Transcriptomic changes associated with maternal care in the brain of mouthbrooding cichlid *Astatotilapia burtoni* reflect adaptation to self-induced metabolic stress

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Abstract

Parental care in *Astatotilapia burtoni* entails females protecting eggs and developing fry in a specialized buccal cavity in the mouth. During this mouthbrooding behavior, which can last 2-3 weeks, mothers undergo voluntary fasting accompanied by loss of body mass and major metabolic changes. Following release of fry, females resume normal feeding behavior and quickly recover body mass as they become reproductively active once again. In order to investigate the molecular underpinnings of such dramatic behavioral and metabolic changes, we sequenced whole brain transcriptomes from females at four time-points throughout their reproductive cycle; 2 days after the start of mouthbrooding, 14 days after the start of mouthbrooding, 2 days after the release of fry, and 14 days after the release of fry. Differential expression analysis and clustering of expression profiles revealed a number of neuropeptides and hormones, including the strong candidate gene, *neurotensin*, that suggest molecular mechanisms underlying parental behaviors may be common across vertebrates despite their *de novo* evolution in certain cichlid lineages. In addition, oxygen transport pathways were found to be dramatically downregulated particularly at later the mouthbrooding stage while certain neuroprotective pathways were upregulated, possibly to mitigate negative consequences of metabolic depression brought about by fasting. Our results offer new insights into the evolution of parental behavior as well as revealing candidate genes that would be of interest for the study of hypoxic ischemia and eating disorders.

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Introduction

Parental behavior has evolved multiple times in animals. Although it is a costly reproductive strategy for parents, it increases fitness through higher offspring survival rate. These parental costs come in the form of time and energy, as time spent caring for and protecting offspring must be balanced against competing behavioral interests such as feeding and mating (Numan and Woodside, 2010; Olazábal and Young, 2006). For most species, the diverse set of activities that compose 'feeding' and 'parental care' must be co-regulated through partially overlapping neuroendocrine circuits, and evidence indicates substantial cross-talk between these circuits in at least some species (e.g. Olszewski *et al.*, 2010; Crossin *et al.*, 2012; Shahjahan, Kitahashi and Parhar, 2014). While some species increase feeding during parental care to compensate for the added demands, others inhibit feeding behavior to favor parental care, a tradeoff that comes at the expense of parental condition (Fischer and O'Connell, 2017; O'Rourke and Renn, 2015).

The neural circuits and molecular mechanisms regulating parental care and feeding behaviors are well studied, primarily in mammalian systems. Ongoing work provides inference regarding homology across vertebrates at both the circuit (Goodson, 2005; Goodson and Kingsbury, 2013; O'Connell and Hofmann, 2011) and molecular signaling (Volkoff, 2016) levels. The close connection between reproduction and energy balance is well-documented in mammals with several nuclei of the "maternal brain" receiving either direct or indirect input from the arcuate nucleus feeding regulatory neurons (Schneider, 2004). Within the arcuate nucleus of the hypothalamus, two specific populations of neurons, one producing orexigenic neuropeptide Y (NPY) and agouti related peptide (AgRP), and the other producing anorexigenic pro-opiomelanotcortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), are directly responsive to the peripheral signals of satiety, leptin (Cone, 2005) and ghrelin (Cowley et al., 2003).

Mouthbrooding, a form of parental care seen in several species of fish in which the parent holds the developing eggs and fry in the buccal cavity, represents an extreme example of food restriction and parental investment. In an ultimate sense, mouthbrooding is an adaptation in response to predation pressure (Kuwamura, 1997) and has evolved independently in cardinalfishes, gobies, betas (Rüber et al., 2004), and several clades of cichlid fishes (Barlow, 2000; Fryer and Iles, 1972) where it is thought to have promoted rapid speciation (Salzburger et al., 2005). In a proximate sense, mouthbrooding severely limits food intake, such that most species completely abstain from food intake while others obtain food used only for provisioning the fry (Yanagisawa et al., 1996). This brooding phase is plastically adjusted in response to

predation threat (Taborsky and Foerster, 2004), noise induced perturbations (Butler and Maruska, 2021), and parental energy reserve as well as the quality and availability of mates (Iwao et al., 2004).

In the African cichlid fish *Astatotilapia burtoni*, it is the female alone that provides parental care in the form of mouthbrooding, abstaining from food intake for 2-3 weeks. This leads to a dramatic reduction in body mass (Grone et al., 2012; Renn et al., 2009), and reduced GSI associated with lowered estradiol and 11-KT (a fish-specific androgen) (Maruska et al., 2020b). Unlike a state of involuntary starvation, feeding motivation is decreased during mouthbrooding even when the developing eggs are removed from the buccal cavity (Mrowka, 1984), thus the female does not anticipate caloric intake. The capacity to withstand and recover from prolonged periods of food deprivation requires behavioral, physiological, morphological and molecular adaptations to conserve energy and maintain immune function (Secor and Carey 2016). We have previously identified an enhanced reduction in gut epithelial turnover during mouthbrooding as one such potentially adaptive mechanism for energy conservation (Faber-Hammond *et al.*, 2019). While the full extent of metabolic changes during mouthbrooding is not known, it may share similarity to changes seen in animals that engage in hibernation or other related extreme adjustments when caloric intake is not anticipated. During hibernation, shifts in mitochondrial ATP production and calcium uptake may improve energy efficiency (Barger et al., 2003), neuroprotective mechanisms may mitigate oxidative damage (Yin et al., 2016), and changes to brain water homeostasis can also impact neuronal signal transduction (Osborne and Hashimoto, 2008).

Expression of genes encoding some neuropeptides and their receptors is known to differ between brooding, non-brooding, and starved females, suggesting that different regulatory mechanisms control this special case feeding inhibition (Grone et al., 2012). Furthermore, recent work examining neuronal activation across the brain during mouthbrooding in *A. burtoni* provides evidence for a large degree of shared circuitry involved in the regulation of maternal care, food intake, and energy balance with nuanced differences in specific brain regions (Maruska et al., 2020a). Such work predicts global changes in gene expression across the brain with specifically regulated genes in some regions.

In the current study, we analyze whole brain transcriptomes of female *A. burtoni* from multiple time-points across the reproductive cycle. The time-points selected represent dramatically different stages in terms of nutrition state, energy requirements and parental care behaviors. By conducting differential expression analyses and parallel enrichment analyses for functional gene sets, we identify patterns of upregulation or downregulation at either of two

specific mouthbrooding stages relative to each other and to two post-release time-points, one of which includes intensive maternal care. Genes that are differentially regulated across the maternal stages (both mouthbrooding and post release) may underlie the specific behaviors performed at these time-points, while genes showing trends that correlate with decreasing body condition may reflect energy reserves. In addition to providing valuable insight into the evolution of parental behavior, our results reveal previously unknown changes in the maternal cichlid brain that could have clinical impacts in the study of brain hypoxic ischemia, inflammation, and neuroprotection. Our holistic approach using whole brain samples aims to identify novel mechanisms beyond a candidate gene approach and capture global changes of a metabolically active tissue.

Materials & Methods

Animal Husbandry

Fish used in this experiment were derived from the same lineage as was sequenced for the *Astatotilapia burtoni* reference genome assembly (GCA_000239415). Females of reproductive age were maintained in 30-gallon mixed sex stock tanks with 2-4 males and 8-12 females. Tanks were kept on a 12/12 day/night cycle with a half an hour dawn and dusk settings. The salinity and pH levels of tanks were matched to the average levels of Lake Tanganyika. Tanks were checked daily, and when brooding females were captured and fry development stage was confirmed. Females were assigned to one of four treatment groups: two days mouthbrooding (B02), 14 days mouthbrooding (B14), two days post-release of fry (R02), and 14 days post release (R14) (Fig. 1). To avoid confounding date with treatment groups, females were sequentially assigned one to each group, with the exceptions that only females confirmed to be holding stage 0 fry were assigned to the B02 group. Brooding females were placed into a five-gallon tank with a terracotta pot for shelter and gravel. A single male stimulus fish (~3g + 1g) was housed in the same tank, retained by a cylindrical mesh barrier (10 cm diameter) to allow visual and olfactory contact. The male stimulus fish was fed standard cichlid pellets daily within the confined cylinder. Because brooding females are not expected to eat, the addition of food would have fouled the tank. Therefore, females had visual and olfactory cues regarding food availability without actual access to it. Once females released the fry, they were fed standard fish flakes daily. All protocols complied with Renn lab IACUC approval #01-2017.

Sampling

Females were monitored daily for brooding or release of fry. All fry were removed two days after the initial release date. Any female that cannibalized her fry prior to two days post-release was reassigned to the R14 group, so that all R02 fish were actively caring for free swimming fry. At B02, B14, R02 and R14 time-points females were anesthetized in MS-222, weighed, measured for standard length and rapidly decapitated. Whole brains were removed and preserved in 1mL RNAlater (Ambion) and stored at -80°C. All dissections were done between 1600-1800 hours. While whole brain gene expression profiling avoids the technical variation introduced by brain punch techniques, this approach is not without caveats and limitations. Primarily, region specific regulation and genes expressed in a limited number of cells are less likely to be detected. Certain genes are known to be differentially regulated in brain region specific patterns that are lost with whole brain expression profiling. For example, Butler et al. (Butler et al., 2020) studied the role of galanin in maternal care and infanticide in *A. burtoni*, and found that expression in POA neurons is associated with maternal care while expression in NLT neurons is associated with feeding. While this finer scale resolution is lost in analysis of whole brains, our method does capture a snapshot of the broad changes in the brain throughout the reproductive cycle associated with this metabolically demanding behavior.

RNA extraction / Sequencing

Brains were removed from RNAlater and placed in 200 ųl of 1-Thioglycerol/Homogenization Solution from the MAxwell 16 LEV Simply RNA Tissue Kit (Promega cat# AS1280). Samples were homogenized using a hand-held pestle motor and plastic pestle in a microcentrifuge tube. Homogenized samples were transferred to the MAxwell 16 LEV Simply RNA Tissue Kit cartridge and processed in the Maxwell 16 Instrument (Promega AS1000) using the High Strength LEV Magnetic Rod and Plunger Bar Adaptor (Cat.# SP1070). Standard DNAse treatment was included and samples were eluted in 50 ųl H_20 including RNAseOUT (Invitrogen). RNA quality was initially checked by electrophoresis and quantity by NanoDrop 1000 (Thermo Scientific). RNA (1-2 ug/sample) were sent to Novogene (Sacramento CA) for library prep and paired-end 150 bp sequencing (Illumina Platform PE150).

Bioinformatics

Paired end RNA-seq reads were preprocessed with TrimGalore v0.6.4 (Krueger, 2012) for adaptor trimming and FastQC v0.11.3 (Andrews, 2010) for quality filtering. Remaining paired reads were aligned to the *A. burtoni* reference genome (GCF_000239415.1) with STAR v.2.5.3

(Dobin et al., 2013), using the associated RefSeq GTF file to guide the alignment. Aligned reads were assembled and quantified using StringTie v1.3.4 (Pertea et al., 2015), and count tables were constructed as input for differential expression analysis. All methods, results, and discussion a WGCNA analysis (v1.69: Langfelder and Horvath, 2008) that was conducted in parallel are included in supplementary material.

Data normalization and subsequent differential expression (DE) analysis was performed with DESeq2 v1.20.0 (Love et al., 2014) in R-studio v1.1.383 (RStudio Team, 2020). A likelihood ratio test (LRT) was performed to generate a list of genes that showed significant expression differences between any of the four sampled time-points, followed by pairwise Binomial Wald tests to discover which pair of time-points differ. As a supplementary analysis, brooding (B02, B14) and release (R02, R14) stages were pooled and a pairwise Binomial Wald test was performed to discover candidate genes that differentiate by associated parental behaviors and response to food intake. Significant DE genes from both tests are reported at FDR < 0.1, and for exploratory purposes we report genes trending towards DE at an unadjusted P < 0.05 (results reported as File S1). This less stringent threshold provided sufficient number of genes for downstream functional analyses. LRT DE genes were tested for gene ontology (GO) term enrichment with BiNGO v3.0.4 (Maere et al., 2005), implemented in Cytoscape v3.7.2 (Shannon et al., 2003). While *A. burtoni* transcripts were assigned names based on their NCBI RefSeq GTF annotation, they were reannotated by predicted protein sequence to obtain a comprehensive list of GO terms (File S2). In order of priority, GO annotations were preferentially used from the Swiss-Prot, TrEMBL (The UniProt Consortium, 2019), and EggNOG (Huerta-Cepas et al., 2019) protein databases (accessed Feb-2018).

We performed hierarchical clustering analysis on subsets of significant and DE-trending genes that address original and novel exploratory hypotheses with the goal of discovering co-expression and possible interaction of candidate genes. For all clustering analyses, we used VST (variance stabilizing transformation) normalized count tables generated by DESeq2, and constructed heatmaps using the made4 v1.56.0 R-package (Culhane et al., 2005). This package utilizes the correlation similarity metric and average linkage clustering for expression data, which allows for clustering of expression patterns regardless of the overall magnitude of expression. Clustering was performed on individuals to generate expression-based dendrograms, while the associated heatmaps display time-point medians for easier interpretability of expression data. Bootstrap values for dendrograms were generated with the PVclust v2.2-0 R-package (Suzuki and Shimodaira, 2006) following 10k clustering iterations.

In addition to the detection of individual differentially expressed genes we aimed to identify changes in functional gene categories across time-points, therefore we ran Gene Set Enrichment Analysis (GSEA v4.0.3) for sets of genes representing GO categories (Subramanian et al., 2005). Pairwise comparisons were done with gene-level VST-normalized counts between each of the four time-points in both directions, resulting in 12 possible comparisons. Only six comparisons are possible between four time-points, however reciprocal comparisons (*e.g. B02 vs B14* and *B14 vs B02*) were performed because GSEA results slightly differ based on the direction of the test due to significance being determined by ranking of sets, and the union is reported. For each GSEA comparison, 1000 permutations of gene set randomization were performed because there were fewer than 10 samples per group. Sets with FDR values < 0.05 were considered significantly enriched, and the GO categories had to meet this threshold in either of two reciprocal tests per time-point comparison. GSEA documentation recommends an FDR cutoff of 0.25 for determining significance in exploratory analyses due to false negatives being less desirable than false positives in such analyses, however we chose the more restrictive alpha threshold of 0.05 to limit the number of significant GO sets. GSEA tests were followed by leading edge analysis to determine both direction of change and overlap between enriched gene sets.

Significantly enriched GO gene sets are not fully independent in the gene accessions they contain, as single genes often have a number of associated GO terms. To account for this, we took steps to reduce complexity in the list of significant GO terms by merging terms with minimum 50% overlap of member genes into GO families. Overlap coefficients (CO) were calculated as the proportion of shared genes between pairs of terms divided by the smaller of the two gene sets. The inverse of these coefficients (*1 - CO*) were converted into a distance matrix for all significant GO terms from GSEA, then used to build a neighbor joining tree with PHYLIP v3.698 (Felsenstein, 2005). TreeClust v1.00 (Balaban et al., 2019) was used to cluster nodes that had a maximum distance of 0.5 from each other, equivalent to a minimum 50% overlap coefficient between GO terms. Merged GO families were named using the most significant (lowest FDR) term from all GSEA time-point pairwise comparisons.

For hierarchical clustering of significant GO terms and families, GSEA was re-run to obtain normalized enrichment scores (NES) of each of four time-points vs. all other samples, with positive values representing upregulation and negative values representing downregulation of entire gene sets in an analog to log2 fold change of individual genes. Hierarchical clustering was performed using the Ward.D2 clustering and Euclidean distance methods implemented by the Heatmap tool from the ComplexHeatmaps R package v2.6.2 (Gu et al., 2016; Ward, 1963).

Results

The time-points selected for the current study represent dramatically different states with regard to behavioral, feeding, and metabolic activity. We use the R14 stage as a baseline in which there is no maternal care behavior, active feeding behavior, adequate energy reserves, and normal metabolic activity. The early brooding stage, B02, is characterized by modest buccal-churning as maternal care, no feeding behavior, adequate energy reserves, and potentially reduced metabolic activity to conserve energy, while the later brooding stage, B14 shares the lack of feeding behavior, it is also characterized by more intense buccal based maternal care and dramatically reduced energy reserves, which likely necessitates dramatic regulation of metabolic activity. The early release stage, R02, shares this increased maternal care but of a very different nature in terms of territory defense, while feeding behavior has resumed, the energy stores remain depleted again potentially requiring substantial metabolic regulation.

Transcriptome profile statistics

Twenty-two (22) samples passed QC filters for analysis, with 5 samples representing B02 and R02 time-points and 6 samples representing B14 and R14 time-points. From ~610M total high-quality reads (mean per sample = ~27.7M ± 7.8M), 84.6% reads were aligned to the *A. burtoni* genome. These *A. burtoni* brain transcriptomes yielded hits to 25,572 / 44,653 (57.3%) of all RefSeq annotated genes, which accounted for 92.9% of final gene counts. Conversely, 7.1% of gene counts represented novel unannotated transcripts within the genome assembly, and upon GO annotation for the entire transcriptome we found only a small portion (17.7%) of these novel transcripts to be putative protein coding genes, many of which appeared to code for transposable element machinery. Therefore, only annotated NCBI RefSeq genes in the assembly were used for downstream analyses.

Differential Gene Expression

We found 128 genes to be differentially expressed (DE) across the four reproductive time-points through DESeq2 LRT analysis with an FDR < 0.1, making up 0.5% of RefSeq annotated genes expressed in the brain. For exploratory purposes, we also examined a secondary list of 1,643 genes (1,515 additional genes) trending toward differential expression at an unadjusted $P < 0.05$ (File S1). With *post-hoc* pairwise comparisons, we explored patterns among the less stringent set of trending differentially expressed genes (Table 1). The highest number of DE genes, 105, occurred between late brooding, B14, and early release, R02, stages in the brooding cycle (44 B14-biased; 61 R14-biased) when maternal behavior and feeding are

dramatically changing. Conversely, the fewest DE genes, 20, were detected in the contrast between early brooding, B02, and early release, R02 (11 B02-biased and 9 R02-biased). Among the full set of DE genes identified for a given brooding stage (both up and down regulated), only a small percentage are biased toward that brooding stage relative to all other 3 stages (B02: 2.56% B14: 0.56%, R02: 1.36%, R14: 0%), meaning that there are few stage specific gene expression levels. The same dynamic pattern was seen among the larger list of genes tending toward differential expression with a greater percentage of differential expression being stage specific (2.5% - 6.8%). While the two brooding stages are very different in terms of duration of starvation and other physiological factors, they share a mouthbrooding maternal component that differs from either of the release stages, therefore we also combined the two brooding stages and the two release stages and performed a pairwise pooled brooding vs. pooled release Binomial Wald test. This analysis revealed 31 DE genes at FDR < 0.1, 21 of which were significant DE genes and the remaining 10 were trending towards significance based on LRT analysis. An additional 1124 genes trending towards DE in the brooding vs. release analysis at an unadjusted P < 0.05 (File S1). Functionally interesting candidate genes appearing in both LRT and brooding vs. release DE lists are highlighted in expression heatmaps while the following discussion primarily reports on the LRT DE gene list across all 4 time-points.

The DE genes discussed below include several that are classically associated with parental care such as the teleost ortholog to *oxytocin, isotocin neurophysin* (*itnp*), and others that have more recently emerged as potential candidates in parental care such as *neurotensin* (*nts*). We find strong correlation of expression that clusters genes involved in parental care with others classically associated with feeding, such as *galanin* (*gal*). As expected, given the voluntary starvation phase, many of the known orexigenic genes and anorexigenic genes vary dramatically across the time-points sampled, and we see compensatory response genes that constitute metabolic regulatory mechanisms, suggesting hypoxic conditions and activation of neuroprotective genes.

Gene Ontology Enrichment

GO enrichment for the 128 genes determined to be differentially expressed across stages by LRT analysis (regardless of comparison or directionality) yielded only six significant terms (Table 2). Although none of the enriched GO terms are typically associated with maternal or voluntary fasting behavior in our searches of current literature. Instead, five were related to oxygen transport, and the sixth was “negative regulation of protein phosphatase type 2B activity”. The oxygen transport GO categories were detected based on differential expression of

4 globin genes, and “negative regulation of protein phosphatase type 2B activity” was represented by 2 DE genes, *repressor of calcipressin1-like* (*rcan1l*) and *peptidyl-prolyl cis-trans isomerase fkbp1-like*. Although the number of significant DE genes in both of these groups was low and significance levels may be inflated as a result, there were only 25 and 3 non-significant genes in these GO categories, respectively, among the remaining 22,924 annotated genes in the brain transcriptome. In addition, these GO groups are represented by genes that show dramatic changes across time-points, with globin gene *hemoglobin subunit beta-A-like* as the third most significant DE gene (FDR=4.68E-6) and *rcan1l* as the second most significant gene (FDR=8.75E-14) in the dataset. When running GO enrichment analysis on the 1,643 genes that trend toward differential expression for an unadjusted $P < 0.05$, we found a list of six different enriched terms (Table 2), including “hormone activity” as the top GO term represented by 36 accessions. These findings suggest that observed behavioral changes may not require vast changes in expression of entire gene pathways, but rather they could result from dramatic changes in a small number of genes or minor expression changes in larger gene networks.

GSEA

We used GSEA as a parallel approach to discover enriched pathways that differ between time-points, which allowed us to detect differences in aggregate signal for groups of genes related in function even when individual member genes fail to meet significance thresholds in DE analysis. In fact, there are a number of enriched sets that fail to contain any identified DE genes. Because there is partial overlap of gene accessions between sets, we merged significant GO sets into families, and after this data reduction step we found a total of 23 enriched GO families comprised of 107 GO sets and an additional 32 unmerged GO terms for a total of 55 independent sets (Fig. 2, File S3). These enriched GO terms include several that are potentially involved in modifying behavior, including several related to neuronal growth/death and neuropeptide receptor activity. Other notable enriched families and sets are related to ion channel regulation, oxygen transport, energy metabolism, and inflammatory response. Following normalized enrichment score (NES) clustering for these sets, the two dominant patterns that emerged are both highly dynamic, alternating between upregulation and downregulation across adjacent time-points. The majority of independent GO sets and families (38/55) show these patterns with 19 sets upregulated in B02 and R02 relative to B14 and R14 and another 19 sets showing the opposite pattern. Fourteen (14/55) of the remaining sets show more gradual expression changes across the approximately cyclical time-points with adjacent pairs of time-points having either higher or lower expression than the two distal time-points. The

remaining 3/55 sets are families composed of GO terms with inconsistent NES patterns between time-points.

Discussion

Among cichlids, mouthbrooding is thought to have arisen 10–14 times (Goodwin, Balshine-Earn and Reynolds 1998) and once at the base of the remarkably diverse haplochromine clade (DeVos and Seegers 1998). Thus, the mouthbrooding haplochromine cichlid species *A. burtoni*, an important model system for the physiological and genomics studies of complex social behavior (Maruska and Fernald 2013; Brawand et al. 2014), offers a useful perspective to identify mechanisms underlying mouthbrooding that may have contributed to the diversification of this cichlid clade and possibly also in others.

Across the mouth-brooding reproductive cycle, female *A. burtoni* undergo two important transitions: an initial transition to starvation during the obligatory mouthbrooding phase and a second transition to increased aggression and maternal defense following release of fry (Renn et al., 2009). The care given to the developing offspring during the mouthbrooding phase includes metabolically demanding buccal churning to aerate the fry. As the obligatory phase of mouthbrooding continues, this metabolic demand is coupled with a dramatic reduction in body condition due to prolonged voluntary starvation. Following the release of fry from the buccal cavity the metabolically demanding maternal care is coupled with resumption of feeding behavior. From the perspective of parental care, while considering fundamental distinctions, these two transitions can be compared to initiation of mammalian pregnancy and parturition, or avian incubation of eggs which involves a decrease in activity and early provisioning of chicks with increased maternal activity. From the perspective of feeding behavior, these transitions are dramatically different from mammalian transitions of pregnancy and parturition given the complete lack of food intake and dramatic reduction in body condition during brooding.

The time-points selected for the current study highlight these important transitions that encompass behavioral, feeding, and metabolic changes. The late release time-point, R14, provides a rough baseline during which extensive maternal care and aggression have subsided. The early brooding phase, B02, describes the initial transition to the maternal brooding stage while late brooding stage, B14, describes an individual that has undergone substantial loss of body condition and yet retains the brood care motivation while sustaining suppression of feeding behavior. The early release stage, R02, describes the social stages of maternal care as the female interacts with offspring, shows heightened aggression and has resumed feeding behavior. That we find the greatest number of differentially regulated genes between B14 and

R02 likely reflects the dramatic differences with regard to both maternal behavior, and body condition.

The results discussed below include both known and novel candidates for the regulation of maternal care, feeding behavior, and metabolic regulation. In addition, oxygen transport pathways were found to be dramatically downregulated throughout mouthbrooding while neuroprotective pathways and immune response were upregulated, likely to mitigate negative consequences of metabolic depression brought about by fasting. These results offer new insights into the evolution of mouthbrooding behavior. The individual genes identified by DE analysis are corroborated by GO term and GSEA analysis implicating full pathways in these transitions.

Some of the GO and GSEA analysis results (*e.g.* “equilibrio-perception” and “sensory perception of light”) indicate biological processes known to be controlled by specialized brain regions (Kasumyan, 2004; Rosa Salva et al., 2014), thus pointing to the need for future studies focused on more localized gene expression in the brain using micropunch or single cell approaches. With regard to maternal mouthbrooding, it is known that activation of galanin neuropeptide expressing neurons in the POA plays a role in maternal care and infanticide while activation of neurons expressing the same peptide in the NLT brain region is associated with feeding regulation (Butler et al., 2020). To the extent that neuronal activation correlates with gene regulation, when not studied in isolation, gene expression profiles from these regions controlling different aspects of behavior will be lost.

Parental and reproductive behavior

Numerous studies show positive correlation between parental care and expression of the teleost ortholog to *oxytocin, isotocin neurophysin* (*itnp*) (DeAngelis et al., 2017; Keverne and Kendrick, 1992; O’Connell et al., 2012; Pedersen et al., 1982), making it a candidate gene for regulation in the current study. When compared to baseline, R14, late stage brooding females in B14 show significantly lower expression of *itnp* (FDR = 7.32E-2) (Fig. 3). This negative, rather than positive, correlation between *itnp* and care may be analogous to the reduced expression seen in female stickleback following spawning (Kulczykowska and Kleszczyńska, 2014) associated with oocyte maturation and ovulation, or may simply reflect the low social engagement during this phase and suggest it is not a primary regulator of maternal mouthbrooding behavior in *A. burtoni*.

The neuropeptide *neurotensin* (*nts*) has emerged as a candidate for involvement in parental care, showing significantly elevated expression (FDR = 4.1E-2) throughout the mouthbrooding stages with a sharp decrease after release of fry (Fig. 3, 4). Expression of *nts* is positively and negatively associated with different forms of parental behavior in different taxa. In mice, injected *nts* inversely modulates aggressive behavior potentially through interaction with its receptor *neurotensin receptor 1* (*ntsr1; Fig. 4*) (Gammie et al., 2009) and causes increased neuronal activity in several brain regions including the lateral septal nucleus (LS) and bed nucleus of the stria terminalis (BNST), which are involved in social behavior and anxiety (Clauss et al., 2019; Deng et al., 2019). Localized expression studies in mice suggest a complex relationship with elevated *nts* expression in postpartum females in the medial preoptic area (MPOA) and lower expression in the LS (Driessen et al., 2014). Meanwhile, data from birds, domesticated Japanese quails, show higher *nts* expression in the female hypothalamus associated with parental behavior including reduced aggression towards juveniles (Lopes and de Bruijn, 2021).

The involvement of the *nts* pathway, beyond differential expression of the neuropeptide itself, is corroborated by our GSEA enrichment results that reveal the GO set “G protein-coupled neurotensin receptor activity” to be increased in B14 compared to B02 fish (FDR = 1.18E-2), suggesting receptor expression may be subject to feedback from early expression of the neuropeptide (Fig. 2B, File S3). This GO set represented many genes of low-expression level that failed to meet significance thresholds independently, though 3 of the 17 *nts* receptors or receptor-interacting ligands did reach an unadjusted threshold ($P < 0.05$) (Fig. 4). Two of these genes, *sortilin 1/neurotensin receptor 3* (*sort1*) and *sortilin-related VPS10 domain containing receptor 3-like* (*sorcs3l*) follow a pattern similar to *nts* itself, although their highest expression is in B14 instead of B02. Spatial coexpression of *nts* and *sort1* is also well documented in mammals, with both genes upregulated in the MPOA while being downregulated in the LS brain region of postpartum female mice relative to virgins, (Driessen et al., 2014). These region-specific changes in mice further highlight the need for more targeted analysis in the future in *A. burtoni* in order to disentangle the interactive role of nts and its receptors in the regulation of maternal behavior. In our study, a third *nts* receptor, *neuromedin U receptor 1* (*nmur1*) follows an inverted pattern relative to *nts* with its highest expression at release, R02. This gene’s functional relationship to *nts* signaling is not well studied as its primary affinity is for the pleiotropic peptide *neuromedin U* (*nmu*), which has a brain expression profile that parallels *nmur1* with highest expression in R02, suggesting they are coexpressed in the maternal brain (Martinez and O’Driscoll, 2015).

Among the DE neuropeptides, hormones, and receptors shown in Fig. 4, hierarchical clustering revealed that expression of *nts* was most strongly correlated with *galanin* (*gal*) with both expressed at significantly higher levels in brooding time-points relative to release time-points (Fig. 4, File S1). Studies of fish, mammals, and amphibians have found gal to be associated with parental behavior (Butler et al., 2020; Fischer et al., 2019; Wu et al., 2014). Specific MPOA galanin neurons are activated during parental behavior in male and female mice, and optogenetic activation induced parental pup-grooming while genetic ablation suppresses parental behavior (Wu et al., 2014). In *A. burtoni*, galanin neuron activity is associated with maternal care in the POA and feeding in the arcuate nucleus (Butler et al., 2020). Correlated increased expression of *gal* and *nts* is also reported in POA of *Dendrobates tinctorius* males involved in tadpole transport (Fischer et al., 2019). That expression of *nts* and *gal* wanes in *A. burtoni,* as maternal aggression increases just prior to and following the release of fry (Renn et al., 2009), suggests a conserved role in recognition and protection of offspring rather than overt defense. Additional parallels with paternal care in frogs include the upregulation of *myelin basic protein-like* (*mbpl*) (unadjusted P = 1.51E-2) and downregulation of *progesterone receptor* (*pgr*) (unadjusted P = 8.83E-3) and *prolactin receptor b* (*prlrb*) (unadjusted P = 8.80E-3) at the two mouthbrooding time-points in *A. burtoni* and in parental *D. rinctorius* males, yet *prolactin* (*prl*) itself was not differentially expressed in either study. Several genes associated with parental behavior in both studies show opposite regulation, including *neuroligin-3* (*nlgn3*) and *secretogranin II* (*scg2*), which are downregulated in the *R. imitator* parental males and upregulated in *A. burtoni* mouthbrooding females, while *androgen receptor* (*ar*) shows the opposite pattern in each species. Assuming these normalized read counts correlate with translated peptide levels, the opposing patterns of differential expression between *A. burtoni* and *D. rinctorius* may reflect the species-specific demands of parental behavior or result from parental behavior being expressed in the opposite sex.

While inconsistent patterns of regulation between *D. rinctorius* and *A. burtoni* may result from the different techniques, brain punch versus whole brain, or taxa-specific parental strategies, the similarity in suites of genes at play is of note. In the frog POA, *cocaine- and amphetamine-regulated transcript (cart)* is upregulated in parental males while *corticotropin-releasing hormone binding protein* (*crhbp*) is downregulated and in the MP *corticotropin-releasing factor receptor 2* (*crhr2*) as well as *cart* are upregulated while *pro-thyrotropin-releasing hormone* and *thyrotropin-releasing hormone* (*trh*) *receptor* is downregulated (Fischer et al., 2019). In *A. burtoni,* homologs of these ligands and receptors (*cartl*, *trh*, *crhbpl,* and *crh*) are strongly correlated in expression with increased expression in B02 and R02 surrounding the

transition from mouth-brooding care to defense of fry. In mice, *Crhbp* inhibits endogenous *Crh* to stimulate maternal aggression and deletion of the gene inhibits maternal aggression (Gammie et al., 2008), suggesting it may play a similar role in the *A. burtoni* maternal transition.

Fasting and Metabolic Regulation

Under normal conditions, the arcuate nucleus contains two specific populations of neurons, one producing orexigenic neuropeptide Y (NPY) and agouti related peptide (AgRP) to stimulate feeding behavior, and the other producing anorexigenic pro-opiomelanotcortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) to inhibit feeding, which are directly responsive to the peripheral signals of satiety, leptin (Cone, 2005) and ghrelin (Cowley et al., 2003), and indicate energy reserves and body condition. However, in brooding females the reduction in body mass (Grone et al., 2012; Renn et al., 2009) that should signal to increase orexigenic signals do not result in feeding behavior during the mouthbrooding time-points. Among the many hormone/neuropeptide genes related to feeding behavior, we find several to be among those trending toward differential expression across the reproductive time-points, including *relaxin-3-like* (*rln3l*), *gal*, *cartl, cholecystokinin-like* (*cckl*), *pro-opiomelanocortin B* (*pomcb*), and *peptide YY* (*pyy*) (Fig. 3). While many of these show large variances within time-points, they appear to be responsive to the resumption of feeding post-release.

The anorexigenic genes *pomcb* and *cart* show highest expression after resumption of feeding, R02, and are low during brooding, again reflecting energetic state. These genes are regulated by peripheral signals leptin and insulin (Lau and Herzog, 2014; Valen et al., 2011). Orexigenic genes *gal* and *rln3l* cluster with *nts* (Fig. 3) and show increased expression during mouthbrooding when females are not eating. As these genes are expected to promote food intake (Ganella et al., 2013; Volkoff and Peter, 2001) their upregulation during brooding suggests that the brains may be receiving the typical signals of hunger from the periphery, although the females suppress or do not respond to the signals with increased feeding. Alternatively, these genes may be playing a role directly promoting maternal mouthbrooding care.

While anorexigenic *neuropeptide Y* (*npy*) does not show differential expression across time-points in the current study, a related peptide, *pyy*, is elevated in mouthbrooding stages (unadjusted P = 0.01). In the digestive system *pyy* expression increases with food intake to signal satiety (Karra et al., 2009), however in the brain its response to fasting is variable across species (Volkoff, 2016). In a study of the mouse enteroendocrine system *pyy* was shown to be coexpressed and co-secreted with both *nts* and *glucagon-like peptide-1* (*glp1*) (Grunddal et al.,

2016). The genes *pyy* and *glp1* were each found to work synergistically with *nts* in these mice to inhibit gastric emptying and decrease food intake. In *A. burtoni* maternal brains, *pyy* expression correlates with *nts* expression (Fig. 3) across samples through a clustering of candidate DE neurohormones and neuropeptides. Its role in this system and potential interaction with *nts* in regulation of fasting behavior warrants further study.

In addition to the potential role of *nts* in parental behavior, this neuropeptide has a pleiotropic role and also can regulate fasting. In a number of studies in mice and rats, researchers have shown that increases in *nts* in the brain suppressed feeding behavior through interaction with its associated receptor, *ntsr1* (Kim et al., 2008; Schroeder and Leinninger, 2018). In *A. burtoni*, we see the highest expression of *nts* in mouthbrooding fish and a sizable drop in non-brooders but no change in low-expressed *ntsr1* (Fig. 4). This pattern is consistent with the hypothesis that *nts* plays a role in fasting behavior in mouthbrooding females by eliciting an anorectic effect despite static expression of *ntsr1*. It is possible that mouthbrooding *A. burtoni* may actually be suppressing signals that would normally increase hunger and feeding behavior. Such suppression has been suggested by studies addressing the expression of feeding behavior-associated hormones, neuropeptides, and their receptors in *A. burtoni* and *O. niloticus* brains (Das et al., 2019; Grone et al., 2012).

Many of our DE genes point to compensatory metabolic mechanisms to allow fish to withstand long periods of fasting. For example, the pyruvate dehydrogenase kinases *pdk2* (FDR = 9.71E-5) and *pdk4* (FDR = 0.024) show significantly higher expression in the brain in the two mouthbrooding time-points relative to the two non-brooding time-points. These genes are associated with food deprivation in fish, mammals, and other taxa, and have been shown to increase during periods of starvation then decrease after resumption of feeding (Wu et al., 2000; Yang et al., 2019). This energy intensive pathway mediates the interaction of glycolysis and the tricarboxylic acid cycle (Sugden and Holness, 2003). The functional conservation of pyruvate dehydrogenase kinase activity across taxa may have been a necessary mechanism allowing for the evolution of mouthbrooding behavior in cichlids and other independent fish lineages due to the associated fasting behavior.

GSEA results also corroborate this change in mitochondrial function with GO sets related to mitochondrial ATP production and electron transport showing upregulation in B02 fish relative to R14 fish (Fig. 2, File S4). These GO terms signal a shift in energy metabolic processes at the start of a long period of prolonged fasting, and include gene sets like “ATP biosynthetic process”, “mitochondrial fusion”, “mitochondrial electron transport, NADH to ubiquinone”, “proton−transporting ATP synthase activity, rotational mechanism”, and

"mitochondrial proton−transporting ATP synthase complex". Metabolic downshifts are common among starving or hibernating animals and are often characterized by reduction in mitochondrial function in liver and skeletal muscle tissue for energy conservation (Barger et al., 2003). That we see these changes anticipated in the periphery to be occurring in brain samples may reflect high metabolic demand of neural tissue.

Hypoxic signaling and Neuroprotection

Late-stage (post-hatching) mouthbrooding cichlids show reduced tolerance to hypoxia (*P. multicolor:* (Corrie et al., 2008)) possibly due to costs of maternal behavior including buccal churning to aerate the eggs. However, we find a dramatic reduction in gene expression for several globin genes, which could also contribute to the observed sensitivity to hypoxia. Four genes reach, and one approaches, statistically significant reduction in expression at the late-brooding B14 and R02 time-points (Fig. 5). These contribute to GO term enrichment (Table 2) and GSEA results (Fig. 2) for oxygen transport related terms. These are among the most dramatic gene expression changes observed, likely reflecting consistent regulation across multiple brain regions. The reduction in globin expression may be a response to nutrient state as seen under starvation in the Seabream (Ntantali et al., 2020). The sustained expression could reflect "carryover", the persistence of acquired neurogenomic states into subsequent stages (Burkhari et al., 2019). However, we also find *hypoxia-inducible factor 1-alpha-like* (FDR = 0.12, unadjusted P = 7.72E-4) elevated in both brooding time-points along with two target genes, *pdk4,* known to be also induced by starvation (Lee et al., 2012), and *round spermatid basic protein 1-like* (*rsbn1l*), a less understood gene known to be expressed in testes as well as breast cancer tissue (Abu-Jamous et al., 2017). The correlated expression of *rsbn1l* and *pdk4* supports the hypothesis that mouthbrooding cichlids are responding to hypoxia-related signals possibly due to reduced globin gene expression.

The reduction in nutrient intake and apparent reduction in neural oxygen transport capacity confers a large metabolic cost that could be mitigated by neuroprotective pathways as suggested by GSEA (Figs 2, 4). We see varied and dynamic patterns among the neuroprotection-related genes, some being upregulated in late brooding and release such as two butyrophilin related DE genes (*butyrophilin subfamily 2 member A1-like* and *V-set domain-containing T-cell activation inhibitor 1-like)* that co-localize on a genome scaffold and are known immune system regulators that may have neuroprotective function in response to hypoxia, reduced nutrient intake, or the action of other DE genes associated with foreign DNA/RNA (Abeler-Dörner et al., 2012). Conversely, other genes involved in neuroprotection are

downregulated at late brooding time-points and upregulated at early brooding time-points. Downregulation of ECM/collagen-related DE genes including matrix metalloproteinases such as *matrix metalloproteinase 9* (*mmp9*) could impact recovery via delayed promotion of neuroblast cell migration (Chen et al., 2009) while regulation of *platelet-derived growth factor receptor-like* (*pdgfrl*), similar to other PDGF proteins seen in cerebral hypoxia of stroke victims (Krupinski et al., 1997), could aid in neural regeneration in response to neuronal damage.

Nutrient and oxygen deficit impact the regulation of ion gradients and ion transport pathways in the brain that require energy and aerobic ATP synthesis. Both ion transport and cell junction genes are upregulated in whole brain samples under starvation in seabream (Ntantali et al., 2020) and hypoxia-tolerant species are known to have evolved means to reduce ion leakage in the CNS (Boutilier, 2001), thus reducing energy demands in such conditions. Similarly, increased myelination mitigates loss of action potentials under hypoxia (Waxman et al.1990). These facts are consistent with the observed upregulation at B14 and R02 time-points of *cell adhesion molecule 4* (*cadm4*) (FDR=2.21E-14 & 3.2-fold change), which regulates myelination of the CNS (Elazar et al., 2019; Golan et al., 2013) and could contribute to the maintenance of action potentials under starvation and hypoxic conditions during mouthbrooding. These and other DE genes, including those coding for myosin, actin, and troponin complex proteins, contribute both to Molecular Function GO terms, like ion transport-related and action potential gradient-related GO sets, and to Biological Process GO terms related to structure and function of skeletal muscle and heart tissue (Fig. 2, File S3), supporting the hypothesis of added demand to regulate ion gradients for neural function during mouthbrooding. Here again, the patterns of regulation among genes contributing to ion transport-related GO terms is highly variable due to the individual genes being negative vs. positive regulators of the same or related processes.

In addition to oxygen transport and ion regulation, our GSEA analysis detected the inflammatory pathway as a large family of 16 GO sets under the umbrella term “interleukin-1 beta production” (File S3) that showed decreased expression at the early brooding B02 time-point and increased expression at the late brooding B14 time-point. While IL1B, a cytokine secreted by microglia in the brain in response to hypoxia or injury to the brain (Hewett et al., 2012), is not itself differentially expressed in our dataset, GSEA identifies genes involved in IL1B suppression via (NF-κB) signaling (*e.g. protein nlrc3-like, NACHT, LRR and PYD domains-containing protein 12-like*) (Allen, 2014). The upregulation of these pathways and static expression of cytokines may be adaptations that help avoid negative physiological consequences of prolonged inflammation associated with hypoxic conditions and starvation experienced during each repeated reproductive cycle (Martin & Król, 2017; Schäfer et al., 2021).

To the best of our knowledge, these immune function pathways have not been implicated in other whole brain studies of fish under starvation (*e.g*. Ntantali et al., 2020; Yang et al., 2019) but have been associated with paternal care in stickleback (Burkhari et al., 2019) suggesting their regulation may represent an adaptation in cichlids that reduce the costs of mouthbrooding.

Conclusions

Using female *A. burtoni*, we present a comprehensive analysis of whole-brain gene expression and how it changes across distinct brooding stages. The gene expression patterns reveal how the female brain may resolve trade-offs between metabolic demands and parental care. Similar conclusions can be drawn from lists of DE genes, GO analysis, and the GSEA results that rely on differing levels of statistical significance for individual genes. In addition to identifying *itnp,* classically associated with parental care, our data support the involvement of *nts*, a gene that has more recently emerged as a candidate in the regulation of social phenotypes. These, and other identified differentially expressed genes, could be subjects for exciting functional analysis with gene editing techniques that are being made available in *A. burtoni* and other emerging model organisms (Junti, 2019). Both GO enrichment and GSEA analysis further support the involvement of receptors and downstream activity of this pathway. Furthermore, correlation of gene expression ties these pathways to important neuropeptides involved in the regulation of feeding behavior and metabolism highlighting the known tradeoffs between care and feeding (O'Rourke and Renn, 2015).

Interestingly, we find both orexigenic and anorexigenic genes that show increased expression during mouthbrooding stages as well as anorexigenic genes that show decreased expression during mouthbrooding, which underscores the complexity of feeding regulation under a situation of voluntary starvation. Future transcriptomic studies should include food-deprived individuals as an additional control group for comparison with mouthbrooding fish. Such a comparison would help elucidate the degree to which our observed increase in neuroprotective pathways and increased expression of negative regulators of inflammation represent evolved mechanisms to counter the hypoxic and metabolic demands of mouthbrooding. Our results underscore that cichlid maternal behavior and fasting are potentially regulated by pleiotropic activity of novel peptides such as *neurotensin* as well as the expected classical neurohormones and neuropeptides. It is noteworthy that these hormonal signals were dwarfed by dramatic changes observed in oxygen transport capacity, neuroprotection, and ionic regulation pathways in the brain that mirror metabolic shifts from animals regularly subjected to starvation or hypoxic conditions. Here, additional time-points through these transitions could be

used to further test the degree to which gene expression changes persist to reflect previous neurogenomic states versus priming the brain for future neurogenomic states (Burkhari et al., 2019). While the observed changes in neurohormones are brain logically brain specific, many of the other gene expression changes are likely not unique to brain tissue and rather reflect systemic response to stresses of starvation and energy demands.

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Data availability

Raw transcriptome data and the processed read count table are available in NCBI's GEO repository under the accession GSE192958. R-code, input files, and supplementary (as well as additional WGCNA analysis) files can be found in GitHub repository: github.com/jfaberha/cichlid_transcriptome/tree/main/lab_stock_analysis.

Author contributions

SCPR conceived of the project, prepared samples, JFH performed the bioinformatics. Both authors conducted analyses and wrote the manuscript.

Competing Interests

No competing interests declared

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Figures and Tables

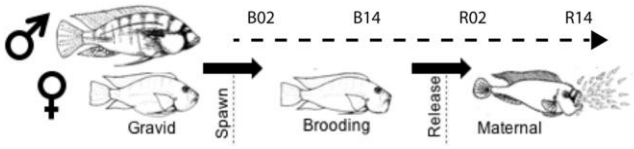

Fig. 1. The four groups of samples in this study consist of whole brains of females harvested two days after the start of mouthbrooding (B02), fourteen days after the start of mouthbrooding (B14), two days post-release of fry (R02), and fourteen days post-release of fry (R14).

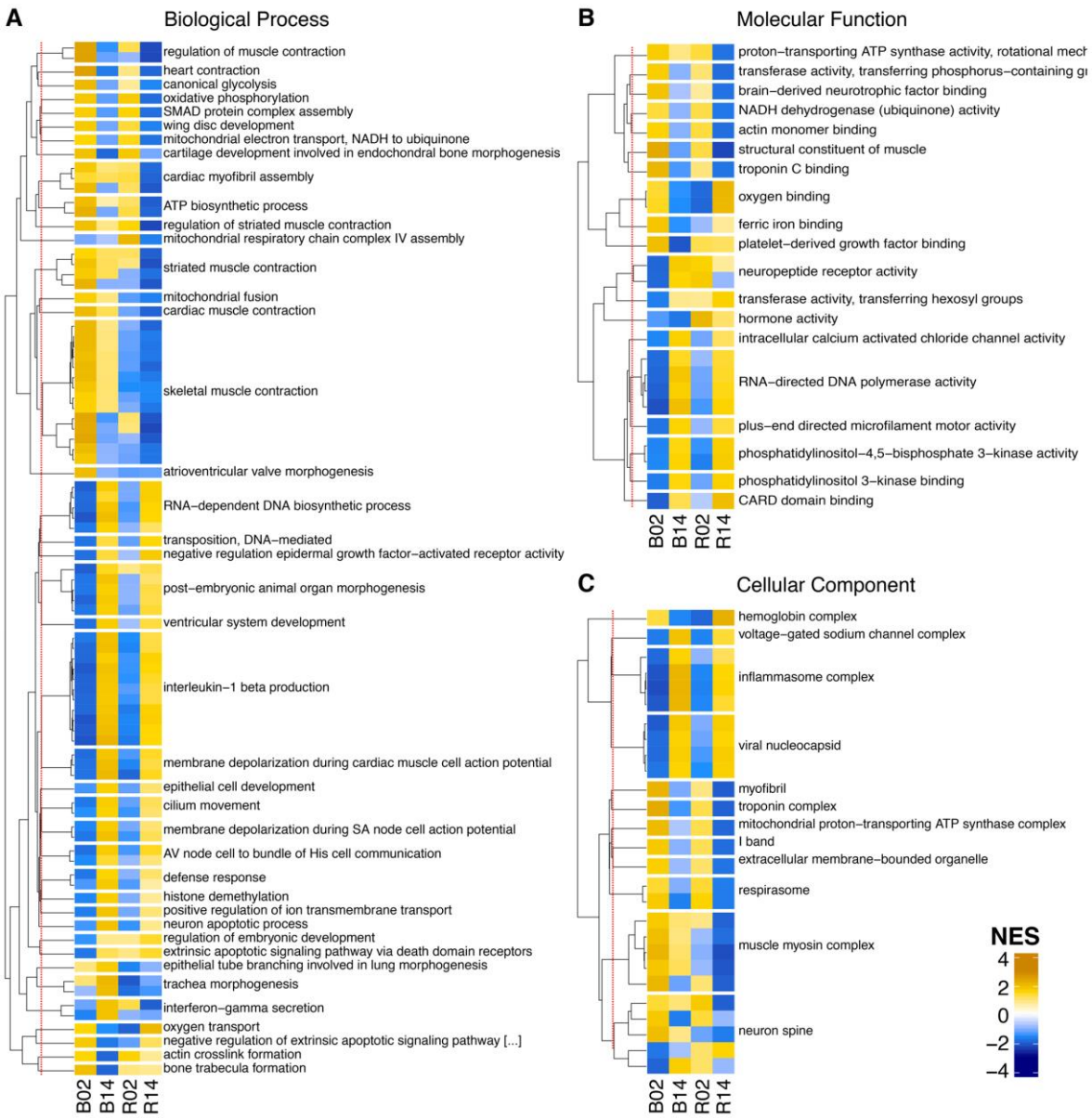

Fig. 2. Heatmap shows clustering of normalized enrichment scores (NES) for significant GO gene sets identified by GSEA. Terms from each of the three GO categories are shown separately; A) biological process, B) molecular function, and C) cellular component. Terms were merged if they had a minimum overlap coefficient of 50%, represented by the dashed red line, for the number of shared member genes and hierarchical clustering of GO terms was performed using the Ward.D2 clustering method. GO terms on the right of each heatmap either represent unclustered GO terms or the most significant GO term within a merged cluster. For B02 and R02 time-points, *n*=5 and for B14 and R14 time-points, *n*=6.

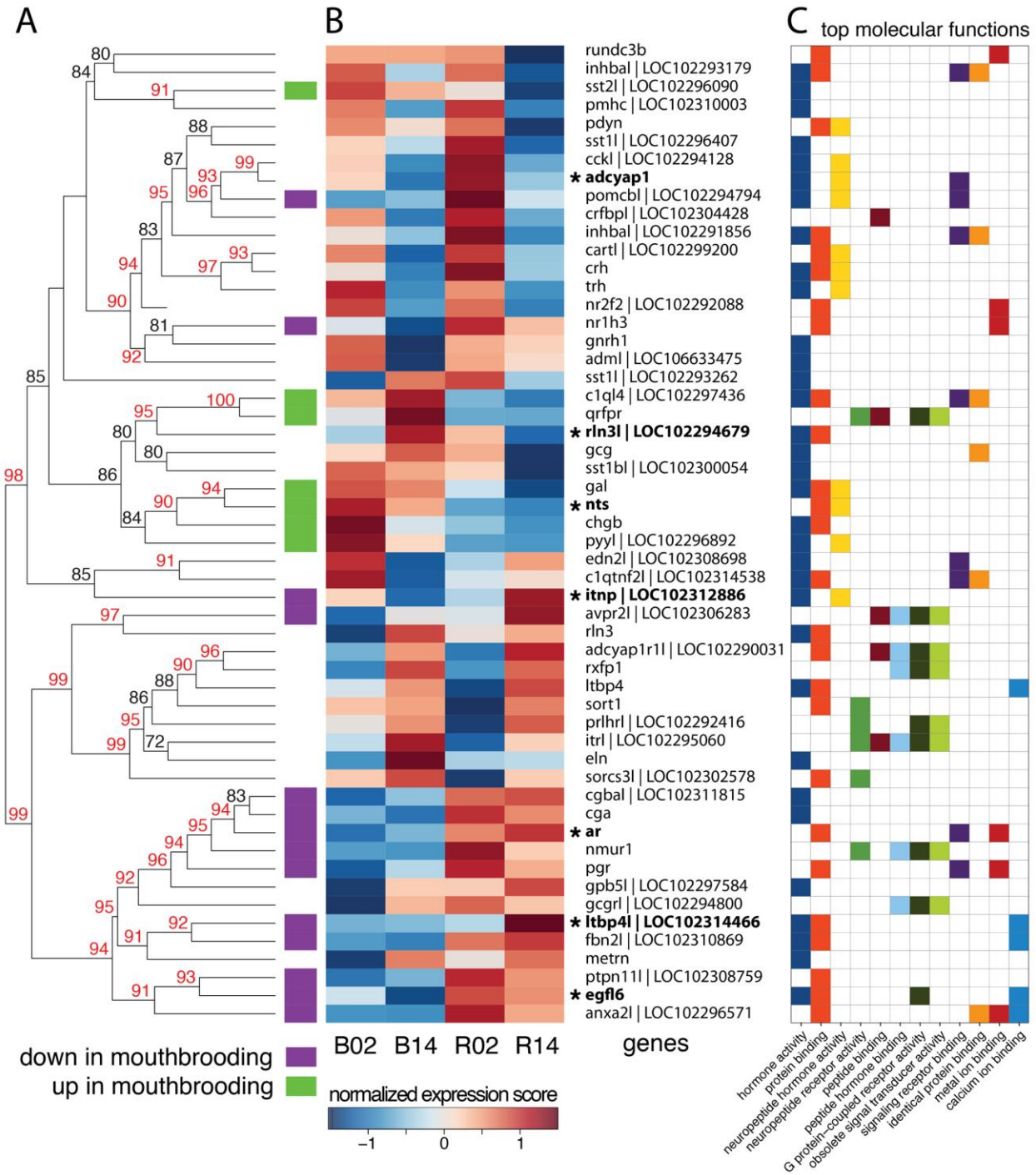

Fig. 3. Heatmap shows the (A) hierarchical clustering of (B) gene expression patterns of neurohormones, neuropeptides, and receptors that were selected from the DE-trending gene list based on their gene onotology (GO) annotations. Gene hierarchical clustering was performed using the average linkage method with a correlation metric distance. Bootstrap values were

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generated in the PVclust R package with red values highlighting nodes conserved in ≥90% of iterations. Heatmap values represent mean VST expression of samples within groups scaled by row for visualization in the made4 R package. Gene abbreviations were manually assigned based on reference gene names for those that include *A. burtoni* accessions. Genes with asterisks and bold are differentially expressed based on LRT analysis at FDR < 0.1, while all other genes show near significance at a less stringent threshold with an unadjusted P < 0.05. Genes marked with side colors are either up (green) or down (purple) in pooled mouthbrooding time-points relative to pooled release time-points based on DESeq2 Binomial Wald results at an unadjusted P < 0.05. (C) The top 12 molecular function GO terms, selected by the abundance of represented genes within this set, are displayed in a binary heatplot with columns clustered by the average linkage method and Euclidean distance. For B02 and R02 time-points, *n*=5 and for B14 and R14 time-points, *n*=6.

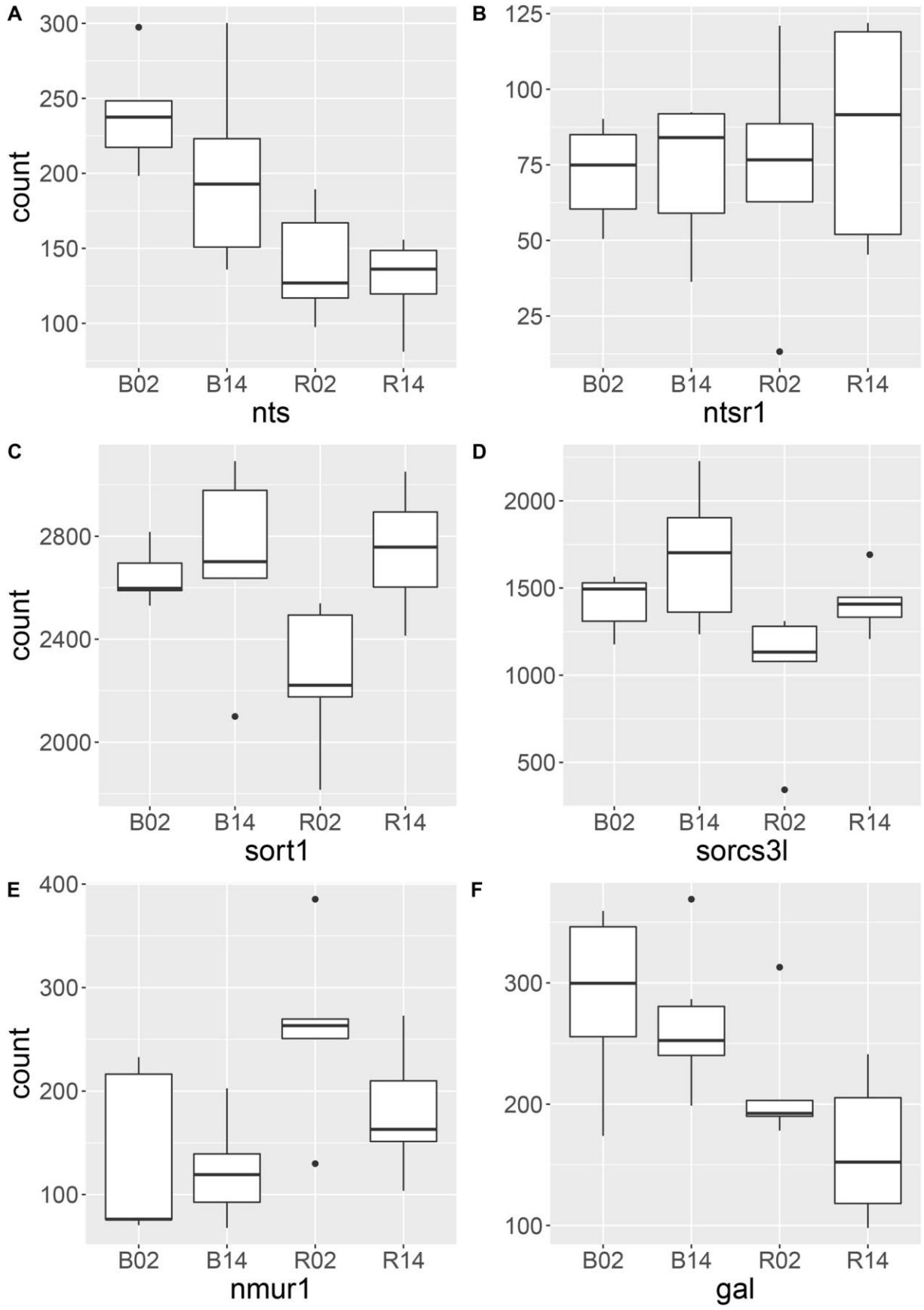

Fig. 4. Boxplots showing expression by time-point for candidate behavioral gene *neurotensin* (A), its receptors (*neurotensin receptor 1*: B; *sortilin 1*: C; *VPS10 domain-containing receptor SorCS3-like*: D; *neuromedin-U receptor 1*: E), and correlated behavioral neurohormone *galanin* (F). Expression is represented as DESeq2 normalized counts.

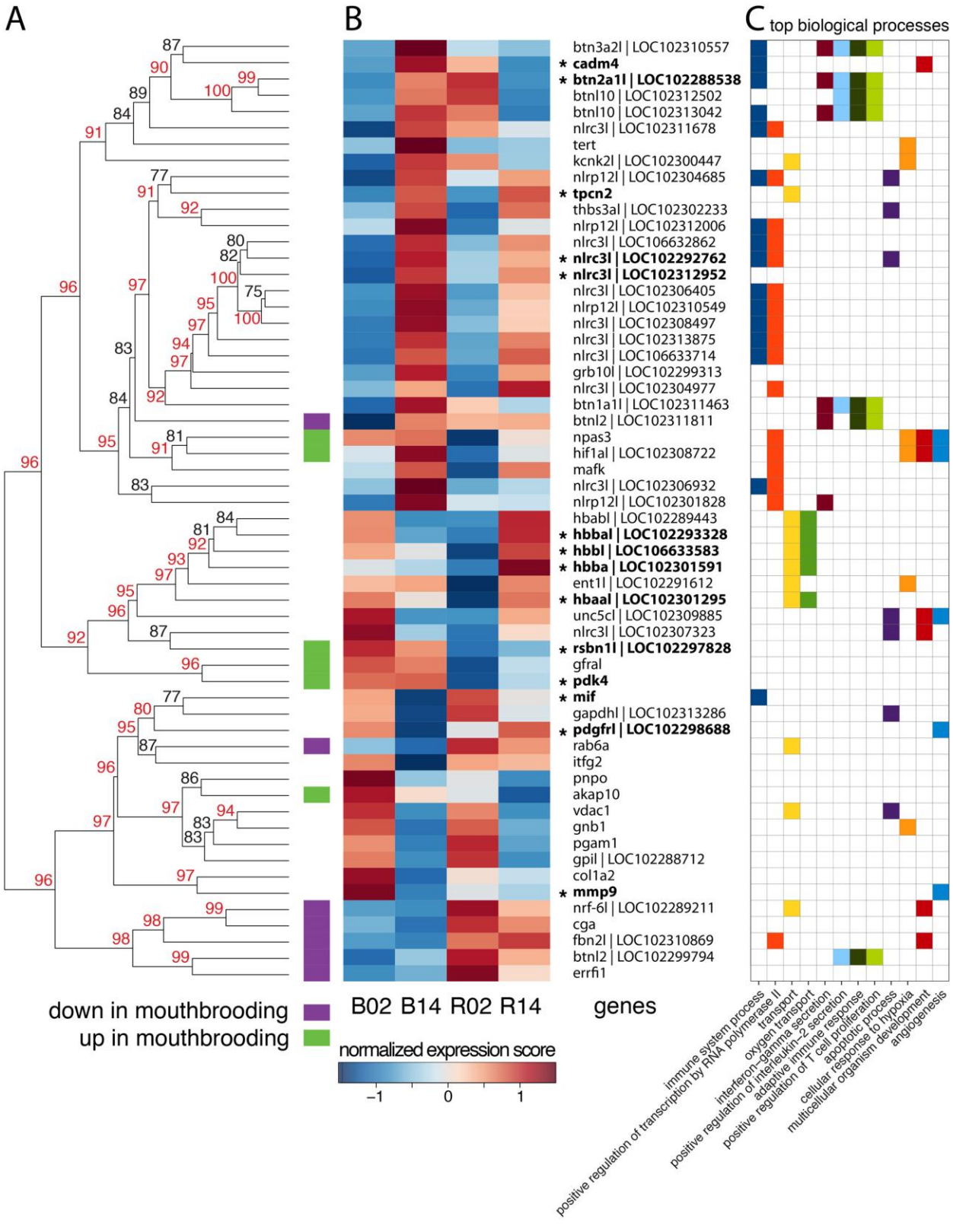

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Fig. 5. Heatmap shows the (A) hierarchical clustering of (B) gene expression patterns of candidate genes involved in response to hypoxia and neuroprotection. Genes in this figure were selected from the DE-trending list for having functional GO annotations related to oxygen-transport, neuroprotection, neuroinflammation, or response to hypoxia, plus many genes from a list of hif1a-interacting genes from Harmonizome database (https://maayanlab.cloud/Harmonizome/; accessed June 2020). Gene hierarchical clustering was performed using the average linkage method with a correlation metric distance. Bootstrap values were generated in the PVclust R package with red values highlighting nodes conserved in ≥90% of iterations. Heatmap values represent mean VST expression of samples within groups scaled by row for visualization in the made4 R package. Gene abbreviations were manually assigned based on reference gene names for those that include *A. burtoni* accessions. Genes with asterisks and bold are differentially expressed based on LRT analysis at FDR < 0.1, while all other genes show near significance at an unadjusted P < 0.05. Genes marked with side colors are either up (green) or down (purple) in pooled mouthbrooding time-points relative to pooled release time-points based on DESeq2 Binomial Wald results at an unadjusted P < 0.05. (C) The top 12 biological process GO terms, selected by the abundance of represented genes within this set, are displayed in a binary heatplot with columns clustered by the average linkage method and Euclidean distance. For B02 and R02 time-points, *n*=5 and for B14 and R14 time-points, *n*=6.

Table 1. Summary of the number of P DE genes between stages identified by pairwise comparison (Walds Binomial FDR p<0.1). Red (above the diagonal) indicates upregulation in the stage named in the row compared to to stage named in the column; blue indicates downregulation in the stage named in the column stage compared to to the stage named in the row. Deeper shades represent DE genes found in all 3 pairwise comparisons and percentage calculation for stage-specific regulation.

	B02	B14	R02	R14	all 3 others	% specific
B02		16	11	5	0	2.56
B14	26		44	7	0	0.56
R02	9	61		7	0	1.36
R14	11	26	15		0	0
all 3 others	2	1	2	0		

Table 2. Gene Ontology enrichment of DE gene lists from LRT analyses performed in DESeq2, interpreted as genes that differ in expression across experimental time-points.

DE test set threshold	GO-ID	Description	corr p-value	ratio of test genes	ratio of ref genes
FDR<0.1	GO:0005833	hemoglobin complex	9.30E-03	4/124	22/22924
FDR<0.1	GO:0005344	oxygen transporter activity	9.30E-03	4/124	25/22924
FDR<0.1	GO:0015671	oxygen transport	9.30E-03	4/124	26/22924
FDR<0.1	GO:0015669	gas transport	9.30E-03	4/124	27/22924
FDR<0.1	GO:0019825	oxygen binding	9.30E-03	4/124	28/22924
FDR<0.1	GO:0032513	negative regulation of protein phosphatase type 2B activity	4.45E-02	2/124	3/22924
P<0.05	GO:0005179	hormone activity	3.46E-03	36/1563	213/22924
P<0.05	GO:0033269	internode region of axon	3.13E-02	6/1563	9/22924
P<0.05	GO:0061077	chaperone-mediated protein folding	3.86E-02	23/1563	126/22924
P<0.05	GO:0005576	extracellular region	4.09E-02	470/1563	5869/22924
P<0.05	GO:0048200	Golgi transport vesicle coating	4.09E-02	5/1563	7/22924
P<0.05	GO:0048205	COPI coating of Golgi vesicle	4.09E-02	5/1563	7/22924

Table S1. Differentially expressed genes identified by LRT in DESeq2 with an unadjusted P-value less than 0.05 are found in the "DESeq2_LRT_results" tab. Pairwise Binomial Wald results for this same gene set are included. Genes are identified by their Stringtie accessions and associated NCBI RefSeq annotations. Cells are highlighted for DE genes with FDR < 0.1 for the respective group contrast. The second tab, "DESeq2_BvsR_results", contains full set of genes for Brooding vs. Release Binomial Wald test at the same significance threshold.

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Table S2. Annotation information for expression data derived from the GTF file associated with NCBI accession GCF_000239415.1, plus Gene Ontology info used in enrichment analysis.

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Table S3. Results for Gene Set Enrichment Analysis (GSEA) using GO terms as gene categories. Rows contain results for a single GO term and pairwise contrast between time-points. Additional columns contain GSEA results for each time-point vs. all other time-points combined. GO terms are clusters to account for gene overlap between lists. Second data spreadsheet contains gene list and annotations for each of the significantly enriched GO terms identified by GSEA.

Click here to download Table S3