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"Early-life manipulation of cortisol and its receptor alters stress axis programming and social competence"

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Summary

In many vertebrate species, early social experience generates long-term effects on later-life social behaviour. These effects are accompanied by persistent modifications in the expression of genes implicated in the stress axis. It is unknown, however, whether stress axis programming can affect the development of social competence, and if so, by which mechanism(s). Here we used pharmacological manipulations to persistently reprogram the hypothalamic-pituitary-interrenal (HPI) axis of juvenile cooperatively breeding cichlids, *Neolamprologus pulcher.* During the first two months of life, juveniles were repeatedly treated with cortisol, mifepristone or control treatments. Three months after the last manipulation, we tested for treatment effects on (i) social competence, (ii) the expression of corticotropin-releasing factor (*crf*), glucocorticoid receptor (*gr1*) and mineralocorticoid receptor (*mr*) in the telencephalon and hypothalamus and (iii) cortisol levels. Social competence in a social challenge was reduced in cortisol-treated juveniles, which is in accordance with previous work applying early-life manipulations using different social experiences. During early life, both cortisol and mifepristone treatments induced a persistent down-regulation of *crf* and upregulation of *mr* in the telencephalon. We suggest that these persistent changes in stress gene expression may represent an effective physiological mechanism for stress coping.

Introduction

Through developmental plasticity, the early social environment can strongly influence animal social behaviour and social competence in later life stages [1,2]. Social competence, the ability of animals to optimise their social behaviour as function of the available social information[3,4], can be shaped by the quantity, quality and diversity of social interactions young animals are exposed to during early life [2]. For example, laboratory mice reared in communal nests that received more intensive maternal care and tactile stimulation [5] or encountered more peer-to-peer interactions [6] before weaning, later in life established social hierarchies faster and behaved more adequately with respect to their social rank compared to mice reared by single mothers. Similar patterns have been reported in young zebra finch (*Taeniopygia guttata*) males [7] and in teleost fish. In the cooperatively-breeding cichlid fish *Neolamprologus pulcher*, individuals reared in larger [8] or more complex [9,10] social groups had an improved social competence both as juveniles and adults compared to individuals that reared in small groups or in groups consisting of a single age class. Improved social competence was indicated by the ability to defend a resource more efficiently [9] and to integrate more easily into a new social group [10].

In vertebrates, differential programming of the stress axis has repeatedly been shown to accompany long-term effects of the early social environment on social behaviour [11–13]. The hypothalamic-pituitary-adrenal (HPA) axis of mammals and birds or its homolog, the hypothalamic-pituitary-interrenal (HPI) axis of fish, is the main physiological mechanism responsible (i) for eliciting and terminating stress responses [14] and, in turn, (ii) for responding and adapting to environmental changes [15]. The presence or absence of *N. pulcher* parents during early life has been shown to affect the expression of genes implicated in the HPI stress axis [16,17]. Early life experience can shape neurobiological pathways involved in stress responsiveness through organizational effects on tissue sensitivity for glucocorticoids [18,19]. Glucocorticoids, such as cortisol (in fish and most mammals) or corticosterone (in rodents and birds) are the major vertebrate stress hormones and are responsible for the regulation of the stress axis [20]. Evidence suggests that the early social environment may generate a cascade of neurobiological changes involving the vertebrate stress axis, which has implications for social behavioural [12].

Following the perception of a stressor, a stress responses is elicited by an endocrine cascade, where the primary steps include the release of catecholamines in the sympathetic nervous system, followed by the hypothalamic release of corticotropin-releasing factor (CRF) [20]; this eventually leads to the secretion of glucocorticoids form the adrenal or interrenal glands into the bloodstream [21]. The control of stress responses is mediated by cortisol signalling through two types of intracellular receptors that act as ligand-dependent transcription factors [22,23]. These receptors mediate the activation or repression of different genes in the target cells [24]. Elevated concentrations of cortisol activate the glucocorticoid receptors (GR) in the nucleus preopticus [25], the hypothalamus and the pituitary gland [18], which attenuate and eventually terminate the stress response via negative feedback loops that lead to suppression of further cortisol release. This suppression happens by blocking further CRF production [25]. The second intracellular receptor type, the mineralocorticoid receptor (MR) in teleost fish [26] and in the nonepithelial limbic neurons of other vertebrates [23], has a high-affinity to cortisol and is activated at basal levels [22,23]. Nuclear MR maintains the integrity and stability of the limbic circuit, determining the threshold or

sensibility of the limbic stress response system [27]. In contrast, low-affinity MR in membranes boosts the initial stress response [27].

Although early social experience alters both stress axis programming and social competence, it is as yet unknown whether stress axis programming in early life *causally* determines the development of social competence, and if so, by which mechanism. Few studies have pharmacologically manipulated the stress axis prenatally or in early life to test for causal links between this axis and behaviour (neophobia [28,29]; aggression [30]; defence against an intruder [31]; migration [32]; predator avoidance [33]; exploration [34]). A well-developed social competence should be particularly important for highly social species, such as cooperative breeders, in which almost all aspects of life include social interactions [4]. Here we aimed to manipulate early-life programming of the principal pathway that regulates the stress axis by pharmacological application of either cortisol or GR blocker in the highly-social cichlid *N. pulcher*. We predicted that our treatments would generate long-term effects on later life social competence, social performance, gene expression in the brain, and fluctuating cortisol.

Mifepristone has been successfully used to block GR1 (see [35] and references therein), which is the teleost homolog of the mammalian GR. Besides its function as GR blocker, it binds also to progesterone receptors, and therefore may have an additional function in reproduction [35]. However, this second function is unlikely to play a role in this study, as the fish were in their earliest juvenile stages when they were treated with mifepristone (i.e., > 6 months before sexual maturity). Previous work revealed contrasting behavioural effects of pharmacological administrations of cortisol and mifepristone. Blocking GR1 by mifepristone in *adult N. pulcher* induced a higher expression of submissive behaviours in intruders aiming to take over the territory from a resident, but nevertheless led to a higher success monopolization of territory [36]. The authors concluded that this increased success in territory acquisition resulted from an enhanced expression of adequate behaviours (social competence) after mifepristone treatment. Adult rainbow trout (*Oncorhynchus mykiss*) decreased aggression levels towards territory intruders [37] after being treated with mifepristone. In contrast, rainbow trout that were exposed to cortisol exhibited increased aggression, both when treated as adults [37] or during early ontogeny [30]. Based on these findings, we hypothesized that mifepristone treatment would increase submission and decrease aggression, but nevertheless lead to a higher success in obtaining or retaining resources (see [36]). Conversely, cortisol treatment was hypothesized to enhance aggression in *N. pulcher* facing a territory intrusion, but not necessarily lead to a higher success in aggressive contests over a resource.

In vertebrates, elevations of cortisol levels generate a negative feedback via GR that eventually blocks further corticotropin-releasing factor (CRF) release (e.g. [24,38,39]). Therefore, we predicted that early-life cortisol treatment would up-regulate *gr1* gene expression, thereby enhancing the negative feedback loop on further cortisol release by decreasing *crf* mRNA levels. Additionally, we predicted up-regulation of *mr* gene expression, as early-life corticosteroid treatment has been shown to up-regulate *mr* expression in Japanese quail (*Coturnix coturnix japonica*) [40]. Conversely, the GR blocker mifepristone has been shown to induce down-regulation of *crf* and *gr* genes in different tissues of the fish brain; for example, in the hypothalamic preoptic area of rainbow trout, *O. mykiss*, [41], telencephalon-preoptic brain region of goldfish, *Carassius*

auratus [42] and in whole body samples of juvenile zebrafish, *Danio rerio* [43]. Consequently, we predicted a decrease of the *gr1* and *crf* expression after early-life GR blocker treatment. However, these predictions are based on studies measuring the immediate effects of mifepristone administration as studies of early-life exposure to mifepristone are not available, and should therefore be considered with caution.

Finally, we predicted that our treatments would translate into different mechanisms of stress response regulation, that is, the response of an individual to a stressor by cortisol release, which in turn gives rise to physiological pathways that aid in returning to a homeostatic state [44,45]. Mifepristone treatment increased endogenous cortisol levels in the fish *Opsanus beta* [46] but, importantly, it suppressed the amplitude of cortisol responses in both fish and rats [41,47]. After a post-natal corticosterone treatment, juvenile Japanese quail (*C. c. japonica*) showed a shorter stress response after a stressful stimulus than control birds [48]. Therefore, we predicted that both mifepristone and cortisol treatment would decrease stress responsiveness. Specifically, mifepristone would reduce the amplitude of the stress response and cortisol reduces the duration of the response.

Materials and methods

Study species

N. pulcher is a cooperatively breeding cichlid fish endemic to Lake Tanganyika, East Africa. Social groups consist of a dominant breeding pair, related and unrelated helpers ranging from 1 up to 25 individuals and the offspring of the current breeding pair. A group will inhabit a joint territory of up to 1 m^2 [49,50]. Helpers stay in the natal territory even after reaching sexual maturity (at a size of ≥3.5 cm SL (standard length)) [51] at about 10 and 12 months of age [36]. In natural populations, reproductive success of the dominant breeding pair increases with the number of helpers due to improved offspring survival [52]. In turn helpers pay-to-stay with breeders by contributing to territory defence and maintenance [53,54].

Offspring start performing social behaviour towards other siblings at about 5 weeks post hatching and frequencies of these behaviours increase over time [9]. At a size of 1.5 to 2 cm, offspring start to join in helping tasks. These duties include the cleaning and fanning of eggs produced by dominants, removing sand from the breeding cavity, and defending the breeder's territory against predators and intruders [55]. Helper task specialization is size dependent [56]. Small helpers usually perform alloparental care in form of egg cleaning and fanning [57], while large helpers engage in territory defence and territory maintenance [56]. The composition and size of social groups during the first months of life strongly affects social behaviours (helping behaviours, social competence, life history decisions and the expression of stress axis genes) [4,9,16,17,58,59].

Animal housing

The experiments were conducted at the Hasli Ethological Station of the Institute of Ecology and Evolution, University of Bern, Switzerland, under license BE 74/15 of the Veterinary Office of Kanton Bern. The

parents of our experimental fish were laboratory-reared 2nd and 3rd generation offspring of wild caught fish from Kasakalawe Point, Mpulungu, Zambia. We created 31 breeding pairs from haphazardly chosen adult males and females from our laboratory stock. Each breeding pair was randomly assigned to produce offspring to be exposed to one of three treatments: (i) the stress hormone cortisol (cor; n= 11), (ii) the glucocorticoid receptor blocker mifepristone (mif; n= 10) and (iii) a blank control treatment (control; n= 10). The breeding pairs were housed in individual 60-L tanks equipped with 2 cm of fine sand, a biological filter, two flowerpot halves serving as potential breeding cavities, and a half-transparent PET bottle mounted near the water surface to serve as refuge. The water temperature was kept at 27 ± 1 ºC with a light dark regime of 13:11 h and a dimmed-light phase of 10 min. The breeding pairs were fed commercial adult flake food (JBL Novo Tanganyika®) 5 days a week and frozen zooplankton 1 day a week. Additionally, frozen krill and *Artemia spp.* nauplia (Artemix, Dohse Aquaristik, Germany) were provided twice a week to stimulate egg production. We waited until the pairs produced a clutch and the larvae had developed into free-swimming fry. The first day of free-swimming was defined as the experimental 'Day 0' (e.g. 10±2 days post-fertilization). After Day 0, each rearing group was fed TetraMin Baby® food the amount of which was adjusted to the number of fry and their age by following the feeding regime describe in [59]. Additional adult flake food was provided for the breeders This feeding regime was adopted to ensure homogeneous growth rates among siblings within and across rearing groups.

Experience phase

Cortisol treatment

For the cortisol treatment, we used a concentration that was half of the cortisol plasma levels reported from stressed *N. pulcher* adults [60]. When this concentration was applied to developing rainbow trout eggs, it generated transitional (<1 day) elevations of cortisol levels and induced a long-term effect on stress sensitivity [61]. To prepare the treatment solution, hydrocortisone (Product number H4001, Sigma-Aldrich, Switzerland) was dissolved in Dimethylsulfoxid (DMSO) to get a stock solution of 1 mg/ml cortisol concentration. The final concentration of 200 ng/ml was obtained by adding 100 µl of cortisol stock solution to 500 ml of tap water. Local tap water had ideal water parameters to hold Tanganyika cichlids and needed no further processing.

Mifepristone treatment

For the mifepristone treatment, we used a concentration that had been previously shown to generate short-term effects on *N. pulcher* social performance [36]. We dissolved mifepristone (RU486; Product number M8046 Sigma-Aldrich, Switzerland) following the modified protocol of [62]. Briefly, a mifepristone stock solution (50 ng/µl) was used to obtain a final concentration of 400 ng/L by adding 4 µl of mifepristone stock solution to 500 ml of water.

Control treatment

In the control treatment we applied the same solvents in the same concentration used to dilute mifepristone (DMSO, PBS, BSA), but without adding mifepristone.

Application of water baths

At experimental Days 10, 20, 30, 40, 50 and 60 (Fig. 1) the assigned treatment was applied to all offspring of a rearing group. Treatments were assigned to rearing groups randomly, on the condition that the same treatment was never assigned to two neighbouring tanks and all treatments were represented equally in the different rows of the tank-rack. The treatments were applied as water baths; this non-invasive method allows repeated applications of hormones at very low manipulation stress levels [62–64]. For the water bath, a maximum of 20 juveniles were placed inside a 2-L glass beaker filled with 500 ml of tap water. All beakers were supplied with oxygen using glass Pasteur pipettes connected to an air stream. The beakers were kept in complete darkness and were isolated against noise during the entire exposure procedure which consisted of (1) a 30 min acclimatization period, (2) 1 hour of exposure to the respective treatment, (3) a first recovery period of 30 min in a beaker with home tank water and (4) a second recovery period of 30 min inside a mesh cage (14.5 x 8.5 x 7 cm) hanging in the home tank. Finally, the juveniles were gently released from the mesh cage into the home tank. The breeder pair always immediately reaccepted their offspring.

We kept the repeated exposure to the drugs short to generate a pulsed exposure (1 hour) followed by a long unexposed period (10 days), because we wanted that the exposure to cortisol to have similar effects to short stressful situations in a natural context. The application of short exposures to cortisol and mifepristone is expected to lead to a temporary increase in the cortisol of juveniles once they developed full functionality of their HPI axis [62]. Veillette et al. 2007 reported that in summer flounders the HPI axis was fully developed at an age of 3 weeks, which is even slightly earlier than the age when we exposed our fish to mifepristone (30 days of age, that is, 20 days after free swimming). Thus, we can assume that the HPI axis in *N. pulcher* was fully functional at first exposure. In 21-d old summer flounders the administration of mifepristone resulted in a rapid (within a few hours) increase of cortisol, and we assume that the same happened in *N. pulcher*.

Behavioural recordings

During the experience phase, which lasted until Day 60, we performed repeated recordings of all spontaneous social behaviours and the general activity of the experimental juveniles in their home tanks. During this time the juveniles were still co-housed with the breeder pair (see 'Animal housing conditions'). The recordings were conducted 9 days after an application of a pharmacological or control treatment (Fig. 1) following the observation methods and ethogram used by [9]; (see ethogram in Supplement, Table S1). Thus, recording took place on days 29, 39, 49, 59 and 69, but not on Day 19, as at this age fish were still very small and did not show noticeable social behaviour. Before each recording, a transparent acetate grid of 14 x 10 cells (4 x 4 cm each cell) was attached to the front wall of the tank, which was used to randomly select the first juvenile for a recording using a random number table, and for estimating its activity. All behavioural recordings were done by an observer who was blind to the treatment. Before starting a recording, juveniles were allowed to acclimatize for 5 min to the presence of the observer, who sat

motionless in front of the tank. A total of three juveniles per rearing were recorded for 5 min each, amounting to a total recording time of 15 min per tank [9].

Recorded behaviours

The recorded behaviours (cf. [9] see Ethogram in Supporting Information, SI) were grouped according to their function for statistical analyses: (1) Restrained aggression, which comprises all threat behaviours not involving physical contact. (2) Overt aggression, which involves physical contact or attempted physical contact (*i.e.* chasing). While overt aggression by larger specimens can have a strong impact and inflict injuries in the receiver, we never observed injuries in the interactions between our experimental juveniles. Overt aggression was rare and thus could not be statistically analysed. (3) Submissive displays, which can be shown spontaneously towards dominant individuals or in response to received aggression, most often consists of a strong vibration of tail and body. (4) Affiliative behaviour, includes swimming in close proximity without showing signs of aggression, and soft body touches ('bumping'; see ethogram in SI), which is mostly performed by subordinates towards dominants. (5) General activity was measured as the amount of locomotion by an individual estimated by the number of lines of the acetate grid a focal individual crossed during a 5 min observation period. All behaviours were recorded as frequencies. The sum of the behaviours shown by the three juveniles per rearing group was used for statistical analyses [9].

Neutral phase

At the end of the 2-months experience phase breeder pairs were removed and returned to the laboratory stock. The experimental juvenile groups were left in their home tanks under the same housing conditions describe above and without any treatment for the next 95 days. At the beginning of the neutral phase, juvenile groups in the home tanks had a mean size of 44.1 (± 3.14 SE).

Two month old N. pulcher juveniles are independent of parental care [4]. When living in a family group, at this age they start to act as brood care helpers [57]. They can specialise in direct brood care, defence or territory maintenance, or develop a submissive non-helper type [59,65,66]. For our study, introducing a neutral phase was therefore critically necessary to prevent individual task specialisation, which would have confounded the effect of the pharmacological manipulation.

Test phase

Selection of focal individuals

At Day 157 individuals were selected for hormone measurement and the social challenge test. We determined the median standard length (SL) for each rearing group and selected four focal individuals that were closest to the mean SL of their rearing group. These focal fish were later used in a social challenge test (see Fig. 1) where they acted in one of two social roles, owners (Ow.) and intruders (Int.; see below). When

selecting the focal fish (*i.e.* two replicate fish per social role), we preassigned them to their role and marked each individual with a fin clip according to social role for later identification. The SL of focal individuals was 3.035 ± 0.34 cm (mean ± SE; Int.) and 2.995 cm ± 0.25 (Ow.). Until and between the manipulations for hormone sampling and behavioural testing (see Fig. 1) the focal juveniles were kept in individual, transparent isolation boxes (10.5 x 10 x 17 cm) floating at the surface of the home tank, which allowed for exchange of visual and olfactory cues with the other siblings housed in the tank. It facilitated repeated, quick catching of the focal individuals to reduce catching stress.

Hormone sampling

During three consecutive days (Days 160-162) at 10:00 h, focal individuals were placed for 30 min each in a separate 2-L glass beaker containing 500 ml of clean tap water. This procedure has been shown to lead to habituation to the handling procedure in other cichlids [67], including *N. pulcher*, thereby minimizing the effect of handling stress on our stress measurements.

At Day 163 we measured basal cortisol of all focal fish using the same manipulations and procedures used during the habituation phase. In a total of 93 individuals (cortisol, Ow.: n=20, Int.: n= 16; mifepristone, Ow.: n=19 Int.: n=10; control, Ow.: n=19, Int.: n=9) we sampled baseline cortisol using the 'fish-holding water method', a non-invasive technique to sample waterborne steroid hormones in small fishes [67–69]. Finally, on Day 164 we sampled cortisol responses after fish had experienced an acute stressor in the same 93 individuals and following the procedure described above. The acute stressor consisted of placing the focal individuals gently in a mesh and exposing them to air for 1 min [70,71] before placing them into the glass beaker to sample their cortisol.

All cortisol samples were obtained between 10:00-11:00 h to minimize variation due to diurnal fluctuations of cortisol excretion [45]. The preparation of hormone samples followed a protocol developed by Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel (see details in the SI). Cortisol content was analysed by ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS).

Social challenge test

We staged an asymmetric contest over a shelter, which is a vital resource for *N. pulcher* in nature, to test the ability of fish to express adequate behaviour according to their preassigned social role. Thus, this test evaluated the social competence of fish in a biologically meaningful context. Preassigned owners should defend the shelter against an intruder and are in a favourable position to maintain the shelter. Therefore a restrained, non-escalated form of aggression (threat display) should suffice for a successful defence [9]. Preassigned intruders are usually not able to take over the shelter, but should aim to achieve tolerance from the resident owner when near the shelter [9,16]. In nature, each subordinate group member defends a private shelter for hiding from predator attacks within the group territory [52] and these shelters are crucial for survival of subordinates [10,72].

A total of 58 individuals were tested in the social challenge test (mif. n=19, cort. n=20, control n=19) in the role of owners, and a total of 46 individuals (mif. n=12, cort. n= 21, control n=13) as intruders. Different

sample sizes between treatments within a social role were caused by the death of some individuals between hormone sampling and behavioural testing due to a temporary deterioration of water conditions in some rearing tanks. Further, more fish were tested in the owner role than in the intruder role; for logistical reasons we could not perform tests in the intruder role for all rearing groups. This is because in *N. pulcher* larger individuals are dominant over smaller ones, so we size-matched the focal fish and their respective opponents to the nearest millimetre (difference mean ± SE, between focal individual and opponent 0.16 ± 0.075 mm SL). Opponents were unrelated, unfamiliar fish from a laboratory stock tank, which had never received any hormonal treatment. Each opponent was used only once, and after the contest it was immediately returned to its home tank.

One day before the social challenge, at Day 167, focal individuals and opponents were acclimatized to the experimental set up, which was a 20-L tank (20 x 20 x 30 cm) equipped with a 2 cm layer of sand divided into two compartments by an opaque partition. The owner's compartment was equipped with a flowerpot halve (5.5 cm outer diameter) and a biological filter. The intruder's compartment only contained an air stone.

At Day 168 we removed the opaque partition to start the asymmetric competition test. The behavioural recording started when one of the individuals crossed the virtual border between the two compartments (*i.e.* where the partition had previously been) and lasted for 20 min. All social behaviours performed by both individuals were recorded using the program Solomon Coder (Copyright © 2017, András Péter). The observer, who was blind to the treatments, was hidden behind a black curtain. After the recording, we determined the time point when the contest had been decided ('end of contest'), and who won or lost the contest following the criteria used by Nyman and colleagues [36]. Briefly, an individual lost when it was evicted from the vicinity of the shelter and did not attempt to gain access to it anymore; an individual was the winner when it had unrestricted access to the shelter and was not attacked by the other fish; contests were rated as 'undecided' when no clear winner or loser existed at the end of the recording. Contests were classified as 'alternative outcome' if the owner chose the aquarium filter as shelter so that the intruder could use the flowerpot without being threatened or attacked by the opponent.

Gene expression

To assess whether the constitutive expression of stress axis genes was altered by our early-life pharmacological manipulations we measured the expression of three key genes of the stress axis, *crf*, *gr1* and *mr*. As outlined above, the products of these three genes play a central role in regulating stress responses.

Between days 171 and 173, we sacrificed two randomly-selected replicate individuals from each rearing group by an overdose of tricaine methanesulfonate (MS-222; Sandoz, Switzerland). None of these fish had taken part in hormone sampling and/or the social challenge test beforehand. Following the procedures described in [16], we dissected the brains with a scalpel under a steromicroscope, and stored the telencephalon and the hypothalamus of each brain separately in RNAlater® (Qiagen, The Netherlands) and kept the vials at -20°C for up to 7 months until RNA extraction. Only one individual from each rearing

group was used for genetic analysis (sample size per treatment and brain area: cortisol n = 11; mifepristone n = 10; control n = 10; size: 2.99 ± 0.26 cm SL) and the other was kept as backup. The two brain regions were chosen because they contain the brain tissues involved in stress regulation, namely the limbic forebrain neurons (telencephalon) and the parvocellular CRH-producing neurons in the paraventricular nucleus (PVN) of the hypothalamus [73]. Gene expression was measured by a standard reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) protocol (details in SI).

Statistical analyses

We analysed treatment effect by GLMMs and LMMs (see details below). If models had non-significant interaction terms they were excluded stepwise [74,75]. Poisson distributed generalized linear-mixed effect models (GLMMs) were corrected in case of over-dispersion by including an observation level random factor. For each factor of the model, estimates and their standard errors, and t or z-values according to the type of model are given in Tables 2-5 of the SI. Significance testing was based on deviance when removing respective terms from the model. The change in likelihood was compared to a chi-square distribution (likelihood ratio test [LRT], see reference [76], therefore χ^2-values are given in the main text and SI tables. Normality assumptions for all linear-mixed effect models (LMMs) were tested by visual inspection of Quantile-Quantile plots and by Shapiro-Wilks and Lilliefors (Kolmogorov-Smirnov) normality tests. If necessary, the dependent variable was boxcox-transformed to achieve normality of the error terms. We checked for potential outliers of model residuals by visual inspection of the Cook's distance score [77], excluding values with a Cook's distance >0.5. In addition we assessed the influence of those values with the Grubb's test [78]. Only in one data set (*crf* in the telencephalon), one high-leverage data point had to be excluded based on these criteria. Post-hoc analyses were done in cases of significant interaction terms in models. Data were analysed using R 3.1.2. and the packages 'lme4' [79] , 'car' [80] , 'nortest' [81], 'MASS' [82], 'multcomp' [83] , and "outliers" [78].

Experience phase

The sums of behavioural frequencies of the three individuals recorded from each rearing group at a given developmental time point, and the sums of line crosses (our estimate of locomotory activity; see section 'Behavioural recordings above), were included as dependent variables of Poisson-distributed GLMMs, which were fitted with a log-link function. 'Treatment', 'Experimental Day' and their interaction term were included as fixed effects, and 'Rearing group of origin' was included as a random effect.

Contest outcome

Contest outcome was analysed by fitting a binomial GLMM with logit link function. The binary outcome refers to either winning or losing the contest over the shelter. The categories 'undecided' and 'alternative outcome' were excluded from this analysis. Because social performance [84], in our case contest outcome, and social status [85] can depend on individual cortisol levels, we included waterborne cortisol levels in the models of contest outcome. We included 'stress responsiveness' as covariate in the models; it was calculated as the difference between cortisol content in the holding water after the acute stressor (Stress

level cortisol) and cortisol content in the water sample of the same individual when not stressed (Basal cortisol level) [44,45]. Note that waterborne cortisol, both basal levels and stress responsiveness, *per se* did not differ between treatments and we do not present these results. They did, however, influence the outcome of fights (see 'Results'). Individuals in both social roles can take over the resource therefore 'Social role' (*i.e.* owner or intruder) was included as a covariate. Furthermore, 'Treatment' was included as fixed factor and 'Rearing group of origin' was included as random factor.

Social behaviour during contest

We only included behaviours performed until the end of the contest in our analysis. While after the end of a contest, social behaviour may still occur, these behaviours would not occur after natural contests, where a loser would either leave the territory or get out of reach of the winner. To account for contest duration, we analysed the behavioural data until the end of contests as rates per minute [9,16] and fitted LMMs for analysis. 'Treatment' was included as a fixed factor and 'Rearing group of origin' as a random factor in all LMMs. 'Total aggression of opponent' was included as a covariate since the conflict can escalate and extend if both opponents behave aggressively, and neither shows submissive behaviour.

We analysed the two social roles of owners and intruders separately, since they afford different appropriate behavioural responses. For owners, we analysed restrained aggression, an appropriate type of behaviour expected in individuals that already own a resource in a competitive situation [9]; we included 'Submissive behaviour of opponent' as covariate as the submissive tendencies of conspecifics in *N. pulcher* influence aggression, and *vice versa* [9,16]. For intruders, submissive behaviour is the most adequate social behaviour in an asymmetric situation to achieve being tolerated near the shelter, because an aggressive takeover of the shelter is nearly impossible [9,16]; we included 'Total aggression of opponent' as covariate because of the above mentioned mutual dependence between submission and aggression. None of the behaviours expressed in the two social roles were influenced by cortisol levels (i.e. basal or acute stress levels or stress responsiveness; data no shown).

Gene expression

In order to test the effect of treatment on the expression of the candidate genes (*crf*, *gr1*, and *mr*) we fitted LMs with the factors 'Treatment' and 'Size' (SL in cm). Size was included as a covariate only if it significantly predicted gene expression, which was only true for *mr*. 'Plate number', that is, the identification number of each plate used for quantification of each gene's transcript copy number was included in the models, because the enzyme mixture used for each plate was prepared separately and could potentially affect the measurement of gene expression.

Results

Experience phase

Treatment and age (*i.e.* experimental Day) interactively influenced affiliative behaviour performed in the home tank (GLMM, n=121, χ^2=16.22, p=0.0003). This effect is mainly due to an interaction between cortisol treatment and age (Cortisol × Day: Estimate= 0.0429 ± 0.0134 SE, z=3.19, p=0.0014; Mifepristone × Day: Estimate= 0.00611 ± 0.0141 SE, z=-0.44, p=0.66; Table S2, Fig. 2). This interaction is unlikely to be explained by locomotory activity of the fish, because activity was not affected by treatment (Table S2) or its interaction with age (non-significant interaction term was dropped from the model). No other social behaviour was affected by treatment or its interaction with age during the experience phase (results not shown).

Social challenge

Contest outcome

Contest outcome (winning or losing) was interactively influenced by treatment and cortisol stress responsiveness (binomial GLMM, n = 44, Treatment × Stress responsiveness: χ^2= 13.71, p=0.0012). This interaction was mostly caused by an interaction between mifepristone and stress responsiveness (Cortisol × Stress responsiveness: Estimate= 1.729 ± 1.321, z=1.31, p=0.19; Mifepristone × Stress responsiveness: Estimate= 5.679 ± 2.262, z=2.51, p=0.012; Table S3). The likelihood of winning a contest increased with stress responsiveness in the mifepristone, whereas in the control treatment fish with a lower stress responsiveness were more likely to win (Fig. 3).

Social behaviour during contest

Treatment significantly affected the amount of restrained aggression shown during a contest by focal owner fish (LMM, n=58, χ^2=13.49, p=0.0012) as well as contest duration (LMM, n= 58, χ^2= 8.49, p=0.014). Focal owner fish, which received the cortisol treatment in early life, showed more restrained aggression during contests (Estimate= 1.441 ± 0.418, z-value = 8.12, p = 0.0019; Fig. 4a, Table S4) and contests lasted longer in these fish (Estimate= 3.867 ± 1.828, t-value = 2.12, p = 0.039; Fig. 4b, Table S4). In contrast, behaviour and contest duration were unaffected in owner fish that had received the mifepristone treatment (Table S4). Both treatments had no effect on intruder behaviour (results not shown).

Gene expression

In the telencephalon the expression of both *crf* and *mr* was affected by treatment (*crf*: LMM, n = 31, χ^2= -780.11, p=0.012; *mr*: LMM, n = 21, χ^2= -240.28, p=0.027). Cortisol (Estimate = -2.175e-06 ± 8.480e-07, t-value = -2.57, p=0.017; Table S5, Fig. 5a) and mifepristone (Estimate = -2.237e-06 ± 9.005e-07, t-value = -2.48, p = 0.02) treated fish had a down-regulated *crf* expression. In contrast, *mr* was upregulated by both treatments (Cortisol treatment: Estimate = 0.018 ± 0.009, t-value = 2.06, p = 0.04973; Table S5, Fig. 5b. Mifepristone treatment: Estimate = 0.021 ± 0.009, t-value = 2.33, p = 0.028; Table S5, Fig. 5b). Body size was negatively correlated with *mr* expression (Spearman's rank correlation, rho= -0.36, p=0.046) but not with the other two genes in the telencephalon. In the hypothalamus, early-life treatment did not significantly affect the expression of *crf* and *mr* (Table S5). G*r1* expression was not affected by either treatment (results not shown).

Discussion

Our experiment showed that social behaviour and social performance, here winning or losing a contest over an important resource, is causally evoked by early life stress axis programming in the cooperatively breeding cichlid *N. pulcher*. Our pharmacological manipulations persistently altered two key players in the regulation of the vertebrate stress axis, (i) the mineralocorticoid receptor and (ii) the corticotropin-releasing factor. The behavioural effects of the manipulations became apparent only over the long-term and only in fish assigned to the role of territory owners. Interestingly, social behaviour was little affected by treatment *during* the phase of drug application. Early cortisol application had several long-term effects. Fish showed more aggressive behaviour and contests took longer to be resolved, *crf* gene expression was downregulated and *mr* gene expression was upregulated in the telencephalon compared to control individuals. Interestingly, mifepristone application affected gene expression in the same way as the cortisol treatment. Moreover, it influenced the likelihood of winning a contest in interaction with stress responsiveness.

Effects of early-life treatments on behaviour

Early-life treatment and developmental day interactively influenced affiliative behaviour during the experience phase. The interactive effect during the first weeks of life it is difficult to interpret as Fig. 2 suggests that the changes of affiliative behaviour change non-linearly with age in the different treatments. Affiliative behaviours and glucocorticoid levels have been shown to be related in other vertebrates [86]. For example, in bonobos (*Pan paniscus*) aggressive conflicts increase HPA axis activity and promote affiliative behaviours between victims of the conflict and conspecifics [86]. Furthermore, in rhesus macaques playful behaviours in young males correlate with lower cortisol levels [87]. The interactive effect on affiliative behaviour was the only effect of treatment on behaviour during the experience phase, whereas all other behavioural effects became apparent only during the late juvenile period.

For the social test performed during later life, we predicted aggression to increase in cortisol treated fish. For instance, juvenile rainbow trout given a cortisol treatment during the egg stage increased their frequency of bites and lateral displays performed towards their mirror image [30]. Accordingly, we found that cortisol treated fish showed higher rates of restrained aggression. If this type of low-level aggressive behaviour, which does not include physical contact with opponents, tends to resolve contests faster, it can be viewed as being the most appropriate behaviour in a contest situation [9]. However, if high rates of aggression go along with longer contest durations, as was the case in our cortisol treated fish, it suggests that cortisol evoked in a decrease of social competence and consequently, decreased social performance. Longer contests should increase energy expenditure, because agonistic interactions and submissive behaviours are energetically demanding [88], and being involved in distracting, aggressive contests reduces vigilance and thereby the chance to detect dangerous predator [89]. Evidence from zebrafish and laboratory rodents suggests that our cortisol treatment had a very similar effect to that of natural stressors. After dyadic interactions of dominant and subordinate zebrafish endogenous cortisol levels increased to levels

sufficiently high to elicit changes in *mr* mRNA levels [90]. Furthermore, in young rodents, early exposure (between postnatal day 28 and 42) to non-social stressors or to social deprivation increased aggressiveness in adulthood [91].

Contrary to our predictions, mifepristone had no detectable influence on social behaviour during the experimental contest. Our predictions were based on findings of immediate effects of mifepristone treatment in adult *N. pulcher*, which have been shown to gain access to a resource by increasing their submissive behaviour when being attacked [36]. The lack of direct effects of mifepristone on behaviour in our study clearly indicates that long-term effects of early-life administration and immediate effects are not directly comparable. As discussed above, this is probably due to the possibility to compensate for early-life impacts on the stress axis by re-programming of this axis. Interestingly, we found a striking interactive effect of mifepristone and stress responsiveness on contest outcome; control fish had an enhanced likelihood of winning a contest if they had a reduced stress response. Nyman and colleagues [36] argued that the likelihood of winning a contest is enhanced by low stress responsiveness, which may explain these results. However, the opposite effect occurred in fish treated with mifepristone in early life. Although this reversal of the relationship between stress responsiveness and winning is difficult to explain, we propose that it was caused by the differential programming of the stress axis of mifepristone-treated fish, which likely qualitatively altered the link between stress axis regulation and behavioural regulation.

Contrary to our findings for owners, we found that early-life treatment did not influence the contest duration or social behaviours for intruders. This finding contrasts previous work in *N. pulcher*, which showed a significant influence of early-life social experience on stress gene expression [17,36] and on the behaviour of both owners and intruders in the same asymmetric competition paradigm [9,16]. In these studies, *N. pulcher* reared with parents and other older group members (helpers) (i) had a higher expression of the *gr1* gene in the telencephalon, (ii) when in the role of intruders they showed more submissive behaviour per received opponent aggression and (iii) they were less neophobic [92] compared to juveniles reared in a socially deprived setting with same aged siblings only; thus also these previous findings involved a re-programming of the stress axis, but with significant differences from the current study. Here, different genes, behaviours and social roles were affected, which suggests that different components of the stress axis were affected by social factors [16,17] and direct application of cortisol and mifepristone. This is perhaps not so surprising, given that during a social challenge social cues and experiences accompany the stress response. In the case of cortisol administration salient cues that can be learned and memorized are absent, and these learning processes are one important function of adaptive stress responses [91].

Effects of early-life treatments on stress axis programming

Early exposure to cortisol and to mifepristone induced persistent changes of the expression of two main stress genes in the telencephalon, suggesting that glucocorticoid signalling in this brain area during later life will differ compared to control treatment fish [91]. The telencephalon is central for behavioural expression as it is involved in social decision making [93,94] and cognitive processing of information [95]. In the following paragraphs we discuss the long-term effects of cortisol and mifepristone, which suggest

that both drugs may have temporarily increased fluctuating cortisol levels after the applications during early life. Therefore, both drugs may have induced similar changes in the telencephalon.

In line with our hypothesis, repeated exposure to cortisol during early development caused a persistently lower constitutive gene expression of *crf* in the telencephalon. In contrast to mammals, in which cortisol regulates CRF release in the hypothalamus [38,39,96], in fish the *crf* gene is also expressed in other brain areas, including the telencephalon. In adult goldfish (*C. auratus*) cortisol implants down-regulate *crf* mRNA levels in the telencephalon [25]. In the cichlid *Oreochromis mossambicus* the principal source of plasma CRF is the ventral telencephalon [97], and these authors suggested that the self-inhibiting negative feedback of cortisol by blocking the secretion of further CRF acts predominantly in this brain region. CRF is related to stress-coping style, which is defined as the physiological and behavioural response to stress [98]. A proactive coping style is characterized by low stress axis activity, whereas the opposite is true for reactive individuals [98,99]. Accordingly, we hypothesize that repeated exposure to cortisol early in life generated fish with an attenuated cortisol response that produce lower levels of CRF under stressful conditions. Possibly these effects on the cortisol response were too subtle to be detected by our rather coarse, non-invasive measurement of waterborne cortisol [69].

The mineralocorticoid receptor gene (*mr*) was up-regulated in cortisol treated-fish. Nuclear MR determines the sensitivity of the limbic stress response system [73]. MR signalling is important for coordinating the initial stress response [21,100] and preparing animals for coping with a stressor (e.g. lower sensory detection thresholds, higher alertness) [101], but MRs are also thought to be involved in the negative feedback on cortisol [102]. In Japanese quail, corticosteroid exposure before or after hatching results in *mr* up-regulation in the hippocampus [40], which is part of the telencephalon. In line with our predictions, cortisol treatment up-regulated *mr* gene expression in experimental fish. We speculate that a higher sensitivity to cortisol in the presence of more MR may help these fish to mount a stress response at a normal speed (*i.e.* a rapid response to a stressor despite their lowered CRF response; discussed above), which is then followed by an attenuated response with lower peak values of cortisol due to lower *crf* expression.

The fact that a similar change of *crf* expression was caused by mifepristone treatment in the telencephalon is in accordance with our hypothesis that the mifepristone applications generated increases of endogenous cortisol [46]. Furthermore, early mifepristone application induced a higher constitutive expression of *mr* in the telencephalon. Although, this finding is opposite to our initial predictions, we speculate that the early mifepristone treatment generated a compensatory effect in the stress axis similar to the one by cortisol. Mifepristone blocks GRs, which are involved in the clearance of cortisol and blocking of further cortisol production. Correspondingly, juvenile *N. pulcher* exposed to mifepristone can be assumed to have experienced temporarily enhanced cortisol concentrations. In fish temporary cortisol increases after application of mifepristone can last 2 (larval summer flounders, *Paralichthys dentatus*, [62]; adult gulf toadfish, *Opsanus beta*, [46]) or 3 days [adult goldfish, *Carassius auratus*, [25]). In our mifepristone treated *N. pulcher*, persistent programming of higher *mr* expression may have developed as a compensatory mechanism to mediate the effects of high plasma cortisol concentrations as previously shown in mice [103].

Socially challenging interactions are known to induce elevations of fluctuating glucocorticoid levels in several vertebrates including fish [90,98] and mammals [104]. In cooperatively breeding species or other species with hierarchical social organization, individuals are exposed to frequent social interactions including repeated challenges of their social rank. For instance, subordinate members of cooperative breeders, both in highly social vertebrate and invertebrate species, recurrently challenged their position in the queue for a breeding position by lower ranking individuals [105]. Moreover subordinate group members have to mediate conflicts with dominants over group membership [56,106] by appeasement behaviours in the form of subordination and helping [54,59,107]. Social challenges will induce frequent stress responses and thus elevations of glucocorticoid levels in the involved social partners [90]. If individuals experience frequent social stressors early in life, a reprogramming of the stress axis involving altered *mr* and *crf* expression might be a mechanism allowing animals to mount normal cortisol responses even in socially stressful situations later in life. However, the potential benefits of programming depend strongly on the later-life social environment. Moreover, programming of the stress axis by early cortisol surges and does not come without cost, as it can negatively affect emotionality and behavioural performance in the long run as shown in laboratory rats [19,108]. Such negative behavioural impact of glucocorticoids might explain why *N. pulcher* had impaired social competence and social performance following early-life cortisol exposure. Finally, further research would have to confirm whether multiple social stressors during early life have comparable effects to multiple pharmacologically induced cortisol surges.

In the past years, evidence is rapidly accumulating that the stress axis of vertebrates can be re-programmed early in life by social [11,16,93] and ecological [94,95] stressors in mammals, birds and fish. Understanding the physiological and behavioural mechanisms of stress coping should greatly increase our understanding of individual life history trajectories and behavioural strategies, both in natural and perturbed environments. Vertebrates, including humans, are increasingly exposed to environmental stressors, be it because of competition for increasingly limited resources or human-made problems such as pollution. Here we uncover a potentially general mechanism that may be involved in stress axis re-programming and stress coping, which regulates stress by increasing the sensitivity of limbic stress response and decreasing stress axis reactivity. Further research is necessary to reveal, which type of environmental social and ecological stressors will elicit this programming mechanism in vertebrates, and whether there are certain sensitive periods when it can happen [112]

Conclusions

The altered programming of the stress axis induced by our pharmacological treatments during early development suggests the generation of a mechanism to cope with stressors. The down-regulation of *crf* and the up-regulation of *mr* in the telencephalon both by early-life mifepristone and cortisol have two potential implications: First, the increase of mineralocorticoid receptor expression could increase the sensitivity of the limbic stress response and result in a faster initial response to stress that is mediated by MR [66]. Second, lower levels of *crf* expression could lower HPI axis activity following the encounter of a stressor [98], which means that it may need stronger stress stimuli to mount a stress response compared to

control fish. This suggests that individuals have developed a physiological mechanism that allows them to better cope with stressors. This may help them to avoid physiological damage caused by increases of allostatic load [113], which is the amount of energy required to maintain homeostasis [113]. However, the stress axis programming by cortisol and mifepristone comes with some costs, as these fish exhibit reduced social competence. Whether or not this stress axis programming results in net fitness benefits depends on the frequency and strength of stressors present in the environment and whether the costs are smaller than the benefits. Our results suggest that early life exposure to stimuli known to trigger elevations in cortisol levels such as social defeat [114] or social deprivation [91] may lead to programming of stress axis genes such that this protective mechanism is implemented.

Additional Information

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Author's Contributions

MR and BT designed the study, MR collected behavioural data and biological samples, MR and GG did the hormone analyses, MR and DR did the gene expression analyses, MR, BT and DR did the statistical analyses, MR and BT wrote the manuscript, all authors edited and approved the manuscript.

Competing Interests

The authors do not declare competing interests

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Figure Captions

Figure 1. Timeline of experimental manipulations between developmental Day 0 (first day of free swimming) and Day 173. White boxes: experience phase; individuals received pharmacological treatments and their behaviour was recorded. Grey boxes: neutral phase; broods were kept under identical conditions without their parents. Black boxes: test phase; experimental individuals were selected for hormonal measurements and social challenge tests; brain were dissected of 'naïve' individuals, i.e. individuals which had not taken part in either hormone measurements or social challenge test.

Figure 2. Frequency of spontaneous affiliative behaviour performed by juveniles towards their siblings (medians ± interquartile ranges are shown). Behaviours were recorded in the home tanks of rearing groups (3 times 5 min per group, see 'Materials and Methods') during the experience phase 9 days after the application of each treatment starting on developmental day 29 until day 59. Treatments: control (black), cortisol (white) and mifepristone (grey).

Figure 3. Interactive effect of mifepristone treatment and stress responsiveness on the likelihood of winning (outcome = 1) or losing (outcome = 0) a contest. Mifepristone treatment (n=20, grey line and triangles) and control treatment (n=22, black line and circles).

Figure 4. Social behaviours in asymmetric contest over a shelter. (a) Total duration (min) of contests when owners were the focal individuals. (b) Rate of restrained aggression per min performed by owners. In both panes control n=19 (black), cortisol n=20 (white) and mifepristone n=19 (grey). Medians and ± interquartile range are shown.

Figure 5. Expression of candidate genes in the telencephalon relative to the reference gene 18s. (a) Expression of *crf* gene. (b) Expression of *mr* gene. In both panels control n= 10 (black), cortisol n=11 (white) and mifepristone n=10 (Black). Means ± S.E. are shown.