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Divergent brain gene expression profiles between alternative behavioural helper types in a cooperative breeder

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Abstract

Juveniles of the cooperatively breeding cichlid fish *Neolamprologus pulcher* either consistently provide help in form of alloparental egg care ("cleaners") or consistently abstain from helping ("noncleaners"). These phenotypes are not based on heritable genetic differences. Instead, they arise during ontogeny, which should lead to differences in brain structure or physiology, a currently untested prediction. We compared brain gene expression profiles of cleaners and noncleaners in two experimental conditions, a helping opportunity and a control condition. We aimed to identify (a) expression differences between cleaners and noncleaners in the control, (b) changes in gene expression induced by the opportunity and (c) differences in plasticity of gene expression between cleaners and noncleaners. Control cleaners and noncleaners differed in the expression of a single gene, *irx2*, which regulates neural differentiation. During the opportunity, cleaners and noncleaners had three upregulated genes in common, which were implicated in neuroplasticity, hormonal signalling and cell proliferation. Thus, the stimulus in the opportunity was sufficiently salient. Cleaners also showed higher expression of seven additional genes that were unique to the opportunity. One of these cleaner-specific genes is implicated in neuropeptide metabolism, indicating that this process is associated with cleaning performance. This suggests that the two types employed different pathways to integrate social information, preparing them for accelerated reaction to future opportunities. Interestingly, three developmental genes were downregulated between the control and the opportunity in cleaners only. Our results indicate that the two behavioural types responded differently to the helping opportunity and that only cleaners responded by downregulating developmental genes.

KEYWORDS
cooperation, genomic reaction norms, *irx2*, phenotypic plasticity, telencephalon, transcriptome profile

1 | INTRODUCTION

Cooperation is widespread in animals and the fitness benefits and specific ecological and social circumstances under which cooperation evolves are increasingly understood (Bourke, 2011; Ghoul, Andersen, & West, 2017; Kasper, Vierbuchen, et al., 2017; Sachs, Mueller, Wilcox, & Bull, 2004; Taborsky, Frommen, & Riehl, 2016; West, Griffin, & Gardner, 2007). In cooperative breeders, where nonbreeding caregivers help raise offspring (Bergmüller, Johnstone, Russell, & Bshary, 2007; Lukas & Clutton-Brock, 2012), individual helpers vary in the amount of alloparental care ("helping") they provide, in contrast to eusocial species in which helping is obligatory. Consistent phenotypic differences in the contribution to direct alloparental care such as feeding or grooming foreign offspring, territory defence and

territory maintenance have been reported (Carter, English, & Clutton-Brock, 2014; English, Nakagawa, & Clutton-Brock, 2010; Kasper, Kölliker, Postma, & Taborsky, 2017; Le Vin, Mable, Taborsky, Heg, & Arnold, 2011). Both environmental influences on development (English, Browning, & Raihani, 2015; Fischer, Bohn, Oberhummer, Nyman, & Taborsky, 2017; Kasper, Kölliker, et al., 2017;) and genetic variation (Charmantier, Keyser, & Promislow, 2007; Sparkman, Adams, Steury, Waits, & Murray, 2012) can underlie those consistent differences.

Plasticity in behaviours can occur on different timescales. Environmental influence can be integrated during development, which results in relatively stable organizational effects, but individuals also remain behaviourally flexible to some degree and are able to immediately react to challenges. In cooperative breeders, ecological and social influences during ontogeny can induce "helper phenotypes," which show a persistently higher propensity to provide alloparental care, for instance through self-reinforcing processes during social niche specialization (Bergmüller & Taborsky, 2010). However, helpers also need to fine-tune their behaviour to short-term environmental conditions (Koenig, Pitelka, Carmen, Mumme, & Stanback, 1992; Stiver, Dierkes, Taborsky, & Balshine, 2004), such as sudden changes of help needed by beneficiaries or demand of help by dominant group members, requiring the maintenance of behavioural flexibility (Holekamp, Swanson, Van Meter, & Holekamp, 2013). Both developmental plasticity and short-term behavioural flexibility could manifest at the molecular level as differences in brain gene expression, which can be quantified (Aubin-Horth & Renn, 2009; Nyman, Fischer, Aubin-Horth, & Taborsky, 2017). Investigating the molecular mechanisms of helping would shed light on the molecular basis of plasticity in cooperatively breeding species. This knowledge could contribute to the identification of genomic "toolkits" for cooperative behaviour (i.e., shared genes or gene networks that underlie repeated evolution of cooperation across the animal kingdom, (Rittschof & Robinson, 2014). This is of particular interest since cooperation has evolved multiple times independently (Sterelny, Joyce, Calcott, & Fraser, 2013), and comparisons of the sets of genes and networks implicated in transitions from noncooperative ancestors to cooperative species could yield interesting insights in the commonalities or disparities of the parallel evolution of cooperation.

Variation in helping due to developmental plasticity as well as behavioural flexibility has been described in the cooperatively breeding African cichlid fish *Neolamprologus pulcher* (Fischer et al., 2017; Kasper, Kölliker, et al., 2017; Zöttl, Heg, Chervet, & Taborsky, 2013). Dominant *N. pulcher* breeder pairs monopolize reproduction, and several subordinate helpers assist with direct brood care (egg cleaning) and other activities, for instance the removal of sand from the breeding chamber and defence against fish and egg predators (Balshine, Leach, Neat, Reid, & Taborsky, 2001; Taborsky, 1984). In this species, consistent individual differences in egg care have been described that are not due to heritable genetic variation, but are shaped by developmental plasticity (Kasper, Kölliker, et al., 2017). Juvenile subordinates consistently invest either in alloparental egg care ("cleaners") or consistently abstain from helping ("noncleaners"). Previous studies suggest that individuals that do not engage in helping behaviours instead display submissive behaviours. This facilitates their acceptance in the territory that provides protection from predation, representing an alternative social strategy to helping (Bergmüller & Taborsky, 2005; Fischer et al., 2017; Kasper, Colombo, Aubin-Horth, & Taborsky, 2018). Theoretical models suggest that the existence of consistent behavioural helper types facilitates the evolution and maintenance of cooperative breeding because individuals thereby reliably signal their commitment to helping and their renunciation of reproduction (Bergmüller, Schürch, & Hamilton, 2010), facilitating partner choice and assortment of cooperation partners (Barta, 2016; McNamara, Barta, & Houston, 2004). However, the molecular and neural underpinnings of these alternative helper types have not yet been identified. Consequently, it is unknown whether brain physiology or structure differs permanently between the types or whether the brains of the two behavioural helper types solely show transient differences during helping opportunities.

In the present study, we quantified brain gene expression differences in juveniles of the cooperatively breeding cichlid fish *N. pulcher* either in a resting (control) condition or when given the opportunity to provide alloparental egg care. We analysed the telencephalon, because it has been implicated in social cognition and cooperation in teleosts (Bshary, Gingins, & Vail, 2014; Pollen et al., 2007) and in a previous study that investigated neural activation during alloparental egg care in *N. pulcher* (Kasper et al., 2018). Since information on the role of single candidate genes or regulatory networks in the cooperative-breeding context is virtually absent, whole transcriptome sequencing techniques are the best tools to identify relevant patterns of gene expression (Calisi & MacManes, 2015). Using a 2×2-design of two different cooperative phenotypes ("cleaners" and "noncleaners") in juvenile subordinates in two experimental conditions ("opportunity" and "control"), we compared whole transcriptomes of the following contrasts. First, we tested whether gene expression profiles of cleaners differed from those of noncleaners within the control condition to gain insights into persistent, long-term transcriptomic differences induced during development between the two types. Second, we investigated transient changes in gene expression that were specifically associated with information processing as well as performing direct brood care behaviour during the helping opportunity. The expression of different sets of genes by cleaners and noncleaners during the opportunity would suggest that those types differ in their genomic reaction norms, that is, "the number and nature of genes that vary in expression" (Aubin-Horth & Renn, 2009). Thus, comparing expression levels of particular genes between the two helper types and their response to the opportunity would lend additional insights whether differences in genomic reaction norms exist between helper types.

2 | MATERIAL AND METHODS

2.1 | Study animals and behavioural tests

To ensure that the study animals represented a natural range of genetic variation found in the wild, we used laboratory-bred F_1 offspring of parents caught in Lake Tanganyika, Zambia. Forty-two

breeding pairs were randomly assigned to 60-L tanks and removed 8–10 days after spawning a clutch, which is approximately the time when they cease to provide direct parental care (defined as "day 0"). Thus, all fish used in the experiment grew up in groups with full-siblings of the same age and were kept under similar temperature, feeding and light conditions. As we used test fish in this experiment that were bred within the scope of a study that investigated the effect of early-life exposure to egg predators on later helping behaviours (Kasper, Kölliker, et al., 2017), the majority (61%) had frequently encountered live egg predators previous to the behavioural tests. On day 85, we randomly selected experimental subjects with an average body length of 18.4 mm (±1.8 mm standard deviation) to be tested later, on day 100, in the alloparental care opportunity ("egg-cleaning task"). The experimental subjects were transferred to 20-L tanks equipped with a sand layer and a clay flowerpot cut in half that served as a shelter. On the following day, we added a larger, unrelated territory owner (Figure 1), which was kept together with the test fish for the following 7 to 14 days to induce a subordinate status in the test fish, as only subordinate individuals show alloparental egg care behaviour (von Siemens, 1990). The behavioural tests took place in the 20-L tanks to avoid handling stress and exposing the test fish to a novel environment before the experiments that could complicate the interpretation of gene expression results. All fish used in this experiment were subjected either twice to the same behavioural test, the "helping opportunity," or first to the opportunity and then to a control situation. The helping opportunity was designed to closely mimic the natural situation of a juvenile helper in a *N. pulcher* group while keeping the social environment as simple as possible: In the presence of a dominant territory owner, the test fish was given the opportunity to perform direct alloparental brood care (cleaning of a clutch) and to defend the brood (attacks and aggressive displays) against the sympatric unspecialized egg predator *Telmatochromis vittatus* that is abundant in *N. pulcher* habitats in the wild (Bruintjes & Taborsky, 2011; Ochi & Yanagisawa, 1998). *Telmatochromis vittatus*, a small snail-brooding cichlid with an elongated shape, is a facultative ovivore but also feeds on plankton (Karino, 1998) and often enters *N. pulcher* breeding cavities and preys on eggs and larvae (Konings, 1998). Due to its small size and jaw morphology, the egg predator posed no threat to juveniles of the size-range we used in this experiment. In addition to performing cleaning and defence behaviours, the test fish and the dominant individual could also interact socially (aggressive displays and attacks of the dominant, submissive displays of the test fish). However, we confined the dominant to a transparent plastic container for the duration of the test to prevent it from directly interacting with the clutch or the egg predator (Figure 1). The dominant was potentially able to see the egg predator, but not the clutch, and also to communicate with the test fish via behavioural or olfactory cues in a limited way (the containers of the dominant and the egg predator were placed on diagonally opposite corners of the tank). In *N. pulcher*, helping behaviour takes place within the context of the social group and group members have been shown to aggressively interact with idle helpers (Fischer, Zöttl, Groenewoud, & Taborsky, 2014). Thus, a potential audience effect of a helper expressing alloparental brood care in the presence of a dominant would represent a natural aspect of the helping opportunity. We directly recorded egg cleaning (nibbling on eggs to remove microbial overgrowth), submissive displays of the test fish towards the tube containing the dominant (i.e., tail quivering, trembling movement with tail or whole body) and defence behaviours towards the egg predator (displays and attacks) for 20 min (see full details of the behavioural tests in Kasper, Kölliker, et al. (2017). All test fish (N = 78) were exposed to the first helping opportunity test at around day 100. This first test was done to preliminarily classify test subjects as "cleaners" or "noncleaners," depending on whether they responded to the presentation of a clutch spawned by an unrelated pair by performing egg cleaning. The repeatability of cleaning propensity, which is performing this behaviour or not, is rather high in this species (51%, Kasper, Kölliker, et al., 2017) compared to the average repeatability of behaviours (37%, Bell, Hankison, & Laskowski, 2009). Indeed, 66% of the fish tested twice in the helping opportunity in the present experiment showed the same egg-cleaning propensity consistently (see below). Thus, we assumed that the classification of individuals assigned to the control groups based on a single behavioural test was sufficiently accurate to predict the helper type after the first test. It is important to note that almost all test fish (90%), regardless of their egg-cleaning behaviour, performed defence behaviours towards the egg predator in a large previous experiment and consistent individual differences between individuals were rather quantitative than qualitative (Kasper, Kölliker, et al., 2017). In the present study, 18 of the 20 test fish that were confronted with the helping opportunity directed at least one defence behaviour against the egg predator, and 16 showed at least one instance of defence. There was no evidence that cleaners were more likely to defend than noncleaners (Chi-square test with simulated p-value; χ^2 = 2.22, p = 0.45, Supporting information Table S1), but cleaners performed more defence behaviours than noncleaners (Welch two sample t test; t = 2.79, df = 10.7, p = 0.02, Supporting information Table S2). It was therefore not possible to define distinct types based on this behaviour. *N. pulcher* reach sexual maturity at a standard length of 30–35 mm. Since the test fish used in the present experiment ranged between 16 and 23 mm in size, we were unable to determine their sex and we assumed that gene expression related to sexual maturation was not yet occurring. After the first helping opportunity, each test fish was uniquely marked with visible implant elastomer (VIE) tags (Northwest Marine Technology, Inc.) and transferred back into its original tank and sibling group. One week later, test fish were again transferred to the test tanks and housed for another week separately with a different dominant individual. For the second behavioural test at around day 115, age-matched individuals were randomly assigned to either the opportunity treatment or the control treatment (Figure 1). This way, 18 "cleaners" and 29 "noncleaners" were tested in the opportunity, and 15 "cleaners" and 16 "noncleaners" were selected for the control treatment (Supporting information Figure S1). The control fish in the second behavioural test received a sham treatment that followed exactly the same procedure of the opportunity

treatment and included a similar amount of disturbance by the experimenter but lacked the presentation of a clutch and an egg predator. Of the 47 individuals tested in the opportunity treatment, 15 cleaned eggs in both helping opportunity treatments, and 16 were consistent noncleaners. The brains of the three inconsistent cleaners and 13 inconsistent noncleaners of the opportunity treatment were not sampled.

2.2 | Tissue preparation

Fish of the opportunity treatment that had shown cleaning or noncleaning in both helping opportunity tests (N = 15 and 16, respectively) were caught with a hand net and immediately euthanized in a beaker containing 1 g MS-222 (ethyl-3-aminobenzoate methanesulphonate salt/tricaine, Sandoz, Switzerland) per 100-ml tank water 45 min after the presentation of the clutch. MS-222 causes cardiac and respiratory arrest by blocking sodium currents (Lalonde-Robert, Beaudry, & Vachon, 2012) and has been widely used as anaesthetic in studies of brain gene expression in poikilotherms before (Kesavan, Chekuru, Machate, & Brand, 2017; Laberge, Feldhoff, Feldhoff, & Houck, 2008; Nyman et al., 2017; Teles et al., 2015). Although the effects of MS-222 on brain gene expression are unknown, we aimed at reducing its potential impact by minimizing the time between respiratory arrest, that is, when opercular movement ceased, dissection and RNA fixation to approximately 30 s. Age-matched control individuals were also sacrificed (N = 15 and 16, for cleaning and noncleaning controls, respectively). In order to remove the telencephalon, we cut the fish in half sagittally, removed the two brain halves, fixated RNA by adding a droplet of RNAlater™ (Sigma-Aldrich), separated the telencephalon tissue and stored it in RNAlater™ at −20°C according to the manufacturer's instructions until RNA extraction up to 6 months later. Telencephalon extractions were done within <10 min after sacrificing the fish to prevent degradation of mRNA. According to the photomicrographs of another African cichlid species, *Astatotilapia burtoni* (Burmeister, Munshi, & Fernald, 2009), our telencephalon samples contained the dorsal (pallial) and the ventral (subpallial) division of the telencephalon, the entopeduncular nucleus, the nucleus taenia and the olfactory bulb. Some substructures of the dorsal division of the telencephalon, most notably the lateral (Dl) and the medial (Dm) parts, are the putative homologs of the mammalian hippocampus and basolateral amygdala, respectively. The dorsal (Vd), central (Vc), ventral (Vv) and supracommissural (Vs) parts of the ventral telencephalon correspond to the mammalian striatum, nucleus accumbens, lateral septum and pallidum, as well as the extended amygdala (O'Connell & Hofmann, 2011).

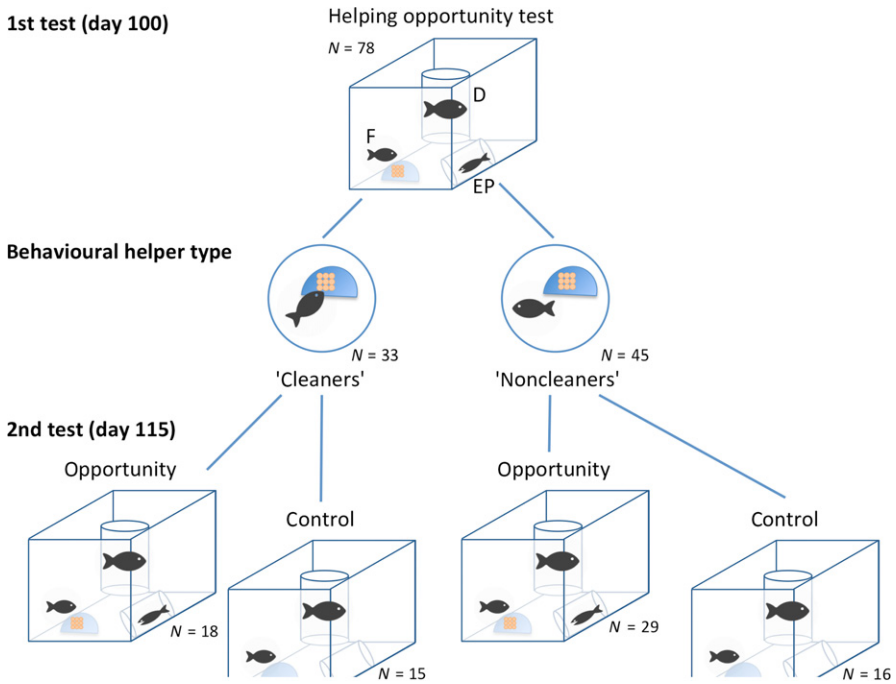

FIGURE 1 Schematic representation of the two behavioural tests that each test fish was subjected to before being sacrificed for brain extraction. The first helping opportunity test was done to preliminarily categorize the test subjects into "cleaner" or "noncleaner" types and took place when fish were approximately 3 months old (day 100). Before the observation started, the dominant territory owner (D) was confined to a vertical transparent tube. At the beginning of a recording, we fixed a portion of a clutch (orange) spawned by unrelated laboratory-stock pairs on a piece of transparent plastic sheet to the inside of the shelter (blue). We directly recorded all instances of egg cleaning of the focal fish (F), as well as its interactions with the dominant individual. After 10 min, an egg predator (EP) in a transparent plastic container was introduced, after which egg cleaning, social interactions and all instances of aggression towards the egg predator were recorded for another 10 min. The second helping opportunity treatment was done on day 115 and followed the same protocol. Fish in the control received a similar treatment, but no eggs and no egg predator were presented. 31 test and 31 control subjects were sacrificed 20 min after the opportunity ended [Colour figure can be viewed at wileyonlinelibrary.com]

2.3 | RNA isolation and library preparation

We extracted RNA only from brain samples that could be dissected and fixated within 10 min and in which we could clearly separate the telencephalon from the remaining brain tissue. This led to the exclusion of 14 samples, leading to a sample size of 12 for each phenotype-condition combination (Supporting information Figure S1). Telencephalon tissue was homogenized using metal beads in Eppendorf tubes containing 700 µl QIAzol reagent (Qiagen), and total RNA was extracted and purified with the miRNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Samples were randomly assigned to batches to minimize systematic batch effects on experimental condition and cleaner type. We assessed RNA quantity using a Nanodrop spectrophotometer (Thermo Scientific) and RNA quality using the Agilent Bioanalyzer 2100 Expert RNA (mean RIN ± *SD* = 9.5 ± 2.7, Supporting information Table S3) and immediately stored RNA at −20°C until sequencing library preparation. We generated barcoded RNA libraries from 48 samples with the KAPA Stranded mRNA-Seq Kit (KAPA Biosystems) following the manufacturer's protocol. Library quality was assessed on a Bioanalyzer High Sensitivity DNA Assay (Agilent), and library size was quantified with a Qubit fluorometer (Thermo Fisher Scientific). Libraries were combined into six pools and brought to a final concentration that was standardized within each pool (mean concentration ± *SD* = 6.14 ± 4.13 ng/µl). Single-end sequencing was performed on six lanes on an Illumina HiSeq 2000 instrument at the Génome Québec Innovation Center at McGill University, Montreal, Canada.

2.4 | Read mapping

In total, 105 Gb of raw sequencing data was generated, which represents 1.047×10^9 single-end Illumina reads of 100 bp (average reads per sample ± *SD* = 20M ± 8M). We assessed the quality of raw reads with FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) and removed adapters and low-quality sequences with Trimmomatic (Bolger, Lohse, & Usadel, 2014; see Supporting information for settings). We excluded ten libraries that had a low quality based on the number of reads, PCR artefacts, per base quality and sequence content (Conesa et al., 2016) according to the criteria described in detail in chapter 4 of the Supporting Information). Consequently, the average reads per sample increased to 23M ± 5M. The sample sizes for the final statistical analyses were thus 10 cleaners and 10 noncleaners in the opportunity and 10 cleaners and 8 noncleaners in the control (Supporting information Figure S1). After trimming, we kept 1.037×10^9 (99%) of the raw reads which we aligned to the genome of a closely related species, the Nile tilapia *Oreochromis niloticus*, using TOPHAT v.2.1.1 (Trapnell, Pachter, & Salzberg, 2009) in order to extract genomewide read counts for every individual library (see, Hebert (2017) for downloadable alignment pipeline). The reference genome (GenBank assembly accession GCA_001858045.2, accession date: March 14, 2017) had been generated with the single-molecule real-time sequencing technology (Pacific Biosciences) and comprised 37 848 coding sequences (Conte, Gammerdinger, Bartie, Penman, & Kocher, 2017). In general, cichlid species show high synteny, strong chromosomal conservation and no major genomic rearrangements between them (Brawand et al., 2014; Mazzuchelli, Kocher, Yang, & Martins, 2012; O'Connor, Marsh-Rollo, Ghio, Balshine, & Aubin-Horth, 2015). Therefore, more reliable results can be achieved by mapping the short single-end reads of *N. pulcher* generated with Illumina HiSeq in the present study to the high-quality genome of *O. niloticus*, instead of aligning them to a de novo transcriptome assembly (see chapter 5 of Supporting information for details). Only unambiguous matches (i.e., unique hits) were kept in the final alignment and all multimapping reads were discarded. Other alignment parameters were kept in the default mode. We obtained read counts for each gene contained in the reference genome based on the individual BAM files using HTSEQ-COUNT v.0.7.2 (mode: *intersection-nonempty*, see Anders, Pyl, & Huber (2015) for details). Mapping results (i.e., raw genomewide read counts) for each individual sequencing library were concatenated using custom Python scripts (Hebert, 2017) in order to produce a complete read-count matrix containing raw gene-specific read counts for each library. Gene annotation was added to the final read-count matrix using the publicly available annotation information associated with the genome version used (NCBI *Oreochromis 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: November 25, 2016, date of submission of annotation to the public databases: December 5, 2016, software version: 7.2). In total, 3.8×10^8 (37%) high-quality trimmed reads successfully mapped to the reference genome with unambiguous matches. The resulting read-count matrix that was used for differential gene expression analysis was comprised of 38,425 transcripts.

2.5 | Differential gene expression analysis

The differential gene expression analyses were performed on 38 libraries (opportunity: 10 cleaners and 10 noncleaners, control: 10 cleaners and 8 noncleaners, the exclusion of samples and the criteria are described in the Supporting information, Table S3 and Figure S1). We filtered genes with a read count of zero in all samples as well as those that were expressed only in a single sample. This led to the exclusion of 9,148 (24%) and 1,140 (3%) genes, respectively. We additionally explored more stringent filters based on criteria that were meaningful to our study, for instance including only those genes that were present in a specific proportion of individuals within this experimental group/cleaner type combination (for details, see chapter 7 of the Supporting information). Here, we only present results based on the first filtering approach. Transcriptomes of both behavioural helper types during control and those within each type between control and opportunity test were compared using DESEQ2 (Love, Huber, & Anders, 2014). We constructed the DESeqDataSet object from the read-count matrix and an additional file containing a concatenated variable "group" that denotes both the experimental condition (control C and opportunity O) and the behavioural type

(cleaners H and noncleaners N). We performed differential gene expression analysis by fitting negative binomial generalized linear models using the Wald statistic and estimating the dispersion with the "parametric" setting. DESEQ2 uses the "median-of-ratios" method to normalize counts which takes into account sequencing depth and RNA composition (Love et al., 2014). Initially, we fitted a model with an interaction term of experimental condition by behavioural type and assessed whether cleaners and noncleaners differed in the direction they regulated gene expression when exposed to the opportunity (see chapter 6 of the Supporting information for the code used). None of the genes showed a significant interaction (Supporting information Table S8), and thus, we proceeded with a simpler model without the interaction term as suggested in the manual of the DESEQ2 package (Love et al., 2014), focussing on pairwise comparisons (see Supporting information for the code used). We computed the following three different contrasts to test for differential gene expression between (a) cleaners and noncleaners in the control (CH vs. CN), (b) cleaners in control and opportunity (CH vs. OH) and (b) noncleaners in control and opportunity (CN vs. ON), automatically applying the built-in "independent filtering" algorithm. This led to the exclusion of six genes in the first and 2,733 genes each in the second and the third comparison. Those excluded genes contained outliers that did not fit a negative binomial distribution as identified by the Cook's distance (Love et al., 2014). Raw *p*-values were corrected for multiple comparisons (p_{adj}) using the false-discovery rate (Benjamini & Hochberg, 1995). We ordered the resulting lists of differentially expressed genes according to their adjusted *p*-values and considered those with $p_{adj} < 0.05$ as significantly differentially expressed (Supporting information Tables S9 and S10). In order to support the results of the DESEQ2 analyses, we conducted a permutation test in which we compared the number of differentially expressed genes (DEGs) found with the real data to a null distribution obtained by computing the number of DEGs found in 2,000 models with a permutated experimental group/cleaning type assignment (for details, see chapter 8 of the Supporting information).

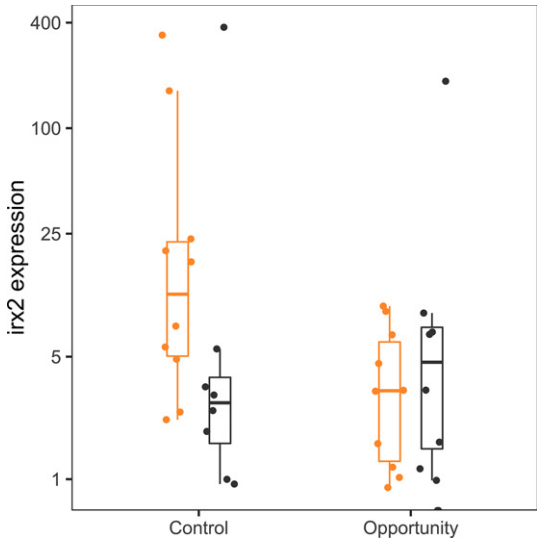

FIGURE 2 Normalized counts of Iroquois homeobox 2 (*irx2*) in cleaners (yellow) and noncleaners (black) in control and opportunity. Individual data points are plotted; Tukey boxplots show the median, the 25th and 75th percentile (lower and upper hinges, respectively), and whiskers extend to the smallest (or largest) value within 1.5 times the interquartile range of the lower (or the upper) hinge [Colour figure can be viewed at wileyonlinelibrary.com]

3 | RESULTS

1. Resting gene expression differences between cleaners and noncleaners

TABLE 1 Genes that were (a) upregulated or (b) downregulated in cleaners during the opportunity with FDR-adjusted *p*-values <0.05

GenBankID	Abbr	Gene name	Mean	FC	log2FC	log2FCSE	Wald	*p*	p_{adj}
(a)									
XM_003439202.4	csrnp1b	Cysteine/serine-rich nuclear protein 1	800.369	1.323	0.403	0.084	4.816	1.47E−06	0.019
XM_003438903.3	**hr38**	**Probable nuclear hormone receptor HR38**	**67.549**	**1.486**	**0.572**	**0.124**	**4.626**	**3.73E−06**	**0.021**
XM_005475227.3	epsti1	Epithelial–stromal interaction 1	13.615	1.372	0.456	0.099	4.609	4.05E−06	0.021
XM_003453237.3	rsad2	Radical S-adenosylmethionine domain-containing 2	6.846	1.303	0.382	0.088	4.349	1.37E−05	0.036
XM_019366159.1	**mb9.15**	**Maternal B9.15 protein**	**50.450**	**1.459**	**0.545**	**0.123**	**4.443**	**8.89E−06**	**0.036**
XM_005457455.3	ido2	Indoleamine 2,3-dioxygenase 2	4.835	1.427	0.513	0.118	4.368	1.25E−05	0.036
XM_003455595.4	**c-fos**	**Proto-oncogene c-fos**	**493.334**	**1.431**	**0.517**	**0.118**	**4.399**	**1.09E−05**	**0.036**
(b)									
XM_005450549.3	irx2	Iroquois homeobox 2	9.136	−1.351	−0.434	0.086	−5.026	5.02E−07	0.013
XM_003451558.4	neurod1	Neuronal differentiation 1	75.538	−1.347	−0.430	0.093	−4.649	3.33E−06	0.021
XM_005474264.3	dach1	Dachshund homolog 1	205.732	−1.369	−0.453	0.104	−4.343	1.40E−05	0.036

Genes that were also upregulated in noncleaners during an opportunity are highlighted in bold. For each gene ID, the mean of the normalized counts of all samples (mean) is given together with their fold change (FC), log2-fold change (log2FC) with its standard error (log2FCSE), the Wald statistic (Wald), the *p*-value (*p*) and the adjusted *p*-value (p_{adj}). We also provide the full gene names and the abbreviation used in the main text.

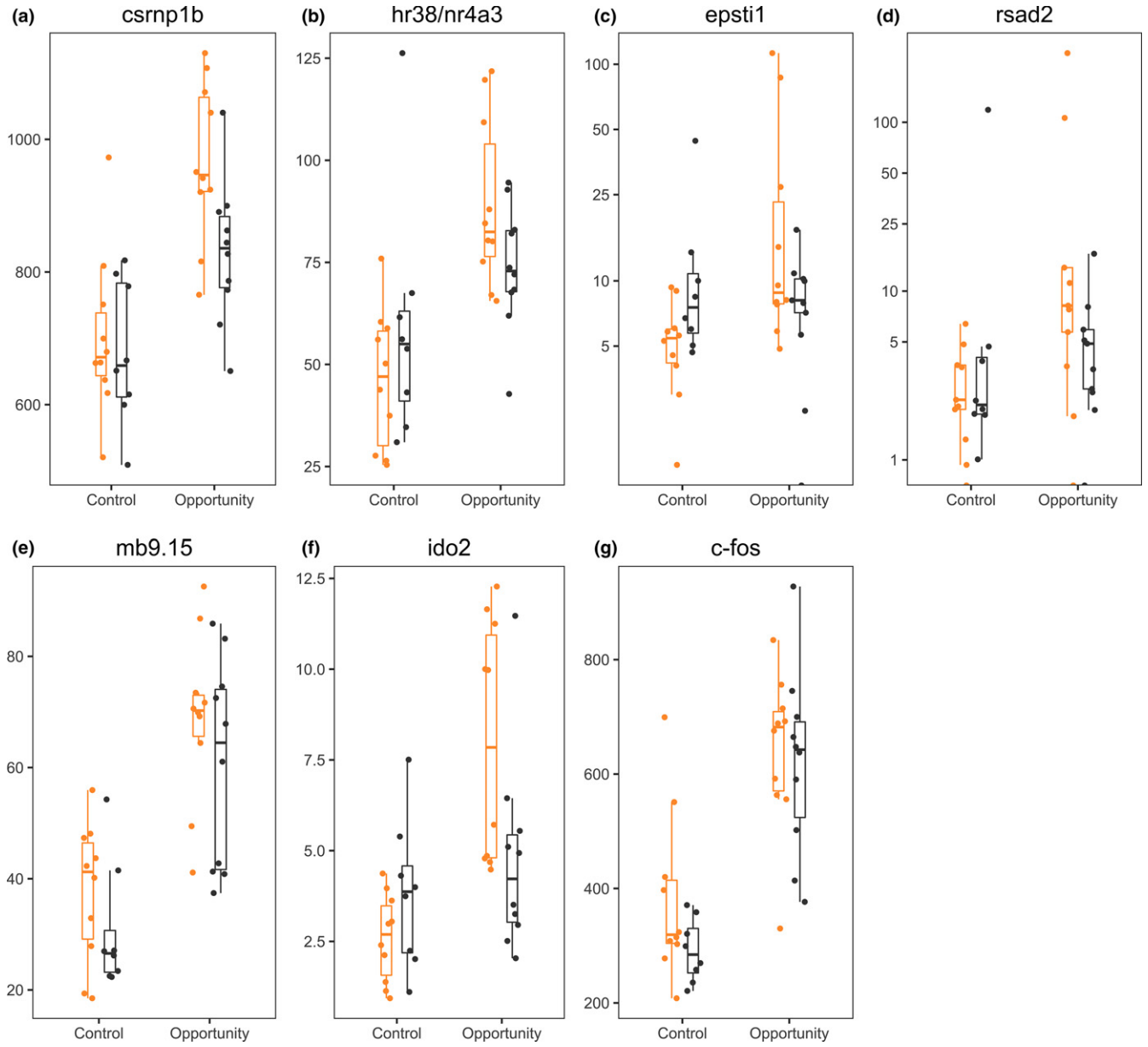

FIGURE 3 Genes that were upregulated during the opportunity in cleaners. Normalized counts shown for both behavioural helper types—cleaners (yellow) and noncleaners (black) in control and opportunity. (a–d [*Csrnp1b*, *epsti1*, *rsad2 and ido2*]) are genes that were only upregulated in cleaners, and (e–g [*hr38*, *mb9.15 and c-fos*]) were also upregulated in noncleaners during the opportunity. See Table 1a for full gene names and Figure 2 for description of box plots[Colour figure can be viewed at wileyonlinelibrary.com]

Resting expression of a single gene, iroquois homeobox 2 (*irx2*), was significantly higher in cleaners than noncleaners (log2-fold change = 1.36, *SE* = 0.08, adjusted *p*-value = 0.002; Figure 2).

2. Genomic reaction norms of cleaners in response to the helping opportunity

Cleaners upregulated seven genes (Table 1a, Figure 3) and downregulated three genes during the opportunity (Table 1b, Figure 4). *Csrnp1b*, *hr38*, *epsti1*, *rsad2*, *mb9.15* (XM_003438903.3), *ido2* and *c-fos* had higher expression levels in cleaners during the opportunity than in cleaners during the control (Table 1a, Figure 3, Supporting information Table S9). Three of these seven upregulated genes, *c-fos*, *hr38* and *mb9.15*, were also upregulated in noncleaners in the opportunity (see Table 1a). Cleaners expressed less *irx2* (Figure 2), *neurod1* and *dach1* during the opportunity than during the control.

3. Genomic reaction norms of noncleaners in response to the helping opportunity

Noncleaners increased the expression of 11 genes in response to the cooperation opportunity (Table 2, Supporting information Table S9). In contrast to cleaners, they downregulated none significantly. Similar to cleaners, noncleaners expressed more *c-fos*, *hr38*

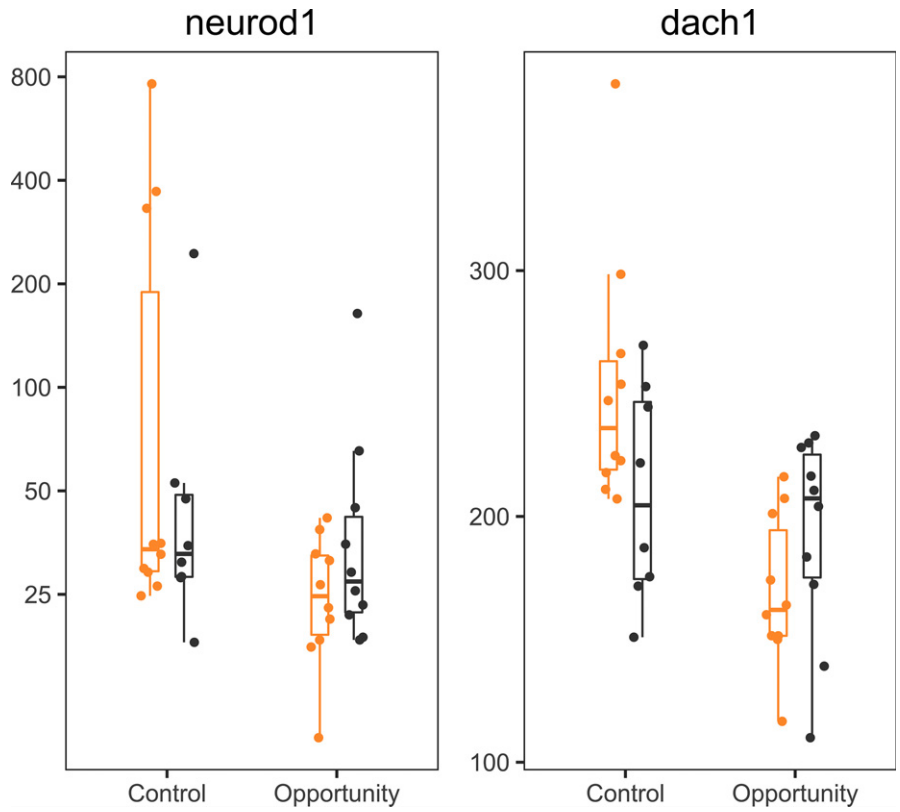

FIGURE 4 Genes that were downregulated during the opportunity in cleaners. Normalized counts shown for both behavioural helper types—cleaners (yellow) and noncleaners (black) in control and opportunity. See Figure 2 for the plot for *irx2* as well as for the description of box plots and Table 1b for full gene names[Colour figure can be viewed at wileyonlinelibrary.com]

and *mb9.15* in the opportunity. Additionally, noncleaners had higher expression levels of three genes that contained the neuronal PAS domain, namely *npas4b*, *npas4* and *npas4bl*; Figure 5). *Egr-1*, *egr-2* and *ier2* were also upregulated, as well as *plk2* and the uncharacterized gene LOC109203933 (XR_002063607.1). Normalized counts of *npas4b* and *npas4bl* correlated strongly (Spearman rank correlation; $r = 0.85$, $p < 0.001$), suggesting that they are either the same gene or recent paralogs, which is corroborated by blast

TABLE 2 Genes upregulated in noncleaners during opportunity with FDR-adjusted *p*-values <0.05

GenBankID	Abbr	Gene name	Mean	FC	log2FC	log2FCSE	Wald	*p*	p_{adj}
XM_003455595.4	**c-fos**	**Proto-oncogene c-Fos**	**493.334**	**1.626**	**0.701**	**0.120**	**5.849**	**4.93E−09**	**<0.001**
XM_019367106.1	npasdc4b	Neuronal PAS domain-containing protein 4B	33.575	1.552	0.634	0.122	5.215	1.84E−07	0.002
XM_003444618.4	npas4	Neuronal PAS domain protein 4	2495.747	1.411	0.497	0.099	5.010	5.43E−07	0.003
XM_005452100.3	plk2	Polo-like kinase 2	2954.527	1.415	0.500	0.100	5.029	4.92E−07	0.003
XM_003454061.4	egr-2/krox-20	Early growth response 2	37.455	1.538	0.621	0.126	4.928	8.30E−07	0.004
XM_003452282.4	egr-1	Early growth response protein 1-B	1512.105	1.397	0.482	0.102	4.725	2.30E−06	0.010
XM_019366159.1	**mb9.15**	**Maternal B9.15 protein**	**50.450**	**1.490**	**0.576**	**0.124**	**4.643**	**3.44E−06**	**0.012**
XM_003452801.4	ier2	Immediate early response gene 2 protein	23.450	1.480	0.566	0.126	4.482	7.38E−06	0.019
XM_003438903.3	**hr38**	**Probable nuclear hormone receptor HR38**	**496.133**	**1.463**	**0.548**	**0.122**	**4.479**	**7.50E−06**	**0.019**
XM_019367107.1	npasdc4bl	Neuronal PAS domain-containing protein 4B-like	27.634	1.478	0.563	0.125	4.503	6.68E−06	0.019
XR_002063607.1	unch	Uncharacterized LOC109203933	13.765	1.277	0.353	0.082	4.297	1.73E−05	0.040

Genes that were also upregulated in noncleaners during an opportunity are highlighted in bold. For each gene ID, the mean of the normalized counts of all samples (mean) is given together with their fold change (FC), log2-fold change (log2FC) with its standard error (log2FCSE), the Wald statistic (Wald), the *p*-value (*p*) and the adjusted *p*-value (p_{adj}). We also provide the full gene names and the abbreviation used in the main text.

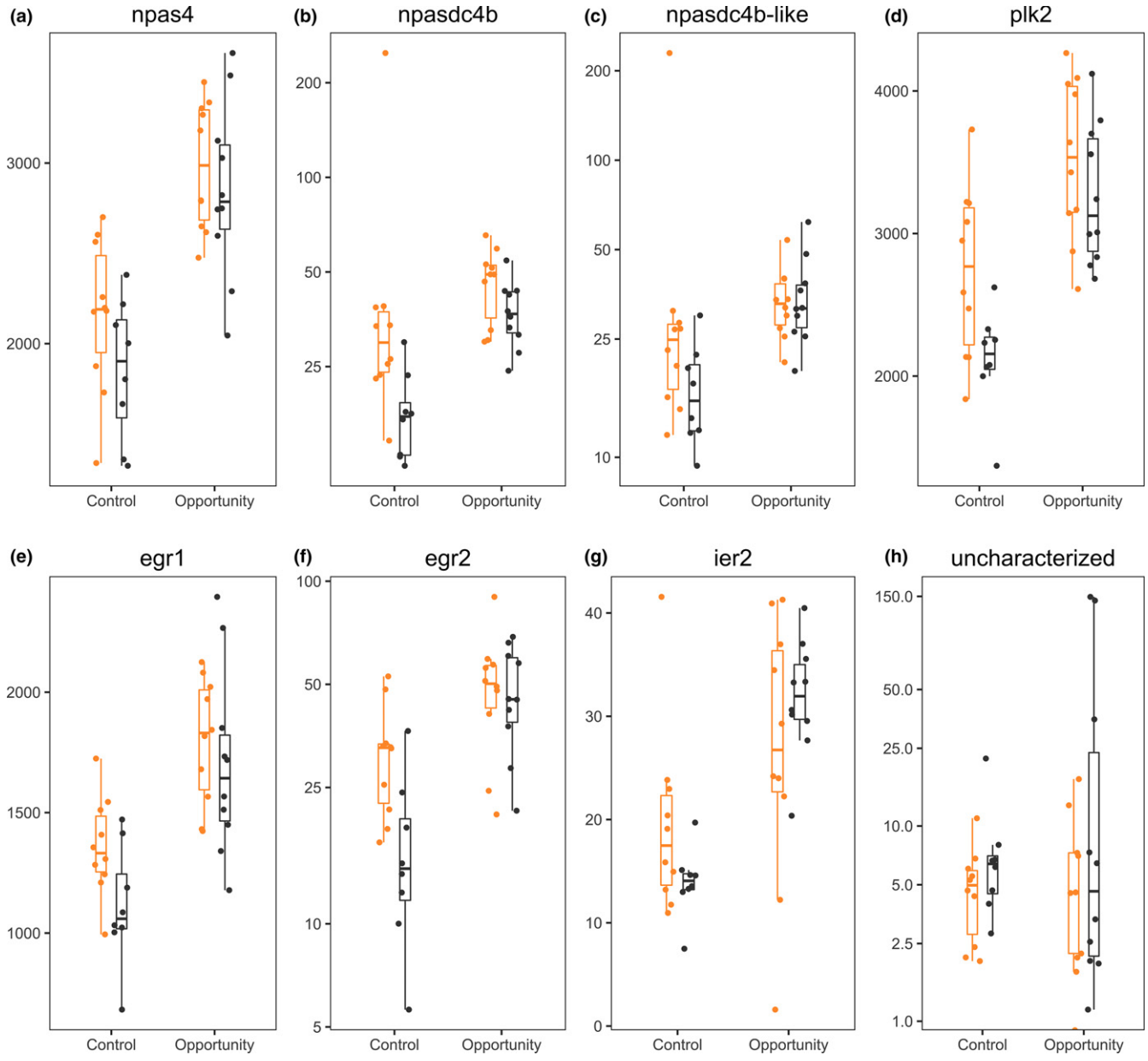

FIGURE 5 Genes that were upregulated in noncleaners during the opportunity. Normalized counts shown for both behavioural helper types—cleaners (yellow) and noncleaners (black) in control and opportunity. See Table 2 for full gene names and Figure 2 for description of box plots[Colour figure can be viewed at wileyonlinelibrary.com]

results. There were no significantly differentially expressed genes when we compared cleaners to noncleaners during the opportunity (Supporting information Table S9). Note that while the binary variable helper type was associated with differences in gene expression, the *number* of reads of the genes identified as differentially expressed in the three conditions above did not correlate with the frequencies of behaviours (Supporting information Table S6).

Permutation tests confirmed that it was unlikely that the overall number of DEGs in this experiment has arisen by chance. The mode of the null distribution was 2, in contrast to the result from the original data, which was 22 DEGs. Furthermore, 70.5% of the null distribution yielded a smaller number of DEGs than the real data (Supporting information Figure S2, panel A). This was corroborated by the permutation tests for the pairwise comparisons of cleaners in control and opportunity, as well as noncleaners in control and opportunity, that resulted in a number of DEGs that were 85.7% and 88.3% smaller than the real data, respectively (Supporting information Figure S2, panels C and D). However, for the comparison of noncleaners and cleaners in control, this was not so clear (61.1% smaller numbers of DEGs, Supporting information Figure S2, panel B), probably due to the fact that the distributions are bounded by zero and for small numbers of DEGs the differences are therefore not as marked as for larger numbers (for details see chapter 8 in the Supporting information).

4 | DISCUSSION

In the present study, we compared gene expression profiles of two alternative behavioural helper types occurring in the cooperatively breeding cichlid *N. pulcher*. We found persistent expression differences in a neuronal differentiation gene, *irx-2*, between cleaners and noncleaners in a resting condition. This suggests that developmental plasticity leads to the divergence of these behavioural helper types in this species. Two neuroplasticity genes were upregulated in both types during the opportunity. These genes, *c-fos* and *hr38*, are used as markers of neuronal activity in vertebrates and insects, respectively (Cruz et al., 2013; Fujita et al., 2013). This leads to the conclusion that the alloparental care stimulus presented in the opportunity (clutch and egg predator) was sufficiently salient. Thus, noncleaners' lack of egg care behaviours was not due to the fact that they were unable to "perceive" the opportunity. Apart from these concordantly upregulated genes, cleaners expressed a different set of genes in response to the opportunity compared to noncleaners. Cleaners also expressed a gene with a potential role in neuropeptide metabolism that we could not detect as significantly upregulated in noncleaners, indicating a signature of their current cleaning performance. Finally, in contrast to cleaners, noncleaners did not downregulate three neuronal differentiation genes in response to the opportunity. Taken together, our results show that gene expression profiles of cleaners and noncleaners differed in a resting condition and after being challenged by a helping opportunity. Furthermore, the types seem to differ in the slopes of the genomic reaction norms of three neuronal differentiation genes.

4.1 | Resting expression difference as indicator of developmental plasticity

Our results show that the behavioural types did not only differ in their egg-cleaning behaviour during the opportunity, but also in constitutive brain gene expression that might have resulted from permanent organizational effects during development. We detected a difference in the expression of *irx2* (*iroquois homeobox 2*) in the telencephalon between control groups of cleaners and noncleaners. *Irx2* seems to have rather long-term organizational effects on brain morphology and physiology via promoting regional identities (Gómez-Skarmeta & Modolell, 2002). Increased *irx2* expression likely characterizes regions in which neurogenesis and neural differentiation take place (Cohen, Cheng, Cheng, & Hui, 2000), because adult patterns of neural differentiation match those during embryonic development (Esposito, 2005; Zapala et al., 2005). The fact that this comparison was made between fish that were categorized into behavioural types in a previous opportunity, but received a sham opportunity before sampling ("resting expression"), is indicative of basic physiological differences between the types. In *N. pulcher*, consistent, but not heritable, individual differences in alloparental brood care have been described previously to arise during ontogeny (Kasper, Kölliker, et al., 2017;) based on social and ecological influences (Fischer et al., 2017). The present study suggests that this developmental specialization is associated with persistent differences in brain gene expression profiles. The findings of consistent behavioural differences that are reflected by divergent resting expression of a gene implicated in neuronal differentiation are consistent with the observation that behavioural helper types in this species arise by developmental plasticity (Schlichting & Pigliuggi, 1993). A recent study reported an upregulation of *irx2* in the telencephalon of sticklebacks two hours after a territorial intrusion, providing evidence for a role of this gene in a social context (Bukhari et al., 2017). However, even though both defending a territory and caring for and defending the clutch of a dominant breeder pair are forms of social interaction, they might represent different functional contexts, resulting in different directions of the gene expression response. In particular, the stickleback territory defence situation represents a social *challenge* including aggressive interactions with conspecifics, whereas in our study the situation is rather an *opportunity*, which includes (allo)parental care (O'Connell & Hofmann, 2011). However, here the helping task also included defence behaviour, albeit against a heterospecific. Therefore, it is conceivable that *irx2* is involved in aggressive as well as cooperative interactions, but the direction of its regulation differs between those contexts. As a caveat, we would like to mention a potential limitation of our study. Finding exactly one gene that is differentially expressed between the two cleaning phenotypes makes the biological interpretation rather difficult, because evidence for differing developmental plasticity is based on the different expression of one out of potentially many markers. It remains an open question why other markers of developmental plasticity are not differentially expressed between the cleaning types in the resting condition. This could be due to a lack of statistical power. Even though we had sample sizes that exceed those of most other studies, the fold-change differences were subtle in general and it could well be that, even though other genes were differentially expressed between those types, they failed to reach the transcriptome-wide significance threshold. It should be mentioned that, in an attempt to increase power, we applied a more stringent filter before differential gene expression analysis with DESEQ2 but this did not result in a larger number of DEGs (Supporting information Table S5).

Interestingly, cleaners and noncleaners did not only differ in their resting expression of *irx2* but also seem to differ in the slopes of the genomic reaction norm of this gene. Cleaners reduced *irx2* expression during the opportunity but noncleaners showed no plastic response, resulting in similar *irx2* expression levels of both behavioural helper types during the opportunity. Glucocorticoid receptor 1 (*gr1*) expression in *N. pulcher* individuals raised under different social conditions showed a similar pattern (Nyman et al., 2017). Individuals from a rich social rearing environment expressed more *gr1* in the control condition, but downregulated its expression during a social challenge, whereas the expression levels of individuals from a poor social environment were not affected by the challenge. In contrast to cleaners, noncleaners were perhaps not able to reduce *irx2* expression because of a floor effect of low resting levels that represent the minimal amount of *irx2* needed for basic brain tissue maintenance and repair.

On the ultimate level, behavioural specialization into alternative helper types may substantially reduce maintenance costs of neural machinery that processes relevant information in each cooperation opportunity (see Snell-Rood, 2013). Integrating over a multitude of environmental cues during development can produce an adapted adult phenotype (Kasumovic, 2013), provided that environments are sufficiently stable over an individual's lifetime or, in this particular case, helper phase, to extrapolate later environmental conditions from the early experience. On the proximate level, early-life social conditions have been shown to affect expression of genes with effects on neuroplasticity or stress response later in life (Branchi, Karpova, Andrea, Castrén, & Alleva, 2011; McGowan & Szyf, 2010; Nyman et al., 2017; Taborsky, Tschirren, Meunier, & Aubin-Horth, 2013).

4.2 | Concordant regulation of neuroplasticity genes in both helper types

The concordant upregulation of immediate early genes (IEGs) in both cleaners and noncleaners indicates that they could be implicated in the perception of the helping opportunity, which is the clutch and the egg predator, in both types, but that this input would then lead to divergent behavioural output in the behavioural helper types. Remarkably, both behavioural helper types concordantly increased the expression of two well-known IEGs, *c-fos* and *hr38*, when facing an alloparental brood care opportunity, indicating that the opportunity activated neurons in the telencephalon. *C-fos* is expressed in neurons following an action potential (Dragunow & Faull, 1989) and has been implicated in processes of activity-dependent neuroplasticity and memory formation. Higher levels of *c-fos* protein are found in the hippocampus of rats that experienced a novel environment compared to those in their home cage (VanElzakker, Fevurly, Breindel, & Spencer, 2008). *Hr38*, as well as its vertebrate homolog *nr4a3* (Rittschof et al., 2014; Shpigler et al., 2017), is implicated in the regulation of ecdysteroid and steroid-hormone receptor signalling and has been associated with caste specialization and division of labour in honeybees (Yamazaki et al., 2006). *Nr4a3* is increased in the auditory forebrains of zebra finches after hearing an unfamiliar conspecific song but not after habituating to the song (Dong et al., 2009). Thus, these genes might be implicated in the learning response to a novel socially relevant cue. It has been demonstrated in a social context that certain IEGs are highly expressed in the brain in reaction to a novel (Dong et al., 2009; Robinson, Fernald, & Clayton, 2008) and/or socially important stimulus (Burmeister, Jarvis, & Fernald, 2005), but less in response to familiar stimuli. Maternal B9.15 was also concordantly expressed in cleaners and noncleaners in the opportunity, but its role in the brain is unclear. However, the *Xenopus mb9.15* was assigned to the BTG family (www.uniprot.org/uniprot/P40745) that is implicated in neuron differentiation (Buanne et al., 2000). Hence, while *c-fos*, *hr38/nr4a3* and *mb9.15* are probably expressed due to the perception of the opportunity, the nonconcordantly expressed genes might be a signature of the behavioural differences in response to that opportunity.

4.3 | Current performance and preparation for future opportunities in cleaners

In addition to the three concordantly upregulated genes, cleaners were characterized by a unique expression profile that differed from the profile of noncleaners. Compared to their control group, cleaners increased expression levels of *csrnp1b* during the opportunity, which regulates the proliferation and survival of neural progenitor cells during vertebrate development (Feijo, Sarrazin, Allende, & Glavic, 2009). Cleaners also upregulated the expression of *ido2*, which is an enzyme that breaks down tryptophan and therefore probably affects the metabolism of serotonin, which is synthesized from tryptophan (Fukunaga et al., 2012; Kim et al., 2012). In humans and rats, this gene is associated with chronic pain and depression (Kim et al., 2012). In mice, fear behaviour due to chronic social stress is reduced by the application of an indoleamine-2,3-deoxygenase 1 (*ido1*) inhibitor (Fuertig et al., 2016). Monoamines modify synaptic transmission and therefore have a crucial function in behavioural flexibility (Burmeister, Rodriguez Moncalvo, & Pfennig, 2017), which has been demonstrated in the cooperative cleaner fish *Labroides dimidiatus* (Messias, Paula, Grutter, Bshary, & Soares, 2016; Paula, Messias, Grutter, Bshary, & Soares, 2015). Thus, *ido2* could reflect the current performance of cleaning behaviours and the individuals' capacity for behavioural flexibility via their implication in the metabolism of neuropeptides, whereas genes implicated in neuroplasticity likely prepare subordinates for future opportunities.

It is important to note that, in contrast to a neural response that results in an instant behavioural reaction to the stimulus, the genes expressed in this context will take effect only in the next encounter via a process termed "genomic action potential" (Clayton, 2000; Robinson et al., 2008). This process enables individuals to integrate social information during brief but consequential experiences, making them prepared to react faster and more appropriate to similar situations with potential impact on fitness in the future (Bukhari et al., 2017). In the cooperative-breeding context, theoretical models predict that the performance of helping behaviours has important implications for the acceptance of helpers by the breeders in the territory (Johnstone & Cant, 1999; Quiñones, van Doorn, Pen, Weissing, & Taborsky, 2016). It has also been demonstrated empirically that subordinates showing more appropriate behaviour in this context face a lower threat of aggression (Bergmüller & Taborsky, 2005) and even eviction (Fischer et al., 2014), which might be crucial for their survival.

While the majority of differentially expressed genes were upregulated during the opportunity, cleaners downregulated three genes. Apart from *irx2*, two additional transcription factors implicated in neural differentiation during the opportunity, *neurod1* and *dach1*, were expressed at lower levels in cleaners. *Dach1* has functions in embryonic brain development and is expressed in proliferating areas of the brain in later life stages (Machon et al., 2002). *Neurod1* is essential for the terminal differentiation, survival, maturation and integration of adult-born neurons (Gao et al., 2009). Winners of

aggressive social encounters in zebrafish express more *neurod1* in the medial part of the dorsal telencephalon (Dm) (Teles, Cardoso, & Oliveira, 2016), which is the teleost homolog of the mammalian basolateral amygdala (Portavella, Torres, & Salas, 2004). This suggests a role for *neurod1* in social memory, learning and recognition, which have been proposed to be crucial proximate "building blocks" of cooperation (Soares et al., 2010). In the present study, however, the direction of the regulation seems counterintuitive. Cleaners expressed those differentiation genes at higher levels in the control condition than during the opportunity, whereas noncleaners did not vary their expression between conditions. More specifically, cleaners' *irx2* (and potentially *dach1*) expression levels in the control exceeded those of noncleaners. It would be interesting to investigate why these genes seem to be important on the long-term in cleaners, but not during the opportunity, where their expression is reduced. For instance, one could speculate that the down-regulation of genes with long-term effects during the opportunity has the effect of enhancing the "signal" of the gene products that are expressed in response to the opportunity by reducing the "noise" of background gene expression. This resting gene expression that functions as a kind of "housekeeping" (i.e., in this particular case, *irx2* might keep functional nodes in the telencephalon intact over time by preventing cell migration between those nodes) could interfere or directly inhibit the expression of genes that have a direct connection with the processes triggered by egg-cleaning behaviour. However, investigating this hypothesis is clearly beyond the scope of the present study.

4.4 | Noncleaners' gene expression response to the opportunity

Apparently the helping opportunity also induced the expression of genes regulating neural plasticity in noncleaners, even though these individuals did not perform alloparental egg care. In noncleaners, we only found upregulated genes in the comparison between the control condition vs the opportunity among the set of significantly differently expressed genes. Noncleaners also responded to the helping opportunity, but they did not show a similar down-regulation of neuronal differentiation genes. Apart from *c-fos*, *nr4a3* and *mb9.15*, which noncleaners overexpressed concordantly with cleaners during the opportunity, several other IEGs were upregulated, some of which are implicated in neural plasticity, memory formation and learning (see GO terms in Supporting information Table S10). *Npas4* seems to take up a pivotal role in these processes because of its rapid induction. It has been proposed that *npas4* regulates the transcription of other transcription factors that play a role in cognition, for instance *c-fos*, *egr-1* and brain-derived neurotrophic factor (*bdnf*) (Benito & Barco, 2015; Lin et al., 2008; Maya-Vetencourt, 2013). Its expression is necessary for fear memory formation and retention (Ploski, Monsey, Nguyen, Dileone, & Schafe, 2011), and its down-regulation during stress impairs neuroplasticity (Yun et al., 2010). A recent study in zebrafish suggested a role of *npas4* in social memory formation (Teles et al., 2016). *Nr4a3/hr38* and *npas4*, among other genes, have been shown to be dysregulated in honeybees, sticklebacks and house mice during a territorial intrusion (Rittschof et al., 2014). Thus, these genes could be part of an evolutionarily conserved neuromolecular regulation mechanism of social behaviour, termed "social toolkit." In our study, *nr4a3* was upregulated in both phenotypes, indicating that it is involved in the perception of the opportunity rather than the egg-cleaning behaviour itself. Similarly, another nuclear receptor, ftz-f1/nr5a3, has been identified as a hub transcription factor transducing the hormonal signals during social challenges in honeybees and mice (Chandrasekaran et al. 2011, Grgurevic, Büdefeld, Rissman, Tobet, & Majdic, 2008). *Plk2*, a regulator of synaptic plasticity for which an association with social stimuli has been documented in mice (Feldker et al., 2006), was also overexpressed in noncleaners in the opportunity. *C-fos*, *egr-1*, *egr-2* and *ier2* are rapid primary response genes, which are expressed after sustained stimulation (Tyssowski et al., 2017). Interestingly, in a previous study, we found an opposite *egr-1*-expression pattern: *egr-1* was decreased in individuals 45 to 60 min after experiencing a helping opportunity for the first time compared to fish in a control group (Kasper et al., 2018). It thus seems that during the very first opportunity, *egr-1* was downregulated in all individuals experiencing the opportunity, whereas at the second opportunity it was upregulated, regardless of helper type (Figure 5). However, here we could detect statistically significant expression differences only in noncleaners, probably due to cleaners' higher resting expression levels. In individuals that did not perform egg-cleaning behaviour despite being presented with the clutch, the opportunity was associated with an increase in the transcription of transcription factors that are related to synaptic transmission, memory and hormone signalling pathways, but also social behaviour (Supporting information Table S10). This represents evidence that those genes could be connected with a situation that necessitates increased responsiveness to social stimuli.

The gene expression differences between the behavioural helper types were rather subtle in terms of fold change and the number of differentially expressed genes. This was expected because of the complex and quantitative nature of behaviour that very likely has a complex genetic basis and strong environmental influence (Boake et al., 2002). Therefore, even if many genes contribute to the phenotype, it is likely that many genes with rather small effect might not reach genomewide statistical significance (Boyle, Li, & Pritchard, 2017). Moreover, the types differed only in their egg-cleaning propensity and no obvious morphological, physiological or other behavioural differences were observed otherwise. The gene expressed differently by noncleaners between control and opportunity seemed to be similarly regulated by cleaners, but the same was not true for the genes that were differently expressed by cleaners. Comparing the set of genes that were significantly upregulated in noncleaners with the gene list ordered by FDR-adjusted *p*-value shows that those genes were regulated in the same direction in cleaners but with less pronounced differences that did not reach the significance threshold (Supporting information Tables S7A–S9). However, for genes differentially regulated in cleaners in the opportunity, we did not find regulation in the same direction in noncleaners, except for *csrnp1b* (Supporting information Table S7B–S9). Thus, the

nonoverlapping portion of the activated gene sets of cleaners and noncleaners might indicate that the opportunity activates partly different neural pathways in the helper types.

4.5 | Conclusion

Our finding of resting differences indicates that, even when not challenged, alternative behavioural helper types differ in their brain physiology or structure. It is likely that these differences might have arisen through developmental plasticity because, in this species, helping propensity is not shaped by genetic variation. Our study highlights the importance of transcriptional regulation in the response to a helping opportunity, as was suggested for behaviours in general by previous findings in bees (Ament, Blatti et al., 2012; Ament, Wang et al., 2012), sticklebacks (Bukhari et al., 2017) and burying beetles (Benowitz, McKinney, Cunningham, & Moore, 2017). In the present study, we also show that even though behaviourally different, both types expressed a common set of neuroplasticity genes that might therefore be implicated in the perception and integration of the alloparental care stimulus. Several of the genes we found differentially expressed in this study are implicated in a conserved neuromolecular regulation mechanism of social behaviour ("social toolkit," Rittschof et al., 2014). Analysing the whole transcriptome of the telencephalon at an early stage of the gene regulation cascade following a helping opportunity enabled us to suggest additional candidate genes beyond those already identified as "social toolkit genes" whose functions in the brain are so far unknown but which are potentially important in a cooperation context.

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AUTHOR CONTRIBUTIONS

C.K., B.T. and N.A.H. conceived and designed the experiment; C.K. carried out the behavioural observations, brain sampling, RNA extractions, prepared RNA-Seq libraries, performed differential gene expression analysis; C.K. and F.O.H. mapped the transcripts to the genome; C.K., N.A.H. and B.T. drafted the manuscript; and all authors approved the final version of the manuscript.

DATA ACCESSIBILITY

All sequence data for this study were archived at the Sequence Read Archive (SRA) on NCBI, BioProject number PRJNA480218 and BioSample numbers SAMN09630713 to SAMN09630760. Gene expression data (number of reads mapped to each reference sequence) are archived at Dryad (https://doi.org/10.5061/dryad.797kg). Lists of differentially expressed genes and GO terms associated with differentially expressed genes are provided as online Supporting information with this article (Tables S8–S10).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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