Evolutionary conserved neural signature of early life stress affects animal social competence

Cecilia Nyman[1], Stefan Fischer[2], Nadia-Aubin-Horth[3] & Barbara Taborsky[1]

[1]Div. of Behavioural Ecology, Institute of Ecology and Evolution, University of Bern, Switzerland

[2]Institute of Integrative Biology, University of Liverpool, Liverpool L69 7ZB, UK

[3]Département de Biologie and Institut de Biologie Intégrative et des Systèmes, Université Laval, Quebec, Canada

Running head: Early life stress and social competence

Corresponding author: Cecilia Nyman, cwikstro@abo.fi

Abstract

In vertebrates the early social environment can persistently influence behaviour and social competence later in life. However, the molecular mechanisms underlying variation in animal social competence are largely unknown. In rats, high-quality maternal care causes an upregulation of hippocampal glucocorticoid receptors (*gr*) and reduces offspring stress responsiveness. This identifies *gr* regulation as a candidate mechanism for maintaining variation in animal social competence. We tested this hypothesis in a highly-social cichlid fish, *Neolamprologus pulcher*, reared with or without caring parents. We find that the molecular pathway translating early social experience into later-life alterations of the stress axis is homologous across vertebrates: fish reared with parents expressed the glucocorticoid receptor *gr1* more in the telencephalon. Furthermore, expression levels of the transcription factor *egr-1* (early growth response 1) were associated with *gr1* expression in the telencephalon and hypothalamus. When blocking glucocorticoid receptors (GR) with an antagonist, mifepristone (RU486), parent-reared individuals showed more socially appropriate, submissive behaviour when intruding a larger conspecific's territory. Remarkably, mifepristone-treated fish were less attacked by owners and had a higher likelihood of territory takeover. Our results indicate that early social-environment effects on stress axis programming are mediated by an evolutionary conserved molecular pathway, which is causally involved in environmentally-induced variation of animal social competence.

Key words: early environment, mifepristone, cooperative breeding, stress axis, glucocorticoid receptor, cichlids

Introduction

Variation in early-life social experience, such as the quality of parental care [1] or natal group composition [2,3] can have profound long-term influences on the emotional, cognitive and social development of vertebrates, including humans (e.g., [1,3–7]), with ensuing marked consequences for Darwinian fitness (reviewed in [8]). In particular, more complex early social experiences generally tends to favour the development of improved social competence in animals, and thereby their performance during social challenges later in life (rev. in [8,9]. 'Animal social competence' is defined in an evolutionary context, and denotes the ability to optimize the expressed social behaviour by a flexible use of social information, thereby improving fitness [10]. However, while we have evidence for triggers from the social environment, which are responsible for variation in animal social competence (reviewed in [9]), as yet the neural molecular causes of this variation are not understood.

Indirect evidence suggests that social influences on the programming of the vertebrate stress axis may co-vary with both stress responsiveness and social competence. Offspring of laboratory rats experiencing high-quality maternal care undergo persistent reprogramming of their stress axis and exhibit low stress responsiveness later in life [1,4,11]. A number of experimental studies reported that more intensive maternal care, for instance caused by the presence of several mothers in communally-breeding laboratory mice, does not only reduce the susceptibility to stress, but also enhances social competence (rev. in [12]). Feedback from the maternal to offspring behaviour is in part caused by a persistent upregulation of hippocampal glucocorticoid receptor (*gr*) gene expression in offspring [5,11,13] [note that for the glucocorticoid receptor italicised lowercase letters refers to genes (*gr* or *gr1*) and capital letters (GR or GR1) to gene products throughout]. Hippocampal GRs exert a negative feedback on glucocorticoid production, thereby contributing to the termination of stress responses and reducing the susceptibility to stress [14]. The expression of *gr* in the mammalian hippocampus is itself regulated by the transcription factor early growth response 1 (*egr-1*) [15,16], a marker for neuronal activity [17] and plasticity [18]. The *egr-1* gene codes for a transcription factor, which has been suggested to target later-acting genes including genes of the stress axis in the dorsolateral telencephalon of the fish brain (the putative homologue of the mammalian hippocampus) [17].

In non-mammalian vertebrates, the corticoid stress axis is similarly organized as in mammals (e.g. [19,20]), and the early social environment persistently affects *gr* expression in the brains

of birds [21,22] and fish [23,24]. For instance, varying early social experience in the cichlid *N. pulcher* affected total brain expression of the gene *gr1*, which is homolog to the mammalian glucocorticoid receptor [25], whereas the expression of the second glucocorticoid receptor (*gr2*) present in *N. pulcher* was not affected by rearing environment [24]. Because of the correlations between maternal care, stress axis programming and social competence in laboratory rats, we hypothesized that the regulation of glucocorticoid receptors is a prime candidate mechanism causally involved in generating variation of social competence in vertebrates. Discovering the key components of social competence is crucial in understanding how social competence has evolved, is maintained and regulated in social species and how it can be modulated during early development.

Three ingredients are needed to determine the causality of the association between *gr* expression levels and social competence in vertebrates in general. First, we need a non-mammalian model species, in which early life stress effects on behaviour are well-documented [3,23,26], and which thus allows to test the generalizability of GR function in social competence in vertebrates. Second, differences in expression of *egr-1* and *gr* following early life stress must be established [15,16,23]. Third, manipulations of the pathway coupled with quantitative behavioural assays must be carried out as a first step to establish the functional role of this candidate pathway. Blocking or enhancing the activity of a specific pathway, for example by using pharmacological manipulations, coupled with measurements of the resulting phenotype changes, allows to test the functional involvement of physiological regulatory pathways [27].

We investigated the mediating role of the GR pathway regulating the variation in social competence in the cooperatively-breeding cichlid fish, *Neolamprologus pulcher*. Individuals of this species reared in larger [28] or more complex [3,23,26] social groups show more appropriate social behaviours in a variety of social contexts and thus better social competence, resulting in advantageous outcomes of social interactions such as reduced contest durations [26]. We first compared gene expression of fish that had been reared either with (+F) or without (–F) parents and a broodcare helper. We analysed the relationship between the expression of the glucocorticoid receptor gene *gr1* and the transcription factor *egr-1*, proposed to regulate *gr* expression [16] in two brain areas, the telencephalon and the hypothalamus. The telencephalon is of interest since in this brain area the putative homologue to the mammalian hippocampus is located [29], which in rats was influenced by maternal care

leading to changes in *gr* expression [4]. Moreover, both telencephalon and the hypothalamus play a key role in the regulation of animal social behaviour [30] and of the hypothalamic-pituitary-interrenal (HPI) stress axis, the stress axis of fish [31], which is homolog to the hypothalamus-pituitary-adrenal (HPA) stress axis of mammals [4,11,32].

Second, we investigated the causal role of the GR pathway in modulating social competence. We compared the social behaviour of blank treated fish (control) and fish treated with mifepristone (RU486), a substance which selectively blocks glucocorticoid but not mineralocorticoid receptors [33,34] in fish (goldfish, *Carassius auratus* [35,36]; rainbow trout, *Oncorhynchos mykiss* [36], medaka [37] and the lined bristletooth *Ctenochaetus striatus* [38]. We predicted that blocking GRs by mifepristone treatment would increase circulating glucocorticoids through impaired negative feedback response (see [35]) thereby increasing stress responsiveness of these fish, which should result in impaired social competence ([12].

Here we first tested whether early social experience in *N. pulcher* affects the activity of the pathway from *egr-1* to *gr1* similarly to mammals. Second, by pharmacologically blocking the GR activity using an antagonist in parent-reared fish, we tested if it is causally involved in the variation of social competence.

Methods

Study species

Neolamprologus pulcher is a cooperatively breeding cichlid endemic to Lake Tanganyika, East Africa, living in large family units of up to 25 fish consisting of a dominant breeder pair, one or several related or unrelated alloparental brood care helpers and fry from recent broods. In the juvenile stage all fish join in brood care, albeit to a different extent [39]. Even after sexual maturity, which occurs around the age of 10-12 months, many *N. pulcher* continue to serve as helpers. Social groups are organized in a strict, linear hierarchy structured by body size [40]. *N. pulcher* possess a fine-scaled repertoire of affiliative, submissive and aggressive social behaviours used to maintain this hierarchy and to solve social conflicts among groups members [41]. The contextual expression of these behaviours is strongly affected by the social environment young experience early in life [3,23,26,28].

Animal husbandry

Both experiments were done at the 'Ethological Station Hasli' of the Institute of Ecology and Evolution (IEE), University of Bern, Switzerland, under licence number 52/12 of Veterinary

Office of the Kanton Bern. All tanks were equipped with a 2 cm sand layer, a biological filter, and clay pot halves and PET bottles serving as shelters. The light:dark cycle was set to 13:11 h with a 10 min dimmed light period in the mornings and evenings, and the water temperature was kept at 27 ±1 ºC. Fish were fed 6 days a week (5 days commercial flake food, 1 day frozen zooplankton). All fish used in experiments 1 and 2, except those sacrificed for brain sampling, were integrated in the *N. pulcher* stock tanks of the IEE at the end of our experimental work.

Experiment 1: Effects of social experience on gene expression

Rearing treatment. Details of the rearing procedure are given in [23] and [42]. In brief, ten breeding pairs, which were 2nd and 3rd generation offspring from *N. pulcher* wild caught at Kasakalawe Point, near Mpulungu, Zambia, produced the experimental broods in ten 200-L tanks. Ten days after a breeder pair had produced a clutch, the hatchlings had reached the free-swimming stage. On that day, we randomly assigned half of each brood to one of two treatments, (i) being either reared with parents, the helper and same age siblings (+F treatment, n = 10 groups), or (ii) with same age siblings only, without presence of older family members (−F treatment, n = 10 groups). Each treatment group was raised in a separate 100-L compartment of a 200-L tank (mean group size, +F fish: 32.6±3.8 SE; –F fish: 35.4±5.1 SE). The social experience treatment lasted for 62 days (see experimental timeline in Fig. 1A). Afterwards we removed the parents and the helper from the +F treatment and transferred them back to our laboratory stock tanks. Fish from both treatments were kept in 100-L compartments under identical conditions for the next 72 ± 2 days.

Tissue sampling. The procedure described is as in [23]. We removed the individuals from their home tank on day 134 (± 2 days), measured their length and weighed them before placing them into a 20-L test tank (30 x 20 cm, 20 cm high) 24 h before brain sampling. We divided the test tank into two compartments by an opaque PVC wall and placed the individual in an empty compartment of the test tank (balanced between right and left side between trials). In the other compartment, we placed a clay pot half serving as shelter in the centre. Our aim was to measure the fish baseline gene expression in the brain after 24 hours without influence of recent social interactions. We used two replicate individuals from each rearing group. The sex of these individuals was unknown, as the genital papillae of the fish at this age is not yet differentiated. Before brain sampling, we removed the divider and let the individual swim freely in the test tank for 20 min before reinstalling the divider again. Then fish was left

undisturbed for another 10 min, and after the total of 30 minutes, we killed the fish with an overdose of Tricaine methanesulfonate (MS222; Sandoz, Switzerland). We collected brains from fish of both conditions: +F (8 groups, 15 fish) and –F fish (10 groups, 20 individuals), for a total of 35 fish. We could use experimental fish from 8 of the original 10 +F rearing groups only, because of a procedural mistake during the first two trials. Moreover, in one +F group only one replicate individual was sampled because the brood was very small and individuals were needed for further behavioural experiments (see [42]). We dissected telencephalon and hypothalamus from the brain tissue, and placed each sample into a 1.5 ml vial with RNAlater (Ambion). Samples in RNA later were left overnight at +6 ºC and then moved to -20 ºC for permanent storage.

Gene expression. We measured the gene expression of *gr1* and *egr-1* in the telencephalon and hypothalamus of *N. pulcher*. The expression of the 'housekeeping' gene 18S was used as a control. Detailed protocols of primers used, RNA sample preparation and qPCR are given in the supplementary information (SI). All qPCR samples were run in three replicates.

Experiment 2: Blocking of GR1

Experimental broods. To create the experimental broods, we formed 10 breeder pairs in separate 60-L tanks by merging unfamiliar adult males and females haphazardly selected from the institute's male and female stock tanks. In this experiment all experimental broods were reared with parents. Parents stayed with the clutch for 72 days (10 days until the hatchlings were free-swimming plus 62 days during the juveniles stage; see experimental timeline, Fig. 1B). Afterwards the parents were removed and transferred back to the institute's breeding stock. During the following 35 ± 2 days ('neutral phase'), the siblings were kept in 30-L compartments under identical, standard housing conditions (see 'Animal husbandry').

Immersion. Following the protocol by [43], mifepristone (RU486, Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO) at 50 mg/mL, then serially diluted in 0.1 M acetic acid (1:10), phosphate-buffered saline (1:100), and finally diluted in distilled water for an immersion concentration of 400ng/L. Controls were appropriately prepared with diluents without mifepristone.

Nine days before each social challenge test, two fish from each sibling group were caught, measured in length and transferred to perforated plastic isolation containers floating in their

home aquaria (N=40 fish). Thus the experimental fish had visual and chemical contact with their siblings. After 7 days in the isolation containers, fish were exposed to an immersion treatment. Fish were singly immersed during 48 hours in 2 L of water in glass containers containing either 400 ng/L of mifepristone or control water. Each fish was exposed to both conditions (mifepristone and control), half of the fish (n=20) received the mifepristone treatment first and the other half of the fish (n=20) first received the control treatment. On day 97, fish underwent the first social challenge test (see below). On day 98 the fish were moved back to the floating plastic container in their home aquaria, where they remained for another 7 days until the second 48-h immersion treatment occurred, followed by the second social test at day 107. We had decided to keep the fish in the isolation boxes during the 7-day periods between treatments to prevent injury of the focal fish. *N. pulcher* live in closed social groups and fish returning after only 1 day into a group would be considered as strangers and might be attacked heavily.

Social challenge test. On day 97 and 107 (± 2 days) in the morning at 0900-1100 hours, two individuals of each of the ten experimental families underwent a staged asymmetric competition over a shelter (for details see [23,26]). The morning hours are supposed to be particularly sensitive to blocking of by mifepristone because of the spontaneous morning rise of cortisol occurring in vertebrates [27]. In preparation of a competition trial, a focal individual was removed from the immersion treatment and placed into a 20-L test tank (30 x 20 x 20cm) where it stayed for a 2-h habituation period before testing. Biological half-life of mifepristone is 18 hours [44] indicating that GRs should still be blocked after this habituation period. The test tank was divided into two compartments by an opaque PVC wall. The focal individual of the challenge was always assigned the role of the territory intruder and was placed in an empty compartment of the test tank (balanced between right and left side between trials). A halved clay pot serving as a shelter was placed in the centre of the other compartment, which represented the contested resource. This compartment was stocked with an unfamiliar same-aged, but slightly larger juvenile *N. pulcher*, which was assigned to become shelter owner and the opponent of the focal fish (opponent was 0.129 cm ± 0.011 cm larger than focal fish). Each shelter owner served as opponent for both trials (mifepristone and control) of a given focal fish. The shelter owner had been already transferred to the experimental tank 24 hours before the onset of a trial, which is sufficiently long for *N. pulcher* individuals to occupy a novel shelter and defend it as core of its territory [3,26].

After the 2-h habituation time, the wall between the compartments was lifted so that the pre-assigned intruder and the shelter owner could interact. The starting point of the trial was defined as the moment when either of the two fish for the first time crossed the previous border between the two compartments, that is, the line where the PVC divider had been before. From that point on the behaviour of the focal individual was recorded for 20 min. The observer (CN) was blind to the exposure treatment of the focal fish. Behaviours of both fish [submissive display (tail quivering), overt aggression (i.e. aggression with attempted body contact, which includes ramming, biting and chasing), restrained aggression (aggression without attempted body contact, which includes fin spread, approach, head down position and opercular spreading), hiding in shelter, locomotion without showing social behaviour)] were recorded continuously using the Observer 5.0 software (Noldus, The Netherlands). Twenty minutes after the start of the contest, we categorized the focal fish as either the winner or the loser of the resource. Fish were classified as winner, if they stayed in or close (< 3 cm) to the shelter and were not attacked by the other fish. Fish were classified as loser, if they were evicted from the vicinity of the shelter and showed submission but no overt aggression towards the other fish, or if they stayed close to the water surface (< 5 cm). The contest was rated as 'undecided' in three cases (1 mifepristone treatment, 2 control treatments) when there was no clear winner or loser after 20 min. These three fish were excluded from further analysis. After the 20 min behavioural recording the two fish were separated by the partition and 1 day later the opponent and the focal fish were transferred back to their home tanks.

Data analysis

We used R 3.0.2 (R Core Development team 2013) for the statistical analyses. The results of Experiments 1 and 2 were analysed by fitting general linear mixed models (LMM) with fish identity and the identity of experimental groups (family of origin) as random factors in each model. In experiment 1 we analysed the effect of treatment (+F or –F) on gene expression. For some individuals, gene expression data for one or both genes had to be discarded, because the coefficient of variation (CV) of the three replicates run for each individual on each gene was too large (a CV cut-off of 5% was used for all genes, see SI). This resulted in sample sizes of N=27 for *egr-1* and *gr1* in the telencephalon, and of N=18 for *egr-1* and N=27 for *gr1* in the hypothalamus. In experiment 2, we tested the effect of treatment (mifepristone or control) on behaviours displayed by intruders and shelter owners. We analysed only the behaviours between the start and the end of contest. Contests were considered to be terminated when the loser retreated to the upper parts of the water column, or a distant corner

of the tank, or when it did not aim to gain access to the shelter. We analysed behavioural rates (behaviour per min) since the duration of these periods varied between trials. Received overt aggression (aggression displayed by initial owner of the shelter) was included as covariate in the LMM on submissive behaviour, as submissive displays in *N. pulcher* are often a direct response to received overt aggression. We ran the models with the command 'mixed' of the R package 'afex' [45]. Error terms were examined for normality by visual inspection of the distribution of the residuals, predicted vs. fitted value plots and Quantile-Quantile plots. If necessary, we log-transformed the data and/or used boxcox transformations in order to achieve a normally distributed error structure. For significance testing of the terms of the mixed models, the 'mixed' function singly removes each term from a model, it compares the reduced model to the full model and it calculates type 3 p-values using a Kenward-Roger approximation for degrees-of-freedom [45]. Models were fitted with sum contrasts. These are orthogonal contrasts, where every level of a factor is compared to the overall factor mean, which is represented by the intercept. P-values of post-hoc analyses of significant interactions were corrected for multiple testing by applying the Benjamini-Hochberg false-discovery rate method [46].

Results

Experiment 1

To study whether the early social environment (+F / –F) influences the expression of *gr1* in the telencephalon and the hypothalamus, we analysed the interaction between brain areas and social rearing conditions effects on gene expression. These two factors interactively influenced the expression of *gr1* (LMM, interaction term: $F = 6.067$, $p = 0.020$, early rearing: $F = 2.518$, $p = 0.133$, brain part: $F = 1.783$, $p = 0.193$, $N = 54$, Figure 2A). Post-hoc tests revealed that the significant interaction was caused by a differential expression of *gr1* in the telencephalon, with +F fish having a higher expression than –F fish (LMM, $F = 7.108$, adjusted-$p = 0.037$, $N = 27$), whereas *gr1* expression did not differ in the hypothalamus (LMM, $F = 0.343$, adjusted-$p = 0.567$, $N = 27$). Because *egr-1* is part of the pathway triggering *gr1* expression in the hippocampus of rats [16], we tested whether *egr-1* expression predicts *gr1* expression across individuals. *Egr-1* expression predicted *gr1* expression in both brain areas (LMM, *egr-1* expression: $F = 8.522$, $p = 0.006$, brain part: $F = 4.585$, $p = 0.042$, interaction: $F = 3.041$, $p = 0.090$, $N = 54$, Figure 2B).

Experiment 2

To dissect the functional link between GR activity and social competence, we analysed the effects of mifepristone on the social behaviour of parent-reared fish in the social challenge test. Focal fish (all assigned to the role of intruders, see 'Methods') exposed to mifepristone showed more submissive displays relative to the amount of received overt aggression from the shelter owner compared to fish of the control treatment as indicated by the significant interaction term of treatment × received overt aggression (LMM, table 1, figure 3A). There was no difference in aggression displayed by a control or a treated intruder, but intruder fish treated with mifepristone received less overt aggression from shelter owner (LMM, table 1, figure 3B). The likelihood of intruder fish to win the contest and to take over the ownership of the shelter was significantly higher when treated with mifepristone (LMM, estimate 7.519±3.675, Chi^2= 15.99, $p<0.0001$, figure 4) than in the control treatment despite the initially adverse ownership asymmetry.

Discussion

Individual variation in the responsiveness of the HPA stress axis to early social experience is widespread across a diversity of vertebrate taxa [4,21,24,47]. Effects have been reported in regions of the telencephalon and the hypothalamus [4,21,23], two brain areas holding many nuclei of the social decision making network (SDMN) [30]. Dysregulation of the HPA axis can influence life-time glucocorticoid levels, causing impaired social behaviour and neuronal dysfunction in the brain [48]. Here we first showed that early social experience affects *gr1* expression in the telencephalon but not the hypothalamus. Furthermore we showed that *egr-1* expression in both brain parts predicts the expression of *gr1*. These results suggest that *egr-1* expression is involved in triggering *gr1* expression as previously shown in laboratory rats [16,49] and that the effects of the early social environment on stress axis programming are mediated by a molecular mechanism that is evolutionary conserved among vertebrates. Second, we blocked GR signalling to test whether the GR pathway is causally involved in the regulation of social behaviour and social competence. We found that short-term blocking of GRs causes an improvement of social competence in parent-reared *N. pulcher*.

Early life has been shown to affect several components of the molecular pathways involved in the stress axis in a variety of vertebrates. In laboratory rats, the programming of the corticoid stress axis of new-born pups depends on the quality of maternal care: if care is poor, offspring are more sensitive to stress later in life [4,13]. This effect, which arises through a reduced expression of the glucocorticoid receptor (*gr*) gene through epigenetic modifications, is now

well understood [16]: lower expression of *gr* in the hippocampus results in a weaker negative feedback and thus a delayed termination of stress responses. As *gr1* was downregulated in the telencephalon of fish reared without older conspecifics in our study, our results suggest that the dysregulation of the negative feedback loop of the stress axis under reduced social stimulation [4] is conserved across vertebrates. The reason for lower *gr1* expression in the telencephalon of *N. pulcher* is still unknown but could possibly be due to epigenetic modifications as seen in rats [16]. In the hypothalamus, where the *gr1* receptor is also part of the HPI axis [25,31] *gr1* expression did not differ between our treatments. In birds, maternally deprived chicks showed lower hypothalamic expression of *gr1* compared to non-deprived chicks, whereas the expression in the hippocampus and cerebellum was not affected by rearing [21]. Furthermore, mineralocorticoid receptors were less expressed in maternally-deprived chicks in the hippocampus. The pattern observed in mammals and fish may thus not extend to all vertebrates, at least in its entirety, potentially partly because of different ligand specificity of mineralocorticoid and glucocorticoid receptors in mammals and birds [36,50]. Our study suggests an evolutionary conserved neural signature for mammals and fish while further studies among reptiles and amphibians are warranted to clarify the extent of HPA conservation across vertebrates.

The expression of the transcription factor *egr-1* plays a significant role in activating effector genes downstream in mammals [51]. For example, increased expression of *egr-1* in the hippocampus correlates with the activation of the serum glucocorticoid-inducible kinase (SGK) gene in rats, a kinase important in the stress response [51]. *Egr-1* also regulates *gr* expression in rats [16,51,52]. Postnatal handling increases both *egr-1* and *gr* expression in the rat hippocampus [53]. Here we show that in *N. pulcher*, *egr-1* expression predicts expression of *gr1* in both the telencephalon and the hypothalamus, suggesting that *egr-1* also regulates the expression of the glucocorticoid receptor in this fish species. Our result thus suggests that a similar *egr1-gr* pathway in rats and fish brain is activated under broadly similar environmental conditions, although interestingly the tactile stimulation by maternal care believed to induce the *gr* gene expression change in rats [54] is absent in fish brood care.

Our pharmacological manipulation showed that GR signalling influences social competence. Bernier and colleagues [35] found that blocking GRs with mifepristone influenced the negative feedback loop, causing prolonged expression of corticotropin-releasing factor in the olfactory bulbs and the telencephalon-preoptic area and resulted in increased cortisol levels.

Hence, we expected that an altered HPI axis in mifepristone-treated +F fish would reduce social competence. However, our results show that this short-term manipulation of GRs had the opposite effect, as indicated by a higher readiness to show submission after being assigned a socially inferior position (intruder), lower received aggression by the dominant opponent and a higher likelihood to gain a resource. These results suggest that GR-blocker treated fish were more likely to win the contested resource as a consequence of their improved social abilities, although they had started as 'designated losers' and were on average slightly smaller than the initial shelter owner. Thus mifepristone treated fish were more efficient in solving the contest than controls. This is the first time that social competence [8,10] has been pharmacologically manipulated by directly interfering in the hormonal pathways controlling social behaviour. Blocking GRs using mifepristone is also known to attenuate the acute stress responses in rats by dampening the ACTH response to a stressor [55] and, as a consequence, reducing glucocorticoid production [56]. In fish, mifepristone application reduces GR protein expression and at the same time leads to a compensatory increase of *gr* mRNA production in rainbow trout [36]. Transcript abundance of the corticotropin-releasing factor is reduced by mifepristone treatment, suggesting a decreased HPI axis capacity [36]. Most importantly, and similar to rats, it almost entirely abolishes stressor-induced cortisol production and thus stress responsiveness [36]. We therefore hypothesize that the improvement of appropriate social behaviour, and thus social competence of mifepristone treated fish was a direct consequence of an attenuated stress response.

Drawing from findings in rats [54], our results suggest that fish from the socially enriched environment with a higher *gr1* expression have a moderate and shorter, “more appropriate” stress responsiveness. This prediction is supported by the finding that these parent-reared fish have weaker neophobic responses [57]. Therefore, it might seem counterintuitive that blocking GR enhanced social competence, mostly likely because their stress responsiveness was attenuated [35,36,55,56]. All of these studies, including our own, however, blocked GR systemically. GR1 does not only occur in the telencephalon and hypothalamus, but in various tissues of fish [58] and we assume that mifepristone inhibited GRs in all of these tissues [36]. In rats, systemic mifepristone treatment enhances synaptic plasticity (in rat hippocampi) [59], increases neuronal activation in the medial prefrontal cortex and ventral subiculum [55], but decreased activity in specific regions of the hippocampus and central amygdala [55]. Furthermore, mifepristone increased GR density in the amygdala and frontal cortex of rats, but reduced it in the hypothalamus [60]. Blocking GRs might also have altered the function of

mineralocorticoid receptors in the brain, which have a much higher affinity for glucocorticoids than GRs, and play an important role in the appreciation of stressors and orchestrating of stress responses [61]. The balance between the density of mineralocorticoid and glucocorticoid receptors ensures the dynamic function of the HPA axis [62]. Thus our mifepristone treatment may have affected several brain regions besides the hippocampus through different mechanisms, and these multiple effects may jointly have induced an improvement of social abilities in our fish.

The link between early environment, GR activity and social competence, as demonstrated by our manipulations, can have important consequences for stress responsiveness. Particularly in social, group-living species, it is crucial to respond quickly and flexibly to a variety of social challenges and opportunities by appropriate behavioural responses [8]. Our results suggest that this can be achieved by a short-term reduction of GR activity. On the other hand, animals with experimentally blocked GR activity failed to mount a full acute stress response [36,56], which in face of certain social stressors (e.g. parent-offspring interaction, social defeat, isolation) may also hamper their fitness [63]. This would be even more detrimental under conditions of prolonged chronic stress causing increased baseline glucocorticoid (GC) levels and a dampened acute stress response [64], which is why a long-term blocking of GR activity leading to a generally overreactive stress axis should not be expected to occur under natural conditions. In conclusion, our results indicate that the influence of early social environment on stress responsiveness and regulation of the GR pathway can have far reaching consequences influencing individual fitness and social dynamics in group-living animals.

Acknowledgements

We are grateful for advice on sample preparation by Sergio Cortez Ghio, statistical advice from Leif Engqvist and logistic support from Evi Zwygart and Danielle Bonfils.

Funding

This research was funded by the Swiss National Science Foundation (SNSF grant 31003A_156881 to BT), the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery program (to NAH) as well as by Ressources Aquatiques Quebec (RAQ) international fellowship program and Ella och Georg Ehrnrooths Stiftelse fund (to CN).

Author contributions

CN, BT and NAH designed the study. SF bred and reared the fish for experiment 1. CN bred and reared the fish for experiment 2. CN performed the behavioural experiments and pharmacological manipulations. CN and NAH did the gene expression laboratory work. CN and BT performed the statistical analysis. CN, BT and NAH drafted the manuscript. All authors have approved the content of the manuscript.

Research ethics

Experiment 1 and 2 were done at the 'Ethological Station Hasli' of the Institute of Ecology and Evolution (IEE), University of Bern, Switzerland, under licence number 52/12 of Veterinary Office of the Kanton Bern.

Data accessibility

Behavioural observation files and gene expression values have been deposited to Dryad, doi: doi:10.5061/dryad.47tc5.

Supplementary material

RNA extraction protocol, primer and qPCR information.

Competing interests

We declare no competing interests.

References

1. Champagne FA. 2010 Epigenetic influence of social experiences across the lifespan. *Dev. Psychobiol.* **52**, 299–311.
2. Branchi I, D'Andrea I, Fiore M, Di Fausto V, Aloe L, Alleva E. 2006 Early social enrichment shapes social behavior and nerve growth factor and brain-derived neurotrophic factor levels in the adult mouse brain. *Biol. Psychiatry* **60**, 690–6. (doi:10.1016/j.biopsych.2006.01.005)
3. Taborsky B, Arnold C, Junker J, Tschopp A. 2012 The early social environment affects social competence in a cooperative breeder. *Anim. Behav.* **83**, 1067–1074. (doi:10.1016/j.anbehav.2012.01.037)
4. Liu D *et al.* 1997 Maternal Care , Hippocampal Glucocorticoid Receptors , and Hypothalamic-Pituitary-Adrenal Responses to Stress. *Science (80-.).* **277**, 1659–1662.
5. Liu D, Diorio J, Day JC, Francis DD, Meaney MJ. 2000 Maternal care, hippocampal synaptogenesis and cognitive development in rats. *Nat. Neurosci.* **3**, 799–806.
6. Lindeyer CM, Meaney MJ, Reader SM. 2013 Early maternal care predicts reliance on social learning about food in adult rats. *Dev. Psychobiol.* **55**, 168–175.
7. Ruploh T, Bischof H-J, von Engelhardt N. 2013 Adolescent social environment shapes sexual and aggressive behaviour of adult male zebra finches (Taeniopygia guttata). *Behav. Ecol. Sociobiol.* **67**, 175–184. (doi:10.1007/s00265-012-1436-y)
8. Taborsky B, Oliveira RF. 2012 Social competence : an evolutionary approach. *Trends Ecol. Evol.* **27**, 679–688.
9. Taborsky B. 2016 Opening the black box of developmental experiments: behavioural mechanisms underlying long-term effects of early social experience. *Ethology* **122**, 267-283.
10. Oliveira RF. 2009 Social behavior in context : Hormonal modulation of behavioral plasticity and social competence. *Integr. Comp. Biol.* **49**, 423–440. (doi:10.1093/icb/icp055)
11. Caldji C, Tannenbaum B, Sharma S, Francis D, Plotsky PM, Meaney MJ. 1998 Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5335–5340. (doi:10.1073/pnas.95.9.5335)
12. Branchi I, Cirulli F. 2014 Early experiences: Building up the tools to face the challenges of adult life. *Dev. Psychobiol.* **56**, 1661–1674. (doi:10.1002/dev.21235)
13. Francis D, Diorio J, Liu D, Meaney MJ. 1999 Nongenomic transmission across generations of maternal behavior and stress responses in the rat. *Science* **286**, 1155–1158. (doi:10.1126/science.286.5442.1155)
14. de Kloet ER. 1991 Brain corticosteroid receptor balance and homeostatic control. *Front. Neuroendocrinol.* **12**, 95–164.
15. Weaver ICG, D'Alessio AC, Brown SE, Hellstrom IC, Dymov S, Sharma S, Szyf M, Meaney MJ. 2007 The transcription factor nerve growth factor-inducible protein a mediates epigenetic programming: altering epigenetic marks by immediate-early genes. *J. Neurosci.* **27**, 1756–68. (doi:10.1523/JNEUROSCI.4164-06.2007)
16. Weaver ICG, Hellstrom IC, Brown SE, Andrews SD, Dymov S, Diorio J, Zhang T,

Szyf M, Meaney MJ. 2014 The methylated-DNA binding protein transcriptional activation of the glucocorticoid receptor. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **369**, 1–11. (doi:20130513)

17. Desjardins JK, Fernald RD. 2010 What do fish make of mirror images? *Biol. Lett.* **6**, 744–7. (doi:10.1098/rsbl.2010.0247)

18. Morgan JI, Curran T. 1995 Review : The Immediate-Early Gene Response and Neuronal Death and Regeneration. *Neurosci.* **1**, 68–75. (doi:10.1177/107385849500100203)

19. Wingfield JC, Sapolsky RM. 2003 Reproduction and resistance to stress: when and how. *J. Neuroendocrinol.* **15**, 711–724.

20. Steenbergen PJ, Richardson MK, Champagne DL. 2011 The use of the zebrafish model in stress research. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **35**, 1432–1451. (doi:10.1016/j.pnpbp.2010.10.010)

21. Banerjee SB, Arterbery AS, Fergus DJ, Adkins-Regan E. 2012 Deprivation of maternal care has long-lasting consequences for the hypothalamic-pituitary-adrenal axis of zebra finches. *Proc. Biol. Sci.* **279**, 759–66. (doi:10.1098/rspb.2011.1265)

22. Zimmer C, Spencer KA. 2014 Modifications of glucocorticoid receptors mRNA expression in the hypothalamic-pituitary-adrenal axis in response to early-life stress in female japanese quail. *J. Neuroendocrinol.* **26**, 853–860. (doi:10.1111/jne.12228)

23. Nyman C, Fischer S, Aubin-Horth N, Taborsky B. 2017 Effect of the early social environment on behavioural and genomic responses to a social challenge in a cooperatively breeding vertebrate. *Mol. Ecol.* **26**, 3186-3203

24. Taborsky B, Tschirren L, Meunier C, Aubin-Horth N. 2013 Stable reprogramming of brain transcription profiles by the early social environment in a cooperatively breeding fish Stable reprogramming of brain transcription profiles by the early social environment in a cooperatively breeding fish. *Proc. Biol. Sci.* **208**, 1–7.

25. Arterbery AS, Fergus DJ, Fogarty EA, Mayberry J, Deitcher DL, Kraus WL, Bass AH. 2011 Evolution of ligand specificity in vertebrate corticosteroid receptors. *BMC Evol Biol* **11**, 1–15.

26. Arnold C, Taborsky B. 2010 Social experience in early ontogeny has lasting effects on social skills in cooperatively breeding cichlids. *Anim. Behav.* **79**, 621–630. (doi:10.1016/j.anbehav.2009.12.008)

27. Rimmele U, Besedovsky L, Lange T, Born J. 2013 Blocking mineralocorticoid receptors impairs, blocking glucocorticoid receptors enhances memory retrieval in humans. *Neuropsychopharmacology* **38**, 884–94. (doi:10.1038/npp.2012.254)

28. Fischer S, Bessert-Nettelbeck M, Kotrschal A, Taborsky B. 2015 Rearing-Group Size Determines Social Competence and Brain Structure in a Cooperatively Breeding Cichlid. *Am. Nat.* **186**, 123–40.

29. O'Connell LA, Hofmann HA. 2012 Evolution of a vertebrate social decision-making network. *Science* **336**, 1154–1157.

30. O'Connell L a, Hofmann H a. 2011 The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *J. Comp. Neurol.* **519**, 3599–639. (doi:10.1002/cne.22735)

31. Wendelaar Bonga SE. 1997 The Stress Response in Fish. *Physiol. Rev.* **77**, 591–625.

32. Champagne FA. 2010 Early Adversity and Developmental Outcomes: Interaction

Between Genetics, Epigenetics, and Social Experiences Across the Life Span. *Perspect. Psychol. Sci.* **5**, 564–574.

33. Schjolden J, Basic D, Winberg S. 2009 Aggression in rainbow trout is inhibited by both MR and GR antagonists. *Physiol. Behav.* **98**, 625–630. (doi:10.1016/j.physbeh.2009.09.018)

34. Wong EYH, Herbert J. 2005 Roles of mineralocorticoid and glucocorticoid receptors in the regulation of progenitor proliferation in the adult hippocampus. *Eur. J. Neurosci.* **22**, 785–792. (doi:10.1111/j.1460-9568.2005.04277.x.Roles)

35. Bernier NJ, Lin X, Peter RE. 1999 Differential expression of corticotropin-releasing factor (CRF) and urotensin I precursor genes, and evidence of CRF gene expression regulated by cortisol in goldfish brain. *Gen. Comp. Endocrinol.* **116**, 461–77. (doi:10.1006/gcen.1999.7386)

36. Alderman SL, McGuire A, Bernier NJ, Vijayan MM. 2012 Central and peripheral glucocorticoid receptors are involved in the plasma cortisol response to an acute stressor in rainbow trout. *Gen. Comp. Endocrinol.* **176**, 79–85. (doi:10.1016/j.ygcen.2011.12.031)

37. Miyagawa S, Lange A, Tohyama S, Ogino Y. 2014 Characterization of Oryzias latipes glucocorticoid receptors and their unique response to progestins. *J. Appl. Toxicol.* **35**, 302–309. (doi:10.1002/jat.3020)

38. Ros AFH, Vullioud P, Bshary R. 2012 Treatment with the glucocorticoid antagonist RU486 reduces cooperative cleaning visits of a common reef fish, the lined bristletooth. *Horm. Behav.* **61**, 37–43. (doi:10.1016/j.yhbeh.2011.09.013)

39. Kasper C, Kölliker M, Postma E, Taborsky B. 2017 Consistent cooperation in a cichlid fish is caused by maternal and developmental effects rather than heritable genetic variation. *Proc. R. Soc. B Biol. Sci.* (doi:10.1098/rspb.2017.0369)

40. Dey CJ, Reddon AR, O'Connor CM, Balshine S. 2013 Network structure is related to social conflict in a cooperatively breeding fish. *Anim. Behav.* **85**, 395–402. (doi:10.1016/j.anbehav.2012.11.012)

41. Taborsky M. 1984 Broodcare helpers in the cichlid fish Lamprologus Brichardi: their costs and benefits. *Anim. Behav.* **32**, 1236–1252.

42. Fischer S, Bohn L, Oberhummer E, Nyman C, Taborsky B. 2017 Divergence of developmental trajectories is triggered interactively by early social and ecological experience in a cooperative breeder. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E9300–E9307.

43. Veillette PA, Serrano X, Garcia MM, Specker JL. 2007 Evidence for the onset of feedback regulation of cortisol in larval summer flounder. *Gen. Comp. Endocrinol.* **154**, 105–110. (doi:10.1016/j.ygcen.2007.05.033)

44. Exelgyn. 2015 'Mifegyne Summary of Product Characteristics (SPC)' (PDF). London: Medicines and Healthcare Products Regulatory Agency (MHRA).

45. Singmann H, Bolker B, Westfall J. 2015 Analysis of Factorial Experiments, package 'afex'. , 1–44.

46. Benjamini Y, Hochberg Y. 1995 Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B* **57**, 289–300.

47. Navailles S, Zimnisky R, Schmauss C. 2010 Expression of glucocorticoid receptor and early growth response gene 1 during postnatal development of two inbred strains of mice exposed to early life stress. *Dev. Neurosci.* **32**, 139–48. (doi:10.1159/000293989)

48. Holmes MC, Seckl JR. 2006 The role of 11beta-hydroxysteroid dehydrogenases in the brain. *Mol. Cell. Endocrinol.* **248**, 9–14. (doi:10.1016/j.mce.2005.12.002)

49. Weaver ICG, Cervoni N, Champagne FA, Alessio ACD, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ. 2004 Epigenetic programming by maternal behavior. *Nat. Neurosci.* **7**, 847–854. (doi:10.1038/nn1276)

50. Proszkowiec-Weglarz M, Porter TE. 2010 Functional characterization of chicken glucocorticoid and mineralocorticoid receptors. *Am J Physiol Regul Integr Comp Physiol* **298**, 1257–1268. (doi:10.1152/ajpregu.00805.2009.)

51. James AB, Conway A, Morris BJ. 2005 Genomic profiling of the neuronal target genes of the plasticity-related transcription factor – Zif268. *J. Neurochem.* **95**, 796–810. (doi:10.1111/j.1471-4159.2005.03400.x)

52. Hellstrom IC, Dhir SK, Diorio JC, Meaney MJ. 2012 Maternal licking regulates hippocampal glucocorticoid receptor transcription through a thyroid hormone-serotonin-NGFI-A signalling cascade. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **367**, 2495–510. (doi:10.1098/rstb.2012.0223)

53. Meaney MJ, Diorio J, Francis D, Weaver S, Yau J, Chapman K, Seckl JR. 2000 Postnatal Handling Increases the Expression of cAMP-Inducible Transcription Factors in the Rat Hippocampus : The Effects of Thyroid Hormones and Serotonin. *J. Neurosci.* **20**, 3926–3935.

54. Meaney MJ, Szyf M. 2005 Maternal care as a model for experience-dependent chromatin plasticity? *Trends Neurosci.* **28**, 456–463.

55. Wulsin AC, Herman JP, Solomon MB. 2010 Mifepristone decreases depression-like behavior and modulates neuroendocrine and central hypothalamic — pituitary — adrenocortical axis responsiveness to stress. *Psychoneuroendocrinology* **35**, 1100–1112. (doi:10.1016/j.psyneuen.2010.01.011)

56. Zalachoras I, Houtman R, Atucha E, Devos R, Tijssen AMI, Hu P, Lockey PM. 2013 Differential targeting of brain stress circuits with a selective glucocorticoid receptor modulator. *PNAS* **110**. (doi:10.1073/pnas.1219411110/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1219411110)

57. Bannier F, Tebbich S, Taborsky B. 2017 Early experience affects learning performance and neophobia in a cooperatively breeding cichlid. *Ethology* **123**, 712–723.

58. Bury NR *et al.* 2003 Evidence for two distinct functional glucocorticoid receptors in teleost fish. *J. Mol. Endocrinol.* **31**, 141–156.

59. Krugers HJ, Goltstein PM, Linden S Van Der, Joe M. 2006 Blockade of glucocorticoid receptors rapidly restores hippocampal CA1 synaptic plasticity after exposure to chronic stress. *Eur. J. Neurosci.* **23**, 3051–3055. (doi:10.1111/j.1460-9568.2006.04842.x)

60. Bachmann CG, Linthorst ACE, Holsboer F, Reul JMHM. 2003 Effect of chronic administration of selective glucocorticoid receptor antagonists on the rat hypothalamic – pituitary – adrenocortical axis. *Neuropsychopharmacology* **28**, 1056–1067. (doi:10.1038/sj.npp.1300158)

61. Joëls M, Baram TZ. 2009 The neuro-symphony of stress. *Nat. Rev. Neurosci.* **10**, 459–466.

62. Joëls M, Karst H, DeRijk R, de Kloet ER. 2008 The coming out of the brain mineralocorticoid receptor. *Trends Neurosci.* **31**, 1–7. (doi:10.1016/j.tins.2007.10.005)

63. Goymann W, Wingfield JC. 2004 Allostatic load, social status and stress hormones:

The costs of social status matter. *Anim. Behav.* **67**, 591–602.

64. Wingfield JC, Maney DL, Breuner CW, Jacobs JD, Lynn S, Ramenofsky M, Richardson RD. 1998 Ecological Bases of Hormone-Behavior Interactions : The ‘ Emergency Life History Stage ’ 1. **38**, 191–206.

Table 1: Results of Experiment 2. Linear mixed models testing the effect of treatment (mifepristone or blank) on submissive displays and total aggression by intruders (restrained and overt), and on overt aggression received by intruders from owners. Received overt aggression was also included as covariate in the LMM on submissive displays. P-values <0.05 are highlighted in bold.

Factors	Estimate±SE	F	p	N
Submissive displays:				37
Treatment	0.111±0.045	5.672	**0.028**	
Received overt aggression	-0.014±0.011	1.362	0.252	
Received overt aggression x Treatment	0.031±0.009	9.900	**0.005**	
Intruder total aggression:				37
Treatment	0.012±0.021	0.316	0.581	
Received overt aggression:				37
Treatment	-0.021±0.008	7.369	**0.014**	

Figure legends

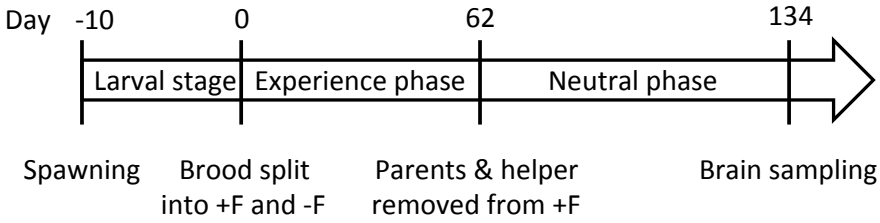
Figure 1: Timeline of (A) experiment 1 and (B) experiment 2. Both experiment started when brood was free swimming (day 0), 10 days after spawning. In experiment 1, half of the clutch was reared for 62 days with parents a helper and same aged siblings (+F), and the other half of the clutch was reared with same aged siblings only (−F; 'experience phase'). During the following 'neutral phase' (72 days) all fish were kept only with siblings. In experiment 2, all clutches were reared with parents for 62 days ('rearing phase'). Immersions in either mifepristone or control solution (in balanced order) started on days 95 and 105 and lasted for 2 days. The social challenges (contest over shelter) started 2 hours after the end of the immersions, on days 97 and 107.

Figure 2: (A) Brain gene expression of *gr1* in telencephalon and hypothalamus; means±SE are shown; asterisk indicates significant difference. (B) *Egr-1* expression as a predictor of *gr1* expression in two brain areas, telencephalon (open triangles, grey line) and the hypothalamus (filled rectangles, black line).

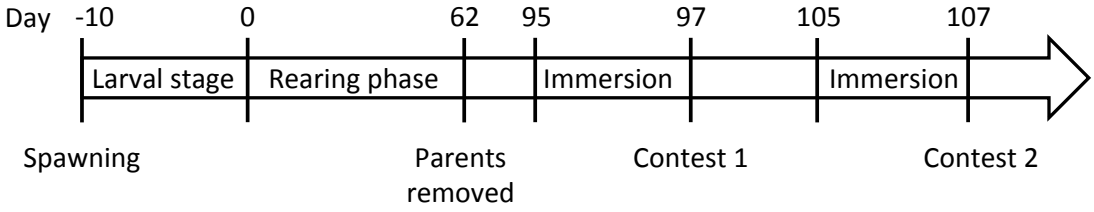
Figure 3: (A) Rate of intruder submission (min^{-1}) relative to the overt aggression received from the initial shelter owner (min^{-1}). (B) Rate of overt aggression the intruder fish received from the initial shelter owner (min^{-1}). Filled circles and oblique line represent mifepristone (mif) treatment; open circles and horizontal line represent control (con) treatment. Figures display means±SE. Asterisk indicates significant difference.

Figure 4: Number of fish in the control (con) and mifepristone (mif) treatment either winning (grey bars) or losing (black bars) the interaction in the social challenge test and winning/losing the access to the shelter.

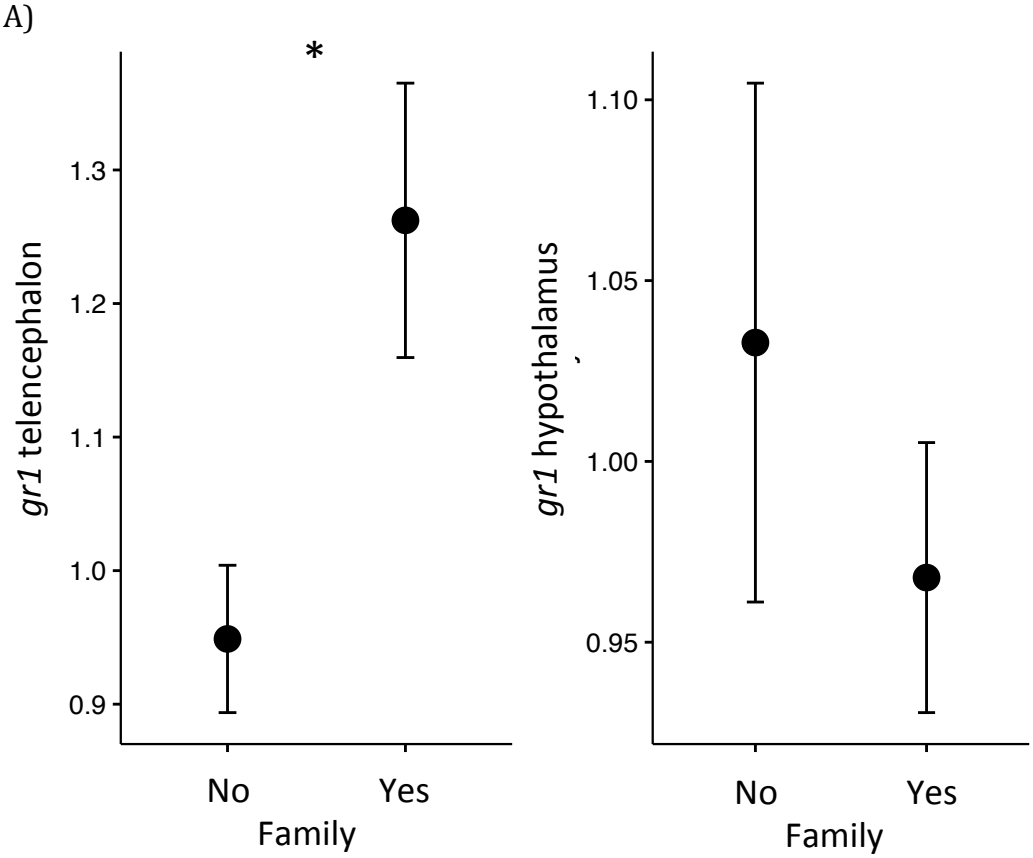
A)


Day
-10
0
62
134
Larval stage
Experience phase
Neutral phase
Spawning
Brood split into +F and -F
Parents & helper removed from +F
Brain sampling

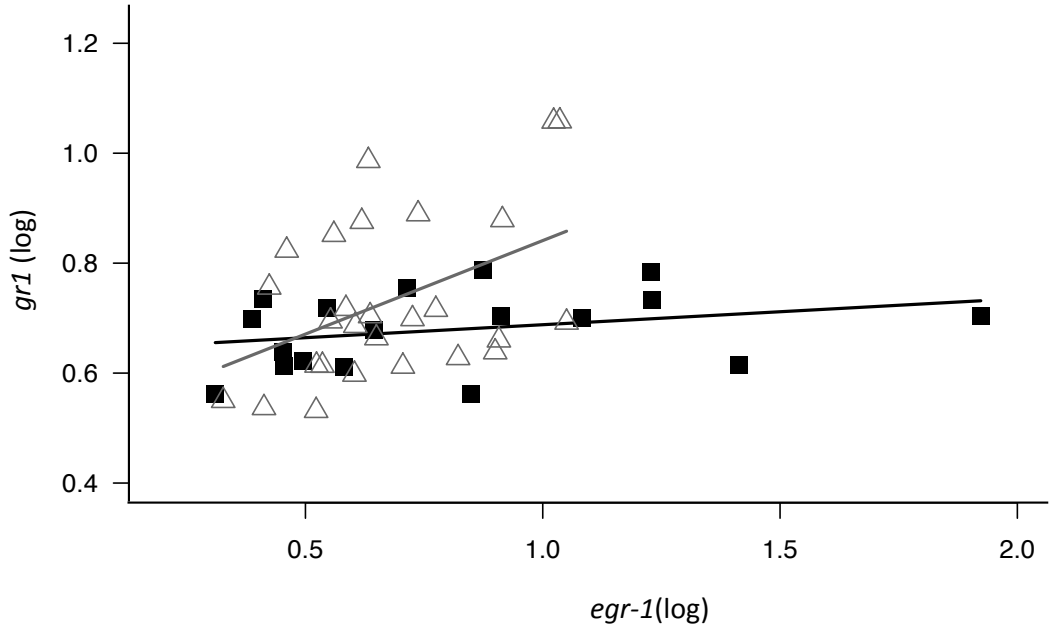
B)


Day
-10
0
62
95
97
105
107
Larval stage
Rearing phase
Immersion
Immersion
Spawning
Parents removed
Contest 1
Contest 2

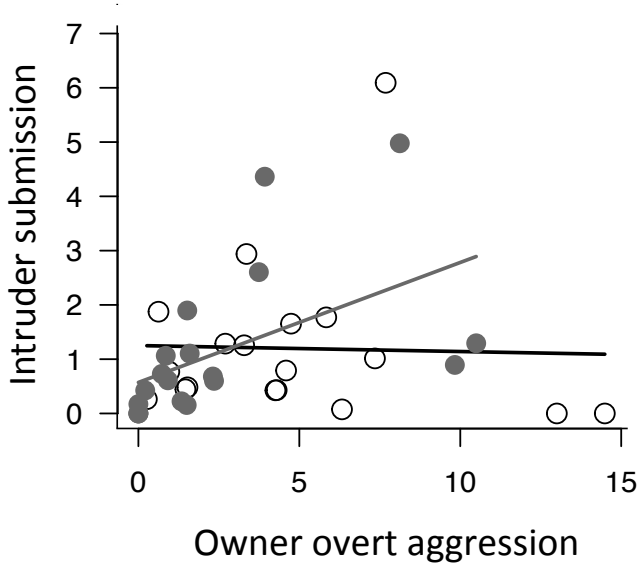
A)


*
gr1 telencephalon
1.3
1.2
1.1
1.0
0.9
No
Yes
Family
gr1 hypothalamus
1.10
1.05
1.00
0.95
No
Yes
Family

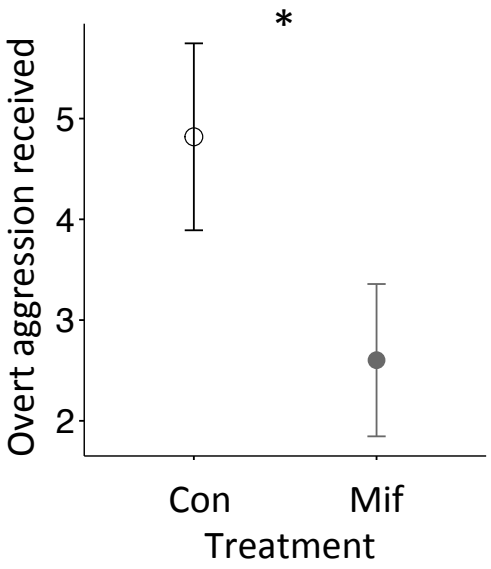
B)

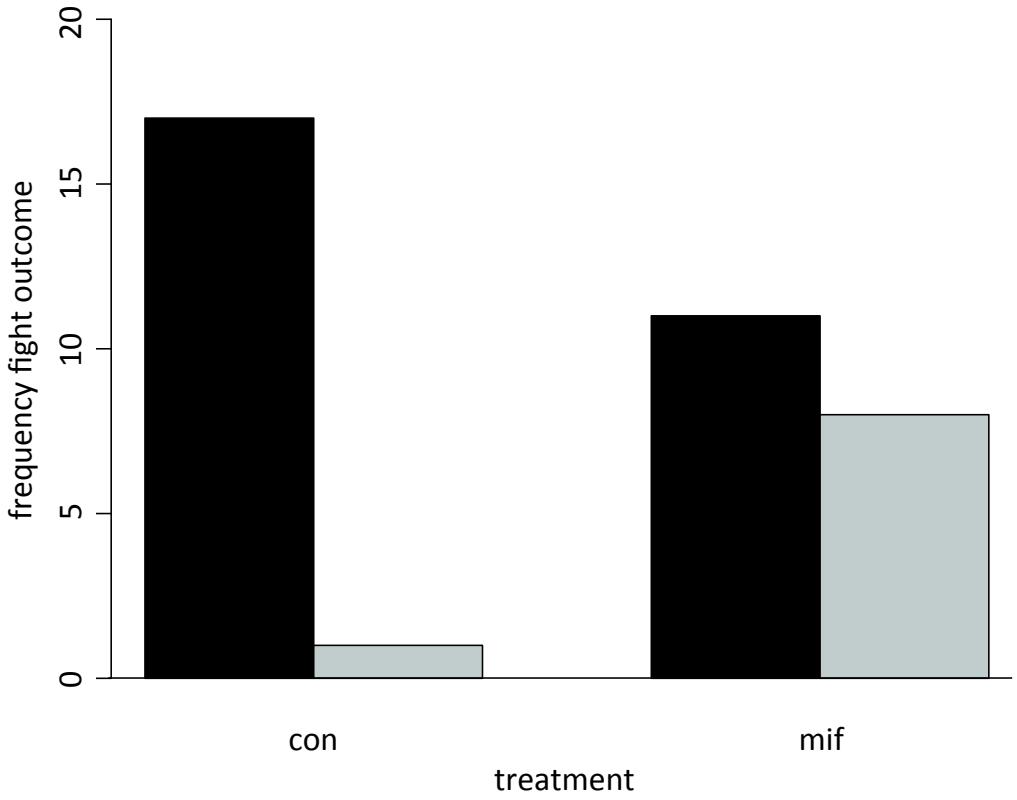

gr1 (log)
1.2
1.0
0.8
0.6
0.4
0.5
1.0
1.5
2.0
egr-1(log)

A)


Intruder submission
0
1
2
3
4
5
6
7
0
5
10
15
Owner overt aggression

B)


*
Overt aggression received
2
3
4
5
Con
Mif
Treatment


frequency fight outcome
0
5
10
15
20
con
mif
treatment

Evolutionary conserved neural signature of early life stress affects animal social competence

Cecilia Nyman[1], Stefan Fischer[1,2], Nadia-Aubin-Horth[3] & Barbara Taborsky[1]

[1]Div. of Behavioural Ecology, Institute of Ecology and Evolution, University of Bern, Switzerland

[2]Institute of Integrative Biology, University of Liverpool, Liverpool L69 7ZB, UK

[3]Département de Biologie and Institut de Biologie Intégrative et des Systèmes, Université Laval, Quebec, Canada

Proceedings of the Royal Society B

Supplementary Information (SI)

Sample preparation and qPCR

Primers for qPCR

We measured the gene expression of *egr-1* and *gr1* in the hypothalamus and in the telencephalon of *N. pulcher*. We used the gene 18S as a control or ' housekeeping' gene. Primers for *gr1* were the same as used in [1], while 18S primers were the same as used in [2]. Primers for *egr-1* were designed from the genome of *N. brichardi* (http://cichlid.umd.edu/cichlidlabs/kocherlab/bouillabase.html) using the *A. burtoni* sequence as a search template (NCBI database ID number: AY493348.1, for-CGGCGATATATCCTAAAATC; rev-TCCCATGCCTATAAACACT).

Sample preparation

RNA extraction was done from hypothalamus and telencephalon, for each brain part separately, using the Qiagen miRNeasy micro kit. We chose this kit, because the samples were very small and yielded low amounts of RNA. The protocol was modified to avoid sampling miRNAs. To make sure to get an end product free of DNA we carried out an DNAse treatment. In brief, we placed the brain tissue in a 1.5 ml Eppendorf tube with 200 µl of QIAzol lysis reagent (Qiagen), homogenized it and added further 500 µl of QIAzol lysis reagent. After that we placed the tube on the benchtop at room temperature (RT) for 5 min, after which we added 140 µl of chloroform, we shook the sample for 1 minute, and left it for 15 min (RT) and centrifuged it at 12 000 g at 4ºC for 20 min. After centrifugation we moved

the upper aqueous phase to a new clean Eppendorf tube, added 140 µl chloroform, shook the sample for 1 minute and centrifuged it at 12 000 g at 4ºC for 20 min for a second phase separation step. After centrifugation we moved again the upper aqueous phase to a new clean Eppendorf tube, added 250 µl of 70% Ethanol, mixed the sample thoroughly, and pipetted the whole sample onto a RNeasy MinElute spin column (SC, Qiagen). Then we centrifuged the SC at 10 000 rpm for 30 s (RT) and discarded the flow-through. After this we carried out an DNAse treatment by pipetting 350 µl of buffer RWT (prepared with isopropanol, Qiagen) onto the spin column, centrifuged the column (10 000 rpm, 30 s, RT), discarded the flow-through, pipetted 80 µl of DNase I incubation mix (10 µl DNase stock solution + 70 µl Buffer RDD, Qiagen) onto the SC and left the tube at RT for 15 min. After the DNAse treatment we pipetted 500 µl of Buffer RWT onto the SC, centrifuged it (10 000 rpm, 30 s, RT), reapplied the flow-through onto the SC, centrifuged the SC (10 000 rpm, 30 s, RT) and discarded the flow-through. Then we pipetted 500 µl buffer RPE (Qiagen) onto the SC, centrifuged the SC (10 000 rpm, 30 s, RT) and discarded the flow-through. To collect total RNA instead of miRNA we pipetted 500 µl of 80% Ethanol onto the SC, left the tube 5 min at RT and then centrifuged the SC (10 000 rpm, 30 s, RT) and discarded the flow-through. We repeated this step once. After the ethanol wash we placed the SC into a new 2 ml collection tube, centrifuged it (15 000 rpm, 5 min, RT) with the lid open, removed the SC and placed in into a final 1.5 ml collection tube, added 14 µl of RNase free water (Qiagen) to the SC and centrifuged the SC (15 000 rpm, 1 min, RT) to elute the RNA. At the end we checked the RNA concentration and sample composition with a Nanodrop microvolume spectrophotometer.

We carried out reverse transcription using the same amount of RNA from each sample (200 ng RNA from hypothalamus and 304 ng RNA from telencephalon) using a standard Superscript protocol (Invitrogen). To confirm the expression of each gene and success of RT, we used a small amount of cDNA from random samples form both treatments in a PCR using both genes and visualised it using an electrophoretic gel. To determine amplification efficiency, the absence of primer dimmers and the specificity of amplification for each primer pair, we run qPCR experiments and melting curves (50 to 90 Celsius) using standard curves consisting of 5 x 10-fold dilutions (of pooled samples) in duplicates (Aubin-Horth *et al.*, 2012). We prepared the primers (Eurofins) and 5 µl of sample cDNA on a 384-well plate (axigen) by using an epmotion liquid handler (Eppendorf) which is used for quantitative real-time PCR experiments following the scaled-down version of the Quantitect SYBRGreen PCR

kit manufacturer's protocol (Qiagen) using a 384-well plate qRT-PCR machine (Light Cycler, Roche). We run each sample (from each individual) for hypothalamus and telencephalon in triplicate for a given gene together with no primers and no template controls. To verify that only a single amplified product was present and that no primer dimers were produced, we also performed a melting curve on each replicate. We calculated the coefficient of variation (CV) of the three replicates and use a CV cut-off of 5% for all genes. The CV value is used for checking the repeatability and precision of the qPCR assay. Using the expression of a control gene (18S) [3], we calculated the relative gene expression for each individual / brain region combination.

References

1. Taborsky B, Tschirren L, Meunier C, Aubin-Horth N. 2013 Stable reprogramming of brain transcription profiles by the early social environment in a cooperatively breeding fish Stable reprogramming of brain transcription profiles by the early social environment in a cooperatively breeding fish. *Proc. Biol. Sci.* **208**, 1–7.
2. O'Connor CM, Marsh-rollo SE, Ghio SC, Balshine S, Aubin-horth N. 2015 Is there convergence in the molecular pathways underlying the repeated evolution of sociality in African cichlids? *Horm. Behav.* **75**, 160–168. (doi:10.1016/j.yhbeh.2015.07.008)
3. Pfaffl MW. 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45. (doi:10.1093/nar/29.9.e45)