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Effect of the early social environment on behavioural and genomic responses to a social challenge in a cooperatively breeding vertebrate

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Abstract

The early social environment can have substantial, lifelong effects on vertebrate social behaviour, which can be mediated by developmental plasticity of brain gene expression. Early-life effects can influence immediate behavioural responses towards later-life social challenges and can activate different gene expression responses. However, while genomic responses to social challenges have been reported frequently, how developmental experience influences the shape of these genomic reaction norms remains largely unexplored. We tested how manipulating the early social environment of juvenile cooperatively breeding cichlids, *Neolamprologus pulcher*, affects their behavioural and brain genomic responses when competing over a resource. Juveniles were reared either with or without a breeder pair and a helper. Fish reared with family members behaved more appropriately in the competition than when reared without. We investigated whether the different social rearing environments also affected the genomic responses to the social challenge. A set of candidate genes, coding for hormones and receptors influencing social behaviour, were measured in the telencephalon and hypothalamus. Social environment and social challenge both influenced gene expression of *egr-1* (early growth response 1) and *gr1* (glucocorticoid receptor 1) in the telencephalon and of *bdnf* (brain-derived neurotrophic factor) in the hypothalamus. A global analysis of the 11 expression patterns in the two brain areas showed that neurogenomic states diverged more strongly between intruder fish and control fish when they had been reared in a natural social setting. Our results show that same molecular pathways may be used differently in response to a social challenge depending on early-life experiences.

Keywords: behavioural flexibility, brain gene expression, cooperative breeder, developmental plasticity, early social environment, genomic reaction norm, neurogenomic state, social challenge, social competence

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Introduction

The early social environment can have important and persisting effects on the development of an animal's emotional (reviewed in Champagne 2010) and behavioural phenotype (reviewed in Kasumovic & Brooks 2011). Long-term effects of the early social environment have been reported in all vertebrate classes (mammals: e.g. Harlow & Zimmermann 1959; Mireault & Bond 1992; Liu *et al.* 1997; Bastian *et al.* 2003; Branchi & Alleva 2006; birds: Adkins-Regan & Krakauer 2000; Ruploh *et al.* 2013, 2014; Schmidt *et al.* 2014; reptiles: Ballen *et al.* 2014; amphibians: Nicieza & Metcalfe 1999;

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fish: e.g. Arnold & Taborsky 2010; Taborsky *et al.* 2012). The social conditions experienced early in life can affect a remarkably broad array of traits including life history traits and reproductive schedules (Kasumovic & Brooks 2011), coloration (Ballen *et al.* 2014) or learning and memory (Liu *et al.* 2000), but most often it affects behaviours in the social domain (reviewed in Taborsky 2016a). For instance, variation in the amount of received maternal care can affect maternal care behaviour of the next generation (Liu *et al.* 1997; Francis *et al.* 1999) or the ability to use social information in effective hierarchy formation (Branchi *et al.* 2006). The sex composition of littermates or social groups during rearing can affect later mate choice decisions (Adkins-Regan & Krakauer 2000) or aggressive tendencies (Benus & Henkelmann 1998).

Lasting effects induced by the early social environment on social behaviours are thought to result from developmental plasticity in the brain (e.g. Fischer *et al.* 2015) and can be mediated by organizational effects of hormones or epigenetic modifications. Organizational effects of the hormonal system (Phoenix *et al.* 1959; Soares *et al.* 2010) impact the neural structural level, are slow and involve mechanisms such as neurogenesis, apoptosis and synaptic plasticity (reviewed in Soares *et al.* 2010). Organizational effects are considered nonreversible and they usually affect a phenotype during specific sensitive periods of development, for example in the perinatal period or during puberty (Rice & Barone 2000; Romeo 2003). Furthermore, early adversity can result in socially driven epigenetic modifications (Champagne 2008). These lasting effects can often be measured by persistent alterations of gene expression profiles in different brain areas, including effects on hormonal ligands and receptors related to the stress response and social recognition [e.g. corticosteroids, serum oxytocin and oxytocin and estrogen receptors (Zimmer *et al.* 2013; Cao *et al.* 2014); glucocorticoid receptors (*gr*, *gr1*, Zimmer & Spencer 2014); and corticotropin-releasing factor (*crf*) (Liu *et al.* 1997; McGowan *et al.* 2009; Banerjee *et al.* 2012; Taborsky *et al.* 2013)]. The early social environment might also have long-lasting consequences for the individual by influencing and modulating neuronal plasticity of the brain and related gene expression pathways [brain-derived neurotrophic factor (*bdnf*) and nerve growth factor (*ngf*) (Zhang *et al.* 2002; Roceri *et al.* 2004)].

Behavioural flexibility, a form of plasticity that should be distinguished from developmental plasticity, is expressed as a response to an environmental trigger and is immediate and reversible (Taborsky & Oliveira 2012). For example, in the social domain, individuals perceive and use social information to flexibly adjust their behaviour to the present social context ('social competence', Taborsky & Oliveira 2012). Behavioural flexibility is mediated in part by the activational effects of the hormonal system (Soares *et al.* 2010). Activational effects work at the functional level by changing the activity of neural circuits and are rapid and transient. Social challenges and opportunities can activate different patterns of gene expression in specific brain areas, which can be measured as genomic reaction norms (Aubin-Horth & Renn 2009). For example, when previously subordinate cichlid fish, *Astatotilapia burtoni*, change their social rank, changes in behaviour and coloration are accompanied by an activation of different brain areas through expression of the immediate early genes (IEGs) *egr-1* and *c-fos* (Burmeister *et al.* 2005; Maruska *et al.* 2013) and changed expression of genes coding for hormones and their receptors in different brain areas associated with social behaviour (Huffman *et al.* 2012a, 2015).

We predict that developmental plasticity and behavioural flexibility will jointly shape social behaviour, resulting in different slopes of behavioural reaction norms based on social rearing conditions (e.g. Dettling *et al.* 2002). This means that the shape of an immediate behavioural response towards a social challenge (e.g. the slope between baseline and challenged condition) would differ depending on the early-rearing environment. For example, rhesus monkeys separated from their mothers early in life respond to peer presence with much lower frequencies of affiliation behaviour than do mother-reared peers, even after years of living in normal social conditions (Feng *et al.* 2011). This difference in short-term behavioural response of individuals that experienced divergent rearing environments should correspond to changes in components of the underlying control mechanisms, in particular long-term and short-term alterations of gene expression. At the molecular level, this can best be studied by measuring brain genomic reaction norms in response to an environmental challenge (behavioural flexibility) of individuals reared in different environmental conditions (developmental plasticity). With genomic reaction norms, we measure how an individual of a particular phenotype responds to a specific situation at the gene expression level, within a specific tissue, brain area or cell type, depending on the question asked. As a hypothetical example, an individual that experienced benign early-life conditions might respond by high brain glucocorticoid receptor (*gr*) expression towards a social stimulus, whereas an individual that grew up under adverse conditions may mount a much smaller *gr* response (or might not respond at all). Individuals reared in socially more complex early environments generally behave more socially competent in a range of different social challenges compared to when reared in

more simple environments (reviewed in Taborsky 2016a). Furthermore, to capture the change in the overall pattern of expression after a social challenge in individuals from the two contrasting early-rearing environments, a neurogenomic state can be defined using the expression of all genes in all surveyed brain regions at once (Robinson *et al.* 2008). Such differences in molecular responses to a behavioural challenge between individuals that faced different early social environments have so far been only demonstrated in laboratory strains of rodents (measured at the mRNA or protein level, Plotsky & Meaney 1993; Wigger & Neumann 1999; Ago *et al.* 2013). For example, male mice reared in isolation show higher *c-fos* protein levels in the cortex when faced with a social challenge than group-reared males (Ago *et al.* 2013). There is, however, no published explicit test of the effect of the early-rearing environment on gene expression levels in response to a short-term challenge.

Finally, the consistent finding that variation in the early social environment of animals results in different behavioural responses to social challenges and opportunities (Taborsky 2016a) gives rise to the question whether changes in behaviour relate to changes in gene expression patterns. Testing such a relationship is an important first attempt to decipher the functional significance of this variation at the gene expression level (Williams 2008). For instance, Cummings *et al.* 2008 show that specific genes are turned on in the females swordtail fish, *Xiphophorus nigrensis*, interacting with attractive males but then turned off when interacting with other females. Further aggressive behaviour in threespine stickleback, *Gasterosteus aculeatus*, was shown to be positively correlated with gene expression of glucocorticoid receptors (Aubin-Horth *et al.* 2012). However, whether one always expects a linear relationship between a phenotype and the underlying endocrine pathways, or whether individuals from different context (age, sex, status, environment) should show the same relationship is less certain (Williams 2008).

To understand how brain genomic reaction norms have evolved in the social domain under natural conditions when confronted with biologically relevant challenges, we need information from a broader array of taxonomic groups and, in particular, also from natural study organisms (as opposed to organisms artificially selected for a certain purpose), because they can be expected to display naturally evolved reaction norms (Groothuis & Taborsky 2015). Here, we chose a highly social fish species as study system, the cooperatively breeding cichlid *Neolamprologus pulcher*. This species, which has become a key organism for the study of vertebrate social evolution (e.g. Wong & Balshine 2011; Taborsky 2016b), is now also studied within an ecological genomics framework (Aubin-Horth *et al.* 2007; Taborsky *et al.* 2013; Brawand *et al.* 2014; O'Connor *et al.* 2015, 2016; Reddon *et al.* 2015). We investigated the association between behavioural and genomic reaction norms in this species by comparing the response to a social challenge (a contest over a resource) of individuals whose early-rearing environment differed in levels of social complexity. As previous experiments showed that *N. pulcher* reared in different social environments display altered behavioural responses to social challenges (Arnold & Taborsky 2010; Taborsky *et al.* 2012), we predicted that social rearing and social challenge would jointly influence genomic reaction norms in the brain of these fish.

We aimed to answer two questions: (i) How do genomic reaction norms measured in fish exposed to a social challenge or a control situation differ between fish reared in different social environments? (ii) Is the observed behaviour and the early social environment related to the genomic response? To answer the first question, we measured gene expression in the telencephalon and hypothalamus of socially challenged and control fish. These two brain areas play a key role in social behaviour and decision-making in fish (O'Connell & Hofmann 2011) and in their hypothalamic–pituitary–interrenal (HPI) stress axis. The HPI is homologous to the mammalian hypothalamic–pituitary–adrenal (HPA) stress axis, which has been shown to be strongly impacted by the early social environment across different vertebrate classes (Meaney & Szyf 2005; Banerjee *et al.* 2012; Taborsky *et al.* 2013). In the telencephalon, we measured expression of *egr-1*, *bdnf*, *gr1*, *crf* and *neuroserpin*, and in the hypothalamus the expression of *egr-1*, *bdnf*, *gr1*, *crf*, *avt* and its V1a2 receptor (*avtr*). The product of these genes are known to be involved in the modulation of social behaviour or social dominance relationships and/or to be affected by early social experience in vertebrates (Liu *et al.* 1997; Young *et al.* 1999; Zhang *et al.* 2002; Madani *et al.* 2003; Burmeister *et al.* 2005; Branchi *et al.* 2006; Aubin-Horth *et al.* 2007). To answer our second question, we analysed the relationship between social behaviours expressed during the social challenge and gene expression.

Methods

Study species

Neolamprologus pulcher is a cooperatively breeding cichlid endemic to Lake Tanganyika, East Africa. It lives in large family units of up to 25 fish consisting of a dominant breeder pair, one or several related or unrelated helpers and fry from recent broods. Subordinates provide help in form of direct brood care of the dominants'

offspring and of territory defence and maintenance. In turn, they remain accepted by dominants at a territory, at which they have access to critical resources ('pay-to-stay'; Taborsky 1985; Balshine-Earn *et al.* 1998; Bergmüller & Taborsky 2005; Stiver *et al.* 2005; Heg & Taborsky 2010; Zöttl *et al.* 2013b; Fischer *et al.* 2014). By being accepted at a territory, helpers benefit particularly from protection from predators and access to high-quality shelters (Balshine-Earn *et al.* 1998; Heg *et al.* 2004), and they might eventually get a chance to inherit a breeder position (Stiver *et al.* 2004). *N. pulcher* groups are organized in size-based linear hierarchies (Dey *et al.* 2013) and the fish have a large, fine-scaled repertoire of social behaviours to establish and maintain these hierarchies (Taborsky 1984). Higher ranking fish show an array of open and restrained aggressive displays towards lower ranking fish, which in turn show different submissive behaviours.

The early social environment influences the development of social behaviour and social competence of *N. pulcher*. When young are reared either with the breeding pair, a helper and their siblings (+F treatment), or with their siblings only (−F treatment), +F fish show more adequate social behaviour and solve social conflicts more efficiently than −F fish (Arnold & Taborsky 2010; Taborsky *et al.* 2012). Analysis of whole-brain gene expression in adult individuals has shown that the stress axis of these fish is stably reprogrammed by the early social rearing treatment. +F fish had a lower expression of *gr1* and *crf* compared to fish from the −F treatment (Taborsky *et al.* 2013).

Housing conditions

The experiment was carried out at the 'Ethologische Station Hasli' of the Institute of Ecology and Evolution, University of Bern, Switzerland, under licence number 52/12 of the Veterinary Office of the Kanton Bern. The breeding pairs used to generate the experimental fish were second- and third-generation offspring of wild-caught *N. pulcher* from Kasakalawe Point, Mpulungu, Zambia. Rearing tanks of 200 L were equipped with a 2-cm sand layer, and eight clay pot halves and two PET bottles serving as shelters. The light:dark cycle was set to 13:11 h with a 10-min dimmed light period in the morning and evening to simulate the light conditions of Lake Tanganyika. Fish were fed ad libitum 6 days a week (5 days commercial flake food, 1 day frozen zooplankton). Water temperature was held constant at 27 ± 1 °C.

Early social environment treatments

We used two early social environments: being reared (i) with parents, one helper and same-aged siblings (+F treatment), or (ii) with same-aged siblings only, but no older family members (−F treatment). We first created the experimental broods, by forming 20 social groups in separate 200-L tanks, consisting of a breeder male, a breeder female and an immature helper by haphazardly selecting unfamiliar fish from the institute's breeding stock. Ten days after a breeder pair had spawned a clutch, the offspring had reached the free-swimming stage and were used to form 20 experimental groups. Each experimental group was placed in a 100-L compartment of a 200-L tank, separated from neighbouring groups by an opaque PVC sheet. Offspring of each experimental group were assigned randomly to one of the two early social environment treatments. Mean group size was 32.6 fish ±3.8 SEM in the +F treatment and 35.4 fish ±5.1 SEM in the −F treatment. Groups receiving the +F treatment were moved to an empty 100-L compartment together with their parents and helper, whereas groups receiving the −F treatment were moved to another empty 100-L compartment without their parents and the helper. The early social environment treatment lasted for 62 days in accordance with earlier studies (Arnold & Taborsky 2010; Taborsky *et al.* 2012, 2013; Fischer *et al.* 2015). Afterwards, the parents and the helper were removed from the +F treatment and were transferred back to the institute's breeding stock. During the following 72 ± 2 days ('neutral phase'), the sibling groups of both treatments were kept in their original 100-L compartments under identical, standard housing conditions (following Taborsky *et al.* 2012).

Social challenge test

As a social challenge, we chose a test situation that juvenile fish encounter regularly in natural territories, where they have to defend a private shelter against other juvenile family members (Taborsky 2016b). On day 134 (±2 days), four individuals per experimental group were used in this social challenge test. Two fish were assigned to the challenge treatment and two fish to a control treatment. Behavioural data were collected from a total of 80 fish (36 +F individuals from nine groups and 44 −F individuals from 11 groups). Brain samples were taken from a total of 71 fish; 36 challenged individuals (16 +F and 20 −F fish) and 35 controls (15 +F and 20 −F fish). We staged an asymmetric contest over a shelter (for details, see Arnold & Taborsky 2010). Briefly, a 20-L test tank (30 × 20 × 20 cm) was divided into two compartments by an opaque PVC wall. One compartment was empty and the other compartment contained a small clay pot half placed in the centre, which served as a shelter. The focal individual of the challenge test was always

assigned the role of a territory intruder; that is, initially it did not own the shelter. Twenty-four hours before testing, a focal juvenile (2.303 cm ±0.012 SEM) was removed from its home tank, measured, weighed and placed into the empty compartment of the test tank (balanced between right side and left side between trials). At the same time, an unfamiliar *N. pulcher* of the same age was placed in the compartment with shelter to become the pre-assigned shelter owner (2.303 cm ±0.645 SEM) and, thereby, the territory owner. Sizes were matched between the two individuals as close as possible (size difference 0.038 cm ±0.006 SEM). The shelter owner, which served only as an opponent for the focal fish, was always a fish reared in a social group consisting of a breeder pair and a helper (+F condition). Each shelter owner was used only once. In the control treatment, juveniles were exposed to the same handling procedures as the challenged fish and placed in the empty compartment of tanks equally equipped as the test tanks of the challenged fish, but without any opponent present.

The asymmetric competition trials were carried out on the day after the fish had been placed in the experimental tank, between 12:00 and 14:00 h. Previous studies have shown that 24 h is sufficiently long for *N. pulcher* individuals to occupy a novel shelter and defend it as its core territory (Arnold & Taborsky 2010; Taborsky *et al.* 2012). Before the start of a trial, the divider between the compartments was lifted so that the pre-assigned intruder and the shelter owner could interact. The starting point of the trial was set to the moment when either of the two fish crossed the virtual, vertical border between the two compartments (the place where the PVC divider had been before) for the first time. From that moment onwards, the behaviour of the focal individual was recorded for 20 min from behind a black curtain with an observation slit. The observer (CN) was blind to the rearing treatment of the focal fish. The behaviour of both fish (submission, overt aggression, restrained aggression, hiding in shelter and swimming activity) was recorded continuously using the OBSERVER 5.0 software (Noldus, The Netherlands). After 20 min, the winner and loser of the contest were determined. A fish was considered as winner when it stayed in or close (<3 cm) to the shelter and when it was not attacked by its conspecific. Conversely, it was regarded as loser when it was evicted from the vicinity of the shelter and showed submission, but no overt aggression, towards the other fish, or if it stayed close to the water surface (<5 cm; see Taborsky *et al.* 2012). In seven cases (2 +F fish and 5 −F fish), there was no clear winner or loser after 20 min, in which case this contest was rated as 'undecided' and these trials were excluded from further behavioural analysis. After 20 min, the two fish were separated again by the divider and the winner was allowed to use the shelter for 10 min. For the control trials, we followed the same procedures as in the challenge test, but the focal fish in the control situation was not exposed to a shelter owner. In these trials, after the divider had been removed, the control fish could swim freely in the test tank for 20 min while we recorded its activity (swimming or in pot). At the end of the observation, the opaque wall was put back in and the control fish was left 10 min on the side with the shelter if it had entered the shelter during the experiment, otherwise it was left on the opposite side in the aquarium.

Tissue sampling

A 30-min interval from the start of the trial to brain collection was chosen as this protocol has been used successfully before (Cummings *et al.* 2008). It could thus safely be assumed that changes in gene activation patterns could be measured after this time. After the opaque divider was put back in place following the 20-min behavioural recording, a 10-min period without social contact followed for both the challenged and the control fish before the brain tissue was sampled. In the challenge treatment, only brains of the intruder fish (the focal fish) were sampled. In the control treatment, all control fish were sampled. Individuals were sacrificed with an overdose of buffered tricaine methanesulfonate (MS222; Sandoz, Switzerland) within 30 s of catching and the brain was quickly dissected under a binocular microscope (magnification: 16×). The brain was divided into five brain areas: telencephalon, hypothalamus, cerebellum, optic tectum and hind brain. After the dissection, each part was put into a 1.5-mL vial and immersed in RNAlater (Ambion). Further analysis focused on the telencephalon and hypothalamus regions. Samples in RNA later were left overnight at +6 °C and then moved to −20 °C for permanent storage. The sex of the individuals could not be determined as in *N. pulcher* the sex can only be determined when the fish start to become sexually mature, which occurs around lengths of 3.5 cm, while our test subjects ranged between 2.1 and 2.4 cm standard length.

Sample preparation

We performed RNA extraction from telencephalon and hypothalamus, for each brain part separately, using a miRNeasy micro kit (Qiagen) using a modified manufacturer protocol (see Data S1) so that the miRNAs were discarded. The RNA concentration and sample composition were checked with a Nanodrop microvolume spectrophotometer (samples ranged between 27 and

139 ng/μL). Reverse transcription was done 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 candidate gene and success of RT, a small amount of cDNA from random samples from both treatments was used in a PCR using all the different candidate genes and visualized using an electrophoretic gel.

Candidate genes

We measured the expression of five genes in the telencephalon (*egr-1*, *bdnf*, *gr1*, *crf* and *neuroserpin*) and six genes in the hypothalamus (*egr-1*, *bdnf*, *gr1*, *crf*, *avt* and *avtr*) of *N. pulcher*. We were interested in the reaction norm of these genes, that is, if their expression level is different in fish facing a control vs. a challenge condition, and if these reaction norms differed between fish reared in +F or −F social conditions. The gene 18S was used as a control gene. *egr-1* (early growth response 1, also known as NGFI_A, Krox-24, zif268, ZENK and TIS8) is an immediate early gene coding for a transcription factor used as a marker for neuronal activity (Desjardins & Fernald 2010) and plasticity (Morgan & Curran 1995). The gene is activated in different brain areas in response to a novel or changing social cue (Burmeister *et al.* 2005), and this property has been used to determine which brain areas respond to a certain stimulus. In the lateral part of the dorsal telencephalon (Dl), which is thought to be the fish homologue of the mammalian hippocampus (Folgueira *et al.* 2004), *egr-1* has been proposed to act as a transcription factor targeting later-acting genes involved in stress responses (Desjardins & Fernald 2010). *bdnf* (brain-derived neurotrophic factor) is a molecule influencing neuronal proliferation, differentiation and synaptogenesis (McAllister *et al.* 1999) and is therefore assumed to impact brain function and structure (Branchi *et al.* 2004). Rat pups facing repeated maternal deprivation show persistently altered *bdnf* expression in the hippocampus and prefrontal cortex compared to control (undisturbed) pups (Roceri *et al.* 2004). In *A. burtoni*, a higher *bdnf* expression was observed in the Dl of fish learning a task (finding shelter and a female) compared to nonlearners (Wood *et al.* 2011). *gr1* (glucocorticoid receptor 1) is a ligand-activated nuclear receptor that is part of the HPI stress axis in fish and is activated by glucocorticoids. Acting as a transcription factor, it is involved in modulating stress responses in different tissues and in the negative feedback of corticosteroids on stress responses taking place in the hippocampus (Jacobson & Sapolsky 1991; De Kloet *et al.* 1998). Previous work showed that adult *N. pulcher* reared in −F conditions have higher *gr1* expression in whole-brain samples than +F individuals (Taborsky *et al.* 2013). *crf* (corticotropin-releasing factor) plays a role in activating the stress response, and in modulating social behaviours associated with parental care, social memory, as well as prosocial and affiliative behaviours (review in Hostetler & Ryabinin 2013). *crf* was higher expressed in whole-brain samples of *N. pulcher* reared in −F conditions (Taborsky *et al.* 2013). Neuroserpin is a serine protease inhibitor that is assumed to play a role in synaptic plasticity and is most prominently expressed in areas of the brain that participate in learning, memory and behaviour (review in Miranda & Lomas 2006). Thus, this gene might be implicated in plastic behavioural responses in fish. The neuropeptide arginine vasotocin (*avt*), the fish homologue to the mammalian arginine vasopressin (AVP), is involved in osmoregulation, the regulation of the stress response, and in reproductive and social behaviours (reviewed in Godwin & Thompson 2012). Aubin-Horth *et al.* (2007) showed that dominant individuals of *N. pulcher* had higher levels of whole-brain *avt* gene expression, compared to subordinate conspecifics, and its expression is higher in wild-caught males of the social cichlid *N. pulcher* than that of the nonsocial cichlid *Telmatochromis temporalis* (O'Connor *et al.* 2015), but this difference was not repeated in a laboratory study (O'Connor *et al.* 2016). The V1a2 receptor for *avt* (*avtr*) is implicated in social behaviour in fish by mediating aggressive and mating behaviour (Lema 2010; Kline *et al.* 2011; Huffman *et al.* 2012b, 2015; Oldfield *et al.* 2013).

Quantitative real-time PCR

Primers for *gr1* and *crf* were as in Taborsky *et al.* (2013); the *avt*, *avtr* and 18S primers were as in O'Connor *et al.* (2015), while primers for the other genes were designed using the sequences available from the genome of *N. brichardi* (http://cichlid.umd.edu/cichlidlabs/kocherlab/bouillabase.html). The sequences are as follows: *egr-1* (using the *A. burtoni* sequence as a search template, NCBI database ID number: AY493348.1, *N. brichardi* NCBI database ID number XM_006781510.1, forward: CGGCGATATATCCTAAAATC; reverse: TCCCATGCC TATAAACACT), *bdnf* (using the *A. burtoni* sequence as a template, NCBI database ID number: HQ398161.1, *N. brichardi* NCBI database ID number XM_006780270.1, forward: GGGTGACAGCTGTGGATAAAA; reverse: GGGGTTGCATTTGGTCTCATA) and *neuroserpin* (using the *Oreochromis mossambicus* sequence as a template, NCBI database ID number: HQ667766.1, *N. brichardi* NCBI database ID number XM_006799864, forward: -GGATG GACCCTGTTCTCC; reverse: TTGCCCTGACCAGGAC TCT). To determine amplification efficiency, the absence

of primer dimers and the specificity of amplification for each primer pair, qPCR experiments and melting curves (50–90 °C) were run using standard curves consisting of 5 × 10-fold dilutions (of pooled samples) in duplicates (Aubin-Horth *et al.* 2012). The primers (Eurofins) and 5 μL of sample cDNA were prepared on a 384-well plate (axigen) using an epMotion liquid handler (Eppendorf) and used for a quantitative real-time PCR experiment following the scaled-down version of the Quantitect SYBR Green PCR kit manufacturer's protocol (Qiagen) using a 384-well plate qRT–PCR machine (Light Cycler, Roche). Each sample for hypothalamus and telencephalon was run 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, a melting curve was also performed on each replicate. Relative gene expression for each individual-brain area combination was calculated using the expression of a control gene (18S) (Pfaffl 2001).

Data analysis

We used two different data sets to answer our questions. To analyse genomic reaction norms and neurogenomic states of individuals from the different early social environment and social challenge treatments, we included all intruder and all control fish (data set 1). To analyse (i) the expressed behaviours during the challenge of intruders and owners and (ii) the relationship between intruder behaviour and gene expression, we only analysed intruder fish that either won or lost the contest over the shelter (data set 2). Furthermore, we analysed only the interactions between the start and the end of a contest. Contests were considered to be terminated when the loser did not aim to gain access to the shelter and retreated either to the upper parts of the water column or to a distant corner of the tank. As the duration of these periods varied between trials, we analysed behavioural rates (per min). We used this subset of the data (data set 2) for two reasons. (i) Controls could not be included because they could not show any social behaviour; (ii) contests which were still undecided after 20-min observation time were excluded, because behavioural frequencies are expected to vary with the eventual fight outcome (e.g. the loser should show submission). By including fights that were ongoing at the end of the observation time behaviours would be biased towards higher aggression relative to submission rates.

Statistical analyses were conducted with R 3.0.2 (R Core Development team 2013) including the package 'LME4' (Bates *et al.* 2013) and 'AFEX' (Singmann *et al.* 2015). Linear mixed models (LMM) were built to analyse the influence of the two rearing treatments (+F and −F fish) on fish behaviour. We used intruder behaviour as our dependent variable and rearing treatment (+F/−F) as our independent variable. In the LMM with intruder submission as dependent variable, owner-aggressive behaviour was included as covariate, as in *N. pulcher* submission is often an immediate response to received aggression. In the LMMs with intruder overt aggression and restraint aggression as dependent variable, the contest outcome (winning/losing) was included as covariate. In a further set of LMMs, we analysed the influence of the two rearing treatments (+F and −F fish), the social challenge treatments (intruder vs control fish) and their interactions on the expression levels of each single gene. If the interaction term 'rearing treatment × social challenge' was significant, we conducted post hoc analyses by testing for gene expression differences between the two social challenge situations, separately within +F fish and −F fish, respectively. For all post hoc analyses, we present adjusted *P*-values after applying the Benjamini–Hochberg false-discovery rate method (Benjamini & Hochberg 1995) to correct for multiple testing. For some individuals, gene expression data were missing for one or more genes because the coefficient of variation (CV) of the three replicates was too large. A CV cut-off of 5% was used for all genes. The sample sizes for each gene are as follows: in telencephalon: *egr-1*, *bdnf*, *gr1*, *crf*, *neuroserpin* $N = 57$ (of them −F control = 14, −F intruder = 16, +F control = 13 and +F intruder = 14) and in hypothalamus: *egr-1* $N = 40$ (of them −F control = 12, −F intruder = 12, +F control = 6 and +F intruder = 10), *bdnf*, *gr1*, *crf*, *avtr* $N = 56$ (of them −F control = 17, −F intruder = 17, +F control = 10 and +F intruder = 12) and *avt* $N = 54$ (of them −F control = 17, −F intruder = 17, +F control = 9 and +F intruder = 11). In addition, a principal component analysis (PCA) was performed to reduce the complexity of the gene data set and thus to obtain a 'neurogenomic state' (Robinson *et al.* 2008) for each individual that summarizes the information on all genes in both brain areas. The PCA was done with 70 individuals as observations and expression levels of seven different candidate genes, with a total of 11 measures of gene expression (five in telencephalon, six in hypothalamus) as variables using the R package 'PSYCH' (function 'principal'). A correlation matrix for the 11 measures of gene expression was used as input (Pearson correlation coefficients). To be able to include individuals with missing data (see above) in the analysis, the mean gene expression of that gene for a given combination of rearing environment and social challenge was used in the data analysis for these individuals (Zar 1999). A varimax rotation was applied to the data. Loadings of individual genes on each principal component (PC) were determined, and the PC scores for individual fish were calculated. LMMs were built to analyse the

influence of the early social environment and social challenge treatments and their interactions on the first two principal components (see below). All models assumed a Gaussian error structure, which was validated by visual inspection of the distributions of residuals, predicted vs. fitted values and quantile–quantile (Q–Q) plots. Some variables were log-transformed to achieve a normally distributed error structure. Experimental group was included as random factor in each model. To account for possible effects of intruder size, the intruder standard length (I_SL) was included as covariate in all behavioural models. For significance testing, each term was singly removed from the model and the reduced model was compared to the full model. To do so, we used the command 'mixed' in the R package 'AFEX', which calculates type 3 *P*-values using a Kenward–Roger approximation for degrees-of-freedom (Singmann *et al.* 2015). 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.

Ethical note

Fish interacted directly with each other in the asymmetric competition. We observed carefully that no fish was injured during the experiment, in which case the trial would have been immediately interrupted. This never happened. Some fish showed overt aggression towards each other (i.e. aggression that involves body contact, Taborsky 1984). Probably due to the small size and low weights of the fish, these direct body contacts never caused any injuries in the opponent. A fish subject to overt aggression usually responded by showing submissive tail quivering and/or by retreating out of reach of the aggressor, which stopped aggression immediately.

Results

Effect of early social environment on behavioural phenotype

To test whether our early social environment treatment was effective to influence the phenotypic development of the fish, we tested whether the rearing treatment influenced the later-life social behaviour of our experimental fish. Intruder fish of the +F treatment displayed more submissive behaviour relative to the amount of received owner aggression than did fish from the −F treatment (Fig. 1, LMM, interaction term: $F = 7.2413$, $P = 0.013$, treatment: $F = 1.269$, $P = 0.270$, received aggression: $F = 22.599$, $P < 0.0001$, $N = 31$). In contrast, intruder overt aggression did not differ between the rearing treatments, but winners showed more overt aggression than losers (LMM, treatment: $F = 0.759$, $P = 0.397$, contest outcome: $F = 4.381$, $P = 0.048$, $N = 31$). Intruder restraint aggression (i.e. threat displays towards the opponent without body contact) was not influenced by the rearing treatment or by contest outcome (LMM, treatment: $F = 0.203$, $P = 0.658$, contest outcome: $F = 0.001$, $P = 0.992$, $N = 31$).

Genomic reaction norms in response to early social environment and social challenge treatments

Telencephalon. The early social environment (+F/−F) and the social challenge (intruder/control) treatments interactively influenced the expression of *egr-1* and *gr1* in the telencephalon (Table 1; Fig. 2). Post hoc analysis revealed that −F fish had a lower *egr-1* expression in the control than in the intruder situation (LMM, −F fish: $F = 11.372$ adjusted $P = 0.006$, $N = 30$), whereas in +F fish there was no difference in *egr-1* expression with respect to the social challenge (LMM, +F fish: $F = 0.215$, adjusted $P = 0.648$, $N = 27$). In +F fish, *gr1* expression tended to be lower in the intruder than in the control situation (LMM, +F fish: $F = 5.355$, adjusted $P = 0.063$, $N = 27$), whereas −F fish did not differ with respect to the social challenge (LMM, −F fish: $F = 0.124$, adjusted $P = 0.728$, $N = 30$). The early social environment and social challenge did not significantly influence gene expression levels of *bdnf*, *crf* and *neuroserpin* in the telencephalon (Table 1; Fig. 2; Table S1, Supporting information).

Hypothalamus. The early social environment (+F/−F) and the social challenge (intruder/control) treatments interactively influenced the expression of *bdnf* in the hypothalamus (Table 1; Fig. 3). Post hoc analysis showed that +F fish had a higher *bdnf* expression in the control than in the intruder situation (LMM, +F fish:

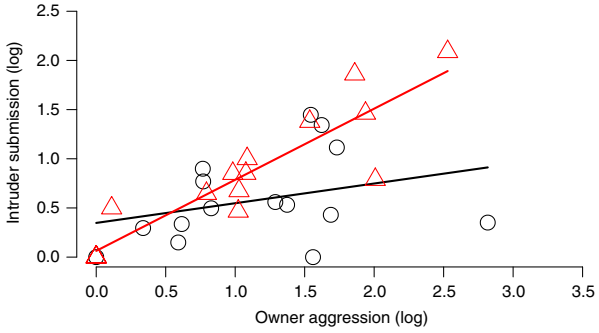

Fig. 1 Intruder submission (log-transformed) in relation to received owner aggression (log-transformed). Behaviours are expressed as rates per minute. Circles and lower line represent the −F treatment; triangles and upper line represent the +F treatment. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1 Results of the linear mixed models testing the effect of rearing environment (−F or +F) and social challenge (intruder or control situation) on the expression of candidate genes in *N. pulcher*. For sample sizes, see section 'Data analysis'. *P*-values <0.05 are highlighted in bold

Brain area	Factors	Estimate ± SE	*F*-value	*P*-value
Telencephalon	*egr-1* (log)			
	Rearing	−0.064 ± 0.025	6.473	**0.023**
	Challenge	−0.034 ± 0.025	1.787	0.189
	Rearing × challenge	−0.055 ± 0.025	4.684	**0.036**
	bdnf			
	Rearing	−0.016 ± 0.049	0.101	0.756
	Challenge	0.030 ± 0.044	0.457	0.503
	gr1			
	Rearing	−0.080 ± 0.037	4.627	**0.048**
	Challenge	0.062 ± 0.036	2.959	0.093
	Rearing × challenge	−0.077 ± 0.036	4.577	**0.038**
	crf			
	Rearing	−0.101 ± 0.056	3.260	0.090
	Challenge	0.022 ± 0.040	0.315	0.578
	neuroserpin			
	Rearing	−0.028 ± 0.043	0.425	0.524
	Challenge	−0.001 ± 0.041	0.001	0.980
Hypothalamus	*egr-1* (log)			
	Rearing	−0.157 ± 0.070	4.880	**0.044**
	Challenge	0.060 ± 0.068	0.756	0.392
	bdnf			
	Rearing	0.036 ± 0.045	0.643	0.435
	Challenge	−0.023 ± 0.042	0.281	0.600
	Rearing × challenge	−0.181 ± 0.042	18.195	**0.0001**
	gr1 (log)			
	Rearing	0.011 ± 0.019	0.360	0.557
	Challenge	−0.010 ± 0.017	0.352	0.556
	crf (log)			
	Rearing	0.015 ± 0.029	0.181	0.676
	Challenge	0.025 ± 0.025	1.077	0.3055
	avt (log)			
	Rearing	0.076 ± 0.123	0.379	0.547
	Challenge	0.084 ± 0.076	1.199	0.280
	avtr			
	Rearing	−0.057 ± 0.052	1.196	0.291
	Challenge	−0.022 ± 0.050	0.191	0.665

$F = 5.815$, adjusted $P = 0.029$, $N = 22$), whereas the reverse was found in −F fish, which had a higher *bdnf* expression in the intruder than in the control situation (LMM, treatment: $F = 15.007$, adjusted $P = 0.001$, $N = 34$). Moreover, fish reared in the +F social environment had a higher expression of *egr-1* than in the −F condition, whereas the social challenge did not influence its expression (Table 1; Fig. 3). The early social environment and social challenge did not influence the expression of *gr1*, CFR, *avt* and *avtr* in the hypothalamus (Table 1; Fig. 3; Table S1, Supporting information).

Neurogenomic states

We used a PCA analysis to define a neurogenomic state that synthetizes gene expression patterns in the two brain areas studied for each individual. The first two principal components of the PCA accounted for a total of 45% of the variance in gene expression (PC 1: 27 %; PC 2: 18%, Table 2). All genes analysed in the telencephalon (*egr-1*, *bdnf*, *gr1*, *crf* and *neuroserpin*) loaded positively on PC1. The genes analysed in the hypothalamus loaded negatively (*egr-1*, *gr1*) or positively (*bdnf*, *crf*, *avt* and *avt*) on PC2 (Table 2). We extracted the individual PC scores for each fish for the two-first principal components and investigated the effects of early social environment and social challenge treatment on these two components by LMMs (Fig. 4). For example, a positive score for an individual on PC1 indicates higher expression in the telencephalon of the five genes studied. The early social environment and the social challenge jointly influenced PC1 and PC2

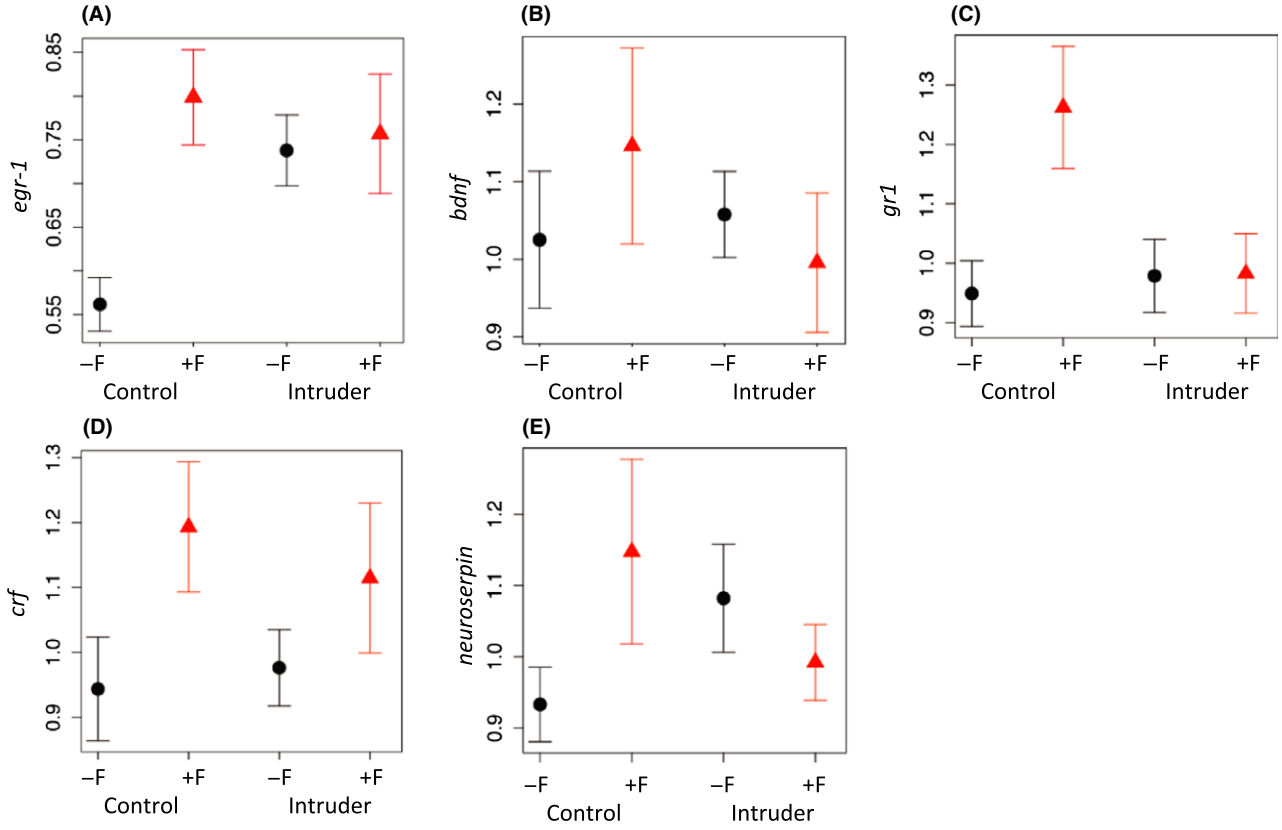

Fig. 2 Gene expression for control and intruder fish for five genes in the telencephalon. (A) immediate early gene *egr-1*, (B) brain-derived neurotrophic factor (*bdnf*), (C) glucocorticoid receptor (*gr1*), (D) corticotropin-releasing factor (*crf*) and (E) *neuroserpin*. Gene expression of *egr-1* is log-transformed as it was done in the linear mixed model. Filled circles represent −F treatment, and filled triangles represent +F treatment. Figures display means ± SE. [Colour figure can be viewed at wileyonlinelibrary.com]

(Table 3). This significant interaction was reflected in a larger divergence of neurogenomic state (PC scores) between control and intruder fish from the +F rearing treatment as compared to −F fish, along both PC axes (Fig. 4).

Behaviour and gene expression

The expression levels of two of the analysed genes were associated with behavioural variation among individuals (Table 4). In the telencephalon, *crf* expression was interactively influenced by the early social environment and intruder submission. In +F intruders, the expressed *crf* levels decreased with increasing amounts of displayed submissive behaviours, whereas no such relationship was present in −F intruders (Fig. 5A). In the hypothalamus, *gr1* expression decreased with intruder submission, with no effect of early social environment (Fig. 5B). Gene expression was not influenced by intruder overt and restrained aggression. Winning or losing the contest did not impact expression of any of the genes, nor was gene expression of winners vs. losers influenced by the social treatment. None of the other analysed genes were significantly related to any social behaviour.

Discussion

In this experimental study, we aimed to understand how the early social rearing environment of a cooperatively breeding fish species influences brain genomic responses to a short-term social challenge. We found that early social environment and social challenge treatments interactively influenced the expression of an immediate early gene (*egr-1*) and a glucocorticoid receptor (*gr1*) in the telencephalon, and of a neural plasticity gene (*bdnf*) in the hypothalamus. Moreover, *egr-1* in the hypothalamus was more expressed in fish reared in the +F environment, independently of their exposure to a social challenge. A global analysis of the 11 measures of gene expression patterns in the brain showed that the neurogenomic state diverged more between intruder fish and control fish from the +F rearing treatment than in −F fish. Finally, we showed that with increasing submissive behaviour of intruders the expression of *crf* in the telencephalon decreased, but only in fish from the

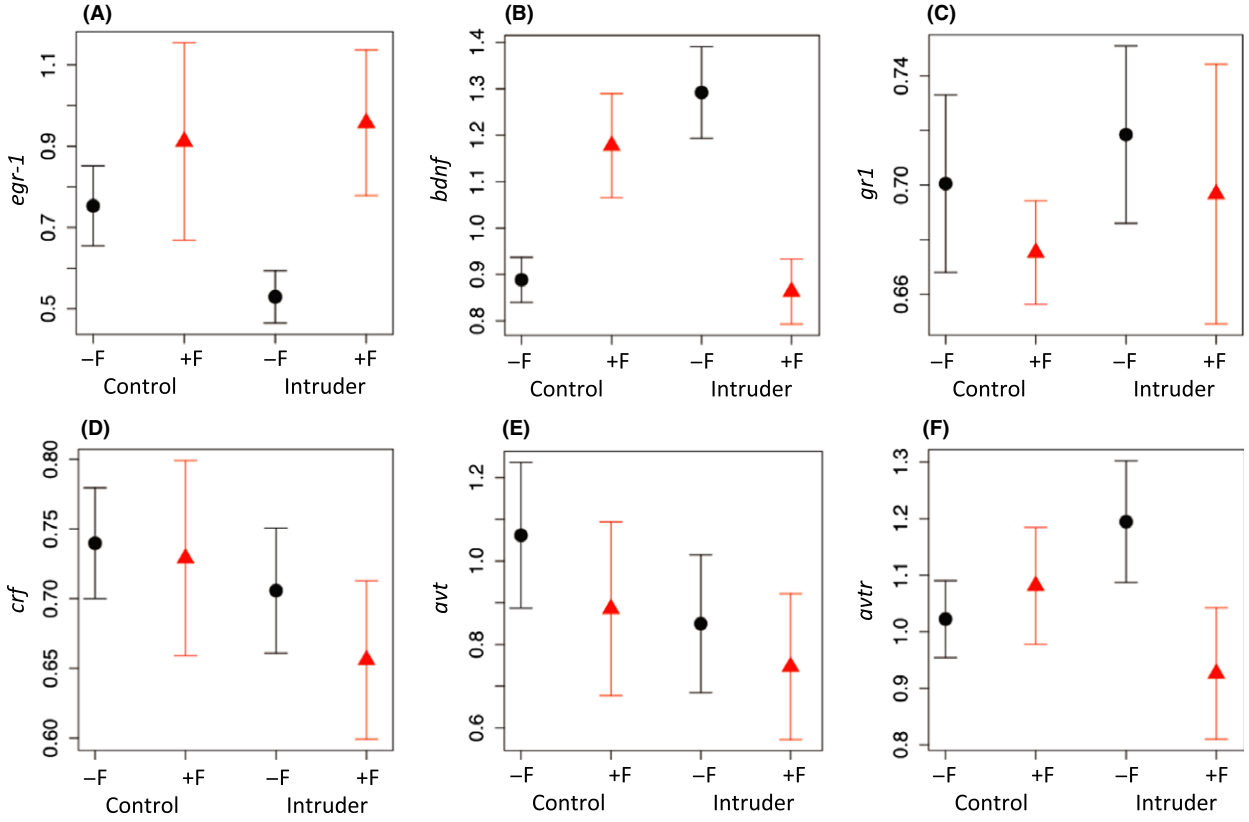

Fig. 3 Gene expression for control and intruder fish for six genes in the hypothalamus. (A) immediate early gene *egr-1*, (B) brain-derived neurotrophic factor (*bdnf*), (C) glucocorticoid receptor (*gr1*), (D) corticotropin-releasing factor (*crf*), (E) arginine vasotocin (*avt*), and (F) arginine vasotocin receptor V1a2 (*avtr*). Gene expression of *egr-1*, *gr1*, *crf* and *avt* is log-transformed as it was done in the linear mixed models. Filled circles represent −F treatment, and filled triangles represent +F treatment. Figures display means ± SE. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2 Factor loadings for the seven different candidate genes, with a total of 11 measures of gene expression (five in telencephalon, six in hypothalamus) on the first two principal components (PC). The respective higher loadings among the two PCs are highlighted in bold. $N = 70$

Brain area	Gene	PC1	PC2
Telencephalon	*egr-1*	**0.66**	−0.04
	bdnf	**0.80**	−0.13
	gr1	**0.79**	0.05
	crf	**0.74**	−0.14
	neuroserpin	**0.83**	−0.11
Variance explained		27%	
Hypothalamus	*egr-1*	0.11	**−0.22**
	bdnf	0.15	**0.78**
	gr1	0.08	**−0.21**
	crf	0.03	**0.43**
	avt	−0.14	**0.56**
	avtr	−0.03	**0.86**
Variance explained			18%

+F rearing treatment. In the hypothalamus, *gr1* expression decreased with increasing amounts of submissive behaviour of the intruder.

We first established that the behavioural response of a fish to a social challenge was markedly affected by the rearing treatment. During the social challenge, intruder fish reared with parents and a helper showed more submissive behaviour per received aggression. If in a natural context an intruder cannot monopolize its own shelter, the adequate response is to submit towards other shelter owners (Taborsky 1985; Zöttl *et al.* 2013a). The latter are then willing to tolerate the subordinate fish close to the shelter (Taborsky *et al.* 2012), which would enable the subordinate to share the access to the shelter in case of a predator attack. Our result therefore suggests that +F fish showed better social competence, confirming earlier findings by Arnold & Taborsky (2010) from a similar behavioural experiment.

The early-rearing environment influenced the gene expression response to a social challenge of several genes in both the telencephalon and the hypothalamus. First, the telencephalon expression of *egr-1* was relatively high in +F fish in both social situations (control or intruder), while in −F fish this gene was highly expressed only after taking part in the contest over a

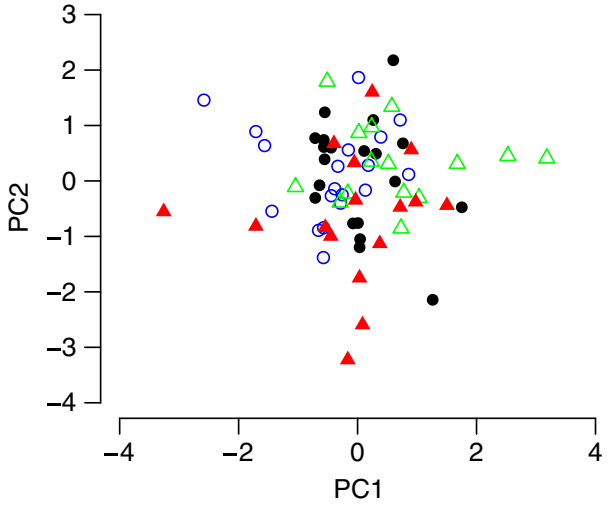

Fig. 4 Relationship between individual PC1 and PC2 scores representing the neurogenomic states of individuals from each combination of early social environment and social challenge. Triangles represent +F rearing treatment fish and circles −F individuals. Open symbols represent control individual in the social challenge, and filled symbols represent intruders. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 3 Results of the linear mixed models testing the effect of rearing environment (−F or +F) and social challenge (intruder or control situation) using the PC scores of the first two principal components. $N = 70$. P-values <0.05 are highlighted in bold

Factors	Estimate ± SE	F-value	P-value
PC1			
Rearing	−0.234 ± 0.128	3.337	0.09
Challenge	0.059 ± 0.109	0.290	0.59
Rearing × challenge	−0.313 ± 0.109	8.262	**0.006**
PC2			
Rearing	0.156 ± 0.117	1.766	0.2
Challenge	0.250 ± 0.114	4.794	**0.03**
Rearing × challenge	−0.231 ± 0.114	4.126	**0.05**

Table 4 Effect of rearing environment, submissive behaviour and size of intruders on brain gene expression in fish facing a social challenge. *crf*: $N = 22$, *gr1*: $N = 21$. P-values <0.05 are highlighted in bold

Brain area	Factors	Estimate ± SE	F-value	P-value
Telencephalon				
crf				
	Rearing	−0.318 ± 0.101	9.002	**0.009**
	Submission	−0.003 ± 0.002	2.832	0.128
	Intruder size	−1.241 ± 0.968	1.381	0.263
	Rearing × submission	0.006 ± 0.002	8.995	**0.014**
Hypothalamus				
gr1				
	Rearing	−0.060 ± 0.042	1.917	0.191
	Submission	−0.003 ± 0.001	8.121	**0.012**
	Intruder size	0.171 ± 0.494	0.097	0.759

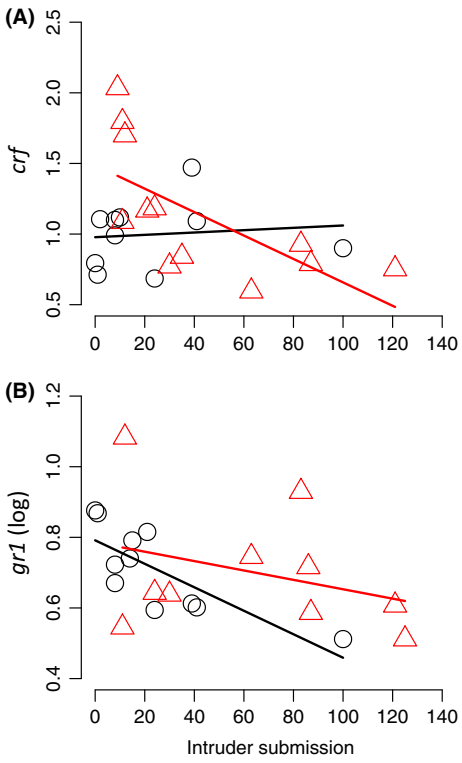

Fig. 5 Association of intruder submission and gene expression of (A) *crf* in the telencephalon and (B) *gr1* in the hypothalamus. Gene expression of *gr1* is log-transformed as it was done in the linear mixed model. Sample sizes *crf*: $N = 22$, *gr1*: $N = 21$. 5A: Circles and horizontal line represent −F treatment; triangles and oblique line represent +F treatment. 5B: Circles and lower line represent −F treatment; triangles and upper line represent +F treatment. [Colour figure can be viewed at wileyonlinelibrary.com]

shelter. Environmental stimulation activates the expression of *egr-1* (Burmeister & Fernald 2005; Goerlich *et al.* 2012). Higher *egr-1* expression of −F intruders after the challenge compared to the −F control suggests a short-term response to the challenge, while there is a lack of an *egr-1* response to the challenge in the +F intruders which keep a higher baseline *egr-1* expression. Similarly, isolation-reared, but not group-reared, male mice had a significant rise in expression levels of *c-Fos*, another immediate early gene, in the prefrontal cortex 2 h after facing a social challenge (Ago *et al.* 2013). Together, these studies suggest that the transcription response of *egr-1* to a social challenge can be affected by the early social environment in vertebrates. These changes can have far-ranging consequences. As *egr-1* is a transcription factor mediating the expression of downstream genes belonging to many different pathways, it

is likely that entirely different networks are activated under the two social rearing conditions. Higher *egr-1* expression measured in +F fish and in challenged −F fish could increase their behavioural and neuronal plasticity (Donovan *et al.* 1999), activate effector genes downstream (e.g. by regulating GR expression by binding to its promoter (Weaver *et al.* 2007, 2014) and increase learning and memory capabilities (Joëls *et al.* 2006; Roozendaal & McGaugh 2011).

Second, like *egr-1* expression, expression levels of *gr1* in the telencephalon were influenced by the combined effect of rearing environment and social challenge treatments. In +F fish, *gr1* was downregulated in the intruder challenge group compared to the control situation, whereas in −F fish, *gr1* expression was generally low and unaffected by the social challenge. Fewer glucocorticoid receptors in specific brain regions are known to reduce the efficiency of negative feedback to return cortisol levels to normal, prestress levels (Ladd *et al.* 2004). In rats, for instance, decreased quality of maternal care leads to lifelong reduction of *gr* expression (the functional homologue of the *gr1* gene in fish, Bury *et al.* 2003) in the hippocampus and prefrontal cortex (telencephalon in fish), impairing their negative feedback inhibition of the HPA axis (Liu *et al.* 1997; Ladd *et al.* 2004; Navailles *et al.* 2010). Interestingly, after the social challenge, +F and −F fish had similarly low *gr1* levels. Poststress downregulation of glucocorticoid receptor gene expression has been recently quantified in mammals. A 15-min forced swim test in rats quickly resulted in lower levels of *gr* mRNA in the hippocampus, which was suggested to be a mechanism protecting neurons from repeated stress (Mifsud *et al.* 2016). The response to the social challenge observed in +F fish is similar suggesting that this could be a 'normal' vertebrate-wide transcriptional response to challenging situations, which is disturbed by early rearing in a socially deprived environment, as seen in −F fish.

Finally, *bdnf* expression levels in the hypothalamus showed crossing reaction norms, as there were both developmental and short-term environmental effects. After the contest, +F fish had a lower *bdnf* expression than in the control situation, whereas the reverse pattern was present in −F fish. Thus, the response in −F individuals was opposite to that of +F fish, suggesting that the same activational pathways were used differently in the same situation by fish from the two rearing treatments. *bdnf* is implicated in several important functions, including the stress response. Rats subjected to stress show increased hypothalamic *bdnf* mRNA levels (Smith *et al.* 1995), and conversely, strong cerebral *bdnf* inhibition decreases HPA activity in mice (Naert *et al.* 2015). Our results would thus suggest that −F fish may be subject to a higher stress response when socially challenged. Moreover, +F fish might have been more stressed while being alone in the control situation. However, increased *bdnf* expression is also expected to enhance synaptic plasticity (Alder *et al.* 2003). Therefore, we would have predicted +F fish, which are known to behave more flexibly in social encounters (Taborsky & Oliveira 2012, this study), to show higher expression when socially challenged. +F individuals had a higher *bdnf* expression only in the control situation, suggesting that their basic state, that is, before a social challenge, may be inherently more amenable to plasticity. However, the fact that we found lower expression after the challenge may mean that the role of *bdnf* in the stress response is more prominent in this system. Measuring *bdnf* levels after a nonsocial stress could help disentangle these two effects.

Gene expression was not always influenced by both the early-rearing environment and the short-term social challenge. In the hypothalamus, *egr-1* was only influenced by the rearing treatments. The hypothalamus is a key area regulating many different social behaviours, including aggression, parental care, sexual behaviour and social cognition, and the activity of the HPA axis (O'Connell & Hofmann 2011; Wolkers *et al.* 2015). Because of the broad effect of *egr-1* on many different pathways, the higher *egr-1* hypothalamus expression in +F fish compared to −F fish might indicate that +F fish are able to show a greater extent of plasticity than −F fish in a wide array of social behaviours and social contexts. Furthermore, contrary to our expectations, the early social environment and social challenge did not influence gene expression of *crf*, *bdnf* and *neuroserpin* in the telencephalon, or *gr1*, *crf*, *avt* and *avtr* in the hypothalamus. There are several possible reasons to explain the lack of treatment difference in expression of these genes. First, the timing of sampling is crucial (see Liu *et al.* 2000). If we sample the brain too early, some later-acting genes have possibly not been activated yet, whereas when sampling too late we might miss the window for early-activated genes. Furthermore, it is possible that differential gene expression in opposite directions in different subregions of the complex 'social decision-making (SDM) network' might have masked an effect (Greenwood *et al.* 2008). The telencephalon contains six important nodes of the SDM network and the hypothalamus holds two nodes (O'Connell & Hofmann 2011). As we sampled the whole telencephalon and hypothalamus, we might have lost some valuable information on gene expression at the level of the subregions (Wood *et al.* 2011). Finally, while the control fish in our experiment did not meet an opponent in the control situation, we nevertheless cannot exclude that they perceived the

control environment as novel experience, which influenced brain gene expression.

The pattern of expression of several genes can define the neurogenomic state associated with a particular behaviour (Robinson *et al.* 2008; Aubin-Horth *et al.* 2009). In addition to our analysis of effects on single genes, we investigated the neurogenomic state of fish reared in each type of environment. Fish reared in the more natural +F environment showed a larger shift in neurogenomic state when faced with a social challenge compared with fish that experienced a −F rearing environment. The principal component analysis suggests that the expression of candidate genes is strongly coordinated within each of the targeted brain areas. The larger overall change observed in fish reared in the natural, +F environment thus suggests that the social challenge we chose has significant consequences for the coordinated activation of the molecular networks of these genes. This result also raises the intriguing possibility that −F fish do exhibit a genomic response, but that it is delayed. Quantifying such a potential time shift in genomic response was beyond the scope of the study but could also potentially result in the altered behavioural response observed in these fish. In any cases, these concerted genomic modifications may be linked to the modulation of behaviour in response to the social challenge (reviewed in Robinson *et al.* 2008; Taborsky & Oliveira 2012).

The observation that a behavioural response to a social challenge is accompanied by changes in the average level of gene expression can reasonably lead to the prediction that behaviour and gene expression will covary at the individual level (Williams 2008). This is supported by our results on the link between gene expression and the expression of submissive displays, a social behaviour, which is of particular importance for *N. pulcher* to maintain the stability of its social system. The amount of submissive displays by intruders decreased with the expression of *crf* in the telencephalon, and *gr1* in the hypothalamus. Showing more submissive displays represents an adequate behavioural response when being in the intruder role, as most intruders were not able to take over the shelter. For *crf*, the interaction between social rearing and amount of submission was significant; intruders of +F treatments showing more submission had lower *crf* expression, while in −F intruders this trend was absent. For *gr1*, intruders from both rearing treatments showed more submission with a lower expression of the gene. It is possible that the amount of submission an intruder shows influences the expression of these genes or that the gene expression itself regulates the submissive behaviour. The lower *crf* expression in intruders showing more submission could be related to social defeat stress (SDS) as seen in rats (Panksepp *et al.* 2007), as submissive intruders are the defeated contestants in our social challenge test. Rats facing SDS have lower hippocampal *crf* mRNA expression 6 h after an encounter compared to nondefeated rats (Panksepp *et al.* 2007). *N. pulcher* intruders with higher *gr1* expression might be more bold and risk-prone, as it has been observed in sticklebacks (Aubin-Horth *et al.* 2012), which might explain their lower submission tendencies.

In conclusion, our results highlight the importance to incorporate the environmental conditions experienced during development when we aim to understand the genomic basis of social behaviour. Furthermore, it shows how integrative biology approaches can help understanding the evolution of complex social behaviour, by jointly investigating molecular, neuroendocrine and behavioural responses to environmental conditions in ecologically relevant contexts (Aubin-Horth & Renn 2009; Taborsky & Taborsky 2015). Future studies should aim to obtain a more complete picture of the genes and the gene networks involved in the development and regulation of social behaviour.

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Data accessibility

Behavioural observation files and gene expression values have been deposited to Dryad, doi:10.5061/dryad.9c2j1. Information on primers is provided in the Methods section.

C.N., B.T. and N.A.H. designed the study. C.N and S.F. reared and handled the fish. C.N. performed the behavioural experiments. C.N. and N.A.H. did the gene expression laboratory work. C.N. performed the statistical analysis. C.N., B.T. and N.A.H. drafted the manuscript. All authors have approved the content of the manuscript.

Supporting information

Additional supporting information may be found in the online version of this article.

Data S1 RNA extraction protocol

Table S1 Results of the full linear mixed models including nonsignificant interactions testing the effect of rearing environment and social challenge on the expression of candidate genes.