Mechanisms and behavioural consequences of egg-mediated maternal effects

Inaugural dissertation
of the Faculty of Science,
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presented by

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from Venezuela

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General introduction

Solitary animals form aggregations when the benefits outweigh the cost (Korb & Heinze, 2016). In aggregation, each individual increases its chances to gain fitness benefits, such as survival and reproduction. For example, animals that forage in groups increase their survival probability because some group members are vigilant while others forage ("many-eyes-effect"), or the number of individuals in a group decreases the risk of mortality of single individuals by dilution and confusion effects (Roberts, 1996). Similarly, the gathering of sexually mature conspecifics, such as fish, guaranty individual reproduction in mating systems consisting of aggregations (Domeier & Colin, 1997). Hence, these kinds of fitness benefits are the starting point for the formation of animal societies (Korb & Heinze, 2016).

One of the major transitions in evolution is the origin of societies (Maynard Smith & Eörs, 1995). Animal societies evolved several times independently in invertebrates and vertebrates (Maynard Smith & Eörs, 1995). Among the traits that characterise animal societies, I focus on one characteristic which constitutes the framework of this work, namely group size (Kappeler et al., 2019).

Group size is a trait that has been fundamental for the description of vertebrate animal societies (Kappeler, 2019). Group size is influenced by predation risk and resource availability, and these two factors are the selective force that drives the evolution of animal societies (Pollard & Blumstein, 2008). Group size is one important component of animal societies because it influences group cohesion and composition and allows the formation of social structures (e.g., dominance hierarchies and social bond). Those social structures are mediated by social interactions (Kappeler, 2019). In this work, I focus on the proximate mechanism underpinning the influences of group size on social behaviours (e.g., helping and submissive behaviour) that maintain the structure of one type of animal society which is the cooperative breeding species *Neolamprologus pulcher*.

The size of a group determines the chance of survival of all group members. Large groups increase the survival of adults in suricates (*Suricata suricatta*) (Clutton-Brock et al., 1999) and dependent young of the cichlid fish *N. pulcher* (Heg et al., 2005). Nevertheless, a large group size does not always provide fitness benefits. For example, adult survival of the

African wild dogs (*Lycaon pictus*) decreases with group size (Creel & Creel, 2015). Hence, an optimal group size is determined by the synergetic effect of selective pressures (Pollard & Blumstein, 2008) and the dynamics within a group, such as social interactions among group members (Aureli & Schino, 2019).

Group size determines the number of social interactions between group members and the number of dyads that can be formed (Kappeler, 2019). Among the social interactions between group members, aggressive and affiliative interactions have been widely studied in vertebrate animal societies (Lehmann et al., 2007; Maldonado-Chaparro et al., 2015; Turner et al., 2018). Dyadic interactions, such as social bonding and dominance, provide the formation of social structures that characterize some animal societies (Kappeler, 2019). Social structures, such as hierarchies, are characterized by agonistic dyads, in which one individual exhibits aggressive behaviour as, for example, punishment and threats (Tibbetts et al., 2022), and the other either response with the same strategy or can show submissive behaviour to surrender (Reddon et al., 2021). Importantly, the ability to choose the type of behavioural display in a dyadic interaction, this means being behaviourally flexible (i.e., social competence (Taborsky & Oliveira, 2012)) should be a capability of the interacting partners. Behavioural flexibility in such a context helps to establish the hierarchy faster and therefore decrease the energy expenditure. The energetic cost associated with agonistic interactions (Briffa & Sneddon, 2007) allows the maintenance of hierarchies over time, this is a fundamental aspect to maintain group cohesion and to resolve conflicts between group members in some animal society such as cooperative breeders.

Cooperative breeders are an example of an animal society that contains all the components of a social system proposed by Kappeler 2019. Cooperative breeders have evolved independently several times across the tree of life due to environmental constrains, such as limited breeding territories (Koenig et al., 1992), predation pressure (Groenewoud et al., 2016) and environmental variability (Rubenstein & Lovette, 2007). Cooperative breeder groups consist of a dominant breeding pair and several kin and non-kin subordinate individuals that jointly cooperate to raise young of the dominant breeding pair (Koenig et al., 1992). The number of individuals inside a group is variable. It ranges from seventeen individuals in the cooperative breeder African wild dog (Gusset & Macdonald, 2010) up to more than fifty individuals in the plural cooperative breeder super starling (*Lamprotornis superbus*) (Guindre-Parker & Rubenstein, 2020). The social structure of the group is maintained through individual

social interactions, linear hierarchies, cooperation and division of labour (Lukas & Clutton-Brock, 2018). And this kind of complex animal society is stable in several animal taxa because the associated fitness benefits outweigh the cost of maintaining this type of social groups.

In a social group, dominants and subordinates are protected against predators due to the joint territory defence by all group members (Groenewoud et al., 2016) and partly also by the many eyes effect and group dilution (Fischer & Frommen, 2018). Dominants increase the survival of their current offspring by recruiting subordinates that defend the brood (Teunissen et al., 2019) and provide for the dependent young (Koenig & Walters, 2012). Furthermore, they may save energy for a future reproductive event by decreasing their workload on energetically costly tasks, such as foraging or territory defence (Tanaka et al., 2018). Subordinates gain direct fitness benefits if they inherit the breeding territory (Koenig et al., 1992) and indirect fitness benefits by raising their kin (Hamilton, 1964). Offspring raised inside a cooperative breeder group gain a higher chance to survive until reaching sexual maturity and they can either invest energy on helping to raise a new cohort of siblings or not. Then, sexually mature offspring can either remain philopatric and queue for the breeding position or disperse to another group (Koenig, 2017). However, the common interest of increased survival chances between dominants, subordinates and offspring, in a cooperative breeder group, does not prevent intra-group conflicts (Komdeur, 2006).

Conflicts of interest between members of a cooperative breeder group arise because there is competition and the fitness benefits are not always aligned between dominants, subordinates and dependent young (Trivers, 1974). The prevention of those conflicts to cooperate for the good of the whole group is a major transition in evolution, and cooperative breeders have an ideal social system to study the mechanisms underlying this type of conflict and their resolution (Taborsky et al., 2021). Asymmetries in the fitness interests of parents and offspring can result in a parent-offspring conflict. Dominant breeders value the survival of all current and future offspring (Kuijper & Johnstone, 2018), while offspring value their own survival (Kuijper & Johnstone, 2018) more than its siblings' survival, and with time the benefits of being a subordinate group member devaluate because they are more likely to survive and reproduce outside the natal group (Trivers, 1974). In addition, parents and offspring may disagree on the resource allocation on offspring production and duration of parental investment (Trivers, 1974). They can even disagree on offspring dispersal time from the natal territory when dominants need help to raise a new brood. Hence, parents might have evolved means to

influence the life trajectories of their offspring, whereas offspring might have evolved mechanisms to resist parental programming (Kuijper & Johnstone, 2018).

During offspring production, parents have first-hand access to information about the environment; however, this information is not necessarily available to the developing offspring. Furthermore, parents can affect the phenotype of their offspring regardless of offspring genotype (Danchin et al., 2011). For example, parents can assess environmental conditions to predict future conditions where offspring will live (Uller, 2008), and the environment where parents live can influence offspring phenotype (Day & Bonduriansky, 2011). Similarly, offspring can obtain cues from physical conditions of parents as proxy for the environmental conditions where they will live (Gluckman et al., 2005). Hence, parental effects on offspring and offspring responses (or lack of response) to those effects are strategies that can be studied in cooperative breeder animal societies because fitness optima for parents and offspring may not be always aligned.

Parental effects are a phenomenon defined as the effects of parental phenotype and environment on offspring phenotype that cannot be explained by inherited genes because it is a form of plasticity that spans over generations (Danchin et al., 2011; Uller, 2008). Fathers and mothers can influence offspring phenotype, but in this work, I focus on maternal effects, which are widespread across animal taxa (Marshall & Uller, 2007) and play an important role in evolutionary processes such as in life-history evolution (Wolf & Wade, 2009).

Non-genetic maternal effects are defined as the causal influence of maternal phenotype or genotype or maternal environment on offspring phenotype, and this effect is not mediated by genetic mechanisms (Kuijper & Johnstone, 2018; Wolf & Wade, 2009). Maternal effects can be adaptive for offspring, for mothers, or both. They can be adaptive for offspring when maternal effects provide information enabling offspring to adjust their phenotype to the predicted conditions encountered after birth (Kuijper & Johnstone, 2018); if this enhances maternal reproductive success, in this case maternal and offspring fitness optima are aligned. They may also be adaptive only for mothers, at the cost for offspring fitness, if they allow mothers to reduce the investment per single offspring and to have either more offspring in the current brood or higher survival and reproductive success in the future. The latter maternal effects may result in offspring evolving resistance to maternal effects (Kuijper & Johnstone, 2018). The mechanisms underlying adaptive maternal effects are important to elucidate the

potential conflict between mothers and offspring in cooperative breeder groups on aspects, such as dispersal from the natal nest and helping to raise mothers' current offspring. Hence, in this work, I investigate the mechanisms and functions of maternal effects which may lead to behavioural specialization in offspring of the cooperatively breeding cichlid fish *N. pulcher*.

Female reproductive life history traits, such as reproductive effort, offspring and egg and clutch size can be adjusted in response to environmental cues (Baker et al., 2015). In oviparous species, the energetic cost associated with the pre-natal production of offspring is certainly higher for females than for males because oocyte production and growth comprise most of the maternal investment (Brooks et al., 1997). For example, the metabolic cost of the female lizards *Sceloporus undulatus* increases 122% when they are gravid (Angilletta & Sears, 2000). In laying barn swallows (*Hirundo rustica*), the daily energy expenditure during egg production increases the basal metabolic rate by 370% (Monaghan & Nager, 1997). Hence, it is expected that females will fine-tune the allocation of resources in each reproductive event according to different internal and external parameters and future reproductive events. Then, female reproductive life history traits and resource allocation strategies could serve as a mechanism for egg-mediated maternal effects.

Egg-mediated maternal effects have been described in insects (Russell & Lummaa, 2009), birds (Groothuis & Schwabl, 2008), amphibians, (Pakkasmaa et al., 2003), reptiles (Uller et al., 2007) and fish (Adrian-Kalchhauser et al., 2018). Maternal effects occur when an egg composition is adjusted by the mother and this adjustment shapes the offspring phenotype. For example, egg size can be a proxy for the quantity of proteins and nutrients present in the egg (Sharda et al., 2021) and in birds it has been positively correlated with offspring lifetime fitness (Krist, 2011). Then, mothers can provide a different amount of nutrients to individual eggs (Bernardo, 1996) and produce larger eggs, which is often correlated with offspring size after hatching (Segers & Taborsky, 2011). Large offspring are more mobile (Schürch & Taborsky, 2005) and more likely to survive early life stages (Kamler, 2005; Williams, 1994) when predators prey on small individuals (Sogard, 1997). Furthermore, unsaturated fatty acids are known to enhance scape response in the red drum (*Sciaenops ocellatus*) fish larvae (Fuiman & Ojanguren, 2011). As an alternative to nutrients allocation, mothers may use hormones to shape different traits of offspring phenotype, including behaviour later in life (Groothuis & Schwabl, 2008; Hsu et al., 2016).

During oocyte production, hormones in the female circulation system have a dual function. They promote oocyte production (Davies & Ryan, 1972; Groothuis & Schwabl, 2008) and embryonic development after fertilization (Brown et al., 2014). Then, hormonal allocation into the oocytes is an important mechanism that could shape offspring phenotype (Groothuis et al., 2005) because hormones have long-term organizational effects during embryonic development (Brooks et al., 1997; Mouton & Duckworth, 2021; Seckl, 2001; von Engelhardt & Groothuis, 2011). In addition, the maternal environment during oocyte production is known to influence the hormonal state of the mother which in turn influences offspring growth, gene expression, and behaviour (Champagne, 2020; Groothuis et al., 2005; von Engelhardt & Groothuis, 2011; Welberg & Seckl, 2001). The social environment is known to shape morphology (Linksvayer & Wade, 2005), physiology (Russell & Lummaa, 2009) and behaviour (Raulo & Dantzer, 2018). In consequence, the social environment of social species, such as cooperative breeders, is an influential context for maternal effects (Russell & Lummaa, 2009) and early life social experiences for offspring (Branchi et al., 2006; Fischer et al., 2017). Both maternal effects and early life social experiences can shape the social behaviour of young individuals. Hence, to understand how maternal effects and the offspring's own experiences interact to modify behaviour, it is important to study the mechanisms underlying maternal effects and early life experiences. In this work, I focus on the hypothalamic-pituitary-adrenal/interrenal axis as a candidate mechanism that can be shaped by maternal effects and early life social experiences (Nesan & Vijayan, 2013; Welberg et al., 2001).

The hypothalamic-pituitary-interrenal axis is the fish homolog to the mammalian hypothalamic-pituitary-adrenal axis (Mommsen et al., 1999). This axis, also called the stress axis, allows animals to deal with stressors. Stressors are defined as noxious or unpredictable stimuli that cause physiological, hormonal, and behavioural changes in an organism (Romero, 2004). After the exposure to stressors, vertebrates cope by mounting a stress response, which is a highly conserved response across vertebrate taxa (Romero, 2004). The stress response has been defined as the activation of coordinated neurophysiological responses in the brain and periphery to overcome stressors (Taborsky et al., 2021)

The neurophysiological response to stressors starts with the release of catecholamines by the sympathetic nervous system followed by the hypothalamic release of corticotropin releasing factor (CRF), which leads to a signalling cascade to promote the release of glucocorticoids (GCs) by the interrenal tissue (i.e., fish homolog to the adrenal gland)

(Sapolsky et al., 2000). The main GC in mammals and fish is cortisol, whereas in birds and rodents it is corticosterone (Romero, 2004). The effect of GCs depends on the type of receptor they bind to and the receptor numbers (Romero, 2004). They can bind to either mineralocorticoid receptors (MRs) or glucocorticoid receptors (GRs), while the GRs are responsible for the negative feedback loop that terminates stress response by suppressing the further release of GCs by the interrenal tissue (O'Regan et al., 2001). This conserved physiological mechanism in vertebrates (Romero, 2004) is the focus of this work for two reasons.

First, it is a key candidate mechanism that modulates cognitive processes, environmental adaptation and behaviour. Learning is a process known to be integrated in brain areas such as the hippocampus, amygdala and prefrontal cortex, and those areas are enriched on GRs (Finsterwald & Alberini, 2014). GCs concentrations can either facilitate or inhibit memory formation in humans and rodents (Finsterwald & Alberini, 2014; Sapolsky et al., 2000). Adaptation to novel environments can offer survival benefits to individuals, and the stress axis is a physiological mechanism that allows animals to adapt to changing environments. One important type of environment is the social environment in cooperative breeder groups because individuals must be flexible and adjust their behaviour in social interactions (i.e., behavioural competence (Taborsky et al., 2012; Taborsky & Oliveira, 2012)) to maintain their membership inside the group. Behaviour is a trait that integrates brain process, neurophysiological status and environmental inputs such as social interactions (Tinbergen, 1963). Social and non-social behaviour is modulated by GCs, GRs and MRs. For example, GCs are known to increase locomotion activity in rodents (Falkenstein et al., 2000). Furthermore, GC levels seem to positively influence cooperative behaviours among group members (Soares et al., 2010). Hence, stress axis can modulate cognitive processes involved in behavioural flexibility, which is indispensable to be able to navigate in changing social and non-social environments.

Second, the stress axis can be shaped by maternal effects and early life social experiences. For example, laboratory rat mothers that provide high licking and grooming behaviour to their pups, enhance GC negative feedback sensitivity of pups' stress axis (Meaney & Szyf, 2005). In the cooperative breeder marmoset (*Callithrix geoffroyi*), early life rejection by adult members leads to a higher urinary cortisol release during a social separation later in

life (Birnie et al., 2013). Hence, stress axis programming either by maternal effects or early life experiences in a social group could lead to the modulation behaviour.

Social interactions could modulate behaviour. In cooperative breeder groups, social interactions maintain social structure because they create hierarchies and promote helping behaviours by subordinate individuals. In most cooperative breeder groups, helping behaviour is raised as a consequence of delayed dispersal (Koenig, 1981; Wild & Korb, 2017). Hence, the decision whether to remain philopatric and to help dominants to raise siblings or to disperse the natal territory is a life-history decision, faced once individuals are big enough to reproduce. This life-history decision depends on environmental cues (e.g., availability of territories) and the individual behavioural phenotype (Wey et al., 2015). Individual behavioural phenotype in the cooperative breeder *N. pulcher* is shaped by the composition of the social group (Fischer et al., 2017), social experience (Arnold & Taborsky, 2010; Taborsky et al., 2012) and stress axis programming (Nyman et al., 2017; Reyes-Contreras et al., 2019). Laboratory evidence shows that *N. pulcher* specialize in two alternative behavioural phenotypes. The first one is a philopatric individual with a high frequency of showing submissive behaviour (Fischer et al., 2017), which is an honest signal that appeases dominant breeders (Bergmüller & Taborsky, 2005; Reddon et al., 2021). The second is an early dispersal that demonstrates a higher frequency of alloparental care in the form of cleaning and defending the eggs of the dominant breeders, which appeases and increases dominant breeders' fitness (Fischer et al., 2017; Kasper et al., 2018). Then, it is sensible to ask if in natural populations of *N.pulcher* such behavioural phenotypes exist and if they are a function of group size.

This work aims to investigate if stress axis programming during early life is a mechanism underlaying social behavioural flexibility in the cooperative breeder *N. pulcher*, which is a behavioural trait required to maintain group structure and to gain fitness benefits. A further aim is to investigate if the composition of the social environment (i.e., number of group members) is a key factor that offers mothers the opportunity to implement maternal effects to adjust offspring phenotype, for example by shaping offspring stress axis, and/or to provide juveniles an opportunity to adjust their phenotype according to their own experiences.

Chapters overview

Chapter 1. *"Stress axis programming generates long-term effects on cognitive abilities in a cooperative breeder".*

This chapter has been published in Proceedings of the Royal Society B.

In this chapter I investigate if social and non-social behavioural flexibility (i.e., the ability to adjust behaviour to new contexts) share a common underlying cognitive mechanism. The prediction was that if the hypothalamic-pituitary-interrenal (HPI) axis is the underlying mechanism shared between the non-social and social domains; then, early life HPI axis programming will affect equally behavioural flexibility in a non-social and social task. Evidence was found that early-life HPI axis programming with cortisol reduces both social and non-social behavioural flexibility, suggesting a shared cognitive basis of behavioural flexibility.

Chapter 2: "Egg-mediated maternal effects in a cooperatively breeding cichlid fish".

This chapter has been submitted to Scientific Reports.

In this chapter I investigate the potential mechanism of egg-mediated maternal effects and if embryos use those maternal cues. In small groups mothers have a high mortality risk and need helpers to raise the next brood. Hence, mothers may use egg-mediated maternal effects to shape offspring phenotype. Then, offspring may develop a philopatric phenotype that help in the natal territory. The prediction was that mothers in a small social group produce large eggs to increase offspring survival and allocate a high corticosteroid metabolites concentration. Corticosteroid metabolites may shape offspring stress axis and decrease explorative behaviour. There is no evidence for egg-mediated maternal effects. This suggest that mother and offspring fitness benefits may be aligned during offspring early developmental period. Alternatively, that offspring phenotypic plasticity, rather than an egg-mediated maternal effect, is a possible mechanism for the behavioural phenotype specialization found in this species.

Chapter 3: "Behavioural phenotypes in a wild population of a cooperatively breeding cichlid".

Manuscript in preparation.

In a natural population, I investigated if the size of the social group offers the opportunity to subordinate individuals to specialize in two behavioural phenotypes, namely philopatric with high propensity to show submissive behaviour or early dispersal with high frequency of helping

in the natal territory. The prediction was that in a small social group with a higher demand to help, subordinates will be more likely to maintain their group membership by showing high frequency of helping behaviour in the form of territory maintenance and defence, whereas in large social groups subordinates will be more likely to have a high frequency of submissive behaviour. Evidence was found that in small social groups, large subordinate individuals tended to show a lower frequency of submissive behaviour and a higher frequency of sand digging. Although dispersal propensity did not differ between subordinates in different group sizes, the results suggest that subordinates' own social experiences inside a social group can shape at least the frequency of submissive behaviour and sand digging, which two energetically costly behaviours that guarantees group membership.

Appendix 1. Statistical analysis to test if egg-mediated maternal effects influence social behaviour and hormonal status of offspring.

In this appendix I describe the method used to test the behaviour of juveniles that were produced by mothers in either a small or a large social group. The aim of this was to test if egg-mediated maternal effects influence offspring behaviour in different social contexts. The social contexts were spontaneous behaviours among siblings, asymmetric competition over a resource, family integration and prospecting frequency (i.e., proxy for dispersal). In addition, hormonal measurements were taken at different time points to assess if egg-mediated maternal effects program the physiological status of offspring. The hormones assessed were cortisol, estradiol, testosterone, 11-ketotestosterone, and progesterone. The statistical analysis described the influence of maternal social environment on the behaviour and hormonal status of offspring.

Appendix 2. Methods and statistical analysis to test if maternal transcripts are shaped by the size of the social environment.

This is a collaborative work with PhD candidate Carlos Ernesto Rodríguez Ramírez to investigate if maternal transcripts are a form of egg-mediated maternal effects. In this appendix is the description of the breeding design, collection and storage of samples, RNA extraction method, RNA sequency parameter, and the bioinformatics used to analyse the data generated in the RNA sequency facility of University of Bern.

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Chapter 1

Stress axis programming generates long-term effects on cognitive abilities in a cooperative breeder

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Abstract

The ability to flexibly adjust behaviour to social and non-social challenges is important for successfully navigating variable environments. Social competence, i.e., adaptive behavioural flexibility in the social domain, allows individuals to optimize their expression of social behaviour. Behavioural flexibility outside the social domain aids in coping with ecological challenges. However, it is unknown if social and non-social behavioural flexibility share common underlying cognitive mechanisms. Support for such shared mechanism would be provided if the same neural mechanisms in the brain affected social and non-social behavioural flexibility similarly. We used individuals of the cooperatively-breeding fish *Neolamprologus pulcher* that had undergone early-life programming of the hypothalamic-pituitary-interrenal (HPI) axis by exposure to (i) cortisol, (ii) the glucocorticoid-receptor antagonist mifepristone or (iii) control treatments, and where effects of stress-axis programming on social flexibility occurred. One year after the treatments, adults learned a colour-discrimination task, and subsequently, a reversal-learning task testing for behavioural flexibility. Early-life mifepristone treatment only marginally affected learning performance, whereas cortisol treatment significantly reduced behavioural flexibility. Thus, early-life cortisol treatment reduced both social and non-social behavioural flexibility, suggesting a shared cognitive basis of behavioural flexibility. Further our findings imply that early-life stress programming affects the ability of organisms to flexibly cope with environmental stressors.

Key words: stress-axis programming, social competence, behavioural flexibility, colour discrimination, reversal learning, cichlid.

Introduction

Individuals that can flexibly adjust their social and non-social behaviour to different contexts may obtain fitness advantages over individuals expressing fixed behaviours. For instance, in the social domain, individuals flexibly adjusting their social behaviour according to social information, such as own and others' rank and/or a partner's fighting experience (Arnold & Taborsky, 2010; Taborsky et al., 2012; Taborsky & Oliveira, 2012), may solve contests faster by showing less energy-costly behaviour (Lehner et al., 2011). Social flexibility based on the optimal use of social information is also referred to as social competence (Taborsky & Oliveira, 2012). Also, outside the social domain, behavioural flexibility can enhance fitness (e.g., (Cauchard et al., 2017)). Individuals can benefit from behavioural flexibility to manage decision-making, for instance when feeding or evading predators (Lea et al., 2020). Behavioural flexibility is expected to be especially beneficial when adapting to changeable environments, for instance, to urbanization (rev. in (Lea et al., 2020)).

Both social and non-social behavioural flexibility are based on learning and memory (e.g., (Audet & Lefebvre, 2017; Taborsky & Oliveira, 2012)). It remains unclear, however, whether social and non-social cognition are based on shared brain mechanisms (Ashton et al., 2018; Shaw et al., 2015) or whether there are special-purpose mechanisms for the social and the non-social domains (see discussions in (Kendal et al., 2018; Lotem & Halpern, 2012)). In a recent conceptual paper, Varela et al. (Varela et al., 2020) proposed three alternative models on how cognition systems might be organized. Firstly, cognitive lower-level traits underlying behavioural flexibility, which include the input, encoding, storage and retrieval of information, are all domain-general (Varela et al., 2020). Secondly, lower-level traits may be specialized for social and non-social information processing, suggesting a modular cognitive system (Taborsky & Oliveira, 2012; Varela et al., 2020). Thirdly, cognitive mechanisms may be mixed, with some cognitive lower-level traits being specialized for a domain and others being domain-general (Munger et al., 2010).

Here we ask whether non-social and social behavioural flexibility, that is, the ability to adjust behaviour to new contexts, is affected in a similar or a different way in a vertebrate after the programming of a key physiological system, the HPA/HPI axis (also referred to as 'stress axis'). If there were common effects of early-life programming of the stress axis on social and

non-social behavioural flexibility, this would support the existence of a general underlying cognition mechanism (cf. (Varela et al., 2020)). The vertebrate stress axis modulates social behaviour (Falkenstein et al., 2000; Schjolden et al., 2009; Soares et al., 2010; Spencer, 2017) and social competence (Nyman et al., 2017, 2018; Reyes-Contreras et al., 2019) during ontogeny. It is also an important determinant of cognition and brain development (Bebus et al., 2016; Lupien et al., 2009). The vertebrate HPA/HPI axis is regulated by glucocorticoids (GCs) and their receptors, the mineralocorticoid (MRs) and glucocorticoid receptors (GRs) (Greenwood et al., 2003; Sapolsky et al., 2000). Both receptor types are important to acquire, store, consolidate, and retrieve information. For instance, MRs are involved in the initial phase of memory encoding; they increase hippocampal excitability and produce emotional hippocampal long-term potentiation reinforcement allowing memory formation in rats (Sapolsky et al., 2000; Wang et al., 2013; Whitlock et al., 2006). GRs activated, at moderate GC levels, are important for memory consolidation (Finsterwald & Alberini, 2014), whereas very high GC levels rather inhibit memory in rodents and humans (Finsterwald & Alberini, 2014; Sapolsky et al., 2000) and cognitive flexibility in a non-social domain (Lui et al., 2017). Conversely, blocking GRs by the glucocorticoid-receptor antagonist mifepristone (Ros et al., 2012), enhanced memory consolidation in humans (Roat-Shumway et al., 2018) and mice (Rimmele et al., 2013).

To answer the question whether social and non-social flexibility are modulated similarly by stress axis programming we used the cooperatively breeding cichlid fish, *Neolamprologus pulcher* as model system. In adults, manipulation of the HPI axis by applying the GR-blocker mifepristone resulted in a short-term enhancement of social competence (Nyman et al., 2018). Repeated exposure to cortisol during early-life decreased the social competence in these fish (Reyes-Contreras et al., 2019). In addition, early-life exposure to cortisol or mifepristone resulted in altered stress-axis programming. Both treatments induced a long-term up-regulation of the MR gene and down-regulation of the corticotropin releasing factor (CRF) gene in the telencephalon of adults (Reyes-Contreras et al., 2019). Hence in *N. pulcher*, stress axis programming modulates the development of social flexibility. However, we do not yet know whether early-life HPI axis programming also affects non-social behavioural flexibility in this fish species. Behavioural flexibility in non-social contexts has been most often evidenced by the ability of animals to override previously formed associations, e.g., by reversal learning (Audet & Lefebvre, 2017; Lea et al., 2020; Rasolofoniaina et al., 2021). Here we exposed *N. pulcher* adults that had been treated with cortisol and mifepristone

during early life (Reyes-Contreras et al., 2019) to a two-colour discrimination task at age of 1.5 years to test their learning ability followed by a reversal-learning task to test their behavioural flexibility. We predicted that early-life exposure to cortisol will impair performance in these tasks (Lui et al., 2017), whereas mifepristone exposure will increase performance (Rimmele et al., 2013; Roat-Shumway et al., 2018). If the underlying mechanism (i.e. stress axis programming) that modulates behavioural flexibility is shared between the non-social and social domains, then we predict that stress axis programming changes behavioural flexibility in a non-social task in a similar way than it did for social flexibility (Reyes-Contreras et al., 2019).

Methods

Study species

N. pulcher is a cooperatively-breeding cichlid fish endemic to Lake Tanganyika, East-Africa. Its social groups comprise a dominant breeding pair and related and unrelated subordinate individuals, structured in sized-based linear social hierarchies (Taborsky, 1984). All group members engage in frequent and diverse social interactions to establish or maintain the social hierarchy or to jointly defend the territory and juveniles against intruders (Fischer et al., 2014; Groenewoud et al., 2016). Subordinates can achieve tolerance by dominants by either showing helping behaviour or by showing submission (Bergmüller & Taborsky, 2005; Fischer et al., 2017; Kasper et al., 2017, 2018). Socially more competent individuals (i.e., more socially flexible individuals) have a higher propensity to respond to breeder aggression by submission (Arnold & Taborsky, 2010; Nyman et al., 2017; Taborsky et al., 2012) and are more likely to be accepted as group member by dominants, which is indispensable for survival in the wild (Fischer et al., 2017; Groenewoud et al., 2016; Taborsky et al., 2012). The development of social competence is influenced by the social environment present during rearing of individuals, such as presence vs. absence of adults (Arnold & Taborsky, 2010; Nyman et al., 2017, 2018; Taborsky et al., 2012) or group size (Fischer et al., 2015) during early life.

Early-life treatments

For our experiment, we used fish that received the following pharmacological treatments during their first two months of life during the study by Reyes-Contreras et al. (see

methods in (Reyes-Contreras et al., 2019) and ESM): (i) cortisol (the glucocorticoid hormone of fish [200 ng ml-1] (Reyes-Contreras et al., 2019)), (ii) mifepristone (a glucocorticoid-receptor blocker of fish [400 ng l-1], (Ros et al., 2012)) or (iii) control treatment. Afterwards, until the learning tests, fish were housed in single-sex aggregations of maximally 60 fish in two 200-L compartments of a 400-L tank (for housing conditions, see ESM).

Experimental design

Forty-eight fish were tested in the two learning tasks, 16 of each early life treatment. The sex of the individuals was balanced across treatments; hence, from each early-life treatment eight males and eight females were chosen. The age of the fish during the experiment did not differ significantly between the three early life treatments (mifepristone: 533.1 d ± 28.1 mean ± SE; cortisol: 581.4 d ± 24.0; control: 603.3 d ± 23.1; GLMM comparing control (intercept) vs. each drug treatment: cortisol: estimate ± s.e. = -0.0892 ± 0.0680, z = -1.31, p = 0.19; mifepristone: estimate ± s.e. = -0.0894 ± 0.0695, z = -1.29, p = 0.2). During the time of the learning tasks, each individual was housed separately in a 25-L tank, which was equipped with 2 cm of gravel sand, one biological filter, and half of a flowerpot in the back of the tank serving as shelter. They could not see fish in adjacent tanks to prevent (i) that they use social cues to solve a learning task, and (ii) that territorial aggression between neighbours interferes with learning. At no point of time, fish showed any signs of stress (freezing behaviour, dark skin spots) and they participated deliberately in all trials. Near the front screen of the tank, we placed the experimental apparatus, a grey PVC plate with four rows of holes and five holes per row (Fig S1 a, b). Each fish was first habituated to the presence of the plate, which was left permanently inside the tank. Then, we trained the fish to use the experimental apparatus (section 'Training phase'). Subsequently, fish were exposed to a colour discrimination learning task (section 'Acquisition of colour discrimination'), followed by a reversal learning task (section 'Reversal of colour discrimination'). The experiments were conducted at the Hasli Ethological Station of the Institute of Ecology and Evolution, University of Bern, Switzerland. All experimental procedures were approved by the Veterinary Office of the Kanton Bern, Switzerland, licence number BE 93/18.

Training phase

Individuals were trained to (i) dislodge a green plastic disc covering a hole of the grey PVC-plate and (ii) to eat a food reward hidden below the disc inside a hole following methods

in (Buechel et al., 2018). Three consecutive holes from any of the rows of the plate were selected randomly. Inside the first and the third hole, a small piece of krill was placed, while the hole in the middle was left empty. The training was done stepwise, with increasing difficulty to retrieve the food (Buechel et al., 2018): Two green plastic discs of 15 mm diameter (Lucon-Xiccato & Bisazza, 2014) were used to progressively cover the holes, in the following sequence: (1st) entirely open, (2nd) one quarter covered, (3rd) half covered, (4th) completely covered. During the training phase, the two green discs were always removable by the fish and thus the food reward was always accessible.

Each individual received a maximum of four trials per day. For the first and second level of difficulty, individuals were allowed 1 hour to complete the task. For the third and fourth difficulty level, they were given 45 min to complete the task. Trials with the fourth level of difficulty, with completely covered food holes, were video recorded to assess the time required to dislodge both discs and eat the rewards. During the following trial, this time was allowed as maximum time for a given individual to solve the training task. We continued this procedure iteratively, gradually decreasing the time needed to solve a training trial, until all individuals solved the task in 5 min.

Before each trial, an opaque partition was placed in the middle of the tank, temporarily separating the back half of the tank, containing the focal fish and its shelter, from the frontal half with the experimental apparatus. This allowed the experimenter (MRC) to set up the task without the focal fish seeing the procedure. At the beginning of each trial, a few drops of water containing the smell of krill was added to the tank to provide an incentive for the fish to search for the food item; then, the opaque partition was lifted, and the trial started. The same procedures were carried out in the colour acquisition and reversal learning tasks (see below).

Acquisition of colour discrimination

The fish had to learn to discriminate yellow from blue discs (Fig S1 c, d). These two colours were chosen because *N. pulcher*, and its closely related congener Neolamprologus brichardi, attend to the face of conspecifics during social encounters (Hotta et al., 2019), which contain yellow and blue marks (Bachmann et al., 2017) (Fig. S2). To standardize the distance to reach the discs, we always placed the rewards and discs in the row of the hole-plate closest to the shelter of the fish. Again, two holes were filled with a piece of krill and covered with the

discs, and an empty hole was left in between. Either the yellow or the blue disc was rewarded. The rewarded colour was balanced across sex and early-life treatments. The fish could easily dislodge the rewarded disc (Fig. S1c) by either pushing it away or lifting it, as they learned it during the training task. However, they could not move the unrewarded disk, as it was blocked by a plastic knob glued to its bottom side that tightly fitted in the holes of the PVC-plate (Fig. S1d). This allowed us to place a piece of krill under both the rewarded and the unrewarded disc to control for olfactory cues (Buechel et al., 2018).

In each trial, an individual was allowed up to 5 min to make a choice, dislodge the rewarded disc, and eat the food reward. The first disc that was touched by a fish with its mouth was considered as chosen. The trial was terminated by placing the opaque divider between the fish and the hole-plate as soon as the fish had dislodged the rewarded disc and had eaten the reward. In the few cases, in which a fish did not eat the food reward within 5 min (i.e., the fish had made a wrong choice or no choice, but did not uncover the reward), the rewarded disc was moved to open the hole halfway. Then, the fish was given one additional minute to retrieve the reward (see (Buechel et al., 2018)). If the fish did not succeed by then, the food item was removed from the hole by tweezers and provided directly to the fish. This protocol assured that per trial one piece of krill was eaten and all fish had a similar satiation and motivation level (Buechel et al., 2018).

Each individual received six trials a day. At the beginning of each experimental day, we used a dice to select the position of the rewarded disc (left or right) for the six trials for each fish separately. We used a pseudo-random rule adjusting the ratio of the rewarded:unrewarded side (left or right) to be at maximum 4:2 to avoid that fish were unintendedly trained for a side bias. Thus, when the dice determined the same side for the rewarded colour four times in a row, by rule we placed the reward at the other side for the remaining two trials of a day.

Reversal of colour discrimination

To test for behavioural flexibility, all the fish that successfully reached the learning criterion in the colour acquisition task were exposed to a reversal-learning task. We followed the exact same procedures and learning criterion described for the acquisition task, except that the rewarded colour was reversed for each individual.

Learning criterion

To assess whether fish from different early-life treatments differ in their ability to learn, both in the colour acquisition and the reversal-learning tasks, we recorded the number of blocks needed to reach the learning criterion. To reach the learning criterion a fish had to make at least 5 out of 6 correct choices (i.e., 80% of correct choices) in two consecutive blocks (Bannier et al., 2017; Buechel et al., 2018). One block consisted of six trials in which the fish had made a correct or wrong choice. If the fish had not made a choice in a given trial, this trial was not counted when evaluating the criterion. Therefore, one block could last for more than one day, i.e., until six trials with choice were done. When no-choice trials occurred, we compensated for these by increasing the number of trials. After two consecutive blocks had passed, the learning criterion was assessed. Only three individuals did not reach the learning criterion after 36 trials and were assumed to not have learned the colour acquisition task. These three fish were excluded from the subsequent reversal-learning task.

Statistical analyses

The statistical analyses were done using the program R, version 3.5.1 (Team, 2018). To test the effect of early life treatment on number of blocks needed to reach the learning criterion in the colour acquisition task and the reversal-learning tasks, cox regression proportional hazard models were fitted using the package 'survival' (Therneau & Grambsch, 2019), and the coefficients were estimated by likelihood ratios (Fox & Weisberg, 2011). In those models, we included the frailty term 'family of origin' as random effect (Landes et al., 2020). Except the factor 'rearing treatment', factors that did not significantly influence the probability to reach the learning criterion were removed from the model by stepwise elimination.

For the colour acquisition task, the initial model (Table S1) included rearing treatment (cortisol, mifepristone or control) and colour of the rewarded disc to test for possible colour preferences. In addition, we included sex and age of the fish as covariates. Age has been previously shown to influence learning in this species (Bannier et al., 2017). We stepwise backwards-deleted age and sex of the focal fish from the final model, because they did not significantly influence the number of blocks the fish needed to reach the learning criterion in the acquisition of colour discrimination task (Table 1). In the model for the reversal task, the rearing treatment, rewarded colour, sex and age were included in the initial model (Table S1).

Sex of the focal fish did not significantly affect the probability to reach the learning criterion and was dropped from the final model (Table 1). For each fixed factor in the initial and final models the proportion of hazard assumptions were fulfilled (Tables 2 and S2).

The effect of early life treatment on the number of blocks needed to reach the learning criterion was plotted as the inverse Kaplan Meier curves using 'survminer' package (Kassambara et al., 2020) (Fig. 1 a, b). Further, we tested the treatment effects on the latencies to make the first choice (see ESM).

Results

During the acquisition of colour discrimination, individuals that were treated with mifepristone during early life did not differ significantly in their learning performance from the control treatment (p=0.06, Table 1); however, mifepristone treated fish have roughly twice the chance to pass the learning criterion in the next block compared to control fish (hazard ratio, HR = 2.1; see Table S3) . Early-life cortisol treatment and control individuals did not differ in the acquisition task (HR=1.62). During the reversal-learning task, fish that had received a cortisol treatment during early life needed longer to reach the learning criterion, which indicates a lower behavioural flexibility of this treatment group (Table 1, Fig. 1b, HR=0.30). In addition, older individuals needed less time to reach the learning criterion in the reversal task than younger ones (Table 1, HR=0.99). Fish of the early-life mifepristone treatment did not differ from control individuals in the reversal task (HR=0.61). In both tasks, fish reached the learning criterion faster when the rewarded colour was yellow (Table 1, HRs=3.25 and 1.99, see Table S3). The latency to perform the first choice in the acquisition task, which can be regarded as measure of the motivation to participate in learning tasks, did not differ between treatments (see ESM, Table S4).

Discussion

We had proposed that if there are common effects of early-life stress axis programming on behavioural flexibility in the social and non-social domains, this would suggest the existence of a shared cross-domain cognitive mechanism (cf. (Varela et al., 2020)). In support of this proposal, we showed that early-life exposure to cortisol impairs behavioural flexibility in a

non-social context (green solid arrows in Fig. 2) in a cichlid fish species. Previous research revealed that the same treatment, i.e., early-life exposure to cortisol, also hampered social flexibility in these fish (Reyes-Contreras et al., 2019), and that their social flexibility is based on social learning (Arnold & Taborsky, 2010). This implies that exposure to cortisol impaired social learning in *N. pulcher* (green dashed arrow in Fig. 2). More generally, our results imply that (1) social and non-social flexibility can share a neural substrate in the brain, and (2) that early-life stress programming affects the ability of organisms to flexibly cope with environmental stressors.

Effects of cortisol

Contrary to our predictions, fish that received a cortisol treatment early in life did not differ from control individuals in their learning abilities during the colour acquisition task. A previous experiment showed that cortisol can have detrimental effects on learning performance. Rats that had received a cortisol implant for a period of twelve weeks performed poorly in a maze test compared to control animals (Endo et al., 1996). However, cortisol treated fish exhibited reduced behavioural flexibility, as shown by their poorer performance in a reversal-learning task. This long-term effect was present 1.5 years after the end of the exogenous cortisol treatment. In a previous study, we had reported that early-life cortisol treatment reduced social competence of the fish. It led to an increase of aggressive behaviour during contests over a resource, which extended contest duration while not increasing the chances to win a resource (Reyes-Contreras et al., 2019). As aggression is energetically very costly in *N. pulcher* (Grantner & Taborsky, 1998) such prolonged contests will increase energy expenditure. Our result that cortisol application impairs behavioural flexibility is in line with findings in other vertebrates. It has been shown that GCs negatively affect neurogenesis, which may be required for fear memory extinction and thus behaviourally flexible adjustment to changing conditions (Anacker & Hen, 2017; Seehagen et al., 2015). In rats, chronic cortisol treatment significantly impaired cognitive flexibility in the water maze task (Lui et al., 2017). Finally, human infants were less able to flexibly adjust their behaviour after exposure to a stressor (Seehagen et al., 2015). After a stressor, infants continued to show a previously rewarded behaviour for longer than non-stressed controls, even if now this behaviour did not produce a reward anymore.

Effects of mifepristone

Early-life exposure to the GR-blocker mifepristone marginally improved later-life learning abilities compared to control fish, but did not affect behavioural flexibility. The direction of this marginal result agrees with previous studies measuring short-term effects of mifepristone learning abilities in vertebrates (Rimmele et al., 2013; Schwabe et al., 2013). For example, a 28-day mifepristone treatment in humans with mood disorders improved their attention and learning ability (Roat-Shumway et al., 2018). Similarly, mifepristone treatment applied 4 h before a memory retrieval test significantly enhanced how humans recalled picture details compared to a placebo group (Rimmele et al., 2013).

Mechanistic link between the stress axis and cognitive abilities

In vertebrates, cognitive performance, and the activity of the HPA/HPI stress axis are both modulated by the activation of the two major receptor types of glucocorticoids, the GRs and the MRs. These receptors are expressed in the hippocampus and limbic brain areas and also modulate memory formation (Datson et al., 2012). The formation of memory requires two processes, long-term potentiation (LTP), i.e., a persistent strengthening of synapses based on recent patterns of activity (Shavit Stein et al., 2017), and the expression of the Cyclic AMP Response Element-Binding protein (CREB) (Datson et al., 2012). Both processes can be modulated by pharmacological manipulations of MRs and GRs (Datson et al., 2012; Shavit Stein et al., 2017). Importantly, MR expression was shown to influence the behavioural flexibility in a non-social task in rodents. Rats with an overexpression of MR in the forebrain (independent of their GR expression) had an impaired ability to solve a reversal learning task (Harris et al., 2013). In the fish used in the present study, early-life applications of mifepristone and of exogenous cortisol both generated a permanent upregulation of the gene coding for MRs in the telencephalon (Reyes-Contreras et al., 2019). Together, these findings suggest that a persistently altered expression of the MRs is involved in the mechanistic link between early life programming of the stress axis and non-social behavioural flexibly *N. pulcher*. Further research in different vertebrate taxa is needed to show whether glucocorticoid receptors are generally involved in the mechanistic basis of vertebrate behavioural flexibility.

Additional factors influencing cognitive performance

Younger individuals took significantly longer to reach the learning criterion in the reversal task, as reported previously in *N. pulcher* (Bannier et al., 2017). These results in *N. pulcher* differ from a general tendency found in other vertebrates, in which behavioural flexibility decreased with age (dogs (Piotti et al., 2018), monkeys (Bartus et al., 1979), and rats (Schoenbaum et al., 2002)). Both studies in *N. pulcher* were performed at an age when in nature these fish approach dispersal from their natal groups and/or achieving breeder status, i.e., at around 2-3 years (Jungwirth et al., MS), and thus they are about to face a drastic change of their environment. Therefore, it seems plausible that *N. pulcher* exhibit greater flexibility the closer they reach to the age of independent reproduction, whereas in the above-mentioned mammalian experiments, the reported age-related decrease of flexibility reflected senescence.

In both the acquisition and reversal of colour discrimination, *N. pulcher* reached the learning criterion faster when the rewarded colour was yellow, which agrees with previous experiments in this species in non-social colour discrimination tasks (Culbert et al., 2020; Fischer et al., 2021). Surprisingly, a preference for yellow was absent in a social context, when yellow facial marks were experimentally enhanced (Culbert et al., 2020). The non-social, ecological relevance of yellow in the natural environment of *N. pulcher* is yet unknown. As we fully balanced the rewarded colour across trials and treatments, the effect of colour on cognitive performance should not systematically bias our results.

Ultimate implications of a shared cognitive mechanism for behavioural flexibility

In their natural environments, animals encounter numerous non-social and social challenges, during which the ability to express behavioural flexibility may be beneficial. For instance, in the non-social context, individuals should use information about predation risk to adjust anti-predator behaviour (Fischer et al., 2017; Stratmann & Taborsky, 2014; Watve & Taborsky, 2019). In the presence of predators, Japanese minnows (*Pseudorasbora parva*) increase foraging activity during night-time when predation risk is lower (Takashi Asaeda & Manatunge, 2005). In *N. pulcher*, juveniles adjust their fear behaviour towards heterospecifics based on information about danger learned early in life (Watve & Taborsky, 2019). In the social domain, animals are exposed to frequent and diverse social interactions with different categories of conspecifics, such as potential mates, group mates, cooperation partners and competitors, which all may have different ranks or resource holding potentials. In the

cooperative breeder *N. pulcher* maintaining or achieving (Bergmüller et al., 2005; Jungwirth et al., 2015) group membership is indispensable for survival (Brouwer et al., 2005; Groenewoud et al., 2016; Heg et al., 2004, 2005). High social competence enables them to show appropriate social behaviour during the multitude of possible social interactions, which increases their likelihood to be accepted in a group (Fischer et al., 2017).

Evidence from learning experiments for or against the existence of a cognitive mechanism spanning social and non-social domains is thus far equivocal. In laboratory rodents, early social experience modulates social learning but not the performance in non-social learning tasks (Branchi, 2009; D'Andrea et al., 2007; Lévy et al., 2003), suggesting specialized cognitive mechanisms. In narrow-striped mongooses (*Mungotictis decemlineata*) social learning opportunities in groups affected reversal learning speed negatively (Rasolofoniaina et al., 2021). Conversely, in cooperatively-breeding Western Australian magpies (*Cracticus tibicen dorsalis*), performance in several non-social cognition tasks was influenced positively by the complexity of the social environment: performance in these tasks improved with increasing group size (Ashton et al., 2018).

General implications

Our results have two general implications. Firstly, our results imply that social and non-social flexibility can share a neural substrate in the brain. Evidence of whether social and non-social flexibility are based either on a shared or on specialized, modular cognitive mechanisms has been largely lacking (Varela et al., 2020) and existing evidence is controversial and rather indirect, resting on studies comparing non-social cognition between individuals exposed to different social conditions (Ashton et al., 2018; Bannier et al., 2017; Lévy et al., 2003). What could be the advantage of evolving a shared cognitive mechanism? It has been argued that one advantage of a shared cognitive mechanism is that it is less subject to energetic constraints than multiple special-purpose mechanisms, given the finite energy available for parallel brain activity (Varela et al., 2020). However, a shared cognitive mechanism used for multiple purposes is likely to be constrained by temporal trade-offs (Varela et al., 2020), e.g., when attention towards different environmental cues need to be processed simultaneously by the same cognitive system (Varela et al., 2020). Thus, whether shared or special-purpose mechanisms evolve should depend on the relative strength of temporal and energetic constraints present in a species, and thus on a species' ecology.

Secondly, social and non-social flexibility are impacted by stress programming. Organisms require behavioural flexibility to deal with challenges in their social and non-social environments such as during encounters with competitors, when there are changes in resource availability, or when fending off predators. Physiological stress systems, which are present in all organisms including animals, plants and microbes, are integral in coping with such environmental challenges (Taborsky et al., 2021). Programming of the physiological stress system as shown in our study occurs by parental effects or own early experience, and has been documented in most major vertebrate taxa, including mammals (Champagne, 2020; Curley et al., 2011; Weaver et al., 2004), birds (Banerjee et al., 2012), amphibians (Hu et al., 2008) and fish (Alsop and Vijayan, 2008; Nesan and Vijayan, 2013a; Taborsky et al., 2013; Reyes-Contreras et al., 2019) as well as invertebrates (rev. in (Hime et al., 2021)). Therefore, our finding that non-social and social behavioural flexibility is affected by stress programming points towards an important link between the exposure to environmental stressors, which can lead to stress programming (Henry et al., 1994; Meaney et al., 1996), and the ability of organisms to flexibly cope with non-social and social stressors.

Data availability: Data available from the Dryad Digital Repository: doi:https://doi.org/10.5061/dryad.qfttdz0jr (Reyes-Contreras & Taborsky, 2022)

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Tables and figures

Table 1. Summary table of the cox regression proportional hazard model testing the effects of early-life treatment, colour of the rewarded disc and age on the number of blocks needed to reach the learning criterion during the acquisition of the colour discrimination task and the reversal task. The coefficients were estimated using likelihood ratios. Significant results in bold.

	Coefficient ± SE	χ^2	p
Acquisition of colour discrimination			
Rearing treatment (cortisol)	0.482 ± 0.384	1.58	0.21
Rearing treatment (mifepristone)	0.739 ± 0.396	0.39	*0.062*
Rewarded colour (yellow)	1.178 ± 0.334	0.33	**0.00042**
Frailty (family)		0	0.94
Reversal of colour discrimination			
Rearing treatment (cortisol)	- 1.197 ± 0.439	7.42	**0.0065**
Rearing treatment (mifepristone)	- 0.496 ± 0.416	1.42	0.23
Rewarded colour (yellow)	0.689 ± 0.331	4.35	**0.037**
Age (days)	- 0.00787 ± 0.00211	13.93	**0.00019**
Frailty (family)		0	0.94

Table 2. Summary table of the proportion of hazard assumptions of the models in Table 1.

	χ^2	p
Acquisition of colour discrimination		
Rearing treatment (cortisol)	1.57	0.21
Rearing treatment (mifepristone)	0.00083	0.98
Rewarded colour (yellow)	2.46	0.12
Global	4.36	0.23
Reversal of colour discrimination		
Rearing treatment (cortisol)	0.24	0.63
Rearing treatment (mifepristone)	1.08	0.29
Rewarded colour (yellow)	0.96	0.33
Age (days)	0.95	0.33

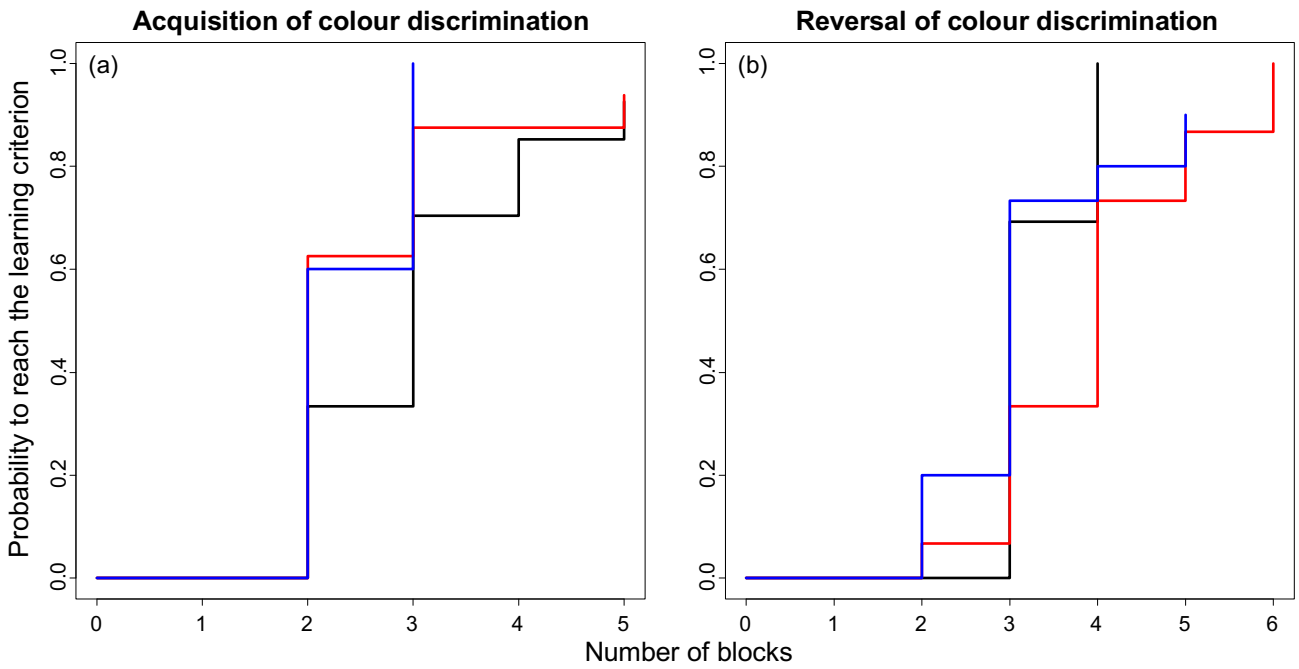

Figure 1. Inverse Kaplan-Meier curves showing the results of (a) the acquisition of colour discrimination and (b) of the reversal-learning task. Black lines: control treatment; red lines: cortisol treatment; blue lines: mifepristone treatment.

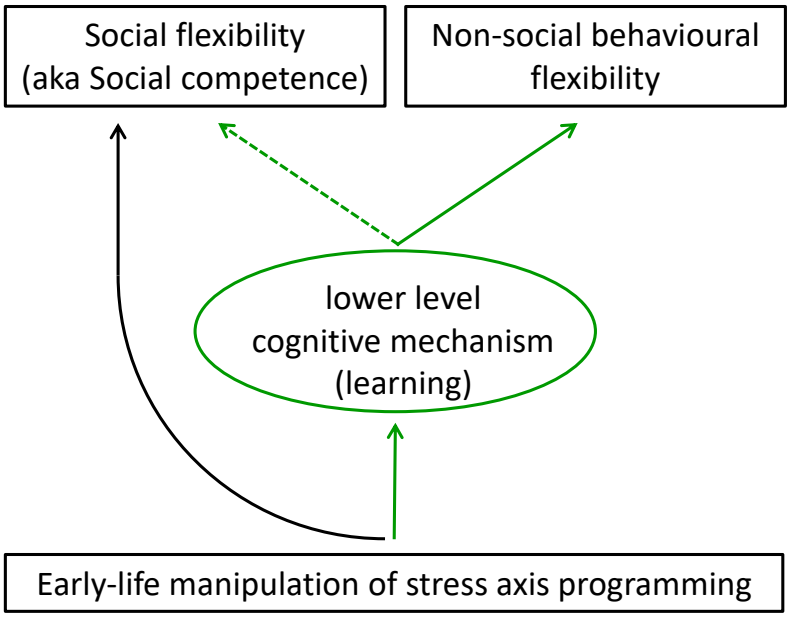

Figure 2. Hypothesis resulting from this study that social and non-social flexibility share common lower-level cognitive traits. Black: results shown in (Reyes-Contreras et al., 2019). Green solid arrows: pathway shown in this study. Dashed green arrow: inference drawn from (Arnold & Taborsky, 2010), that social flexibility is based on social learning.

Supplementary Information

Stress axis programming generates long-term effects on cognitive abilities in a cooperative breeder

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Contains Electronic Supplementary Material: Supplementary Methods and Results, and Tables S1- S4 and Figures S1- S3

Supplementary Methods

Sourcing and general housing conditions

The animals used were the fifth-generation offspring from wild caught *Neolamprologus pulcher* population originating from a population near Kasakalawe at the south-eastern tip of Lake Tanganyika, Zambia. In our aquarium facility of the Ethological Station Hasli, all fish tanks are equipped with a 2-cm layer of river sand, one biological filter and structures for sheltering to minimize aggression among conspecifics. The water temperature is kept at 27±1 °C and the L:D cycle was to 13:11 hours. The fish are fed five days a week with JBL Novo Tanganyika food flakes and once a week they receive a mix of frozen invertebrates, except during learning trials, when fish are fed six pieces of krill per day during the trials.

In *N. pulcher* adults the mean baseline cortisol plasma levels range from 20-35 ng ml-1 (Mileva et al., 2009) and after an acute stressor plasma cortisol level reaches 500 ng ml-1 (Mileva et al., 2009).

Early life treatment

The fish used in this study were reared and received their early-life treatments during a previous study (Reyes-Contreras et al., 2019); the treatments used to manipulate the development of the stress axis in the juvenile *N. pulcher* used in the current study were described in full detail in (Reyes-Contreras et al., 2019). The fish used in the current study had been kept in aggregations ever since the early-life treatments (see main text) and were never used in any experimental tests before they were used in the current experiment. For the early-life treatments, Reyes-Contreras et al. (Reyes-Contreras et al., 2019) had randomly assigned 31 unrelated broods of *N. pulcher* to one of three pharmacological treatments. The drugs used and their concetrations are given in the main text (preparation of drug solutions and supplier information see (Reyes-Contreras et al., 2019)). Each early life treatment was applied by a water bath every ten days, starting the first day of free swimming until day 60 after free swimming (see (Reyes-Contreras et al., 2019)). The concentration of cortisol treatment was choosen because it generates long-term effects on stress sensitivity in developing rainbow trout (*Oncorhynchus mykiss*) (Auperin & Geslin, 2008). The control treatment contained only the solvents used in the mifepristone treatment (see (Reyes-Contreras et al., 2019)).

Ethical information, sample size and blinding procedures

After early-life treatment, mortality rates, and growth rates did not differ between treatments. The sample size for the learning and reversal learning experiment was chosen to allow for sufficient statistical power based on effect sizes reported in other learning experiments in our study species (Bannier et al., 2017; Fischer et al., 2021)). Each individual was assigned a number. During the trials, only this individual number was known to the experimenter (MRC), but not the early life treatment. During the trials of both tasks the experimenter stayed quietly behind a black curtain to avoid disturbance of the fish. Videos of the trials were recorded with handycams and identified with the recording day. This allowed experimenter to later analysed choice and latency while being blind to the treatment and sex of the fish.

At the end of the study, fish from the control treatment were integrated in the breeding stock of our aquarium facility at the Ethologische Station Hasli. All individuals treated with cortisol and mifepristone were sacrificed in accordance with the regulations of our animal facility and the Veterinary Office of the Kanton Bern, Switzerland, licence no. 93/18.

Supplementary results

Latencies to first choice

To assess whether fish from different early-life treatments differ in their motivation to take part in the learning trials, which might have affected their learning success, we measured the latency to make the first choice in the colour acquisition task for a subset of our data. The subset contained eight individuals of each of the three treatments (N=24), balanced for rewarded colour. We analysed the latencies during the first 12 trials of the colour acquisition task. We fitted a generalized linear mixed model GLMM assuming a gamma distribution using the packages ‘car’ (Fox & Weisberg, 2011), ‘MASS’ (Venables et al., 2002), ‘afex’ (Singmann et al., 2018) and ‘nlme’ (Bates et al., 2015). The model included ‘rearing treatment’, ‘first choice’ (i.e., either correct or incorrect choice) and ‘colour of the rewarded disc’ as fixed factors and the identity of ‘family of origin’ as random factor (see Table S4).

Supplementary Tables

Table S1. Summary table of the initial cox regression proportional hazard model before stepwise deletion of non-significant fixed factors, testing for the effects of early-life treatment on the number of blocks to reach the learning criterion during the colour discrimination acquisition and reversal task. Significant effects are given in bold.

	Coefficient ± SE	χ^2	p
Acquisition of colour discrimination			
Rearing treatment (cortisol)	0.481 ± 0.384	0.38	0.21
Rearing treatment (mifepristone)	0.623 ± 0.414	0.41	0.13
Rewarded colour (yellow)	1.201 ± 0.338	**0.34**	**0.00038**
Sex (male)	0.469 ± 0.328	0.33	0.15
Age (days)	- 0.000559 ± 0.00163	0.0016	0.73
Frailty (family)		0	0.94
Reversal of colour discrimination			
Rearing treatment (cortisol)	- 1.197 ± 0.441	0.44	**0.0066**
Rearing treatment (mifepristone)	- 0.474 ± 0.418	0.418	0.26
Rewarded colour (yellow)	0.683 ± 0.332	0.33	**0.039**
Sex (male)	0.272 ± 0.339	0.34	0.42
Age (days)	- 0.00822 ± 0.00219	0.0022	**0.00017**
Frailty (family)		0	0.94

Table S2. Summary table of the proportion hazard assumption for the model presented in Table S1.

	χ^2	p
Acquisition of colour discrimination		
Rearing treatment (cortisol)	1.21	0.27
Rearing treatment (mifepristone)	0.0067	0.94
Rewarded colour (yellow)	2.20	0.14
Sex (male)	0.752	0.39
Age (days)	0.039	0.84
Global	4.77	0.45
Reversal of colour discrimination		
Rearing treatment (cortisol)	0.21	0.64
Rearing treatment (mifepristone)	0.8	0.37
Rewarded colour (yellow)	1.14	0.29
Sex (male)	2.79	0.095
Age (days)	0.28	0.59
Global	6.56	0.26

Table S3. Summary table of the hazard ratios of the models in Table 1 of the main text. The exponential coefficients (Exp (coef)) give the hazard ratios, which provides the effect sizes of the fixed factors of the models. The lower c.i and upper c.i are the 95% of confidence intervals of the hazard ratios.

	Exp(coef)	Lower c.i. 95%	Upper c.i. 95%
Acquisition of colour discrimination			
Rearing treatment (cortisol)	1.62	0.76	3.43
Rearing treatment (mifepristone)	2.1	0.96	4.56
Rewarded colour (yellow)	3.25	1.69	6.25
Reversal of colour discrimination			
Rearing treatment (cortisol)	0.30	0.13	0.72
Rearing treatment (mifepristone)	0.61	0.27	1.38
Rewarded colour (yellow)	1.99	1.04	3.81
Age (days)	0.99	0.99	0.99

Table S4. Summary table of the gamma generalized linear mixed model testing for the effects of early-life treatment on the latency to make the first choice in the colour acquisition phase. Estimates refer to the factor level in brackets.

Factor	Estimate ± SE	t	p
Intercept	0.0225 ± 0.00235	9.56	< 0.0001
Rearing treatment (cortisol)	0.00037 ± 0.00284	0.13	0.90
Rearing treatment (mifepristone)	0.00171 ± 0.00280	0.61	0.54
First choice (incorrect)	0.00413 ± 0.00331	1.25	0.21
Rewarded colour (yellow)	0.000509 ± 0.00226	0.23	0.82

Supplementary Figures

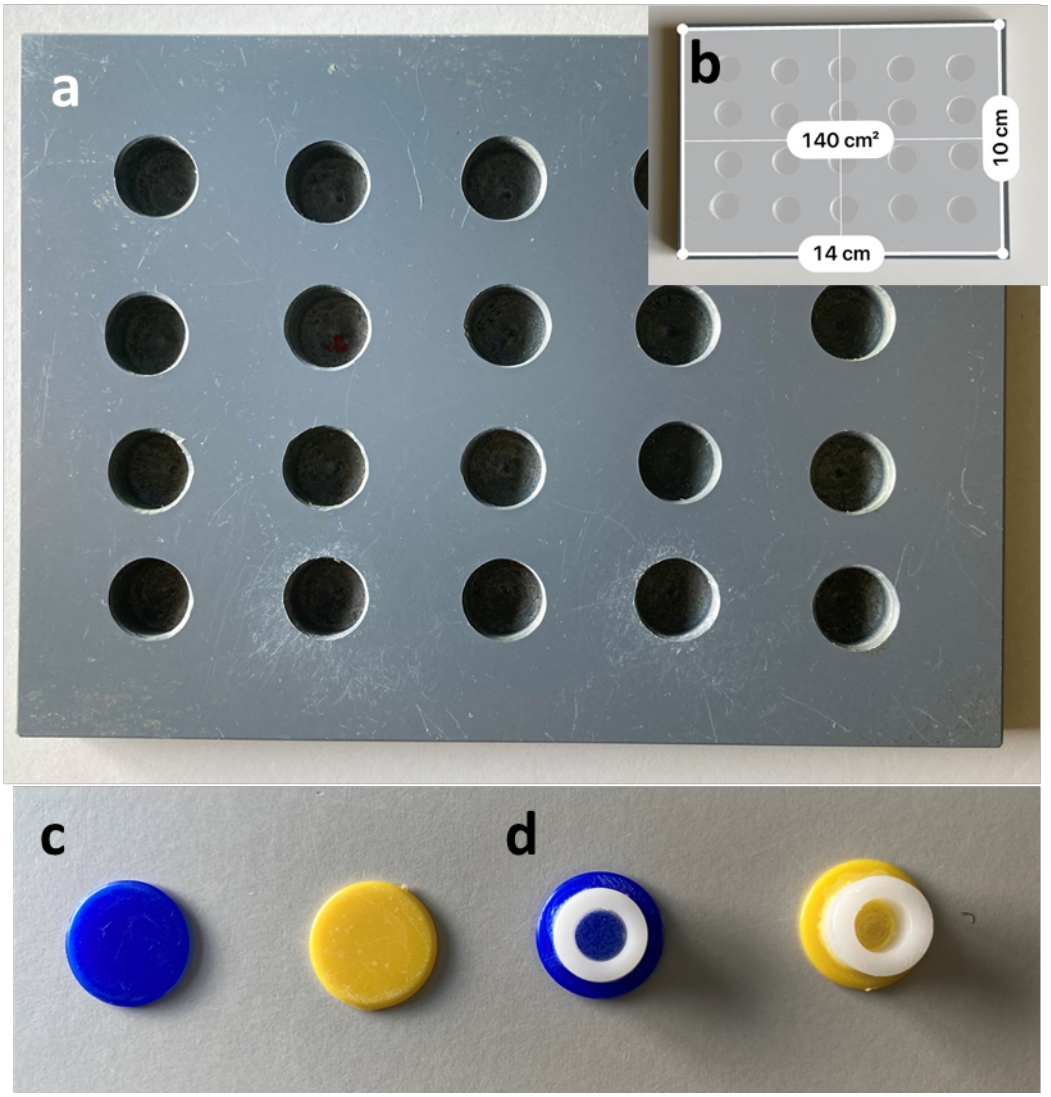

Figure S1. (a, b) The learning apparatus consisted of a grey PVC plate (140 x 100 x 10 mm) with 20 holes (11 mm in diameter, 7 mm deep) arranged in four rows and five columns (cf. (Buechel et al., 2018)). (c) Rewarded discs that can be dislodged. (d) Bottom view of the unrewarded discs, the white plastic knob is glued to the disc and fits tightly in a hole, such that the disc cannot be pushed to the side by the fish.

Figure S2. Lateral view of *Neolamprologus pulcher* head. The colours blue and yellow are characteristics of the facial features of this species.

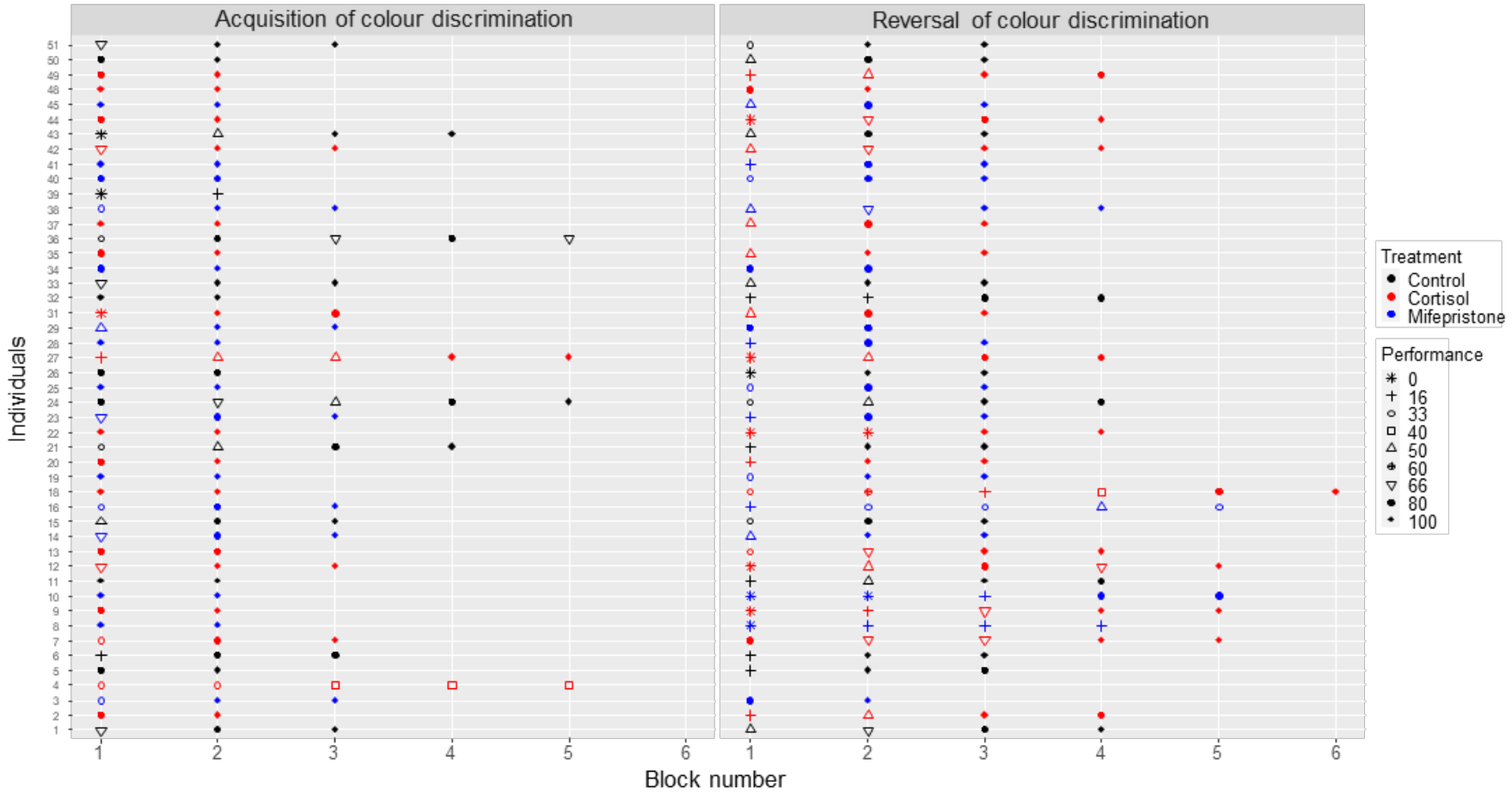

Figure S3. Individual performance in each block during the acquisition phase (left) and reversal phase (right) of the colour discrimination task. The percentages of correct choices made in each block are represented by different symbols (see figure legend). Individuals had reached our learning criterion if they had either 80% (filled circle) or 100% (filled diamond) correct choices in two consecutive blocks. Each individual (y-axis) was exposed to one of the following treatments early in life: control (black), cortisol (red) and mifepristone (blue)

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Chapter 2

Egg-mediated maternal effects in a cooperatively breeding cichlid fish

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Abstract

Mothers can influence offspring phenotype through egg-mediated maternal effects, which can be influenced by cues mothers obtain from their environment during offspring production. Developing embryos use these components but have mechanisms to alter maternal signals. Here we aimed to understand the role of mothers and embryo in how maternal effects might shape offspring social phenotype. In the cooperatively-breeding fish *Neolamprologus pulcher* different social phenotypes develop in large and small social groups differing in predation risk and social complexity. We manipulated the maternal social environment of *N. pulcher* females during egg laying by allocating them either to a small or a large social group. We compared egg mass and clutch size and the concentration of corticosteroid metabolites between social environments, and between fertilized and unfertilized eggs to investigate how embryos deal with maternal signalling. Mothers in small groups produced larger clutches but neither laid smaller eggs nor bestowed eggs differently with corticosteroids. Fertilized eggs scored lower on a principal component representing three corticosteroid metabolites, namely 11-deoxycortisol, cortisone, and 11-deoxycorticosterone. We did not detect egg-mediated maternal effects induced by the maternal social environment. We discuss that divergent social phenotypes induced by different group sizes may be triggered by own offspring experience.

Key words: developmental plasticity, corticosteroids, maternal effects, clutch size, cooperative breeding, cichlids

Introduction

Egg-mediated maternal effects, where mothers influence the size or composition of eggs, can shape offspring phenotype. These effects are taxonomically widespread (insects (Russell & Lummaa, 2009), birds (Groothuis & Schwabl, 2008), fish (Adrian-Kalchhauser et al., 2018), amphibians (Pakkasmaa et al., 2003), and reptiles (Uller et al., 2007)). These maternal effects can occur, for instance, by the adjustment of egg mass (Bernardo, 1996), which is often considered as proxy for the amount of nutrients present in eggs such as lipids and proteins (e.g., in fish eggs (Sharda et al., 2021)), or by adding hormones (Groothuis & Schwabl, 2008), and antioxidants (carotenoids, vitamins) as shown in bird eggs (Blount et al., 2002). Egg-mediated maternal effects can be adaptive for offspring, for mothers, or both. They can be adaptive for offspring when maternal effects provide information enabling offspring to adjust their phenotype to the predicted conditions encountered after birth (Kuijper & Johnstone, 2018); if this enhances maternal reproductive success, in this case maternal and offspring fitness optima are aligned. They may also be adaptive only for mothers, at the cost for offspring fitness, if they allow mothers to reduce the investment per single offspring and to have either more offspring in the current brood or higher survival and reproductive success in the future. The latter maternal effects may result in offspring evolving resistance to maternal effects (Kuijper & Johnstone, 2018).

Mothers can influence offspring lifetime fitness by enhancing their investment in egg quality. For example, egg mass typically correlates positively with offspring size after birth (rev. in (Segers & Taborsky, 2011)). Larger offspring often have better survival prospects because they are more mobile (Schürch & Taborsky, 2005) and are more likely to survive the earliest life stages (Kamler, 2005; Williams, 1994), if their main predators are gape-size limited (Sogard, 1997). Eggs can also be endowed with particular nutrients enhancing predator evasion. For instance, in a teleost fish, the red drum (*Sciaenops ocellatus*), a higher content of certain unsaturated essential fatty acids in their eggs resulted in larvae with faster escape responses (Fuiman & Ojanguren, 2011). In birds, egg mass was positively correlated with offspring lifetime fitness (Krist, 2011). A higher investment in the offspring quality may have to be traded for quantity, as documented for instance in anseriform birds (Figuerola & Green, 2006). In the post-hatching phase, other factors such as sibling competition and received amount of food, which influence growth rate, will shape offspring phenotype. Alternatively, to clutch size and egg nutrient allocation, mothers can also use hormone signalling via

modification of egg composition to shape offspring phenotype in various ways (von Engelhardt & Groothuis, 2011), including their later behaviour (Hsu et al., 2016).

Hormones are known to have long term organizational effects during early embryonic developmental periods. Maternal hormone signalling plays an important role in shaping the hormonal environment of developing embryos (Brooks et al., 1997; Mouton & Duckworth, 2021; Seckl, 2001; von Engelhardt & Groothuis, 2011). It is influenced by environmental cues perceived by mothers, such as the nutritional quality of the environment, risk, or social stability, which in turn can influence offspring growth, gene expression, and behaviour (Champagne, 2020; Groothuis et al., 2005; von Engelhardt & Groothuis, 2011; Welberg et al., 2001). In mammals, embryos can be influenced by maternal hormones via the placental blood stream during the entire gestation period (Fowden et al., 2009). In contrast, in oviparous species, the maternal influence on the embryonal hormonal endowment is restricted to the egg formation period, which allows studying maternal and embryonal influences on offspring phenotype separately.

In highly social species like cooperative breeders, communal breeders and eusocial species, the social environment is key in shaping morphology (Linksvayer & Wade, 2005), physiology (Russell & Lummaa, 2009), and behaviour (Raulo & Dantzer, 2018). Both own early social experience (e.g.,(Branchi et al., 2006; Fischer et al., 2017)) and maternal effects can shape the social phenotype of offspring (Russell & Lummaa, 2009). In cooperative breeders, conflict between parents and offspring may arise over offspring dispersal tendencies, if parents need help at their territory, while offspring might prefer to breed independently. Dispersal propensity in cooperative breeders can be influenced by early social experience such as the experienced group composition (Fischer et al., 2017). However, there is also a wide range of maternal effects that can act during the pre- , early, late, and post-reproductive phases, which may allow mothers to influence whether their offspring develop into a philopatric helper type or into dispersive individuals (reviewed in Table 2 of (Russell & Lummaa, 2009)).

A key candidate mechanism for modulating social phenotype in dependence of maternal and early social cues is the activity of the vertebrate ‘stress axis’ (i.e., the hypothalamic-pituitary-adrenal/interrenal axis (Nesan & Vijayan, 2013; Welberg et al., 2001)) with glucocorticoid (GC) hormones as major signalling hormones (Hill et al., 2021). GCs and glucocorticoid receptors (GRs) facilitate social behaviour of vertebrates in multiple ways (Raulo & Dantzer, 2018). GCs favour the propensity to show alloparental care in wild

vertebrates (Raulo & Dantzer, 2018); in social cichlids, being reared in a socially more complex environment leads to an increase in GR mRNA expression in the brain as well as a higher ability to express appropriate social responses (i.e., higher social competence (Taborsky & Oliveira, 2012)) during contests (Nyman et al., 2017) and an enhanced tendency to engage socially with conspecifics (Solomon-Lane & Hofmann, 2019). Finally, in laboratory rats, GR expression is involved in the transgenerational, non-genetic transmission of stress axis reactivity, which is mediated by the intensity of tactile contact between mothers and pups (Francis et al., 1999; Meaney & Szyf, 2005). Glucocorticoid metabolites can have organizational effects during early development (Mouton & Duckworth, 2021). However, it is unknown whether mothers of cooperative breeders allocate glucocorticoids differentially depending on their need of help to influence offspring social and dispersive phenotype, and in case of a parent-offspring conflict over dispersal, what the role of the embryo is in using them (e.g., see (Paitz et al., 2016) for a mechanism of teleost embryos to eliminate maternal GCs).

Thus, to understand how maternal effects ultimately shape offspring social phenotype in cooperative breeders, the role of mothers - endowing eggs with different hormones - and of embryos using them needs to be experimentally disentangled. The first step to this end is to compare egg content by mothers in relatively high (small groups) vs. low (large groups) need of help and without vs. with embryonic development ongoing. A second step would then be to experimentally manipulate the hormones in the eggs to test for causal relationships in the offspring. Here we addressed the first step by experimentally varying the group size of mothers of the cooperatively-breeding cichlid *Neolamprologus pulcher* to elicit environment-induced maternal effects in the eggs. Next, we compared egg mass and clutch size between the two social environments, and we compared the composition of glucocorticoid metabolites without embryonic development (unfertilized eggs) with glucocorticoids present in developing eggs in the two social environments to investigate the fate of those hormones after fertilization and hence the role of the embryo itself. We predicted that females reproducing in small groups lay larger eggs than in large groups (Taborsky et al., 2007), as they have fewer helpers assisting them in defending the offspring against predators. Larger young are more mobile (Schürch & Taborsky, 2005) and have an advantage under size-dependent predation risk as it is common in aquatic environments (Sogard, 1997). Furthermore, we predicted that in small groups, in which there is a higher predation risk and a higher need of help (compared to large groups), females use egg-mediated maternal effects, such as an increased glucocorticoid deposition. The latter is known to affect offspring behaviour, including increased fear response to predators

and potentially reduce explorative behaviour (Redfern et al., 2017), which in turn could potentially decrease offspring propensity to disperse.

Methods

Study species

Neolamprologus pulcher is a cooperatively-breeding cichlid endemic to Lake Tanganyika, East Africa (Taborsky, 2016). The groups consist of a dominant breeding pair and a variable number of subordinate individuals of different sizes and ages (Taborsky, 2016), which help to raise the offspring of the current breeding pair ('brood care helpers'). Helpers can obtain inclusive fitness benefits if they are related to the breeder's offspring (Taborsky, 2016). Moreover, all helpers obtain direct benefits by access to shelters in the breeders' territory, which is indispensable for survival because predation risk is high (Groenewoud et al., 2016). Individual survival (Brouwer et al., 2005) and the persistence of groups over time (Heg et al., 2005) increase with group size. Breeding females reduce their egg mass with increasing group size (Taborsky et al., 2007) and increase it under perceived predation risk during egg maturation (Sharda et al., 2021). Also, the behaviour of fish later in life is shaped by the early social environment (i.e., group size and composition) (Arnold & Taborsky, 2010; Fischer et al., 2017; Nyman et al., 2017, 2018; Taborsky et al., 2012) and perceived predation threat (Fischer et al., 2017). The latter occurs both by way of egg-mediated maternal effects (Sharda et al., 2021) and own offspring experience with predator cues (Fischer et al., 2017; Watve & Taborsky, 2019). Offspring raised in the presence of more adults and perceived a higher predation risk had a better social competence and were more likely to disperse from social groups for independent breeding (Fischer et al., 2017).

Ethical statement

The experiments were approved by the Veterinary Office of the Kanton Bern, Switzerland and conducted in the aquarium facilities of the Ethological Station Hasli of the University of Bern, Switzerland, under the licence number BE 93/18. The methods and experiments were performed in accordance with the Swiss Animal Welfare law and followed the ARRIVE guidelines. The fish used to constitute large and small social groups were taken from the breeding laboratory stock of the aquarium, which originally was derived from wild

caught fish from the Kasakalawe point population near Mpulungu, Zambia. At the end of the experiments social group members were reintegrated in the breeding stock. Offspring born in the groups were assigned to another experiment (La Loggia et al. in prep).

Experimental groups and housing conditions

We set up small and large breeding groups by selecting unrelated fish from the stock tanks of our aquarium facility. A small group consisted of one breeding pair and one helper, which corresponds to the minimal natural group size (Bergmuller et al., 2005). In the natural environment, most *N. pulcher* groups contain several helpers of different body sizes and ages (Bruintjes & Taborsky, 2011). Correspondingly, large groups consisted of a breeder pair and eight helpers of different sizes and sexes (see Table S1 in Supplementary Material); see also (Fischer et al., 2015). Breeding pairs were assigned to breed either first in a small and subsequently in a large group, or other way round, with the order of group size treatments being balanced across tanks.

The breeding groups were housed in 400-L tanks that were divided in two compartments by opaque, water-tight dividers, one small 100-L compartment for small groups (33 x 65 x 50 cm length x depth x height) and one large 300-L compartment (97 x 65 x 50 cm length x depth x height) for large groups. All compartments were equipped with a 2-cm sand layer, one half a flowerpot per fish on the tank bottom as shelters and breeding sites, and additional hiding places mounted near the water surface (empty, semi-transparent plastic bottles). In natural territories all group members have their own hiding place, which they defend against other group members (Werner et al., 2003). The water temperature was kept at 27±1 °C and the light-dark cycle was 13:11h with dimmed-light phases of 10 min in between to simulate natural light conditions. All groups were fed commercial adult flake food (JBL Novo Tanganyika®) five days a week and they received fresh food twice per week. Additional TetraMin Baby® powdered flake food was provided when free-swimming fry were present in a tank.

In natural populations, *N. pulcher* breed in colonies, and territories are always established in close vicinity to neighbouring groups (Jungwirth et al., 2015). These neighbouring conspecifics, and heterospecific space competitors, opportunistic egg predators (*Telmatochromis vitattus*), and dangerous piscivorous predators (*Lepidiolamprologus elongatus*) frequently intrude natural territories. Hence, breeders and subordinate helpers are

constantly defending their territory against the various competitors and predators (Groenewoud et al., 2016). The presence of these threats increases the need for help for the dominant breeders and, in turn, raises their readiness to accept helpers (Zöttl et al., 2013). To mimic natural conditions and to elicit helping behaviours by subordinates, which increases their likelihood to be accepted by the dominant breeders (Bergmüller & Taborsky, 2005), once a week, we exposed all groups to one of the following helping tasks, where the order of presentations was balanced across tanks. (a) Defence against an egg predator, which consisted of presenting one *T. vitattus* inside a transparent tube during 5 min near the centre of the territory (Bruintjes & Taborsky, 2011). (b) Territory maintenance, which consisted of digging out sand from the shelter(s) used by the dominant breeders for breeding and/or hiding (shelter use by dominants was established directly before the task, and depending on these observations, one or two shelters were filled with sand) (Bruintjes & Taborsky, 2011). (c) Defence against an unfamiliar conspecific, presented inside a transparent tube for 5 min near the centre of the territory (Desjardins et al., 2008).

Production of experimental broods

In each group, breeding pairs were allowed to produce at least four clutches (Fig. 1). The 1st , 2nd and 4th clutch were all fertilized and not used for analysis in this study. Only the 3rd clutch generated the samples for this study. It was either unfertilized or freshly fertilized (Table 1).

The 1st clutch was removed and discarded; the time to first spawning served to establish new groups and achieve and monitor group stability. Group stability was defined as (i) the absence of evicted individuals, (ii) all group members having access to the bottom of the territory, and (iii) the absence of overtly aggressive interactions between group members. If those criteria were not met before the 1st clutch was laid, the group was re-structured by exchanging members or move them to a different aquarium, which sometimes helps to stabilize groups. The 2nd and 4th clutches were allowed to develop into broods that grew up within their respective group until an age of 2 months and received brood care by all group members (egg cleaning, fanning, guarding). These young were used in a different study (La Loggia MS). The 3rd clutch was collected for analysis of this study (‘spawning 1’).

After producing a 4th clutch, the dominant breeders were moved to another tank where they were merged with a new set of unrelated, unfamiliar subordinate individuals taken from

our stock tanks to obtain ‘spawning 2’ (Fig. 1). If a breeder pair had spawned before in a small group, it was now placed in a large group, and, conversely, if it had been in a large group, it was now placed in a small group. Also in this new social group, we collected the 3rd clutch (‘spawning 2’). Hence, spawning 1 and 2 correspond to the laying sequence at which females spawned in a particular social group. We included the spawning sequence in the data analysis, because carry-over effects between clutches may exist, which affect the maternal reproductive strategy in her current social group.

Production of unfertilized eggs

We obtained unfertilized eggs to enable us to analyse maternal hormone deposition to eggs, which is unaltered by embryonic development. We prevented fertilization by separating a female ready to lay eggs, further termed ‘gravid female’ from the rest of the group. This was in most cases the dominant breeder female, and in a few cases the large helper female. A gravid female was recognized by her protruded genital papilla and an inflated belly. To collect the eggs of spawning 1 and 2, female reproductive status was checked twice per day for these signs of an approaching spawning. When this occurred, we added one transparent divider to separate the breeder male and the gravid female, and another transparent divider to separate the female from the rest of the group (Fig. S1). Next to the divider that separated the gravid female and dominant male, we placed two adjacent flowerpot halves leaning against each side of the transparent partition such that they formed a “shared shelter” (see Fig. S1). It could be visited by the female and the male simultaneously for spawning, but still prevented physical contact between the breeders so that the sperm released by the male could not reach the eggs (Maldonado, 2017). This method has proven successful for collecting unfertilized eggs.

Production of fertilized eggs

If females did not spawn within 10 days, we removed the transparent partition, but we continued monitoring the female and in case a spawning occurred, we collected the fertilized eggs as soon as possible (23.23 ± 5.89 hours, mean ± s.e.) after they were laid. Those samples were stored for further analysis of hormonal content to analyse the fate of corticosteroid metabolites after fertilization (see sample sizes for group size treatments and fertilization state in Table 1).

Sample collection

The unfertilized and freshly fertilized eggs of spawning 1 (i.e., clutch 3 in first social environment) and spawning 2 (i.e., clutch 3 in second social environment) were collected with help of a tweezer, which we used to detach each single egg individually carefully from the surfaces, where we had detected them (e.g., flowerpot, partition, filter). For each clutch we counted the number of eggs.

Furthermore, from all unfertilized clutches, we randomly collected ten eggs and weighed each individual egg to the nearest mg to obtain their fresh weights. Out of those ten eggs, five eggs were randomly selected, dried at 60 °C for 12 hours, and then weighed individually to the nearest mg to obtain their dry weight, which was used as proxy of egg mass (Antunes & Taborsky, 2020).

The remaining eggs of each unfertilized clutch as well as all eggs from the fertilized clutches were placed in a cryo pore tubes of 1.6 ml, which were immediately flash frozen in liquid nitrogen, and stored at -80 °C until corticosteroid extraction. In addition, we measured the length and weight of the female, which had produced the clutch to calculate Fulton's body condition index, because body condition can influence the number and size of eggs(Taborsky et al., 2007).

Steroid analysis

Background information on teleost steroid pathways

The steroidogenesis pathway in teleost resembles mammalian pathways. It starts by the conversion of cholesterol to pregnenolone. One final metabolite resulting from pregnenolone is 11-deoxycortisol which is further metabolized to cortisol by cholesterol side-chain cleavage enzyme cytochrome P450 (Tokarz et al., 2015a). Cortisol has been widely reported to be present in teleost eggs in stressed (Mccormick, 1998; Mileva et al., 2011) and in non-stressed females (Alsop & Vijayan, 2008). Cortisol can be further metabolized to cortisone (Tokarz et al., 2015a). The presence of cortisone has been previously reported in unfertilized eggs of tilapia cichlids (*O. mossambicus*) (Tagawa et al., 2000). Following another path, pregnenolone can be metabolized to 11-deoxycorticosterone and further to corticosterone (Tokarz et al., 2015a). The corticosteroid metabolite 11-deoxycorticosterone has not been reported in

unfertilized teleost oocytes but is an important regulator of female's oocyte maturation (Milla et al., 2009).

Steroid extraction and measurement in eggs

We had used a paired design for clutch collection with the same females laying eggs both in a large and a small group, as we aimed to control for between-female variability in egg mass and clutch size. However, in some cases also large helper females spawned, and importantly many of the clutches were too small to provide enough material for the corticosteroid analysis. Therefore, we had to pool clutches in this analysis including eggs from multiple females within the same group size treatment and the same fertilization state (Table 1) to reach approximately 100 mg per sample. The final mass of the samples was 92.59 ± 5.25 g (mean ± standard deviation).

The frozen eggs of the pooled samples were grinded using a TissueLyserII, weighed and diluted to 600 mg with DPBS (Gibco DPBS (1x); Dulbecco's Phosphate Buffered Saline; REF14190-094). To each unfrozen sample 75 µl mixture of internal standard work solution was added. The internal work solution used contained 11-deoxycortisol[1]13C3 (14,3 nmol/L), corticosterone-d4 (28,5 nmol/L), 21-deoxycortisol-d4 (14,3 nmol/L), and 11-deoxycorticosteron[1]13C3 (9,0 nmol/L), which were diluted 25x in 20% methanol. The samples were subsequently left for one hour at room temperature. Each sample was extracted twice in 1 ml methanol by using a vortex (2000 rpm), followed by centrifuging at 12000xg for 10 min at room temperature. The supernatant was transferred to tubes containing 200 mg of solid ZnCl2 for lipid precipitation (Wang et al., 2010). The total volume of the combined supernatants was made to 4 ml by adding 2 ml methanol, and centrifuged at 12000xg for 10 min at 4°C. The supernatant was dried under nitrogen gas in a water bath at 50°C, re-suspended in 1 ml methanol, centrifuged at 12000xg for 10 min at room temperature, followed by addition of 1.8 ml water to the supernatant. This mixture was centrifuged at 12000xg for 10 min at 4°C. The supernatant was loaded on C-18 SPE columns (SEClute™ SPE C18-Aq 500mg/3mL, code 5138775, Aurora Borealis Control BV, Schoonebeek, The Netherlands) pre-equilibrated with 3 ml of methanol, followed by 3 ml of water. After loading the supernatant, eluding the cartridge, the flow through was collected, columns were washed with 3 ml water, and then eluted with 2 ml methanol. The eluent was dried under nitrogen gas in a water bath at 50°C and re-suspended in 80 µl methanol followed by addition of 120 µl water to make a final

concentration of 40% methanol. Standards were prepared using dilution series from pre-prepared stock and ranged from 0.05-6.96 nmol/l cortisone. The standards were treated according to the same extraction procedure as described for fish eggs.

The samples were analysed using the Waters Acquity system ultra-performance liquid chromatography (uPLC) coupled with a cartridge of type XBridge™. In addition, samples were analysed with Waters TQ-S Xevo system tandem mass spectrometry (MS-MS).

Statistical analysis

Egg mass and clutch size

Statistical analyses were done with R version 4.1.2 (R Core Team, 2021). To assess the difference in egg mass between females reproducing in large and small group we run linear mixed models (LMMs), using the package 'lme4' 1.1-27.1 (Bates et al., 2015). The normality assumptions of the LMM and the normal distribution of corticosteroid metabolites were confirmed with Shapiro-Wilk tests and Kolmogorov-Smirnov tests with Lilliefors correction together with a visual inspection of the quantile-quantile (Q-Q) plots of the model residuals (Houseman et al., 2006) using the packages 'nortest' 1.0-4 (Gross & Ligges, 2015) and 'afex' 1.0-1 (Singmann et al., 2021). To calculate the differences in egg mass, we used the mean egg dry weights from unfertilized clutches. We fitted an LMM which included egg mas as dependent variable and 'group size', 'spawning sequence' (i.e., spawning 1 or 2), and their interaction, and 'female body condition' as fixed factors. We included the identity of the breeding pair as a random factor to account for the repeated spawns by the same pairs. The interaction term did not significantly explain egg mass (Table S2) and the simplified model without the interaction had a lower AIC; therefore, we dropped the interaction term from the final model. In addition, we fitted a generalized mixed effect model (GLMM), assuming a negative binomial distribution, which included clutch size as dependent variable and 'group size', 'spawning sequence', their interaction, and 'female body condition' as fixed factors. 'Breeder pair identity' was included as random factor. The interaction term did not significantly explain the clutch size (Table S3) and the AIC of the model was similar to the simplified model without interaction, so both models are equivalent; therefore, the interaction term was dropped from the final model.

Model selection was based on the Akaike's information criterion (Engqvist, 2005). If a fixed term had no significant effect on the response variable and the simplified model had a lower AIC than the model including this factor, it was dropped from the final model. We started model simplification by removing non-significant interaction terms, followed by main effects, with group size and fertilization state always being retained in the final model per default. Full initial models before simplification are shown in Tables S2-S6. Effect sizes were obtained by converting the statistical values (i.e., t and z) to the effect size statistic 'Cohens' d value' using the package 'effectsize' version 0.7.0.5 (Ben-Shachar et al., 2020).

Egg hormone concentration

We set a signal to noise (S/N) ratio equal or higher than 10 as cut-off to select the corticosteroid metabolites analysed further in this study. These were cortisone, cortisol like-compound (95% like cortisol), 11-deoxycortisol, and 11-deoxycorticosterone.

The corticosteroid metabolites 11-deoxycortisol, cortisol-like, and cortisone were log-transformed to achieve normality. Afterwards, the variables were scaled to unit variance using the function 'scale.unit', followed by a principal component analysis (PCA) using the 'PCA' function and the package "factoextra v. 1.0.7" (Kassambara, 2020). In addition, a graphical representation of the first two principal components (PC) was constructed using the two PCs that explained most of the variance of the data set, by using the package "FactoMineR v. 2.4" (Husson et al., 2020). The loadings of each individual sample for each steroid metabolite were extracted from the PCA, and linear models (LM) were done to determine the influence of group size and fertilization status on the individual scores of PC1 and PC2.

Results

Egg mass and clutch size

Egg dry weight did not differ between females reproducing in large and small groups (d = 0.20; c.i.= - 0.45 to 0.85, Table 2; full model on Table S2), and spawning sequence (i.e., spawning 1 and 2) did not significantly affect egg mass (d = 0.50, c.i.= - 0.11 to 1.08) (Fig. 2). Female body condition tended to affect egg mass (Table 2).

The interaction between group size and spawning sequence did not significantly influence clutch size (d = 0.34; c.i.= - 0.10 to 0.78, see full model in Table S3). Females in small groups laid significantly more eggs (d = 0.59; c.i.= 0.15 to 1.03, Table 2) and females in the second spawning laid significantly larger clutches irrespective of female body condition (d = 0.80; c.i.= 0.36 to 1.24, Table 2). In addition, female body condition positively affected clutch size (d = 0.68; c.i.= 0.24 to 1.12; Fig. 3a-b, Table 2).

Finally, we tested whether egg mass and clutch size were negatively correlated, as expected in case of a trade-off between these traits, but this was not the case (Pearson correlation, r = - 0.042, p = 0.86).

Egg corticosteroid concentration

In the PCA of corticosteroid concentrations in the eggs, PC1 explained 64.96% of the variation, and PC2 explained 26.87% while the other two PCs together explained only 8.17% of the variance (Table S4). We further analysed the individual scores of PC1 and PC2. Three metabolites explain a similar amount of the variance explained by PC1, 11-deoxycortisol (34.85%), 11-deoxycorticosterone (32.45%), and cortisone (27.33%). Instead, the cortisol-like compound explains 78.29% of variance explained by PC2 (Table 3).

Next, we analysed the individual scores of the PCA for effects of group size and fertilization status. Group size did not affect the PC1 scores, (d = 0.08; c.i.= - 0.31 to 0.46; Fig. 4a) and corticosteroid metabolites between unfertilized and fertilized eggs for PC1 did just not reach statistical significance (d = 0.39, c.i.= - 0.02 to 0.78; Fig. 4b see Table S5 for full initial model, final model Table 4). Because three of the four metabolites explained a similarly high percentage of variation in PC1 (11-deoxycortisol, 11-deoxycorticosterone, and cortisone, in total 94.63%), and all three load positively on PC1 (Fig. S2), this result show that less of these hormones tended to be present in fertilized than in unfertilized eggs. For PC2, steroid metabolite composition did not significantly differ between group size treatments or fertilization status (effect size: group sized d = 0.16, c.i.= - 0.24 to 0.55; fertilization status d = 0.14; c.i.= - 0.26 to 0.53; LM, group size treatment: estimates ± s.e. = - 0.265 ± 0.412, t =- 0.642, p = 0.50; fertilization status: estimates ± s.e. = 0.277 ± 0.408, t = 0.678, p = 0.48, see Table S6 for full initial model)

Discussion

In this study, we asked whether social group size, determined by the number of brood care helpers present in a group, and the fertilization state influence egg traits of females that may influence helper behaviour of the offspring in the cooperatively-breeding cichlid *N. pulcher*. We had hypothesized that females with few helpers (i.e., in small groups) produce larger offspring, which have a survival advantage, and may use egg-mediated maternal effects to decrease offspring probability to disperse from the natal territory. In brief, the size of social groups, the spawning sequence, and female body condition significantly influenced clutch size. Females with fewer helpers laid larger clutches than females in large groups. Females that were re-allocated to a second social group laid larger clutches in comparison with females in the first social group. Females with a higher body condition laid larger clutches irrespective of the group size. Contrary to clutch size, group size did not influence egg mass, and there was no indication of a trade-off between clutch size and egg mass. The concentration of the yolk corticosteroid metabolites 11-deoxycortisol, 11-deoxycorticosterone, and cortisone all loaded on the same principal component and tended to be lower in fertilized eggs in comparison with unfertilized egg, although it did just not reach statistical significance. In addition, there was an unknown corticosteroid metabolite present in eggs, which we identified as a 11-deoxy metabolite. It lacks hydrogens in the 11 positions, and it is 95% similar to cortisol.

A possible explanation for females producing larger clutches when being in small groups is the lower survival prospects for offspring in small groups (Brouwer et al., 2005; Groenewoud & Clutton-Brock, 2021; Mumme et al., 2015; Rood, 1990). Females in small group may produce more offspring to ensure the survival of at least some offspring because of these lowered survival prospects. At the same time, the need for more helpers to grow up and to join in brood care in the juvenile stage is higher in small than in large groups (Angulo et al., 2013; Heg et al., 2005), which also should favour larger clutches to be produced by females in small groups. Thus, lower survival prospects and a higher need for more helpers together may explain larger clutch sizes laid in small groups.

Females had larger clutches also in the second spawning of the spawning sequence. It is possible that there is a positive effect of female age on reproductive traits. For example, six-month-old zebra finches (*Taeniopygia guttata*) female produced larger clutches in comparison with three-month-old females (Williams & Christians Williams, 2003) and in female Artic

charr (*Salvelinus alpinus*) egg mass is positive correlated with female age (Lasne et al., 2018). *N. pulcher* females can produce a new clutch every 15-30 days and we collected four clutches in each spawning sequence. Hence, in the second spawning (i.e., in a second social group) females were at least two months older that the first spawning, which might explain the production of larger clutches in the second spawning, irrespective of female body condition, which was statistically controlled for.

In addition, clutch size increased with female body condition regardless of group size and spawning sequence. This positive relationship has been previously reported for threespine stickleback fish (*Gasterosteus aculeatus*) (Baker et al., 2015). It seems plausible that *N. pulcher* females with better condition laid larger clutches because they are able to divert more of their energetic resources into egg production.

In an earlier study, *N. pulcher* females were shown to lay smaller eggs in the presence of more helpers, presumably allowing females to save energy for the next reproductive event (Taborsky et al., 2007). More recently, a meta-analysis (Dixit et al., 2017) confirmed that in cooperatively breeding fish and birds there is a general tendency of breeder females to reduce egg mass if they received more help, suggesting load-lightening by a higher number of helpers. To the contrary, in our study mothers adjusted clutch size instead of egg mass to group size, with no evidence for a trade-off between clutch size and egg mass (see absence of trade-off also in previous work in *N. pulcher* (Taborsky et al., 2007) and in lizards (Warne & Charnov, 2008). Possibly females are able to plastically adjust egg size (Baker et al., 2015) in response to short-term changes of group size. Although females were able to lay at least four clutches in each group size treatment, longer-term measurements of more reproductive events may be necessary to detect egg mass adjustment to the size of the social group. Alternatively, dominant breeder females may use other mechanism different from egg mass to increase offspring probability to remain philopatric and become broodcare helpers, such as varying the egg composition by provision offspring with differential concentrations of proteins, lipids, vitamins, hormones, or maternal transcripts to shape offspring phenotype.

The physiological mechanisms underlying adaptive maternal effects are often cryptic and therefore poorly understood (Richardson, 2021). There is ample evidence, however, that maternal effects can be mediated by hormones deposited in eggs (Groothuis et al., 2019; Groothuis & Schwabl, 2008; Pfannkuche et al., 2011) or, in mammals, they can be transmitted to the embryos by the maternal blood stream (Kosten & Nielsen, 2014; O'Regan et al., 2001).

During reproduction of oviparous species, maternal hormones have a dual function: in (i) promoting offspring developmental processes (Brown et al., 2014) and (ii) fine tuning physiological functions in the maternal body during the reproductive phase (Davies & Ryan, 1972; Groothuis & Schwabl, 2008). The corticosteroid hormonal profiles of mothers and of the hormones deposited in eggs are often correlated. In fish, maternal circulating cortisol enters the vitellogenic follicle either via diffusion or by binding to yolk proteins, which suggests that a high corticosteroid hormone concentration in maternal plasma due to stressors may spill over to the embryo and may generate long-term effects on embryo phenotypic traits (Sopinka et al., 2017).

The corticosteroid metabolites detected in fertilized and unfertilized eggs of our study may have a relevant biological function before and after fertilization. The metabolite 11-deoxycortisol is a maturation-inducing steroid of teleost oocytes (Lubzens et al., 2010). Cortisone can be converted to cortisol in the last weeks of human fatal development (Pearson Murphy, 1979), but teleost embryos lack the enzyme 11β-hydroxysteroid dehydrogenases type 1, which metabolize cortisone to cortisol (Tsachaki et al., 2017); hence, it is difficult to hypothesize about a biological function of cortisone in teleost eggs. The metabolite 11-deoxycorticosterone is a ligand for the mineralocorticoid receptor (MR) in teleost fish (Tokarz et al., 2015b). In zebrafish, maternal RNA expression of mineralocorticoid receptor (mr RNA) has a low abundance, but after twelve hours of fertilization it increases, which makes MRs available for binding to 11-deoxycorticosterone (Pikulkaew et al., 2010). Additionally, it has been suggested that the 11-deoxycorticosterone-MR axis may be involved in the early developmental process and regulation of development after hatching in teleost (Kiilerich et al., 2018). In contrast to previous teleost egg analyses (Alsop & Vijayan, 2008; Mileva et al., 2011; Szisch et al., 2005), in our corticosteroid profiles, cortisol was not detected. Possibly cortisol was converted to the inactive form ‘cortisone’, as it has been reported from unfertilized oocytes of tilapia cichlids (*O. mossambicus*). In these fish, radio labelled cortisol is completely converted to cortisone once it enters the oocytes. This means that if cortisol diffuses into the oocyte from the maternal circulation (Sopinka et al., 2017), it is converted to cortisone while the oocyte is still inside the mother and once it is ovulated it lacks cortisol. This may explain the absence of cortisol in unfertilized and fertilized eggs in our experiment. As a fourth corticosteroid, we detected an unknown metabolite that was by 95% similar to cortisol and the function of which is not known.

Contrary to our predictions, we did not detect differences between corticosteroid concentrations in eggs produced by females in different group sizes. It is possible that a difference in group sizes per se does not represent a strong enough stressor leading to an increase circulating maternal corticosteroid concentration that will differentially affect the oocytes. Alternatively, even if maternal corticosteroid concentrations were high enough to spill over to oocytes, the lack of differences between social groups may be explained by the fact that teleost oocytes, which are inside the female ovarian follicle (Lubzens et al., 2010), are protected against high levels of maternal circulation levels of cortisol. This is because high levels initiate the transcription of the enzyme 11β-hydroxysteroid dehydrogenase type 2 in the theca and granulosa cells, which are monolayers of cells that surrounds the oocyte(Lubzens et al., 2010) being responsible for the conversion of cortisol to cortisone (Faught & Vijayan, 2018).

Analysing the individual scores of our PCA on corticosteroid metabolites revealed that the three corticosteroid metabolites loading on PC1 were present in lower amounts in fertilized eggs than in unfertilized eggs (Fig. 4b), even if the difference just not reached statistical difference. This marginal difference of corticosteroid concentration between fertilized and unfertilized eggs may have several possible explanations. First, it can be attributed to metabolization of the three corticosteroids after fertilization (Kumar et al., 2018). Embryos inside an egg may have converted maternally deposited corticosteroids. For example, chicken embryos convert glucocorticoid hormones to 20-b-dihydrocortisol (von Engelhardt et al., 2009), which requires a set of enzymes (Groothuis & Schwabl, 2008). In zebrafish, cortisol is metabolized to cortisone, which is further metabolized to 20β-hydroxycortisone, and the latter is excreted (Tokarz et al., 2012, 2013). Second, the metabolites can be converted to another corticosteroid molecule before fertilization (Tagawa et al., 2000; von Engelhardt et al., 2009). Third, they can be excreted from the oocyte before (Tagawa et al., 2000) and after fertilization (Paitz et al., 2016).

In summary, while we found an effect of group size during reproduction on clutch size, we do not have evidence that the social environment induces egg-mass differences or corticosteroid-mediated maternal effects to shape offspring phenotype. The social environment of *N. pulcher* females may induce other egg-mediated maternal effects such as endowment with vitamins (Blount et al., 2002), other hormones (Mouton & Duckworth, 2021) or maternal transcripts (Adrian-Kalchhauser et al., 2018). Alternatively or in addition, in our study species,

the social environment shapes offspring phenotype directly by way of developmental plasticity: In *N. pulcher* offspring social, and helping behaviours as well as dispersal propensity are plastically adjusted to the composition and size of groups they experience early in life (Arnold & Taborsky, 2010; Fischer et al., 2015; Nyman et al., 2017, 2018; Taborsky et al., 2012). Theoretical models predict that at least under strong selection offspring plasticity decreases the magnitude of maternal effects because by being plastic offspring can use direct environmental information (Kuijper & Hoyle, 2015). Developmental plasticity allows individuals to integrate cues during their development such that their phenotype is adapted to local conditions. Hence, offspring may actively scan their own social environment for informative cues to plastically adjust their phenotype, and either to strive for independent reproduction or to forego reproduction and help raising breeders' offspring. This may render egg-mediated maternal effects relatively less important. Instead, mothers may influence the behaviour of offspring in the juvenile stage, by preventing dispersal or enforcing help (Bergmüller & Taborsky, 2005; Naef & Taborsky, 2020a, 2020b; Quiñones et al., 2016).

Conclusions

The social environment can modulate offspring phenotype via maternal effect and offspring own experience. The absence of egg-mediated maternal effects that provide a head-start and may shape offspring stress axis by hormonal endowment suggest that mother and offspring fitness benefits may be aligned during offspring early developmental period. Later in life, the same social environment may shift mother and offspring fitness optima with the possibility of conflict of interest between parties.

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Data availability

The datasets analysed during the current study are available in the "Egg-mediated maternal effects in a cooperatively breeding cichlid fish" repository, https://figshare.com/s/e1e0b92a214f229d0a2b

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Tables and figures

Table 1. Number of independent samples collected to assess clutch size, egg mass, and corticosteroid metabolites in large and small groups, separated by spawning sequence (i.e., laying sequence) and fertilization status.

Measurement	Small group	Large group	Small group	Large group	Small group		Large group	
	Spawning 1 Unfertilized	Spawning 1 Unfertilized	Spawning 2 Unfertilized	Spawning 2 Unfertilized	Fertilized	Unfertilized	Fertilized	Unfertilized
Egg mass	7	2	4	7	-	-	-	-
Clutch size	7	3	3	7	-	-	-	-
Corticosteroid metabolites	-	-	-	-	9	8	4	8

Table 2. Results of the final two models, first linear mixed model (LMM) to test the effect of group size, spawning sequence (i.e., spawning 1 or 2), and female body condition on egg mass. Sample size: small groups n = 11 clutches (spawning 1: n = 7, spawning 2: n = 4); large groups: n = 9 clutches, (spawning 1: n = 2, spawning 2: n = 7). Second the generalized linear mixed-effect model (GLMM) to test the effect of group size, spawning sequence (i.e., spawning 1 or 2), and female body condition on egg dry weight and clutch size. Sample size: small groups n = 10 clutches (spawning 1: n = 7, spawning 2: n = 3); large groups: n = 11 clutches, (spawning 1: n = 4, spawning 2: n = 7). Estimates refer to the factor levels given in brackets. Significant p-values are in bold (except for the intercept).

	Estimate ± S.E.	t	z	p
Egg mass				
Intercept	0.0003 ± 0.00009	3.23		0.0089
Spawning sequence (spawning 2)	0.00005 ± 0.00003	1.73		0.11
Group size (small)	0.00002 ± 0.00003	0.61		0.56
Female body condition	0.00005 ± 0.00003	1.86		*0.088*
Clutch size				
Intercept	2.3531 ± 0.5968		3.943	< 0.001
Spawning sequence (spawning 2)	0.57 ± 0.175		3.25	**0.0012**
Group size (small)	0.348 ± 0.174		2	**0.045**
Female body condition	0.54 ± 0.179		3.02	**0.0025**

Table 3. Contribution of each steroid hormone to the first and second dimension of the PCA (PC1 and PC2) of maternal steroid allocation to unfertilized and fertilized eggs laid in large and small groups.

Dimensions	Variable	Variance explained in %
PC1	11-Deoxycortisol	34.85
	Cortisone	27.33
	11-Deoxycorticosterone	32.45
	Cortisol-like	5.37
Total of the variance explained		64.96%
PC2	11-Deoxycortisol	1.91
	Cortisone	18.66
	11-Deoxycorticosterone	1.14
	Cortisol-like	78.29
Total of the variance explained		26.87%

Table 4. Summary table of linear model that tested the effect of group size and fertilization status on the PC1 scores.

	Estimate ± S.E	t	p
Intercept	-0.934 ± 0.609	-1.53	0.14
Group size (small)	0.478 ± 0.609	0.79	0.44
Fertilization status (unfertilized)	1.184 ± 0.603	1.96	*0.06*

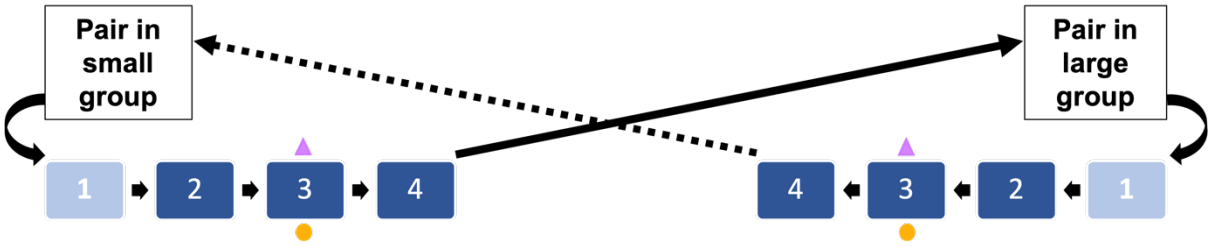

Figure 1. Schematic representation of the egg collection sequence. A breeder pair was assigned either to a small or to a large social group. In each group, breeders produced up to four clutches (i.e., 1,2,3, and 4). The first clutch (light blue box) was removed and discarded. The 2nd and 4th clutches were allowed to hatch and young to grow up in the social groups (dark blue boxes). The 3rd clutch (spawning 1, i.e., orange circle) was either unfertilized or freshly fertilized and collected for analysis. After the 4th clutch, the breeding pair was assigned to a new set of helpers either in a small (dotted arrow) or large (solid arrow) and the eggs of spawning 2 (i.e., purple triangle; unfertilized or freshly fertilized) were collected following same procedure described for spawning 1.

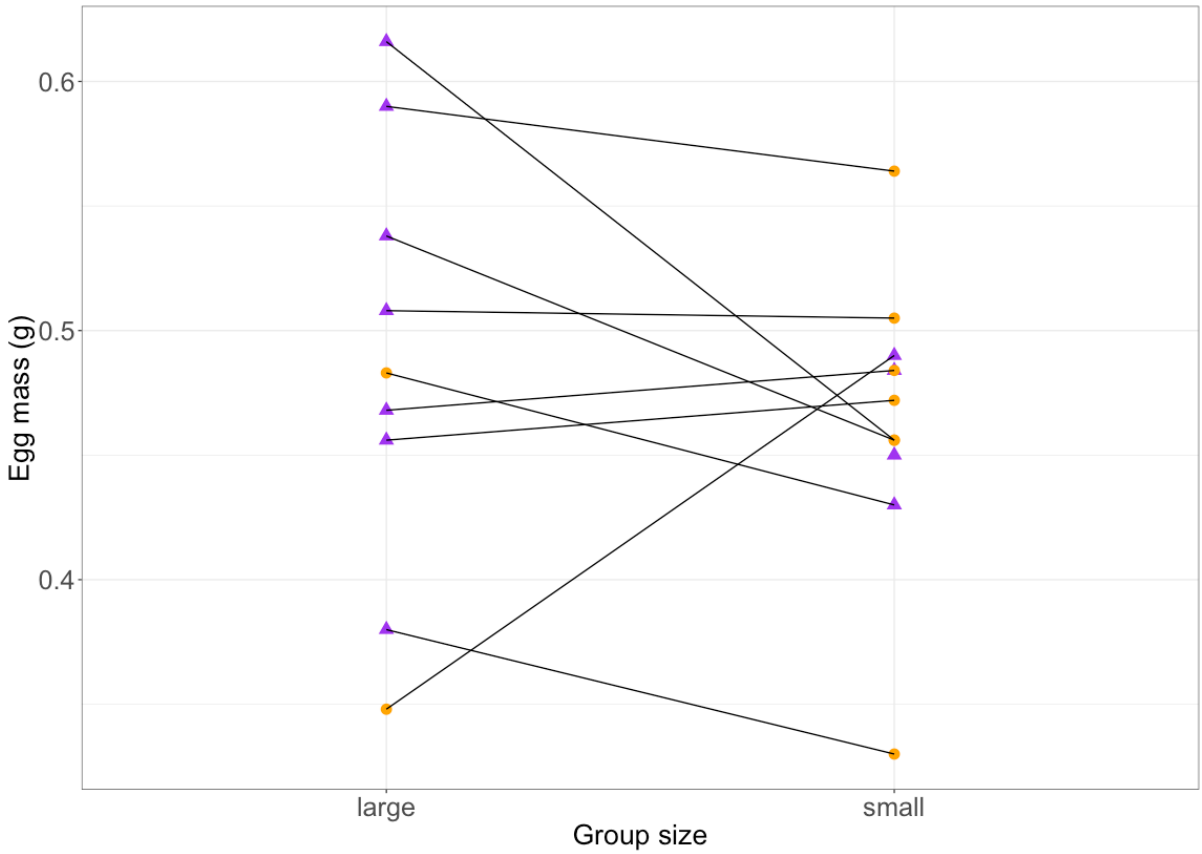

Figure 2. Egg mass of individual females under the two different group size conditions. The clutches of spawning 1 are represented by orange circles and the ones from spawning 2 by purple triangles.

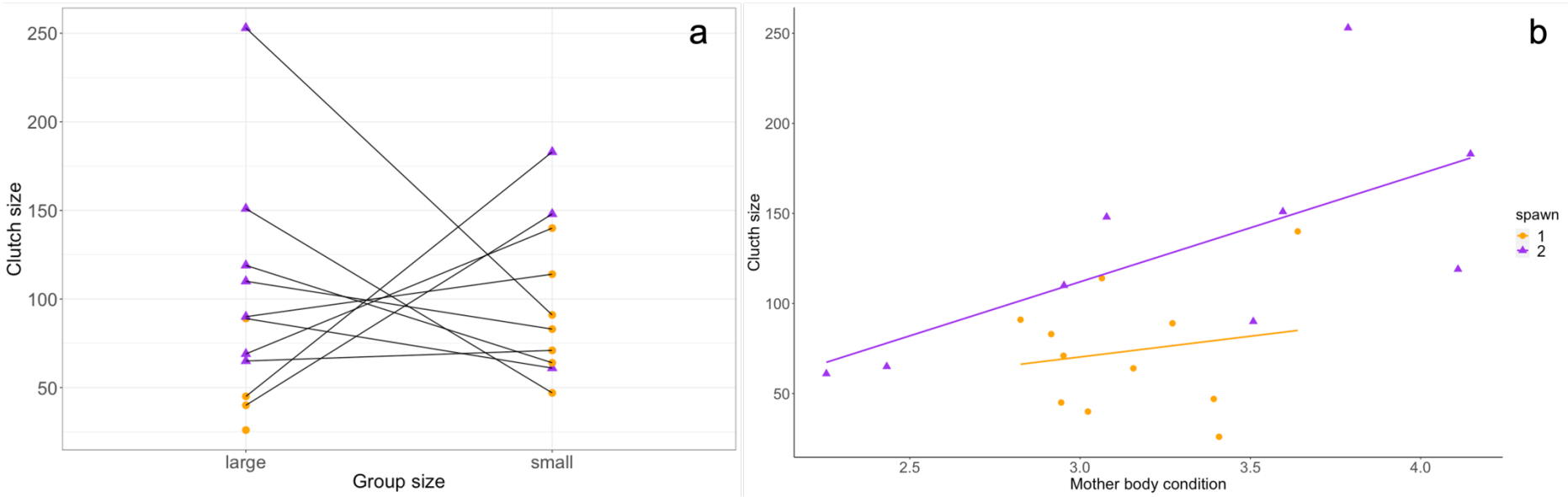

Figure 3. (a) Clutch sizes in the different group size treatments. The clutches of spawning 1 are represented by orange circles and the ones from spawning 2 by purple triangles. (b) Clutch size as a function of mother body condition. The body condition was calculated using Fulton's index.

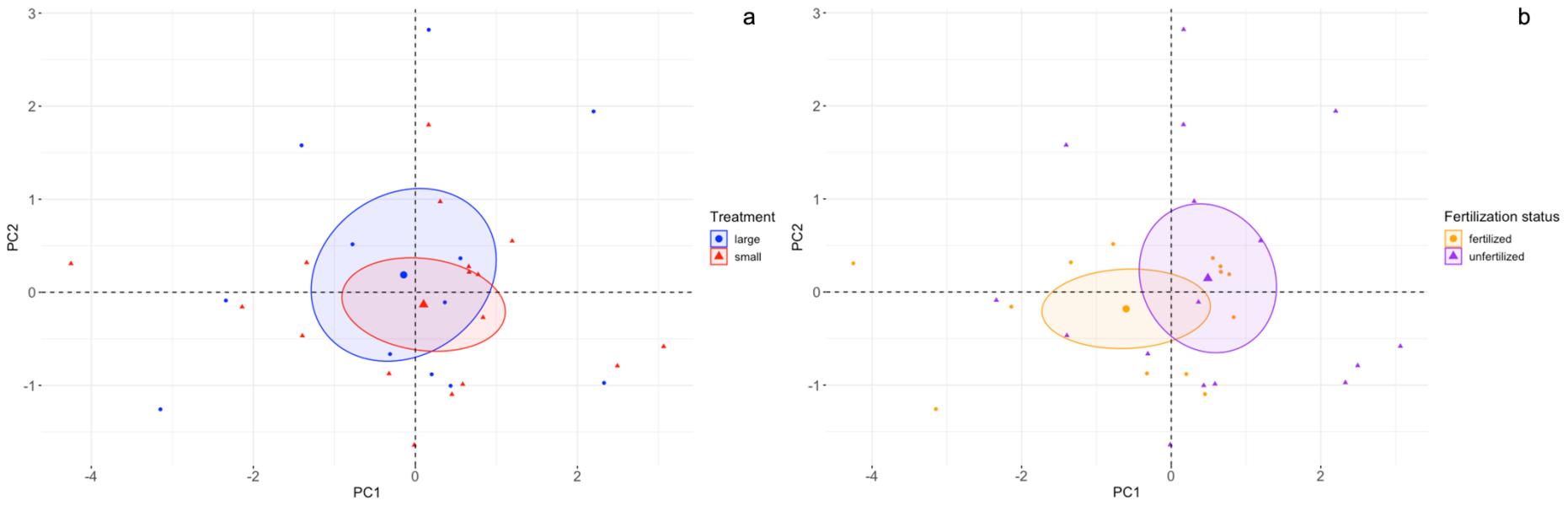

Figure 4. PCA of the four corticosteroid metabolites identified the fish eggs for samples of both group sizes and fertilization states. (a) Large (blue circles) and small (red triangles) groups. (b) Fertilized (orange circles) and unfertilized (purple triangles) eggs. In both panels, individual samples are depicted with small symbols whereas the mean value of each group size is depicted with large symbols.

Supplementary information

Egg-mediated maternal effects in a cooperatively breeding cichlid fish

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Supplementary Information Figure S1-S2, Table S1-S6

Supplementary Tables

Table S1. Initial individual size in large groups when the groups are first established in their home tanks. The group structure was taken from Fischer et al. 2015.

Size range (cm)	Status	Number	Sex
1.5-2.5	Small helper	2	Unknown
2.6-3.5	Medium Helper	2	Female
3.6-4.0	Large helper	1	Male
3.9-4.0	Large helper	1	Female
4.1-4.6	Large helper	1	Male
4.7-5.2	Large helper	1	Female
5.2-5.5	Dominant breeder	1	Female
5.5-6.0	Dominant breeder	1	Male

Table S2. Results of the full, initial linear mixed model (LMM), to test the effect of group size, spawning sequence and their interaction, and female body condition on egg mass. Sample sizes: small groups n = 11 clutches (spawning sequence 1: n = 7, spawning sequence 2, n = 4). Sample size: large groups n = 9 clutches, (spawning sequence 1: n = 2, spawning sequence 2: n = 7).

	Estimate ± S.E.	t	p
Model AIC: -193.3			
Intercept	0.0002 ± 0.00009	2.96	0.014
Spawning sequence (spawning 2)	0.00009 ± 0.00006	1.48	0.17
Group size (small)	0.0000 ± 0.00006	0.93	0.37
Female body condition	0.00005 ± 0.00003	1.67	0.12
Spawning sequence (spawning 2) x group size (small)	-0.00007 ± 0.00009	- 0.74	0.48

Table S3. Generalized linear mixed-effect model (GLMM), to test the effect of group size, spawning sequence, and female body condition on clutch size. Sample sizes: small groups: n = 10 clutches (spawning 1: n = 7, spawning 2: n = 3); large groups, n = 11 clutches (spawning 1: n =4, spawning 2: n = 7). Estimates refer to factor levels given in brackets. Significant p-values are in bold except for the intercept.

	Estimate ± S.E.	z	p
Intercept	2.292 ± 0.558	4.11	< 0.001
Spawning sequence (spawning 2)	0.804 ± 0.224	3.59	**0.00034**
Group size (small)	0.565 ± 0.215	2.63	**0.0086**
Female body condition	0.513 ± 0.168	3.05	**0.0023**
Spawning sequence (spawning 2) x group size (small)	-0.487 ± 0.319	-1.53	0.13

Table S4. Percentage of explained variance for each dimension of the PCA on egg corticosteroids for the full data set including fertilized and unfertilized eggs from large and small groups.

	Eigenvalue	Variance (%)	Cumulative variance (%)
Dimension 1	2.60	64.96	64.96
Dimension 2	1.08	26.87	91.84
Dimension 3	0.22	5.55	97.38
Dimension 4	0.11	2.62	100

Table S5. Summary table of the full initial linear model (LM), of the individual scores along PC1 investigating the effect of group size and fertilization state on corticosteroid content in eggs. Reference categories for the estimates are given in brackets.

	Estimate ± S.E	t	p
Intercept	-0.791 ± 0.807	-0.98	0.34
Group size (small)	0.272 ± 0.969	0.28	0.78
Fertilization state (unfertilized)	0.971 ± 0.988	0.98	0.34
Group size (small) x fertilization state (unfertilized)	0.348 ± 1.261	0.28	0.79

Table S6. Summary table of the full, initial LM, of the individual scores along PC2 of the PCA investigating the effect of group size and fertilization state on corticosteroid metabolite content in eggs. Reference categories for the estimates are given in brackets.

	Estimate ± S.E	t	p
Intercept	-0.315 ± 0.538	-0.59	0.56
Group size (small)	0.193 ± 0.646	0.3	0.77
Fertilization state (unfertilized)	0.753 ± 0.659	1.14	0.26
Group size (small) x fertilization state (unfertilized)	-0.775 ± 0.841	-0.92	0.33

Supplementary figures

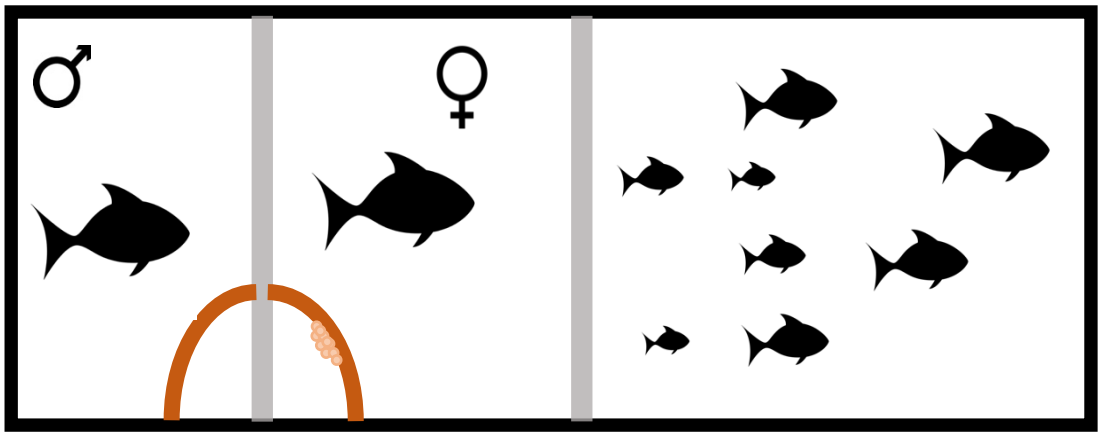

Figure S1. Experimental set-up used to collect unfertilized clutches ('spawning 1 and 2'). For spawning, *N. pulcher* females and males jointly visit the breeding chamber, where the female deposits eggs on the chamber walls, which are immediately fertilized by males. In the set-up we built a "shared shelter" around a transparent divider, which could be visited simultaneously by the breeder male and the female (i.e., breeder or large helper), and where they could court each other, which stimulate females spawning. Yet, the breeders had no physical contact. This method allowed to collect unfertilized eggs from females, since the transparent divider prevented the fertilization of the eggs by the male. The breeder male (left compartment) was separated from the female (middle compartment) by a transparent partition (left grey vertical line). The rest of the group was separated from the breeders by another transparent partition (right grey vertical line; set-up adapted from (Maldonado, 2017).

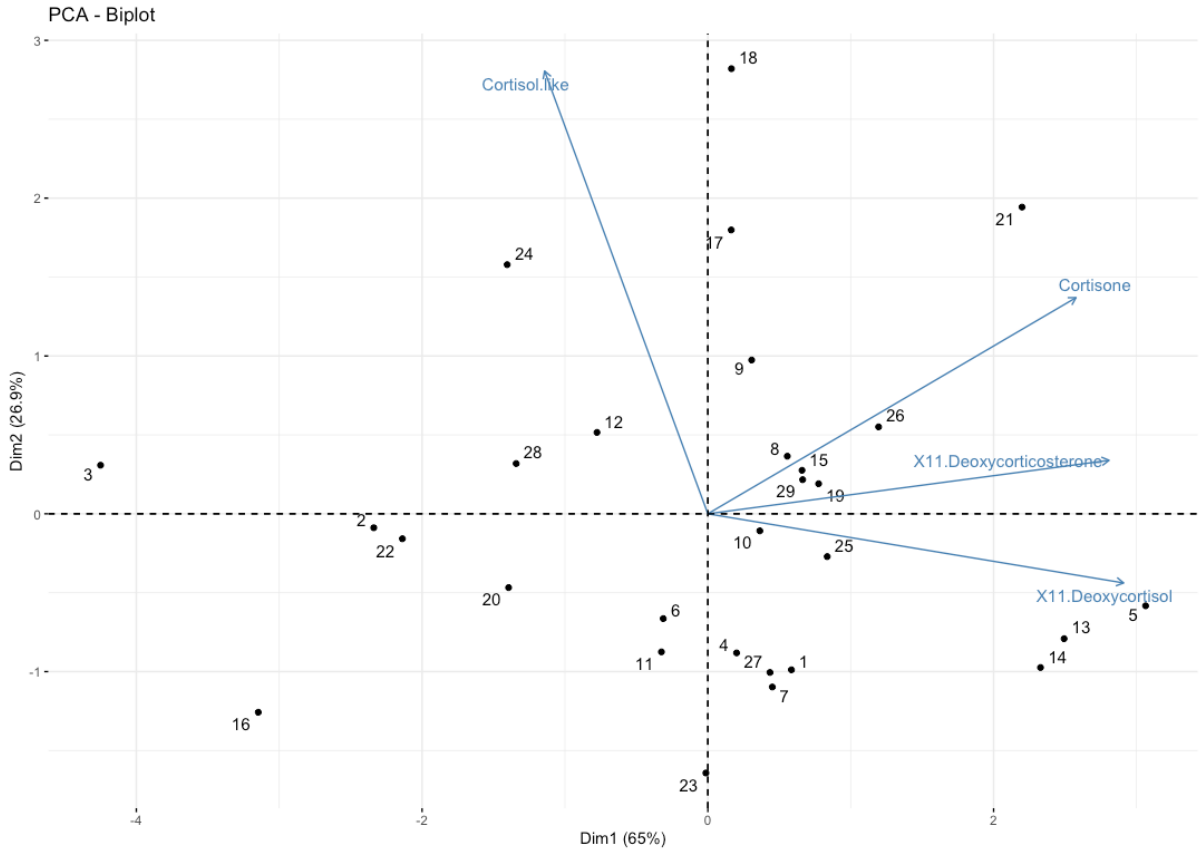

Figure S2. Biplot of the dimension 1 of the principal component (PC1), cortisone, 11-deoxycorticosterone, and 11-deoxycortisol load positively in this dimension whereas the cortisol-like metabolite loads in dimension 2 of the principal component (PC2).

References

Maldonado, M. (2017). *Mate choice in a cooperative breeder*.

Chapter 3

Behavioural phenotypes in a wild population of a cooperatively breeder

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Abstract

In cooperatively breeding species, subordinates can obtain group membership through social interactions with other group members or providing services such as helping with territory defence. Large subordinate individuals which can reproduce are expected to adjust their behaviour as a function of the demand of help and group size because if the environmental conditions allow, they may either leave the group to start breeding or queue for the breeding position in their natal group. There are few studies that investigate the effect of group size on the behavioural strategies used by large subordinate helpers in wild populations. We conducted behavioural observations of a wild population of the cooperatively breeding species *Neolamprologus pulcher* to assess if group size determines the social behavioural strategy of large subordinate individuals. We found evidence that in small social groups, large subordinate individuals tended to show a lower frequency of submissive behaviour and a higher frequency of sand digging. However, they did not increase their territory defence in the presence of a heterospecific egg and larvae predator. There was no evidence that dispersal propensity, measured as prospecting frequency, was shaped by group size. A PCA revealed that prospecting is uncorrelated with submissive behaviour and helping behaviour. Our results suggest that group size may be involved in shaping behavioural phenotypes of juvenile subordinates.

Key words: cooperative breeding, group size, dispersal, social behaviour, helping, cichlid

Introduction

Membership in social groups of cooperatively breeding animals is regulated through social interactions between members, such as aggression and submissive behaviour, which serve to build and maintain the social hierarchy within a group (Lukas & Clutton-Brock, 2018). Importantly, the behavioural traits expressed during social interactions should be adjusted such that they are appropriate in a given social context (Arnold & Taborsky, 2010; Oliveira, 2009; Taborsky et al., 2012; Taborsky & Oliveira, 2012). For example, subordinate individuals, which express a higher frequency of submissive behaviour towards a dominant group member may gain access to a shelter to hide from predators (Bergmuller et al., 2005; Taborsky et al., 2012). Submissive behaviour is an honest signal to communicate submission to a recipient and consequently can be used to avoid or reduce the latter's aggression (Reddon et al., 2021). Alternatively, subordinates can achieve tolerance by dominants by investing energy and resources in favour of breeder's offspring, such as defending the territory against predators (Bergmüller & Taborsky, 2005; Naef & Taborsky, 2020; Zöttl, Heg, et al., 2013).

If both strategies, helping and showing submissive behaviour, are effective in appeasing the aggression by dominants, which of these behaviours is shown should be influenced by the group members' needs, which are often influenced by group size, as well as the cost of expression the behaviour by the actor and individual behavioural specialization. In different socially living taxa, subordinate individuals adjust their helping effort as a function of group size and their own proximity to a breeding vacancy (e.g., paper wasps (Cant & Field, 2001), cichlid fish (Zöttl, Chapuis, et al., 2013)). In large social groups, high ranked individuals may interact more with similarly ranked individuals to maintain their rank in the hierarchy, which may lead to a higher relative workload for lower ranked individuals (Fischer et al., 2014). More generally, the demand by dominants for help may increase when the social group is small because the workload per individual is higher (Josi et al., 2020; Kingma et al., 2010). However, an additional member in a large group does not always increase the fitness of dominants, or the increase may be incremental (Kingma et al., 2014). Theoretical models predict that the direct fitness benefits reach a plateau when the group size increases (Powers & Lehmann, 2017). Hence, from a certain number of helpers being present onwards, benefits of a larger group size may diminish, but the cost for breeders increase, for instance by higher local resource competition (Brouwer et al., 2006; Mumme et al., 2015), helpers may steal fertilizations from

dominant breeders (Dierkes et al., 2005) or because the ability of breeders to control the contributions by helpers and to enforce help decreases (Koenig et al., 1992; Powers & Lehmann, 2017). Hence, individual contributions to helping behaviour should depend on group size.

Group size may shape subordinate behaviour, which is important to retain membership in social groups (Fischer et al., 2015). Group size can also give rise to divergent behavioural phenotypes (Taborsky, 2021) as demonstrated in a laboratory study on the cooperatively-breeding fish *Neolamprologus pulcher* (Fischer et al., 2017). In this species juveniles specialize in two types of social behaviours: helping behaviour and submissive behaviour. This is correlated with two emerging life history trajectories, namely, to remain as helper at the natal territory ('natal philopatry') or to disperse and breed elsewhere (Fischer et al., 2015, 2017). A full factorial experiment conducted in the laboratory demonstrated that early in life the presence or absence of adults in a social group triggers a higher frequency of submissive behaviour and a higher propensity to remain philopatric in an environment with low predation (i.e., low need of help) (Fischer et al., 2017). In contrast, the propensity to show helping behaviour early in life was high when adults were absent, and these individuals were more likely to leave the natal territory (natal dispersal, (Fischer et al., 2017)). In addition, several field studies tested how much helping or submissive behaviour *N. pulcher* group members showed when the demand of help (Fischer et al., 2014), the predation risk (Heg & Taborsky, 2010) or the number of group members is manipulated (Bruintjes & Taborsky, 2011). However, so far it has not been demonstrated that the two behavioural phenotypes are present in large and small groups of *N. pulcher* in their natural environment.

We conducted a field experiment in a wild population of the cooperatively breeding fish *N. pulcher* to assess if group size influences their social behavioural phenotype. We focused on large subordinate individuals, which possess a relatively high rank in the size-based hierarchy of these fish (Dey et al., 2013) and are sexually mature. In this species, the evolution of sociality is driven by high predation pressure, and group size across populations varies with local predation risk (Groenewoud et al., 2016). Breeding pairs alone cannot sustain an own territory due to high predation risk, and a field experiment showed that fish are more philopatric when predation risk is high (Heg et al., 2004). Hence to be a member of a social group with access to the safety of a territory providing shelter and defence by breeders and other helpers is key for the survival of subordinates. We predicted that in small groups, where

dominant breeders have a higher demand for help, the subordinate individuals will show a higher frequency of helping behaviour to achieve acceptance at a territory by the dominant breeders, and in large groups, with lower demand for help, we predicted a higher frequency of submissive behaviour relative to the received aggression by other group members to achieve acceptance on the territory. We further predicted that the propensity to disperse from the natal territory and to join larger, safer groups (Reddon et al., 2011) or to breed independently (Fischer et al., 2017; Jungwirth, Walker, et al., 2015) is higher in small groups with high predation risk compared to large groups.

Methods

Study species

N. pulcher inhabit territories that contain hiding structures such rock and gastropod shells (Groenewoud et al., 2016), which they defend from predators and competitors (Taborsky & Limberger, 1981). Breeders lay their eggs in a central shelter (the 'breeding chamber'), and subordinate individuals often have a private shelter to hide from predators (Werner et al., 2003). Subordinate brood care helpers may be allowed access to the breeding chamber to provide alloparental care for the breeder's brood by fanning and cleaning the eggs. Helpers also maintain the territory by removing sand from shelters (Bergmüller & Taborsky, 2005). In addition, they defend the territory against piscivorous (e.g., Neolamprologus elongatus) and egg predators (e.g., *Telmatochromis vitattus*), as well as con- and heterospecific space competitors (Groenewoud et al., 2016).

Groups of this cooperatively breeding species yield stability over years by increasing their size (Heg et al., 2005) and increasing the survival of new recruits (i.e., dominants' offspring) (Brouwer et al., 2005). Breeders and subordinates are organized in a linear, size-based hierarchy (Dey et al. 2013), which comprise up to 25 members that differ in size, sex, and relatedness (Taborsky, 2016). Large and medium-sized individuals do most of the territory defence against piscivore predators and territory maintenance, whereas small subordinates do most of the alloparental egg care and defence of the breeding chamber from egg predators (Bruintjes & Taborsky, 2011).

There is a multitude of social behaviours used during social interactions among group members and neighbours that allow maintaining the social structure and stability of groups. The most common social behaviours are (i) aggressive displays, which are threat displays not involving physical contact; (ii) overt aggression involving physical contact like ramming and biting; and (iii) submissive behaviour that is often shown in response to threat displays and overt aggression and serve to appease aggression by other group members (Arnold & Taborsky, 2010; Bergmüller & Taborsky, 2005). In addition, subordinate individuals perform affiliative behaviour towards breeders and other group members; this behaviour consists of light touches of the belly of a dominant with the mouth (Taborsky, 1984).

Study population

All data were collected by SCUBA diving at the southern tip of Lake Tanganyika in Zambia, East Africa, in the population of Chikonde (Groenewoud et al., 2016), which is located at the coast of Mutondwe Island. In this population we chose one colony that lived between a depth of 7.6 and 9.3 m and measured 50.7 x 40.6 x 22 x 25.1 m in area. The habitat consisted of a sandy bottom with scattered rocks and empty gastropod shells.

Selection of focal groups and focal fish

We selected 26 groups, 13 small and 13 large groups. Group size was classified following Fischer et al. (2014). Groups of 5 ± 0.59 members (mean ± standard error) were classified as small, groups of 14.92 ± 0.57 members were classified as large. Group sizes in between small and large groups (i.e., groups with 10-12 members), were not used since the difference in group members between large and small group was marginal. As focal fish, in each group we caught one helper of a standard length of 4.38 ± 0.037 cm (mean ± s. e.), which was sexed and measured (i.e., standard length), a tiny 3mm-long fin clip was taken of the tip of the anal fin stored for future parentage analysis, and they were tagged underwater using a unique elastomer mark (Jungwirth et al., 2019). The combination of fin clip and unique elastomer mark allowed us to quickly detect and identify the focal individual in its group when arriving at a territory, to record its behaviours and for catching before behavioural experiments. Only one individual disappeared from the colony during the entire study period; therefore, we caught a second individual with a similar size from the same social group as a replacement.

Study design

To characterize the behavioural phenotype of focal individuals, we video recorded and live scored all the behaviours between the focal individual and (i) other group members, (ii) conspecific non-group members, (iii) competitors, and (iv) predators. The behaviours of the focal fish were scored in five different tasks set in a consecutive order (Fig. 1). First, there was the baseline behavioural observation, in which individuals could interact with others without any experimental manipulation, and which allowed us to estimate the spontaneous occurrence of behaviours in large and small groups. Second, an experimental manipulation of submissive behaviour was done twice (termed 'elicited-submissive-behaviour (Esb) task' 1 and 2), which aimed at increasing the frequency of submissive behaviour (Fischer et al., 2014). Third, a behavioural manipulation of demand of help that consisted in two different 'helping tasks' that served as an opportunity for focal individuals to provide more help in two different ways. The first task was defending the territory against an egg and larvae predator that may eat the breeders' current brood. The second task consisted of maintaining the territory by digging away sand from the breeders' shelters (Bruintjes & Taborsky, 2011; Taborsky, 1984).

Behavioural observations

As defence behaviour occurs frequently and at high speed, and typically many group members contribute to defend against a particular predator or space competitor, we decided to score the defence behaviour of the focal individual from video recordings made during the territory defence task. All other behaviours were live recorded while SCUBA diving, and they were noted by pencil on writing slates.

Spontaneous behaviour to obtain baseline behaviour

Before any of the experimental manipulations (see below), we recorded 20 min of the spontaneous behaviour of the focal fish to obtain an undisturbed baseline (i.e., 'baseline observation).

Experimental manipulation of behaviour

Submissive behaviour

To elicit submissive behaviour of the focal fish, we applied the same method as described by Fischer et al. (2014). In brief, focal individuals were caught and placed inside a transparent cylinder (15 cm length and x 7 cm diameter). The bottom of the cylinder and the top lid were perforated, allowing full visual and olfactory exchange between all group members and the focal fish. The cylinder was left during a period of 24-h close to the observed private shelter of the focal individual. In the study by (Fischer et al., 2014), this manipulation led to an increase of the frequency of submissive and helping behaviours of *N. pulcher* subordinates after they were released from the cylinder after 24-h. Twenty-four hours after the focal individual was released from the cylinder, we immediately recorded all the behaviours performed during 10 min. The same procedure was repeated for a second time after 2 days (see Fig. 1). We named these two tasks 'elicited submissive behaviour (Esb) 1 and 2'. We did not sum the behaviours of these two tasks, because we performed the first and the second helping tasks (see below) in between the two 'elicited submissive behaviour' tasks (Fig. 1); therefore, the frequency of submissive behaviour might have been adjusted after the first helping task. Submissive behaviour occurs in a low frequency, and the tasks 'elicited submissive behaviour 1 and 2' represent only a total of 20 min behavioural observation. Therefore, to increase the statistical power that allows to detect differences in submissive behaviour between group sizes we also analysed the frequency of submissive behaviour across tasks (i.e., baseline, elicited submissive behaviour 1 and 2, territory maintenance and defence), which accounts for a total of 60 min observation time.

Helping tasks

To elicit helping behaviour, we exposed the groups to two different 'helping tasks' known to prompt either sand digging ('territory maintenance' task) or defence behaviour against an egg-predator ('territory defence' task). Each task was presented only once in each group. In Fig. 1, both are denoted as 'helping task 1 and 2', because the order of the territory defence and territory maintenance tasks was balanced across large and small groups to prevent a sequence effect; half of the large and half of the small groups first received the territory defence task ('Helping task 1' in Fig. 1) and second the territory maintenance task ('Helping task 2' in Fig.1), and in the other half of the groups it was the reverse order.

Territory maintenance. To increase the frequency of territory maintenance behaviour by the focal fish, which in *N. pulcher* consists mostly of digging sand out of the breeding chamber (Taborsky, 1984), we added sand to the breeding chamber of our groups (see Bruintjes and Taborsky, 2011; Taborsky and Riebli, 2020). This reduces the hiding space for the breeders, but not for the focal subordinate itself, as subordinates have their own hiding places at the territory (Werner et al., 2003). Therefore, removing sand from the breeding chamber by subordinates is considered as helping behaviour (Bruintjes & Taborsky, 2011). In this helping task we scored the frequency of digging by the focal fish for 10 min immediately after the sand was added.

Sand digging across tasks. Sand digging out of the breeding chamber by the focal fish occurred in a low frequency, which decreases the statistical power to detect difference between group sizes. Hence, we scored sand digging by the focal fish across all other tasks (i.e., baseline, elicited submissive behaviour 1 and 2 and territory defence). This is an energetically costly behaviour (Grantner & Taborsky, 1998) use to pay-to-stay in the territory (Bergmuller et al., 2005; Zöttl, Heg, et al., 2013) and it could be traded-off with other equally energetically costly behaviour such as submissive behaviour.

Territory defence. *T. vittatus* predates on eggs and larvae produced by the dominant breeders, and defence against these egg predators is assumed to raise the survival of the breeders' offspring. Hence, defence by subordinate individuals against *T. vittatus* is considered as helping behaviour (Bruintjes & Taborsky, 2011; Kasper et al., 2017). To elicit territory defence by the focal fish, we placed one *T. vittatus* (4.82 ± 0.035 cm; mean ± s. e.) as stimulus fish in a transparent Plexiglas tube of 15 cm length and 11 cm diameter, which was located close to the breeding chamber of the territory. In this task, we observed the focal fish during 10 min and scored live all the behaviours of the focal individual towards the stimulus fish.

In this task the behaviours occurred at a very high speed and live observations may not capture the complete range of aggressive behaviours of the focal fish towards the presented T. vitattus. Therefore, during our live observations, we simultaneously also recorded 10-min videos, which were further analysed by MRC using the software 'Solomon coder' (András, 2019) to quantify the frequency of aggressive behaviours by the focal fish towards the stimulus fish. Then, we compared the aggressive behaviours scored live by both observers (MRC, CS) and the behaviours scored in the videos by MRC to corroborate the accuracy of the

measurements. The two measurements, behaviours scored live and from the videos, were highly positively correlated (Spearman rank correlation, rho = 0.86, $p < 0.001$). Therefore, we used the aggressive behaviours scored from the videos when analysing focal defence behaviour against *T. vitattus* in in the territory defence task. We calculated the sum of all restrained and overt aggressive behaviours by the focal fish towards the presented T. vitattus, and used this sum, i.e., the total aggression towards the presented *T. vitattus* as measure of the focal fish's defence effort.

To assess the effect of the activity of the *T. vitattus* while being presented in the tube on the frequency of total aggression of the focal fish, we measured the activity of *T. vitattus* from the 'territory defence' task videos. This measurement was done in the following way. Every 30 seconds we recorded if the *T. vitattus* inside the tube was moving or not. Then, we divided the number of times the stimulus fish moved by the total duration of the video where we scored the behaviour. This score was called 'activity *T. vittatus*' and it was used for analysis because higher activity of *T. vittatus* it is known to elicit more defence behaviour in *N. pulcher* (Jungwirth, Josi, et al., 2015).

Prospecting behaviour

Previous studies showed that prospecting behaviour of subordinate *N. pulcher* in neighbouring groups precedes dispersal and increases their chance of successful dispersal (Jungwirth, Walker, et al., 2015). Therefore, during all tasks (in total 60 min), we counted how often the focal individual stopped interacting with the group members and swam to a neighbouring group. We carefully followed the fish by observing it and if it was close to getting out of sight, we followed it from a distance. We waited until the focal fish returned to its territory to continue counting the behavioural interactions between the focal fish and other group members.

Statistical analyses

We fitted General Linear Models (GLMs) with negative binomial distribution and Generalized Linear Mixed-effect Models (GLMMs), where we assumed either Poisson or binomial or negative binomial distributions as indicated below. The significance of fixed factors was tested by Likelihood Ratio Tests (LRT). We used the statistical software R, version 4.1.2. (R Core Team, 2021) and the R packages "car" (Fox & Weisberg, 2019), "MASS"

(Venables & Ripley, 2002), “lme4” (Bates et al., 2015) and “lmerTest” (Kuznetsova et al., 2017).

For fixed factors with more than two levels (i.e., type of task) we performed pairwise Post-Hoc tests to assess how the means of the levels differed, using the package “emmeans” (Lenth, 2022).

Model selection was done using the Akaike’s Information Criterion (AIC) (Engqvist, 2005). The factors in the full models were stepwise backward selected, starting with two-way interaction terms. If a term did not significantly explain the variance of the dependent variable ($p > 0.05$), we fitted a model with and without the non-significant factor and kept the model with the lower AIC value if it was at least lower by a value of 2. In case the models had a similar AIC we consider the models were equivalent. Thus, if the interaction term was not significant and the models were equivalent, we backward selected the interaction term and kept the simplified model. Group size and type of task (except in models where the data of only one task was included) were always kept as factors in the models by default. Initial full models are in the SI section (Table S1 and S2). All data plots were done using the package “ggplot2” (Wickham, 2016).

Submissive behaviour

We first assessed if the frequency of submissive behaviour by the focal fish towards other group members was correlated with the frequency of aggression received by all other group members. Then, we run a GLMM with Poisson distribution to assess if the frequency of submissive behaviour in the two elicited-submissive-behaviour tasks was explained by ‘group size’, ‘task’ and the interaction between the two, ‘total aggression received by other group members’ and ‘observer’ (Full model Table S1).

Furthermore, we fitted a GLMM with Poisson distribution to assess if the frequency of submissive behaviour across all tasks (i.e., baseline, elicited submissive behaviour 1 and 2, territory maintenance and defence) was explained by any of the following fixed factors: ‘group size’, ‘task’ and their interaction , ‘observer’, ‘sex of the focal fish’, ‘standard length of the focal fish’, ‘total aggression received by other group members’ and ‘frequency of sand digging

in all tasks' (full model in Table S2); the latter two fixed terms were included, because received aggression (i.e., total aggression) and the frequency of sand removal by digging during territory maintenance are known to influence submissive behaviour towards dominants (Naef & Taborsky, 2020). Group identity was included in both models as random factor because we evaluated the behaviour of the same fish in multiple tasks.

Territory maintenance task and sand digging across tasks

As digging behaviour occurred at very low frequencies in the territory maintenance task and across all tasks as well, yielding low statistical power, we did a Fisher exact test to assess if sand digging in territory maintenance and across tasks were associated with group size.

Territory defence

A GLM with negative binomial distribution was fitted to test if the defence by only the focal fish was explained by 'group size', 'standard length of *T. vittatus*', and '*T. vittatus* activity'. Both, the activity and the of size of *T. vittatus* elicited defence behaviours by *N. pulcher* in previous field studies (Bruintjes & Taborsky, 2011). In the model of this task we included a smaller number of fixed factors to avoid overfitting.

Prospecting

A GLMM with negative binomial distribution was fitted to assess how much of the variance in prospecting behaviour done by the focal fish was explained by 'group size', 'task', 'sex' and 'standard length' of focal individual', 'received aggression by other group members', 'submissive behaviour towards other group members', and observer. Submissive behaviour appeases dominant breeders and facilitates acceptance in the group; conversely received aggression can lead to eviction from the territory (Arnold & Taborsky, 2010). Few individuals did prospect in the 60 min behavioural observation, which correspond to the observation time in all tasks. To avoid overfitting of the models, we did not include any interaction terms between fixed factors. To account for multiple observations in the five tasks, we included group identity as random factor

Correlation between behaviours across tasks

To assess which behaviours better explain the behavioural profile of focal individuals a principal component analysis (PCA) was done by using the package "factoextra v. 1.0.7" (Kassambara, 2020); for a graphical representation of the first two principal components (PC 1 and 2) we used the package "FactoMineR v. 2.4" (Houseman et al., 2006). In addition, a correlational matrix was drawn to show how behaviours are correlated between different tasks irrespective of group size and within each group size. The visual representation of the matrices was done using the package "ggbeeswarm" (Clarke & Sherrill-Mix, 2017) (Fig. S1-S3).

Results

Submissive behaviour

The submissive behaviour focal fish did towards other group members was positively correlated with received aggression (Spearman, rho=0.54, $p < 0.001$; Fig. 2). In both elicited-submissive-behaviour (Esb) tasks, focal fish from small groups tended to be less submissive than focal fish in large groups (Table 1, Fig. 3. See full model in Table S1). Furthermore, the task where focal fish showed submissive behaviour has a significant positive influence on the frequency of submissive behaviour (Table 1, Fig. 4. See full models in Table S2). Focal fish from large and small groups showed significantly less submissive behaviour in the territory defence task in comparison with each of the following tasks: baseline, Esb 1, Esb 2 (Table 2).

Territory maintenance task

After filling up the breeding chamber with sand, focal fish of large and small groups did not differ in their frequency of territory maintenance (Fisher exact test, $p = 1$, Fig. 5a).

Sand digging in all tasks

Focal fish from small groups tended to dig more across tasks than their counterparts in large group (Fisher exact test, $p = 0.07$, Fig. 5b).

Territory defence

Focal fish in large and small groups did not differ in their defence behaviour towards the stimulus fish (Table 1, Fig. 6).

Prospecting

The frequency of prospecting across tasks did not differ between focal fish in large and small groups (Table 3, Fig. 7), neither between males nor females (Fig. S4). The comparison between tasks did not reveal a significant difference in prospecting behaviour, irrespective of the group size (Table S3). However, bigger focal fish that did prospect were more likely to show submissive behaviour towards other group members and received less aggression from other group members (Table 3).

Correlation between behaviours across tasks

The behavioural profile from focal individual belonging to a large group did not differ from fish in small groups (Fig. 8). The first 3 principal component (PC) explained similar amount of the variance PC1(27.72%), PC2 (23.54%), PC3 (19.97%). The other two components (PC4 and 5) explained 28.77% of the variance together. In territory defence (31.82%) and submissive behaviour (4.08%) loaded positively on PC1, and affiliative behaviour (29.24%) together with digging (29.01%) loaded negatively (Fig. 9). In addition, prospecting behaviour has the highest loading (54.23%) on PC2 (Table 4).

Discussion

Group size tended to influence the behavioural profile of subordinate individuals of a wild population of *N. pulcher*. In small social group with few helpers, focal fish tended to show less submissive behaviour towards others group members, but tended to increase sand digging behaviour. Territory defence and prospecting behaviour were not influenced by the size of the social group. However, prospecting behaviour explained most of the variance in the second dimension of the principal component which suggest that prospecting is different from other behaviours, namely territory defence, digging, submissive and affiliative behaviour.

Submissive behaviour and sand digging can appease dominants and increase the acceptance of subordinate individuals inside a territory (Bergmüller & Taborsky, 2005) but they have a different function. In a dyadic social interaction, a submissive animal is surrendering to an aggressive social partner. Here submissive behaviour is an honest signal to mediate potential conflicts between differently ranked individuals (Reddon et al., 2021). Physical postures to surrender are efficient to terminate a fight in mature fallow deer (*Dama dama*), pinguins (*Eudyptula minor*) (Reddon et al., 2021) and in cichlid fish (*N. pulcher*) (Arnold & Taborsky, 2010; Taborsky et al., 2012). In *N. pulcher*, digging sand underneath stones creates new hiding places that can be used by the digging individual and also by any group member. These hiding places, called shelters, are indispensable for the survival of *N. pulcher* because the predation risk is high (Groenewoud et al., 2016). Sand digging is particularly important, if the territories of *N. pulcher* have sandy bottoms, as there the sand can enter shelters by water movements, and thereby make them inaccessible from hiding for predators attack.

We had predicted that subordinates have higher helping propensity in small as compared to large groups, because the need for help per helper is higher with few subordinates present and also the predation risk is higher in small groups (Groenewoud et al., 2016; Heg et al., 2005). Indeed, focal fish in small groups tended to contribute more to digging away sand underneath stones to create or maintain shelters than did subordinate individuals in large groups. There are two possible explanations for this. First, large subordinate fish that invest energy in digging (Grantner & Taborsky, 1998) instead of using energy to growth or reproduce, may employ this behaviour as a mechanism to pay for being allowed to stay inside the territory (Bergmuller et al., 2005; Fischer et al., 2014). Large subordinates are sexually mature and would be large enough to disperse into another group, but often opt to stay in the current group because dispersal is risky in population with high predation risk, as it is the case in our study population (Groenewoud et al., 2016). Second, if large subordinates remain inside a small group, they may have a higher probability to inherit the breeding position because the reproductive queue is shorter.

The size of the social group tended also to shape the frequency of submissive behaviour. In large groups, subordinates tended to show a higher frequency of submissive behaviour in comparison with subordinates in small groups. In a previous field experiment done with large and small social groups, subordinates were prevented to interact with other group members by

being confined in a transparent container; after release, when the interaction was possible again, submissive behaviour per received aggression was higher in large than in small groups (Fischer et al., 2014). In addition, semi-natural laboratory experiments reported that being in relatively larger social groups, more submissive behaviour per received aggression was shown, with likely positive effects on fitness, because it increased tolerance inside a territory and thus enhanced access to hiding places (Arnold & Taborsky, 2010; Fischer et al., 2017;Taborsky et al., 2012). Hence, our results provide further evidence that in large social group, the frequency of submissive behaviour, which represents an honest signal (Reddon et al., 2021) of non-challenging the breeding position, is higher than in small groups.

A conceptual review by Taborsky 2021 suggests that some behaviours, such helping and submissive behaviour together with philopatry may be part of the same behavioural phenotype. The review further proposed that individual behavioural phenotype and the size of the social group, to which an individual belongs, influence each other (Taborsky, 2021). Individual behavioural phenotype can influence group size, because it determines the acceptance rate of other group members. In turn, the social interactions among group members, which depend on the number of individuals (Kappeler, 2019), will shape individual behavioural phenotype. There is evidence on how social interactions influence the physiological mechanisms that modulate social behaviour in a social interaction. For example, when there is a high energetic cost of maintaining a high rank in a social hierarchy, which could be a product of a high frequency of dyadic interactions, dominant individuals have a high glucocorticoid level (Goymann & Wingfield, 2004). Furthermore, juveniles of the cooperatively-breeding marmoset (*Callithrix geoffroyi*) that experienced frequent rejections by other group members during early life had an increased stress axis reactivity to social isolation later in life (Birnie et al., 2013).

Philopatry is a behaviour than can be estimated by prospecting behaviour. Prospecting behaviour is a proxy for philopatry because individuals with higher frequency of prospecting are more likely successfully join another group (Jungwirth, Walker, et al., 2015). Interestingly, prospecting behaviour was significantly explained by submissive behaviour towards other group members and less received aggression by other group members. Contrary to our predictions, subordinate large helpers in small and large groups did not differ in their prospecting behaviour, possibly because one field season is not sufficient to obtain enough data to detect differences in group size. Alternatively, prospecting depends on the outside options

of individuals and environmental conditions (Guindre-Parker & Rubenstein, 2020), which is the case for most cooperatively breeding species (Koenig et al., 1992). Our results suggest that prospecting behaviour in *N. pulcher* is different from other behaviours like submissive behaviour because is represented in a different axis of the principal component analysis. Prospecting in subordinate *N. pulcher* involves interactions with members from neighbouring groups, while at the same time they act as subordinate helpers in their group of origin. Prospecting thus may prepare dispersal to another group, while a subordinate is still benefitting from the safety in their group of origin. This may indicate that prospecting is adjusted according to outside options rather than inside group demand (Zöttl, Chapuis, et al., 2013). Dispersal propensity to be associated with behavioural phenotype has been observed in females of cooperatively-breeding yellow-bellied marmots (*Marmota flaviventris*). Females that are more affiliated and embedded within the social network are more likely to remain philopatric (Blumstein et al., 2009). However, there is lack of studies that demonstrate the influence of social group size on individual behavioural phenotype and how the behavioural phenotype influences dispersal patterns (Wey et al., 2015).

Group size may be a selective force that determine a suite of social behaviours, which can result in a behavioural phenotype. The reason is that group size influences survival (Guindre-Parker & Rubenstein, 2020), the amount of care the dependent young will receive (Pike et al., 2019), the number and diversity of interactions among group members (Taborsky, 2021), and a modulator of juvenile' physiology (Creel et al., 2013), brain architecture (Solomon-Lane & Hofmann, 2019), and behaviour (Arnold & Taborsky, 2010; Fischer et al., 2017).

Conclusions

In a wild population of a cooperatively breeding fish, group size tended to influence two energetically costly behaviours (Grantner & Taborsky, 1998) known to enhance the probability to be accepted in a social group: submissive behaviour and sand digging. Interestingly, the performance of those behaviours did not prevent subordinates from assessing neighbouring territories. This suggests that individuals may opt to either to 'pay-to-stay' in the natal territory or show intensive submissive behaviour, which both appeases the aggression by dominants, and possibly wait until conditions are favourable for dispersal.

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Tables and figures

Table 1. Results after model selection: (1) Submissive behaviour in the two 'elicited submissive behaviour' tasks: A GLMM with Poisson distribution that tests how much of the variance in the frequency of submissive behaviour was explained by group size, task, total aggression received by other group members and observer. (2) Submissive behaviour across tasks: A GLMM with Poisson distribution that tests how much of the variance in submissive behaviour is explained by group size, the task where the behaviour was observed (i.e., baseline, elicited submissive behaviour 1 and 2, territory maintenance and defence), standard length and sex of the focal individual, sand digging in all tasks, observer and the received aggression by other group members. (3) Territory defence task: A GLM with negative binomial distribution that tests how much of the variance in territory defence by the focal fish was explained by group size, standard length and activity of the stimulus fish *T. vittatus*. Estimates refer to the factor levels given in brackets. The χ^2 values are in denoted with $^{(a)}$. Significant effects are in bold.

	Estimate ± S. E.	$z/\chi^{(a)}$	p
Submissive behaviour in the elicited submissive behaviour tasks, AIC 205.3			
Intercept	0.629 ± 0.243	2.59	< 0.001
Group size (small)	-0.445 ± 0.221	$3.51^{(a)}$	*0.061*
Elicited submissive behaviour task 2	0.198 ± 0.185	$1.15^{(a)}$	0.29
Total received aggression	0.1169 ± 0.0268	$14.66^{(a)}$	**< 0.001**
Observer (MRC)	0.2181 ± 0.2262	0.96	0.33
Submissive behaviour across tasks, AIC 441.5			
Intercept	0.928 ± 1.150	0.81	0.42
Group size (small)	- 0.316 ± 0.197	$2.35^{(a)}$	0.13
Task		$40.04^{(a)}$	**< 0.001**
(Elicit submissive behaviour task 1)	-0.005 ± 0.187		
(Elicit submissive behaviour task 2)	0.205 ± 0.181		
(Territory maintenance task)	-0.51 ± 0.225		
(Territory defence task)	-1.408 ± 0.329		
Sex (male)	0.088 ± 0.215	$0.17^{(a)}$	0.68
Total received aggression	0.135 ± 0.022	$33.85^{(a)}$	**< 0.001**
Sand digging across tasks	-0.0587 ± 0.101	$0.35^{(a)}$	0.56

Standard length focal fish (cm)	-0.0756 ± 0.254	0.09[(a)]	0.77
Focal fish territory defence, AIC 158.14			
Intercept	- 4.079 ± 7.042	-0.58	0.56
Group size (small)	0.297 ± 0.952	0.092[(a)]	0.76
Standard length *T. vittatus*	1.524 ± 1.578	0.69[(a)]	0.41
Activity *T. vittatus*	0.072 ± 1.394	0.0018[(a)]	0.97

Table 2. Submissive behaviour by the focal fish across tasks. Comparison between different tasks where focal fish did submissive behaviour towards other group members. Significant p values are highlighted in bold.

Comparison between tasks	Estimate	Standard Error	p
Baseline - Elicited submissive behaviour 1	0.005	0.187	1.00
Baseline - Elicited submissive behaviour 2	-0.205	0.181	0.79
Baseline - Territory maintenance	0.510	0.225	0.16
Baseline – Territory defence	1.408	0.329	**0.0002**
Elicited submissive behaviour 1 - Elicited submissive behaviour 2	-0.210	0.183	0.78
Elicited submissive behaviour 1 - Territory maintenance	0.505	0.232	0.19
Elicited submissive behaviour 1 – Territory defence	1.404	0.334	**0.0003**
Elicited submissive behaviour 2 - Territory maintenance	0.714	0.221	0.01
Elicited submissive behaviour 2 – Territory defence	1.613	0.325	**< 0.001**
Territory maintenance – Territory defence	0.899	0.352	*0.08*

Table 3. Prospecting behaviour. Summary table of the GLMM (AIC 181.3) with negative binomial distribution that tests for the effects of group size, task, standard length and sex of the focal fish, total aggression received by other group members, observer, and submissive behaviour shown towards other group members on the frequency of prospecting behaviour across tasks. Estimates refer to the factor levels given in brackets. The χ^2 values are denoted with the letter[(a)]. Significant effects are in bold except for the intercept.

	Estimate ± S. E.	$z/\chi^{(a)}$	p
Intercept	-9.687 ± 2.85	-3.40	0.001
Group size (small)	-0.443 ± 0.501	$0.78^{(a)}$	0.38
Tasks		$8.34^{(a)}$	*0.08*
(Elicited submissive behaviour task 1)	0.957 ± 0.533		
(Elicited submissive behaviour task 2)	1.123 ± 0.493		
(Territory maintenance task)	0.506 ± 0.555		
(Territory defence task)	-0.177 ± 0.733		
Standard length focal fish (cm)	1.401 ± 0.572	$4.75^{(a)}$	**0.029**
Sex (male)	0.247 ± 0.55	$0.2^{(a)}$	0.65
Received aggression	-0.565 ± 0.222	$9.43^{(a)}$	**0.002**
Submissive behaviour towards other group members	0.294 ± 0.142	$4.61^{(a)}$	**0.032**
Observer (MRC)	1.808 ± 0.488	$4.96^{(a)}$	**0.0001**

Table 4. Contribution to the first and second dimension of the PCA of each behaviour scored from focal fish in large and small groups.

Dimensions	Variable	Contribution to the dimension (%)
Dimension 1	Defence	31.82
	Submissive behaviour	4.08
	Affiliative behaviour	29.24
	Prospecting	5.84
	Digging	29.01
Total of the variance explained		27.72
Dimension 2	Defence	14.09
	Submissive behaviour	1.67
	Affiliative behaviour	11.91
	Prospecting	54.23
	Digging	18.10
Total of the variance explained		23.54

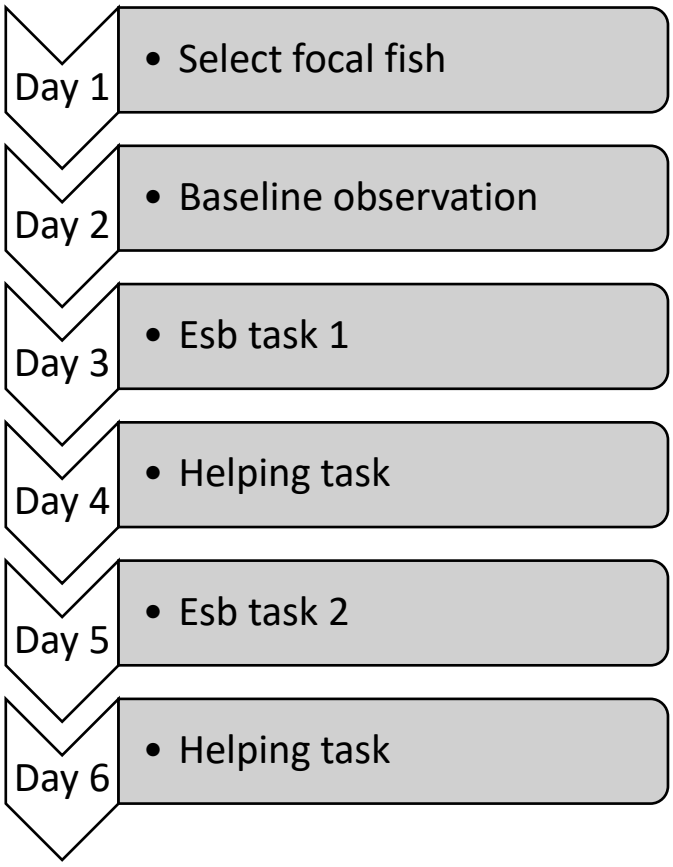

Figure 1. Order of the behavioural observations scored for the focal individuals in each task, including the baseline observation, the 'elicited-submissive-behaviour' (Esb) tasks 1 and 2, and two different 'helping tasks': 'territory defence' against a *T. vittatus* presented in a tube and 'territory maintenance', in which we counted sand digging events to clean the breeding chamber filled with sand. The order of the 'territory defence' task and the 'territory maintenance' task was balanced across large and small groups (see 'Methods') to prevent a sequence effect. Each 'helping tasks' was presented only once.

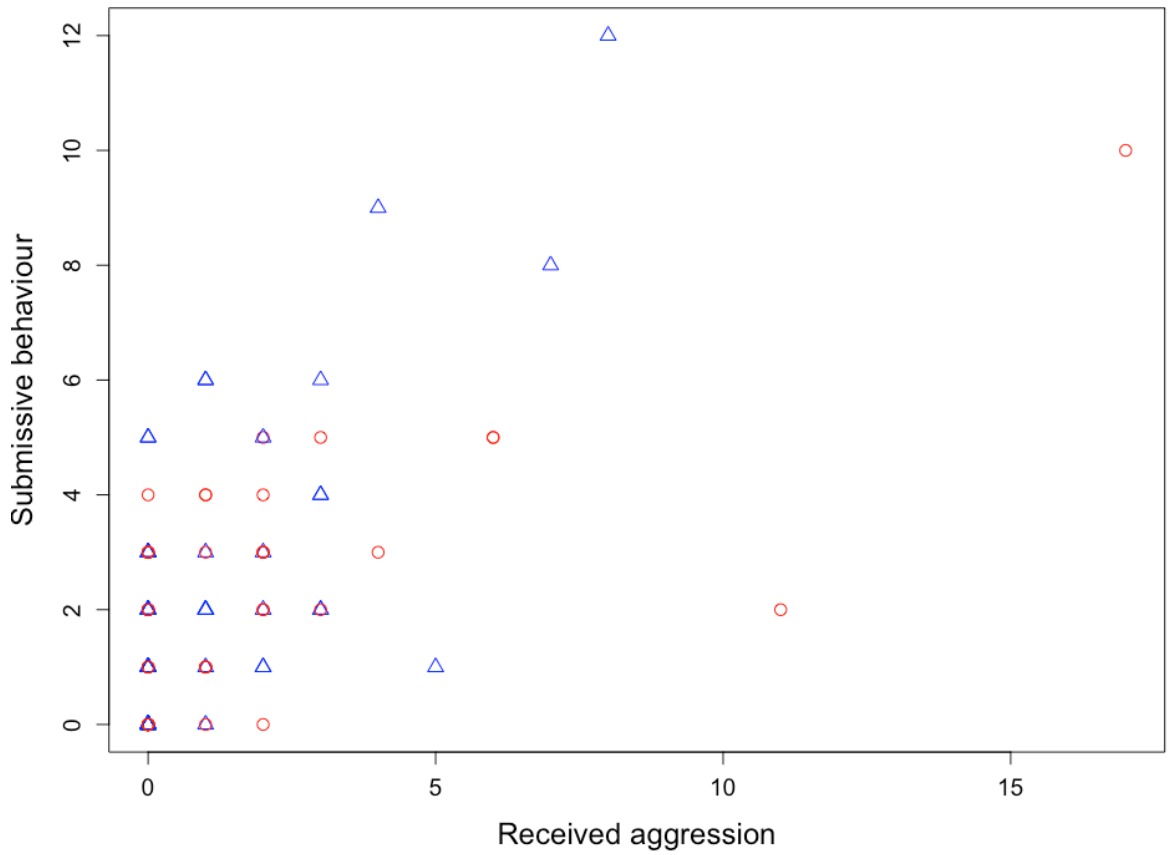

Figure 2. Correlation between submissive behaviour shown towards other group members and received aggression. The focal fish in large groups are depicted in red circles and small groups in blue triangles.

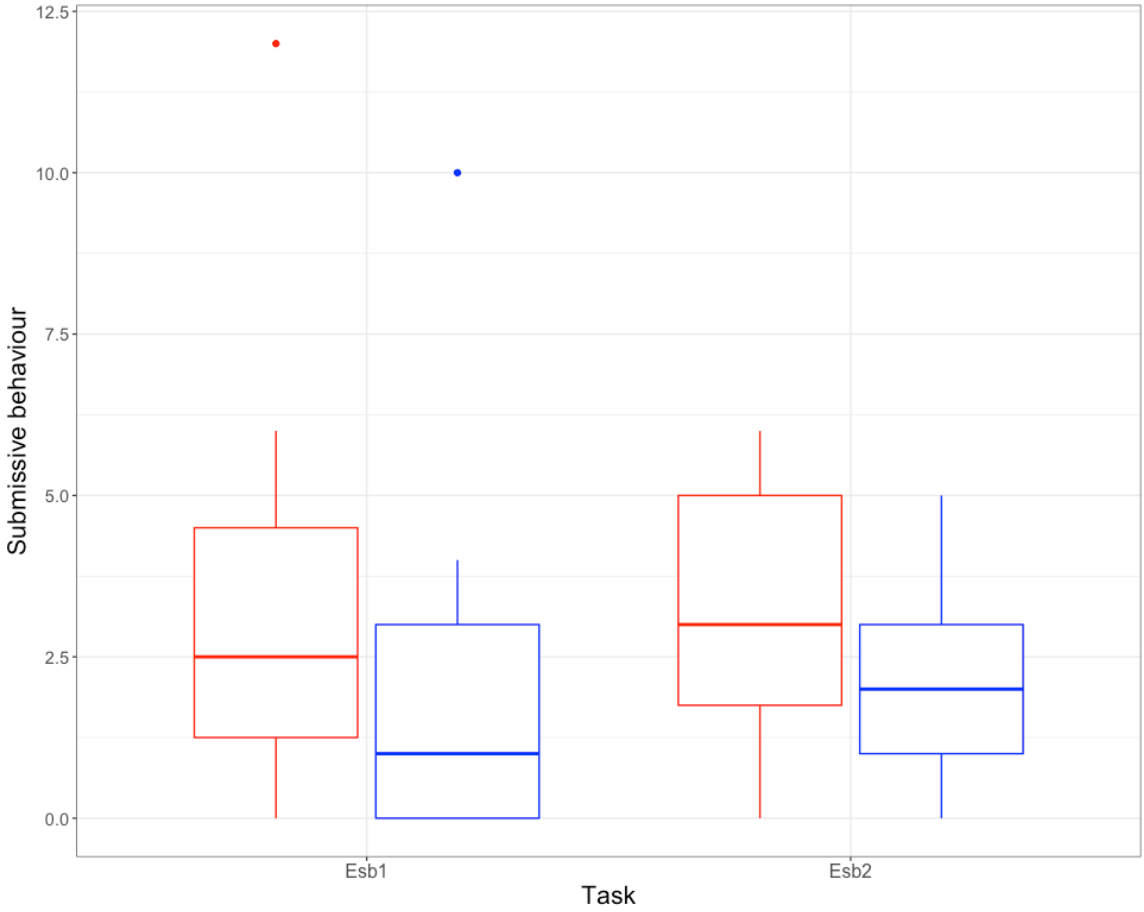

Figure 3. Submissive behaviour by the focal fish to other group members in the two elicited submissive behaviour tasks (Esb 1 and Esb2). Focal fish from small group are depicted in blue whereas large groups are in red.

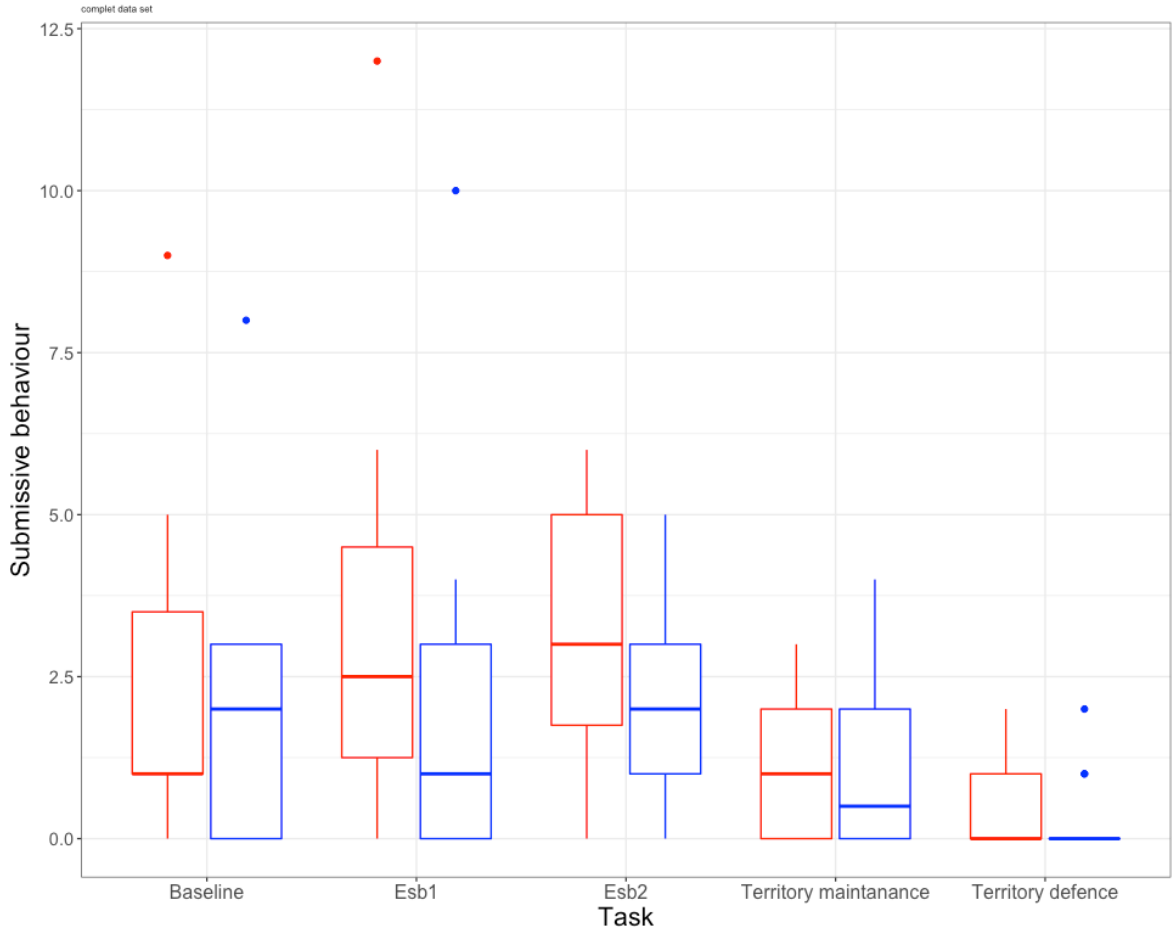

Figure 4. Frequency of submissive behaviour by focal individuals directed to another group members in the five different tasks: baseline, elicited submissive behaviour (Esb) 1 and 2, territory defence and maintenance. Focal individuals from large groups are depicted in red and focal individuals from small groups in blue.

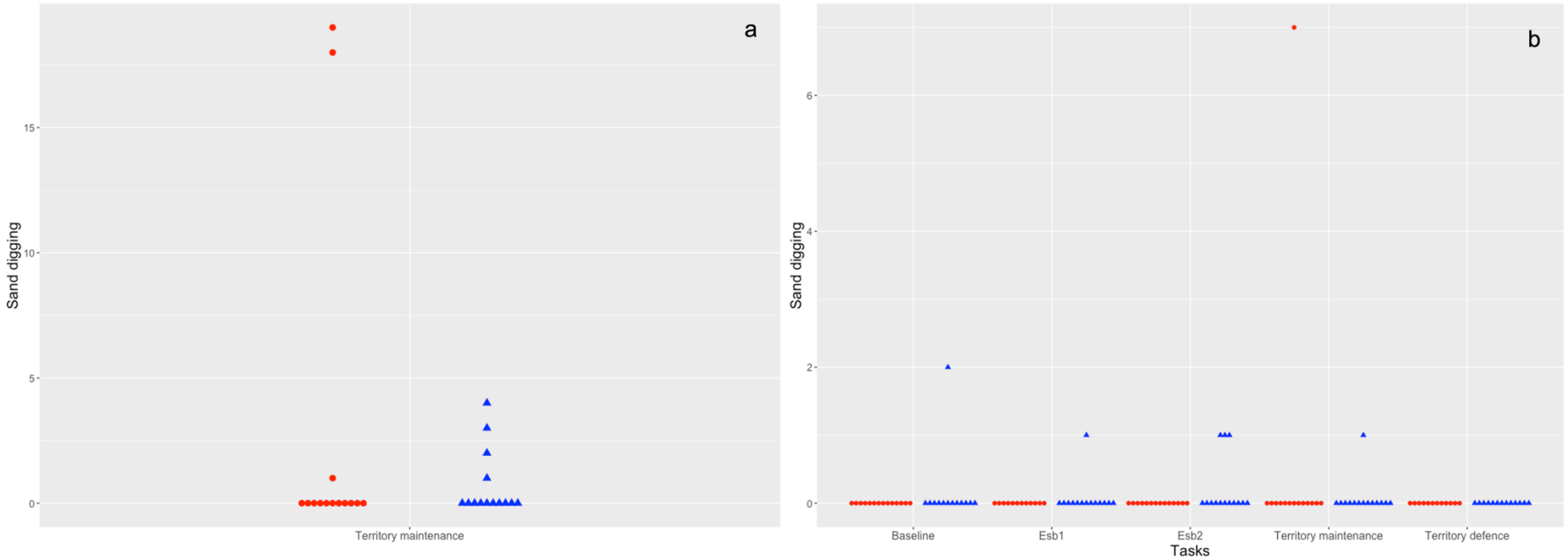

Figure 5. Sand digging by the focal fish. Focal fish in large (red triangles) and small (blue circles) groups. Raw data of (a) sand digging in the territory maintenance task (b) sand digging behaviour across tasks.

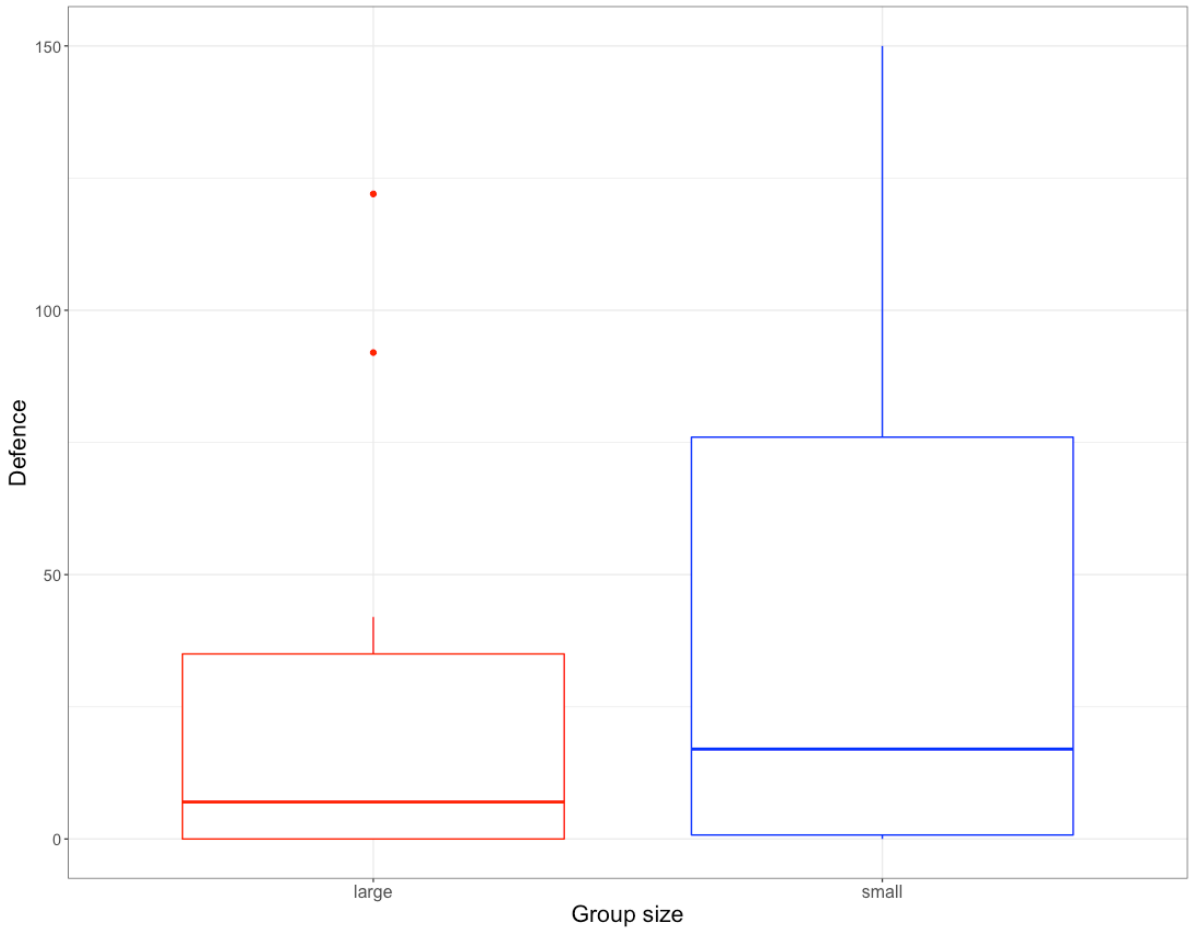

Figure 6. Territory defence by the focal fish. Boxplots of the frequency of total aggression towards the presented *T. vittatus*.

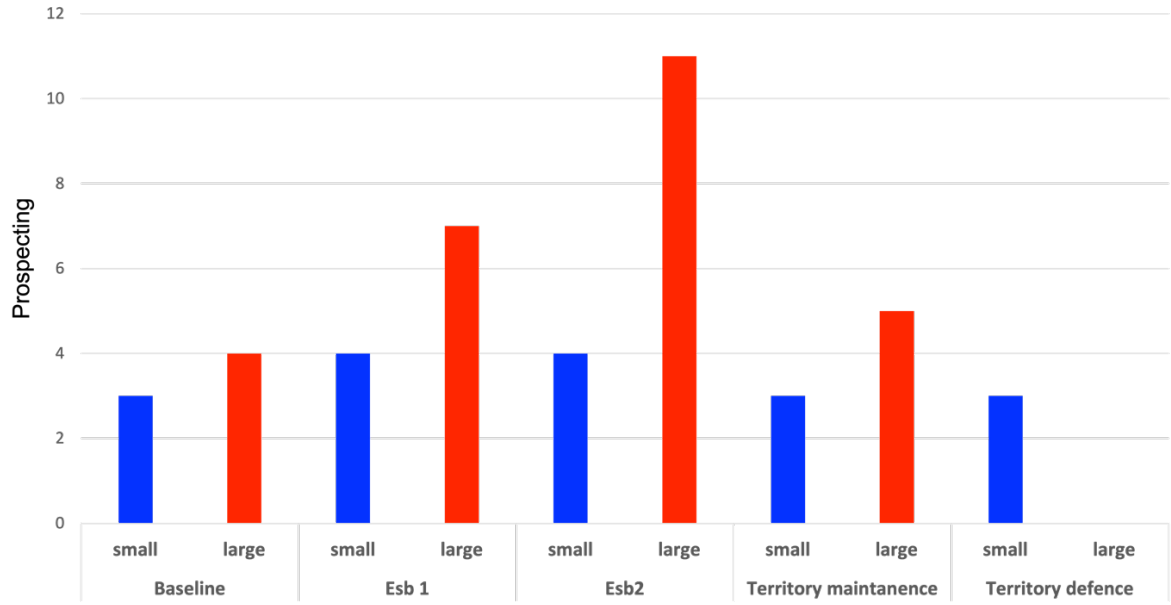

Figure 7. Prospecting behaviour by the focal fish. Bar plot of the frequency of prospecting behaviour of focal fish in large (red) and small (blue) groups.

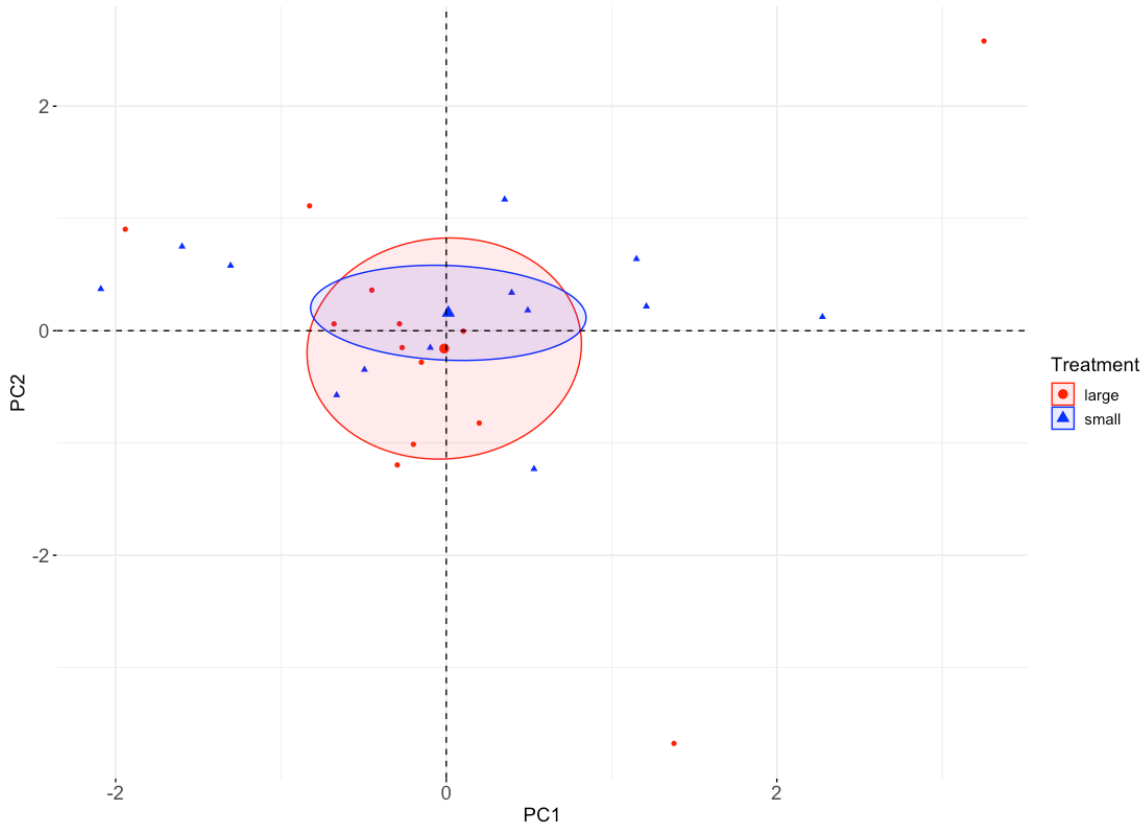

Figure 8. Behavioural profile (i.e., affiliative behaviour, submissive behaviour, prospecting, territory maintenance and defence) of focal individuals in large (red circles) and small (blue triangles) groups. The two main axes of the principal component analysis are depicted in the coordinate axis. Large symbols represent mean values, and small symbols are individual fish.

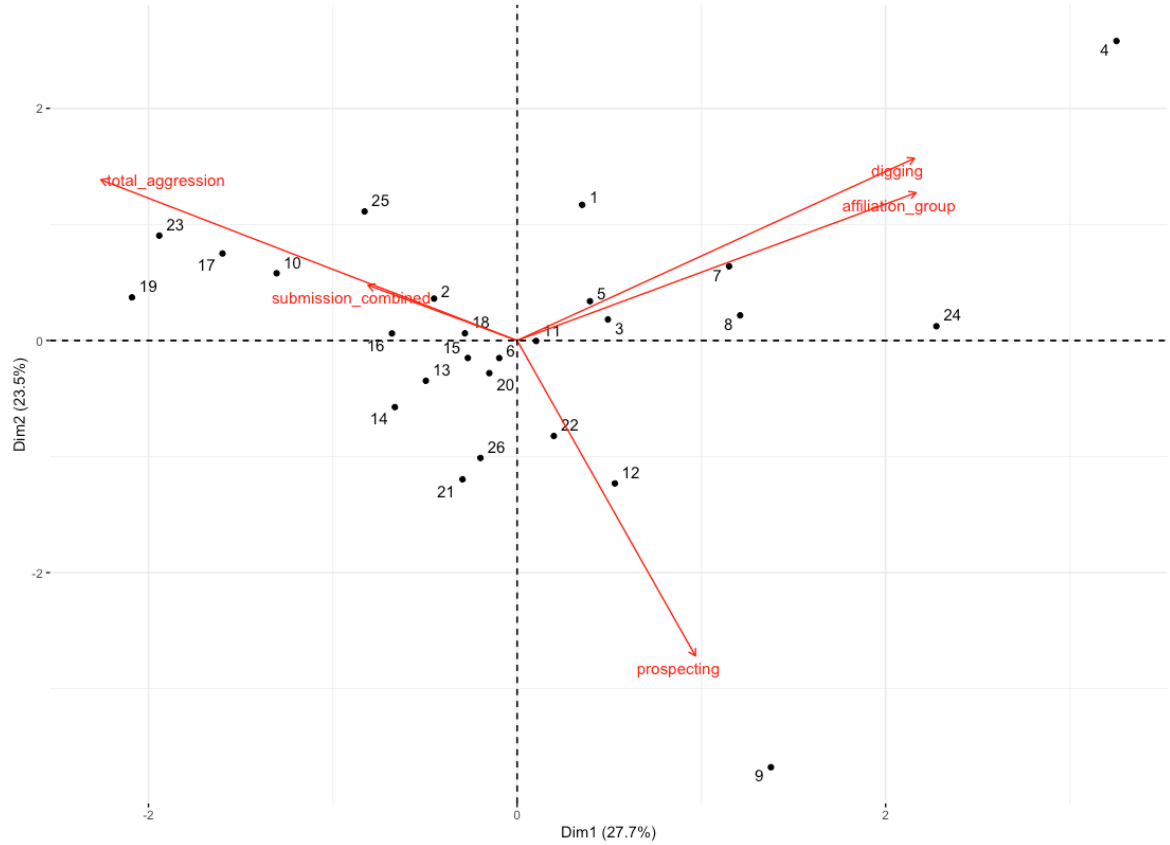

Figure 9. Biplot of the principal components (PC) 1 and 2 that depict the following behaviours: submissive behaviour, territory defence (i.e., total aggression against *T. vitattus*), affiliative behaviour, territory maintenance and prospecting. Each point represents one focal fish.

Supplementary Information

Behavioural phenotypes in a wild population of a cooperatively breeder

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Supplementary Information

Contains: Tables S1-S4 and Figures S1-S5.

Supplementary Tables

Table S1. Elicited submissive behaviour task 1 and 2. Full Generalized Mixed-Effect Model (GLMM, AIC 205.9) with Poisson distribution that test if the frequency of submissive behaviour towards another group members, in these two tasks, was explained by group size, task and their interaction, total aggression received by other group members and observer. Estimates refer to the factor levels given in brackets. The χ^2 values are in denote with the letter[(a)]. Significant effects are in bold except for the intercept.

	Estimate ± S. E.	$z/\chi^{(a)}$	p
Intercept	0.699 ± 0.244	2.87	0.0041
Group size (small)	- 0.671 ± 0.296	-2.26	**0.024**
Elicited submissive behaviour task (2)	0.043 ± 0.229	0.19	0.85
Total received aggression	0.128 ± 0.028	15.99[(a)]	**< 0.001**
Observer (MRC)	0.196 ± 0.224	0.78[(a)]	0.38
Group size (small) * Task	0.432 ± 0.374	1.37[(a)]	0.25
(Group size (small) * Elicited submissive behaviour task (2))	0.432 ± 0.374		

Table S2. Submissive behaviour across tasks. Full GLMMs with Poisson distribution that test if focal fish submissive behaviour towards other group members, across tasks, was explained by group size, task and their interaction, sex and standard length of the focal fish, total aggression received by other group members, observer and frequency of sand digging in all tasks. Estimates refer to the factor levels given in brackets. The χ^2 values are in denote with the letter[(a)]. Significant effects are in bold except for the intercept.

	Estimate ± S. E.	$z/\chi^{(a)}$	p
GLMM Model 1, AIC 446.9			
Intercept	0.642 ± 1.176	0.55	0.59
Group size (small)	-0.094 ± 0.301	-0.31	0.75
Elicited submissive behaviour task 1	0.227 ± 0.232	0.98	0.33
Elicited submissive behaviour task 2	0.272 ± 0.245	1.108	0.27
Territory maintenance task	-0.565 ± 0.325	-1.74	*0.08*
Territory defence task	-1.188 ± 0.418	-2.85	**0.004**
Sex (male)	0.098 ± 0.214	0.21[(a)]	0.65
Standard length focal fish (cm)	- 0.044 ± 0.253	0.031[(a)]	0.86
Total received aggression	0.146 ± 0.024	33.12[(a)]	**< 0.001**
Sand digging across all tasks	- 0.058 ± 0.107	0.30[(a)]	0.58
Observer (MRC)	0.041 ± 0.179	0.05[(a)]	0.82
Group size (small) * Task		4.53[(a)]	0.34
Group size (small) * Elicited submissive behaviour task 1	-0.66 ± 0.384		
Group size (small) * Elicited submissive behaviour task 2	-0.139 ± 0.364		
Group size (small) * Territory maintenance task	0.121 ± 0.461		
Group size (small) * Territory defence task	-0.489 ± 0.678		
GLMM Model 2, AIC 443.4			
Intercept	0.847 ± 1.185	0.72	0.48
Group size (small)	- 0.315 ± 0.198	2.34[(a)]	0.13
Task		40.07[(a)]	**< 0.001**
Elicited submissive behaviour task 1	-0.002 ± 0.188		
Elicited submissive behaviour task 2	0.214 ± 0.185		
Territory maintenance task	-0.502 ± 0.227		
Territory defence task	-1.405 ± 0.329		
Sex (male)	0.094 ± 0.217	0.19[(a)]	0.67
Total received aggression	0.133 ± 0.023	30.67[(a)]	**< 0.001**
Standard length focal fish (cm)	- 0.067 ± 0.256	0.07[(a)]	0.79
Frequency of sand digging in all tasks	- 0.057 ± 0.101	0.33[(a)]	0.56
Observer (MRC)	0.038 ± 0.177	0.05[(a)]	0.83

Table S3. Prospecting behaviour across tasks. Comparison between tasks (i.e., baseline, elicited submissive behaviour 1 and 2, territory maintenance and defence) where we scored focal fish prospecting behaviour. The focal fish belong to large and small groups.

Comparison between tasks	Estimate	Standard Error	p
Baseline - Elicited submissive behaviour 1	-1.903	0.886	0.20
Baseline - Elicited submissive behaviour 2	-1.201	0.914	0.68
Baseline - Territory maintenance	-0.197	0.926	1.00
Baseline – Territory defence	0.339	1.062	1.00
Elicited submissive behaviour 1 - Elicited submissive behaviour 2	0.703	0.810	0.91
Elicited submissive behaviour 1 - Territory maintenance	1.707	0.906	0.33
Elicited submissive behaviour 1 – Territory defence	2.243	1.042	0.20
Elicited submissive behaviour 2 - Territory maintenance	1.004	0.931	0.82
Elicited submissive behaviour 2 – Territory defence	1.540	1.082	0.61
Territory maintenance – Territory defence	0.536	1.039	0.99

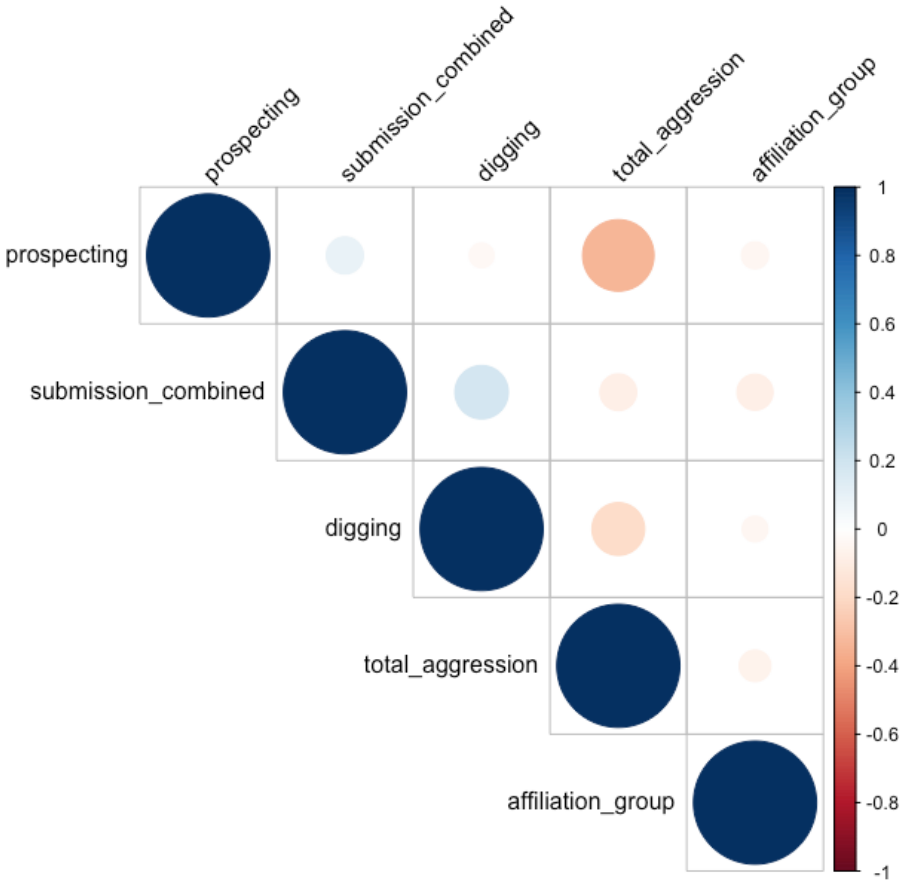

Figure S1. Correlogram of behaviours scored during different tasks (i.e., baseline, elicited submissive behaviour 1 and 2, territory maintenance and defence) from focal individuals belonging to large and small groups. The submissive combined behaviour is the sum of all submissive behaviours done by the focal fish towards other group members across all tasks including territory defence task.

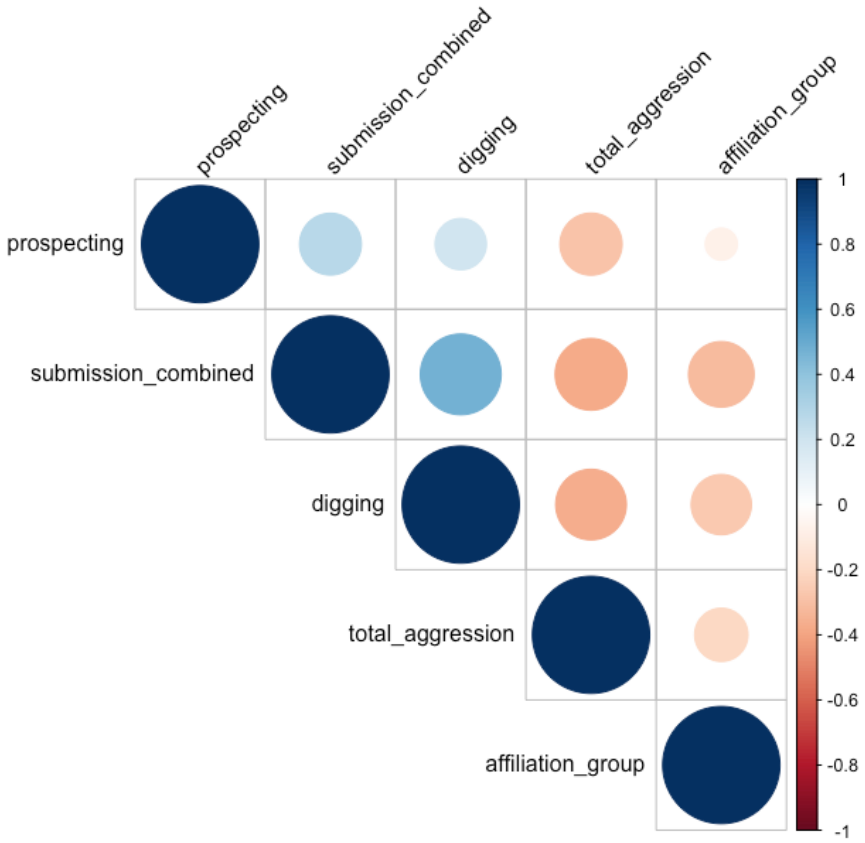

Figure S2. Correlogram of behaviours scored during different tasks (i.e., baseline, elicited submissive behaviour 1 and 2, territory maintenance and defence) from focal individuals belonging small groups. The submissive combined behaviour is the sum of all submissive behaviours done by the focal fish towards other group members across all tasks including territory defence task.

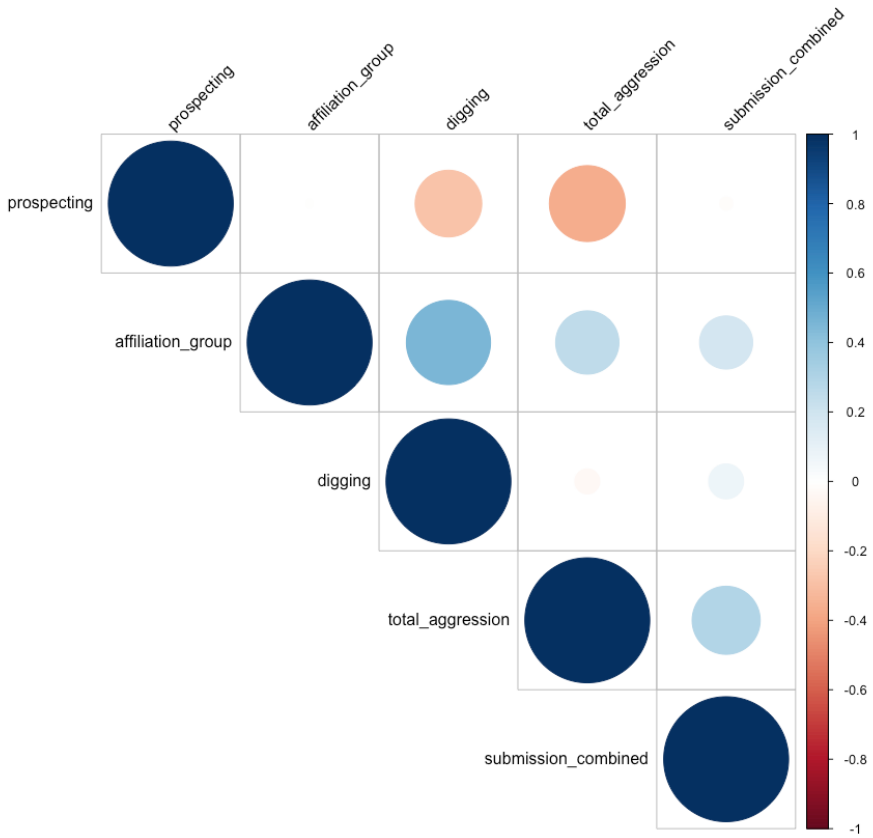

Figure S3. Correlogram of behaviours scored during different tasks (i.e., baseline, elicited submissive behaviour 1 and 2, territory maintenance and defence) from focal individuals belonging large groups. The submissive combined behaviour is the sum of all submissive behaviours done by the focal fish towards other group members across all tasks including territory defence task.

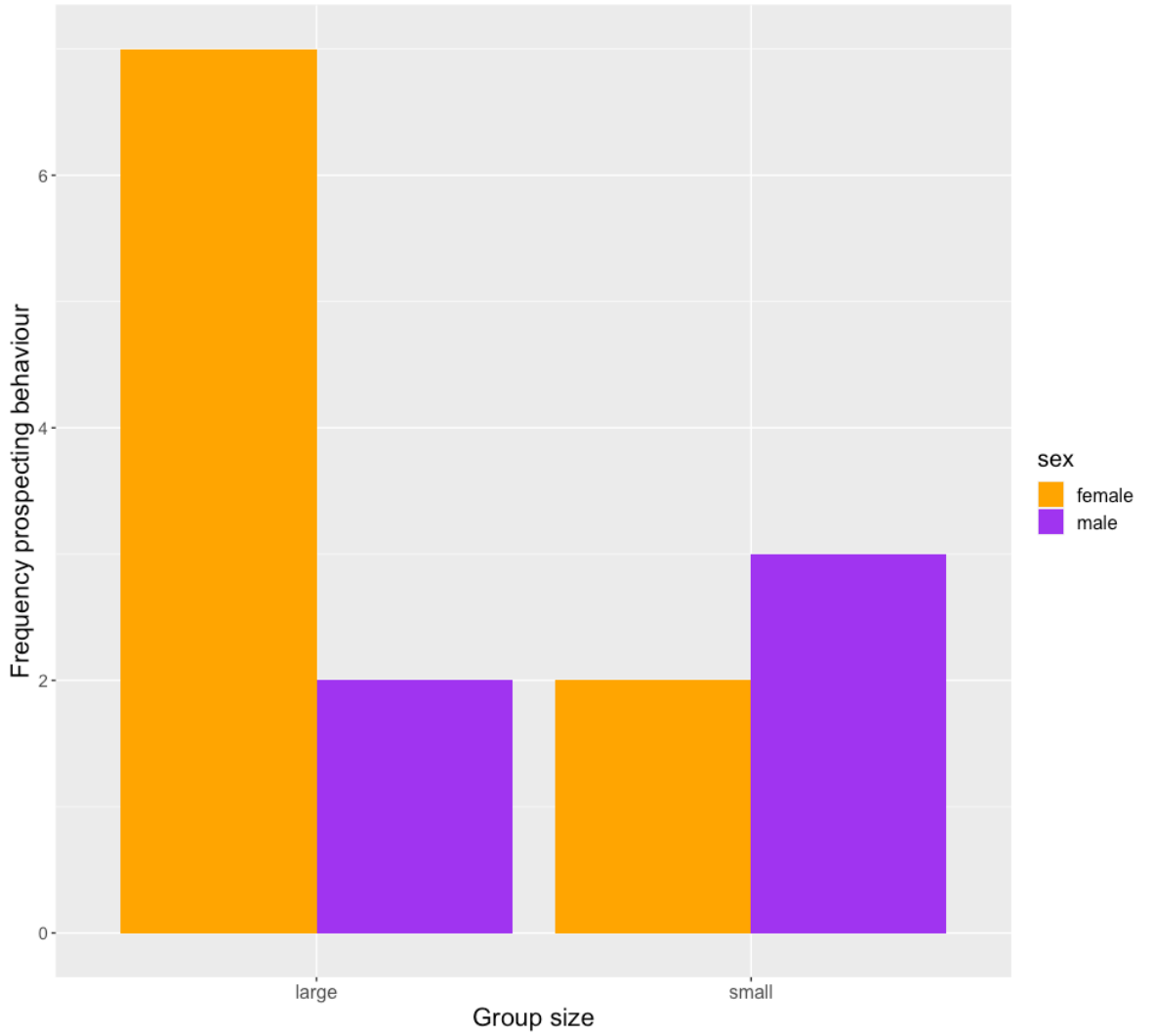

Figure S4. Bar plot of the frequency of prospecting behaviour between females (orange) and males (purple) in large and small groups regardless of the tasks where the focal subordinates were observed.

General discussion

By using the cooperatively breeding species *Neolamprologus pulcher* in my research, I first examined if early life programming of the vertebrate stress axis is the underlying neurophysiological mechanism of behavioural flexibility. Behavioural flexibility is an important trait that allows animals to adapt to changing non-social and social environments. In the social context it allows the establishment of social hierarchies, which is indispensable to maintain group stability in cooperative breeder animal societies. Second, I investigated if group size, an important characteristic of animal societies, serves two functions: (i) whether it influences the egg-mediated maternal effect in a way that allows mothers to shape offspring phenotype and (ii) if it provides individuals with the opportunity to adjust their own phenotype according to the social interactions they may experience with other group members.

The understanding of the mechanisms that underline behaviour offers the opportunity to ask questions about the evolution of the trait and how it is conserved across animal taxa. Learning has often been described as a precondition for social interactions and problem solving; therefore, the understanding of a candidate mechanism underlaying this cognitive process can shade light into whether this trait is required for overcoming non-social and social challenges.

Chapter 1 is an important contribution on the shared function of the stress axis (i.e., hypothalamic-pituitary-interrenal axis is the fish homolog to the mammalian hypothalamic-pituitary-adrenal axis (Mommsen et al., 1999)) in the social and non-social domain. This well conserved neurophysiological mechanism across vertebrates has two relevant characteristics that allow to modulate learning and therefore behavioural flexibility. First, the glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) are expressed in the hippocampus, a brain area that is known to modulate memory formation (Datson et al., 2012) and social behaviour (O'Connell & Hofmann, 2011). Second, glucocorticoids (GCs) regulate physiological and cognitive processes. Hence, it is plausible to find evidence that the stress axis has a modulatory role in social and non-social contexts.

There is evidence in favour of and against a share mechanism between social and non-social behavioural flexibility (Brosnan et al., 2010; Varela et al., 2020). In species that live in social groups, the finding of a share mechanism between social and non-social flexibility has

important implications. First, it implies that having hierarchies to maintain a social structure inside a group is a stable strategy because individuals can use behavioural flexibility to keep their position and to reduce the energetic cost of such interactions. Second, if an individual's stress axis is strongly shaped and regulated by the social environment (Creel et al., 2013; Taborsky et al., 2013), then social interactions among members are fundamental to shape an individual's social behaviour because they shape the underlying neurophysiological mechanism of learning and memory, and behavioural flexibility. Furthermore, if GCs regulate the stress axis and social interactions modulate GCs concentrations in individuals that hold different status in the social hierarchy (Goymann & Wingfield, 2004); then, the social environment will generate long-term effects on individuals' behavioural flexibility, and this may feedback on the social structure of the group (Taborsky, 2021). A future research area in this direction could be to study how an individual's behavioural flexibility in a social context impacts the structure of the social group. For example, how does behavioural flexibility influence the performance of helping tasks in different class categories in the social hierarchy? How does behavioural flexibility of dominant breeders affect the group size? A third implication is that the early-life programming of the stress axis by frequent stimuli that increase glucocorticoids levels may lead to an impairment of behavioural flexibility, which may hamper individuals' ability to adapt to a changing environment.

The evidence that there is a mechanism that allows individuals to response flexibly to environmental challenges, raises the question if environmental challenges, such as the social environment with social interactions, may provide enough information for mothers to shape the offspring's phenotype.

In chapter 2, I examine the mechanisms of egg-mediated maternal effects and whether embryos use maternal information or not, and if the social environment is a reliable source of information for mothers to use egg-mediated maternal effects. The importance of the social environment has been described throughout this work, but survival is one aspect worth to emphasise to understand egg-mediated effects in cooperative breeders. Although the increase of survival as a function of the social group size is species specific, it should be in the interest of all group members to attain survival. Hence, group size may provide mothers with information about the survival probability of her current and future offspring. A small group size signals two things, namely, low survival probability (Brouwer et al., 2005; Mumme et al., 2015; Rood, 1990) and the need of helpers to raise the depended young (Angulo et al., 2013;

Heg et al., 2005). In this chapter, I describe that mothers should attend to increase clutch size to increase the survival chances of at least some offspring.

The contribution of this research to elucidate egg-mediated maternal effects as a function of the social environment indicates that neither egg size nor hormonal mediated maternal effects are mechanisms used by *N. pulcher* females. Females' current body condition is a stronger positive predictor for clutch size, which is a reproductive female trait that can be adjusted in other oviparous species (Baker et al., 2015; Donelson et al., 2008; Ford & Seigel, 1989). Theoretical models predicted that females in cooperative breeder groups should evolve adaptive maternal effects that provide a head start for offspring (Savage et al., 2015). However, other theoretical models state that maternal effects will evolve if the maternal phenotype is sufficiently correlated with the environment where offspring will live (Kuijper & Hoyle, 2015). Hence, a possibility for the absence of egg-mediated maternal effects in *N. pulcher* could be that there is a mismatch between females and offspring's social environment, which might constrain egg-mediated maternal effects. Therefore, further research should be done on how maternal early social environment contributes to egg-mediated maternal effects when offspring and maternal environment are correlated.

The ultimate explanation for this finding is that perhaps dispersal may hinder maternal effects. *N. pulcher* with an early dispersal behavioural phenotype has a lower reproductive performance in comparison with the philopatric phenotype (Antunes & Taborsky, 2020). In red-cockaded woodpeckers (*Picoides borealis*) a similar pattern has previously been reported (Walters et al., 1992). Hence, if reproductive output decreases with dispersal and the new social environment does not match with the maternal phenotype, offspring may rely on phenotypic plasticity rather than maternal effects to specialize in either the philopatric or in the early dispersal behavioural phenotype. To conclude, the result of this research does not support the prediction that embryos use hormonal mediated maternal effects.

Phenotypic plasticity allows animals to match their phenotype to the current environment (Taborsky, 2017). This kind of plasticity has been extensively reported in semi-natural laboratory experiments using *N. pulcher* as a model system (Arnold & Taborsky, 2010; Fischer et al., 2015; Nyman et al., 2017, 2018; Taborsky et al., 2012). Nevertheless, phenotypic adaptation to the size of the social group has been investigated only once in wild populations (Fischer et al., 2014). In chapter 3, I address if the two behavioural phenotypes described for

N. pulcher that are formed during early life development (Fischer et al., 2017) are present in a wild population, and if those phenotypes, namely, philopatric and early dispersal, can be explained by the size of the social groups. The prediction was that sexually mature large subordinates in small social groups are more likely to have the early dispersal and helpful behavioural phenotype. In small groups, the demand for help is higher and the survival probability is lower (Heg et al., 2005). Accordingly, in large social groups, subordinates should rather have the philopatric submissive behavioural phenotype. Indeed, the results show that in small social groups, large subordinates tend to help more by digging sand out of the hiding places to make shelters accessible for other group members. In large social groups, large subordinate individuals tend to express a higher frequency of submissive behaviour. This result provides insight into the effects of the social environment on shaping submissive and helping behaviour of individuals, and supports the previous laboratory findings on the behavioural specialization of this cooperative breeder species.

In a social environment, the size of the group is a characteristic that determines the number and frequency of social interactions among group members. In addition, it provides a context for individuals to have a behavioural specialization to retain its group membership (Bergmüller & Taborsky, 2010). In many cooperative breeder species, group memberships are acquired by paying a rent to the dominant breeders (Kokko et al., 2002). In *N. pulcher* the payment, which appeases dominants, is either help or high frequency of submissive behaviour (Arnold & Taborsky, 2010; Bergmüller & Taborsky, 2005), both are energetically costly (Grantner & Taborsky, 1998); therefore, shaping the behavioural phenotype according to the current social environment seems to be an evolutionary stable strategy to gain long term fitness benefits, such as survival and reproduction.

To conclude, this work provides important insight on the mechanisms that regulate behavioural flexibility and the importance of the social environment to further shape individuals' behaviour. In this research, I demonstrate that the early life programming of the stress axis by environmental factors affects non-social and social behavioural flexibility in the same way. This suggests an interaction between environment and individual in the following way, social and non-social environmental stimuli can program behavioural flexibility; in consequence, the interaction between the individual and the social and non-social environment can be affected by this programming. Furthermore, this work highlights the importance of the social environment for both mothers and offspring. Although, there is no evidence for egg-

mediated maternal effects as a function of social environment, the size of the social environment does shape the behaviour of subordinate individuals which live in a wild population. This corroborates previous findings that *N. pulcher* uses environmental cues to adjust its phenotype to the current environment. These findings have important implications for understanding the mechanism underlying the maintenance and evolution of complex animal societies, and highlight the importance of the social environment on shaping behavioural traits.

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Appendix 1

Statistical analysis to test if egg-mediated maternal effects influence social behaviour and hormonal status of offspring

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Description

We defined social environment as the number of individuals in a group. A group contains one breeding pair and eight (i.e., large group) or only one (i.e., small group) unrelated subordinate(s). The size of the social group has long term effects on the brain architecture (Nyman et al., 2017) and social behaviour of *Neolamprologus pulcher* (Arnold & Taborsky, 2010; Fischer et al., 2017; Taborsky et al., 2012). Furthermore, the interactive effect of the composition of the social group and predator exposure leads to behavioural specialization in this species. Juveniles raised with several group members are known to specialize in a submissive and philopatric phenotype later in life. Whereas juveniles that lack such a diverse social structure during development are more likely to increase their helping propensity and disperse early from the natal territory (Fischer et al., 2017). This behavioural specialization has been described as phenotypic plasticity (Taborsky, 2017) but maternal effects may also contribute to shape *N. pulcher* behavioural phenotype early in life (Kasper et al., 2017; Sharda et al., 2021).

In this study, we aim to test if the social environment where a breeding pair lives, provides cues that may induce parental effects to shape offspring's behavioural phenotype. Consequently, offspring may develop a behavioural specialization and have a physiological programming. We predicted that breeding pairs in a large group may produce offspring with a submissive and philopatric behavioural phenotype, whereas breeders in small groups may produce a helper and early dispersal offspring. We further predicted that the physiology of fish that come from parents in small groups could be shaped in a way that signals breeders that they are not reproductive competitors. Therefore, in fish from small groups, cortisol basal levels may be higher and the sexual hormones (i.e., androgen, testosterone and progesterone) may be lower in comparison with fish that come from breeders in large groups.

This appendix contains a brief description of the methods used for data collection, the data and statistical analyses used in this study.

Methods

We created ten small and ten large breeding groups. All individuals in a group were unrelated and unfamiliar to each other. We collected one clutch of fertilized eggs from the breeding pair and reared it outside the group to disentangle parental effects from behavioural effects. Offspring of one breeding pair were raised in sibling groups in the absence of adults. We started to collect samples from the sibling group when they were 0 days old (i.e., first day of free swimming). We collected brain samples at the age of 0, 30 and 60 days to assess possible candidate genes that may explain the behavioural specialization. We evaluated the spontaneous social interactions between siblings in each sibling group at the age between 20 and 60 days. We sampled the following hormones: cortisol, testosterone, estradiol, progesterone and 11 Keto-Testosterone. Furthermore, we did a series of behavioural tests to assess fish behavioural competence. Behavioural competence is defined as an individual's ability to flexibly adjust its own behaviour in accordance with the social partner in a social interaction (Taborsky & Oliveira, 2012). This has short term fitness benefits that can accumulate over time and translate in survival and reproduction (Arnold & Taborsky, 2010; Taborsky et al., 2012).

Spontaneous behaviours

We assessed the frequency of all behaviours among siblings every ten days, starting at 20 until 60 days old. This period is known to be important for the development of social competence in *N. pulcher* (Arnold & Taborsky, 2010). We followed the procedure described in (Reyes-Contreras et al., 2019). Briefly, in each sibling tank the observer (MRC) randomly selected one fish and scored all its behaviour toward other siblings during 5 min. The procedure was repeated with two more fish. Then, we summed up the behaviours of the three fish (i.e., 15 min total observation time) for further statistical analyses.

Social competence in an asymmetric competition test

We assessed social competence in an asymmetric competition test that consisted of a contest over a shelter (Arnold & Taborsky, 2010). Shelters are indispensable for survival because *N. pulcher* use them to hide from predators' attacks (Bergmüller et al., 2005). In the contest, the owner defences the shelter against the intruder. The intruder is the experimental fish which can gain access to the shelter by increasing the frequency of submissive behaviour. We followed the procedure describe in (Nyman et al., 2018) to assess the end of the contest,

the winner and the loser of the contest. We assessed two siblings of each sibling group, and this behavioural experiment was done when the fish were 212-328 days old.

Family integration

We assessed social competence and acceptance by an unrelated and unfamiliar dominant breeding pair. Subordinate individuals in the wild either remain in the natal territory or disperse to a neighbouring group (Bergmüller et al., 2005). To be accepted in the group, subordinates may show submissive behaviour or help the breeding pair on different tasks, both behaviours appease dominants and increase acceptance (Bergmüller & Taborsky, 2005). Hence, we assessed the acceptance rate by the dominant pair, the frequency of submissive behaviour from the experimental fish towards the breeding pair and if any hormone may explain these two factors. This test lasted two weeks and the observer (MRC) did three behavioural observations in first week of the test. In each observation the observer (MRC) scored the acceptance status and the behavioural interactions between the dominant breeders and the focal fish. We used the sum of the three observations for the statistical analysis.

If the fish gained acceptance (see Supplementary Information in (Fischer et al., 2017) for details about assessing acceptance), we assessed philopatry. We use prospecting as a proxy for philopatry because prospecting propensity proceeds dispersal (Jungwirth et al., 2015). This test was done when the fish were 309-350 days old.

Hormonal profile

We used a modify protocol of the fish-holding water method described in (Reyes-Contreras et al., 2019). This is a non-invasive technique to sample waterborne steroid hormones in small fish (Bender et al., 2008; Scott & Ellis, 2007; Wong et al., 2008). We quantified the following hormones: cortisol, testosterone, 11Keto-Testosterone, estradiol and progesterone (Table 1 and 2).

We collected three hormonal samples from fish that were produced by parents in large and small groups (Table 2). The first sample was taken before the asymmetric competition test (206-322 days old) and served as baseline because the fish had not jet undergone any

behavioural assessment. The second sample was taken when the fish had gained acceptance by the breeding pair and served to assess if the hormonal status had influenced the behaviour of the focal fish towards the breeding pair. The third sample was taken when the fish were 251-342 days old. The third sample was taken at the end of the experiment when the focal fish had been accepted by the breeding pair and had the opportunity to prospect to a neighbouring group. This sample provided information about the hormonal status of the fish while being a subordinate in a group that had the potential to prospect into a neighbouring group.

Statistical analysis

We fitted General Linear Models (GLMs) and Generalized Linear Mixed-effect Models (GLMMs), where we assumed either Poisson or binomial distributions as indicated below. The significance of interaction between two fixed factors was tested by Likelihood Ratio Tests (LRT). We used the statistical software R, version 4.1.2. (R Core Team, 2021) and the R packages "car" (Fox & Weisberg, 2019), "MASS" (Venables & Ripley, 2002), "lme4" (Bates et al., 2015) and "lmerTest" (Kuznetsova et al., 2017).

The factors in the full models were stepwise backward selected, starting with two-way interaction terms. If a term did not significantly explain the variance of the dependent variable ($p > 0.05$), we fitted a model with and without the non-significant factor and kept the model with the lower AIC value if it was at least lower by a value of 2. In case the models had a similar AIC we consider the models were equivalent. Thus, if the interaction term was not significant and the models were equivalent, we backward selected the interaction term and kept the simplified model. Group size was always kept as factor in the models. We used identity of the group of origin has random factor because we assessed behaviours from siblings that had the same group of origin.

Spontaneous behaviour of juveniles

We fitted GLMM with Poisson distribution to test if the variance in spontaneous submissive behaviour among siblings was explained by 'group size', 'age' and their interaction. The interaction did not explain the variance of submissive behaviour; therefore, it was backward selected from the final model. Furthermore, we analyse within each age class the spontaneous submissive behaviours among siblings because *N. pulcher* behaviour changes with age and the presence or absence of adult members (Fischer et al., 2015). The data use for this analysis is in Table 3 and 4.

Asymmetric competition test

In the contest, only five out of 39 intruders won access to the shelter; therefore, the response variable "outcome of the contest" was transformed to a binomial variable. First, A GLM with binomial distribution was fitted to test the effect of body mass and standard length of owners and intruders in the outcome of the contest. This was done to assess if those factors

should be included in the following models. Second, a GLMM with binomial distribution was fitted to assess if the outcome of the contest was explained by 'group size', 'cortisol baseline' and 'contest duration'. The last two factors were included because in *N. pulcher* the stress axis responsiveness is known to influence the outcome of the asymmetric competition contest (Reyes-Contreras et al., 2019) and more socially competent individuals resolve the contest faster (Arnold & Taborsky, 2010). The data used in this analysis are in Tables 5-9.

Family integration

Submissive behaviour of subordinates towards dominants leads to the acceptance by dominant breeders. Conversely, aggressive behaviour leads to eviction (Arnold & Taborsky, 2010). Hence, we analyse how those two behaviours vary according to the physiological status of the focal fish and received aggression by dominant breeders. We fitted a GLMM with Poisson distribution to test if submissive behaviour towards dominant breeders was explained by 'group size', 'stress axis responsiveness' and 'received total aggression from both dominant breeders'. Stress axis responsiveness was calculated by subtracting the cortisol sample taken while the fish was with the family minus the baseline cortisol. Stress axis responsiveness may influence the behaviour of the fish. In addition, we fitted a GLMM with Poisson distribution to test if the response variable, submissive behaviour towards dominant breeders, was explained by 'group size', 'testosterone' and 'received total aggression from both dominant breeders'. We did a similar procedure using the response variable aggression towards dominant breeders, we fitted two GLMM with Poisson distribution, in first model we include 'stress axis responsiveness' and in the second model 'testosterone'. The data use in this analysis is in Tables 10-14.

Only four focal fish prospected, the frequency and duration of this behaviour is in Table 15.

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Tables

Table 1. Abbreviations of the column names used in table 2.

Column	Description
fish_ID	identity of the focal fish that was the intruder in the contest
sample_type	*before the manipulation*: sample collected before asymmetric competition contes; *helper_in_group*: sample collected while being in the family integration test; *prospecting_opportunity*: sample collected when the focal fish could prospect
group_size	large or small group
family_ID	identity of the group of origin
cortisol_ng_mL	cortisol concentration (ng/ml)
estradiol_ng_mL	estradiol concentration (ng/ml)
testosterone_ng_mL	testosterone concentration (ng/ml)
KT_ng_mL	11-Keto-Testosterone concentration (ng/ml)
progesterone_ng_mL	progesterone concentration (ng/ml)

Table 2. Hormones concentration (ng/ml) of all the fish that were in the asymmetric competition contest and family integration test. For some fish the hormones concentration was below the detection limit; therefore, the data is not available (NA).

fish_ID	sample_type	group_size	family_ID	cortisol_ng_mL	estradiol_ng_mL	testosterone_ng_mL	KT_ng_mL	progesterone_ng_mL
1	before_manipulation	large	55X	2.793	0	0	0	0.022
2	before_manipulation	large	55X	3.124	0	0	0.137	0.115
3	helper_in_group	small	53C	10.047	1.639	0.356	0	0
3	prospecting_opportunity	small	53C	5.384	0.38	0.127	0	0.038
3	before_manipulation	small	53C	5.425	0.259	0	0.093	0.077
4	helper_in_group	small	53C	1.981818182	1.664646465	0.151515152	0	0
4	prospecting_opportunity	small	53C	3.893	0.715	0.261	0	0
4	before_manipulation	small	53C	1.831	0	0	0	0
5	before_manipulation	large	51BB	1.281	0	0	0	0.026
5	helper_in_group	large	51BB	0.906	0.214	0.077	0	0.014
5	prospecting_opportunity	large	51BB	1.859	0.454	0.058	0	0.009
6	before_manipulation	large	51BB	3.648484848	0	0	0	0.03030303
6	helper_in_group	large	51BB	3.133333333	0.21010101	0.027272727	0	0.012121212
7	before_manipulation	large	52HH	1.748484848	0.346464646	0.06969697	0	0.025252525
8	before_manipulation	large	52HH	2.515151515	0	0	0	0
9	before_manipulation	large	62LL	0.424242424	0	0	0	0.054545455
10	before_manipulation	large	62LL	1.561616162	0	0	0	0.049494949
10	helper_in_group	large	62LL	2.253535354	0.347474747	0.106060606	0	0.016161616
10	prospecting_opportunity	large	62LL	4.937	0.407	0.239	0	0.021
11	before_manipulation	small	58DD	0.933333333	0	0	0	0.051515152
11	helper_in_group	small	58DD	0.629292929	0.83030303	0.107070707	0	0.012121212
11	prospecting_opportunity	small	58DD	0.694949495	0	0	0	0.013131313
12	before_manipulation	small	58DD	6.128282828	0	0	0	0.093939394
13	before_manipulation	large	59U	1.391	0	0	0	0.015
13	helper_in_group	large	59U	0.476767677	0.129292929	0	0	0.016161616
13	prospecting_opportunity	large	59U	0.91010101	0	0	0	0.009090909
14	before_manipulation	large	59U	1.038	0	0	0	0.017
15	before_manipulation	large	57JJ	1.137373737	0	0	0	0
16	before_manipulation	large	57JJ	0.809090909	0	0	0	0.017171717

fish_ID	sample_type	group_size	family_ID	cortisol_ng_mL	estradiol_ng_mL	testosterone_ng_mL	KT_ng_mL	progesterone_ng_mL
17	before_manipulation	small	63EE	0.793	0.223	0.063	0	0.01
17	helper_in_group	small	63EE	0.004874747	0.000313131	4.94949E-05	0	1.41414E-05
17	prospecting_opportunity	small	63EE	8.981818182	1.560606061	0.124242424	0	0.013131313
18	before_manipulation	small	63EE	0.97	0	0	0	0.009
19	before_manipulation	small	23V	1.517	0	0	0	0.008
20	before_manipulation	small	23V	1.568	0	0	0	0.012
21	before_manipulation	small	65MM	1.936	0	0	0	0.008
22	before_manipulation	small	65MM	1.987	0	0	0	0.011
22	helper_in_group	small	65MM	0.836363636	0	0	0	0.006060606
22	prospecting_opportunity	small	65MM	2.424	0	0	0	0.011
23	helper_in_group	large	68PP	2.54	0.116	0	0	0
23	before_manipulation	large	68PP	1.328	0.151	0.061	0	0.01
23	prospecting_opportunity	large	68PP	2.491	0.264	0.11	0	0
24	before_manipulation	large	68PP	1.772727273	0	0	0.114141414	0.011111111
25	before_manipulation	large	69QQ	3.66969697	0	0	0	0.006060606
25	helper_in_group	large	69QQ	0.839	0.206	0	0	0.012
25	prospecting_opportunity	large	69QQ	0.901010101	0.436363636	0.039393939	0	0.008080808
26	before_manipulation	large	69QQ	2.909090909	0	0	0	0.009090909
26	helper_in_group	large	69QQ	2.325252525	0	0	0	0
26	prospecting_opportunity	large	69QQ	0	0	0	0	0
27	before_manipulation	large	70RR	2.607070707	0	0	0	0.007070707
28	before_manipulation	large	70RR	2.171	0.089	0	0	0.009
28	helper_in_group	large	70RR	1.901	0.281	0.049	0	0.014
28	prospecting_opportunity	large	70RR	3.446	0.199	0.067	0	0
29	before_manipulation	small	60KK	0.365656566	0	0	0	0
29	helper_in_group	small	60KK	1.353535354	0.53030303	0.109090909	0	0.012121212
29	prospecting_opportunity	small	60KK	6.727272727	0.152525253	0	0	0.013131313
30	before_manipulation	small	60KK	0.373	0.143	0	0	0
30	helper_in_group	small	60KK	1.169	0.356	0.371	0	0.012
30	prospecting_opportunity	small	60KK	4.235	1.871	0.23	0	0.009
31	prospecting_opportunity	small	75WW	1.442	0.402	0.074	0	0
31	before_manipulation	small	75WW	0.217171717	0.265656566	0.023232323	0	0.008080808
31	helper_in_group	small	75WW	1.25	0.195	0.035	0	0.012
32	prospecting_opportunity	small	75WW	4.771	0.199	0.068	0	0
32	before_manipulation	small	75WW	0.961616162	0	0	0	0.013131313

fish_ID	sample_type	group_size	family_ID	cortisol_ng_mL	estradiol_ng_mL	testosterone_ng_mL	KT_ng_mL	progesterone_ng_mL
32	helper_in_group	small	75WW	4.445	0.561	0	0	0.015
33	prospecting_opportunity	small	72TT	9.38	1.095	0.175	0	0
33	before_manipulation	small	72TT	0.346464646	0.117171717	0.017171717	0	0.008080808
33	helper_in_group	small	72TT	10.353	0.816	0.173	0	0.019
34	prospecting_opportunity	small	72TT	8.32	1.287	0.128	0	0
34	before_manipulation	small	72TT	1.186	0.17	0	0	0.007
34	helper_in_group	small	72TT	9.857	0.295	0.242	0	0.014
35	before_manipulation	large	71SS	0.848484848	0	0	0	0
36	helper_in_group	large	71SS	8.255555556	0	0	0	0
36	prospecting_opportunity	large	71SS	8.928	0.165	0	0	0
36	before_manipulation	large	71SS	3.309	0	0	0	0
37	helper_in_group	small	77ZZ	3.515	0.531	0	0	0
37	prospecting_opportunity	small	77ZZ	11.55555556	0.344444444	0.25959596	0	0.039393939
37	before_manipulation	small	77ZZ	0.761	0.125	0.05	0	0.009
38	before_manipulation	small	77ZZ	2.362	0.362	0.084	0	0
40	helper_in_group	small	60KK	0.908	0	0	0	0
40	prospecting_opportunity	small	60KK	4.658	0.523	0.089	0	0.045
40	before_manipulation	small	60KK	2.116	0	0	0	0.007

Table 3. Abbreviations of the column names used in table 4.

Column name	Description
origin	treatment, large or small group
tank	number of the sibling tank
Fa	identity of the group of origin
pair	identity of the breeding pair that laid the eggs
age_dph	the number of days after the first day of free swimming
activity	the number of lines a fish crossed
dig	the fish pick up sand with his mouth
ov_agg	over aggression (sum of bite, chase and ram)
r_agg	restrained aggression (sum of frontal approach, head down, s-bend, opercula spread, tail beat and head jolt)
t_agg	total aggression, the sum of overt and restrained aggression
aff	affiliative behaviour (sum of bump and follow)
sub	submissive behaviour (sum of tail quiver and hook)
av_flee	sum of avoid and flee
over_ID	individual number assigned to each row

Table 4. Frequency the spontaneous behaviour among siblings. The spontaneous behaviours were scored every ten days during the two first months of development.

over_ID	origen	tank	Fa	pair	age_dph	ov_agg	r_agg	t_agg	aff	sub	av_flee	activity	dig
1	large	4000	69	QQ	30	8	6	14	1	3	5	43	0
2	large	4000	69	QQ	40	7	14	21	2	8	2	42	0
3	large	4000	69	QQ	50	1	11	12	0	10	2	50	0
4	large	4000	69	QQ	60	9	1	10	4	17	0	66	0
5	large	4005	68	PP	30	0	10	10	1	0	3	30	0
6	large	4005	68	PP	50	1	2	3	2	16	2	85	0
7	large	4005	68	PP	60	4	3	7	2	4	0	96	0
8	large	4009	70	RR	30	1	14	15	2	9	4	48	0
9	large	4009	70	RR	40	1	6	7	0	1	1	54	1
10	large	4009	70	RR	50	2	9	11	1	15	3	28	2
11	large	4009	70	RR	60	2	8	10	4	3	1	15	7
12	large	6015	71	SS	50	12	17	29	3	8	5	70	0
13	large	6015	71	SS	60	4	13	17	4	3	0	47	1
14	large	6016	71	SS	30	0	1	1	0	0	2	71	0
15	large	6016	71	SS	40	0	5	5	2	2	4	41	1
16	small	6019	72	TT	30	22	9	31	1	0	2	41	0
17	small	6019	72	TT	40	0	6	6	0	2	3	27	0
18	small	6019	72	TT	50	1	13	14	7	5	3	51	2
19	small	6019	72	TT	60	4	6	10	3	18	3	31	4
20	large	6028	70	RR	30	1	2	3	0	0	0	81	0

over_ID	origen	tank	Fa	pair	age_dph	ov_agg	r_agg	t_agg	aff	sub	av_flee	activity	dig
21	large	6028	70	RR	40	0	0	0	1	2	4	50	0
22	large	6028	70	RR	50	1	6	7	0	0	0	44	0
23	large	6028	70	RR	60	2	10	12	2	15	1	45	0
24	small	6028	66	SI	30	2	0	2	0	2	5	68	0
25	small	6028	66	SI	40	1	4	5	2	1	0	57	0
26	small	6028	66	SI	60	2	6	8	5	12	0	51	0
27	small	6037	60	KK	30	2	10	12	0	4	0	41	0
28	small	6037	60	KK	40	4	4	8	1	2	0	47	0
29	small	6037	60	KK	50	1	1	2	4	16	0	68	0
30	small	6037	60	KK	60	0	3	3	7	12	2	82	0
31	small	6041	75	WW	30	0	1	1	2	0	2	78	0
32	small	6041	75	WW	40	4	10	14	0	8	1	26	0
33	small	6041	75	WW	50	0	4	4	4	4	3	81	0
34	small	6041	75	WW	60	0	9	9	1	7	2	28	1
35	small	6046	77	ZZ	30	5	15	20	4	4	3	51	0
36	small	6046	77	ZZ	40	0	10	10	1	8	2	42	0
37	small	6046	77	ZZ	50	4	15	19	5	16	1	20	1
38	small	6046	77	ZZ	60	8	7	15	8	12	0	50	0
39	large	6100	80	CCC	30	5	7	12	1	6	6	41	0
40	large	6100	80	CCC	40	2	10	12	5	4	2	34	0
41	large	6100	80	CCC	50	3	4	7	4	7	0	88	0
42	small	6101	78	AAA	30	4	5	9	4	4	4	18	0
43	small	6101	78	AAA	40	1	11	12	3	15	2	37	0
44	small	6101	78	AAA	50	3	3	6	9	6	1	51	0
45	small	7105	73	UU	30	4	3	7	0	3	9	52	0
46	small	7105	73	UU	40	11	8	19	1	3	7	73	0
47	small	7105	73	UU	50	0	2	2	3	11	4	83	0
48	large	7103	59	U	30	0	2	2	3	1	3	77	0

over_ID	origen	tank	Fa	pair	age_dph	ov_agg	r_agg	t_agg	aff	sub	av_flee	activity	dig
49	large	7103	59	U	40	4	2	6	2	8	1	33	0
50	large	7103	59	U	50	1	11	12	4	16	0	62	0
51	large	7103	59	U	60	8	9	17	5	3	2	37	1
52	small	7101	58	DD	30	0	5	5	3	2	2	60	0
53	small	7101	58	DD	40	5	5	10	0	6	1	80	0
54	small	7101	58	DD	50	5	1	6	6	6	0	33	0
55	small	7101	58	DD	60	12	7	19	5	18	0	40	0
56	small	7100	53	C	30	2	0	2	0	1	3	44	0
57	small	7100	53	C	40	1	5	6	1	1	2	57	0
58	small	7100	53	C	50	0	3	3	1	7	2	75	0
59	small	7100	53	C	60	3	5	8	0	9	1	54	1
60	small	7012	65	MM	40	3	5	8	0	0	6	13	0
61	small	7012	65	MM	50	3	8	11	1	3	1	18	0
62	small	7012	65	MM	60	5	12	17	0	19	0	37	0
63	large	7010	51	BB	30	2	10	12	1	3	1	47	0
64	large	7010	51	BB	40	4	2	6	3	15	1	28	0
65	large	7010	51	BB	50	4	5	9	2	18	1	24	0
66	large	7010	51	BB	60	2	1	3	5	18	0	35	0
67	small	7008	63	EE	30	2	1	3	0	1	6	63	0
68	small	7008	63	EE	40	0	3	3	2	1	5	66	1
69	small	7008	63	EE	50	8	26	34	1	11	2	35	0
70	small	7008	63	EE	60	1	11	12	5	12	2	40	1
71	large	7007	62	LL	30	3	3	6	6	1	2	53	0
72	large	7007	62	LL	40	0	3	3	0	10	2	54	1
73	large	7007	62	LL	50	6	4	10	1	5	1	32	0
74	large	7007	62	LL	60	0	5	5	7	3	10	71	0
75	large	7006	52	HH	30	1	4	5	0	8	3	25	0
76	large	7006	52	HH	40	4	3	7	0	2	3	54	0

over_ID	origen	tank	Fa	pair	age_dph	ov_agg	r_agg	t_agg	aff	sub	av_flee	activity	dig
77	large	7006	52	HH	50	1	3	4	2	9	0	59	1
78	large	7006	52	HH	60	10	5	15	1	6	1	59	0
79	large	7004	55	X	30	2	7	9	0	1	2	42	0
80	large	7004	55	X	40	0	0	0	0	1	3	46	0
81	large	7004	55	X	50	7	5	12	0	9	1	37	0
82	large	7005	55	X	60	1	1	2	0	3	0	66	0
83	small	7003	23	V	30	0	7	7	0	5	8	66	1
84	small	7003	23	V	40	0	5	5	1	4	3	57	3
85	small	7003	23	V	60	6	15	21	3	5	5	43	0
86	large	7002	57	JJ	30	1	3	4	0	0	3	36	0
87	large	7002	57	JJ	40	1	0	1	1	3	1	46	0
88	large	7002	57	JJ	50	3	6	9	6	6	2	57	0
89	large	7002	57	JJ	60	1	6	7	3	8	3	15	3

Table 5. Abbreviations of the column names used in table 6, 7, 8 and 9.

Column name	Description
owner_SL	standard length (cm) of the owner
owner_sex	male or female
owner_W	body mass (g) of the owner
intruder_SL	standard length (cm) of the intruder
intruder_sex	male or female
intruder_W	body mass (g) of the intruder
winner	either the owner or the intruder won the contest
intruder_won_binomial	a binomial variable that described if the intruder won (1) or not (0) the asymmetric competition contest
treatment	large or small group
focal_id	identity of the focal fish that was the intruder in the contest
id_siblings	identity of the group of origin
contest_duration_min	lenght of the contest in minutes
avoid_flee_owner	frequency of avoid and flee done by the owner
shelter_owner	the number of times the owner entered the shelter
evicted_owner	the number of times the owner was evicted
restrained_aggression_owner	aggressive displays done by the owner towards the intruder (sum of frontal approach, head down, s-bend, opercula spread, tail beat and head jolt)
overt_aggression_owner	aggressive displays with physical contact done by the owner towards the intruder (sum of bite, chase, mouth fight and ram)
total_aggression_owner	the sum of restrained and overt aggression done by the owner towards the intruder
submissive_behaviour_owner	submissive behaviour done by the owner towards the intruder (sum of tail quiver and hook)
affiliative_owner	affiliative behaviour done by the owner towards the intruder (sum of bump, follow and join)
avoid_flee_intruder	frequency of avoid and flee done by the intruder
shelter_intruder	the number of times the intruder entered the shelter
evicted_intruder	the number of times the intruder was evicted

restrained_aggression_intruder	aggressive displays done by the intruder towards the owner (sum of frontal approach, head down, s-bend, opercula spread, tail beat and head jolt)
overt_aggression_intruder	aggressive displays with physical contact done by the intruder towards the owner (sum of bite, chase, mouth fight and ram)
total_aggression_intruder	the sum of restrained and overt aggression done by the intruder toward the owner
submissive_behaviour_intruder	submissive behaviour done by the intruder towards the owner (sum of tail quiver and hook)
affiliative_intruder	affiliative behaviour done by the intruder towards the owner (sum of bump, follow and join)
cortisol_ng_mL_1	first cortisol sample taken before asymmetric competition
estradiol_ng_mL_1	first estradiol sample taken before asymmetric competition
testosterone_ng_mL_1	first testosterone sample taken before asymmetric competition
KT_ng_mL_1	first 11-Keto-Testosterone sample taken before asymmetric competition
progesterone_ng_mL_1	first progesterone sample taken before asymmetric competition

Table 6. Details of the asymmetric competition contest over a shelter.

owner_SL	owner_sex	owner_W	intruder_SL	intruder_sex	intruder_W	winner	intruder_won_binomial	treatment	focal_id	id_siblings	contest_duration_min
3.6	male	1.217	3.6	male	1.461	owner	0	large	1	55X	14.165
4	male	1.846	4	male	1.819	owner	0	large	2	55X	11.775
3.8	female	1.587	3.8	female	1.488	intruder	1	small	3	53C	17.37666667
3.55	female	1.267	3.55	female	1.426	owner	0	small	4	53C	16.66333333
4	female	1.81	4	female	1.922	owner	0	large	5	51BB	7.435
4.2	female	2.128	4.2	female	2.146	owner	0	large	6	51BB	13.12833333
3.8	female	1.722	3.8	female	1.909	owner	0	large	7	52HH	4.683333333
3.3	female	0.953	3.3	female	1.043	undecided	0	large	8	52HH	20.00833333
3.8	female	1.61	3.8	female	1.563	owner	0	large	9	62LL	8.278333333
3.45	female	1.265	3.45	female	1.105	owner	0	large	10	62LL	18.89833333
3.6	female	1.385	3.6	female	1.572	owner	0	small	11	58DD	20.00833333
4.1	male	1.521	4.1	male	1.909	owner	0	small	12	58DD	19.635
3.5	female	1.532	3.5	female	1.397	undecided	0	large	13	59U	20.00833333
4	female	2.531	4	female	2.101	owner	0	large	15	57JJ	10.96333333
3.75	male	1.449	3.8	male	1.426	owner	0	large	16	57JJ	20.00833333
4	female	1.728	4	female	1.813	intruder	1	small	17	63EE	19.79166667
3.8	female	1.61	3.8	female	1.857	owner	0	small	18	63EE	8.413333333
4	female	1.961	4	female	1.8	owner	0	small	19	23V	13.39666667
4.3	female	2.377	4.3	female	2.223	intruder	1	small	20	23V	15.61
3.55	female	1.046	3.6	female	1.046	owner	0	small	21	65MM	6.828333333
3.5	female	1.229	3.5	female	1.371	intruder	1	small	22	65MM	10.11
3.75	female	1.467	3.8	female	1.763	owner	0	large	23	68PP	3.92

owner_SL	owner_sex	owner_W	intruder_SL	intruder_sex	intruder_W	winner	intruder_won_binomial	treatment	focal_id	id_siblings	contest_duration_min
4.3	female	2.468	4.3	female	2.243	owner	0	large	24	68PP	19.69833333
4.2	female	2.411	4.2	female	2.599	owner	0	large	25	69QQ	3.69
3.85	female	1.61	3.85	male	1.641	owner	0	large	26	69QQ	11.765
3.5	male	1.305	3.5	male	1.326	owner	0	large	27	70RR	20.00833333
3.8	female	1.677	3.7	female	1.485	undecided	0	large	28	70RR	19.865
3.5	female	1.386	3.5	female	1.445	owner	0	small	29	60KK	4.838333333
3.8	female	1.666	3.7	female	1.4	undecided	0	small	30	60KK	10.35666667
3.5	female	1.397	3.45	female	1.342	owner	0	small	31	75WW	8.32
3.6	female	1.525	3.5	female	1.508	owner	0	small	32	75WW	8.423333333
3.2	female	1.123	3.2	female	0.984	owner	0	small	33	72TT	9.496666667
2.9	female	0.768	2.8	female	0.925	owner	0	small	34	72TT	8.568333333
3.5	female	1.351	3.5	female	1.25	intruder	1	large	35	71SS	19.52833333
3.55	male	1.008	3.55	female	1.351	owner	0	large	36	71SS	6.066666667
3.3	female	1.158	3.2	female	1.106	owner	0	small	37	66SI	8.606666667
3.45	female	1.347	3.4	female	1.262	owner	0	small	38	77ZZ	4.213333333
3.15	female	0.933	3.1	female	1.336	owner	0	small	39	77ZZ	20.00833333
3.2	female	0.976	3.2	female	1.143	owner	0	small	40	60KK	16.58

Table 7. Frequency of the behaviours done by the owner of the shelter towards the intruder in the asymmetric competition contest.

avoid_flee_owner	shelter_owner	evicted_owner	restrained_aggression_owner	overt_aggression_owner	total_aggression_owner	submissive_behaviour_owner	affiliative_owner
1	3	0	22	12	34	0	0
2	2	0	30	5	35	0	1
4	8	0	29	8	37	1	1
7	1	1	13	4	17	0	0
0	9	0	3	15	18	0	0
1	9	0	27	9	36	0	0
0	5	0	13	1	14	0	0
14	4	1	34	22	56	0	1
25	1	1	17	5	22	0	1
0	11	0	8	16	24	0	4
3	1	0	45	17	62	0	0
0	19	0	24	127	151	0	0
6	0	0	51	72	123	2	0
1	4	0	20	30	50	0	1
0	28	0	73	17	90	0	0
0	6	0	30	53	83	0	0
0	4	0	29	10	39	0	0
0	13	0	29	32	61	0	0
8	4	0	19	8	27	0	1
0	3	0	22	15	37	0	0
15	2	1	5	2	7	3	0
2	0	0	23	3	26	13	0

avoid_flee_owner	shelter_owner	evicted_owner	restrained_aggression_owner	overt_aggression_owner	total_aggression_owner	submissive_behaviour_owner	affiliative_owner
0	13	0	25	25	50	0	0
0	5	0	4	10	14	0	0
0	4	0	16	2	18	0	0
0	7	0	22	15	37	0	0
0	2	0	111	49	160	0	1
0	4	0	11	10	21	0	0
0	13	0	4	2	6	2	0
0	1	0	24	4	28	0	0
0	5	0	2	11	13	0	1
0	15	0	27	13	40	0	0
0	2	0	13	11	24	0	0
38	0	1	10	15	25	0	1
0	6	0	7	0	7	0	1
0	7	0	9	15	24	0	0
0	1	0	16	10	26	0	0
5	19	0	29	8	37	0	0
1	1	0	16	61	77	0	0

Table 8. Frequency of the behaviours done by the intruder towards the owner in the asymmetric competition contest.

avoid_flee_intruder	shelter_intruder	evicted_intruder	restrained_aggression_intruder	overt_aggression_intruder	total_agression_intruder	submissive_behaviour_intruder	affiliative_intruder
2	0	0	17	2	19	0	1
8	0	0	20	1	21	0	0
15	7	0	18	2	20	1	3
2	0	0	77	9	86	0	0
3	0	1	2	4	6	0	0
1	1	0	46	5	51	0	0
1	0	0	4	0	4	0	0
14	0	0	85	11	96	0	1
8	0	1	54	15	69	0	0
11	0	0	11	2	13	13	1
4	0	0	174	7	181	0	0
3	1	0	103	45	148	1	0
32	0	1	55	9	64	23	0
10	0	1	38	15	53	0	1
22	0	0	110	2	112	1	0
3	2	0	39	21	60	1	0
5	0	1	12	1	13	1	0
8	1	0	50	20	70	0	0
1	16	0	44	9	53	1	3
8	0	1	7	1	8	0	0
0	8	0	23	28	51	0	0
0	1	0	5	3	8	0	0

avoid_flee_intruder	shelter_intruder	evicted_intruder	restrained_aggression_intruder	overt_aggression_intruder	total_agression_intruder	submissive_behaviour_intruder	affiliative_intruder
11	0	0	19	0	19	27	11
5	0	0	3	0	3	0	0
4	2	0	21	5	26	1	0
16	0	0	19	4	23	1	1
51	0	1	95	24	119	0	0
8	0	1	2	0	2	0	0
0	2	0	13	0	13	2	2
1	0	0	47	0	47	0	1
6	0	0	21	0	21	0	1
13	0	0	19	1	20	2	0
5	0	0	18	0	18	8	2
1	29	0	58	46	104	0	1
0	0	0	11	0	11	0	0
6	0	0	17	1	18	0	0
13	0	1	6	0	6	4	0
5	0	0	89	11	100	2	0
6	2	0	47	52	99	3	0

Table 9. Hormones concentration (ng/ml) of the intruders that participated in the asymmetric competition contest. For some fish the hormones concentration was below the detection limit; therefore, the data is not available (NA).

cortisol_ng_mL_1	estradiol_ng_mL_1	testosterone_ng_mL_1	KT_ng_mL_1	progesterone_ng_mL_1
2.793	NA	NA	NA	0.022
3.124	NA	NA	0.137	0.115
5.425	0.259	NA	0.093	0.077
1.831	NA	NA	NA	NA
1.281	NA	NA	NA	0.026
3.648484848	NA	NA	NA	0.03030303
1.748484848	0.346464646	0.06969697	NA	0.025252525
2.515151515	NA	NA	NA	NA
0.424242424	NA	NA	NA	0.054545455
1.561616162	NA	NA	NA	0.049494949
0.933333333	NA	NA	NA	0.051515152
6.128282828	NA	NA	NA	0.093939394
1.391	NA	NA	NA	0.015
1.137373737	NA	NA	NA	NA
0.809090909	NA	NA	NA	0.017171717
0.793	0.223	0.063	NA	0.01
0.97	NA	NA	NA	0.009
1.517	NA	NA	NA	0.008
1.568	NA	NA	NA	0.012
1.936	NA	NA	NA	0.008
1.987	NA	NA	NA	0.011
1.328	0.151	0.061	NA	0.01
1.772727273	NA	NA	0.114141414	0.011111111
3.66969697	NA	NA	NA	0.006060606
2.909090909	NA	NA	NA	0.009090909
2.607070707	NA	NA	NA	0.007070707
2.171	0.089	NA	NA	0.009
0.365656566	NA	NA	NA	NA
0.373	0.143	NA	NA	NA
0.217171717	0.265656566	0.023232323	NA	0.008080808
0.961616162	NA	NA	NA	0.013131313
0.346464646	0.117171717	0.017171717	NA	0.008080808
1.186	0.17	NA	NA	0.007
0.848484848	NA	NA	NA	NA

cortisol_ng_mL_1	estradiol_ng_mL_1	testosterone_ng_mL_1	KT_ng_mL_1	progesterone_ng_mL_1
3.309	NA	NA	NA	NA
0.761	0.125	0.05	NA	0.009
2.362	0.362	0.084	NA	NA
NA	NA	NA	NA	NA
2.116	NA	NA	NA	0.007

Table 10. Abbreviations of the column names used in table 11, 12,13 and 14.

Column	Description
focal_id	individual identity of the focal fish that was in the family integration test
sex	sex of the focal fish
SL_focal	standard lenght of the focal fish
weight	body mass of the focal fish
id_siblings	where the focal fish come from? The identity of the sibling tank. I use the number of the tank to be blind to the treatments
acceptance_day1	acceptance status in the firts day of observation
acceptance_day2	acceptance status in the second day of observation this observation was done after the second hormone sample was taken
acceptance_day3	acceptance status in the second third of observation this observation was done some hours before the focal fish was allowed to prospect
treatment	small or large group
submissive_behaviour	submissive behaviour by the focal fish toward both breeders (sum of tail quiver and hook)
overt_aggression	aggressive displays with physical contact done by the focal towards the breeders (sum of bite, chase, mouth fight and ram)
restrained_aggression_fin	aggressive displays done by the focal fish towards the breeders (sum of frontal approach, head down, s-bend, opercula spread, tail beat, head jolt and fin spread)
restrained_aggression	aggressive displays done by the focal fish towards the breeders (sum of frontal approach, head down, s-bend, opercula spread, tail beat and head jolt)
total_aggression_fin	this is the sum of overt and restrained aggression toward both breeders including find spread
total_aggression	this is the sum of overt and restrained aggression toward both breeders
total_avoid_flee	Total number of times the focal fish fled breeders' attacks and avoided to be in close to the breeders
affiliative	Affiliative behaviour done by the focal towards the breeders (sum of bumping, follow and join)
received_overt_aggression	aggressive displays with physical contact done by breeders towards the focal (sum of bite, chase, mouth fight and ram)
received_restrained_aggression_fin	aggressive displays done by the breeder towards the focal (sum of frontal approach, head down, s-bend, opercula spread, tail beat, head jolt and fin spread)
received_restrained_aggression	aggressive displays done by the breeder towards the focal (sum of frontal approach, head down, s-bend, opercula spread, tail beat and head jolt)
received_total_aggression_fin	this is the sum of overt and restrained aggression done by the breeders towards the focal fish, including find spread

Column	Description
received_total_aggression	this is the sum of over and restrained aggression done by breeders towards the focal fish
cortisol_ng_mL_1	cortisol sample 1 taken before asymmetric competition and family integration.
cortisol_ng_mL_2	cortisol sample 2 taken while being in the family
cortisol_ng_mL_3	cortisol sample 3 taken at the end of the experiment, when the focal was 2 weeks and could prospect
cortisol_responsivenes_2_1	cortisol sample 2- cortisol sample 1
cortisol_responsivenes_3_1	cortisol sample 3 - cortisol sample 1
estradiol_ng_mL_1	estradiol sample 1 taken before asymmetric competition and family integration.
estradiol_ng_mL_2	estradiol sample 2 taken while being in the family
estradiol_ng_mL_3	estradiol sample 3 taken at the end of the experiment, when the focal was 2 weeks and could prospect
testosterone_ng_mL_1	testosterone sample 1 taken before asymmetric competition and family integration.
testosterone_ng_mL_2	testosterone sample 2 taken while being in the family
testosterone_ng_mL_3	testosterone sample 3 taken at the end of the experiment, when the focal was 2 weeks and could prospect
progesterone_ng_mL_1	progesterone sample 1 taken before asymmetric competition and family integration.
progesterone_ng_mL_2	progesterone sample 2 taken while being in the family
progesterone_ng_mL_3	progesterone sample 3 taken at the end of the experiment, when the focal was 2 weeks and could prospect

Table 11. Details of the focal fish that were in the family integration test.

focal_id	sex	SL_focal	weight	id_siblings	acceptance_day1	acceptance_day2	acceptance_day3	treatment
1	male	3.6	1.222	55X	NA	NA	NA	large
2	male	4	1.929	55x	NA	NA	NA	large
3	female	3.8	1.605	53C	NA	NA	fully_accepted	small
4	female	3.55	1.534	53C	NA	NA	tolerated	small
5	female	4	1.922	51BB	NA	NA	NA	large
6	female	4.2	2.146	51BB	evicted	fully_accepted	fully_accepted	large
8	female	3.5	1.043	52HH	evicted	evicted	NA	large
9	female	3.75	1.289	62LL	evicted	evicted	NA	large
10	female	3.5	1.105	62LL	fully_tolarated	fully_tolarated	fully_tolarated	large
11	female	3.6	1.449	58DD	accepted	fully_accepted	fully_accepted	small
13	female	3.7	1.418	59U	evicted	NA	accepted	large
15	female	4.1	2.007	57JJ	evicted	NA	NA	large
16	male	3.8	1.592	57JJ	evicted	NA	NA	large
17	female	4	1.813	63EE	evicted	tolerated	tolerated	small
19	female	4.05	1.964	23V	evicted	tolerated	fully_tolarated	small
20	female	4.3	1.931	23V	tolerated	NA	NA	small
21	female	3.6	1.329	65MM	evicted	NA	NA	small
22	female	3.5	1.223	65MM	fully_tolarated	fully_tolarated	evicted	small
23	female	3.8	1.763	68PP	fully_tolarated	tolerated	accepted	large
24	female	4.3	2.243	68PP	evicted	evicted	NA	large
25	female	4.1	2.599	69QQ	accepted	accepted	accepted	large
26	male	3.8	1.641	69QQ	fully_tolarated	fully_tolarated	NA	large
27	male	3.5	1.385	70RR	evicted	tolerated	NA	large
28	female	3.8	1.485	70RR	evicted	evicted	evicted	large
29	female	3.5	1.468	60KK	fully_accepted	fully_accepted	fully_accepted	small
30	female	3.7	1.464	60KK	fully_tolarated	accepted	accepted	small
31	female	3.5	1.421	75WW	NA	tolerated	tolerated	small
32	female	3.45	1.601	75WW	NA	fully_accepted	fully_accepted	small
33	female	3.3	1.304	72TT	NA	NA	fully_accepted	small
34	female	2.8	0.754	72TT	NA	NA	tolerated	small
35	female	3.6	1.327	71SS	evicted	NA	NA	large
36	female	3.5	1.255	71SS	tolerated	fully_tolarated	fully_accepted	large
37	female	3.1	1.014	66SI	evicted	fully_accepted	fully_accepted	small
38	female	3.4	1.346	77ZZ	evicted	NA	NA	small
40	female	3.2	1.021	60KK	fully_accepted	fully_accepted	accepted	small

Table 12. Frequency of the behaviours done by the focal fish towards the breeders in the family integration test.

submissive_behaviour	overt_aggression	restrained_aggression_fin	restrained_aggression	total_aggression_fin	total_aggression	total_avoid_flee	affiliative
9	0	1	0	1	0	32	1
2	0	4	1	4	1	55	2
11	0	6	5	6	5	10	6
23	2	10	6	12	8	44	19
36	0	2	2	2	2	9	27
14	2	0	0	2	2	23	10
0	0	0	0	0	0	0	0
6	0	0	0	0	0	16	0
29	0	1	1	1	1	1	2
19	0	0	0	0	0	0	7
66	90	3	3	93	93	7	5
0	0	0	0	0	0	3	0
4	0	0	0	0	0	13	0
8	2	13	11	15	13	10	0
3	0	2	1	2	1	7	0
3	0	0	0	0	0	0	0
2	0	0	0	0	0	10	0
18	0	12	1	12	1	3	0
9	1	12	6	13	7	11	4
0	0	0	0	0	0	0	0
31	0	1	0	1	0	3	26
4	1	8	4	9	5	0	0

submissive_behaviour	overt_aggression	restrained_aggression_fin	restrained_aggression	total_aggression_fin	total_aggression	total_avoid_flee	affiliative
1	0	0	0	0	0	0	0
23	0	1	0	1	0	5	0
22	0	3	0	3	0	2	6
16	0	12	5	12	5	2	4
26	0	13	3	13	3	18	6
62	0	5	0	5	0	0	18
33	0	0	0	0	0	6	12
8	0	1	0	1	0	3	1
47	0	3	2	3	2	5	0
62	0	1	0	1	0	25	11
9	0	0	0	0	0	32	9
10	0	0	0	0	0	52	0
34	0	3	0	3	0	7	8

Table 13. Frequency of the behaviours done by breeders towards the focal fish in the family integration test.

received_overt_aggression	received_restrained_aggression_fin	received_restrained_aggression	received_total_aggression_fin	received_total_aggression
16	18	18	34	34
31	30	30	61	61
6	6	6	12	12
32	28	28	60	60
5	15	15	20	20
16	20	18	36	34
0	0	0	0	0
11	12	12	23	23
19	6	6	25	25
0	4	4	4	4
10	21	21	31	31
5	0	0	5	5
11	7	7	18	18
1	23	23	24	24
3	10	7	13	10
4	8	8	12	12
11	0	0	11	11
3	22	22	25	25
0	20	20	20	20
0	0	0	0	0
13	6	6	19	19
0	9	6	9	6

received_overt_aggression	received_restrained_aggression_fin	received_restrained_aggression	received_total_aggression_fin	received_total_aggression
0	0	0	0	0
17	16	14	33	31
12	8	8	20	20
2	8	8	10	10
2	29	29	31	31
91	12	9	103	100
16	9	9	25	25
3	8	7	11	10
45	19	19	64	64
82	8	8	90	90
25	30	30	55	55
93	12	12	105	105
4	21	20	25	24

Table 14. Hormones concentration (ng/ml) of the focal fish that were in the family integration test. For some fish the hormones concentration was below the detection limit; therefore, the data is not available (NA).

cortisol_ng_mL_1	cortisol_ng_mL_2	cortisol_ng_mL_3	cortisol_responsivenes_2_1	cortisol_responsivenes_3_1	testosterone_ng_mL_1	testosterone_ng_mL_2	testosterone_ng_mL_3
2.793	NA	NA	NA	NA	NA	NA	NA
3.124	NA	NA	NA	NA	NA	NA	NA
5.425	10.047	5.384	4.622	-0.041	NA	0.356	0.127
1.831	1.981818182	3.893	0.150818182	2.062	NA	0.151515152	0.261
1.281	0.906	1.859	-0.375	0.578	NA	0.077	0.058
3.648484848	3.133333333	NA	-0.515151515	NA	NA	0.027272727	NA
2.515151515	NA	NA	NA	NA	NA	NA	NA
0.424242424	NA	NA	NA	NA	NA	NA	NA
1.561616162	2.253535354	4.937	0.691919192	3.375383838	NA	0.106060606	0.239
0.933333333	0.629292929	0.694949495	-0.304040404	-0.238383838	NA	0.107070707	NA
1.391	0.476767677	0.91010101	-0.914232323	-0.48089899	NA	NA	NA
1.137373737	NA	NA	NA	NA	NA	NA	NA
0.809090909	NA	NA	NA	NA	NA	NA	NA
0.793	0.004874747	8.981818182	-0.788125253	8.188818182	0.063	0.000049	0.124242424
1.517	NA	NA	NA	NA	NA	NA	NA
1.568	NA	NA	NA	NA	NA	NA	NA
1.936	NA	NA	NA	NA	NA	NA	NA
1.987	0.836363636	2.424	-1.150636364	0.437	NA	NA	NA
1.328	2.54	2.491	1.212	1.163	0.061	NA	0.11
1.772727273	NA	NA	NA	NA	NA	NA	NA

cortisol_ng_mL_1	cortisol_ng_mL_2	cortisol_ng_mL_3	cortisol_responsivenes_2_1	cortisol_responsivenes_3_1	testosterone_ng_mL_1	testosterone_ng_mL_2	testosterone_ng_mL_3
3.66969697	0.839	0.901010101	-2.83069697	-2.768686869	NA	NA	0.039393939
2.909090909	2.325252525	NA	-0.583838384	NA	NA	NA	NA
2.607070707	NA	NA	NA	NA	NA	NA	NA
2.171	1.901	3.446	-0.27	1.275	NA	0.049	0.067
0.365656566	1.353535354	6.727272727	0.987878788	6.361616162	NA	0.109090909	NA
0.373	1.169	4.235	0.796	3.862	NA	0.371	0.23
0.217171717	1.25	1.442	1.032828283	1.224828283	0.023232323	0.035	0.074
0.961616162	4.445	4.771	3.483383838	3.809383838	NA	NA	0.068
0.346464646	10.353	9.38	10.00653535	9.033535354	0.017171717	0.173	0.175
1.186	9.857	8.32	8.671	7.134	NA	0.242	0.128
0.848484848	NA	NA	NA	NA	NA	NA	NA
3.309	8.255555556	8.928	4.946555556	5.619	NA	NA	NA
0.761	3.515	11.55555556	2.754	10.79455556	0.05	NA	0.25959596
2.362	NA	NA	NA	NA	0.084	NA	NA
2.116	0.908	NA	-1.208	NA	NA	NA	0.089

Table 15. Frequency and duration of prospecting behaviour of four focal fish (fish_ID) from large and small groups (treatment). The prospecting behaviour was video recorded for one week. The observer (SW) counted the number of times a fish visited the neighbouring territory (frequency_prospecting) and the total duration of each visit (in seconds, duration_prospecting). The sum of all visits and the total time (total frequency and duration of prospecting) is in red.

tank_number	fish_ID	frequency_prospecting	duration_prospecting	
2012	4	1	42.878	
2013	5	1	400.79	
2013	5	1	81.806	
2013	5	3	525.474	total frequency and duration of prospecting
2011	10	3	93.013	
2011	10	3	114.489	
2011	10	2	191.568	
2011	10	4	145.147	
2011	10	2	285.22	
2011	10	3	133.191	
2011	10	2	164.082	
2011	10	1	29.98	
2011	10	1	37.905	
2011	10	2	73.704	
2011	10	3	97.016	
2011	10	2	92.312	
2011	10	1	18.912	
2011	10	1	44.216	
2011	10	1	68.536	
2011	10	1	43.984	
2011	10	1	78.704	
2011	10	2	82.712	
2011	10	3	87.824	
2011	10	1	284.584	
2011	10	1	24.896	
2011	10	1	41.73	
2011	10	1	66.605	
2011	10	1	49.411	
2011	10	1	12.728	

tank_number	fish_ID	frequency_prospecting	duration_prospecting	
2011	10	1	23.845	
2011	10	1	12.798	
2011	10	1	21.875	
2011	10	3	74.668	
2011	10	2	39.495	
2011	10	1	66.948	
2011	10	1	31.915	
2011	10	1	129.458	
2011	10	1	133.206	
2011	10	1	81.549	
2011	10	1	122.926	
2011	10	2	70.398	
2011	10	1	88.527	
2011	10	2	77.549	
2011	10	1	44.162	
2011	10	1	69.84	
2011	10	1	56.256	
2011	10	1	48.816	
2011	10	1	36.824	
2011	10	1	158.632	
2011	10	1	45.696	
2011	10	1	69.672	
2011	10	2	24.008	
2011	10	1	46.963	
2011	10	1	43.856	
2011	10	1	52.16	
2011	10	1	25.656	
2011	10	1	58.064	
2011	10	1	63.896	
2011	10	2	70.514	
2011	10	1	72.984	
2011	10	1	76.936	
2011	10	1	179.904	
2011	10	84	4582.465	total frequency and duration of prospecting
2012	13	7	543.842	
2012	13	6	363.992	
2012	13	6	285.24	
2012	13	8	555.608	

tank_number	fish_ID	frequency_prospecting	duration_prospecting	
2012	13	20	909.198	
2012	13	19	744.087	
2012	13	19	594.104	
2012	13	13	823.056	
2012	13	13	935.485	
2012	13	12	1062.328	
2012	13	11	1363.57	
2012	13	7	861.792	
2012	13	11	1327.736	
2012	13	10	1747.173	
2012	13	2	120.288	
2012	13	3	168.829	
2012	13	2	161.984	
2012	13	4	606.389	
2012	13	12	873.315	
2012	13	5	835.141	
2012	13	5	1052.624	
2012	13	4	644.352	
2012	13	8	975.732	
2012	13	5	699.544	
2012	13	5	1385.265	
2012	13	5	1064.612	
2012	13	2	278.752	
2012	13	3	438.536	
2012	13	2	282.418	
2012	13	4	288.5	
2012	13	5	570	
2012	13	5	439.28	
2012	13	1	832.992	
2012	13	2	31.752	
2012	13	2	89.488	
2012	13	4	201.232	
2012	13	4	277.264	
2012	13	5	335.251	
2012	13	4	155.056	
2012	13	6	213.02	
2012	13	5	229.69	
2012	13	3	273.939	
2012	13	279	25642.456	total frequency and duration of prospecting

Appendix 2

Methods and statistical analysis to test if maternal transcripts are shaped by the size of the social environment.

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Description

We define social environment as the number of individuals inside a social group. A social group is composed of one breeding pair and one subordinate, whereas a large group contains eight subordinates. There is evidence that social environments have long term effects on the brain architecture (Nyman et al., 2017) and social behaviour of *Neolamprologus pulcher* fish (Arnold & Taborsky, 2010; Fischer et al., 2017; Taborsky et al., 2012). In addition, social environments provide cues to females which may induce egg-mediated maternal effects in the form of maternal transcripts. Maternal transcripts are used during the maternal-zygote transition. Zygote uses maternal transcripts to start its development, and the transcripts degrade a few hours after fertilization when the zygote takes over the development (Yartseva & Giraldez, 2015). Then, maternal transcripts have the potential to modulate the early phases of development, which may underlie the mechanisms that modulate behaviour (e.g., stress axis).

Therefore, we investigate if the social environment where females produce their oocytes (i.e., non-ovulated eggs), shapes maternal transcripts. To test this, breeder females were either in a small or a large social group during oocyte production and we collected

unfertilized eggs. We predicted that there is a differential gene expression between eggs from small and large groups.

This appendix contains the methods used for data collection and the statistical analyses of the RNA sequencing.

Methods for sample collection

Study species

Neolamprologus pulcher is a cooperatively-breeding cichlid endemic to Lake Tanganyika, East Africa (Taborsky, 2016). The groups consist of a dominant breeding pair and a variable number of subordinate individuals of different sizes and ages Taborsky, 2016), which help to raise the offspring of the current breeding pair ('brood care helpers'). Helpers can obtain inclusive fitness benefits if they are related to the breeder's offspring (Taborsky, 2016). Moreover, all helpers obtain direct benefits by access to shelters in the breeders' territory, which is indispensable for survival because predation risk is high (Groenewoud et al., 2016). Individual survival (Brouwer et al., 2005) and the persistence of groups over time (Heg et al., 2005) increase with group size. Breeding females reduce their egg mass with increasing group size (Taborsky et al., 2007) and increase it under perceived predation risk during egg maturation (Sharda et al., 2021). Also, the behaviour of fish later in life is shaped by the early social environment (i.e., group size and composition) (Arnold & Taborsky, 2010; Fischer et al., 2017; Nyman et al., 2017, 2018; Taborsky et al., 2012) and perceived predation threat (Fischer et al., 2017). The latter occurs both by way of egg-mediated maternal effects (Sharda et al., 2021) and own offspring experience with predator cues (Fischer et al., 2017; Watve & Taborsky, 2019). Offspring raised in the presence of more adults and perceived a higher predation risk had a better social competence and were more likely to disperse from social groups for independent breeding (Fischer et al., 2017).

Experimental groups and housing conditions

We set up small (n=8) and large (n=8) breeding groups by selecting unrelated fish from the stock tanks of our aquarium facility. A small group consisted of one breeding pair and one helper, which corresponds to the minimal natural group size (Bergmuller et al., 2005). In the natural environment, most *N. pulcher* groups contain several helpers of different body sizes and ages (Bruintjes & Taborsky, 2011). Correspondingly, large groups consisted of a breeder pair and eight helpers of different sizes and sexes (Fischer et al., 2015). Breeding pairs were assigned to breed either first in a small and subsequently in a large group, or other way round, with the order of group size treatments being balanced across tanks.

The breeding groups were housed in 400-L tanks that were divided in two compartments by opaque, water-tight dividers, one small 100-L compartment for small groups (33 x 65 x 50 cm length x depth x height) and one large 300-L compartment (97 x 65 x 50 cm length x depth x height) for large groups. All compartments were equipped with a 2-cm sand layer, one half a flowerpot per fish on the tank bottom as shelters and breeding sites, and additional hiding places mounted near the water surface (empty, semi-transparent plastic bottles). In natural territories all group members have their own hiding place, which they defend against other group members (Werner et al., 2003). The water temperature was kept at 27±1 °C and the light-dark cycle was 13:11h with dimmed-light phases of 10 min in between to simulate natural light conditions. All groups were fed commercial adult flake food (JBL Novo Tanganyika®) five days a week and they received fresh food twice per week. Additional TetraMin Baby® powdered flake food was provided when free-swimming fry were present in a tank.

In natural populations, *N. pulcher* breed in colonies, and territories are always established in close vicinity to neighbouring groups (Jungwirth et al., 2015). These neighbouring conspecifics, and heterospecific space competitors, opportunistic egg predators (*Telmatochromis vitattus*), and dangerous piscivorous predators (*Lepidiolamprologus elongatus*) frequently intrude natural territories. Hence, breeders and subordinate helpers are constantly defending their territory against the various competitors and predators (Groenewoud et al., 2016). The presence of these threats increases the need for help for the dominant breeders and, in turn, raises their readiness to accept helpers (Zöttl et al., 2013). To mimic natural conditions and to elicit helping behaviours by subordinates, which increases their likelihood to be accepted by the dominant breeders (Bergmüller & Taborsky, 2005), once a week, we exposed all groups to one of the following helping tasks, where the order of presentations was balanced across tanks. (a) Defence against an egg predator, which consisted of presenting one *T. vitattus* inside a transparent tube during 5 min near the centre of the territory (Bruintjes & Taborsky, 2011). (b) Territory maintenance, which consisted of digging out sand from the shelter(s) used by the dominant breeders for breeding and/or hiding (shelter use by dominants was established directly before the task, and depending on these observations, one or two shelters were filled with sand) (Bruintjes & Taborsky, 2011). (c) Defence against an unfamiliar conspecific, presented inside a transparent tube for 5 min near the centre of the territory (Desjardins et al., 2008).

Production of experimental broods

In each group, breeding pairs were allowed to produce at least four clutches (Fig. 1). The 1st , 2nd and 4th clutch were all fertilized and not used for analysis in this study. Only the 3rd clutch generated the samples for this study.

The 1st clutch was removed and discarded; the time to first spawning served to establish new groups and achieve and monitor group stability. Group stability was defined as (i) the absence of evicted individuals, (ii) all group members having access to the bottom of the territory, and (iii) the absence of overtly aggressive interactions between group members. If those criteria were not met before the 1st clutch was laid, the group was re-structured by exchanging members or move them to a different aquarium, which sometimes helps to stabilize groups. The 2nd and 4th clutches were allowed to develop into broods that grew up within their respective group until an age of 2 months and received brood care by all group members (egg cleaning, fanning, guarding). These young were used in a different study (La Loggia MS). The 3rd clutch was collected for analysis of this study ('spawning 1').

After producing a 4th clutch, the dominant breeders were moved to another tank where they were merged with a new set of unrelated, unfamiliar subordinate individuals taken from our stock tanks to obtain 'spawning 2' (Fig. 1). If a breeder pair had spawned before in a small group, it was now placed in a large group, and, conversely, if it had been in a large group, it was now placed in a small group. Also in this new social group, we collected the 3rd clutch ('spawning 2'). Hence, spawning 1 and 2 correspond to the laying sequence at which females spawned in a particular social group. This resulted in a paired data structure with each females laying unfertilized eggs once in a large and once in a small group, allowing to control for between female variability on egg investment, which is known to account for most of the variability in maternal transcripts (Rauwerda et al., 2016). We included the spawning sequence in the data analysis, because carry-over effects between clutches may exist, which affect the maternal reproductive strategy in her current social group.

Production of unfertilized eggs

We obtained unfertilized eggs to enable us to analyse maternal transcripts, which is unaltered by embryonic development. We prevented fertilization by separating a female ready to lay eggs, further termed 'gravid female' from the rest of the group. This was in most cases

the dominant breeder female, and in a few cases the large helper female. A gravid female was recognized by her protruded genital papilla and an inflated belly. To collect the eggs of spawning 1 and 2, female reproductive status was checked twice per day for these signs of an approaching spawning. When this occurred, we added one transparent divider to separate the breeder male and the gravid female, and another transparent divider to separate the female from the rest of the group (Fig. 2). Next to the divider that separated the gravid female and dominant male, we placed two adjacent flowerpot halves leaning against each side of the transparent partition such that they formed a "shared shelter" (see Fig. 2). It could be visited by the female and the male simultaneously for spawning, but still prevented physical contact between the breeders so that the sperm released by the male could not reach the eggs (Maldonado, 2017). This method has proven successful for collecting unfertilized eggs.

Sample collection

The unfertilized eggs of spawning 1 (i.e., clutch 3 in the first social environment) and spawning 2 (i.e., clutch 3 in the second social environment) were collected with the help of a tweezer, which we used to detach each single egg individually, carefully from the surfaces, where we had detected them (e.g., flowerpot, partition, filter).

For each clutch we counted the number of eggs. We observed each clutch to determine a possible spawning pattern. It is likely that eggs close in proximity were spawned consecutively and we aimed to collect eggs with similar spawning times. In each clutch we collected sub samples of 10 eggs that were arranged in a straight line (Fig. 3). If a spawning pattern was not detected, we collected 10 eggs that were next to each other. Each sub sample was placed in a cryo pore tube of 1.6 ml, immediately flash frozen in liquid nitrogen, and stored at -80 °C until RNA extraction. In addition, we measured the length and weight of the female which had produced the clutch to calculate Fulton's body condition index, because body condition can influence the number and size of eggs (Taborsky et al., 2007).

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Methods for RNA extraction, library preparation and RNA-seq data pre-processing

RNA extraction

The RNA material was extracted using the Single Cell RNA Purification Kit from Norgen Biotek Corporation and following the manufacture instructions. Briefly, between 20 to 30 eggs were placed in solution of Buffer RL (350µl) and β-mercaptoethanol (3.5µl). The eggs were grinded using a pestle motor tissue grinder. The homogenate was centrifugated for 1 min at 6'000 rpm to separate the fat content of the eggs, which was often the upper phase. We carefully transferred the lower phase to a new Eppendorf tube with ethanol 100 % (200µl). Then, the lysate was transferred to a single cell RNA spin column (SC). The column was centrifugated for 1min at 6000 RPM and the flowthrough was discarded. We did an on-column DNA removal using RNase-Free DNase I Kit from Norgen Biotek Corporation and followed the manufacture instructions. After the DNA removal the SC was washed three times by adding 400µl of wash solution A to the column, then the SC was centrifuged for 1 min at 14000 RPM and the flowthrough was discarded. The RNA was eluded in two steps. First, we added 8µl of elution buffer, centrifuged for 1 min at 2'000 rpm followed by 1 min at 14'000 rpm. Second, we added 12µl of elution buffer, centrifuged for 1 min at 2'000 rpm followed by 1 min at 14'000 rpm. The RNA samples were storaged at -80 until further analysis.

Sequence of the RNA material

A total of 16 samples (Fig. 4) were sequenced in the Sequencing Facility of University of Bern. Eight samples of small (spawning 1 n = 4, spawning 2 n=4) and 8 samples of large groups (spawning 1 n = 4, spawning 2 n=4).

The paired ended library preparation was done in the Sequencing Facility of University of Bern using an illumina TruSeq Stranded Total RNA Library Prep Kit. The rRNA was removed using Ribo-Zero Plus. Following rRNA removal, the remaining RNA was chemically fragmented and random primers were added for reverse transcription. The sequencing was done with 16 pooled cDNA libraries in 1 lane of a NovaSeq 6000 SP flow cell, 100 cycles, 2 x 50 bp with a coverage of 30 M read/library.

RNA-seq data preparation

The quality of the read of 16 libraries was verified using *FastQC* v0.11.7 (https://www.bioinformatics. babraham.ac.uk/projects/fastqc/). We found evidence for ribosomal RNA contamination on the read libraries, so we used the trimFilterPE module of *FasqPuri* v1.0.5 (Pérez-Rubio, Lottaz, & Engelmann, 2019) to remove any reads mapping to ribosomal genes in the *Oreochromis niloticus* genome.

We used the O_niloticus_UMD_NMBU assembly in Ensembl (https://www.ensembl.org/Oreochromis_niloticus/Info/Index?db=core). After filtering, we mapped the surviving reads to the genome, using *STAR* v2.7.3a (Dobin et al., 2013) with the following parameters: --outFilterMismatchNmax 10 -- --outFilterMatchNminOverLread 0.5 --outFilterIntronMotifs RemoveNoncanonicalUnannotated --chimSegmentMin 20 --alignIntronMin 21 --alignIntronMax 200000 --alignMatesGapMax 200000. Afterwards, we used *FeatureCounts* v2.0.2 (Liao, Smyth, & Shi, 2014) to count how many reads mapped to each gene and exon in the genome. For this step we only included read pairs where both mates mapped successfully to the genome and that were not mapped into different strands or chromosomes. We used MultiQC v1.8 (Ewels, Magnusson, Lundin, & Käller, 2016) to summarize the quality reports for all samples. All steps were performed on the University of Bern HPC cluster UBELIX (http://www.id. unibe.ch/hpc).

One pair of samples was removed. The pair had the following combination spawning 1 in small group and spawning 2 in a large group. It was removed because the spawning 1 had only 8.6 million read left after filtering the ribosomal RNA which was 54% less than the sample with the second lower number of reads.

Differential expression and splicing analysis

For the differential expression and splicing analysis we used R v3.6.1 (R Core Team, 2019) and the package *edgeR* v3.26.8 (Robinson, McCarthy, & Smyth, 2010) available at the Bioconductor website (http://bioconductor.org). For the identification of differentially expressed genes (DEGs) we used the gene-level read counts and filtered lowly-expressed genes using the edgeR algorithm. Therefore, genes with fewer than 10 read counts in 7 or more samples were removed. We calculated the library normalization factors and used the weighted likelihood Empirical Bayes approach implemented in edgeR to estimate gene expression dispersions. Next, using the *limma* v3.40.6 R package (Ritchie et al., 2015) we run a

Multidimensional Scaling Plots (MDS) based on 500 genes with the highest gene expression fold-changes between each pair of samples. This analysis revealed that two other samples were strong outliers (Fig. 5). This difference could be due to inappropriate storage conditions before the extraction of RNA material. Therefore, these two samples (and their pairs) were removed from the analysis. Hence, we had a total of five replicates per each group size instead of eight.

The data was fitted to a Generalized Linear Model (GLM) with a negative binomial distribution using group size as the main explanatory variable and controlling for breeding pair identity. We were unable to include spawning sequence on the GLM, because the three pairs of outliers had all the same combination of spawning sequence and group size (e.i., spawning 1 in a large group and spawning 2 in a small group). Then, this specific combination only had one replicate. This caused false positives when including spawning sequence in the GLM. Finally, we used a quasi-likelihood F-test to identify differentially expressed genes (DEGs) between group sizes. The p-value cut-off was set to 0.05 and correction for multiple testing was done using the False Discovery Rate (FDR) method (Benjamini & Hochberg, 1995).

To identify differentially spliced genes (DSGs) we tested our data for differential exon usage. This method tests whether the relative expression of exons within a gene changes or not between group sizes. If it changes, this suggests changes in the splicing of that gene that are affecting the exons relative abundances. We used the exon-level count data from *featureCounts* as the input and applied the same pre-processing steps and GLM model as we did for the gene-level data. We then used two complementary methods for differential exon usage analysis implemented in *edgeR*. One method is the exon-level, which identifies individual exons with strong changes in their relative expression to other exons. The gene-level method identifies genes where many exons show changes in their relative expression. For the exon-level method, we converted the exon p-values to gene p-values using *edgeR*'s implementation of the Simes method (Simes, 1986).

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Figures

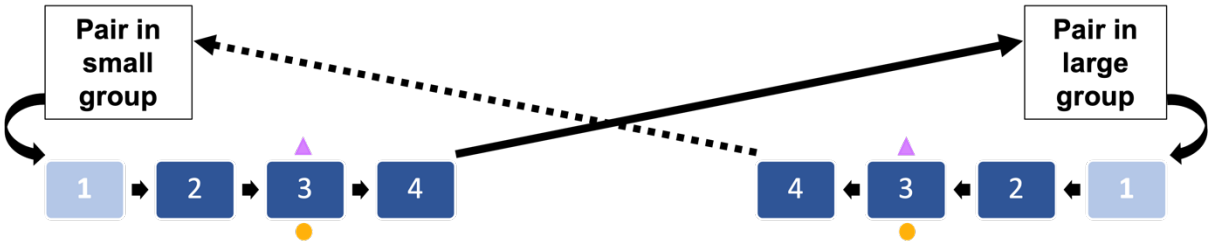


Figure 1. Schematic representation of the egg collection sequence. A breeder pair was assigned either to a small or to a large social group. In each group, breeders produced up to four clutches (i.e., 1,2,3, and 4). The first clutch (light blue box) was removed and discarded. The 2nd and 4th clutches were allowed to hatch and young to grow up in the social groups (dark blue boxes). The 3rd clutch (spawning 1, i.e., orange circle) was either unfertilized or freshly fertilized and collected for analysis. After the 4th clutch, the breeding pair was assigned to a new set of helpers either in a small (dotted arrow) or large (solid arrow) and the eggs of spawning 2 (i.e., purple triangle; unfertilized or freshly fertilized) were collected following same procedure described for spawning 1.

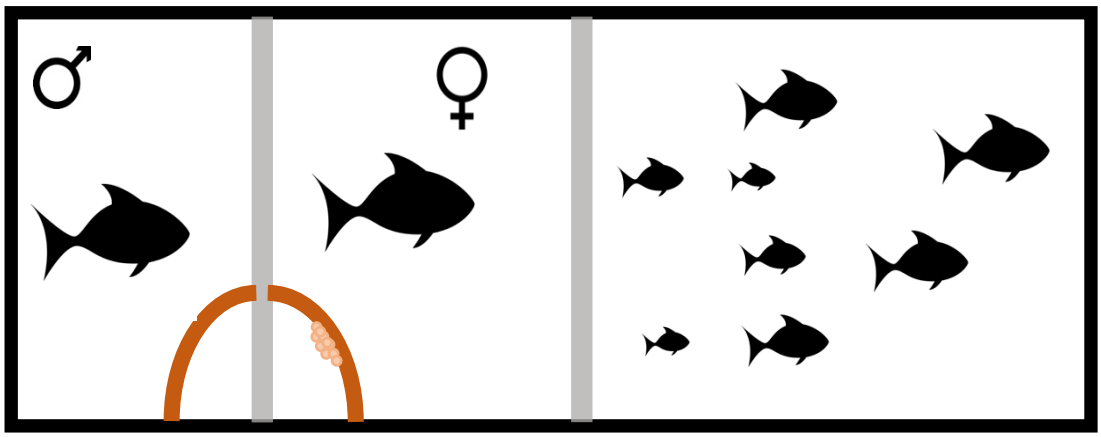


Figure 2. Experimental set-up used to collect unfertilized clutches ('spawning 1 and 2'). For spawning, *N. pulcher* females and males jointly visit the breeding chamber, where the female deposits eggs on the chamber walls, which are immediately fertilized by males. In the set-up we built a "shared shelter" around a transparent divider, which could be visited simultaneously by the breeder male and the female (i.e., breeder or large helper), and where they could court each other, which stimulate females spawning. Yet, the breeders had no physical contact. This method allowed to collect unfertilized eggs from females, since the transparent divider prevented the fertilization of the eggs by the male. The breeder male (left compartment) was separated from the female (middle compartment) by a transparent partition (left grey vertical line). The rest of the group was separated from the breeders by another transparent partition (right grey vertical line; set-up adapted from (Maldonado, 2017).

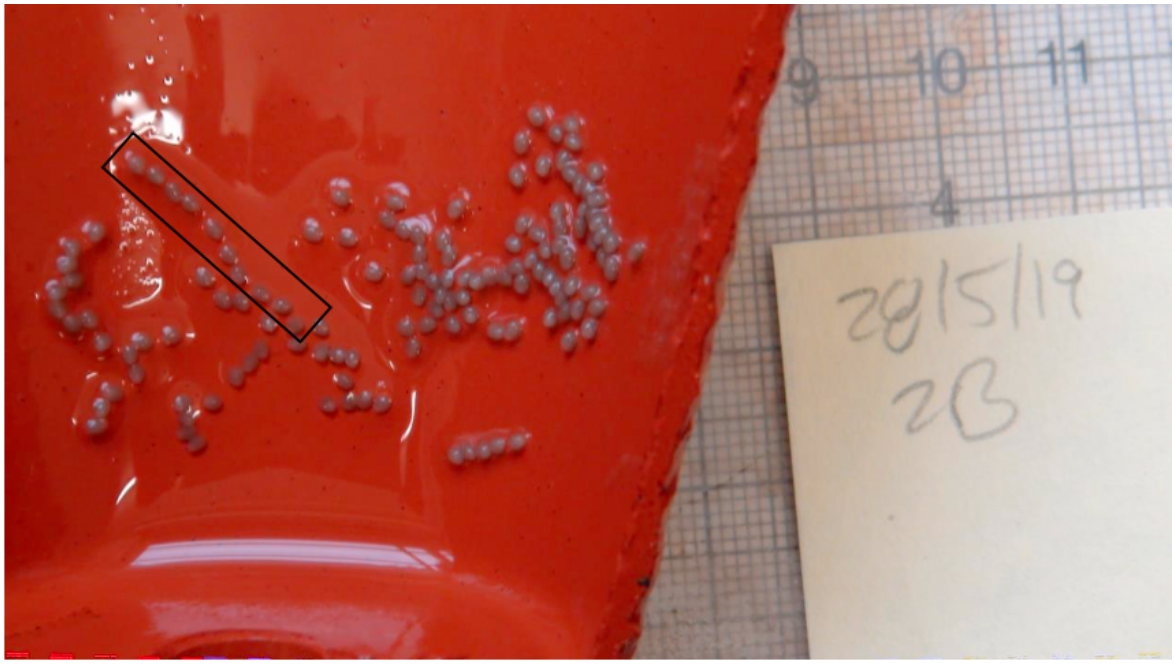


Figure 3. A flowerpot containing one unfertilized clutch. The eggs are arranged either in patches or in a straight line (black rectangle).

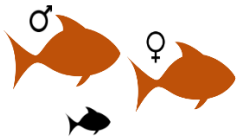
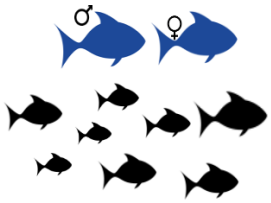
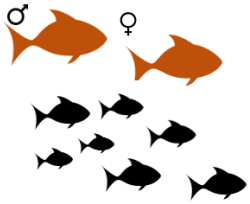
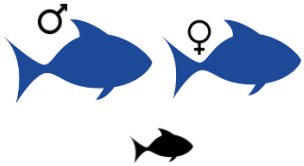
	Group size	Group size
Clutch Number	S1 	L1 
	S1, n = 4	L1, n = 4
Clutch Number	L2 	S2 
	L2, n = 4	S2, n = 4

Figure 4. Schematic representation of the number of samples collected. We collected two spawning for each breeding pair (i.e., spawning 1 and 2). Each spawning was either in a small (S) or a large group (L).

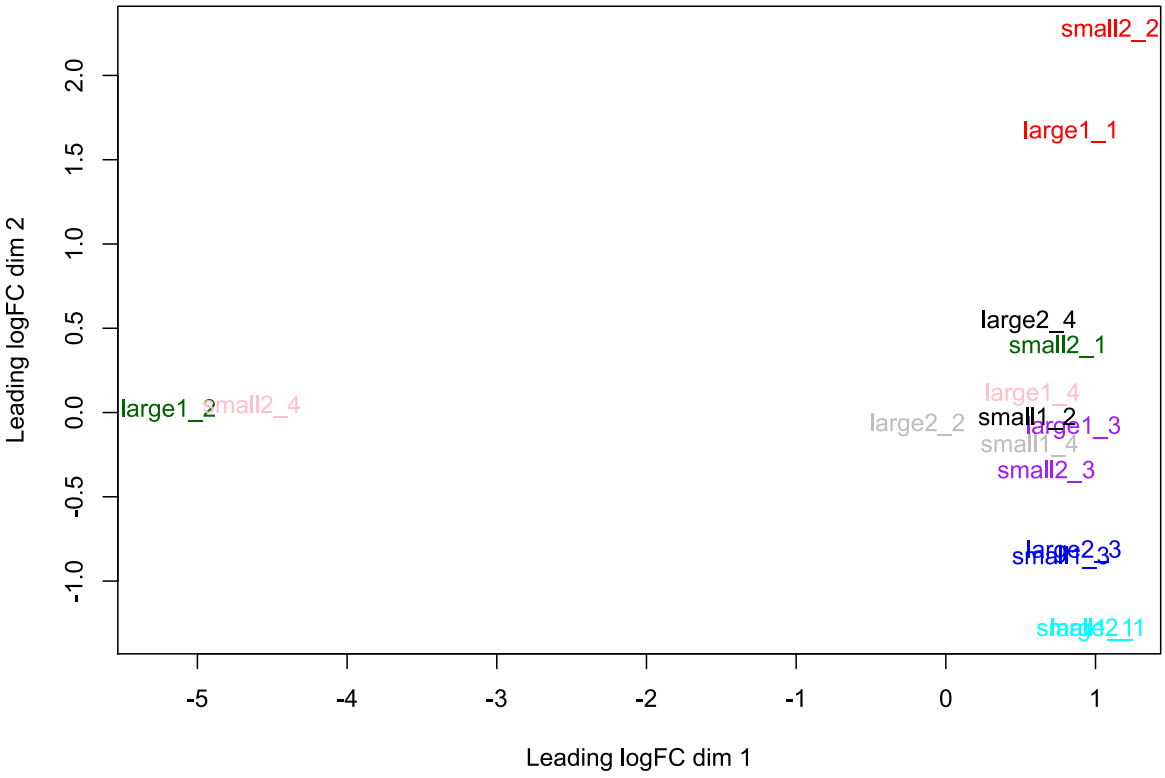


Figure 5. Multidimensional scaling plot (MDS) based on the pairwise gene expression differences between samples. The pair samples are coloured by the identity of the breeding pair. Each sample is labelled with the size of the social group were dominant breeders reproduced, while the two numbers indicate the spawning order and the sample index, respectively. For example, the sample large2_4; large2 indicates that this is the second spawning of the breeding pair that occurred in a large group and the number 4 indicates that this is the 4th replica. The three outlier samples removed from the analysis were small2_2, large1_2, and small2_4.

Declaration of Consent