) the products were sequenced and we run a Blast (NCBI) to confirm the specificity of the primers to the targeted genes.

# Tissue sampling

Tissue sampling was done as in Antunes et al., (2021). We euthanised the fish with an overdose of MS222 (Sigma-Aldrich). The decapitated heads were subsequently embedded in Tissue-Tek (optimal cutting temperature compound, OCT; Sakura) and frozen on dry ice within 3 minutes after euthanasia. The samples were stored and transported on dry ice until processing within the same day. We then sectioned the fish heads in the coronal plane, using disposable R35 microtome blades (Feather) on a Leica CM3050 cryostat. Slices were mounted on glass microscope slides immediately after being cut. The mounted slices were subsequently placed on a cold plate under a WILD M3C stereoscope, using a 24G sample corer tool (Fine Science Tools). The DL, DM, aTn, TPp and POA were dissected. The collected brain tissue was stored for each individual per brain region in an Eppendorf 1.5ml tube with 100µl of DNA/RNA Shield (Zymo Research), and then stored at -80°C until further processing.

## RNA extraction

Brain tissues were digested in Proteinase K for 2h at 55°C to lyse the tissue before RNA extraction. We extracted RNA following the protocol from the Quick-RNA MicroPrep kit (Zymo Research). Samples were treated with DNase I (Zymo Research) to avoid DNA contamination. Extracted RNA was quantified using QuBit RNA HS assay kit (ThermoFischer Scientific) on a QuBit 2.0 fluorometer machine (ThermoFischer Scientific; sample RNA concentration ranged from 10.6 to 70.2 ng  $\mu$ l-1). RNA concentration was too low to be detected in 32 cases out of 88 samples. We reverse transcribed all samples to cDNA using an iSCRIPT cDNA synthesis kit (Bio-Rad).

### Quantitative real-time PCR

Quantitative real-time PCR (q-rt-PCR) experiments and melting curves (ranging from 50 to 90°C) were conducted in triplicate using standard curves for 5 × 10-fold dilutions of all brain RNA. For p75NTR primers we used 5 × 10-fold dilutions of gBlocks® gene fragment synthesised on p75NTR predicted sequence from N. brichardi. These analyses aimed to assess the amplification efficiency (E) of each primer pair, as well as to ensure the absence of primer dimers and the specificity of the amplification (Antunes et al., 2021; Aubin-Horth et al., 2012). The primers (Microsynth) and 1 µl of sample cDNA were prepared in a 96-well plate (Greiner Bio-one). We added in each plate 5× HOT FIREPol EvaGreen qPCR Mix Plus ROX (Solis BioDyne) and performed qPCR on an ABI PRISM 7000 (Applied Biosystems). Triplicate runs were performed for all cDNA samples, including no-template controls. Melting curves were conducted for each replicate to confirm the absence of primer dimers and ensure the production of a single-amplified product. Cycle thresholds (Ct) were determined for each sample, and gene expression for individual brains was calculated using the formula  $1/[(1+E)]^Ct$ . The relative expression was then normalized to the reference gene (18s) (Pfaffl, 2001).

### Statistical analysis

Statistical analyses were conducted using R software version 4.2.2 (R Core Team, 2020). In certain cases, gene expression data for specific genes were excluded from the analysis due to a high coefficient of variation (CV) among the three replicates. To ensure data guality, samples with a CV exceeding the predetermined cut-off of 5% were removed from the analysis. We analysed the effect of early social environment (small vs large groups) on expression of each gene within each node of the SDMN by fitting linear models (LMs). All LMs initially included sex, early-life experience, exposure to behavioural tests and age at sampling as fixed factors. Through stepwise model reduction, factors with an Akaike information criterion (AIC) difference greater than 2 were systematically removed from the models and are not presented in the table. However, early life experience was consistently retained in all models, given its was the experimental factor in this study addressing the research question. To enhance the reliability of the analysis, outliers were identified and removed by calculating Cook's distance. This approach allowed the detection of influential data points, which were subsequently excluded from the analysis. By removing outliers, we ensured that the statistical models were not unduly influenced by extreme observations, resulting in more robust and accurate results. 25 outliers were removed out of 264 observations: 5 in the aTn observations; 4 in the DL observations; 5 in the POA observations and 11 in the TPp observations. Normality assumptions for the error term were evaluated using Shapiro-Wilk tests and visual inspection of quantilequantile plots for skewness and kurtosis. Homogeneity of variance was assessed through Tukey-Anscombe plots. To meet normality assumptions, gene expression levels were logtransformed.

# <u>Results</u>

The results revealed significant differences in gene expression between fish raised in large groups and those raised in small groups across different brain regions. In the anterior tuberal nucleus (aTn), fish raised in large groups exhibited an upregulation of *bdnf* and *TrkB* compared to fish raised in small groups (Table 1a, Figure 1). In the dorsal telencephalon (DL), fish raised in large groups displayed an upregulation of *bdnf* and a downregulation of *TrkB* compared to fish raised in small groups (Table 1b, Figure 2). Furthermore, in the preoptic area (POA), fish raised in large groups showed an upregulation of *bdnf*, *TrkB*, and *p75NTR* compared to fish raised in small groups (Table 1c, Figure 3). In the posterior tuberculum we found no effect of early-life environment on gene expression for the three genes investigated (Table 1d, Figure 4). *bdnf* expression increased with age in the aTn, DL and POA, it decreased with age in the TPp (Table 1a, Table 1b, Table 1c). *p75NTR* increased with age in the DL (Table 1b). *TrkB* and *p75NTR* expression in the aTn (Table 1a), *bdnf* and *p75NTR* expression in the DL (Table 1b) and *TrkB* expression in the TPp (Table 1a).



Figure 1: Relative expression of the three candidate genes in the ATN. L is for fish raised in large groups and S for fish raised in small groups. Bars represent standard error and dots means. Significance bars based on model output: \* p<0.005, \*\* p<0.001, \*\*\* p<0.0001.



Figure 2: Plot of relative expression of the three candidate genes in the DL. L is for fish raised in large groups and S for fish raised in small groups. Bars represent standard error and dots means. Significance bars based on model output: \* p<0.05, \*\* p<0.001, \*\*\* p<0.001.

Table 1: Results from linear models on the effects of early social experience on the relative expression (log2) of three candidate genes in (a) the ATN, (b) the DL, (c) the POA and (d) the TPp. Significant p-values are highlighted in bold.

Brain region	Ν	Target	Factors	Factor Level	Estimate	SE	Df	Sum of Sq	Pr(>Chi)
(a) ATN	16	bdnf	Early-life experience	Small Group	-4.51	1.91	1	74.36	0.017
			Age at sampling		17.08	4.87	1	164.23	0.001
	15	TrkB	Early-life experience	Small Group	-4.62	1.26	1	74.55	0.0004
			Sex				2	64.7	0.003
				Male	0.34	1.51			
				Unknown	5.41	1.64			
			Age at sampling		13.68	3.4	1	90.15	0.0001
	18	p75NTR	Early-life experience	Small Group	-0.53	2.17	1	1.11	0.781
			Sex				2	103	0.048
				Male	-4.11	2.34			
				Unknown	-6.3	3.51			
(b) DL	15	bdnf	Early-life experience	Small Group	-2.89	1.38	1	25.06	0.019
			Sex				2	63.06	0.004
				Male	-1.8	1.49			
				Unknown	-5.46	1.64			
			Age at sampling		8.47	3.25	1	38.5	0.005
	12	TrkB	Early-life experience	Small Group	1.99	0.97	1	11.63	0.04
	11	p75NTR	Early-life experience	Small Group	-2.62	2.37	1	18.99	0.36
			Sex				2	122.54	0.005
				Male	-1.32	2.98			
				Unknown	-9.23	2.96			
			Age at sampling		21.77	8.38	1	85.16	0.004
(c) POA	16	bdnf	Early-life experience	Small Group	-5.68	1.24	1	98.96	<0.0001
			Age at sampling		15.43	3.03	2	122.88	<0.0001
	13	TrkB	Early-life experience	Small Group	-1.92	0.89	1	11.3	0.02
			Sex				2	13.5	0.044
				Male	-0.4	1.16			
				Unknown	1.92	1			
	16	p75NTR	Early-life experience	Small Group	80.21	35.86	1	25070	0.022
			Age at sampling		-140.41	81.33	1	14937	0.069
(d) TPp	17	bdnf	Early-life experience	Small Group	-0.07	0.62	1	0.02	0.91
			Age at sampling		-5.69	1.64	1	18.22	0.001
	17	TrkB	Early-life experience	Small Group	-0.03	0.8	1	0.003	0.97
			Age at sampling		-4.83	1.82	1	16.64	0.008
	15	p75NTR	Early-life experience	Small Group	-0.98	0.86	1	3.39	0.21
			Age at sampling		-4.47	2.1	1	11.74	0.028



Figure 3: Plot of relative expression of the three candidate genes in the POA. L is for fish raised in large groups and S for fish raised in small groups. Bars represent standard error and dots means. Significance bars based on model output: \* p<0.05, \*\* p<0.001, \*\*\* p<0.0001.



Figure 4: Plot of relative expression of the three candidate genes in the TPp. L is for fish raised in large groups and S for fish raised in small groups. Bars represent standard error and dots means. Significance bars based on model output: \* p<0.05, \*\* p<0.001, \*\*\* p<0.001.

### **Discussion**

We investigated the effect of the early social environment on neuroplasticity markers in four nodes of the SDMN. We demonstrated that the early social environment induced long-term differences in expression of *bdnf* and its receptors in three of the four nodes of the SDMN. We show an upregulation of both *bdnf* and *TrkB* in the aTn of fish raised in the larger groups, suggesting that fish raised in large group might invest more in the BDNF/ TrkB pathway in the aTn. Upregulation of *TrkB* means potentially increased number of synapses and dendrites and presence of more neurons (Park & Poo, 2013; Purves et al., 2004; Sakuragi et al., 2013). In the DL we showed that *TrkB* was downregulated in large groups while *bdnf* was upregulated. Blockade of BDNF/TrkB pathway was found to impair LTP in the hippocampus in mammals (Park & Poo, 2013). In the POA all three genes were upregulated in fish raised in large groups compared to fish raised in small groups. Our results demonstrate that social complexity during early development has long-term effects on neuroplasticity.

The BDNF/TrkB pathway on one hand promotes neuronal survival and differentiation, as well as neurite growth (Park & Poo, 2013; Purves et al., 2004). The BDNF/TrkB pathway has been qualified as the synaptic enhancement pathway (Sakuragi et al., 2013). On the other hand, the BDNF/p75NTR pathway provoke apoptosis, decrease neuron survival but also promote neurite growth (Park & Poo, 2013; Purves et al., 2004). The BDNF/p75NTR pathway has been qualified as the synaptic elimination pathway (Sakuragi et al., 2013). In the aTn we found a higher expression of *bdnf* and TrkB in fish raised in large groups compared to fish raised in small groups, In vitro experiments showed that the masking of TrkB in mice hippocampus led to an alternative activation of p75NTR by BDNF (Sakuragi et al., 2013), this suggests that in the DL, large groups fish are more likely to use the synaptic elimination pathway and therefore capable of easily rewiring circuits to novel information or environments. Expression of *bdnf* in the DL in zebrafish was suggested to modulate social memory and allow individuals to better recognise and remember dominant conspecifics (Teles et al., 2016). Taken together this suggests that fish in larger groups could display higher social memory. In the POA we showed that fish raised in large groups have an upregulation of *bdnf* and both receptors. This could mean that both pathways are acting in synergy to promote increased connectivity between neurons while ensuring neuro survival with p75NTR eliminating circuits that are not in use and TrkB ensuring important circuits are enhanced (Sakuragi et al., 2013; Zanin et al., 2019). In the mammalian hippocampus TrkB and p75NTR interact after BDNF attaches to TrkB, and this interaction is important for activating a specific cell survival pathway (Zanin et al., 2019). POA, DL and aTn are interconnected within the SDMN (O'Connell & Hofmann, 2011). The TPp connects to the DL, the DL connects to TPp but also to the POA. The POA connects to DL and aTn and the aTn connects to the DL and POA (O'Connell & Hofmann, 2011). These connections between regions are crucial for information to circulate efficiently in the brain. we can speculate that synaptic plasticity and BDNF plays a very important role here to keep these regions connected and transfer information. In human children, BDNF was found to be of particular importance in modulating the connectivity between brain regions, carrier of BDNF gene variant showed different pattern of connectivity in the hippocampus and between the amygdala, insula and striatal regions (Thomason et al., 2009).

Early social experience is known to influence social competence, social competence is based on behavioural flexibility which could be regulated via neuroplasticity. We know from our previous experiment on the same fish and their siblings that the rearing treatment influenced the expression of social competence (La Loggia et al, in revision). Cardoso et al., (2015) suggested that at the neural level irreversible patterns of social competence can be mediated by two mechanisms: structural reorganisation or switch to relevant neural circuit. It has been suggested that neurotrophins have a huge role in mediating neuro-behavioural plasticity (Branchi et al., 2004). We suggest the structural

reorganisation pathway could be the BDNF/p75NTR pathway, where cell death could lead to restructuring of the neural circuits and therefore help flexibly reallocating space or resources according to changes in the environment (e.g. if there is need for reversal learning). Switching to relevant neural circuit could be achieved through the BDNF/TrKB pathway, with more neurons and/or more connection between these neurons leading to an easier change of neural circuit when needed to respond to changes in the environment. In zebrafish changes in social status resulted in different gene expression pattern across the SDMN, and particularly a differential expression of *bdnf* between winner and losers of fights (Teles et al., 2016). In mice, communal nesting experience in early life leads to higher rates of displayed maternal care as well as higher expression of neurotrophins in the brain (Branchi et al., 2006). Though with have no information on the expression of the BDNF receptors, these studies hint that social plasticity is linked to neuroplasticity. In N. pulcher, both the early social environment and a recent social challenge influenced constitutive gene expression in the hypothalamus (*bdnf*, neuroplasticity marker) (Nyman et al., 2017). While we cannot directly link social competence and *bdnf* pathways expression we have indication that they could be linked, and both influenced by early experience.

While it is possible that neurotrophins are mediating the expression of social behaviour, it is possible that the social environment influences the production of the neurotrophin. Neurotrophin production is experience dependent (Branchi et al., 2004), mRNAs of bdnf are regulated by neural activity so it is possible that different early life experience leads to different neural activity resulting in the differential long lasting expression of *bdnf*. Long-term memory or learning is frequently consolidated through repeated exposure to an experience. Long-term potentiation (LTP) and long-term depression (LTD) are mechanisms underlying learning and the formation of memory (Sakuragi et al., 2013). Repetitive LTP induction leads to long-lasting synaptic enhancement coupled with synaptogenesis (Tominaga-Yoshino et al., 2002, 2008), this is mediated by the BDNF/ TrkB pathway (Sakuragi et al., 2013), while repetitive LTD induction leads to long-lasting synaptic suppression coupled with synapse elimination (Kamikubo et al., 2006; Shinoda et al., 2005) which is mediated by the BDNF/p75NTR pathway (Sakuragi et al., 2013). We hypothesised that large groups face more opportunities to experience repeated social interactions by the presence of more adults interacting with each other in different ways. Therefore, fish raised in larger, more complex groups should be more likely to experience repeated LTP or LTD and form more of one or the other pathway, explaining the results we find here.

While we showed early-life experiences influenced neurotrophin expression, it is important to note that neurotrophin expression could also be influenced by the current environment. In *N. pulcher* there was differential expression of *bdnf* in the hypothalamus in fish that did not experience a challenge but in fish that did expression of *bdnf* the direction of the difference changed between fish raised without adults and fish raised with adults

(Nyman et al., 2017). In zebrafish, expression of *bdnf* was influenced by whether the fish won or lost a fight or if they were fighting a mirror image (Teles et al., 2016). In our case, our fish did not have any challenges prior to sampling so we do not have the information of differential expression of *bdnf* and its receptors after a social challenge. Our results show basal differences in neuroplasticity that can only be explained by the difference in early social environment we induced.

Age and sex influenced relative gene expression in different regions. While our lack of power prevents us to interpret the direction of the differences in gene expression between sexes, we found sex influenced the expression of *bdnf* in the DL, the expression of *TrkB* in the aTn and the POA and the expression of *p75NTR* in the aTn and in the DL. We found that older fish showed increased expression of *bdnf* in the aTn, DL and POA but decreased in the TPp. In the DL older fish had higher expression of *p75NTR*. Finally older fish showed higher expression of *TrkB* in the aTn but lower in the TPp. This suggest that age and sex are important factors to take into consideration when performing gene expression experiments. In rodents, expression of *bdnf* and *TrkB* was lost in the prefrontal cortex of aged individuals (Coria-Lucero et al., 2016). This makes sense in light with theoretical models showing plasticity decreases with age (B. Fischer et al., 2014; Panchanathan & Frankenhuis, 2016).

Our results highlight the importance of the early social experience in programming the basal expression of neuroplasticity markers and the potential long-term effects on neuronal branching. We show that both the expression of two distinct pathways regulating neuronal plasticity are shaped by early social experiences which persist until adulthood. Together with effects on social competence (La Loggia et al. in revision), we hypothesize that early social experiences alter social competence via the BDNF/TrkB and the BDNF/ p75NTR pathways. Future work should focus on testing the influence of social challenges on neuroplasticity gene expression in highly social species. More research is needed to fully understand the mechanisms underlying the expression of social competence.

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### Author contribution statement

Océane La Loggia: Conceptualisation, Methodology, Formal Analysis, Investigation, Data Curation, Writing -Original Draft, Writing – Review and Editing, Visualisation. Diogo Antunes: Conceptualisation, Methodology, Writing – Review and Editing, Visualisation, Supervision. Nadia Aubin-Horth: Conceptualisation, Methodology, Writing - Review & Editing, Supervision. Barbara Taborsky: Conceptualisation, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

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# **General Discussion**



With this thesis, I aimed to shed light on the interplay between the early social environment and the acquisition of social competence. One indicator of social competence in animals is their ability to appropriately adjust their behaviour in response to their partner in social interactions. For example, in sized-based linear hierarchies formation, showing submission when partnered with a larger and more competitive conspecific is an appropriate response to lower the cost of a fight and increase the chances of acceptance within a group (Reddon et al., 2021). To better understand the modalities of acquisition of social competence, I performed a series of experiments using the highly social cichlid Neolamprologus pulcher as a model species. In chapter one I investigated the impact of group size during early life on expression of social behaviour and other social traits in later life. Social competence was shaped by the early social environment with individuals raised in large groups showing increased flexibility in responding to aggression from a larger conspecific. Large-group fish showed repeatability in social competence contrary to small-group fish, suggesting they reliably show social competence across contexts. On the other hand, helping behaviour, exploration and dispersal were not influenced by the early social environment. With sufficient evidence to support previous findings that group size affects social competence, I set out to find the timing of its acquisition. I showed that the first social experience is the most important in shaping social competence, fish raised in large groups showed higher social competence compared to fish raised without adults in the first measure and the second measure regardless of their second social experience. Thirdly, using the same fish as in the first chapter, I investigated whether the behavioural patterns observed were linked to specific patterns of neuroplasticity gene expression across the Social Decision-Making Network (SDMN). I measured the expression of three neuroplasticity genes (bdnf and its receptors TrkB and p75NTR) in four regions of the SDMN. I showed that fish raised in large groups display different neuroplasticity gene expression patterns in the SDMN.

For group-living animals, group size and composition are important components of their environment. A group with more members of various sexes, sizes and social roles (Groenewoud et al., 2016) will be the siege of more diverse and numerous social interactions, which contributes to a more complex social environment (M. Taborsky, 2016). Previous studies have shown that group size can directly impact and promote survival and reproductive success. For instance, in cooperative meerkats, small groups are at higher risk of extinction in high predation environments (Clutton-Brock et al., 1999) and higher group size increases pup body condition and survival (Clutton-Brock et al., 2001). However, the long-term influence of group composition has not been studied so far. There is scarce knowledge about how the variation of group size and complexity experienced by individuals in early life shapes later-life social and life-history trajectories. In chapter one I manipulated the early social environment of *N. pulcher* by raising fish in either large or small groups of adults of various sexes and sizes. The fish could experience their social

environment for two months before being tested for social competence at the age of four months and other life history traits at the age of one year. As my main measure of social competence, I focused on submission behaviour in response to aggression from a larger conspecific and performed repeated test to investigate within individual repeatability. In N. pulcher, submissive behaviour is a very important mechanism to regulate within group aggression and can even function to achieve pre-emptive appeasement of dominants (Bergmüller & Taborsky, 2005; Fischer et al., 2014; Reddon et al., 2019). As such, I consider that submission is a potent signal that can contribute to lowering the cost of agonistic interaction between group members and therefore reduce chances of injury and eviction as well as the energetic cost of fighting (Camerlink et al., 2019; Lehner et al., 2011; Reddon et al., 2021). I hypothesised that fish raised in larger more complex groups would be more socially competent as well as show more consistent socially competent behaviour later in life (Fischer et al., 2015). According to my prediction, I found an effect of the early social environment on the expression of social competence, fish raised in large groups displayed higher levels of flexibility in response to aggression and showed submission faster and more consistently compared to fish raised in small groups. My findings from chapter one on social competence together with prior results obtained with a similar experimental design (Arnold & Taborsky, 2010; Fischer et al., 2015, 2017; Taborsky et al., 2012), showed that group size and complexity experienced in early life enhance social competence in N. pulcher. This emphasizes the importance of early-life social complexity for the development of social competence.

Rearing treatments did not influence the likelihood of being accepted by the dominant fish, the majority of the fish were accepted in the territory four hours after the test. This suggests that the fish behaved in dyadic fights in a range that was still considered competent regardless of their background. In a past experiment featuring fish raised in large and small groups rearing treatment was found to influence the likelihood of being accepted after a dyadic fight (Fischer et al., 2015). It is then unclear how truly the early social environment influences acceptance by a dominant. I did not investigate acceptance within a group, further work could focus on testing how fish from large and small groups behave when integrating into a group. While the fish may have all expressed competent behaviour, social competence is a continuum and fish from large groups still seem to express higher flexibility to respond appropriately to the social situation. This difference while not highlighted by the acceptance of the individuals in my experiment can be greatly beneficial over the many interactions fish have every day, especially in larger groups.

When studying social competence, it is important to consider behavioural repeatability as fitness benefits gained through social competency are small and accumulate over the many social interactions experienced during life (Taborsky, 2021). In the first chapter I introduced the idea that, when studying social competence, it is also important to consider whether these behaviours are repeatable. In the case of species where individuals perform potentially hundreds of social interactions every day, it is therefore crucial to show consistency in social competence. In chapter one, I show that individuals raised in large groups showed repeatable submission behaviour, but also responded appropriately to varying levels of aggression received. My current data is not directly informative for the repeatability of this plastic response by individuals to aggression received but I propose that this is an important question that has thus far been neglected in the study of social competence.

Previous developmental experiments on *N. pulcher* reared with or without older group members showed that the early-life environment influenced adult life-history strategies, with fish raised with adults showing higher philopatry as adults, but instead showed less helping behaviour, compared to fish raised in a socially deprived environment (Fischer et al., 2017). From these findings, I hypothesised that fish raised in larger groups would show less exploration and helping behaviour and remain more philopatric compared to fish raised in smaller, less complex groups. Contrary to my predictions I did not detect any effect of the early social environment on aggression, exploration, helping and dispersal. While broader life-history traits seem unaffected by early group size, social competence expression is shaped by the early social environment following previous work on the same species (Arnold & Taborsky, 2010; Fischer et al., 2015, 2017; Taborsky et al., 2012). Previous work has shown that a higher contrast between rearing environments triggered lifelong divergence in life history strategies (Fischer et al., 2017), in my case I manipulated the size of the group in a more natural range between three and ten individuals while in previous work the comparison was between fish raised in presence or absence of adult group members. Social deprivation from the absence of adults may have led to a higher contrast triggering the difference observed in previous work which did not occur in my own work. However, in nature, juveniles would not reach adulthood in the absence of adults to defend them against predators. The other difference in my approach was that I used younger fish in the dispersal experiment which allowed us to study dispersal patterns of both sexes. I observed very few dispersal events, and most dispersers were males as expected from patterns found in the wild (Jungwirth et al., 2023). My fish were likely too small to consider dispersal for independent breeding, nevertheless, N. pulcher are known to also disperse to other groups where they can rise in rank (Jungwirth et al., 2023). My results suggest that sex rather than early-life experience determines helping, exploration and dispersal propensity, with males showing lower levels of help, and more propensity to explore and disperse compared to females. This is in line with previous findings in other cooperative breeders like meerkats and other studies on *N. pulcher* where sex is determinant of life history strategies (Clutton-Brock et al., 2002; Schürch & Heg, 2010).

In chapter one I highlighted the importance of early life in shaping social competence, which is also found in other bodies of work across a wide range of species (Arnold & Taborsky, 2010; D'Andrea et al., 2007; Kempes et al., 2008; Liedtke & Schneider, 2017;

Sachser et al., 2013; Taborsky et al., 2012). However, it is unclear whether early life is the only sensitive window to gather the necessary information from the environment to acquire social competence. Multiple or extended sensitive periods would be beneficial to overcome potential mismatch with the future environment. To answer this question, in chapter two I manipulated the social environment of *N. pulcher* during their early and later development to simulate changes in the environment before sexual maturation. I chose to increase the contrast between the rearing treatments by having fish raised with a group of ten individuals on one hand and fish raised only among siblings on the other hand, to have the most extreme patterns of social competence expression. Fish raised in large groups showed more submission in response to aggression from a larger conspecific and were most likely accepted in the territory compared to fish raised only among siblings. They also displayed more aggression toward a same-size opponent in the mirror test compared to fish raised without adult group members. This suggests that early development is critical in acquiring the appropriate social skills and that there is no chance of recovering these skills through exposure to a different social environment later in development.

My results suggest that there could be constraints on later life plasticity preventing a reprogramming of the social phenotype. Since early acquisition is crucial for survival, all resources towards gathering information about the environment could be concentrated in the very early life. These resources can be costly, as shown in drosophila where maintenance of plasticity through increased learning abilities leads to lower survival under competition (Mery & Kawecki, 2003). Another explanation could be that in natural conditions, the early social environment is reliable enough to equip individuals with the appropriate skills for the future, hence a second chance at gathering social information did not evolve in this system. In my fish and many other cooperative breeders, most individuals remain philopatric (Bergmüller et al., 2005; Jungwirth et al., 2023; Suh et al., 2022). Philopatry could drive the peak of plasticity in early life as social information remains stable when individuals stay in the same group for extended periods. It could also be that I failed to trigger the opening of a second window. In N. pulcher and other social species prospection is a crucial behaviour to investigate potential territory vacancy and dispersal options (Jungwirth et al., 2015; Kingma et al., 2016; Roper et al., 2003). The period of prospection could be the trigger to open a second sensitive window to gather information on the potential new environment after the dispersal decision. It is also good to note that I only measured one trait in my experiment, this does not exclude the existence of a second plastic window around the age of dispersal decision for other traits that could be more linked to reproduction or dominance status such as aggression, growth or gonadal development.

In chapter three I aimed to uncover the underlying mechanisms behind social competence. I postulate that social competence is achieved through neuromolecular processes mediated by neuroplasticity (Cardoso et al., 2015). The structural and functional

changes in the brain associated with social competence brought by neuroplasticity are thought to be mediated by neurotrophin such as the brain-derived neurotrophic factor (BDNF) (Branchi et al., 2004a). BDNF binds to two receptors leading to two different functional pathways: (i) The BDNF/TrkB pathway, characterised by higher neuronal survival, differentiation and neurite growth (Park & Poo, 2013; Purves et al., 2004), also called synaptic enhancement pathway (Sakuragi et al., 2013); and (ii) the BDNF/p75NTR, also called synaptic elimination pathway (Sakuragi et al., 2013) characterised by apoptosis, lower neuronal survival while also promoting neurite growth (Park & Poo, 2013; Purves et al., 2004). To uncover the link between the expression of BDNF and the expression of social competence I performed a followed-up experiment with the fish I tested in the first chapter where I quantified gene expression of bdnf and its two receptors TrkB and p75NTR across four regions of the SDMN. I focused on four regions involved in the expression of memory (DL: lateral part of the dorsal telencephalon) and social behaviour (aTn anterior tuberal nucleus, POA: preoptic area, TPp: posterior tuberculum) (O'Connell & Hofmann, 2011). I found that early social complexity impacted the expression of *bdnf* and *TrkB* in the aTn and the DL. In the POA, *bdnf* and the two receptors were differentially expressed between fish raised in large and small groups. Patterns of social competence are suggested to be mediated by two mechanisms: structural reorganisation or switch to relevant neural circuits (Cardoso et al., 2015). In chapter three, I suggest that structural reorganisation could be achieved through the BDNF/p75NTR pathway, where cell death could lead to the renewal of the neural circuits and therefore help flexibly reallocate resources after a change in the environment. The BDNF/TrkB pathway could on the other hand be involved in switching to the relevant neural circuit, with more neurons and/or more connection between these neurons leading to an easier change of neural circuit when needed to respond to changes in the environment. In zebrafish (Danio rerio), for example, changes in social status resulted in differential expression of bdnf across the SDMN between winners and losers (Teles et al., 2016). Similar results were found in mice (species) where social status influenced neurotrophin expression levels (Branchi et al., 2006). In N. pulcher, both the early social environment and recent social challenges influenced bdnf expression in the hypothalamus (Nyman et al., 2017). Though with have no information on the expression of the BDNF receptors in these studies, results available so far suggest that social plasticity is linked to neuroplasticity. To sum up, my results suggest that the early social experience has long lasting effects in programming the basal expression of neuroplasticity markers, this hints that the observed differences in social competence shaped by early social exposure may be explained by long term changes on neuroplasticity.

In chapter three I showed that the early social environment triggered life-long changes in basal neuroplasticity gene expression patterns. Together with the results from chapter two on the absence of a second or extended sensitive window for social competence, this can suggest that the necessary neural changes in the SDMN for reintegrating different information about the environment later in life are under constraints. Neurotrophin expression, which mediates neuroplasticity (Branchi et al., 2004b; Huang & Reichardt, 2001), could constrain the ability of the brain to reformat or integrate new information. I explored several alternative explanations as to why the fish did not express a second sensitive window, together with the findings of chapter three which hints towards the constraint hypothesis. However, I cannot exclude that these constraints could also exist because more time to gather information on the environment is not needed. Further work is needed to properly evaluate the role of neurotrophin in shaping plasticity across life stages.

Early acquisition of social competence allows individuals to maximise the fitness gain across social interactions throughout their lives (Taborsky & Oliveira, 2012). Individuals benefit strongly from avoiding injuries or de-escalating fights within group contests (Camerlink et al., 2019). My results show how larger and more complex groups produce more socially competent offspring, therefore suggesting the existence of a positive feedback loop based on individual-to-society feedback (Cantor et al., 2021) enhancing sociality through social competence (Taborsky, 2021). More socially competent individuals are better integrated into the group (Taborsky et al., 2012), find their rank sooner (Branchi et al., 2006) and therefore are more likely to contribute to the growth of the group. In nature, large groups offer many benefits such as increased survival (Clutton-Brock et al., 1999; Heg et al., 2005), increased territory quality (Balshine et al., 2001) and higher reproductive success for breeders (Balshine et al., 2001; Clutton-Brock, 2006; Koenig & Dickinson, 2004, 2016). In cooperatively breeding Australian magpies (*Gymnorhina tibicen*), large groups were found to promote cognitive development and facilitate the transmission of information (Ashton et al., 2018, 2019). In the same species, task performance was positively related to indicators of reproductive success (Ashton et al., 2018), suggesting how the acquisition of better cognitive skills can lead to potential fitness benefits of group living.

Taken together my results provide further evidence of the impact of the early social environment on the acquisition of social behaviour. I demonstrate that the expression of social behaviour is intrinsically linked to neuroplasticity expression across the SDMN. I also showed that early life is the only critical period for acquiring social competence, leaving no chance for recovery in the future. My results suggest that higher social complexity is more likely to yield social phenotypes compatible with group living and thus contribute to maintaining high group size, fuelling sociality in a positive feedback loop (Taborsky, 2021).

While my results provide a better understanding of how and when social competence is acquired in N. pulcher, some questions and hypotheses remain to be explored. For future outlook, I suggest investigating the question of consistency in social competence further. Studies on social competence could benefit from implementing measures of within individual repeatability in showing social competence to strengthen their message on the fitness advantages of social competency. Furthermore, I could not properly show that early life influenced how repeatably individuals expressed social competence. Further work is necessary to fully understand how the early social environment affects the repeatability of the expression of social behaviour. Past studies have always compared social competence along a continuum of behaviour expressed in response to a social situation, however, I propose that another side of social competence is how an individual consistently responds appropriately to a given situation. This could be another way to measure social competence in various social species. Secondly, my results on neuroplasticity gene expression patterns demonstrate basal differences between rearing treatments across the SDMN. However, it would be interesting to link these patterns directly to the expression of social behaviour. Further work could follow up on my study to measure the expression of bdnf and its receptors after a social challenge to investigate if differential patterns are observed when the individuals are required to express social behaviour.

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# **Appendix 1**

Fish can infer relations between colour cues in a non-social learning task.

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# Fish can infer relations between colour cues in a non-social learning task

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# <u>Abstract</u>

Transitive inference (TI) describes the ability to infer relationships between stimuli that have never been seen together before. Social cichlids can use TI in a social setting where observers assess dominance status after witnessing contests between different dyads of conspecifics. If cognitive processes are domain-general, animals should use abilities evolved in a social context also in a non-social context. Therefore, if TI is domain-general in fish, social fish should be able to use TI also in non-social tasks. Here we tested whether the cooperatively-breeding cichlid Neolamprologus pulcher can infer transitive relationships between artificial stimuli in a non-social context. We used an associative learning paradigm where the fish received a food reward when correctly solving a colour discrimination task. Eleven of twelve subjects chose the predicted outcome for TI in the first test trial and five subjects performed with 100% accuracy in six successive test trials. We found no evidence that the fish solved the TI task by value transfer. Our findings show that fish use TI also in non-social tasks with artificial stimuli, thus generalizing past results reported in a social context and hinting toward a domain-general cognitive mechanism.

# **Introduction**

Transitive inference (TI) has been proposed to be an important cognitive ability in social contexts of hierarchy formation and maintenance [1]. TI describes the ability to infer relationships between stimuli that have never been seen together [2,3]. Social species organized in stable groups with rank hierarchies benefit from the ability to infer the social status of others indirectly, and are able to use TI for this [4–6]. Assessing and remembering the ranks of conspecifics allows for appropriate behaviour during interactions, and thus is an important aspect of social competence [7].

It is currently debated whether the brain mechanisms underlying cognition are organised in domain-specific modules or are domain-general [1,8–11]. If cognition is domain-general, the context in which an ability is used should not matter. Conversely, if cognition is organised in modules, animals should not be able to use abilities evolved in a social context also in a non-social context, and vice versa [11]. The usage of TI in animals has been shown either in a social [birds [4], fish [5,12]] or in a non-social context [insects [13], birds[4,14], mammals [15–18]]. Only in a single bird species, the pinyon jay Gymnorhinus cyanocephalus, the use of TI was demonstrated both in a social [4] and a non-social context [19], suggesting that this ability is domain-general in this species. In Tanganyika cichlids, the use of TI was thus far shown only in a social context. After witnessing contests between several dyads of conspecifics, experimental individuals were able to infer their dominance status even if they never observed them interacting directly (Astatotilapia burtoni [5], Julidochromis transcriptus [12]). If we demonstrate that Tanganyika cichlids can use TI in a non-social context, this will support the existence of domain-general cognitive abilities in these fish.

Previous studies suggested two ways to solve a TI task: by logical reasoning [2,20] or by associative learning and value transfer (VT) [1,2,14,21,22]. Logical reasoning has been the main explanation for the human ability to solve TI [2], however, it is difficult to show this in non-human animals. Alternatively, the Value Transfer Theory predicts that animals evaluate a given stimulus depending on the value of a second stimulus presented simultaneously: If a rewarded stimulus A is presented with a second non-rewarded stimulus B, then B will acquire a secondary positive value from A. This means the positive value of A is transferred to some degree to B although B is unrewarded [14,21].

We investigated whether cooperatively-breeding cichlids, Neolamprologus pulcher, can infer transitive relationships between non-social stimuli. It is likely that N. pulcher can infer social rank by using TI as shown in other socially living cichlids [9, 10]. N. pulcher live in groups composed of a dominant breeding pair and subordinate helpers [23], structured by a sized-based linear hierarchy [24]. Finding and maintaining a stable position in the hierarchy is a crucial social skill for N. pulcher, as contests with group members may result in eviction from a group territory imposing a high cost on the evictee [25,26]. In this study, we aimed to test if N. pulcher might use TI in a more general context and can infer the relative value of artificial stimuli in a non-social context. To test this, we trained N. pulcher to solve a colour discrimination task using a well-established conditioning learning paradigm [27–29]. The fish learnt to discriminate four pairs of colours and were trained the five-term series A+B-, B+C-, C+D- and D+E- commonly used in TI experiment (reviewed in [2]; letters are arbitrarily assigned to five stimuli that are rewarded (+) or unrewarded (-)). If N. pulcher can perform TI, we predict that they (1) order the five stimuli in a hierarchical order (e.g. A>B>C>D>E), and consequently, (2) they are able to infer the relationship between B and D, i.e. two colours never seen together before (e.g., to be B>D).

# **Materials and Methods**

### TI training phase

We used 18 N. pulcher, nine males and nine females (see Electronic Supplementary Material (ESM) for fish maintenance). They were trained in a five-term series, consisting of the presentation of four pairs of colour stimuli [2]. We used two batches of nine fish. For the second batch (combination 2), we reversed the order of colour stimuli as compared to the first batch (combination 1) to control for colour preference (Figure S3A). We used a similar learning paradigm as [27] (see ESM).

### TI test phase

All fish (N=12), which reached the learning criterion in the last intermixed session, were tested whether they use transitive inference. The test phase was done on three consecutive days, with two sessions of three trials each day (in total, 18 trials). The first and third trials of a session consisted of a rewarded trial of a randomly chosen, known pair (A+B-, B+C-, C+D-, or D+E-), to avoid extinction learning. Only the second trial was a non-rewarded test trial, where we presented colour B against D. Only second trials were used for the analysis of TI. The fish received a 30-minute break between the two sessions of a day to prevent carry-over effects of experiences between the two sessions. Fish trained with combination 1 succeeded in the task if they chose B over D, fish trained with combination 2 succeeded when they chose D over B. Due to technical issues, one fish only went through two trials and thus we only used the data of the first trial of this fish for analysis.

### Data analysis

All analyses were performed in R (4.0.2) [30]. To analyse the probability of success in the TI test we performed two-tailed binomial tests (i) over all first choices made by fish that participated in the test phase, and (ii) for the individual choices made by every fish over all the six test trials. We compared the choices performed by the fish by fitting three generalised mixed effect models (package 'lmerTest', [31]) assuming binomial distribution, two models to analyse the outcomes during the training periods for the two batches and one model on outcome of the TI test. We used observer, sex, type learning session (in models on training periods), rewarded colour and side as fixed factors. Models were simplified by stepwise exclusion of factors, retaining the more parsimonious model if it had a lower AIC. Individual identity was included as random factor. Post hoc analyses were conducted for pairwise comparison using the Bonferroni method for adjusting P values (package 'emmeans', [32]). To test whether the frequency of exposure to colours B and D affected the choice in the TI test, we calculated the ratio between the number of rewards received from B and D (i.e., rewards received from B/rewards received from D). We then correlated the reward ratio to the error percentage during the TI test and during the training phase, respectively, by Spearman correlation tests.

# <u>Results</u>

# Training phase

The number of correct choices during the training was predicted by the rewarded colour and the side (left or right) of the reward on the well plate (Table 1). Fish in batch 1 made more correct choices when the reward was placed on the right side and fish from batch 2 made more correct choices when the reward was placed on the left side. Post hoc analysis showed that fish strongly preferred the colour that was always rewarded in both batches (i.e., red and purple, Table 1, Figure 1A). In batch 1, there was no preference for colours B and D, i.e., the ones used in the TI test (i.e., black and blue, Table 1). In batch 2, fish preferred black (Table 1), which during the TI test was the incorrect colour (Figure 1A).

### TI test

Out of the twelve tested fish, eleven chose the correct option in the TI test during the first trial (Table 2, p=0.006; Figure 1A). Of eleven fish taking part in all test trial, five fish performed significantly above chance (6/6 correct choices, Table 2), while the other six fish made one between and three mistakes (Table 2, Figure 1B). The reward ratio B/D (batch 1) or D/B (batch 2) did not predict the error rate in the TI test (Spearman correlation, rho=0.28, p=0.41) nor did it predict the error rate in the training phase (rho=-0.27, p=0.22). There was no side or colour effects on the number of correct choices in the TI test (Table 2). There was no statistical difference between sexes in TI performance, which might be attributable to a lack of statistical power (Table 2).

# **Discussion**

Our results demonstrate that N. pulcher use transitive inference when inferring relationships between artificial stimuli in a non-social context. Fish chose the correct colour in the first test trial for TI above chance, and, on the individual level, about half of the fish performed above chance, with 100% accuracy, in the six TI test trials. Our results do not support the use of value transfer as the underlying mechanism of TI in N. pulcher, but our statistical power to draw final conclusions on the mechanism of TI is limited.

While TI in a non-social context was shown in a number of mammals and birds [14,16,33–35], there has been only one such study aiming to test TI in a fish thus far [36]. However, it did not ultimately show the use of TI above chance level, as only four individuals were tested, and not all of these did the correct choice in the first test trial. Moreover, [36] used rewarded test trials, so that the reported indications of the use of TI can alternatively be explained by associative learning during tests. In our study, the TI test trials were not rewarded to exclude associative colour learning and fish performed above chance level. Moreover, our results cannot be explained (i) by simple colour preferences,

as the two batches of fish were trained on the opposite order of the five-term colour series or (ii) by different experience with colour pairs, as error rates during the TI test were not influenced by the reward ratio of test colours during the training. We can therefore safely conclude our fish used TI in a non-social task.

In several taxa, highly social species possess the ability to represent transitive relationships among the hierarchy ranks of conspecifics [4,5,12,19]. It has rarely been tested if this ability can be transferred to a non-social context, which would support domain-general organisation of cognition [11]. In N. pulcher, there is recent evidence that adaptive social flexibility, i.e. social competence [7], and non-social flexibility are affected similarly by the same physiological manipulation [37]. While previously disputed [38], this provided the first evidence of domain-general cognition in a fish [37]. We propose that our results provide further indication of domain-general cognition in fish spanning the social and non-social domain, because TI in a social hierarchy context was shown to exist in the social cichlids A. burtoni and J. transcriptus [5,12], and TI in a non-social context was shown in a social cichlid in this study. Moreover, N. pulcher track the relative ranks of other group members allowing them to reduce their queuing time to territory inheritance when entering group [39], and thus it is likely they employ TI also for inferring ranks. However, to verify domain-general TI in fish, it needs social and non-social TI experiments in the same fish species.

While we demonstrated that fish could make transitive inferences of colour stimuli, the mechanism underlying this ability is yet to be explored. The possibility to use associative learning by value transfer to solve a TI task was first investigated and confirmed in pigeons [14,21,22]. In our study, we performed a separate experiment, in which we trained six fish and tested if they might have used value transfer as a possible mechanism to solve a TI paradigm (see Supplementary Methods in ESM). There was no significant evidence that the fish used value transfer in our experiment (see Figure S4 and Supplementary Results in ESM). This may suggest that a more complex cognitive mechanism is at work. Grosenick et al. [5] discussed that value transfer theory alone cannot explain TI in their study species. However, our negative results for value transfer may also be due to low power, as only one of the six fish failed to show a correct first choice. Nevertheless, none of our repeated individual tests provided evidence for an above-chance use of value transfer.

In conclusion, our study adds to existing evidence suggesting that fish can perform complex cognitive tasks such as TI, but which thus far was only shown in a social [5,12,40] context. As brain structures involved in the Social Decision Making Network are conserved across vertebrates [41], it should not be surprising that fish often show cognitive abilities comparable to other vertebrates [42]. The underlying mechanisms of these abilities are largely understudied, but may often be simpler than assumed (e.g., value transfer), and may differ among different vertebrate species.

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Table 1: GLMM results for the number of correct choices in (a) Batch 1 (N=2477 observation of 9 fish) and (b) Batch 2 (N=2637 observation of 9 fish) during the training phase and (c) in the transitive inference (TI) test only (N=68 observations of 12 fish). Type of session: binary variable indicating if it was a single pair or intermixed session. Rewarded colour: rewarded colour in a trial (factor with four levels). Side: binary variable indicating whether the reward was placed on right or left side of the well plate. (d) and (e): pairwise comparisons between the different colours in the two batches. (f) pairwise comparison between male and female in the TI test.

Factors	df	LRT	Р
a. Number of correct choices in Batch 1			
Rewarded colour	3	48.86	<0.0001
Type of session Side	1	2.46	0.12
b. Number of correct choices in Batch 2	1	7.44	0.006
Rewarded colour			
Type of session			.0.0001
	3	46.66	<0.0001
Side	1	3.00	0.08
c. Number of correct choices in the TI test	1	11 44	0.0007
Side	1	11.11	
Correct colour			
	1	2.16	0.14
Sex	1	2.27	0.13
	1	2.64	0.07
Comparisons	Estimates ± SE	Z ratio	Р
d. Batch 1			
Red – Black	0.87 ± 0.13	6.86	<0.0001
Red – Blue	0.58 ± 0.15	3.86	0.0007
Red – Green	0.76 ± 0.13	5.77	<0.0001
Black – Blue	$-0.29 \pm 0.14$	-2.12	0.20
Black – Green	-0.11 ± 0.12	-0.94	1.00
Blue – Green	$0.18 \pm 0.14$	1.30	1.00
e. Batch 2			
Purple – Black	-0.54 ± 0.15	-3.66	0.0015
Purple – Blue	0.35 ± 0.11	3.07	0.01
Purple – Green	0.27 ± 0.12	2.31	0.12
Black – Blue	$0.89 \pm 0.14$	6.18	<0.0001
Black – Green	0.81 ± 0.15	5.52	<0.0001
Blue – Green	$-0.07 \pm 0.11$	-6.32	1.00

Table 2: Results of binomial tests for the TI test trials (N=12 f	for first choice, N=11 for all test trials).
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Correct choices	N of fish	Probability of success	Р
First choice correct	11	0.92	0.006
6 out of 6	5	1	0.03
5 out of 6	1	0.83	0.22
4 out of 6	3	0.67	0.69
3 out of 6	2	0.5	1



Figure 1: Success in A) the transitive inference test from the first trial of all fish (N=12) and B) every individual that went through the six TI trials (N=11; fish K only went through two trials and therefore was excluded here).

# **Electronic Supplementary Material**

Fish can infer relations between colour cues in a non-social learning task

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Content: Figures S1-S4, Supplementary Methods, Supplementary Results



Figure S1: Picture of the learning set up. A) The plates are made of PVC and contain 20 wells in five lines, in which a food reward can be placed. Only the line closest to the fish compartment was used in the experiment, and the rewards and coloured chips were always placed in the 2nd and 4th hole of this line. The position of chips within this line (left or right) was determined by a random generator (see factor 'side' in Table 1 of the main text). B) Example of rewarded and unrewarded chips. The rewarded chips were placed over a well containing a reward and were easily removable by the fish. The unrewarded chip had a knob glued on the bottom which fitted snugly to the size of the well. Also this chip was placed in a well with a food item to control for chemical cues, but the knob prevented the fish to remove the chip (setup following Buechel et al. 2018).



*Figure S2:* Arrangement of tank (Filter (a) / Shelter (b) / PVC plate (c). The observer could only see the fish from the front side (marked with O). During the experiments, at the beginning of each trial an opaque separation was placed in the tank (d).



Figure S3: A) Combination of colour pairs for the two batches and training procedure. Batch 1 was trained on Combination 1 of colours; the colours were inverted for the second batch (Combination 2). B) Training procedure, the colour pairs on the left correspond to Combination 1 of batch 1, and the pair on the right to Combination 2 of batch 2. Here we only show the colour pairs presented in each type of trial; it does not represent the number of trials per session or the order of presentation (see Methods and ESM for details).



Figure S4: Success in A) the value transfer test from the first trial of all fish and B) every individual in the six value transfer trials (both N=6). Blue: successes; orange: failures.

#### **Supplementary Methods**

#### Fish Maintenance and Habituation

Experimental N. pulcher were laboratory-raised descendants from the Kasakalawe Point population near Mpulungu, Zambia, and were between 35-45 mm standard length (SL) at the time of testing.

The experiment was carried out at the Ethological Station Hasli of the Institute of Ecology and Evolution (IEE), University of Bern, Switzerland. Before the experiments, fish were housed in in 400-litre tanks in conspecific aggregations. These tanks were equipped with a 5 cm layer of river sand, a biological filter, and half-transparent pet bottles mounted near the water surface as shelters.

Eighteen individuals, nine males and nine females, were used in the transitive inference experiment (body size: mean 40.3 mm± 0.7 s.e., range 35 to 45mm) and six individuals, three males and three females, were used in the value transfer experiment (body size: mean 40 mm± 1.29 s.e., range 36 to 44 mm). In the transitive inference experiment, we tested the 18 fish in two batches of nine individuals each. During the experiments, the fish were placed individually in 20-litre tanks, where they were visually and chemically separated from the other participants in the experiments but could see fish kept for another study in the opposite rack of the aquarium. Each tank contained a biological filter, a 2-3 cm layer of sand, and half of a clay flowerpot as a shelter in the back half of the tank (see Fig. S2).

The experimental device to place the stimuli and rewards was a grey PVC plate (dimensions: 20x10x1 cm) containing 20 equally spaced wells of 11 mm diameter and 9 mm depth (Figure S1). This plate was placed in the front half of the tank (Figure S2). To prevent neophobic responses to this device, we left the PVC plate before and during the trial sessions in the tank. The light:dark cycle was set to 13:11 h with a 10 min dimmed light period in the morning and evening to simulate the light conditions of Lake Tanganyika and the water temperature was maintained at  $27 \pm 1$  °C. The fish were fed every day during the trials with krill, except on days with no learning trials, when the fish were fed with commercial flakes food (TetraMin).

#### Training of the motor task

The goal of the training phase was to teach the fish the motor task necessary to take part in the learning phase, i.e. to remove a round plastic chips of 1.5 cm diameter covering a well of the grey PVC plate in order to access a hidden piece of krill (the reward). Yellow chips were used for that task as N. pulcher have a preference for chips of this colour [1,2] For the training phase, the fish had up to four trials per day. Before each trial, an opaque separation was placed between the PVC plate in the front of the tank and the fish sitting in its shelter in the back, to avoid the fish seeing the experimenter setting up the krill and the chips. For each trial two wells were chosen on the row of the PVC plate closest to the fish, with one well left empty in between them (Figure S1). Each of the two wells was filled with a small piece of krill and then covered by a yellow chip. Once the trial was set up, the opaque separation was removed, and simultaneously we added a few drops of water containing the smell of krill by a pipette to the tank water in order to motivate the fish to search for the reward.

The training phase was done in four different steps. In the first step, the chips were placed next to the wells, so that the reward was freely accessible to the fish. In the second step, the chips covered one quarter of the wells; in the third step, the wells were covered to a half with the yellow chips; and in the fourth step, the chip covered the wells completely. In steps 1 and 2, the fish were given 60 min to accomplish the task, in steps 3 and the first trial of step 4, the maximum was 45 min. The trials of step 4 were video recorded; from the

videos we assessed the time required to dislodge both chips and eat the rewards. During the following trial of step 4, this time was allowed as maximum time for a given individual to solve the training task. We iteratively adjusted the maximum time allowed to solve the task in step 4 based on the time a fish needed during its preceding trial, thereby gradually decreasing the time needed to solve the task until all individuals could do this within 5 min. During the training phase, fish did four trials per day. All fish passed the training phase. They needed between two and five days to pass the task within 5 min. We trained the fish until all nine fish within a batch of the TI experiment or all six fish for the value transfer experiment had learnt the motor task, so that they could start the learning phase at the same time.

#### Details on the TI training protocol

The fish had five minutes to choose between two plastic chips of different colours covering a well of a grey well plate (Figure S1). Half of the chips were modified by a gluedon plastic knob that snugly fit the wells of the PVC plate, and thus prevented the fish to move the chip and access the reward (Figure S1). A piece of defrosted krill was placed under each chip but only accessible for the fish when under the movable chip (i.e. the rewarded chip). We trained the fish on four different colour pairs (Figure S3). We used colours that naturally occur on the fish's face and are assumed to be distinguishable by the fish [3]. The training for the TI experiment was done in seven steps. In all sessions, the learning criterion was reached when a fish touched the rewarded chip first either 80% of times on two successive days or 100% in one day. A fish moved on to the next step once it had reached the learning criterion in the current step. The steps comprised single colour pair sessions ("Pair 1", "Pair 2", "Pair 3" and "Pair 4", Figure S3A) and intermixed sessions ("Mix 1", "Mix 2" and "Mix 3", Figure S3B). Each intermixed session comprised two steps: we first presented the single pairs consecutively (e.g. for Mix 2: Pair 1, Pair 1, Pair 2, Pair 2, Pair 3, Pair 3), and after the fish reached the learning criterion, we presented the pairs in randomly mixed order (e.g. for Mix 2: Pair 1, Pair 3, Pair 2, Pair 3, Pair 2, Pair 1). This ensured that the learnt contents were consolidated, before we moved to train the fish a new colour pair (Figure S3B). In the last intermixed session eight trials were done, so that the fish could get an equal exposure to each of the four colour pairs during one session (Figure S3B).

To avoid side biases during learning, in a session of trials, the rewarded side (left or right) within the used row of wells was determined by a random generator at the beginning of each experimental day. However, we used a pseudo-random rule adjusting the rewarded side to ensure that the rewarded well was never more than three times on the same side. To avoid an olfactory bias, krill was placed in both wells, i.e. under the movable (rewarded) and the non-movable (unrewarded) chip (see [4]). A fish was considered to have made a choice when it first touched a chip with the tip of its mouth. The observer waited until the

fish ate the reward to put again the separation and start the next trial. If the fish took more than 5 min to make a choice, the trial was terminated, and the fish was considered to have made no choice. The no-choice trials were not counted, and the experimenter repeated the trial until the fish made a choice.

There were two situations when we gave the fish 'reminder' trials. (1) When a fish made mistakes only on a particular colour pair in an intermixed session during two or more consecutive sessions, we inserted reminder session(s) only for that particular colour pair. When the fish reached the learning criterion in a reminder session, we resumed the intermixed sessions again. (2) If a fish managed to dislodge a chip blocked by the plastic knob and ate the krill from the unrewarded stimulus, which happened rarely, we gave this fish a reminder trial to be sure the fish did not learn the wrong contingency.

#### Value Transfer (VT)

We trained six fish, three males and three females, before testing whether they use VT when choosing between colour stimuli. We trained them on two colour pairs, A/B and C/D. The colours were inverted for half of the fish to control for colour preference. In the first colour pair, A was always rewarded, and B was never rewarded. In the second colour pair, C was rewarded in 50% of the trials and unrewarded in the other 50% of trials, and D was never rewarded. The fish were trained on the colour pairs sequentially. We performed six training trials a day for 6 days a week. We considered that the fish had learnt the colour pairs when they had at least 11 correct trials out of 12 for each colour pair. In VT test, fish were shown colours B and D. If the fish use VT, they should choose B over D, as B was associated with A, which was always rewarded, whereas D was associated with C, which was rewarded only in half of the trials [5]. The test for value transfer used the same scheme as in the TI test phase.

To analyse the probability of success in the VT test we performed two-tailed binomial tests (i) over all first choices made by fish that participated in the test phase, and (ii) for the individual choices made by every fish over all the six test trials.

#### **Supplementary Results**

#### Value Transfer (VT)

When testing for the use of value transfer, 5 out of 6 fish did the correct choice in the first test trial (binomial test, probability of success= 0.83, p=0.22, Figure 1C). None of the individual fish made a correct choice significantly above chance (i.e., 6/6 correct choices; Figure S4).

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# **Appendix 2**

Do rearing group-size and social rank influence the affective state of a cooperatively-breeding cichlid fish?



# <u>Do rearing group-size and social rank influence the affective state of a</u> <u>cooperatively-breeding cichlid fish?</u>

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# Abstract

The affective state of animals, i.e. their mood and emotions, are altered by stressful (negative) or enriching (positive) experiences. In turn, the affective state influences decision making, thereby helping animals to cope with environmental challenges and opportunities. Particularly in the social domain, it is largely unknown how social experiences modulate the affective state. Here, we performed a judgment bias test to study the effects of rearing group-size and experimentally assigned current rank on the affective state, we developed and validated a judgment bias test for this species. Fish learned to discriminate between a positive and a negative stimulus as shown by different latencies to approach the stimulus. Furthermore, the fish showed the response curves expected in judgment bias tests. They showed an intermediate latency to approach an ambiguous stimulus, which significantly differed from the latencies to approach the positive and the negative stimulus. Unexpectedly, there were no significant effects of rearing group size and current social rank on the affective state of *N. pulcher*, despite known effects of these two social parameters on behaviours and physiology of this species.

Keywords: judgment bias test – social group – hierarchy - mood - cooperative breeding – early environment

# **Introduction**

The affective state of animals, i.e. their mood and emotions (Mendl and Paul, 2020), can be altered by negative experiences such as the encounter of an acute (Bateson et al, 2011; Nogueira et la, 2015) or chronic stressor (Harding et al, 2004; Verbeek et al, 2019) or by positive experiences such as rewarding situations (Keen et al, 2013; Perry et al, 2016) or environmental enrichment (Lalot et al, 2017; Matheson et al, 2008). An affective state is a multi-component response to a stimulus or an event that involves physiological and neural activity (Paul and Mendl, 2018). It has always a valence, that is either positive or negative and it can be intense or mild, long lasting or brief (Paul and Mendl, 2018). Affective states guide further physiological, behavioural, and cognitive responses to a stimulus and help animals to avoid harm or to obtain resources (Paul et al. 2005).

One frequently used approach to evaluate how stimuli modulate the valence of an affective state is the judgement bias test. This test quantifies the propensity of a subject to exhibit a certain response to an ambiguous stimulus (Boleij et al., 2012; Mendl et al., 2009). An optimistic bias, indicating a positive affective state, corresponds to a response to an ambiguous stimulus that is similar to a response to a positive stimulus. This suggests that individuals have a higher expectation of reward in the presence of ambiguous information. On the contrary, individuals with a pessimistic bias, indicating a negative affective state, show response to an ambiguous stimulus that is similar to a negative stimulus. This suggests that they have a higher expectation of punishment or lower expectation of reward in the presence of ambiguous information (Nettle and Bateson, 2015). The affective states were almost exclusively investigated after experiencing presumably negative (stressful) or presumably positive (rewarding, enrichment) stimuli. Yet, animals encounter a plethora of stimuli in their social and non-social environments every day. For most of these, the valence assigned to them and effects on their affective state is entirely unknown. In particular, it has rarely been considered how individuals value key social parameters such as group size or social rank.

Thus far, only three studies investigated the influence of social rank on the affective state using judgment bias tests. These studies did not find significant differences between ranks but reported a tendency that dominant individuals were more likely to exhibit a positive bias (chimpanzees, Bateson and Nettle, 2015; tufted capuchin monkeys, Schino et al., 2016; sows during gestation, Horback and Parsons, 2019). It suggests that in these species' dominants tended to be more optimistic. However, this interpretation cannot be generalised across species. In his review, Sapolsky (2005) showed that depending on species and the characteristics of social groups, either top-ranking dominants or low-ranking subordinates are the most stressed, which could lead to a negative affective state.

In addition to rank, also density and social environment may affect the affective state, as they can both indicate higher safety and/or increased resource competition. There is

growing evidence that the social environment experienced early in life is critical in shaping social behaviour (Sachser et al, 2013; Fischer et al, 2015, 2017), but whether early social conditions also alter affective state in the long term is unknown. However, two judgement bias studies targeted the effects of current stock density. They reported tendencies for an optimistic bias for higher stocking density both in rainbow trout (Anderson et al., 2022) and broiler chickens (Anderson et al., 2021).

Here, we asked whether in a cooperatively breeding vertebrate, the cichlid fish *Neolamprologus pulcher*, social rank and rearing group size modulate their affective state. Cooperatively breeding groups are typically structured by pronounced social hierarchies. Rank differences are accompanied by physiological and behavioural differences (mammals: Cavigelli et al., 2002; Creel et al., 1997; birds: Nelson-Flower et al., 2018; fish: Buchner et al., 2004, Bender et al. 2006, Desjardins et al., 2008). In *N. pulcher*, dominants have higher cortisol levels than subordinates (Buchner et al. 2004), and individuals showing more subordinate behaviour have lower levels of cortisol and testosterone (Bender et al., 2006). This suggests higher stress levels to be associated with a dominant rank in these fish, and accordingly we predicted that dominants are significantly more pessimistic than subordinates, regardless of their rearing group size.

In addition to the position in the hierarchy, rearing group size is also a major selective force in *N. pulcher*. Living in large groups has several benefits, including higher fry survival (Brouwer et al., 2005), higher feeding rate of group members (Balshine et al., 2001), lower breeder workloads (Balshine et al., 2001) and lower energetic investment in eggs (Taborsky et al. 2007). However, there are also costs of living in larger groups, for example by a longer queue to achieve a breeding position (Jungwirth et al. 2015) or by higher parentage loss (Hellmann et al., 2015). The composition and size of social groups experienced by *N. pulcher* early in life affects their social and life-history decisions during their entire life (Arnold & Taborsky 2010, Taborsky et al. 2012, Fischer et al. 2015, 2017, Antunes & Taborsky, 2020). Fish reared in large groups express less aggressive and more submissive behaviour towards a dominant conspecific later in life compared to fish reared in small groups (Fischer et al. 2015). This suggests that large-group reared fish are able to form clearer hierarchy relationships and thus and more stable groups, which may imply lower stress levels in these fish. Thus, we predicted that fish reared in large groups will be more optimistic relative to fish reared in small groups.

To test our predictions of how affective state is modulated by rank and rearing group size, we first developed a method to evaluate judgement bias in *N. pulcher*. Second, to study the effect of rearing group size, we compared fish that were reared in large social groups composed of a breeding pair and eight helpers and fish reared in small groups composed of a breeding pair and one helper. Third, to study the effect of rank, adult fish were experimentally assigned an either dominant or subordinate rank, after which they did a first judgement bias test. Subsequently, the fish were assigned to the opposite rank,

before doing a second judgement bias test.

#### **Methods**

#### Study species

*Neolamprologus pulcher* is an obligatory cooperative breeding cichlid endemic to the East African Lake Tanganyika (Konings 1998). In this species, a social group is composed of one breeder pair and 1 to 25 smaller, subordinate individuals (termed 'helpers'). Helpers participate in territory defence, territory maintenance and direct alloparental brood care of eggs and larvae (Taborsky and Limberger 1981). They perform these tasks at least until they reach sexual maturity (around 1 year old). Afterwards, subordinates may choose to either delay dispersal and stay as helpers with the eventual opportunity of natal territory inheritance or disperse and have a breeder position somewhere else (Stiver et al. 2006, Fischer et al., 2017). Helpers vary in relatedness, size, and sex (Taborsky, 2016). In nature, *N. pulcher* groups have a linear size-based hierarchy (Dey et al, 2013), in which same-sized group members cannot stably coexist.

#### Housing conditions

The experiments were conducted at the Ethological Station Hasli of the Institute of Ecology and Evolution, University of Bern, Switzerland, following Swiss Animal Welfare law and were approved by the Veterinary Office of the Kanton Bern, under the licence number BE93/18. Fish were obtained from the University of Bern laboratory stock population from wild caught ancestors from the Kasakalawe Point population at the southern tip of Lake Tanganyika, Zambia. The light:dark cycle (L:D of 13:11 h with 10 min of dimmed light in the mornings and evenings), water temperature (27±1 °C) and the biochemical parameters of the tank mimicked the conditions at the southern end of Lake Tanganyika. The fish were fed once a day with commercial food flakes (5 days a week) and frozen zooplankton (1 day a week), always after the experiments.

#### Experimental procedures

#### Early social experience

The experimental fish had been bred either in small or large social groups during a previous study (La Loggia et al., in prep., Fig. 1). For breeding, nine small groups consisting of one breeder pair and one small unrelated helper were kept in 130-L tanks. Ten large groups consisting of one breeder pair and eight differently sized, unrelated helpers of both sexes were kept in 270-L tanks. The groups produced broods between November 2018 and August 2019, which were reared within their family group for two months ('early social experience phase'). Afterwards, they were kept together with their siblings in 50-L tanks for about one year ('sibling tank'), after which they were individually marked with a small elastomer colour tag (Jungwirth et al. 2019) and transferred to two 200-L aggregation tanks, separated by early-life treatment (small or large group; La Loggia et al., in prep.).



Figure 1: Timeline for our experimental fish from the day the eggs hatched until the judgement bias tests.

#### Training phase

For the training phase, 22 fish (12 females and 10 males) from 9 different broods from the small-group rearing treatment and 25 fish (11 females and 14 males) from 10 different broods from the large-group rearing treatment were removed from the aggregation tanks and singly housed in a 25-L tank (40 x 25 x 25 cm; Fig. 1, Fig. 2a). All tanks were equipped with a 2-cm layer of sand, flowerpot half as shelter, and a biological filter. After one night of acclimation, the fish were trained to remove a chip of 2.5 cm diameter (made from polymer clay), which covered a dark-green plastic cup of 1.9 cm diameter (Fig. 2b). Half of the fish were trained on white chips and half on black chips, with learning colour being equally balanced across fish. For the training, a similar method as in Buechel et al. (2018) and Reyes-Contreras & Taborsky (2022) was used. The front half of the tank was separated from the back half by an opaque divider. A piece of krill was placed in the cup as reward, which was located in the front of the tank. About 2 ml of 'krill water' (water in which krill had been thawed) was added to the tank to motivate the fish to search for krill. Then, the divider was removed, and the fish could access the cup. First, the chip was put next to the cup until the fish had learned to eat the krill out of the open cup. Then 1/4 of the cup was closed, afterwards ½ of the cup and finally, the cups were fully covered. To pass the training phase, the fish had to be able to open the fully covered cup in less than 3 min three times in a row.



Figure 2: (a) Experimental set-up in the 20-L tank for the learning phase and the judgement bias test. At the front side of the tank, P1 and P5 are the positions of the positive or the negative cue, which could be positioned right or left and could be black or white chips (sides and colour were equally balanced). P2, P3 and P4 are the positions where the three ambiguous cues (AP, A, AN) were placed during the tests, using chips in different shades of grey. (b) Photo of the used chips for the five stimuli positioned on the dark green cups designed to hold a reward depending on test condition.

#### Learning phase

Nineteen fish (9 females and 10 males) from the small-group rearing treatment and 21 fish (7 females and 14 males) from the large-group rearing treatment passed the training phase (85% success) and were transferred to the learning phase (Fig. 1). In this phase, the fish had to learn to respond to the positive cue and to avoid the negative cue. The colour (black or white) fish had encountered during the training was used as the positive cue (P) in the learning phase. Chips of the opposite colour were used as negative cue (N). Positive and negative cues were always positioned in positions P1 or P5 (Fig. 2a), which were situated in the left and right corners at the front of the tank. The position (left or right) for the rewarded and unrewarded cue was equally balanced across fish.

All the fish underwent one learning session per day consisting of three trials with the positive cue and three trials with the negative cue. The cues were presented sequentially and in randomized order (on the condition that the same cue was never presented more than twice in a row). When the fish were presented with the positive cue, they could remove the chip from the cup and collect the reward. When they were presented with the negative cue and touched the cup or the chip, a coloured glass marble of 2.5 cm diameter was dropped on the cup to reinforce the negative experience. Under laboratory conditions, *N. pulcher* is very bold. Pilot trials showed that this reinforcement was necessary for *N. pulcher* to differentiate P from N. To this end, a trap door was integrated in the aquarium lid, right above the cups. The observer stood next to the negative stimulus. When the fish touched the negative cue (the chip or the cup underneath), the observer pulled a string

to release a device that held the marble in place, and the marble dropped onto the cup. To ensure that the fish could not distinguish P from N by olfaction alone, both cups were stocked with a krill piece, but during trials with the negative cue, a perforated plastic sheet covered the krill, so that the fish could smell the krill but not eat it.

Before the start of a learning trial, the front half of the tank was blocked by an opaque divider to obstruct the view of the fish during setting up the cup and chip. Afterwards, the divider was removed, and the fish had 3 min to react to the cue. In trials with a positive cue, we measured the latency to open the cup. The divider was put in place again after the fish had eaten the reward, and the next trial was started. If the fish did not touch the cup within 3 min, a maximum latency time of 180 s was recorded, and the experimenter opened the cup so that the fish could eat the reward. In trials with a negative cue, we measured the latency until the fish touched the chip or the cup. If the fish touched the cup or chip within 3 min, the marble was dropped, and the divider was put back in place immediately, and we waited until the 3 min of max trial time had elapsed. If the fish did not touch the cup, a maximum latency time of 180 s was recorded, and the next trial was started. Fish were considered to have learned the difference between the negative and positive cue when the latencies recorded in trials with positive cue were significantly shorter than the latencies in trials with negative cue. To test whether this learning criterion was reached, we combined the trials of two successive days (i.e. trials of day 1 and 2, trials of day 3 and 4, and so on) and tested the differences by Wilcoxon matched-pairs signed-ranks tests (n=6 pairs of P and N trials). The learning criterion was reached on average at 5.6 days (± 0.7 SE).

#### Assignment of rank

Fifteen fish (8 females and 7 males) from the small rearing groups and fifteen fish (4 females and 11 males) from the large rearing groups passed the learning phase (64 % success) and were assigned a rank experimentally (Fig. 2). To this end, half of the fish were placed separately into new 25-L tanks together with a 30% larger unrelated conspecific to induce a subordinate rank in the focal individuals. The other half were combined with a 30% smaller unrelated conspecific to induce a dominant rank. To induce the hierarchy, the pairs of fish stayed in the same tank for 2 days. When the focal fish was to become dominant, the smaller fish, If the focal fish was to become subordinate, we released the two fish at the same time into the tank. Because of the large size differences, fish adopted their respective ranks immediately and no notable aggression between the fish took place.

After rank assignment, all focal fish were put back singly in their original 25-L tank and had 30 min of acclimation before starting the first judgment bias test, which took three days (see next section). Afterwards, they were placed in the opposite rank-giving set-up for two further days (e.g. the fish that were first in the subordinate set-up were placed in

the dominance set-up, and vice versa) before doing the second judgement bias test, again for three days.

#### Judgement bias test

In the training and learning phases and for this test, we used self-made chips of polymer clay using five different grey shades, which were determined with the help of a standardized grey scale. White clay was defined as "0% grey"; black clay was defined as "100% grey" and they were used either as positive or negative cue; then we chose three grey shades as ambiguous cues between 0% and 100%, which represented exactly 25% (AP or AN), 50% (A) and 75% grey (AP or AN; Fig. 2b, Table S1). Fish were tested three days in a row. On each test day only one of the three ambiguous cues was presented (AP, A or AN), with the order of presentation across the three days being balanced between focal fish. On each of the three test days, six trials were always presented in the following order: N, P, AP/A/AN (test trial), P, N, and P (adapted from Laubu et al. 2017). The first two trials in this series (N and P) should serve as a reminder of the positive and negative cues the fish had learned during the 'Learning phase'. Then, as test trial, the chosen ambiguous cue was presented. The ambiguous cue AP and AN were positioned close to the position of the positive and the negative cue, respectively, so either on position P2 or P4 (Fig. 2a), depending on which position the positive or negative cue had for each fish. The intermediate ambiguous cue (A) was positioned exactly in the middle (position P3, Fig. 2a). After the test trial, a P and an N trial were done as another reminder, followed by a final P trial, which should serve to maintain the motivation of the fish to participate in the trials the next day. The ambiguous cue (test trials: AP, A or AN) were always unrewarded, which means that the fish were able to open the cup, but they could not reach the krill, because it was covered by a perforated plastic sheet. We measured the latency to touch the cup or the chip of the ambiguous stimulus with a stopwatch. The fish had a maximum of 3 min to react to the cue. We also video-recorded all trials on the test days and measured the latencies with their help in cases when the response of the fish during the live-recording was not clear.

#### Statistical analyses

Statistical analyses were done with R, 4.0.3 (2020-10-10) (R Core Team, 2019). We fitted Linear Mixed-effects Models (LMM) by using the *lmer* function of the R package *lme4* and obtaining the p-values from *lmerTest* (Kuznetsova et al., 2017) to compare latencies to touch the cup in the learning and judgment bias tests. For all LMMs, we checked for normal distribution of model residuals by normal probability plots, and we checked for homogeneity of variances by plotting the fitted values versus the model residuals (Faraway, 2006). We used backwards stepwise model selection first of the interactions and then the main factors by always removing the term with the highest p-value and choosing the model with the lower Akaike's an Information Criterion (AIC) value by using the *AIC* function of the R package *stats* (Sakamoto et al., 1986). Tables show analyses of

variance with Satterthwaite's method. Following an LMM, we did a contrast analysis of the Estimated Marginal Means (EMMs) for each significant p-value. We used the *emmeans* function of the R package *emmeans* (Lenth, 2020) and p-values were adjusted with the Bonferroni method.

To compare the latencies to respond to the stimuli during the learning phase, the mean latencies to touch the cup for the positive and negative stimulus were calculated and were log-transformed to achieve normality of the residuals. In the initial model, the log mean latency to touch the cup was the response variable and stimulus (P or N), rearing group size (large groups vs. small groups), number of learning day, fish age, sex, and colour, position of the positive stimulus, the interaction between stimulus and number of learning day and the interaction between stimulus and rearing group size were included as fixed factors. The two experimental factors 'stimulus' and 'rearing group size' were by default always retained in the model. Only the variables stimulus, rearing group size, number of learning day, the interaction between stimulus and number of learning day and the interaction between stimulus and rearing group size were retained in the final LMM. Then, for the large rearing group, one LMM was performed to compare the latencies for the positive stimulus across the successive learning sessions and one for the negative stimulus. Two same LMMs were done for the small rearing group. Fish identity was included as random term, since we did repeated measures for each fish and the number of learning days was included as fixed factor for all four LMMs.

For the judgment bias test, we compared log-transformed latencies to touch the cup for each stimulus (P, AP, A, AN, N) on the three test days (latencies for P and N trials were averaged over the three test days). We fitted an LMM with stimulus (P, AP, A, AN, N), rearing group size (large rearing group vs. small rearing group), rank (dominant vs. subordinate), order of the rank assignment, age, sex, colour, position of the positive stimulus as well as the interactions between stimulus and rank, between stimulus and rearing group size and between stimulus, rank, and rearing group size as fixed factors in the initial, full model. Fish identity was included as random term. Model simplification was carried out using the same method as described above for the learning model. Only the terms stimulus, rearing group size, assigned rank and the interaction between stimulus and rearing group size were retained in the final LMM.

Finally, we fitted another LMM with the A-stimulus only and the same fixed factors as described in the model of the judgement bias test with all stimuli. Fish identity was included as random term. Model simplification was carried out using the same method as described above for the learning model. Only the terms rearing group size, rank, age, and colour of the positive stimulus were retained in the final LMM.

#### <u>Results</u>

#### Learning phase

The fish showed significant differences between the latencies to touch the negative and positive stimuli (Fig. 3, Table 1; results of initial, full model see Table S2). There was a significant interaction between stimulus and number of learning day (Table 1). For each learning day, the latencies were significantly longer for the negative stimulus (Table 2). There was also a significant interaction between stimulus and rearing group size (Table 1). For each rearing group size, the latencies were significantly longer for the negative stimulus (Table 3). For fish from both rearing group sizes, the latency towards the positive stimulus significantly decreased with number of learning day (Table 1, Fig. 3; large groups: LMM:  $F_{220,5}$ = 17.65, p < 0.001; small groups: LMM:  $F_{232,3}$ = 8.71.51, p < 0.001) but remained similar for the negative stimulus across successive learning days (Fig. 3; large groups: LMM:  $F_{223}$ = 1.39, p = 0.23; small groups: LMM:  $F_{233,5}$ = 0.54, p = 0.74).

Figure 3: Latencies during the learning phase for the positive and negative stimulus later used in the judgment bias test. The fish were individually trained to discriminate between the positive and negative stimulus. The latencies to touch the cup were measured by a stopwatch and log transformed for analysis. The latency to touch the cup was significantly affected by the interactions between the stimulus (positive or negative) and the number of learning sessions and between the stimulus and the rearing group size (Table 1). For both groups, the latency significantly decreased over time for the positive stimulus and remained constant for the negative stimulus (see Table 1 and 'Results'). Means and standard errors are shown.



Table 1: Results of the final linear mixed-effects model to assess the latency to respond to the positive and negative stimulus during the learning phase. (Results of initial, full model see Table S2). Significant effects are set in bold.

Factors	NumDF	DenDF	F-value	p-value
Stimulus	1	943.11	1053.04	< 0.001
Rearing group size	1	27.09	0.405	0.53
Colour of the positive stimulus	1	27.08	4.131	0.052
Number of learning day	5	945.38	12.419	< 0.001
Stimulus x Number of learning day	5	943.11	12.949	< 0.001
Stimulus x Rearing group size	1	943.11	6.452	0.011

Table 2: Pairwise post-hoc comparisons between the different Estimated Marginal Means (EMMs) of the linear mixed effect model analysing the latencies to touch the different stimuli (positive and negative) during the different learning days. P-values are Bonferroni corrected. Significant effects are set in bold.

Day	Contrast	Estimate	SE	Df	t ratio	p-value
1	Negative - Positive	0.881	0.108	943	8.122	< 0.001
2	Negative - Positive	1.27	0.11	943	11.517	< 0.001
3	Negative - Positive	1.498	0.108	943	13.816	< 0.001
4	Negative - Positive	1.689	0.112	943	15.132	< 0.001
5	Negative - Positive	1.738	0.109	943	15.978	< 0.001
6	Negative - Positive	2.149	0.144	943	14.894	< 0.001

#### Judgment bias test

There were no significant effects of the rank and the interaction between stimulus and rearing group size on the latencies to touch the cup when analysing all stimuli in the same model (P, AP, A, AN, N) (Table 4). There were also no significant effects of the rearing group size and the rank on the latency to touch the cup when analysing only the responses to the ambiguous A-stimulus (Table 5, Fig. 4), although there was a non-significant trend indicating that fish raised in small groups tended to show a lower latency to touch the A stimulus compared to fish from fish raised in large groups (p=0.061, Table 6, see also Fig. 4). Surprisingly, the latencies to touch the A-stimulus were significantly different according to its colour. The latencies were significantly longer for the black stimulus (Pairwise posthoc comparison, estimate= $0.675\pm0.272$  SE, df=26, t-ratio=2.483, p=0.02).

#### Validation of the judgment bias test

The latencies to touch the P and the N stimulus were not affected by rearing group size

(large vs. small rearing groups, Fig. 4) and rank (dominant vs. subordinate, Fig. 5). The latencies to touch the cup were significantly different for each stimulus, except between P and AP and between N and AN (Table 6). This shows that the A-stimulus in our experiment was perceived differently from the P and the N stimulus, while P vs AP and N vs AN could not be clearly distinguished by the fish.

Table 3: Pairwise post-hoc comparisons between the different Estimated Marginal Means (EMMs) of the linear mixed effect model analysing the latencies to touch the different stimuli (positive and negative) for the large and small rearing group size during the learning phase. P-values are Bonferroni corrected. Significant effects are set in bold.

Rearing group size	Contrast	Estimate	SE	Df	t ratio	p-value
Large	Negative - Positive	1.66	0.067	943	24.545	< 0.001
Small	Negative - Positive	1.42	0.065	943	21.766	< 0.001

Table 4: Results of the final linear mixed-effects model to assess the effects of rank assignment (dominant vs. subordinate), rearing group size (large groups vs. small groups) and the interaction between stimulus and rearing group size on the latencies to touch any of the stimuli P, AP, A, AN, or N in the judgement bias tests. (Results of initial, full model see Table S3). Significant effects are set in bold.

Factors	NumDF	DenDF	F-value	p-value
Stimulus	4	261	180.386	< 0.001
Rank assignment	1	261	2.542	0.112
Rearing group size	1	28	0.203	0.656
Stimulus x Rearing group size	4	261	1.867	0.117



Figure 4: Evaluation of individuals' affective state based on the judgement bias test. The latency to touch the cup associated with the positive, ambiguous (AP, A, AN) and negative stimulus were measured, and log transformed in fish reared in large groups (N=15) and in small groups (N=15). Means and standard errors are shown.

Table 5: Results of the final linear mixed-effects model to assess the effects of rank assignment (dominant vs. subordinate), rearing group size (large groups vs. small groups), age and colour of the positive stimulus (black vs. white) on the latencies to touch the intermediary, ambiguous A-stimulus in the judgement bias tests. (Results of initial, full model see Table S4). Significant effects are set in bold.

Factors	NumDF	DenDF	F-value	p-value
Rank assignment	1	29	0.01	0.921
Rearing group size	1	26	3.831	0.061
Age	1	26	3.904	0.059
Colour of the positive stimulus	1	26	6.165	0.02

Table 6: Pairwise post-hoc comparisons between the different Estimated Marginal Means (EMMs) of the linear mixed effect model analysing the latencies to touch the different stimuli (P, AP, A, AN, N) during judgement bias tests. P-values are Bonferroni corrected. Significant effects are set in bold. All pairwise responses towards the stimuli were significantly different except the ones towards the P and AP and the N the AN stimuli.

Contrast	Estimate	SE	Df	t ratio	p-value
P - AP	-0.14	0.117	241	-1.192	1
P - A	-1.145	0.117	241	-9.763	< 0.0001
P - AN	-2.25	0.117	241	-19.191	< 0.0001
P - N	-2.528	0.117	241	-21.566	< 0.0001
AP - A	-1.005	0.117	241	-8.571	< 0.0001
AP - AN	-2.11	0.117	241	-17.999	< 0.0001
AP - N	-2.389	0.117	241	-20.374	< 0.0001
A - AN	-1.105	0.117	241	-9.428	< 0.0001
A - N	-1.384	0.117	241	-11.803	< 0.0001
AN - N	-0.278	0.117	241	-2.375	0.18



Figure 5: Evaluation of individuals' affective state based on the judgement bias test. The latency to touch the cup associated with the positive, ambiguous (AP, A, AN) and negative stimulus were measured, and log transformed for analysis. N=30 fish were assigned a dominant rank or a subordinate rank, in balanced order. Means and standard errors are shown.

## **Discussion**

We developed a judgment bias test for the cichlid *N. pulcher* to test whether rearing group size and social rank influences their affective state. We predicted that (1) dominants are significantly more pessimistic than subordinates, regardless of the rearing group size, and (2) fish reared in large groups are significantly more optimistic than fish reared in small groups. We showed that fish learned to discriminate between the positive and negative stimulus by adjusting their response latency appropriately. The latency to touch the cup for the ambiguous stimulus significantly differed from those for the positive and negative stimulus and was intermediate between these two stimuli. However, there were no significant effects of rearing group size and the current social rank on the affective state of *N. pulcher*. Yet, there was a statistically non-significant trend (p=0.061) for fish reared in small groups to have a shorter latency to touch the cup for the ambiguous stimulus than fish reared in large groups.

We showed that the judgment bias test was suitable for assessing affective state in N. pulcher. The preconditions for validating the cognitive bias are that between the different treatments, the responses to the unambiguous stimuli do not change significantly (Mendl et al., 2009), and that the response to the ambiguous (A) stimulus significantly differ from those to the positive and negative stimulus. Both preconditions held in our study (see Fig. 4). Thus, we showed that the judgment bias test could be used successfully in the fish *N. pulcher*, as it has been used previously in the cichlid *Amatitlania siquia* in a mate choice context (Laubu et al., 2017), and in zebrafish (Espigares et al., 2021) and rainbow trout (Anderson et al., 2021) in a food choice context. However, we had to adapt the design of the test to our study species. Preventing the access to the food reward alone was not sufficient for in *N. pulcher* to learn the negative stimulus; therefore, we reinforced the negative stimulus by punishment. We developed a novel method, applying a mild stressor as additional negative reinforcement by dropping a glass marble on the food cup when the negative stimulus was chosen. This method was less invasive than the negative reinforcement used in Anderson et al. (2021) and Espigares et al. (2021), who chased fish with a net, whereas Laubu et al. (2017) did not use any additional negative reinforcement.

We investigated whether rearing group size modulates the general affective state of *N. pulcher*. We had predicted a change of affective state, as in this species the early social environment changes stress axis programming (Taborsky et al., 2013; Nyman et al., 2017, 2018; Antunes et al., 2021a) and influences the expression social behaviour and social competence until adulthood (Arnold & Taborsky, 2010; Taborsky. et al., 2012; Fischer et al., 2015, 2017; Nyman et al., 2017). Unexpectedly, rearing group size did not affect the outcome of the judgement bias test, irrespective of rank. Both rearing group sizes used in this study exist in natural conditions. In nature, fish growing up in small groups experience higher predation risk and mortality compared to fish reared in large groups (Brouwer

et al., 2005), which is why we expected a higher pessimistic bias in small-group reared fish. One possible reason for this lack of difference is that fish reared in small groups, on the one hand, perceive their environment as more risky (Brouwer et al., 2005), but on the other hand they are exposed to less intraspecific local competition compared to fish growing up in large groups (Taborsky, 2021). Alternatively, the difference in affective state may have been mitigated by the time spent in sibling and aggregation tanks (1 year and 3 months), where the conditions were the same for small and large groups.

Finally, early-life social experiences alter the constitutive brain gene expression of glucocorticoid receptors (Nyman et al., 2017, Antunes et al., 2021b) and of basal cortisol levels (Antunes et al., 2021a), but not stress-induced cortisol levels in *N. pulcher* (Antunes et al., 2021a). These findings may help to explain the absence of effects of rearing group size on the affective state, despite differences in social phenotype between fish reared in large and small groups (La Loggia et al., in revision). Possibly, the affective state can be modulated by stress-induced cortisol levels, but not by basal levels and constitutive receptor expression, which should be tested in future judgement bias tests.

Next to early social experience, we tested the effects of current social rank on the affective state using a judgment bias test. Opposite to our prediction, there were no significant differences between dominants and subordinates, irrespective of their rearing group size. This suggests that the position in a social hierarchy does not influence affective state in *N*. *pulcher*. Alternatively, some aspects of the experimental procedure may have constrained the effects on affective state. In a previous study using the same rank-assignment method as here, it was shown that this method works to establish a hierarchy between individuals (Lerena et al., 2021). Three studies reporting tendencies of dominants to exhibit a positive bias in other species (Bateson and Nettle, 2015; Horback and Parsons, 2019; Schino et al., 2016) used individuals with known rank form existing social group. Instead, in our study, we experimentally induced hierarchies between previously unfamiliar individuals. In addition, the duration of inducing a dominant or subordinate rank in our set-up may not have been long enough to significantly impact the affective state. Cheng et al. (2020) used a crossover rank-and-re-rank design in domestic pigs where piglets formed social units for two weeks and found changes of the dopaminergic system correlating with rank changes.

#### **Conclusions**

To conclude, our results validated that the judgment bias test was suitable for assessing affective state in *N. pulcher*: fish learned to discriminate between the positive and negative stimulus, the responses to these unambiguous stimuli did not differ between treatments, and the latency for the ambiguous stimulus differed from those for the positive and negative stimulus, respectively. Although we report a trend that fish reared in small groups had a more optimistic bias, we lack evidence that the rearing group-size and the current social

rank significantly modulate the affective state of this species. More generally, judgement bias tests have now been successfully used in several fish species and have been suggested to be used when improving welfare and evaluating stressors fish may encounter when used in scientific research, commercial pet trade and fisheries industry (Huntingford et al., 2006; Saxby et al., 2010).

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# **Supplementary Material**

Table S1: Production of the chip: Black and white chips were made out of "black" and "white" FIMO (STAEDTLER Mars GmbH & Co. KG), which is a polymer clay. Note that the three different greys used in the test increase linearly in brightness. The intermediate grey (grey 2) was exactly in the middle between black and white and the other two shades of grey were exactly between the intermediate grey (grey 2) and black respectively white, according to the standardized grey-scale used (see 'Methods'). The different amounts of black and white FIMO were mixed to produce the different shades of grey, and they were compared to a grey scale to equal 0%, 25%, 50%, 75% and 100% grey.

Chip	Black "FIMO" (%)	White "FIMO" (%)
White	0	100
Grey 1	5	95
Grey 2	20	80
Grey 3	50	50
Black	100	0

Table S2: Results of the initial linear mixed-effects model to assess the effects of sex, age, position and colour of the positive stimulus, the interaction between stimulus and number of learning day and the interaction between rearing group size and number of learning day on the latencies to touch the stimuli (positive or negative) during the learning phase. Significant effects are set in bold.

Factors	NumDF	DenDF	F-value	p-value
Stimulus	1	938.07	1049.413	< 0.001
Rearing group size	1	22.23	0.159	0.694
Sex	1	22.06	0.48	0.496
Age	1	22.17	1.564	0.224
Position of the positive stimulus	3	22.26	0.911	0.451
Colour of the positive stimulus	1	22.05	4.312	0.05
Number of learning day	5	939.84	12.382	< 0.001
Stimulus x Number of learning day	5	938.07	12.903	< 0.001
Rearing group size x Number of learning day	5	940.05	0.374	0.867
Stimulus x Rearing group size	1	938.07	6.427	0.011

Table S3: Result of the initial linear mixed-effects model to assess the effects of sex, age, position (left vs. right) and colour (black vs. white) of the positive stimulus, rank assignment order (dominant or subordinate first) and the interaction between stimulus, rank assignment and rearing group size on the latencies to touch all the stimuli (P, AP, A, AN, N) in the judgement bias tests. Significant effects are set in bold.

Factors	NumDF	DenDF	F-value	p-value
Stimulus	4	251	177.305	< 0.001
Rank assignment	1	251	2.585	0.109
Rearing group size	1	22	0.002	0.969
Sex	1	22	0.996	0.329
Age	1	22	0.067	0.8
Position of the positive stimulus	3	22	0.654	0.589
Colour of the positive stimulus	1	22	0.001	0.978
Rank assignment order	1	251	0.213	0.645
Stimulus x Rank assignment	4	251	0.303	0.876
Stimulus x Rearing group size	4	251	1.835	0.123
Rank assignment x Rearing group size	1	251	3.154	0.077
Stimulus x Rank assignment x Rearing group size	4	251	0.241	0.915

Table S4: Results of the initial linear mixed-effects model to assess the effects of rank assignment (dominant vs. subordinate), rearing group size (large groups vs. small groups), sex, age, position (left vs. right) and colour (black vs. white) of the positive stimulus, rank assignment order (dominant or subordinate first) and the interaction between rank assignment and rearing group size on the latencies to touch the intermediary ambiguous A-stimulus in the judgement bias tests. Significant effects are set in bold.

Factors	NumDF	DenDF	F-value	p-value
Rank assignment	1	27	0.024	0.878
Rearing group size	1	22	1.219	0.282
Sex	1	22	0.866	0.362
Age	1	22	3.303	0.083
Position of the positive stimulus	3	22	0.285	0.836
Colour of the positive stimulus	1	22	5.704	0.026
Rank assignment order	1	27	0.717	0.405
Rank assignment x Rearing group size	1	27	0.478	0.495