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# Early social experience has life-long effects on baseline but not stress-induced cortisol levels in a cooperatively breeding fish

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# ABSTRACT

In cooperatively breeding cichlid fish, the early social environment has lifelong effects on the offspring's behaviour, life-history trajectories and brain gene expression. Here, we asked whether the presence or absence of parents and subordinate helpers during early life also shapes fluctuating levels of cortisol, the major stress hormone in the cichlid *Neolamprologus pulcher*. To non-invasively characterize baseline and stress-induced cortisol levels, we adapted the 'static' holding-water method often used to collect waterborne steroid hormones in aquatic organisms by including a flow-through system allowing for repeated sampling without handling of the experimental subjects. We used 8-year-old *N. pulcher* either raised with (+F) or without (-F) parents and helpers in early life. We found that *N. pulcher* have a peak of their circadian cortisol cycle in the early morning, and that they habituated to the experimental procedure after four days. Therefore, we sampled the experimental fish in the afternoon after four days of habituation. -F fish had significantly lower baseline cortisol levels, whereas stress-induced cortisol levels did not differ between treatments. Thus, we show that the early social environment has life-long effects on aspects of the physiological stress system of the Hypothalamic-Pituitary-Interrenal (HPI) axis. We discuss how these differences in physiological state may have contributed to the specialization in different social and life-history trajectories of this species.

#### 1. Introduction

Developmental plasticity is the ability of an organism to adjust its phenotype according to environmental cues perceived during ontogeny (West-Eberhard, 2003). It can affect phenotypic traits at all levels of organismal organisation including morphology, physiology, cognition and behaviour, and those traits are typically irreversible (Piersma and Drent, 2003). Early-life exposure to social or ecological conditions may lead to persistent programming of the physiological stress response (Seckl, 2004; Meaney and Szyf, 2005; Meaney et al., 2007), with ensuing consequences for behavioural performance and fitness.

Animals often perceive changes in the environment as stressors (LaDage, 2015), i.e. stimuli which disrupt or threaten to disrupt an individual's ability to maintain a stable internal state (homeostasis). Typical stressors include the fluctuation of abiotic factors like a cold snap, food shortage, predator encounters, being challenged by a competitor or losing one's territory. Upon the encounter of a stressor, a coordinated neurophysiological response in the brain and its periphery is activated, which includes enhanced metabolism and channels energy to the muscles to restore homeostasis (Sandi and Haller, 2015). In vertebrates, this response is characterized by the activation of the hypothalamic-pituitary-adrenal (HPA) axis (mammals, birds and reptiles) or the hypothalamic-pituitary-interrenal (HPI) axis (amphibians, fish). It is mediated by glucocorticoids (GCs), the main vertebrate stress hormones, which occur as cortisol in most mammals and fish and corticosterone in rodents, birds, reptiles and amphibians. GC concentrations follow a characteristic pattern after a stressor: they rise rapidly from basal levels, reach peak concentrations in the order of minutes or hours (Cockrem, 2013), followed by a decay phase of several hours during which GC levels reach basal levels again (de Kloet et al., 2008). Besides coping with immediate stressors, stress hormones also have long-term consequences. Baseline GC levels are essential for supporting processes associated with metabolism and behaviour, but can also facilitate reproduction (e.g. Bonier et al., 2009; Hau et al., 2016). Stressinduced GC levels play a crucial role in the formation and consolidation of memory related to stressors (De Kloet et al., 1999). However, high stress hormone levels caused by high glucocorticoid baseline levels during environmental disturbances and/or by repeated glucocorticoid

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responses to recurring stressors can impose fitness costs, including tissue damage (Sapolsky, 1986), reproductive suppression (Dulude-de Broin et al., 2019) and reduced survival prospects (Boonstra et al., 1998).

During early life the HPA/HPI axis can undergo lifelong programming by social or non-social experiences altering the response to stressors, as shown in mammals (Champagne, 2010; Liu et al., 1997), birds (Naguib et al., 2006; Zimmer and Spencer, 2014) and fish (Nyman et al., 2018, 2017; Reves-Contreras et al., 2019). For instance, in guppies, Poecilia reticulata, early exposure to predation risk had lifelong effects on cortisol excretion during habituation stress responses (Chouinard-Thuly et al., 2018). The consequences of early social impairment on stress-axis programming have received an increasing interest in human and non-human animals due to its negative health consequences (Lovallo, 2013). For instance, children who experienced a traumatic event during a critical developmental window exhibit lower basal cortisol levels and a blunted cortisol response to a stressor (Desantis et al., 2011; Mangold et al., 2010; Meinlschmidt and Heim, 2005). The absence of maternal care leads to an epigenetic reduction of glucocorticoid receptor (GR) gene expression in the brain of laboratory rats (Liu et al., 1997) and zebra finches, Taeniopygia guttata (Banerjee et al., 2012). Also early social deprivation outside a parental care context can affect HPA/HPI programming by either increasing (mandarin voles Microtus mandarinus; Yu et al., 2013) or decreasing basal cortisol levels (common marmoset Callithrix jacchus, Dettling et al., 2002) or by reducing brain GR expression (Neolamprologus pulcher; Nyman et al., 2017, 2018). In contrast to basal and stress-induced corticosteroid levels, how early social experience affects the course the entire stress response, which also includes the decay phase following a glucocorticoid peak, has so far received the least attention. In wild cavies (Cavia aperea) raised in groups differing in their social stability (i. e. group membership was changed or kept stable) the early social environment does not affect the cavies' stress responsiveness (Sangenstedt et al., 2017).

In the current study, we investigated if and how early social deprivation affects stress responsiveness in the adult life of the cichlid fish N. pulcher. In particular, we asked if the early social environment has long-term effects on individual basal cortisol levels, its response to a stressor by cortisol increase, and its rate of recovery from the stress response. N. pulcher is a cooperatively breeding cichlid, which, in the wild, lives in stable groups consisting of a breeding pair and several subordinate brood care helpers. We used an 8-yr-old laboratory population of N. pulcher of which the early social environment had been manipulated by either being reared with (+F) or without (-F) breeders and a helper during the first 2 months of life. After the early-life experiences, i.e. from an age of 2 months onward, all fish were kept in sexsegregated aggregations without breeders and helpers. Previous work on this laboratory population showed that the early social environment influences N. pulcher's social competence, i.e. the ability to respond appropriately to social information (Taborsky and Oliveira, 2012), their dispersal propensity and their reproductive investment strategy (Fischer et al., 2017; Antunes and Taborsky, 2020). These behavioural changes were accompanied by reprogramming of the HPI axis. Individuals which had been raised without breeders (-F) had a down-regulated GR1 expression in the telencephalon (Nyman et al., 2017, 2018).

Based on these observations, we hypothesized that (i) *N. pulcher* reared in -F conditions will reduce their cortisol levels more slowly after a stressor because of their lower GR1 gene expression in the telencephalon (Nyman et al., 2017, 2018), which suggests they should be less efficient in blocking the excretion of cortisol through a negative feedback mechanism (Ladd et al., 2004; Liu et al., 1997); and (ii) -F fish will have a reduced basal cortisol due to their early social deprivation (Grace and Anderson, 2018; Nephew et al., 2017; Tarullo and Gunnar, 2006).

Fish excrete steroid hormones through the gills, allowing to noninvasively extract waterborne cortisol using the holding-water method (Earley et al., 2008; Scott and Ellis, 2007). To be able to capture

baseline, peak and recovery phase of the stress responses, we adapted the original, static holding-water method (Scott and Ellis, 2007) to a dynamic water flow-through system enabling us to collect repeated water samples from the same fish without disturbing it. We used this method (1) to determine the circadian cortisol cycle of N. pulcher, and thereby to find the period of low diurnal cortisol excretion for our measurements of the stress response, (2) to determine the time the fish need to habituate to the experimental handling procedures and (3) to measure baseline cortisol and stress-induced cortisol response curves in N. pulcher. We also tested whether opercular beat rates (OBRs), which measure the ventilation rate of fish, during a stress response correlate with cortisol excretion (Kim et al., 2018; von Borell et al., 2007), in which case video-recording of OBRs would be a fast and easy alternative to cortisol measurements without involving any disturbance of the animals. When exposed to stressors, such as predator, fish either reduce OBR (Barreto and Volpato, 2011; Di Poi et al., 2016; Stratmann and Taborsky, 2014), or increase OBR (Gibson and Mathis, 2006; Hawkins et al., 2004). Furthermore, OBR has been suggested as a proxy to measure stress-coping styles (Barreto and Volpato, 2011).

#### 2. Methods

#### 2.1. The vertebrate stress response

When a stressor is perceived, the limbic system of the vertebrate brain triggers the release of corticotropin releasing factor (CRF) from the hypothalamus. CRF then stimulates the excretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary to the blood stream, which then triggers the production and secretion of GCs from the adrenal cortex (Joëls and Baram, 2009; Lightman, 2008). The response to a stressor is mainly mediated by changes in circulating GC levels (Sapolsky et al., 2000), although the physiological stress response in fact involves an interaction of numerous physiological systems (Joëls and Baram, 2009, see also Box 1 in Taborsky et al., 2020). There are two types of receptors which regulate the effects of GCs, the glucocorticoid receptors (GR) and the mineralocorticoid receptors (MR). Note that fish have two GRs, GR1 and GR2 due to gene duplication, where GR1 is the functional homologue to the mammalian GR (Arterbery et al., 2011). GR is active when glucocorticoid concentrations are high, for instance during a stress response (Rothuizen et al., 1993). MRs have a 10-fold higher affinity to GCs than GR (Greenwood et al., 2003; Joëls, 2006); therefore, they are activated also during periods of basal glucocorticoid secretions (Greenwood et al., 2003). Together GRs and MRs regulate the HPA/HPI axis by positive and negative feedbacks, and contribute to maintaining and restoring homeostasis (de Kloet et al., 2008; Joëls and Baram, 2009).

# 2.2. Study species

N. pulcher is a cooperatively breeding cichlid fish endemic to Lake Tanganyika living in stable social groups with a size-based hierarchy consisting of a dominant breeder pair and one to 20 subordinate individuals acting as alloparental brood care helpers (Taborsky, 2016). In its natural habitat, N. pulcher can reach ages of up to 6-8 years with modal ages of 2 years for males and 3 years for females (A. Jungwirth et al., in revision). In captivity N. pulcher may attain ages of 10 years (B. Taborsky, pers. obs.). Depending on population of origin, the standard lengths (SL; i.e. the length between the tip of the snout and the end of the caudal peduncle) of this species reach 5.5 to 7.0 cm. Until sexual maturity, which occurs around an age of 1 year and an SL of 3.0-3.5 cm, all subordinate group members delay dispersal from their natal groups and help raising the dominant's offspring. Helping behaviours include direct brood care in form of egg cleaning and oxygen provisioning by fanning, territory maintenance and defence against predators and space competitors (Balshine et al., 2001; Bruintjes and Taborsky, 2011; Heg and Taborsky, 2010; Koenig and Dickinson, 2016; Taborsky, 1985,

1984). Many subordinates stay as helpers at the natal territory long after sexual maturity, whereas others disperse rather soon afterwards (Stiver et al., 2004). In *N. pulcher*, severe predation risk is assumed to have selected for the species' sociality (Heg et al., 2004; Taborsky, 1984), with social group composition driven by variation in predation pressure, social needs and conflicts of interest (Groenewoud et al., 2016).

# 2.3. Ethical approvements and husbandry of experimental fish

All experimental procedures were approved by the Veterinary Office of the Kanton Bern, Switzerland, licence number BE 93/18, and were carried out in accordance to the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, USA as well as the EU Directive 2010/63/EU for animal experiments. All cichlids used in the experiments were bred and housed at the Ethological Station Hasli of the Institute of Ecology and Evolution, University of Bern, which is a licensed breeding facility for cichlid fish (licence number BE 4/11, Veterinary Office of the Kanton Bern).

Prior to an experimental test (i.e., diurnal cycle, habituation or stress response measurements), the involved experimental fish were moved from laboratory stock tanks, where they had been housed in large groups, to 20-L tanks in which they were kept singly until the test ended. This short social isolation was important to reduce variation due to difference in social hierarchy position (Dey et al., 2013) and aggressive encounters during the experiment as far as possible, as changes in hierarchy status affect cortisol excretion in *N. pulcher* (Culbert et al., 2018). Each 20-L tank was equipped with a 2 cm sand layer, a biological filter and a halved flower pot. The light:dark cycle was set to 13:11 h with a 10 min dimmed light period in the morning and evening to simulate the light conditions of Lake Tanganyika. Fish were fed ad libitum with commercial flake food (TetraMin®) 5 days a week and frozen zooplankton one day a week. Water temperature was maintained at 27  $\pm$  1 °C.

### 2.4. Experimental apparatus

To sample the changes of cortisol levels non-invasively over time from the fish, we used a modification of the holding-water method previously used to obtain static measures of waterborne steroid hormones in fish (Earley et al., 2008; Scott and Ellis, 2007). Waterborne cortisol is a reliable proxy for circulating plasma cortisol, because (i) cortisol levels derived from holding water correlate positively with plasma cortisol levels (Scott and Ellis, 2007) and (ii) the lag between cortisol excreted by the gills in the water and plasma cortisol levels is only a few minutes (Ellis et al., 2004). A water flow-through system was established to collect several, consecutive holding water samples from the same individual without disturbing the fish (Fig. 1). The system consisted of a 50-L tank serving as clean tap water reservoir, two 1-L fish-holding containers (except when measuring diurnal cortisol variation, where 2-L containers were used; see below) and two 1-L Erlenmeyer flasks to collect the hormone-water samples. Water with excreted cortisol from the fish-holding containers accumulated in the collecting flasks at a constant rate, while the fish-holding container constantly was refilled by clean tap water from the reservoir at the same rate. Water flow was regulated such that there was one complete exchange of the water volume of the fish-holding container while collecting one sample (3 h for diurnal cortisol fluctuations, 30 min for all other measurements). Two samples were collected in parallel, using a multichannel ISMATEC BVP peristaltic pump (ISMATEC, Switzerland; Fig. 1 shows one of the two parallel sampling systems). Pump and fish-holding container were placed on different racks to avoid transmission of vibrations to the focal fish. Teflon (PFTE) tubing (APSOparts, Switzerland) of 3.28 mm inner diameter was used to pump water between the different compartments. Although water flow was adjusted such that there was a complete exchange of water volume before a sample was taken, some mixture of clean and used water in the fish-holding container may have occurred from the second sampling time point onwards. Hence collected samples are not discrete and entirely independent representations of the cortisol excreted in one sampling period, but should be interpreted in combination of all sampling periods to



Fig. 1. Scheme of the experimental apparatus; blue (dark) lines: flow direction of clean water; red (light) lines: flow of water containing water-borne hormones. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

represent the stress response curve of an individual.

To fill the clean water reservoir, a 50-L tank was carefully cleaned first with pure acetone and subsequently with 99% ethanol; then it was rinsed with distilled water before filling it with tap water. We repeated the cleaning procedure before each refilling of the tank (diurnal cortisol changes: every 2 days; habituation and stress experiment: every 4 days). The water reservoir tank was equipped with an air stone to ensure oxygen saturation in the water.

# 2.5. Experimental procedures

#### 2.5.1. Diurnal cortisol variation

When measuring a cortisol stress response, we have to account for the circadian cortisol cycle of the study species (LaDage, 2015). Typically, there is an increase in basal level secretion of GCs at the beginning of animals active period, and GCs levels then decrease with time reaching their lowest levels towards the end of the active period (Ishida et al., 2005; LaDage, 2015). The diurnal GC peak can vary between species (Lorenzi et al., 2008; Manzaneda et al., 2017). Because we wanted to measure the stress response during the time when the basal diurnal cortisol levels are low (Rao and Androulakis, 2019), as first step of this study, we determined the circadian GC cycle of *N. pulcher*.

To determine the circadian cortisol cycle of *N. pulcher*, we used five fish from our laboratory stock population, which all had grown up within social groups. All fish were caught at 08:00 h from their home tanks. We measured their standard lengths (SL) ( $4.23 \pm 0.6$  cm; mean  $\pm$  SD) and weighed every fish to the nearest 0.01 g on an electronic balance ( $5.24 \pm 0.9$  g). Then, we placed them singly in a fish-holding container of the experimental apparatus, which for this measurement was a 2-L rectangular glass container ( $20 \times 12 \times 12$  cm, L  $\times$  W  $\times$  H) filled with 1.0 L of tap water. The fish were kept in the flow-through apparatus for 24 h for habituation. On the next day, we collected 1.0 L of holding water every 3 h between 08:00 and 20:00 h (8–11 h; 11–13 h; 13–15 h; 15–17 h; 17–20 h). The water samples were processed directly after collection (see section 'Cortisol measurements').

#### 2.5.2. Habituation

The procedures involved in the holding-water method, including catching, measuring, weighing and placing the fish in a novel container is perceived as stressful by the experimental animals (Wong et al., 2008). However, fish habituate to these procedures when exposed to them for several consecutive days (Wong et al., 2008). As we wanted the experimental fish to be at basal cortisol levels when measuring their stress response, we tested how long N. pulcher take to habituate to the apparatus and experimental procedures. For the habituation trials, we used six adult fish from our laboratory stock population of adult N. pulcher (N = 3 males and 3 females). All six fish were reared with adult breeders and helpers when young, so their early life conditions correspond to +F rearing conditions (see Section 2.5.3). One day before the start of the habituation phase, we moved an experimental individual to a 20-L tank. On the next day, we took its SL (5.4  $\pm$  0.2 cm; mean  $\pm$  SD) and weight (5.17  $\pm$  0.9 g), before placing it in the fish-holding container of the experimental apparatus (see Fig. 1), a round 1-L glass beaker (11.5 cm diameter) filled with 500 mL of tap water. We collected 500 mL of holding water every 30 min for 2 h in the same way as described above and processed the samples immediately after collection. After 2 h we placed the fish back in its individual 20-L holding tank, until the next day. We repeated this procedure for four consecutive days. To avoid the diurnal cortisol peak (see Results, section 'Diurnal cortisol variation'), we started the habituation trials every day at 14:00 h. Samples were collected in three batches, with two fish per batch (one male and one female).

# 2.5.3. Stress responses of fish with different social rearing background To investigate if early social deprivation persistently shapes the stress response of adults, we compared the stress responses of fish that

were either raised with (+F) or without (-F) breeders and helper. The fish for this experiment had been reared in one of two early social environments, either (i) with parents, one helper and same-aged siblings (+F treatment), or (ii) with same-aged siblings only (-F treatment; for details on rearing procedures see Fischer et al., 2017). The early-environment treatments were applied for 62 days after larvae had reached the free-swimming stage, which occurs at an age of 10 days; subsequent to the early-environment treatments, fish from all treatments were kept in sex segregated, same age sibling groups in 200-L tanks (Fischer et al., 2017).

We measured stress responses of 20 fish (10 +F and 10 -F fish, balanced for sex), which by the time of our experiment were 8 years old. Like in the habituation experiment, one day before the experiment, we moved the focal fish to a 20-L tank. To let the focal individual habituate to the handling procedures (see Section 2.5.2), we caught it at three consecutive days, and measured its SL and weight (SL: 5.92  $\pm$  0.77 cm; weight: 6.54  $\pm$  3.17 g, N = 20) before placing it in the 1-L fish-holding container of the experimental apparatus, where we kept it in the water flow-through system for 2 h per day. On the fourth day, again after catching and measuring, we placed the focal individual in the holding container and collected a 500-mL sample of holding water for 30 min, to get its baseline cortisol concentration. Afterwards, we applied a mild confinement stressor by moving the focal fish to a new 1-L beaker filled with 1.0 L of tap water, where it was kept within a hand net for 5 min while being fully submerged in the water (adapted from Kittilsen et al., 2009 and Magnhagen et al., 2015). Then we placed the focal fish back to the fish-holding container of the experimental apparatus and collected another four 500-mL samples of holding water for intervals of 30 min over 2 h to measure the stress response. Samples were again processed immediately after collection.

#### 2.5.4. Opercular beat rates (OBR)

We tested if OBR could be a behavioural proxy for stress response, by correlating OBR with cortisol excretion. On the 4th day, during the entire length of the stress response measurements (see Section 2.5.3), all focal fish where video-recorded using a Sony Handycam HDR-PJ260. Out of the 2.5 h of video recordings, the number of opercular beats during the last 2 min of the baseline cortisol sampling (i.e. minutes 28-30 of the first 30 min of the water sampling described in Section 2.5.3) were counted from the videos. Furthermore, the focal's opercular beats were counted every 5 min for 2-min periods during the first 35 min of videos recorded after applying the confinement stressor. Opercular beats were counted using the behavioural analysis software Boris v.7.8.2 (Friard and Gamba, 2016). To get the OBR, the number of opercular beats was divided by the actual time opercular beats could be seen and counted from the video. In a few recordings, the time intervals were not exactly 2 min, for instance, because a fish was moving so that for some seconds opercular beats were not visible. Due to failure of the video equipment during the trials of two fish, OBRs were calculated only for 18 fish.

### 2.6. Cortisol measurements

Water samples were filtered (paper filter 1/2595 grade, diameter 320 mm, Whatman, Sigma-Aldrich, Switzerland) to remove solid particles, and hormones were extracted using solid phase extraction (SPE). Prior to SPE, an internal standard was added to all samples by adding 100  $\mu$ L of 40 ng/mL cortisol-D4 solution. After the hormone samples were loaded onto a cartridge (SPE column Isolute C18 (EC), 500 mg/6 mL, Biotage, Sweden), the cartridges were stored at -20 °C until further processing at the Neuchâtel Platform of Analytical Chemistry (University of Neuchâtel). Cartridges were eluted in 6 mL of ethyl-acetate, which was evaporated at 35 °C by centrifugal evaporation until dry. Samples were then re-suspended in 500  $\mu$ L of MilliQ H<sub>2</sub>O:methanol HPLC grade (50:50) and filtered through 20 mm PTFE hydrophilic syringe filters (BGB Analytik AG, Switzerland) into vials containing 250  $\mu$ L conical glass inserts. Cortisol and cortisone contents were measured by ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Full details on the sample processing are given in Reyes-Contreras et al. (2019).

# 2.7. Statistical analysis

Basal cortisol concentrations refer to the water samples taken on the 4th day of the stress response trials, during 30 min before the fish were exposed to the confinement stressor (see Section 2.5.3). Cortisol 'peak' values refer to the water sample of an individual with the *maximum* cortisol excretion after the stressor. A correlation between basal cortisol and body mass was performed to test if, similarly to other fish species, body mass correlates with excreted cortisol (Ellis et al., 2004; Scott and Ellis, 2007). As previously shown in *N. pulcher* (Bender et al., 2006), there was no significant correlation between basal cortisol and body mass (Pearson's correlation: r = 0.2, p = 0.39; see Supplementary Information (SI), Fig. S1). For this reason, cortisol concentration was not corrected for individual body mass in the further analysis (see Bender et al., 2006). Cortisol concentrations were calculated as per fish per sample.

Statistical analyses were performed using the software R version 3.5.2 (R Core Team, 2018). Diurnal cortisol variation, habituation to experimental procedure, baseline cortisol, cortisol excretion after the stressor and OBR were analysed by fitting Linear Mixed-effects Models (LMM) using the package "lme4" (Bates et al., 2015). All initial models contained the rearing treatment, sampling time point and sex as fixed effects. In the model analysing the habituation data (Section 2.5.2), we also included the interaction between day and sampling time point. When analysing the entire stress response curve, the quadratic term 'time point<sup>2</sup>' was included as additional fixed factor. Rearing treatment and sampling time point were always retained in the models, whereas sex was dropped from the initial models by backwards selection if it was not significant (Bates et al., 2015). Furthermore, in all models the identity of the parents of our experimental fish was included as random term to account for genetic background (some experimental fish were bred from the same parents). Whenever data of the same individual at different time points were analysed, fish identity was included as additional random term. The assumptions of normality of the error term were checked by Shapiro-Wilk tests, and visual inspection of quantilequantile plot of model residuals to detect skew and kurtosis, as well as Tukey-Anscombe plots to check for homogeneity of variance. Cortisol concentration was always log-transformed to fit the normal distribution.

We calculated the difference between (i) OBRs measured 2 min after the stressor and OBRs measured 2 min before the stressor to get the OBR response to the stressor for each individual; and between (ii) cortisol concentration 30 min after the stressor and baseline cortisol to get the cortisol response to the stressor for each individual. OBR response was analysed by fitting a linear model, as family identity explained zero variation of OBR. A Pearson's correlation was performed between OBR response and cortisol response.

Marginal and conditional  $R^2$  were calculated from all models as described in (Nakagawa et al., 2017; Nakagawa and Schielzeth, 2013) using the R package MuMIn (Barton, 2020).  $R^2_{GLMM}$  marginal is the proportion of the total variance explained by the fixed effects only, whereas  $R^2_{GLMM}$  conditional is the proportion of the variance explained by fixed and random effects (Nakagawa et al., 2017).

#### 3. Results

# 3.1. Diurnal cortisol variation

*N. pulcher* had the highest baseline values of diurnal cortisol in the morning between 8 and 11 h (Fig. 2), with cortisol concentrations steadily decreasing during the day. These results indicate that, like in other vertebrates, the circadian cortisol cycle of *N. pulcher* reaches its



Fig. 2. Diurnal cortisol concentrations measured at 4 time intervals, to study the circadian cortisol cycle of *N. pulcher* (N = 5 individuals). Plot shows medians and interquartile ranges.

peak soon after the onset of the activity period. Cortisol concentrations significantly decreased from the interval of 8–11 h to all three of the other measuring intervals, respectively (11–14 h, 14–17 h and 17–20 h; see Table 1a). Excreted cortisol concentrations reached stable, low levels between 14:00 h and 20:00 h (Fig. 2).

# 3.2. Habituation

*N. pulcher* showed the strongest response to the handling procedure on day 2 of the habituation trials as indicated by a significant increase of overall cortisol concentrations between the first and the second day (Fig. 3a,b, Table 1c). Individuals habituated to the handling and experimental procedures on the 4th day (Fig. 3d), where they had overall low cortisol levels throughout the sampling period (Table 1c). The interaction between day and sampling time point was not significant (LMM: Df = 75.1; F = 2.39; p = 0.075) and was removed by backward selection from the final model. Post-hoc analysis using pairwise comparisons showed a significant decrease of overall cortisol concentrations between days 2 and 3, and between days 2 and 4 (Table 1c).

# 3.3. Stress responses of fish with different social rearing background

Fish that had been reared under -F conditions as young had significantly lower basal cortisol concentrations compared to +F fish (Fig. 4, Table 2a). The cortisol response to the stressor was not affected by the early-life treatment (Fig. 5, Table 2b). There was large individual variation between individuals within a treatment (see Fig. 5). A model testing for treatment effects on the stress response that includes baseline cortisol as covariate suggests that a large amount of this variation is explained by variation in basal cortisol levels (see Table 2c). In accordance with the results of the entire stress response, there were also no early-life treatment effects on the peak levels of the cortisol response to the stressor (Table 2d).

#### 3.4. Opercular beat rates (OBR)

Fish reared in -F conditions as young had a significantly higher OBR response (i.e., difference between OBR after and before the stressor; see Methods; Fig. 6, Table 2e). There were no treatment effects on overall OBR (SI, Fig. S3, Table S2). Moreover, there was no significant correlation between OBR and cortisol responses (see Methods) (Pearson's correlation: t = -0.101, df = 15, *p*-value = 0.92; SI, Fig. S4).

#### Table 1

Results of LMMs testing for the effects of (a) diurnal cortisol variation (N = 20 samples of 5 individuals); and (b) habituation to the experimental procedure (N = 96 samples of 6 individuals over 4 days and 4 time intervals per day). (a, b) t-values: results from LMM fitted by restricted maximum likelihood (REML); F values: testing based on deviance after single term deletions using Satterthwaite's method for calculation of degrees of freedom. (c) Pair-wise post-hoc comparisons between habituation days; *p*-values are Bonferroni corrected. Significant p-values are indicated in bold.

Factor	Estimate SE	Df	t	F	p-value	$R^2_{GLMM\ marginal}$	$R^2_{GLMM}$ conditional
a) Diurnal cortisol variation						0.26	0.91
Time point		11.0		17.404	0.00017		
11–14 h <sup>a</sup>	$-0.327 \pm 0.091$	11.0	-3.581		0.0043		
14–17 h <sup>a</sup>	$-0.511 \pm 0.091$	11.0	-5.588		0.00016		
17–20 h <sup>a</sup>	$-0.623 \pm 0.091$	11.0	-6.817		<0.00001		
b) Habituation						0.49	0.62
Day		76.9		10.262	<0.001		
Time point		76.1		26.772	<0.001		
c) Post-hoc comparison	s						
Day 1–Day 2	$-0.840 \pm 0.266$	76.8	-4.361		0.0002		
Day 1–Day 3	$-0.0362 \pm 0.128$	76	-0.203		1.0		
Day 1–Day 4	$0.1896 \pm 0.190$	76.8	1.0		1.0		
Day 2–Day3	$0.7909 \pm 0.190$	76.8	4.17		0.0005		
Day 2–Day 4	$1.0168 \pm 0.202$	77.8	5.039		<0.0001		
Day 3–Day 4	$0.2258 \pm 0.190$	76.8	1.191		1.0		

<sup>a</sup> Reference category for single time points: 8–11 h.



**Fig. 3.** Habituation to the experimental set-up over four consecutive days. (A) Day 1; (B) Day 2; (C) Day 3; (D) Day 4. Plots show a fitted linear model with a degree 2 polynomial smoothing function. Grey shadings represent the standard errors. 'Time points' refers to sample collection, which occurred every 30 min during a period of 2 h: 1 = after 30 min; 2 = after 60 min; 3 = after 90 min; 4 = after 120 min.

# 4. Discussion

We modified the holding-water method for steroid hormone sampling to non-invasively measure cortisol stress response curves in fish. This modification allows measuring the entire course of a stress response in small aquatic organisms, that is, the basal levels prior to a stressor, the rise of stress-induced cortisol up to peak levels and the recovery phase from a stressor when cortisol levels decrease again. The sampling intervals are flexibly adjustable to a given study species. We also showed that the fish habituated to the experimental procedures of the modified method over the course of 4 days, which is a crucial prerequisite for the measurement of basal cortisol levels (Earley et al., 2008; Wong et al., 2008). The cortisol levels were highest only on the second day of the habituation period, which is in line with findings from male convict cichlids (*Amatitlania nigrofasciata*; Wong et al., 2008). Our modified holding-water method should increase our general understanding of individual responses to environmental stressors in small aquatic organisms. For instance, this method allows for non-invasive studies of the repeatability of baseline and reactivity to a stressor (Houslay et al., 2019) and for furthering our understanding of stress resilience and HPI axis sensitivity. The comparison between the cortisol concentration shown in this study and previous studies should be done carefully, due to the differences in sampling time (30 min vs 60 min) and cortisol quantification methods (ELISA vs UHPLC-MS/MS). However, the holdingwater cortisol concentrations of this study are in a similar range than reported in previous studies (rev. in Scott and Ellis, 2007). This opens similar opportunities of repeated, non-invasive stress hormone measurements as already available in terrestrial vertebrates, for instance, from salivary glucocorticoids (Ash et al., 2018; Escribano et al., 2015; Ruis et al., 1997).



**Fig. 4.** Baseline cortisol concentrations of -F (raised with siblings) and +F fish (raised with parents, a helper and siblings). Medians and interquartile ranges are shown. Baseline cortisol concentration refers to cortisol concentration during the 30 min before the stressor was applied.

This modified holding-water method enabled us to compare the stress responses of adult *N. pulcher* exposed to different early-life social experiences. Fish that had been socially deprived of the presence of parents and helpers in early life had a reduced basal cortisol concentration at an age of 8 years (Fig. 4), whereas their early experience did not affect stress-induced cortisol levels (Fig. 5). Our results are not confounded by the circadian cortisol peak as we collected all samples in the afternoon when basal diurnal cortisol levels were the lowest; the natural, circadian cortisol peak was in the early in the morning (8–11 h, see Fig. 2). Fish that experienced social deprivation early in life had an increased opercular beat rate (OBR) response to the stressor (Fig. 6), whereas overall OBR did not differ between rearing treatments, and OBR response was not correlated to cortisol response.

# 4.1. Effects of early life social deprivation

The lower cortisol baseline of -F fish indicates that the early social deprivation of the presence of adults caused reprogramming and life-

#### Table 2

Results from LMMs testing for effects of the early life treatment on (a) baseline cortisol (N = 20), (b) cortisol excretion across the four time points after the stressor (i.e. excluding the baseline; N = 78 cortisol samples from 20 individuals); (c) cortisol excretion across the four time points after the stressor including basal cortisol as covariate (N = 78 cortisol samples from 20 individuals); (d) cortisol stress peak (N = 19); and (e) OBR response (N = 18). Estimates refer to the factor levels given in brackets. The t-values are from LMMs fitted by restricted maximum likelihood (REML); F-values are based on deviance after single term deletions using Satterthwaite's method for calculation of degrees of freedom. Significant p-values are indicated in bold.

Factor	Estimates $\pm SE$	Df	t	F	р	$R^2_{GLMM\ marginal}$	$R^2_{GLMM}$ conditional
(a) Baseline CORT						0.31	0.78
Treatment (+F)	$0.827\pm0.328$	8.0	2.519	6.34	0.036		
Sex (male)	$-0.565 \pm 0.223$	4.1	-2.525	6.37	0.064		
(b) CORT response						0.06	0.93
Treatment (+F)	$0.540\pm0.547$	18.0	0.987	0.97	0.34		
Time	$0.779 \pm 0.256$	56.0	3.038	9.22	< 0.001		
Time <sup>2</sup>	$-0.128 \pm 0.036$	56.0	-3.501	12.25	< 0.001		
(c) CORT response (w/ baseline as covariate)						0.69	0.93
Treatment (+F)	$-0.029\pm0.3$	17.0	-0.099	0.0098	0.92		
Time	$0.776 \pm 0.256$	56.1	3.026	9.15	< 0.001		
Time <sup>2</sup>	$-0.127 \pm 0.036$	56.1	-3.487	12.15	< 0.0001		
Baseline	$1.323\pm0.191$	16.9	6.907	47.71	< 0.0001		
d) Cortisol peak						0.21	0.46
Treatment (+F)	$0.836\pm0.553$	9.9	1.513	2.28	0.16		
Sex (male)	$-0.807 \pm 0.451$	8.4	-1.788	3.19	0.11		
e) OBR response						0.41	0.41
Treatment (+F)	$-0.171 \pm 0.051$	15	-3.348		0.0044		

long dampening of basal cortisol levels, which is the first evidence of early life effects on the stress response persisting for almost a decade in a small vertebrate. In the natural habitat of N. pulcher, group composition and size are crucial for juvenile survival (Brouwer et al., 2005) and the survival of entire groups (Heg et al., 2005). Moreover, offspring growing up in the presence of adults (Arnold and Taborsky, 2010; Taborsky et al., 2012; Fischer et al., 2017; Nyman et al., 2017) or in large groups (Fischer et al., 2015) develop better social competence, an important ability that reduces the costs of a social life style (Taborsky and Oliveira, 2012). Like in N. pulcher, early social deprivation led to reduced basal cortisol levels in cooperatively-breeding common marmosets, Callithrix jacchus (Dettling et al., 2002) and zebrafish, Danio rerio (Parker et al., 2012). Also in humans, childhood abuse or neglect induced hyporeactivity of the HPA axis, characterized by a reduced basal cortisol and reduced cortisol excretion after a standardized stressor (Carpenter et al., 2007). In N. pulcher, the dampening of the basal HPI axis activity might be a consequence of the reduced number of social interactions -F fish have during their early rearing experience (see Arnold and Taborsky, 2010). Focal fish were individually housed during the experiment to standardize their social status and experience during the experiment. N. pulcher exhibits a linear social hierarchy, and changes to their position in the rank order alters cortisol excretion (Culbert et al., 2018). Even though social isolation is known to influences stress responsiveness (Blanchard et al., 2001; Giacomini et al., 2016; Heynen et al., 2016), such standardization was necessary to be able to evaluate the effects of early social experience (rather than of current social challenges) on N. pulcher stress response, and to reduce individual variance.

In general, effects of early experiences on adult stress responses are highly context-dependent and possibly also species-specific. For instance, while a recent meta-analysis in humans showed that on average there is no correlation between effects of early life stress and, respectively, the cortisol awakening response, baseline cortisol and cortisol reactivity (Fogelman and Canli, 2018), the results of the individual studies included in this analysis were highly heterogeneous and the direction of their results depended, among others, on the kind of stressor and sampling method (Fogelman and Canli, 2018). Also in juvenile zebrafish, stress induced cortisol levels changed in a contextdependent way, depending on previous social experience and the type of stressor used (Forsatkar et al., 2017). These studies highlight the need to account for the nature of the early life experiences and the type of stressor when investigating stress responses. 0.3

0.2

5

0.0

**OBR** response



h

+F

Hormones and Behavior 128 (2021) 104910

**Fig. 5.** Cortisol response curve to a confinement stressor the experimental fish were exposed to after a habituation period of four days. Plots show a fitted linear model with a degree 3 polynomial, grey shading represents the standard error. Red (light): -F fish (raised with siblings); blue (dark): +F fish (raised with parents, a helper and siblings). 'Time points' refer to sample collection after a 30 min water collecting interval. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



#### 4.3. Stress response and opercular beat rate

Opercular beat rates, or ventilation frequency, have been used in fish to assess their response to stressors (Barreto and Volpato, 2011; Di Poi et al., 2016). The opercular beat rate is mediated by the sympathetic response to environmental disturbances (Priede, 1985), which can be modulated by stress hormones (Barreto and Volpato, 2004). Some fish species reduce their OBR when challenged by stressors, such as chemical predator cues (Stratmann and Taborsky, 2014), possibly because this 'freezing response' makes them less conspicuous to predators (King and Adamo, 2006), whereas other species increase this rate in face of danger (Gibson and Mathis, 2006; Hawkins et al., 2004), possibly to prepare for flight (Hawkins et al., 2004). In addition, OBR correlates positively with metabolic rate in some fish (Dalla Valle et al., 2003), including N. pulcher (Grantner and Taborsky, 1998). Here we showed that in N. pulcher early social deprivation significantly increases the OBR response to a stressor. The increased OBR of -F fish in response to disturbance might be explained by the fact that these fish perceived their early environment as dangerous because they grew up without guarding parents and helpers (Arnold and Taborsky, 2010). As OBR has been reported to be regulated by CRF (Mével et al., 2009; Solomon-Lane and Grober, 2012), it is possible that early social environment has increased the excretion of CRF in -F fish leading to an OBR increase. In dangerous environments a higher OBR response might be beneficial to evade danger as it increases oxygen intake (Fernandes and Rantin, 1994) and enhances the energy turnover necessary for a flight response. OBR and cortisol responses were not correlated, however, and therefore OBR does not seem to be a suitable proxy for stress cortisol.



### 4.2. Characterization of the stress response of N. pulcher

-F

The stress curves of *N. pulcher* measured by the dynamic holdingwater method reflect the general shape of stress response curves reported in a wide variety of vertebrate species (humans: Gunnar et al., 2009; dogs, Rothuizen et al., 1993; rats, Spiga et al., 2017; birds, Littin and Cockrem, 2001; reptiles and amphibians, Moore and Jessop, 2003; fish, Cockrem, 2013). They contain the baseline level, peak level and the first part of the recovery phase. Cortisol peak values were reached on average 30–60 min after exposure to the stressor (see Fig. 5), which is comparable to results from sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) (Ellis et al., 2004; Fanouraki et al., 2008). Cortisol levels had not reached basal levels within 2 h after the stressor, indicating that we captured the first part of the stress recovery phase only; extrapolation of the cortisol curve in Fig. 5 suggests that it may have taken the fish another 2–3 h to return to basal stress levels.

There was substantial variation between individual stress response curves within rearing treatments (Fig. 5). Such variation has been

# 4.4. Possible mechanisms underlying the early-life effect on baseline cortisol

The reduced basal cortisol levels in N. pulcher induced by the early social deprivation might result from a differential regulation of the mineralocorticoid receptors (MRs). Several hormones (ACTH, CRF) and receptors (GR, MR) are involved in the regulation of circulating cortisol levels (de Kloet et al., 2008; see 'Methods'). MR is activated at basal cortisol levels, while GR is activated at high circulating cortisol levels as they occur during a stress response (Greenwood et al., 2003; Rothuizen et al., 1993). Social deprivation or instability during early development can change the expression of both GR and MR in the brain, resulting in reprogramming of the stress response (Ladd et al., 2004; Liu et al., 1997). In rats maternal separation lead to epigenetic modification resulting in downregulation of the genes coding for GR (Liu et al., 1997) and of CRF (Plotsky and Meaney, 1993) in the hippocampus. Similarly, *N. pulcher* growing up without adults have a persistent downregulation of the GR1 gene (homologous to the mammalian GR) in the telencephalon (Nyman et al., 2018, 2017). Furthermore, N. pulcher, which were repeatedly exposed to a systemic cortisol treatment during their first two months of life had a constitutive downregulation of the CRF gene and upregulation of the MR gene in the telencephalon (Reves-Contreras et al., 2019). In the light of these findings, there are three alternative mechanistic hypotheses to explain the observed reduced basal cortisol in -F fish. (1) -F fish may excrete the same amount of cortisol during basal conditions than +F fish, but may have a higher concentration of MRs in the brain. These may bind more cortisol, resulting in reduced basal cortisol levels. In support of this hypothesis, pharmacologically blocking MRs in rats led to an increase in basal corticosterone (Ratka et al., 1989). (2) -F fish may produce and excrete less cortisol than +F fish, which could be achieved by a downregulation of CRF or ACTH. A previous study in N. pulcher did not find a significant difference in CRF brain expression in -F fish (Nyman et al., 2017), but fish that received repeated cortisol treatments early in life had lower CRF gene expression (Reyes-Contreras et al., 2019). Moreover, peer-reared rhesus macaques had a lower HPA reactivity represented by a reduced basal ACTH and a lower cortisol response to a mild stressor than mother-reared macaques (Clarke, 1993). (3) Excreted cortisol may be converted to cortisone by the enzyme 11β-hydroxysteroid dehydrogenase as reported in different fish species (Baker, 2004; Pottinger and Moran, 1993). We can safely exclude this possibility as we did not find differences in cortisone concentrations between the two early life treatments, neither at baseline levels nor in the response phase (SI, Fig. S2).

# 4.5. Possible ultimate causation of the early-life effect on baseline cortisol

The perception of a dangerous early environment in the absence of guarding group members (see discussion on OBRs above; Arnold and Taborsky, 2010; Watve and Taborsky, 2019) may have affected the stress axis programming of -F fish such that they developed lower cortisol baseline levels, whereas stress-induced cortisol levels were not affected. The differences in basal cortisol are accompanied by a specialization into distinct social behaviour types and life-history trajectories between -F and +F fish (Fischer et al., 2017; Nyman et al., 2017; Antunes and Taborsky, 2020). We speculate that there is a sensitive period during early development of N. pulcher, during which they can adapt behaviour and physiology to cues of their envisaged future conditions. If phenotypic adjustments to early-life cues result in a good match with adult conditions, such predictive adaptive responses can enhance individual fitness (Bateson et al., 2014). Predictive adaptive responses are expected to evolve in species living in variable, but predictable environments (Bateson et al., 2014; Fawcett and Frankenhuis, 2015), conditions that hold for the social environment of N. pulcher: while group sizes are highly variable within populations, group size is predictable across years (Heg et al., 2005). Thus under the predictive adaptive response hypothesis, we predict that by developing a reduced

cortisol baseline –F fish prepared for future, dangerous conditions (Antunes and Taborsky, 2020).

Alternatively, the development of a lower baseline in –F fish might represent a constraint rather than an adaptation. The development the HPA/HPI axis is under strong social influence (Cirulli, 2001; Spencer, 2017). Thus –F fish that grew up in a socially deprived environment may have developed an impaired HPA/HPI axis as has been previously reported in rats (McCormick et al., 2001; Meaney and Aitken, 1985).

# 4.6. Conclusions

We established a method to non-invasively measure cortisol responses to a stressor in small aquatic organisms. Using this method, we showed that early social environment has life-long effects on the physiological state of a highly social cichlid by changing its cortisol baseline, but not stress-induced cortisol levels, for lifetime. Fish exposed to earlylife social deprivation had lower cortisol baselines as 8-year old adults, which adds to a suite of behavioural and reproductive traits induced by the same early-life experiences. The underlying mechanisms and the fitness consequences of the reduced basal cortisol levels in socially deprived fish warrant further investigation. Future research should further involve the study of neurodevelopmental effects of the early social environment at the level of hormone receptors in the brain, for instance, on the expression of the glucocorticoid receptor genes in targeted brain nuclei involved in behavioural decision making (e.g., Antunes et al. MS).

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#### Appendix A. Supplementary data

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D.F. Antunes et al.

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