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Transcriptomic signatures of social experience during early development in a highly social cichlid fish

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Abstract

The social environment encountered early during development can temporarily or permanently influence life history decisions and behaviour of individuals and correspondingly shape molecular pathways. In the highly social cichlid fish *Neolamprologus pulcher*, deprivation of brood care permanently affects social behaviour and alters the expression of stress axis genes in juveniles and adults. It is unclear when gene expression patterns change during early life depending on social experience, and which genes are involved. We compared brain gene expression of *N. pulcher* at two time points during the social experience phase when juveniles were reared either with or without brood care, and one time point shortly afterwards. We compared (a) whole transcriptomes and (b) expression of 79 genes related to stress regulation, in order to define a neurogenomic state of stress for each fish. At developmental day 75, that is, after the social experience phase, 43 genes were down-regulated in fish having experienced social deprivation, while two genes involved in learning and memory and in post-translational modifications of proteins (PTM), respectively, were up-regulated. Down-regulated genes were mainly associated with immunity, PTM and brain function. In contrast, during the experience phase no genes were differentially expressed when assessing the whole transcriptome. When focusing on the neurogenomic state associated with the stress response, we found that individuals from the two social treatments differed in how their brain gene expression profiles changed over developmental stages. Our results indicate that the early social environment influences the transcriptional activation in fish brains, both during and after an early social experience, possibly affecting plasticity, immune system function and stress axis regulation.

KEYWORDS

cichlids, cooperative breeding, early environment, stress axis, time series, transcriptomics

1 | INTRODUCTION

Through developmental plasticity, the social environment experienced during the early life of an individual can persistently shape its phenotype (Adkins-Regan & Krakauer, 2000; Arnold & Taborsky, 2010; Feng et al., 2011; Francis, Diorio, Liu, & Meaney, 1999; Liu et al., 1997; Nyman, Fischer, Aubin-Horth, & Taborsky, 2017; Taborsky, Arnold, Junker, & Tschopp, 2012; see review Taborsky, 2016a). For instance, early social experience can influence individual life history decisions (Fischer, Bohn, Oberhummer, Nyman, & Taborsky, 2017),

Aubin-Horth and Taborsky equally contributed to this work.

maternal behaviour (Francis et al., 1999), learning and memory (Champagne et al., 2008), mate choice decisions (Adkins-Regan & Krakauer, 2000), alcohol abuse (Higley, Hasert, Suomi, & Linnoila, 1991) and fitness (reviewed in Taborsky, 2017).

The early social environment may cause long-term alterations of large-scale gene expression patterns in the brain (Marasco, Herzyk, Robinson, & Spencer, 2016; Sabatini et al., 2007; Weaver, Meaney, & Szyf, 2006; Zhang et al., 2018). For example, a socially enriched early-life environment increases the hippocampus volume in mice and is also associated with altered large-scale gene expression patterns in two parts of the dentate gyrus in the hippocampus in adult mice (Zhang et al., 2018). Likewise in rats, maternal deprivation during early life induces large-scale changes in adult hippocampus gene expression associated with brain formation and function (Weaver et al., 2006). Finally, in rhesus monkeys, postnatal deprivation of maternal care of different durations during early development induces differences in amygdala gene expression as measured later during juvenile development (Sabatini et al., 2007). While these experiments measured long-term effects of early experience on gene expression, we lack information on changes of genome-wide gene expression while or shortly after animals are exposed to divergent social experiences. Studying the effects of early-life social experience on large-scale gene expression data during the course of early ontogeny will provide valuable information about functions altered at the life stages when the substrate necessary for the expression of social behaviours is still developing (Aubin-Horth & Renn, 2009; Bar-Joseph, 2004).

Both transient and permanent changes of gene expression patterns can potentially contribute to long-term behavioural alterations (Hammock, 2013; Weaver et al., 2007, 2014). Genes differentially expressed in a transient manner could lead to a reorganization of brain tissue by specific cell growth and increased synaptic plasticity leading to reorganization of brain circuitry (Zupanc & Lamprecht, 2000), with potential long-term effects on social behaviour. For instance, in rats maternal separation increases expression of the brain-derived neurotrophic factor (*bdnf*), a gene involved in brain plasticity, in a transient manner in the prefrontal cortex during early development (Roceri et al., 2004). Furthermore, time series experiments on candidate gene expression in laboratory rodents showed that permanent gene expression changes can strongly depend on the timing when a social experience is received. For instance, the age when maternal separation was experienced affects the expression of the immediate-early gene *c-fos* and of corticotropin-releasing hormone (*crh*) expression the paraventricular nucleus (PVN) of the hypothalamus (Van Oers, de Kloet, & Levine, 1998). In another time series experiment, 24 hr of maternal separation detected a specific time-course of components of the HPA axis, like glucocorticoid receptor (*gr*) and *crh* expression, in two brain regions of the hypothalamus (Schmidt et al., 2004), which went in opposite directions during the first versus the second half of the separation period. Further, the effects on gene expression in time series experiments differ not only depending on when but also how often an experience is encountered (Horii-Hayashi et al., 2013). Casting a wider net by studying large-scale gene expression will yield information about the temporal pattern of gene expression of both predicted candidate pathways and unsuspected functional categories that are affected by early experience.

In vertebrates, the hypothalamus–pituitary–adrenal/interrenal (HPA/HPI) axis has been proposed as a central mechanism responsible for long-term effects of early social experience (Arnold & Taborsky, 2010; Banerjee, Arterbery, Fergus, & Adkins-Regan, 2012; Fischer et al., 2017; Francis et al., 1999; Liu et al., 1997; Nyman et al., 2017; Nyman, Fischer, Aubin-Horth, & Taborsky, 2018; Taborsky, Tschirren, Meunier, & Aubin-Horth, 2013). Hippocampal glucocorticoid receptors (GRs) are implicated in the negative feedback loop controlling glucocorticoid production, which promotes termination of the stress response (de Kloet, 1991). In rat pups, gene expression of *gr* in the hippocampus is increased with higher quality of maternal care (Liu et al., 1997) resulting in more stress-resilient individuals. Likewise, in the highly social cichlid fish *Neolamprologus pulcher*, individuals reared with (vs. without) brood caring group members are less neophobic (Bannier, Tebbich, & Taborsky, 2017) and more socially competent as juveniles (Arnold & Taborsky, 2010; Nyman et al., 2017) and adults (Taborsky et al., 2012) and feature a persistently altered stress gene expression in the brain (Nyman et al., 2017, 2018; Taborsky et al., 2013). In Japanese quails, pharmacologically activating the stress response causes large-scale changes in gene expression in adults, including hormone receptors implicated in the regulation of serotonin, somatostatin and corticotrophin-releasing factor (Marasco et al., 2016). Developmental experiments tend to study the effect of early experiences on altered stress gene expression measured *after* the experience. How this altered stress gene expression is affected on the large scale, and how it arises *during* the actual social experience phase is, however, largely a black box with unexplored content (Taborsky, 2016a). It is necessary to open this box if we aim to understand the origins of long-term early-life effects.

We performed a time series experiment with three time points to investigate changes in brain gene expression during and after juvenile exposure to two social environments in the cichlid fish *Neolamprologus pulcher*. *N. pulcher* is a cooperative breeder living in size-structured social groups consisting of a dominant breeder pair, their offspring and juvenile and adult alloparental brood care helpers (Balshine-Earn, Neat, Reid, & Taborsky, 1998; Taborsky, 1984, 1985). *N. pulcher* growing up with (+F) or without parents and helpers (−F) start to differ in major sociopositive and aggressive social behaviours already from day 49 after reaching the free-swimming stage (Arnold & Taborsky, 2010). Around day 75, fry start acting as brood care helpers by cleaning and fanning the eggs of the dominant breeding pair (Kasper, Kölliker, Postma, & Taborsky, 2017). Developmental plasticity of behaviour seen in *N. pulcher* (Arnold & Taborsky, 2010; Taborsky et al., 2012) could be regulated transiently or permanently by a variety of genes involved in social behaviour (Goodson, 2005; Oliveira, 2009; Robinson, Fernald, & Clayton, 2008). Therefore, we compared brain gene expression (GE) between juveniles reared with

parents, a helper and same-aged siblings (+F) to juveniles reared with siblings only (−F). In particular, we asked whether differential GE appears during the social experience phase (day 46 and day 56) or afterwards (day 75). We chose a whole transcriptome analysis using RNA sequencing (RNAseq) to allow querying all potential implicated biological functions without a priori predictions. Concurrently, it also allows us to test how the early social environment affects the stress axis, by focusing on a subset of 79 genes related to stress response regulation in fish. By using multidimensional scaling on the 79 genes, we compared the neurogenomic state of stress of each individual (Bukhari et al., 2017; Robinson et al., 2008) between treatments and time points. Based on earlier studies in this species, we predicted differences between rearing environments in expression of genes connected to neuronal plasticity, brain development and the stress response (Fischer, Bessert-Nettelbeck, Kotrschal, & Taborsky, 2015; Nyman et al., 2017, 2018; Taborsky et al., 2013). Using this time series approach, we can answer two important questions: (a) Is there a difference in gene expression at any point in time in *N. pulcher* raised with and without parents? (b) How does the early social environment affect the neurogenomic state of stress during early development?

2 | METHODS

2.1 | Study species

N. pulcher is a cooperatively breeding cichlid endemic to Lake Tanganyika, East Africa. Social groups consist of up to 25 juvenile and adult fish of both sexes organized in a linear, size-structured hierarchy (Taborsky, 2016b). A large, dominant breeder pair and several related or unrelated alloparental brood care helpers jointly defend a territory of 0.5–1 m in diameter. Breeders and all juvenile subordinates join in caring for offspring produced by the breeder pair about every 4 weeks (Kasper et al., 2017). Many subordinates stay at the territory and continue to act as helpers even long after sexual maturity (Taborsky, 2016b; Taborsky & Limberger, 1981). *N. pulcher* possess a large and refined repertoire of affiliative, submissive and aggressive social behaviours used to navigate the complexities of their social environment (Taborsky & Oliveira, 2012), helping to resolve within-group conflict and to maintain group stability (Taborsky, 1984; Taborsky & Taborsky, 2015). The early social environment during the first two months after reaching the fry stage strongly influences the ability to express this behavioural repertoire in an appropriate, context-specific manner (Arnold & Taborsky, 2010; Fischer et al., 2015; Nyman et al., 2017, 2018; Taborsky et al., 2012).

2.2 | Animal husbandry

The experiment was carried out 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. In five 200-L tanks, we set up five social groups consisting of an adult breeder pair (females were ≥5.0 cm standard length, SL; males were ≥0.5 cm larger than females) and two immature subordinates acting as brood care helpers (SL ~ 2 cm and ~3 cm, respectively). All tanks were provided with a 2 cm sand layer, a biological filter, and clay pot halves at the bottom and PET bottles mounted near the surface as shelters. The light:dark cycle was set to 13:11 hr including a 10-min dimmed light period in the mornings and evenings. The water temperature was kept at 27 ± 1°C. The social groups were fed 6 days a week ad libitum with commercial flake dry food ("Tetramin tropical flakes").

2.3 | Experimental broods

We started the experimental manipulation as soon as the five breeder pairs had produced a clutch and the eggs had developed into free-swimming fry (i.e. 10 days after egg laying). This time point was defined as day 0 of the experiment (see Figure 1). The respective tank was separated into two 100-L compartments; the five broods were captured and randomly distributed in equal numbers over the two compartments, creating 10 experimental groups. During

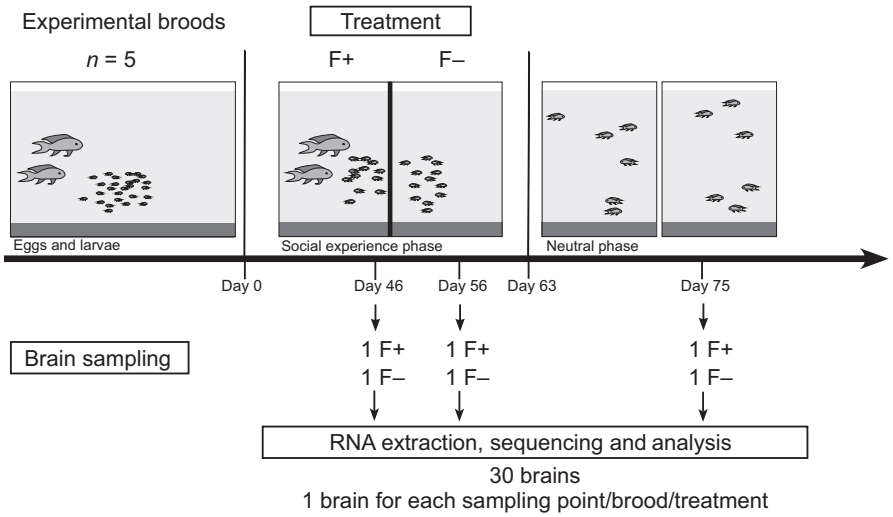

FIGURE 1 Experimental set-up and timeline of early-life experience and brain sampling

the following 62 days, the experimental juveniles were exposed to either of two social experiences. Half of a brood was reared with the parents and the two helpers (+F condition), and the other half was reared without any older conspecifics (same-aged siblings only; −F condition; see Arnold & Taborsky, 2010). Following the rearing protocol established by Arnold and Taborsky (2010), on day 63 the older group members were removed from the +F treatment and both treatments were reared under standardized social conditions only together with their siblings of the same treatment until the end of the experiment at day 75 ("neutral phase"). The neutral phase is important to distinguish whether changes in behaviour or gene expression are a developmentally plastic response to early-life conditions or whether they are just an immediate response to a stimulus (in our case the presence or absence of older fish). Juveniles were fed fine-grained "Tetramin Baby" flake food or live *artemia* nauplia, the amount of which was adjusted to brood size. When fry numbers started to differ between the two 100-L compartments of a tank, it was equalized by randomly selecting excess juveniles for culling at days 46 and 56.

2.4 | Brain sampling

We sampled brains at three time points during development: two time points during the social experience phase (days 46 and 56) and one time point shortly afterwards (day 75; Figure 1). Day 46 was chosen as the first sampling date, because at this age the brains were large enough for RNA extraction and juvenile *N. pulcher* start to show identifiable social behaviours (Arnold & Taborsky, 2010). Day 56, that is 10 days after the onset of the expression of social behaviours, was chosen as the second sampling date, because we expected that this onset of social activities would lead to an increased differentiation of the gene expression in +F and −F fish. Finally, we sampled brains at day 75 to test whether there is still a signature of early experience detectable in differential gene expression after young were kept for almost two weeks under standardized social conditions.

At each sampling day, all fry of a given brood from both treatment compartments (+F and −F treatment) were caught. Two individuals per treatment were randomly chosen with the help of a random number table. After sacrificing them with MS222, brains were dissected by a transversal cut with a razor blade and all brain tissue was extracted from the two resulting halves of the carcass and transferred into 1.5-ml Eppendorf vials filled with RNAlater. After 24 hr at 4°C, the brain samples were transferred to −18°C for storage until analysis. One brain was used for analysis (see below), and one brain was kept as a substitute.

2.5 | RNA extraction, library construction and sequencing

Total RNA was extracted from 30 whole-brain samples. We sampled fish from five broods, with one brain sampled per family and rearing treatment (+F and −F) per time point, yielding five brains per time point and treatment (in total 30 brains). RNA extraction was done from each individual brain separately. RNA extraction of samples from day 46 was performed with a different protocol than the other samples, because day 46 brains yielded less total RNA than day 56 and 75 brains. RNA extractions of day 46 brain tissues were carried out with the miRNA Easy Micro kit (Qiagen). The protocol was modified to avoid sampling miRNAs. For details on this protocol, see Nyman et al. (2017). RNA extraction of day 56 and day 75 brains followed a standard TRIzol RNA extraction protocol (Invitrogen).

RNA concentration was verified with a NanoDrop microvolume spectrophotometer (Thermo Scientific) for all brain samples. In some samples, the spectra indicated possible contamination of TRIzol/phenol in the end product. Therefore, an RNA clean-up kit (Qiagen) was applied to all samples, according to the manufacturer's instructions. After the clean-up procedure, we re-checked RNA concentration and confirmed clean composition with NanoDrop for all brain samples, and checked RNA quality using a Bioanalyzer 2100 instrument and an RNA 6000 Nano kit (Agilent). Samples were further quantified using a Quant-iT RiboGreen RNA Assay Kit (Invitrogen) to determine exact concentration of each sample post-clean-up protocol. Samples were stored at −20°C until the preparation of sequencing libraries.

We generated RNA libraries with barcodes from 30 samples using the "Illumina TruSeq RNA Sample Preparation version 2 Kit" following the manufacturer's instructions. We assessed library quality using a Bioanalyzer High Sensitivity DNA Assay (Agilent). Libraries were combined into five pools and brought to a final concentration that was standardized within each pool (mean concentration ± *SE* = 19.944 ± 0.153 ng/μl). Samples were sequenced on an Illumina HiSeq 2000 platform at the Génome Québec Innovation Center (Montréal, Québec, Canada). Eight samples were sequenced in a given lane, for a total of 40 samples (30 from this study and 10 additional ones from a separate study). Reads were paired-end 100 bp, with a separate barcode to sequence the sample index.

2.6 | Reference and mapping

A total of 185 GB of raw sequencing data were generated, which represents a total number of 3.82×10^9 Illumina reads of 100 bp (paired-end, total average million reads per sample ± std =23.920 M ± 0.424 M). Read quality was determined using FASTQC version 0.11.4 (https://www.bioinformatics.abraham.ac.uk/projects/fastqc/), and adapters, along with low-quality sequences, were removed using TRIMMOMATIC version 0.36 (Bolger, Lohse, & Usadel, 2014), with ILLUMINACLIP:2:30:10, SLIDINGWINDOW:20:2, LEADING:2, TRAILING:2, MINLEN:60. Trimmed reads were aligned to the genome of the Nile tilapia *Oreochromis niloticus* using TOPHAT version 2.1.1 (Trapnell, Pachter, & Salzberg, 2009). Based on the resulting alignment files, genome-wide read counts were extracted for every individual library (alignment pipeline available in Hebert, 2017). The reference genome sequence (accession ID: GCA_001858045.2, downloaded on 14 March 2017) was generated using the single-molecule real-time

sequencing technology (Pacific Biosciences) and comprised 37,848 coding sequences (Conte, Gammerdinger, Bartie, Penman, & Kocher, 2017). Only uniquely mapped reads were kept in the final alignment, while all multimapping reads were discarded, and all other alignment parameters kept to default mode. Raw read counts were calculated for each gene in the reference genome using HTSEQ-COUNT version 0.7.2 (mode: *intersection-nonempty*, see Anders, Pyl, & Huber, 2015 for details). The final numbers of reads obtained for each gene were considered as raw expression levels. Raw expression levels of all sequencing libraries were ultimately concatenated using custom PYTHON scripts (Hebert, 2017) and arranged in a read count matrix displaying, for each sample, the number of reads obtained for every gene in the genome. Gene annotation was added to the final read count matrix, based on the annotation information published with the genome (NCBI *O. niloticus*, Annotation Release 103, GCA_001858045.2, https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Oreochromis_niloticus/103/, date of Entrez queries for transcripts and proteins: 25 November 2016, date of submission of annotation to the public databases: 5 December 2016, software version: 7.2). In total, 1.6×10^9 (42%) high-quality trimmed reads successfully mapped to the reference genome with unambiguous matches ($1.33 \times 10^7 \pm 1.5 \times 10^6$ mapped reads per sample). The resulting read count matrix that was used for differential gene expression analysis displayed 38,425 transcripts (data deposited at Dryad, https://doi.org/10.5061/dryad.nk98sf7q6).

2.7 | Data analysis

Before analysing differential gene expression, we performed a principal component analysis (PCA) on all expressed genes and plotted all libraries along the PC1 and PC2 axes. This revealed two outlier libraries in the dataset, one +F and one −F sample of day 56, which were labelled as poor-quality libraries and excluded from further analysis, leaving in total 28 libraries for analysis. Genes with a read count of zero in all samples (n = 8,428) or with expression only in a single sample (n = 9,512) were excluded ahead of the analysis. In the analysis of the neurogenomic state of stress (see below), this led to the exclusion of three genes (GeneID: 100709371, GeneID: 100709917 and GeneID: 102082854).

2.7.1 | Expression during and after exposure to a social experience

To test for a difference in gene expression at any time point in *N. pulcher* raised with and without parents, differential expression analysis was run with the package DESEQ2 (Love, Anders, & Huber, 2014) with the BIOCONDUCTOR version 3.2. To normalize counts, DESEQ2 uses the "median-of-ratios" method taking into account sequencing depth and RNA composition and for filtering it uses an automatic built-in "independent filtering" algorithm (Love, Huber, et al., 2014). We analysed the time series data following the protocol reported in Love (2019). First, we tested for a significant interaction effect between time (days 46, 56 and 75) and treatment (+F and −F). We performed the differential gene expression analysis by fitting negative binomial generalized linear models using the likelihood ratio test (LRT) (recommended for time series data) and estimating the dispersion with the "parametric" setting. We also performed this analysis using a subset of the data containing only samples from day 56 and day 75, to verify that results were not affected by a different RNA extraction protocol that had to be used for samples from day 46. Second, we carried out comparisons of the treatments within each time point by fitting negative binomial generalized linear models applying the Wald test. We ran the following three contrasts: (a) day46+F versus day46−F, (b) day56+F versus day56−F and (c) day75+F versus day75−F. This analysis led to the exclusion of 266 genes identified as outliers in each of the three comparisons (not fitting the binomial distribution). p-Values were corrected for multiple testing using the false discovery rate (Benjamini & Hochberg, 1995). We used these adjusted p-values with a cut-off of .05 to identify significantly differentially expressed genes. We performed a GO enrichment analysis using the PYTHON package GOATOOLS (https://github.com/tanghaibao/goatools) on the differentially expressed genes (Fisher's exact test, p-value <.05) to identify specific biological processes or molecular functions significantly over-represented in this set of genes, as compared to all the expressed genes detected in the experiment.

2.7.2 | Neurogenomic state of stress

The neurogenomic state of stress is defined as the combined expression of genes associated with the stress response. Seventy-nine genes related to the stress response were selected from the literature (Table S1), including genes coding for receptors for dopamine, glucocorticoids (*gr*), mineralocorticoids (*mr*), oxytocin, neuropeptide Y, corticotropin-releasing hormone (*crh*), urotensin, arginine vasotocin (*avt*), pro-opiomelanocortin, serotonin, thyrotropin, histamine and receptors for the adrenergic system (Bonga, 1997; Lim, Porteus, & Bernier, 2013; Pavlidis, Sundvik, Chen, & Panula, 2011). Through its receptors, dopamine has both inhibitory and stimulatory effects on the HPI axis, while oxytocin, CRH, AVT, neuropeptide Y and thyrotropin have stimulatory effects (Bonga, 1997). For instance, head kidney tissue superfused with serotonin stimulates cortisol secretion from the interrenals in goldfish (Lim et al., 2013), and serotonin further stimulates CRF (corticotropin-releasing factor, homologue to CRH) gene expression (Heisler et al., 2007) and stimulates corticotropin (ACTH) from the pituitary (Jorgensen, Knigge, Kjaer, Moller, & Warberg, 2002). GR binds glucocorticoids with low affinity and shuts down the stress response through negative feedback, while MR also binds glucocorticoids with high affinity and is responsible for maintaining basal HPA axis activity (de Kloet, 1991). Urotensin has ACTH-stimulating activity (Arnold-Reed & Balment, 1994). Adrenergic receptors, through binding of catecholamine, maintain the fight-or-flight response (Plotsky, Cunningham, & Cunningham, 1989). Histamine release is increased by a mild stressor (Westerink et al., 2002), and higher histidine carboxylase (HDC, needed for histamine biosynthesis) levels were found in dominant zebrafish (Pavlidis et al., 2011).

TABLE 1 Genes differently expressed in our two treatments at time point day 75. Forty-three genes were down-regulated and two genes were up-regulated in the treatment −F compared to treatment +F

Gene_ID	Annotation	baseMean	FC	log2FC	lfcSE	stat	pvalue	padj	up/down
GeneID: 100711719	Probable E3 ubiquitin-protein ligase HERC3	5.209	−1.331	−0.413	0.061	−6.708	1.969e−11	5.642e−07	down
GeneID: 100709769	Interferon-induced GTP-binding protein Mx	54.768	−1.311	−0.391	0.063	−6.242	4.318e−10	3.388e−06	down
GeneID: 102077096	E3 ubiquitin-protein ligase rnf213-beta	73.049	−1.425	−0.511	0.081	−6.277	3.441e−10	3.388e−06	down
GeneID: 102080970	Probable E3 ubiquitin-protein ligase HERC3	14.292	−1.386	−0.471	0.076	−6.228	4.730e−10	3.388e−06	down
GeneID: 100700068	Uncharacterized LOC100700068	25.253	−1.463	−0.549	0.090	−6.069	1.290e−09	7.390e−06	down
GeneID: 100712569	Cytidine/uridine monophosphate kinase 2	52.060	−1.358	−0.441	0.076	−5.795	6.820e−09	3.256e−05	down
GeneID: 100697587	Probable E3 ubiquitin-protein ligase HERC6	45.294	−1.368	−0.452	0.079	−5.725	1.036e−08	4.063e−05	down
GeneID: 100708023	XIAP-associated factor 1	6.410	−1.300	−0.379	0.066	−5.709	1.135e−08	4.063e−05	down
GeneID: 106098388	Sacsin	9.230	−1.371	−0.455	0.081	−5.609	2.038e−08	6.485e−05	down
GeneID: 102078358	NLR family CARD domain containing 5	14.539	−1.384	−0.469	0.085	−5.518	3.430e−08	8.953e−05	down
GeneID: 109200992	Sacsin-like	9.506	−1.335	−0.417	0.075	−5.518	3.438e−08	8.953e−05	down
GeneID: 100694742	Probable E3 ubiquitin-protein ligase HERC4	7.429	−1.315	−0.395	0.072	−5.481	4.224e−08	0.0001	down
GeneID: 109204592	Polyubiquitin-like	8.198	−1.249	−0.321	0.061	−5.269	1.374e−07	0.0003	down
GeneID: 102081493	Protein asteroid homolog 1	6.833	−1.310	−0.389	0.075	−5.170	2.336e−07	0.0005	down
GeneID: 100702014	Ubiquitin-like modifier-activating enzyme 1	13.549	−1.298	−0.376	0.075	−5.033	4.835e−07	0.0009	down
GeneID: 100689935	Interferon alpha-inducible protein 27-like protein 2A	12.487	−1.296	−0.374	0.076	−4.940	7.806e−07	0.001	down
GeneID: 100701926	Interferon-induced protein with tetratricopeptide repeats 5	27.084	−1.362	−0.446	0.090	−4.949	7.477e−07	0.001	down
GeneID: 100703877	Receptor-transporting protein 3	7.626	−1.279	−0.355	0.072	−4.929	8.267e−07	0.001	down
GeneID: 109197443	Sacsin-like	11.655	−1.296	−0.374	0.076	−4.892	9.992e−07	0.002	down
GeneID: 100709784	Helicase with zinc finger 2	20.075	−1.257	−0.330	0.069	−4.797	1.607e−06	0.002	down
GeneID: 106098710	Polyubiquitin-B-like	3.824	−1.235	−0.305	0.064	−4.786	1.704e−06	0.002	down
GeneID: 102082855	Epithelial stromal interaction 1	35.537	−1.315	−0.395	0.083	−4.767	1.868e−06	0.002	down
GeneID: 100710414	Interferon-induced protein 44	34.078	−1.255	−0.327	0.069	−4.740	2.132e−06	0.003	down

(Continues)

TABLE 1 (Continued)

Gene_ID	Annotation	baseMean	FC	log2FC	lfcSE	stat	pvalue	padj	up/down
GeneID: 100697780	C-X-C motif chemokine 10	9.702	−1.146	−0.197	0.042	−4.693	2.687e−06	0.003	down
GeneID: 100703276	GTPase IMAP family member 7	5.504	−1.214	−0.280	0.060	−4.679	2.878e−06	0.003	down
GeneID: 106098386	Sacsin	4.600	−1.237	−0.307	0.066	−4.654	3.249e−06	0.004	down
GeneID: 109198210	Sacsin-like	8.952	−1.277	−0.352	0.076	−4.635	3.574e−06	0.004	down
GeneID: 102082289	Interferon alpha-inducible protein 27-like protein 2A	27.145	−1.331	−0.412	0.089	−4.612	3.998e−06	0.004	down
GeneID: 100710944	Receptor-transporting protein 2	7.806	−1.142	−0.192	0.042	−4.562	5.060e−06	0.005	down
GeneID: 100695838	Poly(ADP-ribose) polymerase family member 12	100.375	−1.380	−0.464	0.104	−4.479	7.506e−06	0.007	down
GeneID: 100694586	Glutamate ionotropic receptor NMDA-type subunit 2B	2,220.346	−1.246	−0.317	0.072	−4.437	9.108e−06	0.008	down
GeneID: 100693739	Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit A	1,084.572	1.255	0.328	0.075	4.391	1.129e−05	0.010	up
GeneID: 109195964	Interferon alpha-inducible protein 27-like protein 2A	21.000	−1.328	−0.410	0.094	−4.342	1.414e−05	0.012	down
GeneID: 109200726	Sacsin-like	4.332	−1.219	−0.286	0.068	−4.221	2.430e−05	0.020	down
GeneID: 102080780	Probable E3 ubiquitin-protein ligase HERC3	3.554	−1.202	−0.265	0.063	−4.197	2.709e−05	0.022	down
GeneID: 100699154	Cell adhesion molecule 1	1,318.789	−1.189	−0.250	0.060	−4.164	3.127e−05	0.024	down
GeneID: 100707300	Polyubiquitin	1.655	−1.156	−0.210	0.050	−4.165	3.120e−05	0.024	down
GeneID: 100703374	Uncharacterized LOC100703374	1.174	−1.108	−0.148	0.036	−4.133	3.576e−05	0.027	down
GeneID: 100705426	Low-density lipoprotein receptor-related protein 6	193.811	−1.332	−0.414	0.101	−4.085	4.402e−05	0.032	down
GeneID: 106097615	Sacsin	6.589	−1.205	−0.269	0.066	−4.067	4.756e−05	0.034	down
GeneID: 100692457	Short transient receptor potential channel 4	14.549	1.409	0.494	0.122	4.051	5.100e−05	0.036	up
GeneID: 100700242	Talin-2	1737.778	−1.199	−0.262	0.065	−4.035	5.462e−05	0.037	down
GeneID: 100696359	Signal transducer and activator of transcription 1	909.394	−1.375	−0.459	0.114	−4.024	5.730e−05	0.038	down
GeneID: 102081053	Interferon-induced protein with tetratricopeptide repeats 5	11.993	−1.275	−0.350	0.087	−4.018	5.876e−05	0.038	down
GeneID: 100699831	Nuclear receptor corepressor 2	2,479.319	−1.196	−0.259	0.065	−3.986	6.706e−05	0.043	down

Note: We used adjusted *p*-values (padj) with a cut-off of .05 to identify significantly differentially expressed genes. Gene_ID and annotation refer to the identity of the genes according to the Nile tilapia genome, accession ID: GCA_001858045.2. "baseMean": the average of the normalized count values, dividing by size factors and taken over all samples. "FC": fold change. "log2FC": log2 foldchange. "lfcSE": standard error estimate for the log2 fold change estimate. "stat": Wald statistic. pvalue: *p*-value. "padj": adjusted *p*-value. "up/down": gene up- or down-regulated in the −F fish condition compared to the +F condition.

We performed a nonlinear multidimensional scaling (NMDS) analysis on the 79 genes, in order to collapse the information from these genes in only two dimensions, using the resulting clustering patterns to compare the neurogenomic state of stress between individuals from different treatments and time points. NMDS is a robust unconstrained ordination method used frequently in community ecology (Minchin, 1987). This analysis aimed to highlight differences between the neurogenomic state of stress of the two treatments at each of the three time points. We used the function "monoMDS" of the R package "VEGAN 2.5-5" (Oksanen et al., 2019). This function performs nonmetric multidimensional scaling (NMDS) and tries to find a stable solution using several random starts. In addition, it standardizes the scaling in the result, so that the configurations are easier to interpret. We ran an NMDS with two dimensions. The scaled data accounted for 95% of the variance of the raw data (nonmetric fit, R^2 = .95, Figure S1).

We determined whether NMDS scores differed significantly between treatments and time points by running two separate LMMs on the NMDS scores for each of the two dimensions (NMDS1 and NMDS2) using the package "LME4" of the statistical software "R" (Bates, Maechler, Bolker, & Walker, 2015). For both initial models, we included treatment (2 levels), time point (3 levels), and their interaction as fixed factors, and experimental brood (5 levels) as random factor. The model had a Gaussian error structure. Conformance with a normal error structure was evaluated by visual inspection of the distributions of residuals, quantile–quantile (Q–Q) plots and plots of predicted versus. fitted values as well as by a Shapiro–Wilk test and a Kolmogorov–Smirnov test with Lilliefors correction. Significance testing was based on deviance when removing respective terms from the model using the R command "drop1" (a using a Satterthwaite approximation for degrees-of-freedom yielding *F*-values (Singmann, Bolker, & Westfall, 2015) and (b) a likelihood ratio test (LRT) giving chi-square values. All statistical analyses were performed using R version 3.5.0 (R Core Development team, 2018).

3 | RESULTS

3.1 | Differentially expressed genes

Modelling the effect of social rearing treatment, the three time points and their interaction did not reveal any significantly differently expressed genes (DEGs). A subsequent, separate analysis for each time point revealed 45 DEGs between +F and −F, after the end of the social experience (day 75), whereas no genes were differentially expressed between treatments *during* the social experience (days 46 and 56). In the −F treatment, 43 genes were down-regulated at day 75, whereas only two genes were up-regulated compared to the +F condition (Table 1). One of the up-regulated genes, TRPC4 (short transient receptor potential channel 4), is a calcium-permeable cation channel, regulating Ca(2+) homeostasis, suggested to be important for learning and memory (Fowler, Sidiropoulou, Ozkan, Phillips, & Cooper, 2007). The other up-regulated gene ANKRD28 (serine threonine-protein phosphatase 6 regulatory ankyrin repeat subunit A) is a protein-coding gene important for vesicle coating. Gene ontology (GO) term(s) of each DEG is listed in the Supporting Information (SI, Table S2). Based on GO terms for biological processes on the UniProt website (www.uniprot.org) and pertinent literature (Engert et al., 2000; Fowler et al., 2007; Liu et al., 2014; Mabb & Ehlers, 2010; Ness et al., 2011), we assigned the 45 DEGs to one of eight general functional categories (Table S3). The down-regulated genes are, among others, associated with immunity, post-translational modifications of proteins and brain function. In total, we were able to assign 38 GO terms to 10 different DEGs. We did not detect any enrichment among the GO terms assigned to DEGs, as compared to the rest of the transcriptome. None of the predefined genes associated with stress regulation were differentially expressed in this whole transcriptome analysis.

3.2 | Neurogenomic state of stress

The neurogenomic state of stress differed between the time points sampled, and the change in expression levels across time points seems to be specific to each social treatment (Figure 2). To quantify this visual trend, we analysed the scores of the first and second dimension of the NMDS analysis for the 79 genes related to stress regulation. The LMMs on the NMDS1 scores of each individual sample revealed a significant interaction between the social treatment (+F or −F) and the time points (Table 2). Figure 2 suggests that the interaction is mostly due to NMDS1 scores of +F fish sampled at day 75. While overall the NMDS1 scores becomes smaller with increasing age, in +F fish at day 75, the shift towards smaller scores is stalled (note that the scaling of the scores along the NMDS axes is arbitrary and just indicates how different individuals are in their neurogenomic state, i.e. it does not relate to higher or lower gene expression).

4 | DISCUSSION

Lasting effects of the early social environment on behaviour by means of developmental plasticity have been documented in a range of vertebrates (reviewed in Taborsky, 2016a) and invertebrates (e.g. Kasumovic & Brooks, 2011). However, information on gene expression changes influencing phenotypic development during ontogeny remains scarce (Horii-Hayashi et al., 2013; Schmidt et al., 2004; Vazquez et al., 2006). Using a time series approach, we showed that in the cichlid fish *Neolamprologus pulcher* social experience influences whole-brain gene expression during a stage when these fish have already developed a social behaviour repertoire (see Arnold & Taborsky, 2010) and are at the verge of becoming brood care helpers (Kasper et al., 2017). At developmental day 75, that is, *after* the experimental experience phase, all but two of 45 DEGs were down-regulated in fish having experienced social deprivation. In contrast, *during* the experience phase no genes were differentially expressed when assessing the whole transcriptome. When focusing on the neurogenomic state associated with the stress response, we found that the first dimension (NMDS1) was interactively influenced by social treatment and time point sampled, meaning that individuals from

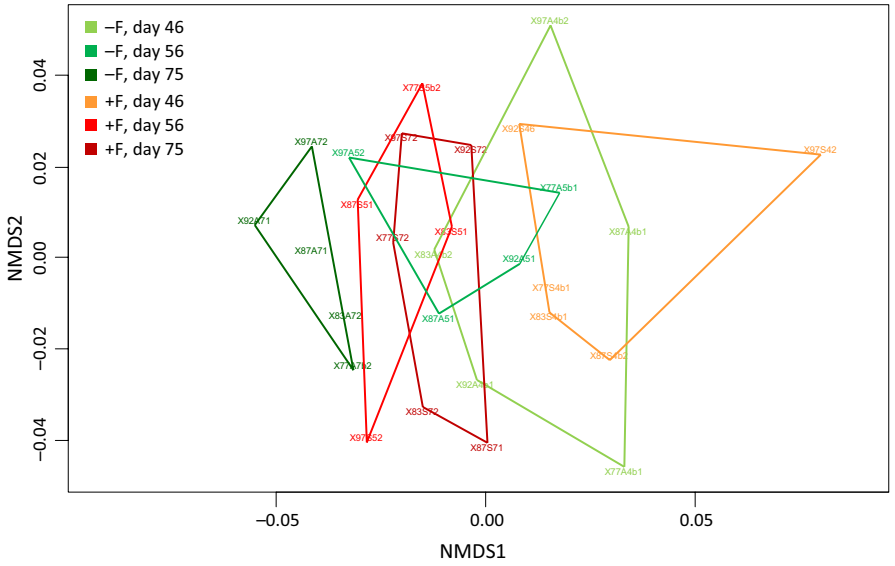

FIGURE 2 NMDS map of the 28 brain samples. Shades of green: −F treatment, shades of red: +F treatments. Increasing age (sampling time points) of the fish is indicated by increasingly darker colours

TABLE 2 Linear mixed models testing the effect of treatment (+F or −F) and sampling time points on the scores of NMDS dimensions 1 and 2

	Estimate	*SE*	Single term deletion	*F*	*p*	X^2	*p*
NMDS dimension 1							
Full model							
Treatment	0.016	0.012					
Time point 56	−0.019	0.013					
Time point 75	−0.055	0.012					
Treatment × Time point 56	−0.032	0.018	Treatment × Time point	3.31	*.056*	7.36	**.025**
Treatment × Time point 75	0.012	0.017					
NMDS dimension 2							
Model without interaction							
Treatment	0.00013	0.0098	Treatment	0.0002	.99	0.00003	.99
Time point 56	0.0054	0.012	Time point	0.17	.84	0.40	.82
Time point 75	−0.0017	0.012					

Note: Estimates and standard errors (*SE*) are taken from the R summary table of a model; *F* and their respective *p* value are determined by the Satterthwaite method; and chi-square and their respective *p*-values are determined by LRT. *p* Values <.05 are highlighted in bold, and values .05 < *p* < 1.0 are highlighted in italics.

the two social treatments differed in how their brain gene expression profiles changed over developmental stages. While overall the NMDS1 scores become smaller with increasing age, in +F fish, the shift towards smaller scores is deferred at day 75.

4.1 | Is there a difference in the transcriptome at any point in time in *N. pulcher* raised with and without parents?

4.1.1 | Differentially expressed genes after the experience phase

In our time series, the 45 genes that were differentially expressed between treatments at the sampling time point shortly after the experience phase (day 75) were broadly assigned to eight general functional categories including immunity, brain function and the post-translational modifications of proteins (Tables S2 and S3 in SI). Considering the functions of the DEGs, we suggest that fish reared in the natural +F condition develop skills that might improve their physical state and immune function as well as behavioural flexibility and memory after being reared with older group members. Our findings indicate that in the stage when fish develop and increasingly express social behaviours (Arnold & Taborsky, 2010), important modifications of key neural networks take place, with likely consequences for their further behavioural development. This interpretation based on putative annotations associated with these genes will necessitate further testing. All genes except two of the differentially expressed genes were down-regulated in −F fish, relative to +F fish. Visual inspection of normalized count plots for the six combinations

of treatments x time points revealed that lower expression values in −F fish came about by two patterns: (a) expression in −F fish remained low at all three time points as well as days 46 and 56 in +F fish, while +F fish at day 75 have higher expression, suggesting a lack of activation of these genes on day 75 in −F fish (b) the expression of genes in −F fish is reduced at day 75 as compared to the first two time points, whereas the expression in +F fish stays high at all time points. Similar patterns of relative low gene expression after incurring social stress have been demonstrated in birds (Marasco et al., 2016). This down-regulation relative to +F fish, if we equate mRNA levels with subsequent protein quantity, suggests that the −F fish have a reduced activation of important neuronal pathways, possibly influencing social behavioural plasticity. This interpretation would be true for genes that code for proteins that have a positive effect on the function they are associated with, and exceptions should be verified, for example, proteins acting as repressors of a function.

Down-regulated genes

Twelve of the DEGs down-regulated at day 75 have described functions connected to immune function. Among these, the C-X-C motif chemokine 10 (CXCL10) and cell adhesion molecule 1 (CADM1) are involved in social behaviour in mice. Blank et al. (2016) detected that treating brain endothelial cells with CXCL10 causes behavioural changes through decreased synaptic plasticity in male mice, while cell treatment with interferons (proteins released upon the presence of pathogens) caused up-regulation of CXCL10. CADM1 knockout in mice has been shown to cause disruption in the regulation of social and emotional behaviours (Takayanagi et al., 2010). Down-regulation of immunity genes in *N. pulcher* possibly indicates altered behavioural regulation and impaired immune system function in juveniles reared in socially deprived condition. Differentially expressed genes at day 75 also include 12 genes related to post-translational modifications of proteins. These genes include the polyubiquitin genes and HERC genes, involved in ubiquitination and cell differentiation. Behavioural studies in mice indicate that in memory consolidation, synaptic plasticity (reviewed in Bailey, Bartsch, & Kandel, 1996) and ubiquitin-dependant protein degradation (Artinian et al., 2008) are crucial elements. All genes involved in post-translational modifications of proteins, except ANKRD28, were down-regulated in −F fish, possibly contributing to reduced synaptic plasticity and hence reduced memory formation. Another ten DEGs were categorized to have a function in brain development, memory and behavioural regulation. Genes down-regulated in −F fish in this category contain different sacsin genes, the glutamate receptor subunit gene (GRIN2B) and the low-density lipoprotein receptor-related protein 6 (LRP6). GRIN2B is involved in behavioural fear response, memory and cerebral cortex development among other functions. This gene has been found to be connected to risky decision-making (Ness et al., 2011), obsessive–compulsive disorders (Alonso et al., 2012) and attention-deficit/hyperactivity disorder (Dorval et al., 2007). Mutations in the sacsin genes are associated with a neurodegenerative disease called autosomal recessive spastic ataxia of Charlevoix–Saguenay (ARSACS or SACS) in humans (Engert et al., 2000), and the gene LRP6 has been linked to memory function and Alzheimer's disease (Liu et al., 2014). Down-regulation of these genes in −F fish may suggest potentially altered memory functions and behavioural regulation.

Up-regulated genes

The genes TRPC4 (short transient receptor potential channel 4) and ANKRD28 (serine threonine-protein phosphatase 6 regulatory ankyrin repeat subunit A) were both up-regulated in the −F treatment. TRPC4 is associated with oligodendrocyte differentiation and ion transport (Table S2, SI). It is expressed in the brain and mediates transfer of sensory information (Clapham, 2007). This receptor channel has been proposed to be a tool for regulation of social and fear-related behaviours but also plasticity. For instance, TRPC4 knockout in mice leads to decreased anxious-related behaviour (Riccio et al., 2014) and to reduced social anxiety and less exploration in rats (Rasmus et al., 2011). TRPC4 channels are suggested to modify synaptic plasticity involved in learning and memory (Fowler et al., 2007). Up-regulation of this gene could therefore contribute to an increased anxiousness of −F fish, which also show more neophobic responses in behavioural tests (Bannier et al., 2017), but it might also reflect increased neuronal plasticity. ANKRD28 is involved in COPII vesicle coating, but a role of this gene in social behaviour has not been reported.

4.1.2 | Expression during exposure to a social experience

During the social experience phase (days 46 and 56), there were no significant transcriptomic differences between the two treatments, which is striking considering the previously described behavioural differences between +F and −F fish observed during the social experience phase (Arnold & Taborsky, 2010; Fischer et al., 2017). Moreover, candidate gene studies in rodents showed an effect of maternal separation on gene expression during the phase when early experiences were made. In maternal separation experiments in mouse pups, time series experiments showed that separation induces temporal gene expression changes of key components of the HPA axis (Schmidt et al., 2004) and *c-fos* gene expression in various brain regions (Horii-Hayashi et al., 2013). In rat pups, maternal deprivation across different time points influenced the expression of the CRF gene in different brain regions, with treatment and time affecting CRF expression interactively (Vazquez et al., 2006). Our contradictory findings could be due to variability of gene expression in different regions of the brain. It may be difficult to detect differentially expressed genes during the developmental experience phases, possibly because gene expression is more diverse and changeable between individuals while they are experiencing different social environments, whereas differences become apparent once all animals are kept under standardized conditions. Our statistical power to detect biologically meaningful changes in a low number of genes in the early phases of development could also result in this null result. The challenge for future studies will be to produce a more detailed

description of developmental changes in region-specific gene expression. For that, a brain atlas for *N. pulcher* is under progress and will be used to guide region-specific tissue sampling prior to gene expression analysis.

4.2 | How does the early social environment affect the neurogenomic state of stress during early development?

The effect of developmental age on the neurogenomic state of stress differed between −F and +F fish, as indicated by the score along the first dimension of the NMDS analysis. Visual inspection of the NMDS map suggests that this statistical interaction is related to the stress state of fish at day 75. While overall the NMDS1 scores become smaller with increasing age, in +F fish at day 75, the shift towards smaller scores is stalled. Our results suggest that alterations of gene expression and the neurogenomic state of stress reported here influence the dissimilar developmental trajectories between juveniles of +F and −F fish (Fischer et al., 2017) and may explain why these trajectories already start to diverge very early in development. Moreover, our results corroborate previous findings of behavioural changes during the first two months of life in *N. pulcher* (Arnold & Taborsky, 2010; Fischer et al., 2015, 2017; Taborsky et al., 2012). Future studies will reveal whether the observed expression differences are also *causally* related to age-dependent social behavioural changes during early development, and whether these gene expression differences are transient or permanent. Previous studies in *N. pulcher* established that the early social environment affects molecular mechanisms of stress regulation much later in life and that the effects on stress axis programming are mediated by an evolutionary conserved molecular pathway in these fish (Nyman et al., 2017, 2018; Taborsky et al., 2013). These studies, however, treated molecular processes *during* the important social experience phase essentially as a black box. Our study opens this black box for the first time, and although there were no genes differentially expressed during this phase when quantifying the entire transcriptome at once (see discussion above), the neurogenomic state of stress differed between the two social treatments. This shift in neurogenomic state might implicate consequences for the coordinated activation of the molecular networks involved in regulation of stress, possibly with ensuing long-term effects on stress reactivity and social performance of *N. pulcher* (Bannier et al., 2017; Nyman et al., 2018; Taborsky & Oliveira, 2012).

5 | CONCLUSIONS

This study has two major results. (a) We found gene expression differences appearing after the social experience phase, which are related to molecular networks possibly affecting behaviour, memory and immune system function differently in the two treatments. (b) We detected dynamic changes of the neurogenomic state of stress during early life, with the most important of these changes again occurring after the social experience phase, which may be involved in developmentally induced changes of stress axis regulation observed in later life stages of vertebrates (Nyman et al., 2018; Sabatini et al., 2007; Taborsky et al., 2013; Weaver et al., 2006). The early social environment has been shown to induce altered stress axis function in mammals, and now also in fish. Whether these findings can be applicable to other vertebrates still awaits further studies. Sampling large-scale gene expression changes at different time points during development is of great importance for future studies if we aim to understand how early social experience shapes developmental plasticity giving rise to various phenotypes.

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AUTHOR CONTRIBUTIONS

M.B.N., B.T. and N.A.H. designed the study. M.B.N. bred and reared the fish as well as performed brain sampling. C.N. did the gene expression laboratory work. Mapping and read count analysis were prepared by F.O.H. C.N. and B.T. performed the statistical analysis. C.N., B.T., N.A.H. and F.O.H. drafted the manuscript. All authors have approved the content of the manuscript.

DATA AVAILABILITY STATEMENT

Files with gene expression values have been deposited at Dryad, https://doi.org/10.1111/mec.15335).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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