A peer-reviewed version of this preprint was published in PeerJ on 20 January 2020.

View the peer-reviewed version (peerj.com/articles/8375), which is the preferred citable publication unless you specifically need to cite this preprint.

Ahi EP, Duenser A, Singh P, Gessl W, Sturmbauer C. 2020. Appetite regulating genes may contribute to herbivory versus carnivory trophic divergence in haplochromine cichlids. PeerJ 8:e8375 https://doi.org/10.7717/peerj.8375

Appetite regulating genes may contribute to herbivory versus carnivory trophic divergence in haplochromine cichlids

Ehsan P Ahi [Corresp., 1, 2], **Anna Duenser** [2], **Pooja Singh** [2, 3], **Wolfgang Gessl** [2], **Christian Sturmbauer** [2]

[1] Evolutionary Biology Centre, Norbyvägen 18A, Uppsala Universitet, Uppsala, Sweden

[2] Institute of Biology, Universitätsplatz 2, Universität Graz, Graz, Austria

[3] Institute of Biological Sciences, University of Calgary, Calgary, Canada

Corresponding Author: Ehsan P Ahi
Email address: ehsanpashayahi@gmail.com

Feeding is a complex behaviour comprised of satiety control, foraging, ingestion and subsequent digestion. Cichlids from the East African Great Lakes are renowned for their diverse trophic specializations, largely predicated on highly variable jaw morphologies. Thus, most research has focused on dissecting the genetic, morphological and regulatory basis of jaw and teeth development in these species. Here for the first time we explore another aspect of feeding, the regulation of appetite related genes that are expressed in the brain and control satiety in cichlid fishes. Using qPCR analysis, we first validate stably expressed reference genes in the brain of six haplochromine cichlid species at the end of larval development prior to foraging. We next evaluate the expression of 16 appetite related genes in herbivorous and carnivorous species from the parallel radiations of Lake Tanganyika, Malawi and Victoria. Interestingly, we find increased expression of two anorexigenic genes, *cart* and *npy2r,* in the brain of carnivorous species in all the lakes. This supports the notion that herbivory compared to carnivory requires stronger appetite stimulation in order to feed larger quantity of food and to compensate for the relatively poorer nutritional quality of a plant- and algae-based diet. Our study contributes to the limited body of knowledge on the neurological circuitry that controls feeding transitions and adaptations and in cichlids and other teleosts.

Appetite regulating genes may contribute to herbivory versus carnivory trophic divergence in haplochromine cichlids

Authors

Ehsan Pashay Ahi[1,2],
Email: ehsanpashayahi@gmail.com

Anna Duenser[1],
Email: anna.duenser@gmail.com

Pooja Singh[1,3],
Email: pooja.singh09@gmail.com

Wolfgang Gessl[1],
Email: wolfgang.gessl@uni-graz.at

Christian Sturmbauer[1],
Email: christian.sturmbauer@uni-graz.at

1. Institute of Biology, University of Graz, Universitätsplatz 2, A-8010 Graz, Austria.
2. Evolutionary Biology Centre, Uppsala University, Norbyvägen 18A, 75236 Uppsala, Sweden.
3. Institute of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

Corresponding Author: Ehsan Pashay Ahi,
Email: ehsanpashayahi@gmail.com

Abstract

Feeding is a complex behaviour comprised of satiety control, foraging, ingestion and subsequent digestion. Cichlids from the East African Great Lakes are renowned for their diverse trophic specializations, largely predicated on highly variable jaw morphologies. Thus, most research has focused on dissecting the genetic, morphological and regulatory basis of jaw and teeth development in these species. Here for the first time we explore another aspect of feeding, the regulation of appetite related genes that are expressed in the brain and control satiety in cichlid fishes. Using qPCR analysis, we first validate stably expressed reference genes in the brain of six haplochromine cichlid species at the end of larval development prior to foraging. We next evaluate the expression of 16 appetite related genes in herbivorous and carnivorous species from the parallel radiations of Lake Tanganyika, Malawi and Victoria. Interestingly, we find increased expression of two anorexigenic genes, *cart* and *npy2r,* in the brain of carnivorous species in all the lakes. This supports the notion that herbivory compared to carnivory requires stronger appetite stimulation in order to feed larger quantity of food and to compensate for the relatively poorer nutritional quality of a plant- and algae-based diet. Our study contributes to the limited body of knowledge on the neurological circuitry that controls feeding transitions and adaptations and in cichlids and other teleosts.

Keywords

Appetite regulation; Trophic specialization; Adaptive radiation; East African Lakes

Background

Little is known about the molecular mechanisms taking place in the central nervous system which evolved in conjunction with herbivorous and carnivorous trophic specialization in teleost fishes. Besides morphological changes in the feeding apparatus, divergence of feeding behaviour

might be another key feature of differential trophic adaptation since the two diet habits require foraging on different quantities of food to balance nutritional requirements due to the unequal quality of these diets. An immediate approach to investigate such molecular mechanisms is transcriptional analysis of genes involved in regulation of feeding behaviour through the central nervous system in fish (Volkoff et al., 2005). To date, only one study in grass carp (a species of Cypriniformes), which shows the transition from carnivory to herbivory during its ontogeny, has addressed gene expression changes in the brain between the two contrasting feeding habits (He et al., 2015). Interestingly, the authors found that few appetite-regulating genes which inhibit food intake (anorexigenic genes) had reduced expression in the brain at the herbivorous life stage, whereas few other genes with opposite effects (orexigenic genes) had increased expression at this stage (He et al., 2015). This finding was consistent with the notion that herbivory requires prolonged insatiety and more active feeding behaviour compared to carnivory in order to compensate for the relatively poorer nutritional quality of a plant-based diet (He et al., 2015). Although, a comprehensive list of potential appetite-regulating genes has been provided mainly from studies on cyprinid model species, such as zebrafish and goldfish, it has turned out that the regulatory function of many of these genes can vary across the orders of teleost fishes (Volkoff, 2016). In addition, only a small subset of the genes are confirmed to have similar appetite-regulating functions in other fish orders including Cichliformes and Perciformes (Volkoff, 2016).

Cichlids of the East African Great Lakes Tanganyika, Malawi and Victoria are well known for their stunning rates of speciation and adaptive radiation (Fryer and Iles, 1972; Kocher, 2004). Lake Tanganyika, being the oldest of the three lakes, shows the most diversity in ecomorphology, behaviour and genetics compared to Lake Malawi, the intermediate, and Lake Victoria, the youngest of the three lakes (Young et al., 2009; Salzburger et al., 2014). The Haplochromini are the most species rich tribe, having seeded the entire species flocks of Lake Malawi and Victoria and having recolonized Lake Tanganyika, giving rise to the tribe Tropheini (Salzburger et al., 2005). It is hypothesised that similar trophic ecomorphologies evolved in all three lakes in response to similar selection pressures as they were derived from a common generalist riverine ancestor (Kocher et al., 1993; Salzburger et al., 2005; Cooper et al., 2010).

Interestingly, haplochromine cichlids are mostly maternal mouthbrooders so the fry start feeding independently at a more mature stage, at the end of larval development (stage 26), compared to

non-mouthbrooders (Fujimura & Okada, 2007, 2008). Due to the high trophic phenotypic plasticity in haplochromine cichlids (Gunter et al., 2013; Schneider et al., 2014), it is important to decipher whether gene regulatory circuitry of appetite-regulating genes that triggers feeding behaviour can be already observed upon completion of the larval development prior to the onset of food intake or is activated once the larvae start feeding. The dietary plasticity, mouthbrooding behaviour and immense diversity of trophic specializations and foraging in cichlid fishes of East African species flocks provide an excellent opportunity to investigate the role of appetite-regulating genes in differential trophic adaptations associated with species divergence.

Here, we hypothesize that appetite-regulating genes might be already differentially regulated in the brain of distinctly adapted haplochromine cichlids at the end of larval development, before the fry is released from the mother's mouth to forage on their own. Our hypothesis advocates for low plasticity and high genetic wiring of feeding behaviour in these fish. Therefore, we selected 12 appetite-regulating genes and analysed their expression level in the brain in a set of three herbivorous and three carnivorous haplochromine cichlid fish species at stage 26 (Fujimura & Okada, 2007, 2008), which marks the end of larval development and the initiation of exogenous feeding. The selected candidate genes are known to have brain expression in fish and are involved in regulation of feeding behaviour by enhancing or inhibiting food intake in teleost fishes (Table 1). The study species belong to two major trophic niches in the three Great East African Lakes; Lake Tanganyika (LT), Lake Malawi (LM) and Lake Victoria (LV). We test whether the differential expression of appetite-regulating genes in the brain predicts the divergence in trophic specialization in differentially adapted species pairs prior to the actual searching for food resources. The study also addresses this possibility in the context of parallel trophic specialization across three independent adaptive radiations. This study reports the results of a first step by validation of stably expressed reference genes in the brain at the end of the larval stage, which allows us to accurately compare inter-species expression of the appetite regulating-genes in haplochromine cichlids. Our results suggest that expression differences of the candidate genes might predict the feeding behaviour of herbivore versus carnivore species before the onset of plastic molecular responses emanating from contrasting feeding diets.

Methods

Fish husbandry and sampling

Six haplochromine cichlid species belonging to two major trophic niches from Lakes Tanganyika (LT), Malawi (LM) and Victoria (LV), were chosen for studying brain gene expression. In order to compare divergent trophic niches, we used one carnivorous species (a piscivore/insectivore) and one herbivorous species (an algae-grazer) for each lake (Fig 1A), based upon previous phylogenetic studies (Koblmüller et al., 2008; Irissari et al., 2018). The parental fish were reared under standardized aquarium conditions and diet (Spirulina flakes with average protein content) until sexual maturation. The spawning pairs were colsely observed and 24 hours after mating their eggs were collected from the mouth of the females through exerting mild manual pressure to their cheeks. Then, the eggs of each species were placed in a standard glass jar with constant gentle shaking for an incubation period until hatching stage. After hatching, larvae were transferred to small floating tanks and kept until stage 26, the time of yolk sac absorption, marking the end of larval development (Fujimura & Okada, 2007, 2008). The rearing and incubation temperature was kept constant at 25.8 degrees centigrade. For each species six larvae were euthanized in water containing 0.2 gram MS-222 per litre, and the entire brain was carefully dissected using a stereomicroscope. The brain tissue from each individual represents one biological replicate, and therefore, six biological replicates per species were used for further analysis of gene expression. Moreover, by the end of the study the parents of the six haplochromine species were sacrificed in water containing 0.8 gram MS-222/litre.

RNA isolation and cDNA synthesis

The entire brain from each individual was dissected as one biological replicate and transferred into a tube with 250 µL of a lysis buffer, specific for RNA isolation from tissue, provided by Reliaprep RNA tissue miniprep system (Promega, #Z6111, USA). A 1.4 mm ceramic bead was added to shred the brain tissue. The brains were homogenized in a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, CA, USA) and total RNA content was extracted following the manufacturer protocol. The protocol has several relatively quick steps; mixing of the

142 homogenized tissue (in the lysis buffer) with isopropyl alcohol and filtering it through a column
143 provided by the kit, RNA washings and gDNA removal. Next, the RNA quantity was measured
144 using a Nanophotometer (IMPLEN GmbH, Munich, Germany) and the quality was evaluated
145 with RNA ScreenTapes on an Agilent 2200 TapeStation (Agilent Technologies). The extracted
146 RNAs with a RIN (RNA integrity number) above seven were used for first strand cDNA
147 synthesis using 500ng total RNA input and High Capacity cDNA Reverse Transcription kit
148 (Applied Biosystems). The cDNAs were diluted 1:10 times in RNase-free water in order to
149 proceed with qPCR. It is worth emphasizing that the Reliaprep RNA kit was successful in
150 extracting high quality RNA from brain tissue regardless of the high level of fat content, thus the
151 kit can be recommend for RNA extraction from other fatty tissues (e.g. oocyte and adipose
152 tissues (Ahi et al., 2018)) for which the conventional methods might not yield acceptable RNA
153 quality for gene expression studies.

154

155 Gene selection and primer design

156 In this study, we selected a non-exhaustive list of 16 target genes that are well-studied in teleost
157 fish, mainly in Cypriniformes, for their role in regulation of appetite and food intake (Table 1).
158 In addition, the function of most of these genes have been investigated in other fish species with
159 evolutionary closer relatedness than members of Cypriniformes to cichlids, such as members of
160 Perciformes or other species of Cichliformes. We also included five genes playing a role in food
161 habit transition from carnivory to herbivory in grass carp which is the only species studied for
162 such a transition at gene expression level across teleost fishes (He et al., 2015) (Table 1).
163 Furthermore, we chose eight candidate genes which are frequently used as reference genes in
164 qPCR studies of different tissues in East African cichlids (Yang et al., 2013; Ahi & Sefc,
165 2017a,b; Ahi, Richter & Sefc, 2017). To design primers, we used conserved coding sequence
166 regions based on the transcriptomes of several East African haplochromine species (*Pundamilia*
167 *nyererei*, *Simochromis diagramma*, *Gnathochromis pfefferi*, *Metriaclima zebra*, and
168 *Astatotilapia burtoni*) and two other cichlid species belonging to distant tribes (*Oreochromis*
169 *niloticus* and *Neolamprologus brichardi*) (Brawand et al., 2014; Singh et al., 2017). The
170 sequences from all species were first aligned in CLC Genomic Workbench, version 7.5 (CLC

Bio, Aarhus, Denmark) and exon/exon junctions were identified through the annotated genome of *Astatotilapia burtoni* in the Ensembl database (http://www.ensembl.org) (Zerbino et al., 2018). The designed primers were spanning the exon/exon with small amplicon size (<200 bp) which is optimal for qPCR quantification (Fleige & Pfaffl, 2006). We used OligoAnalyzer V3.1 software (www.idtdna.com/analyzer/Applications/OligoAnalyzer) and Primer Express V3.0 (Applied Biosystems, CA, USA) to design the primers with minimal dimerization and secondary structures.

qPCR and expression data analysis

In order to prepare qPCR reactions, we followed the protocol suggested by manufacturer; Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, Germany). The qPCR amplifications were conducted in 96 well-PCR plates using ABI 7500 real-time PCR System (Applied Biosystems) with two technical replicates for each biological replicate and observing the experimental set-up known as sample maximization method to attain optimal qPCR conditions (Hellemans et al., 2007). The qPCR program and a dissociation step were performed as described in a previous gene expression study of cichlids (Ahi & Sefc, 2018), and the amplification efficiency of each primer pair was calculated through LinRegPCR v11.0 programme (http://LinRegPCR.nl) (Ramakers et al., 2003) (Table S1).

Three common algorithms for reference validation, BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen, Jensen & Ørntoft, 2004) and geNorm (Vandesompele et al., 2002) were used to rank the most stably expressed reference genes. BestKeeper calculate an index which considers the lowest standard deviations (SD) of Cq values for its ranking, whereas geNorm and NormFinder calculate mean expression values (M) and stability expression values (SV) which respectively take into account gene co-expression and inter-group variations in order to rank the candidate reference genes. The Cq values of the reference gene(s) validated by the three algorithms was used for expression data normalization ($Cq_{reference}$) through obtaining ΔCq for each gene ($\Delta Cq_{target} = Cq_{target} - Cq_{reference}$). For gene expression comparisons within each lake, a replicate of an algae-grazer species was set as a calibrator sample and rest of the samples were normalized according to its ΔCq value ($\Delta\Delta Cq_{target} = \Delta Cq_{target} - \Delta Cq_{calibrator}$). In expression comparisons

between the trophic niches across the lakes, the lowest expressed replicate for each target gene was used as a calibrator sample. Relative expression quantities (RQ) were calculated though $E^{-\Delta\Delta Cq}$ method (Pfaffl, 2001) and their fold difference values (FD), after transformation of RQ values to logarithmic base 2 values, were used to perform statistical analysis (Bergkvist et al., 2010). The significant expression differences were determined using ANOVA statistical tests, followed by Tukey's HSD *post hoc* tests. To assess the species similarities in expression signature of the appetite regulating genes a dendrogram clustering was conducted using expression correlations calculated through Pearson correlation coefficients (r) using R (http://www.r-project.org).

Results

Validation of reference genes for expression analysis

In order to precisely measure the expression of the appetite-regulating genes in the brains, identification of stably expressed reference gene(s) with minimum expression variation among the samples is considered as first crucial step in qPCR (Kubista et al., 2006). The eight candidate reference genes were among the frequently used reference genes in qPCR studies of different tissues in East African cichlids (Yang et al., 2013; Gunter & Meyer, 2014; Ahi & Sefc, 2017a,b; Ahi, Richter & Sefc, 2017). The expression levels of candidate reference genes were variable; from the lowest expression level (highest Cq value) of *tbp* to the highest expression level (lowest Cq value) of *actb1* (Figure 1B). Based on NormFinder, which takes into account the inter-group expression variations, *actb1*, *ef1a* and *rps11*, were ranked as the most stable genes in the brain of our study species from LM, LV and LT, respectively (Table 2). It should be noted that the only *rps11* was always ranked among the top three genes across the lakes according to the NormFinder rankings. geNorm identified *actb1*, *ef1a* and *tuba1* as the most stable genes in LM, LV and LT, respectively. However, *rps11* appeared again to be the only gene ranked among the top three genes in all the lakes (ranked second in all the lakes) (Table 2). Finally, BestKeeper, which calculates expression stabilities through standard deviations in expression, ranked *rps11* as the most stable reference genes among the candidates in all the lakes (Table 2). Based on the

findings by the three algorithms, *rps11* was found to have the most consistent expression stability, and therefore, its expression in the brain samples was selected as normalization factor (NF) for expression analyses of the appetite-regulating genes.

Expression differences between herbivores and carnivores

At first, we used the relative expressions of all 16 target genes in each species in order to construct a dendrogram cluster representing the similarities between species in brain expression of appetite-regulating genes (Fig. 1C). The results showed that the similarities between the species are mainly determined by evolutionary relatedness by which species from the same lake (for Malawi or Victoria) are paired together. However, an interesting difference was observed for the LT species where the carnivore species (C.h) was clustered with the LM species and the herbivore species (P.p) branched distantly from the other clusters (Fig. 1C). This might indicate that the LT species with their much older evolutionary divergence have more distinct expression pattern for appetite regulating genes prior to foraging, as outlined in more detail in the discussion. It also appears that the herbivore brain might have more distinct gene expression patterns for appetite-regulating genes in LT.

When the overall expression levels of the appetite-regulating genes were compared between herbivores and carnivores across the lakes six genes, *cart*, *drd1*, *gabra1*, *npy2r*, *pyy* and *trh* appeared to have differential expression (Fig. 2). Among these, *cart*, *gabra1* and *npy2r* displayed strong expression differences, and all of the genes, except *pyy*, had shown higher expression in the carnivores than herbivores (Fig. 2). These results demonstrate expression differences of certain appetite-regulating genes in herbivorous versus carnivorous haplochromine cichlids prior to initiation of their feeding. This also suggests that feeding behaviour can be already determined in the brain by differential expression of appetite-regulating genes before exposure to available food resources. However, considering the opposing appetite-regulating functions of these genes, i.e. *cart*, *drd1* and *npy2r* are anorexigenic whereas *gabra1* and *trh* are orexigenic genes (Table 1), it appears to be too complicated at this stage to interpret the behavioural outcome of such transcriptional differences across the lakes.

Next, we compared the expression levels of each gene between the herbivorous and carnivorous species within the lakes. All of the genes, except *cck* and *npy*, showed differential expression between the two trophic niches in at least one lake (Fig. 3). Out of the 16 tested genes, 11, 12 and 13 genes were differentially expressed in LM, LV and LT, respectively. In LT, all of the 13 differentially expressed genes showed higher expression in the carnivore species, but this number declined by the age of divergence between the trophic niches in each lake, *i.e.* seven out of the 11 genes for LM and five out of the 12 genes for LV (Fig. 3).When comparing the lakes, seven genes showed similar expression difference between LT and LM, four genes between LM and LV, and four genes between LT and LV. Importantly, only two anorexigenic genes, *cart* and *npy2r*, showed similar expression difference across the lakes; with higher expression in the carnivore species (Fig. 3). The differential expression of *cart* appeared to be increased in the carnivore brains according to the age of divergence between the contrasting species of each lake (*i.e.* LT > LM > LV). The expression results of *cart* and *npy2r* suggest that carnivory versus herbivory and possibly their related feeding behaviour in Haplochromine cichlids might be pre-determined by divergence in brain expression of the anorexigenic genes prior to initiation of feeding.

Discussion

Diversity in cichlid diet and foraging behaviours is thought to be a key factor facilitating their rapid divergence by enabling effective trophic specialization and ecological speciation (Liem, 1973). Plasticity in trophic morphology and physiology, manifested in jaw shape, intestine length and enzymatic activities, are believed to have played an important role in the adaptation to new habitats and the optimization of feeding during ontogeny (Sturmbauer, Mark & Dallinger, 1992; Takahashi & Koblmüller, 2011). But little is known about the link between the brain and foraging behaviours. In particular, the appetite-regulating genes in the brain that might contribute to different dietary habits prior to the onset of feeding have not been studied. Here we investigated the expression of appetite regulating genes in the brains of cichlids adapted to herbivorous and carnivorous trophic niches and identified two appetite-regulating genes, *cart* (or *cartpt1*) and *npy2r*, to have higher expression in the carnivore brains prior to the initiation of

feeding across all three lakes. Both genes are indicated to have anorexigenic function in different groups of teleost fishes (Matsuda et al., 2012; Babichuk & Volkoff, 2013; Wang et al., 2014; He et al., 2015; Volkoff, 2016; Porter, Roberts & Maruska, 2017). The first gene, *cart*, or cocaine- and amphetamine-regulated transcript, encodes a pre-proprotein which proteolyzes to multiple active peptides and participates in biological processes related to regulation of appetite, energy balance, stress response, and reward and addiction responses (Volkoff, 2006, 2016; Koylu, Balkan & Pogun, 2006; Vicentic et al., 2007; Rogge et al., 2008). In most teleost fish including Perciformes, Salmoniformes and Gasterosteiforme only one *cart* isoform has been found (Murashita et al., 2009; Figueiredo-Silva et al., 2012; Striberny et al., 2015), whereas, in two model fish species; medaka and zebrafish (Beloniforme and Cypriniforme, respectively) more than one *cart* isoforms have been characterized (Murashita & Kurokawa, 2011; Akash et al., 2014). In a Haplochromine cichlid, *Astatotilapia burtoni*, six *cart* isoforms have been described and among them *cart*/*cartpt1* show the greatest similarity to mammalian *CART* gene (Hu et al., 2016). The brain expression pattern of *cart* appeared to be similar to its orthologues in other teleosts in the lateral posterior part of the hypothalamus (or lateral tuberal nucleus), which is also similar to the expression of mammalian *CART* in a comparable region called arcuate nucleus (Porter, Roberts & Maruska, 2017).

Studies of Cypriniformes have demonstrated that *cart* induction inhibits food intake and increases locomotion and responsiveness to different sensory stimuli, and thus affecting feeding behavioral activity (Volkoff & Peter, 2000; Woods et al., 2014). It has been long known that predatory behavior is directly influenced by ability to respond to a range of sensory stimuli mediated by vision, olfaction and lateral line in fish (Adams & Johnsen, 1986; Gehrke, 1988; Carr et al., 1996; Montgomery & Hamilton, 1997; LIAO & CHANG, 2003; del Mar Palacios, Warren & McCormick, 2016). In addition, the decrease in brain expression of anorexigenic genes has been linked to the transition from carnivory to herbivory feeding behavior in grass carp (He et al., 2015). In our study, the increased *cart* expression in the carnivore brains prior to feeding may indicate less appetite and a predisposition for more environmental responsiveness in the carnivores, which may be a favorable behavior for predatory-based trophic specialization. Furthermore, the conserved anorexigenic role of CART peptides in teleost fish has been demonstrated in a wide range of species during fasting and re-feeding experiments (reviewed in (Volkoff, 2016)). Interestingly, we found that the difference in *cart* expression level between the

herbivorous and carnivorous species in each lake to be associated with the age of divergence in each lake, *i.e.* the older divergence had the highest difference in *cart* expression levels (Fig. 3). This is especially interesting as the cichlids from older lakes have longer larval developmental periods as they have larger yolk sacs that provide nourishment for longer, so food intake may need to be inhibited for longer (Dreo and Gallaun, 2018, unpublished data).

The second gene, *npy2r*, encodes a receptor of Neuropeptide Y (*npy*), and interestingly, an orthologue of the same receptor has been identified to have reduced expression during the transition from carnivory to herbivory in grass carp (He et al., 2015). The ligand of this receptor, *npy*, is expressed in different tissues, particularly in brain and intestine, and its encoded peptide (NPY) has been one of the first studied appetite-regulating factors in fish (Volkoff, 2016). In this study we found reduced expression of *npy2r* in the brain of herbivores which is consistent with the suggested anorexigenic role of *npy2r* in in grass carp (He et al., 2015). Although, the ligand of *npy2r*, NPY peptide, acts as an orexigenic factor in most teleost fish species (reviewed in (Volkoff, 2016)), but *npy2r* is among the NPY receptors in vertebrates that functions as inhibitory auto-receptor, and thus playing an opposite role to NPY in appetite regulation (Chen et al., 1997; Naveilhan et al., 1999).

Overall, most selected appetite-regulating genes showed no consistent expression differences between herbivores and carnivores across the three lakes indicating that most of these genes do not participate in determination of feeding behavior prior to foraging in haplochromine cichlids. Moreover, their expression differences between the two trophic niches showed the most discrepancies between the species of the youngest and oldest lake adaptive radiations (LV versus LT). Although, the consistently increased expression of the two anorexigenic genes, *cart* and *npy2r*, in carnivores could imply on their potential role in determination of the feeding behaviors prior to foraging, further functional investigations are required to confirm such role for appetite regulating genes in fish. In addition, it is not clear if the peptides encoded by these genes interact with other appetite-regulating factors and whether they override the effects of the other differentially expressed factors in the brain.

Conclusions

Diet is a major factor mediating adaptive divergence in the adaptive radiation of cichlids fishes. Here we took the first step towards delineating the genes involved in regulating appetite in herbivorous and carnivorous cichlids prior to the onset of independent feeding. We identified two anorexigenic genes, *cart* and *npy2r*, to be differentially expressed between the two trophic categories in three parallel cichlid radiations, which is suggestive of their role in controlling satiety in these species. It might also imply that appetite gene regulation is genetically hardwired and not a plastic phenotype. In conclusion, we present a first glimpse into an important aspect of feeding in cichlids that is the regulatory control of appetite. In the future it would be essential to use whole transcriptome sequencing approaches to validate and add to our findings.

List of abbreviations

LT: Lake Tanganyika, LM: Lake Malawi, LV: Lake Victoria.

Declarations

Authors' contributions

EPA, AD, CS, and PS designed the study. EPA and AD conducted the laboratory experiment. EPA analysed the data and prepared the figures. EPA, PS, AD and CS wrote the manuscript. WG and AD performed fish breeding and sampling. WG photographed the adult fishes used in Figure 1A. All authors reviewed the manuscript and approve its content.

Acknowledgements

The authors thank Martin Grube and his lab for technical assistance and access to their real-time PCR System in the Institute of Biology at University of Graz.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Studies of sacrificed fish do not require ethics approval or consent to participate. This is due to the fact that no experiments were carried out with the fish prior to sampling. Fish keeping and sampling was carried out according to the Austrian animal welfare law.

Funding

This study was funded by the Austrian Science Fund (Grant P29838). The Austrian Science Fund requires clarification of all legal issues concerning animal keeping, animal experiments and sampling design prior to grant submission and evaluation, but does not interfere in writing and data interpretation, but funds open access of the resulting publications.

References

Adams MA, Johnsen PB. 1986. Chemical Control of Feeding in Herbivorous and Carnivorous Fish. In: *Chemical Signals in Vertebrates 4*. Boston, MA: Springer US, 45–61. DOI: 10.1007/978-1-4613-2235-1_5.

Ahi EP, Richter F, Sefc KM. 2017. A gene expression study of ornamental fin shape in Neolamprologus brichardi, an African cichlid species. *Scientific Reports* 7:17398. DOI: 10.1038/s41598-017-17778-0.

Ahi EP, Sefc KM. 2017a. A gene expression study of dorso-ventrally restricted pigment pattern in adult fins of *Neolamprologus meeli* , an African cichlid species. *PeerJ* 5:e2843. DOI: 10.7717/peerj.2843.

Ahi EP, Sefc KM. 2017b. Anterior-posterior gene expression differences in three Lake Malawi cichlid fishes with variation in body stripe orientation. *PeerJ* 5:e4080. DOI: 10.7717/peerj.4080.

Ahi EP, Sefc KM. 2018. Towards a gene regulatory network shaping the fins of the Princess cichlid. *Scientific Reports* 8:9602.

Ahi EP, Singh P, Lecaudey LA, Gessl W, Sturmbauer C. 2018. Maternal mRNA input of growth and stress-response-related genes in cichlids in relation to egg size and trophic specialization. *EvoDevo* 9:23. DOI: 10.1186/s13227-018-0112-3.

Akash G, Kaniganti T, Tiwari NK, Subhedar NK, Ghose A. 2014. Differential distribution and energy status-dependent regulation of the four CART neuropeptide genes in the zebrafish brain. *Journal of Comparative Neurology* 522:2266–2285. DOI: 10.1002/cne.23532.

Andersen CL, Jensen JL, Ørntoft TF. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer research* 64:5245–50. DOI: 10.1158/0008-5472.CAN-04-0496.

Babichuk NA, Volkoff H. 2013. Changes in expression of appetite-regulating hormones in the cunner (Tautogolabrus adspersus) during short-term fasting and winter torpor. *Physiology & Behavior* 120:54–63. DOI: 10.1016/j.physbeh.2013.06.022.

Bergkvist A, Rusnakova V, Sindelka R, Garda JMA, Sjögreen B, Lindh D, Forootan A, Kubista M. 2010. Gene expression profiling--Clusters of possibilities. *Methods* 50:323–35. DOI: 10.1016/j.ymeth.2010.01.009.

Brawand D, Wagner CE, Li YI, Malinsky M, Keller I, Fan S, Simakov O, Ng AY, Lim ZW, Bezault E, Turner-Maier J, Johnson J, Alcazar R, Noh HJ, Russell P, Aken B, Alföldi J, Amemiya C, Azzouzi N, Baroiller J-F, Barloy-Hubler F, Berlin A, Bloomquist R, Carleton KL, Conte M a., D'Cotta H, Eshel O, Gaffney L, Galibert F, Gante HF, Gnerre S, Greuter L, Guyon R, Haddad NS, Haerty W, Harris RM, Hofmann H a., Hourlier T, Hulata G, Jaffe DB, Lara M, Lee AP, MacCallum I, Mwaiko S, Nikaido M, Nishihara H, Ozouf-Costaz C, Penman DJ, Przybylski D, Rakotomanga M, Renn SCP, Ribeiro FJ, Ron M, Salzburger W, Sanchez-Pulido L, Santos ME, Searle S, Sharpe T, Swofford R, Tan FJ, Williams L, Young S, Yin S, Okada N, Kocher TD, Miska E a., Lander ES, Venkatesh B, Fernald RD, Meyer A, Ponting CP, Streelman JT, Lindblad-Toh K, Seehausen O, Di Palma F. 2014. The genomic substrate for adaptive radiation in African cichlid fish. *Nature* 513:375–381. DOI: 10.1038/nature13726.

Carr WES, Netherton Iii JC, Gleeson RA, Derby CD. 1996. Stimulants of Feeding Behavior in Fish: Analyses of Tissues of Diverse Marine Organisms. *The Biological bulletin* 190:149–160. DOI: 10.2307/1542535.

Chen X, Dimaggio DA, Han SP, Westfall TC. 1997. Autoreceptor-induced inhibition of neuropeptide Y release from PC-12 cells is mediated by Y_2 receptors. *American Journal of Physiology-Heart and Circulatory Physiology* 273:H1737–H1744. DOI: 10.1152/ajpheart.1997.273.4.H1737.

Figueiredo-Silva AC, Saravanan S, Schrama JW, Kaushik S, Geurden I. 2012. Macronutrient-induced differences in food intake relate with hepatic oxidative metabolism and hypothalamic regulatory neuropeptides in rainbow trout (Oncorhynchus mykiss). *Physiology & Behavior* 106:499–505. DOI: 10.1016/J.PHYSBEH.2012.03.027.

Fleige S, Pfaffl MW. 2006. RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine* 27:126–139. DOI: 10.1016/J.MAM.2005.12.003.

Fujimura K, Okada N. 2007. Development of the embryo, larva and early juvenile of Nile tilapia Oreochromis niloticus (Pisces: Cichlidae). Developmental staging system. *Development Growth and Differentiation* 49:301–324. DOI: 10.1111/j.1440-169X.2007.00926.x.

Fujimura K, Okada N. 2008. Shaping of the lower jaw bone during growth of Nile tilapia Oreochromis niloticus and a Lake Victoria cichlid Haplochromis chilotes: A geometric morphometric approach. *Development Growth and Differentiation* 50:653–663. DOI: 10.1111/j.1440-169X.2008.01063.x.

Gehrke PC. 1988. Influence of gut morphology, sensory cues and hunger on feeding behaviour of spangled perch, Leiopotherapon unicolor (Gunther, 1859), (Percoidei, Teraponidae). *Journal of Fish Biology* 33:189–201. DOI: 10.1111/j.1095-8649.1988.tb05462.x.

Gunter HM, Fan S, Xiong F, Franchini P, Fruciano C, Meyer A. 2013. Shaping development through mechanical strain: the transcriptional basis of diet-induced phenotypic plasticity in a cichlid fish. *Molecular ecology* 22:4516–31. DOI: 10.1111/mec.12417.

Gunter HM, Meyer A. 2014. Molecular investigation of mechanical strain-induced phenotypic plasticity in the ecologically important pharyngeal jaws of cichlid fish. *Journal of Applied Ichthyology* 30:630–635.

He S, Liang X-F, Li L, Sun J, Wen Z-Y, Cheng X-Y, Li A-X, Cai W-J, He Y-H, Wang Y-P, Tao Y-X, Yuan X-C. 2015. Transcriptome analysis of food habit transition from carnivory to herbivory in a typical vertebrate herbivore, grass carp Ctenopharyngodon idella. *BMC Genomics* 16:15. DOI: 10.1186/s12864-015-1217-x.

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome biology* 8:R19. DOI: 10.1186/gb-2007-8-2-r19.

Hu CK, Southey BR, Romanova E V., Maruska KP, Sweedler J V., Fernald RD. 2016. Identification of prohormones and pituitary neuropeptides in the African cichlid, Astatotilapia burtoni. *BMC Genomics* 17:660. DOI: 10.1186/s12864-016-2914-9.

Irissari I, Singh P, Koblmüller S, Torres-Dowdall J, Henning F, Franchini P, Fischer C, Lemmon A, Lemmon E, Thallinger G, Sturmbauer C, Meyer A. 2018. Anchored phylogenomics uncovers deep inter-tribal hybridizations in the Lake Tanganyika cichlid radiation and highlights adaptive loci shaping species' ecology. *Nature communications* 9:3159.

Koblmüller S, Schliewen UK, Duftner N, Sefc KM, Katongo C, Sturmbauer C. 2008. Age and spread of the haplochromine cichlid fishes in Africa. *Molecular Phylogenetics and Evolution* 49:153–169. DOI: 10.1016/J.YMPEV.2008.05.045.

Koylu EO, Balkan B, Pogun S. 2006. Cocaine and amphetamine regulated transcript (CART) and the stress response. *Peptides* 27:1956–1969. DOI: 10.1016/J.PEPTIDES.2006.03.032.

Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N. 2006. The real-time polymerase chain reaction. *Molecular aspects of medicine* 27:95–125. DOI: 10.1016/j.mam.2005.12.007.

LIAO IC, CHANG EY. 2003. Role of sensory mechanisms in predatory feeding behaviorof juvenile red drum Sciaenops ocellatus. *Fisheries Science* 69:317–322. DOI: 10.1046/j.1444-

2906.2003.00623.x.

Liem KF. 1973. Evolutionary Strategies and Morphological Innovations: Cichlid Pharyngeal Jaws. *Systematic Zoology* 22:425. DOI: 10.2307/2412950.

del Mar Palacios M, Warren DT, McCormick MI. 2016. Sensory cues of a top-predator indirectly control a reef fish mesopredator. *Oikos* 125:201–209. DOI: 10.1111/oik.02116.

Matsuda K, Sakashita A, Yokobori E, Azuma M. 2012. Neuroendocrine control of feeding behavior and psychomotor activity by neuropeptideY in fish. *Neuropeptides* 46:275–283. DOI: 10.1016/J.NPEP.2012.09.006.

Montgomery JC, Hamilton AR. 1997. Sensory contributions to nocturnal prey capture in the dwarf scorpion fish *(Scorpaena papillosus)*. *Marine and Freshwater Behaviour and Physiology* 30:209–223. DOI: 10.1080/10236249709379026.

Murashita K, Kurokawa T. 2011. Multiple cocaine- and amphetamine-regulated transcript (CART) genes in medaka, Oryzias latipes: Cloning, tissue distribution and effect of starvation. *General and Comparative Endocrinology* 170:494–500. DOI: 10.1016/J.YGCEN.2010.11.005.

Murashita K, Kurokawa T, Ebbesson LOE, Stefansson SO, Rønnestad I. 2009. Characterization, tissue distribution, and regulation of agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY) in Atlantic salmon (Salmo salar). *General and Comparative Endocrinology* 162:160–171. DOI: 10.1016/J.YGCEN.2009.03.015.

Naveilhan P, Hassani H, Canals JM, Ekstrand AJ, Larefalk Å, Chhajlani V, Arenas E, Gedda K, Svensson L, Thoren P, Ernfors P. 1999. Normal feeding behavior, body weight and leptin response require the neuropeptide Y Y2 receptor. *Nature Medicine* 5:1188–1193. DOI: 10.1038/13514.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research* 29:e45.

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnology letters* 26:509–15.

Porter DT, Roberts DA, Maruska KP. 2017. Distribution and female reproductive state differences in orexigenic and anorexigenic neurons in the brain of the mouth brooding African cichlid fish, *Astatotilapia burtoni*. *Journal of Comparative Neurology* 525:3126–3157. DOI: 10.1002/cne.24268.

Ramakers C, Ruijter JM, Deprez RHL, Moorman AFM. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience letters* 339:62–6.

Rogge G, Jones D, Hubert GW, Lin Y, Kuhar MJ. 2008. CART peptides: regulators of body weight, reward and other functions. *Nature Reviews Neuroscience* 9:747–758. DOI: 10.1038/nrn2493.

Schneider RF, Li Y, Meyer A, Gunter HM. 2014. Regulatory gene networks that shape the

development of adaptive phenotypic plasticity in a cichlid fish. *Molecular Ecology* 23:4511–4526. DOI: 10.1111/mec.12851.

Singh P, Börger C, More H, Sturmbauer C. 2017. The Role of Alternative Splicing and Differential Gene Expression in Cichlid Adaptive Radiation. *Genome Biology and Evolution* 9:2764–2781. DOI: 10.1093/gbe/evx204.

Striberny A, Ravuri CS, Jobling M, Jørgensen EH. 2015. Seasonal Differences in Relative Gene Expression of Putative Central Appetite Regulators in Arctic Charr (Salvelinus alpinus) Do Not Reflect Its Annual Feeding Cycle. *PLOS ONE* 10:e0138857. DOI: 10.1371/journal.pone.0138857.

Sturmbauer C, Mark W, Dallinger R. 1992. Ecophysiology of Aufwuchs-eating cichlids in Lake Tanganyika: niche separation by trophic specialization. *Environmental Biology of Fishes* 35:283–290. DOI: 10.1007/BF00001895.

Takahashi T, Koblmüller S. 2011. The adaptive radiation of cichlid fish in lake tanganyika: a morphological perspective. *International journal of evolutionary biology* 2011:620754. DOI: 10.4061/2011/620754.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* 3:RESEARCH0034.

Vicentic A, Jones DC, Vechia SD, Hunter RG, Kuhar MJ. 2007. The CART (cocaine- and amphetamine-regulated transcript) system in appetite and drug addiction. *The Journal of pharmacology and experimental therapeutics* 320:499–506. DOI: 10.1124/jpet.105.091512.

Volkoff H. 2006. The role of neuropeptide Y, orexins, cocaine and amphetamine-related transcript, cholecystokinin, amylin and leptin in the regulation of feeding in fish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 144:325–331. DOI: 10.1016/J.CBPA.2005.10.026.

Volkoff H. 2016. The Neuroendocrine Regulation of Food Intake in Fish: A Review of Current Knowledge. *Frontiers in Neuroscience* 10:540. DOI: 10.3389/fnins.2016.00540.

Volkoff H, Canosa LF, Unniappan S, Cerdá-Reverter JM, Bernier NJ, Kelly SP, Peter RE. 2005. Neuropeptides and the control of food intake in fish. *General and Comparative Endocrinology* 142:3–19. DOI: 10.1016/J.YGCEN.2004.11.001.

Volkoff H, Peter RE. 2000. Effects of CART peptides on food consumption, feeding and associated behaviors in the goldfish, Carassius auratus: actions on neuropeptide Y- and orexin A-induced feeding. *Brain research* 887:125–33.

Wang F, Chen W, Lin H, Li W. 2014. Cloning, expression, and ligand-binding characterization of two neuropeptide Y receptor subtypes in orange-spotted grouper, Epinephelus coioides. *Fish Physiology and Biochemistry* 40:1693–1707. DOI: 10.1007/s10695-014-9960-5.

Woods IG, Schoppik D, Shi VJ, Zimmerman S, Coleman HA, Greenwood J, Soucy ER, Schier AF. 2014. Neuropeptidergic signaling partitions arousal behaviors in zebrafish. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 34:3142–60. DOI: 10.1523/JNEUROSCI.3529-13.2014.

565 Yang CG, Wang XL, Tian J, Liu W, Wu F, Jiang M, Wen H. 2013. Evaluation of reference
566 genes for quantitative real-time RT-PCR analysis of gene expression in Nile tilapia
567 (Oreochromis niloticus). *Gene* 527:183–192. DOI: 10.1016/j.gene.2013.06.013.

568 Zerbino DR, Achuthan P, Akanni W, Amode MR, Barrell D, Bhai J, Billis K, Cummins C, Gall
569 A, Girón CG, Gil L, Gordon L, Haggerty L, Haskell E, Hourlier T, Izuogu OG, Janacek SH,
570 Juettemann T, To JK, Laird MR, Lavidas I, Liu Z, Loveland JE, Maurel T, McLaren W,
571 Moore B, Mudge J, Murphy DN, Newman V, Nuhn M, Ogeh D, Ong CK, Parker A,
572 Patricio M, Riat HS, Schuilenburg H, Sheppard D, Sparrow H, Taylor K, Thormann A,
573 Vullo A, Walts B, Zadissa A, Frankish A, Hunt SE, Kostadima M, Langridge N, Martin FJ,
574 Muffato M, Perry E, Ruffier M, Staines DM, Trevanion SJ, Aken BL, Cunningham F, Yates
575 A, Flicek P. 2018. Ensembl 2018. *Nucleic Acids Research* 46:D754–D761. DOI:
576 10.1093/nar/gkx1098.

577

578

579

580

581

Figure 1

The haplochromine cichlid species in this study, expression levels of the reference genes and a hierarchical clustering based on expression pattern of appetite-regulating genes in the brains.

(**A**) A simplified phylogenetic tree of the six East African haplochromine cichlids representing their relatedness specified by inhabiting lakes and trophic specializations. The colour of symbol beside each species indicates trophic niche and its shape refers to inhabiting lake. (**B**) Expression levels of a selected set of reference genes using their Cq values in brain across the species. The middle line in each box plot represents the median together with the 25/75 percentiles. (**B**) A dendrogram clustering of species based similarity in expression levels of 16 appetite regulating genes in larval brain prior to foraging.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27865v1 | CC BY 4.0 Open Access | rec: 16 Jul 2019, publ: 16 Jul 2019

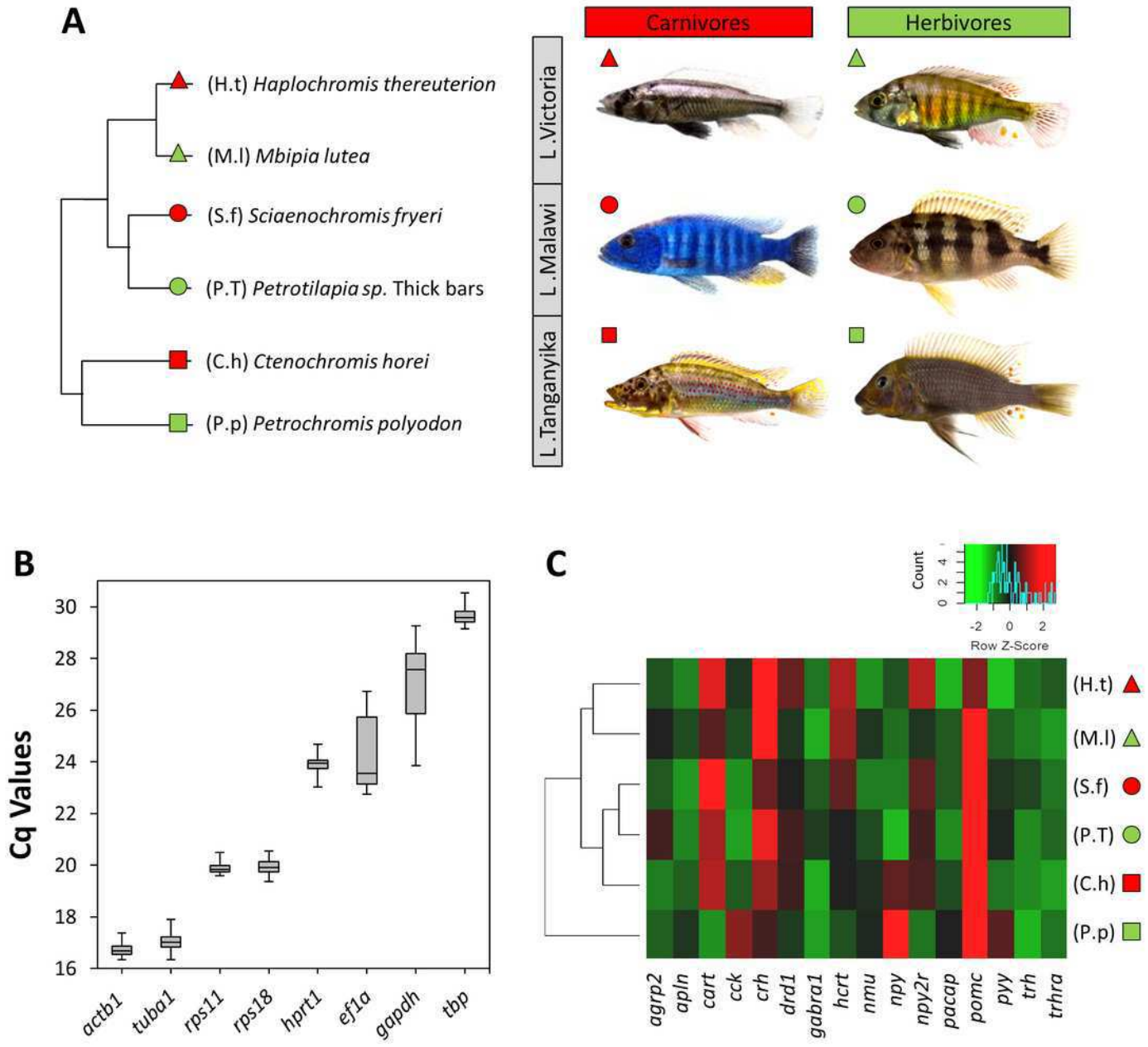

Figure 2

The herbivores versus carnivores expression differences of appetite-regulating genes in the brains of haplochromine cichlids at the end of larval phase.

Comparisons of relative expression levels of 16 appetite-regulating genes in brain, all herbivore species from the three lakes combined versus all the carnivore species, at the end of larval development and prior to foraging. The statistical differences are shown by one, two and three asterisks above bars indicating $P < 0.05$, 0.01 and 0.001, respectively. The middle line in each box plot represents the median together with the 25/75 percentiles.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27865v1 | CC BY 4.0 Open Access | rec: 16 Jul 2019, publ: 16 Jul 2019

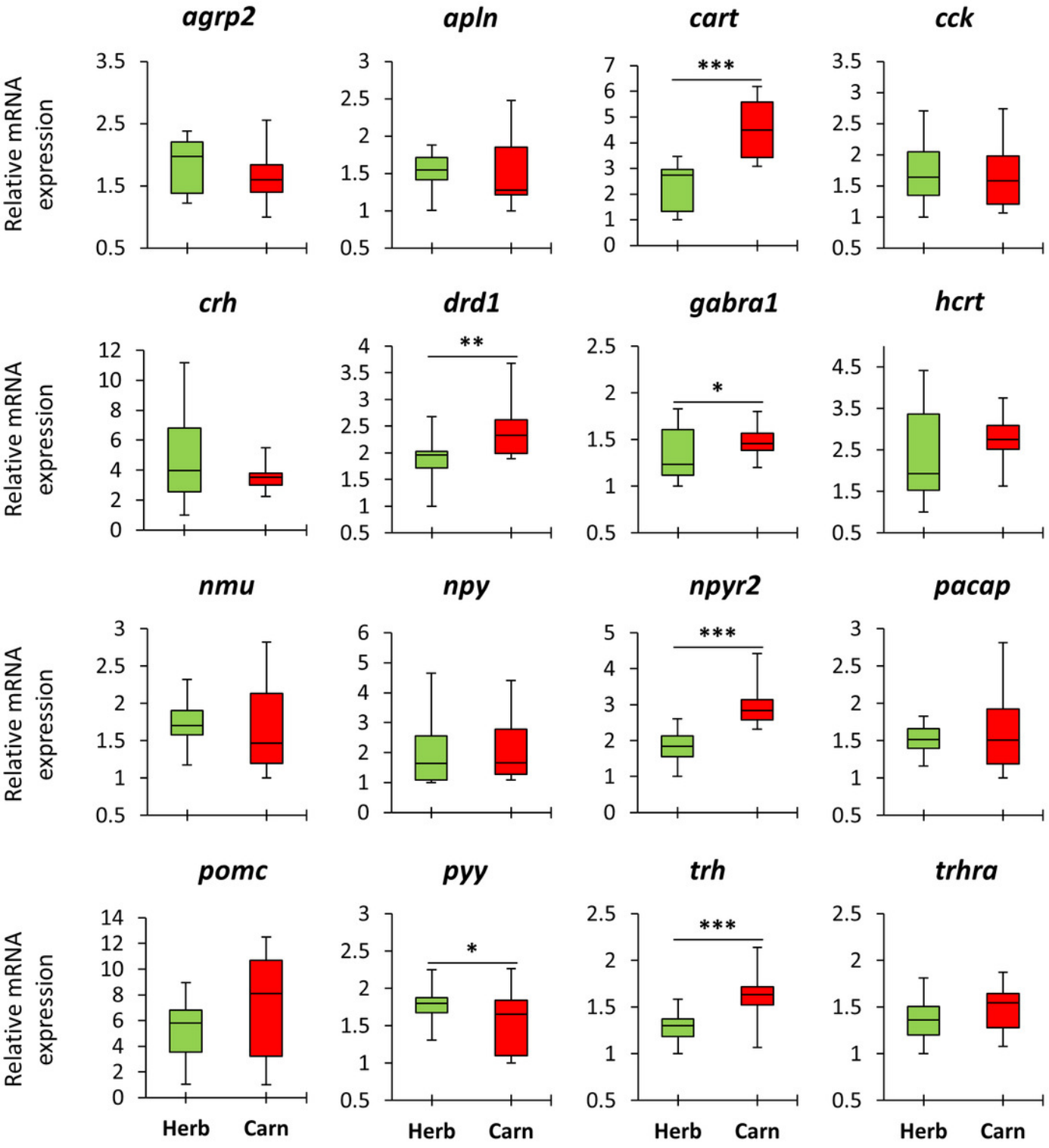

Figure 3

Within lake brain expression differences of appetite-regulating genes between herbivorous and carnivorous haplochromine cichlids at the end of the larval phase.

Comparisons of relative expression levels of 16 appetite-regulating genes in brain, between the herbivorous and carnivorous species of each lake, at the end of the larval development and prior to foraging. The statistical differences are shown by one, two and three asterisks above the bars indicating $P < 0.05$, 0.01 and 0.001, respectively. Error bars represent standard deviations calculated from six biological replicates.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27865v1 | CC BY 4.0 Open Access | rec: 16 Jul 2019, publ: 16 Jul 2019

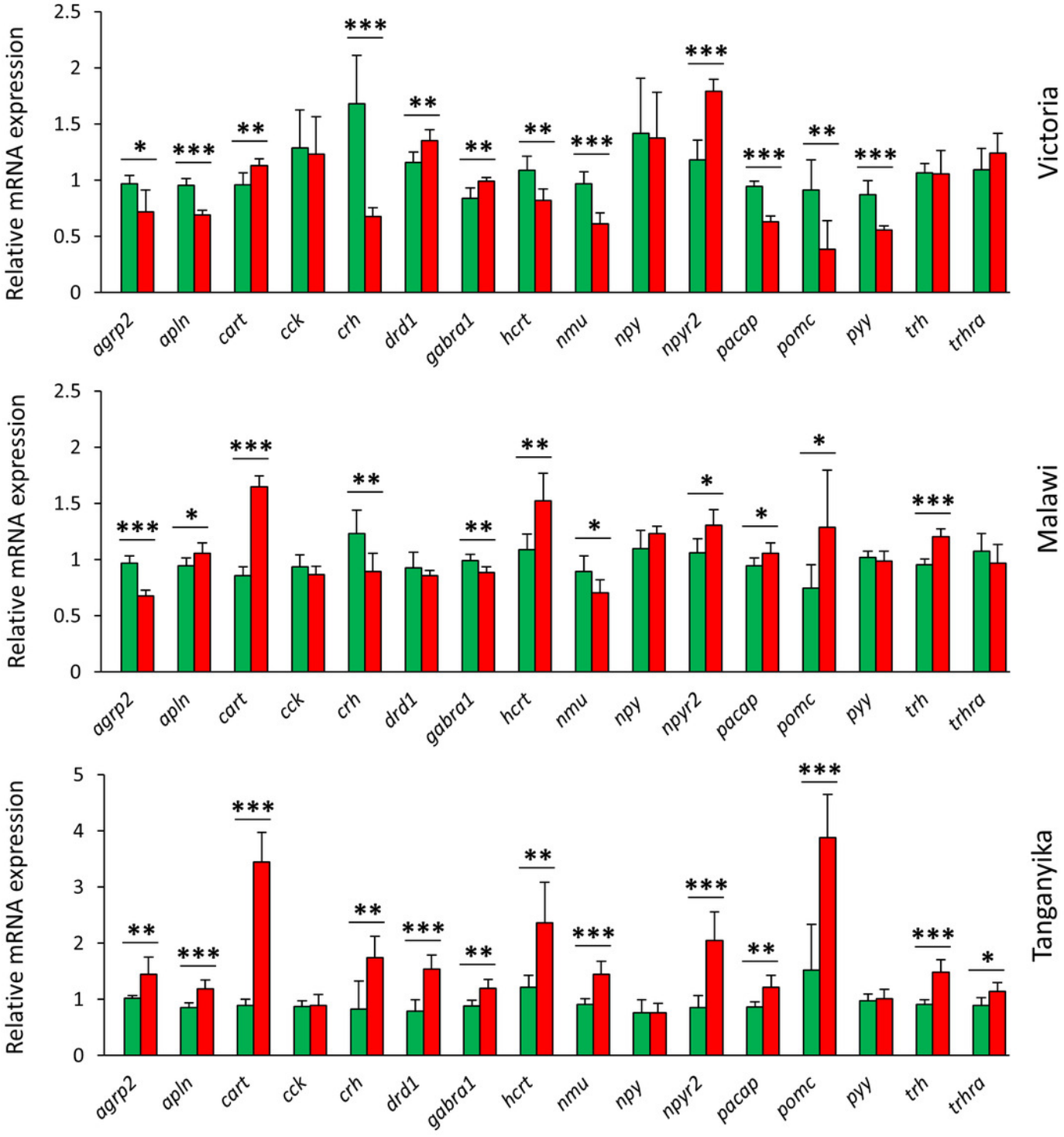

Table 1(on next page)

Selected appetite-regulating genes in this study.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27865v1 | CC BY 4.0 Open Access | rec: 16 Jul 2019, publ: 16 Jul 2019

1

Gene	Description	Organisms	Effects	References
agrp2	Agouti related neuropeptide 2	Perciformes Cypriniformes	Orexigenic Diet transition	(Agulleiro et al., 2014; He et al., 2015)
apln	Apelin, agtrl1 Ligand	Perciformes Cypriniformes	Orexigenic	(Hayes & Volkoff, 2014; Volkoff, 2016)
cart	Cocaine and amphetamine regulated transcript	Cichliformes Perciformes Cypriniformes	Anorexigenic	(Babichuk & Volkoff, 2013; Volkoff, 2016; Porter, Roberts & Maruska, 2017)
cck	Cholecystokinin triacontatriapeptide	Cichliformes Perciformes Cypriniformes	Anorexigenic	(Grone et al., 2012; Babichuk & Volkoff, 2013; Volkoff, 2016)
crh	Corticotropin-releasing hormone	Salmoniformes Cypriniformes	Anorexigenic	(Bernier & Craig, 2005; Volkoff, 2016)
drd1	Dopamine receptor D1	Cypriniformes	Anorexigenic Diet transition	(He et al., 2015)
gabra1	Gamma-aminobutyric acid A receptor alpha-1	Cypriniformes	Orexigenic Diet transition	(Trudeau, Sloley & Peter, 1993; Matsuda et al., 2011; He et al., 2015)
hcrt	Orexin, hypocretin neuropeptide precursor	Cichliformes Perciformes Cypriniformes	Orexigenic	(Yan et al., 2011; Grone et al., 2012; Volkoff, 2016)
nmu	Neuromedin U preproprotein	Perciformes Cypriniformes	Anorexigenic	(Kono et al., 2012; Li et al., 2015; Volkoff, 2016)
npy	Prepro-neuropeptide Y	Cichliformes Perciformes Cypriniformes	Anorexigenic? Orexigenic	(Grone et al., 2012; Matsuda et al., 2012; Babichuk & Volkoff, 2013; Volkoff, 2016; Das et al., 2019)
npy2r	Neuropeptide Y receptor type 2	Perciformes Cypriniformes	Anorexigenic Diet transition	(Matsuda et al., 2012; Wang et al., 2014; He et al., 2015)
pacap	Pituitary adenylate cyclase activating polypeptide	Cichliformes Cypriniformes	Anorexigenic	(Matsuda et al., 2005; Zhou et al., 2013; Costa et al., 2016)
pomc	Pro-opiomelanocortin preproprotein	Cichliformes Cypriniformes	Anorexigenic	(Volkoff, 2016; Porter, Roberts & Maruska, 2017)
pyy	Prepro-peptide YY	Perciformes Cypriniformes	Orexigenic Anorexigenic	(Murashita et al., 2006; Volkoff, 2016)
trh, trhra	Thyrotropin-releasing hormone and its receptor	Cypriniformes	Orexigenic Diet transition	(He et al., 2015; Volkoff, 2016)

2

Table 2(on next page)

Ranking and statistical analyses of reference genes in brain of six haplochromine species from three East African lakes.

	BestKeeper		geNorm		NormFinder	
	Ranking	**I**	**Ranking**	**M**	**Ranking**	**SV**
Lake Malawi	*rps11*	0.080	*actb1*	0.374	*actb1*	0.148
	tuba1	0.134	*rps11*	0.384	*hprt1*	0.176
	rps18	0.153	*tuba1*	0.392	*rps11*	0.210
	actb1	0.171	*hprt1*	0.400	*tuba1*	0.280
	hprt1	0.176	*rps18*	0.422	*rps18*	0.284
	ef1a	0.348	*ef1a*	0.491	*ef1a*	0.295
	tbp	0.349	*tbp*	0.577	*tbp*	0.519
	gapdh	0.935	*gapdh*	0.978	*gapdh*	1.168
Lake Victoria	*rps11*	0.076	*ef1a*	0.387	*ef1a*	0.228
	actb1	0.159	*rps11*	0.393	*actb1*	0.283
	tbp	0.167	*tbp*	0.403	*rps11*	0.295
	ef1a	0.194	*actb1*	0.408	*rps18*	0.386
	hprt1	0.204	*rps18*	0.429	*tbp*	0.413
	rps18	0.208	*hprt1*	0.490	*hprt1*	0.525
	tuba1	0.218	*tuba1*	0.516	*tuba1*	0.656
	gapdh	0.963	*gapdh*	1.298	*gapdh*	2.923
Lake Tanganyika	*rps11*	0.197	*tuba1*	0.535	*rps11*	0.033
	actb1	0.248	*rps11*	0.539	*rps18*	0.036
	rps18	0.257	*rps18*	0.549	*tbp*	0.087
	tbp	0.292	*tbp*	0.599	*tuba1*	0.138
	tuba1	0.300	*hprt1*	0.604	*actb1*	0.158
	ef1a	0.399	*ef1a*	0.643	*hprt1*	0.160
	hprt1	0.400	*actb1*	0.731	*ef1a*	0.386
	gapdh	1.867	*gapdh*	1.996	*gapdh*	4.896

1

Abbreviations: I = BestKeeper index calculated through standard deviations in expression, SV = stability value, M = M value of stability.