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RESEARCH ARTICLE

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Comparative ontogenetic and transcriptomic analyses shed light on color pattern divergence in cichlid fishes

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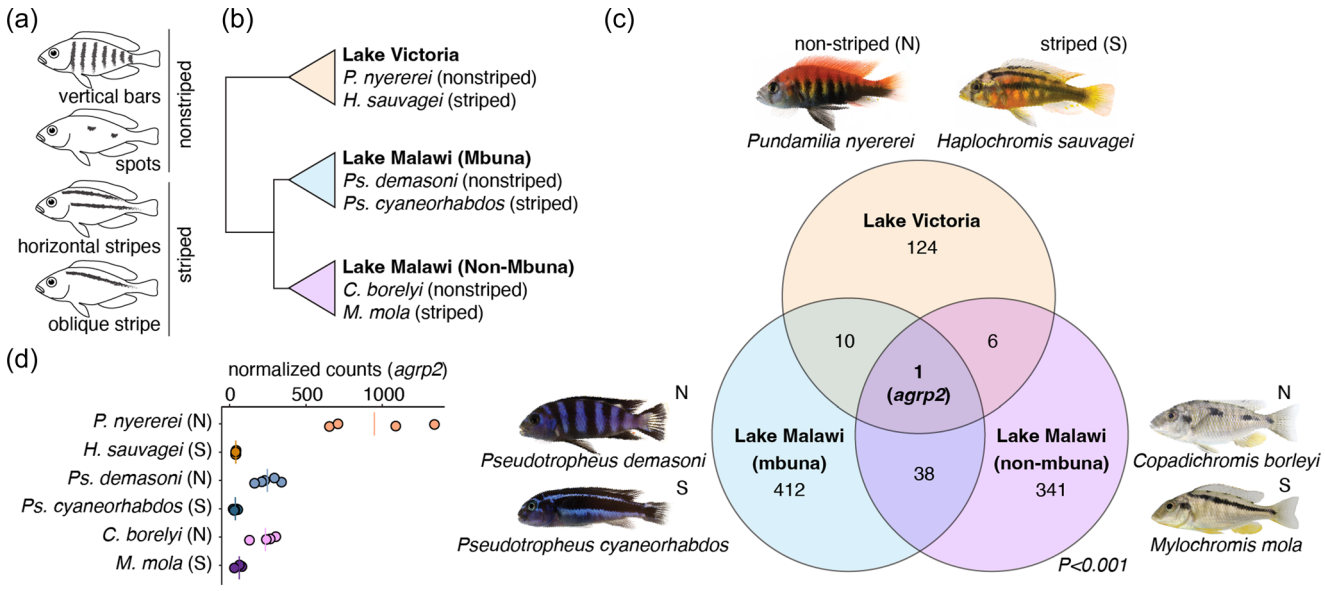
Abstract

Stripe patterns are a striking example for a repeatedly evolved color pattern. In the African adaptive radiations of cichlid fishes, stripes evolved several times independently. Previously, it has been suggested that regulatory evolution of a single gene, *agouti-related-peptide 2* (*agrp2*), explains the evolutionary lability of this trait. Here, using a comparative transcriptomic approach, we performed comparisons between (adult) striped and nonstriped cichlid fishes of representatives of Lake Victoria and the two major clades of Lake Malawi (mbuna and non-mbuna lineage). We identify *agrp2* to be differentially expressed across all pairwise comparisons, reaffirming its association with stripe pattern divergence. We therefore also provide evidence that *agrp2* is associated with the loss of the nonstereotypic oblique stripe of *Mylochromis mola*. Complementary ontogenetic data give insights into the development of stripe patterns as well as vertical bar patterns that both develop postembryonically. Lastly, using the Lake Victoria species pair *Haplochromis sauvagei* and *Pundamilia nyererei*, we investigated the differences between melanic and non-melanic regions to identify additional genes that contribute to the formation of stripes. Expression differences—that most importantly also do not include *agrp2*—are surprisingly small. This suggests, at least in this species pair, that the stripe phenotype might be caused by a combination of more subtle transcriptomic differences or cellular changes without transcriptional correlates. In summary, our comprehensive analysis highlights the ontogenetic and adult transcriptomic differences between cichlids with different color patterns and serves as a basis for further investigation of the mechanistic underpinnings of their diversification.

KEYWORDS
agouti gene family, agrp2, asip2b, Cichlidae, coloration, ontogeny, pigmentation, teleosts

1 | INTRODUCTION

Color patterns often vary widely among closely related species and can provide insights into the evolution of genetic, molecular, and developmental basis of natural diversity (Cuthill et al., 2017; Hubbard et al., 2010). Especially teleost fishes are well known for their richness in colors and pigment patterns (Irion & Nüsslein-Volhard, 2019; Patterson & Parichy, 2019). Although traditionally most insights into the genetic and transcriptional basis of fish color patterns come from model systems such as the zebrafish *Danio rerio* (Irion & Nüsslein-Volhard, 2019; Meyer et al., 1993, 1995; Patterson & Parichy, 2019) and medaka *Oryzias latipes* (Lamoreux et al., 2005), more recently other traits and lineages have been investigated including red ornaments in the three-spined stickleback (*Gasterosteus aculeatus*; Yong et al., 2016), white barring in clown fish (*Amphiprion ocellaris*; Salis et al., 2019), pigmentation loss in cave fish (*Astyanax mexicanus*; Stahl & Gross, 2015), and ornamental patterns in guppies (*Poecilia reticulata*; Kawamoto et al., 2021; Kottler et al., 2013). Another family of fish in which the genetic basis and transcriptional correlates of several pigment pattern and coloration phenotypes have been thoroughly investigated are cichlid fishes. The phenotypes that have been studied from a genetic and/or transcriptomic perspective include egg spot patterns (Henning & Meyer, 2012; Salzburger et al., 2007; Santos et al., 2014), morphological color change (Henning et al., 2013; Kautt et al., 2020; Kratochwil et al., 2022), blotch patterns (Roberts et al., 2017, 2009), general differences in pigment cell distribution (Albertson et al., 2014), yellow, carotenoid-based coloration (Ahi et al., 2020), and vertical bar patterns (Gerwin et al., 2021; Liang et al., 2020). Another common phenotype that has been investigated molecularly are horizontal stripe patterns in cichlids. Most striped cichlids are characterized by two longitudinal stripes: the dorsolateral stripe (DLS) and the midlateral stripe (MLS; Figure 1a). In contrast, only a small number of species of the non-mbuna cichlids of Lake Malawi are characterized by a single oblique stripe



(e)

ENSEMBL ID	Gene ID	Description	p<0.01	p<0.001	p<0.0001
ENSACLG00000010090	*agrp2/asip2b*	*agouti-related protein 2 / agouti-signaling protein 2b*	●	●	●
ENSACLG00000023699	*eif2ak2l*	*eukaryotic translation initiation factor 2 alpha kinase 2-like*	●	○	○
ENSACLG00000006407	*si:ch211-237i5.4*	*insulin-like growth factor-binding protein complex acid labile subunit*	●	○	○
ENSACLG00000026475	*abcc4l*	*multidrug resistance-associated protein 4-like*	●	○	○
ENSACLG00000021770	*NA*	*novel gene*	●	○	○

FIGURE 1 Comparative transcriptomics across cichlid radiations identifies *agouti-related-peptide 2* (*agrp2*) as the single shared differentially expressed (DE) gene in adult skin tissue. (a) Classification of pigmentation patterns. The most common patterns in cichlids are vertical bar patterns, spot patterns, two horizontal stripes and one oblique stripe. (b) Simplified phylogeny of the analyzed species from Lake Victoria and mbuna and non-mbuna cichlids from Lake Malawi showing the phylogenetic relationships of the analyzed species. (c) Venn diagram illustrating the intersection of three pairwise comparisons of striped [S] and nonstriped [N] species. Only a single gene, *agrp2*, is DE in all pairwise comparisons (individual pairwise comparisons have been filtered with $p < .001$). (d) Normalized counts (DeSeq. 2 normalization; median of ratios) of *agrp2* across six species of the Lakes Victoria and Malawi. (e) Conserved DE genes between striped and nonstriped cichlids with p filter thresholds of .01, .001, and .0001.

that starts anteriorly at a dorsal position and then extends diagonally towards the caudal peduncle (Figure 1a).

Based on genetic mapping data and a CRISPR-Cas9 knockout in *P. nyererei*, it has been recently shown that the loss of stripe patterns links to a single gene, the *agouti-related-peptide 2* (*agrp2*; Kratochwil et al., 2018; Liang et al., 2021; Urban et al., 2020). High expression of *agrp2* represses the pattern as also suggested by the fact that stripe patterns are reproducibly associated with low adult expression of *agrp2*, even across the repeated radiations of cichlids of Lakes Victoria and Malawi with their 500 and 800 species, respectively. In Lake Victoria, the phenotype maps to the *agrp2* locus and is Mendelian. In Lake Malawi cichlids *agrp2*, as major effect locus explains more than half of the variance in stripe patterns in F_2 individuals from a striped–nonstriped species cross (Kratochwil et al., 2018). The position of associated *cis*-regulatory intervals (Urban et al., 2020) as well as the presence of additional modifier loci (Gerwin et al., 2021) has been described and varies between the Lake Malawi and Victoria radiations.

Many important aspects of the evolution and development of stripe patterns remain unknown. The first issue we addressed here is whether a whole (adult) transcriptome data set will demonstrate that *agrp2* is, or among, the top differentially expressed (DE) gene between pairs of striped and nonstriped cichlid. An important corollary of this is, if we observe the same between a species with an oblique stripe (*Mylochromis mola*) and closely related species that lacks this stripe (*Copadichromis borleyi*). Secondly, we tested the hypothesis whether the convergently evolved color patterns in all striped and nonstriped cichlids across the East African radiations (Figure 1b) are due to an ancient retention of a set of genes with conserved differential expression. Third, we describe the development of stripe patterns and compare them and their onset with vertical melanic patterns. Lastly, in the species pair of Lake Victoria, we also analyzed different melanic (i.e., stripes and bars) and nonmelanic (i.e., interstripes [INTs] and interbars) parts of the body to test whether we identify genes that link to the spatial variation in melanic pigmentation, and if and how they related to *agrp2*. This could provide new insights into the gene regulator network that underly the striking dynamics of color pattern evolution in cichlid fishes.

2 | MATERIALS AND METHODS

2.1 | RNA extraction and library preparation

All fish in this study were from stocks of the animal facility, at the University of Konstanz, Germany. Animals were killed with an overdose of MS-222. Experiments were performed in accordance with animal research regulations (Regierungspräsidium Freiburg, Reference number: G-17/110). The integument (skin and scales) of the whole skin (or specific pigmented regions for bar and stripe comparison) of adult individuals was dissected as previously described (Liang et al., 2020) and kept in RNAlater (Invitrogen) at 4°C overnight. Tissues were transferred to −20°C for long-term storage. RNAlater was removed before homogenization. TRIzol (Invitrogen) was added (1 ml TRIzol per 0.1 g sample) and the tissue was homogenized in 2 ml Lysing Matrix A tube (MP Biomedicals) using FastPrep-24 Classic Instrument (MP Biomedicals). RNA was extracted following the instructions of the manufacturer. We added one additional 75% ethanol wash step. Purification and on-column DNase treatment was done using the Rneasy Mini Kit and Rnase-Free Dnase Set (Qiagen). After the extraction and purification, we quantified RNA using the Qubit RNA HS Assay Kit (Invitrogen) with a Qubit v2.0 Fluorometer (Life Technologies). First-strand complementary DNA was synthesized using the GoScript Reverse Transcription System (Promega) and 1 μg of total RNA. RNA-sequening (RNA-seq) libraries have been prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina) according to protocol. The final libraries were amplified using 15 polymerase chain reaction (PCR) cycles. Quantification and quality assessment was performed using a Bioanalyzer 2100 (Agilent Technologies). Indexed DNA libraries were normalized and pooled in equal volumes. Paired-end 50 bp (*Pundamilia nyererei* and *Haplochromis sauvagei*; $n = 5$ for each; 24.0 ± 2.6 million read pairs per sample) and 150 bp (*Pseudotropheus demasoni*, *Pseudotropheus cyaneorhabdos*, *Melanochromis auratus*, *Copadichromis boreleyi* and *M. mola*; $n = 4$ for each; 32.9 ± 10.7 million read pairs per sample) libraries were sequenced on an Illumina HiSeq.2500 (*P. nyererei* and *H. sauvagei*) or HiSeq X Ten platform (*P. demasoni*, *P. cyaneorhabdos*, *M. auratus*, *C. boreleyi*, and *M. mola*).

2.2 | Mapping and transcript quantification

After removing adapters using Trimmomatic 0.39 (Bolger et al., 2014), reads from all samples were mapped to the *Astatotilapia calliptera* genome (fAstCal1.2, Ensembl release 99) using STAR 2.7.3a (Dobin et al., 2013) as previously described (Kratochwil et al., 2019). To quantify the expression of transcripts included in the Ensembl *A. calliptera* annotation, we used RSEM 1.3.3 (Li & Dewey, 2011) with default settings. Quality statistics were summarized using MultiQC 1.8 (Ewels

et al., 2016). One sample was removed from subsequent analysis, because it had only 0.1 million (M) reads (Pnye_Skin_P2_Int_04). Overall, we obtained 28.5 ± 8.9 M reads per sample with 84.3 ± 1.6% of the reads aligning to the genome.

2.3 | RNA-seq analysis

Analysis was performed using the DESeq. 2 1.22.2 pipeline (Love et al., 2014) in the R environment (R Development Core Team, 2019).

To perform pairwise comparisons between species and screen for DE genes, we included all samples and used individual contrasts between the Lake Victoria, Malawi mbuna, and Malawi non-mbuna species, respectively. For this analysis, we combined the read data from different regions (i.e., stripe, bar, and nonpigmented regions) of the same individuals (see description below) of the two Lake Victoria species *H. sauvagei* and *P. nyererei* using the collapseReplicates function. As the total dissected area of the skin was comparable (and so were read numbers) between the two species, this should result in comparable results as if one would have combined the material before sequencing. For the comparison of melanic and nonmelanic regions, we reran the analysis with the *H. sauvagei* and *P. nyererei* samples only and used contrasts between melanic and nonmelanic regions by species. All *p* are corrected for multiple testing (false discovery rate) as implemented in the Deseq. 2 pipeline.

To identify gene that are correlated with agrp2 expression in the respective pairwise comparisons, we performed co-expression network analyses that were performed based on the WGCNA package (Langfelder & Horvath, 2008) and as previously described (https://github.com/iscb-dc-rsg/2016-summer-workshop/blob/master/3B-Hughitt-RNASeq-Coex-Network-Analysis/tutorial/README.md). Briefly, we $\log_2$-transformed the count data. Next, all genes that did not have at least a 1.05 expression difference in any of the pairwise comparisons were removed ($n = 2{,}951$), leaving us with 18,011 genes. Using this data, we constructed a similarity matrix with a combination of Pearson correlation and Euclidean distance. The matrix was converted into an adjacency matrix and co-expression modules were detected using the default weight threshold of 0.5 (however, also more relaxed settings or use of subsets of species or all species revealed any coexpression modules that included *agrp2*).

The following additional R packages were used: BiocParallel 1.16.2 (Morgan et al., 2019), tximport 1.10.1 (Soneson et al., 2015), stringR 1.4.0 (Wickham, 2019), vsn 3.50.0 (Huber et al., 2002), ggplot2 3.1.1 (Wickham et al., 2019), RcolorBrewer 1.1-2 (Neuwirth, 2014), pheatmap 1.0.12 (Kolde, 2019), cowplot 0.9.4 (Wilke, 2019), and VennDiagram 1.6.20 (Chen, 2018).

2.4 | Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Embryos and juveniles of the F_3 generation of a hybrid cross between *P. demasoni* and *P. cyaneorhabdos* were sampled and the trunk region isolated (Kratochwil et al., 2018). At these stages, it was not possible to reproducibly dissect solely the skin. The tissue was kept in RNAlater (Invitrogen) at 4°C overnight and then transferred to −20°C for long-term storage. RNA extraction and RT-qPCR analysis was performed as previously described in detail (Liang et al., 2020). The following primers were used: agrp2_F: 5′-GCG AAG AAT AGG CGG CTG TTT G-3′; agrp2_R: 5′-CGA CGC GCC GGA GTT ACG AG-3′; gapfh_F: 5′-CAC ACA AGC CCA ACC CAT AGT CAT-3′; gapdh_R: 5′-AAA CAC ACT GCT GCT GCC TAC ATA-3′; bactin_F: 5′-TGA CAT GGA GAA GAT CTG GC-3′; bactin_R: 5′-TGG CAG GAG TGT TGA AGG T-3′. For genotyping of the *agrp2* locus, the previously known microsatellite MS05 was used. It shows a length polymorphism in the parental species (*P. demasoni*: 322/330, *P. cyaneorhabdos*: 250/266), enabling clear identification (Kratochwil et al., 2018).

2.5 | Photography of embryos and juveniles

Photographs of embryos and juveniles were captured with a stereomicroscope (Leica MZ10F) with a Leica DMC2900 color camera. Fish were first anesthetized with 0.04% tricaine (MS-222, Sigma-Aldrich). A more detailed description of the imaging methodology has been previously published (Kratochwil et al., 2015, 2017).

3 | RESULTS

3.1 | Only *agrp2* shows significant differential expression in the adult skin across all nonstriped versus striped species comparisons

Previous studies demonstrated that adult *agrp2* expression differences are highly correlated with the loss (high *agrp2* expression) and gain (low *agrp2* expression) of stripe patterns in cichlids (Kratochwil et al., 2018),

suggesting a causal role of expression differences of this gene. To further test this hypothesis, we analyzed adult skin tissue of three independent species pairs (Figure 1b), one from Lake Victoria (*P. nyererei*—nonstriped; *H. sauvagei*—striped), one from the mbuna lineage of Lake Malawi cichlids (*P. demasoni*—nonstriped; *P. cyaneorhabdos*—two-striped) and one from the non-mbuna lineage of Lake Malawi cichlids (*C. borleyi*—nonstriped; *M. mola*—oblique-striped). We first performed differential expression analysis and identified 141 (Lake Victoria), 461 (Lake Malawi, mbuna), and 386 (Lake Malawi, non-mbuna) DE genes ($p < .001$; Figure 1c). Of these, 39 genes were shared between the Malawi species comparisons (Lake Malawi, mbuna and non-mbuna) and 11 between the species comparisons including two-striped species (Lake Victoria and Lake Malawi mbuna). Interestingly only one gene was DE ($p < .001$) among all three pairwise comparisons (Figure 1c). This gene is the previously described gene *agrp2* (ENSACLG00000010090). Our comparative analyses therefore confirm previous results (Kratochwil et al., 2018) and might have been expected, as differential expression has been shown for two of the species pairs by RT-PCR (Kratochwil et al., 2018). Yet, two things have been noted: the first observation is the only gene that shows conserved differential expression between nonstriped and striped species (out of the 25,683 genes for which we find expression in the integument). This clearly identifies *agrp2* as an outlier and that it is not a common pattern, as it could, for example, occur due to incomplete lineage sorting of regulatory active genomic elements. Second, the gene expression differences between *C. borleyi* and *M. mola* had previously not been investigated. As aforementioned, the stripe pattern (i.e., one oblique stripe) of *M. mola* differs morphologically from the stereotypic two-stripe pattern of most Lake Malawi and Lake Victoria cichlids. The fact that *agrp2* is among the top DE genes ($p = 5.95 \times 10^{-5}$) makes the gene an interesting candidate to be also involved in controlling the presence/absence of this nonstereotypic, oblique stripe pattern.

Additionally, we also performed pairwise comparisons between integument transcriptomes of *P. demasoni* and another mbuna cichlid, *M. auratus* (Supporting Information: Figure 1) for which transcriptome data are available (this striped species occurs in a dark and yellow morph, we therefore performed two comparisons: one with the dark and one with the yellow morph). In addition, here, *agrp2* turns out to be DE ($p = 3.69 \times 10^{-8}$ in comparison with *P. demasoni* and 1.80×10^{-10} in the comparison with *M. auratus*) and therefore is—including the other three comparisons—DE between all five comparisons (Supporting Information: Figure 1).

3.2 | Pair-wise comparisons provide insights into gene expression divergence between striped and nonstriped species

As we found a large number of DE genes in the individual pairwise comparisons (from 141 in the Lake Victoria comparison with 735 in the Lake Malawi comparison between *P. demasoni* and the dark morph of *M. auratus*), we investigated our data set in three steps to find additional genes that are correlated with the repeatedly evolved striped versus nonstriped divergence (or at least in divergence in coloration, more generally), as well as genes that might be acting downstream of *agrp2*. First, we analyzed the top DE genes (based on *p*-value) of each pairwise comparison. Second, we screened for known pigmentation genes in all significantly DE genes. Third, we searched for genes that are in regulatory relationships with *agrp2*.

Between *P. nyererei* and *H. sauvagei* (Figure 2a), the species from Lake Victoria, *agrp2* is among the top DE genes ($p = 1.8 \times 10^{-31}$, rank based on *p*: 4th, fold change: 23.9). Beyond *agrp2*, we found one additional color gene to be DE between the species: the *kit ligand a* (ENSACLG00000020690; *kitlga*) that can trigger ectopic melanophore development (Parichy & Spiewak, 2015). The gene with the highest statistical support of differential expression is *gbgt1l2* (ENSACLG00000014495; *globoside alpha-1,3-N-acetylgalactosaminyltransferase 1-like*) a member of the glycosyltransferase-6 gene family that is involved in the biosynthesis of antigens (Evanovich et al., 2016; Jacob et al., 2014). It is not surprising to discover immune-related genes among the DE genes between species, as immune genes have been shown to be particularly fast-evolving (Star et al., 2011; Wang et al., 2015). One might speculate that this is not only true for sequence evolution but also regulatory evolution.

In the comparisons between the Malawi species (Figure 2b,c) *agrp2* can be also found among the highly DE genes (mbuna: $p = 1.7 \times 10^{-12}$, rank based on *p*: 60th, fold change: 6.6; non-mbuna: $p = 6 \times 10^{-5}$, rank based on *p*: 181th, fold change: 3.8). The top DE genes in the two comparisons have unknown function with respect to pigmentation: zgc: 110,239 in the mbuna comparison (Figure 2b), a gene with domains suggesting cysteine-type endopeptidase activity, and ENSACLG00000025594 and ENSACLG00000025567 in the non-mbuna comparison (Figure 2c), the latter having similarities to a mannose-binding protein and therefore also is a gene with immune-related function. Within the mbuna pairwise comparison, we also uncovered DE pigmentation and coloration-related genes (Figure 2b and Supporting Information: Figure 2), which link to melanin synthesis (ENSACLG00000011752; *tyrp1b*) and melanosomes

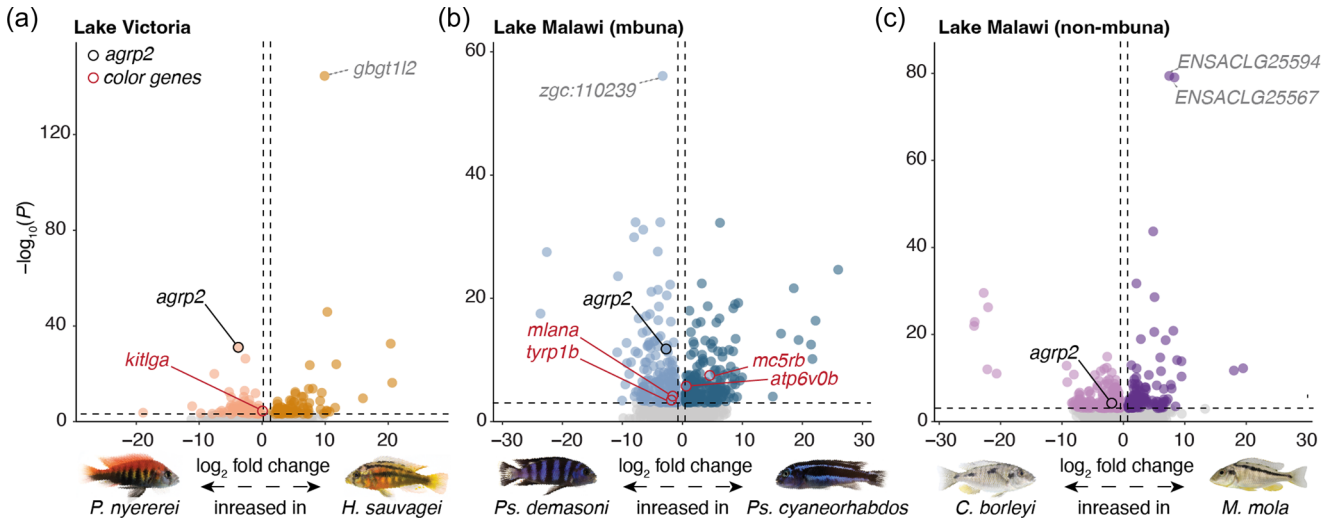


FIGURE 2 Volcano plots of gene expression differences between striped and nonstriped species. (a) Comparison between *P. nyererei* and *H. sauvagei* from Lake Victoria. (b) Comparison between *P. demasoni* and *P. cyaneorhabdos* from the mbuna lineage of Lake Malawi. (c) Comparison between *C. borleyi* and *M. mola* from the non-mbuna lineage of Lake Malawi. On the left side are always genes with higher expression in the nonstriped species, on the right side are genes with higher expression in the striped species. Genes above significance ($p = .001$) and fold change threshold (FC > 1.5) are color coded (lighter colors: higher expression in nonstriped species; darker colors, higher expression in striped species). The point with black outline indicates *agrp2*, the points with red outlines coloration and pigmentation-related genes.

(ENSACLG00000014786; *atp6v0b*) or constitute melanocyte/melanophore markers (ENSACLG00000013936; *mlana*). One particularly interesting DE gene is the melanocortin receptor *mc5rb* (ENSACLG00000008710), as melanocortin receptors are known to be antagonized by the ligand *agrp2* (Supporting Information: Figure 2 and Section 4).

To screen for genes whose expression correlates with *agrp2* expression, and thus indicating that they act, for example, downstream of *agrp2*, we performed co-expression network analyses for the three pairwise comparisons. Interestingly, in none of the data sets was *agrp2* found to be a part of any co-expression module, although a substantial number of genes were found to be (Lake Victoria: 1670/18,011 genes; Lake Malawi mbuna: 9076/18,011 genes; Lake Malawi non-mbuna: 11,537/18,011 genes). This is either suggesting that the effect on gene expression is highly multifaceted and dynamic, and therefore not picked up by our analysis or that the predominant effects of *agrp2* are cellular responses (see Section 4). Both would make sense in the context of a paracrine factor acting on melanocortin signaling.

A caveat of our transcriptomic analysis is the limitation to adult tissues. It is certainly possible that it is the adult differential expression of *agrp2* directly underlies the phenotype by constantly repressing melanophore differentiation, melanin production, and/or melanosome dispersal. However, although previous and the current work show a strong association between adult *agrp2* expression and stripe patterns, we cannot rule out a (causal) developmental role of *agrp2*. To investigate *agrp2* expression differences in embryos/juveniles, we took advantage of a hybrid cross between the striped species *P. cyaneorhabdos* and the nonstriped species *P. demasoni* in which we performed qPCR for *agrp2*. The advantage is that we can compare individuals within the same clutch that will become striped and nonstriped based on their genotype at the *agpr2* allele (Gerwin et al., 2021; Kratochwil et al., 2018). Neither at a late embryonal stage (10 days postfertilization [dpf]) nor at a later juvenile stage, shortly before stripes form (20 dpf), could we find differential expression of *agrp2* between individuals homozygous for the striped *P. cyanorhabdos* allele and individuals homozygous for the nonstriped *P. demasoni* allele (Supporting Information: Figure 4). These results do not support a developmental role of *agrp2*, although a more fine-grained analysis and an analysis across species will be needed to confirm these findings.

3.3 | Bar and especially stripe patterns develop postembryonically

An interesting feature of cichlid fishes is that they develop directly into the adult form, without an prolonged intermediate larval stage (as, e.g., zebrafish; Woltering et al., 2018). Adult color patterns in cichlid

fishes therefore essentially form directly without an intermediate, embryonal pigmentation pattern (Hendrick et al., 2019; Liang et al., 2020). To investigate the onset of pattern formation across the focal species more comprehensively, we documented its development within the first 2 months (Figure 3a–t). Interestingly, bar patterns generally form at late embryonal stages (between ~10 and before ~20 dpf, depending on the species; Figure 3u), whereas stripe patterns form exclusively in juveniles between ~20 dpf in *P. cyaneorhabdos* and ~40 dpf in Lake Victoria species (e.g., *H. sauvagei*; Figure 3v). The melanic patterns of the Lake Victoria species are generally much less pronounced at these juvenile stages (Figure 3a–h and Supporting Information: Figure 5).

3.4 | Transcriptomic differences between adult melanic and nonmelanic integument regions

Although it seems well-supported now that *agrp2* constitutes the central genetic determinant of stripe pattern absence/presence, what remains to be shown is what the molecular underpinnings of the pattern itself are and if *agrp2* plays also a role for shaping melanic patterns (and not only acts as a general repressor). To obtain more in-depth insights into the transcriptional correlates linking to the melanin and nonmelanic regions, we analyzed transcriptomes of four skin regions of adults of each of a species with vertical bars (*P. nyererei*, Figure 4a) and horizontal stripes (*H. sauvagei*, Figure 4d). Previously, it has been shown via qPCR that genes involved in melanin synthesis, including tyrosinase, are DE between bar and interbar regions of *Haplochromis latifasciatus* (Liang et al., 2020), suggesting that RNA-seq on melanic and nonmelanic regions constitutes a valid approach.

Surprisingly though, we found only a small number of DE genes (Figure 4a–c and Supporting Information: Figure 4). We performed pairwise comparisons between all melanic (bars) and nonmelanic (interbars) regions of *P. nyererei* (see positions in Figure 4a). Even with a relaxed significance threshold of $p < .05$, no gene with differential expression between all comparisons could be identified (Figure 4b). Furthermore, *agrp2* showed no differential expression (Figure 4c). As expected (Swalla, 2006), we do however identify *hox* gene (i.e., *hoxa9a*, *hoxa10a*, and *hox11b*) expression differences, with expression being significantly higher in the most posterior region (Interbar 2) than the most anterior region (Bar 1; Supporting Information: Figure 4). In addition, within the striped species, *H. sauvagei*, we document only small expression differences that set apart melanic (DLS; MLS) and nonmelanic (INT; ventral region, VEN; Figure 4a,e). As expected, we found the dorso-ventral patterning gene *zic1* to have higher expression in the dorsal areas (DLS, INT; Figure 4e; Supporting Information: Figure 4). Further, the agouti gene family gene *asip1*, which has been previously described to have high expression in ventral regions (and thereby controlling the pale ventral pigmentation, i.e., countershading; Cal et al., 2019; Liang et al., 2021), shows the expected pattern with high expression in VEN (Figure 4e and Supporting Information: Figure 4). In one of the comparisons (DLS vs. VEN), *agrp2* shows differential expression as well; this, however, mainly seems to reflect a slight dorso-ventral gradient (Supporting Information: Figure 4). Given the little expression differences, we did identify between VEN and the neighboring MLS it seems unlikely that *agrp2* contributes to shaping the pattern itself, confirming what was previously suggested based on qPCR data (Kratochwil et al., 2018).

The most unexpected result of this transcriptomic analysis is the evident lack of expression differences in melanophore or melanin-synthesis-linked genes (Liang et al., 2020; Figure 4b,e and Supporting Information: Figure 3). It might suggest that the differences are based on an additive effect of many small changes in gene expression. Alternatively, differences in pigmentation might not be explained by differences in melanophore number and melanin production but are due to differences in cell distribution and/or pigment aggregation and dispersal. This would be supported by previous findings (Liang et al., 2020) and the described role of *agrp2* as a regulator of melanosome dispersal (Cal et al., 2017). Imaging data (Figure 3 and Supporting Information: Figure S5), however, demonstrates that the differences are very subtle in the two analyzed species, which might lead to the lack of clear signals of differential expression in the transcriptomic comparison. A more rigorous analysis will be needed to investigate the cellular basis of these phenotypes and how they are shaped by *agrp2* expression differences.

4 | DISCUSSION

Here we analyzed a comprehensive data set of transcriptomes of the adult integument of six species of African cichlids as well as provide new insights into the development of the patterns during ontogeny. Using pairwise comparisons of species with and without stripes from three adaptive radiations (Lake Victoria, Lake Malawi mbuna, and Lake Malawi non-mbuna), we show that the gene *agrp2*, which has been previously linked to

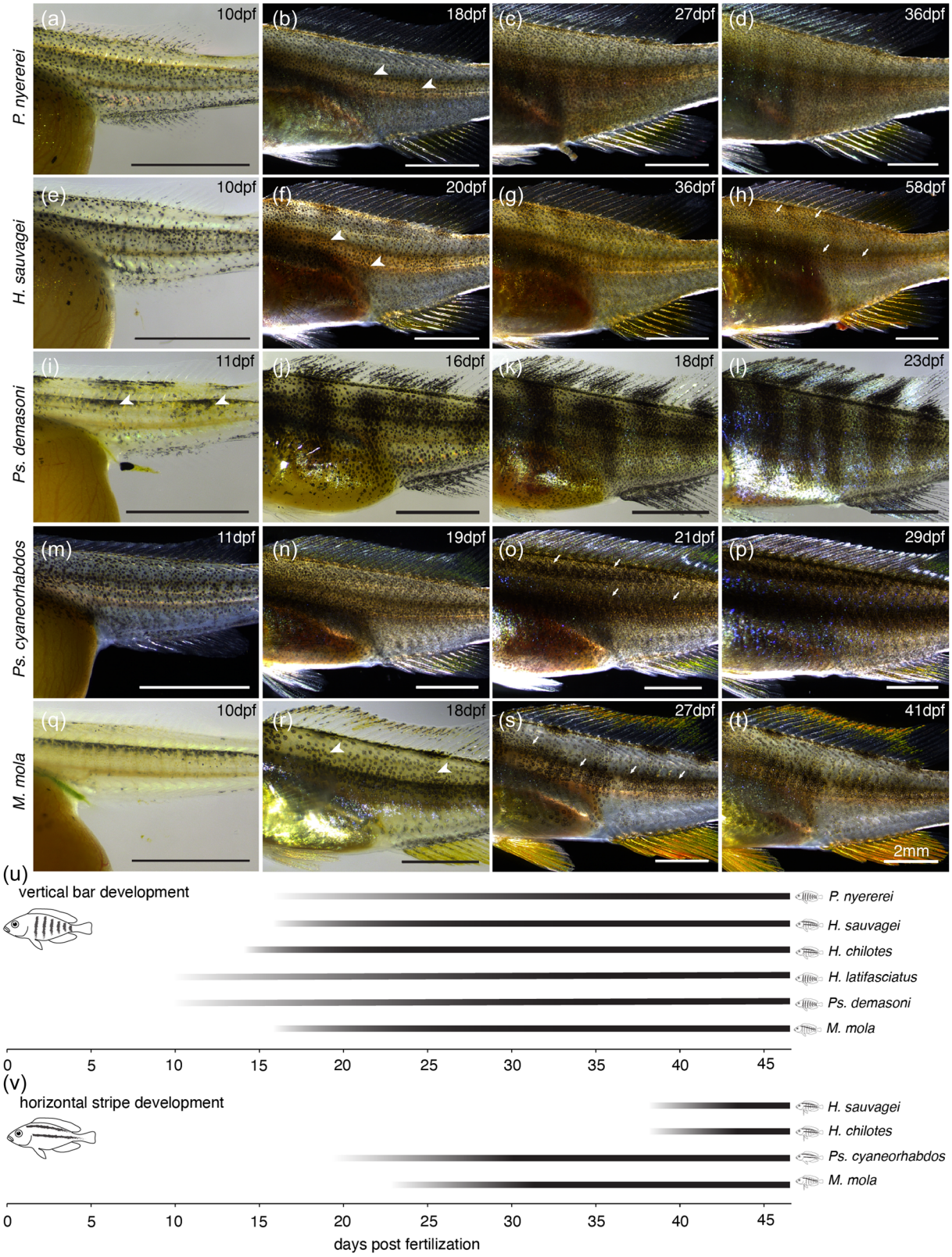


FIGURE 3 Comparative ontogeny of bar and stripe patterns in African cichlid fishes. (a–t) Selected developmental stages before and after the formation of bars (a–l; q–t) and stripes (e–h; m–t) in the Lake Victoria species *P. nyererei* (a–d) and *H. sauvagei* (e–h), as well as the Lake Malawi species *P. demasoni* (i–l), *P. cyaneorhabdos* (m–p), and *M. mola* (q–t). Arrowheads (bars) and arrows (stripes) show the earliest stage where the respective pattern can be seen. (u, v) Summary of the onset of pattern formation for bars (u) and stripes (v).

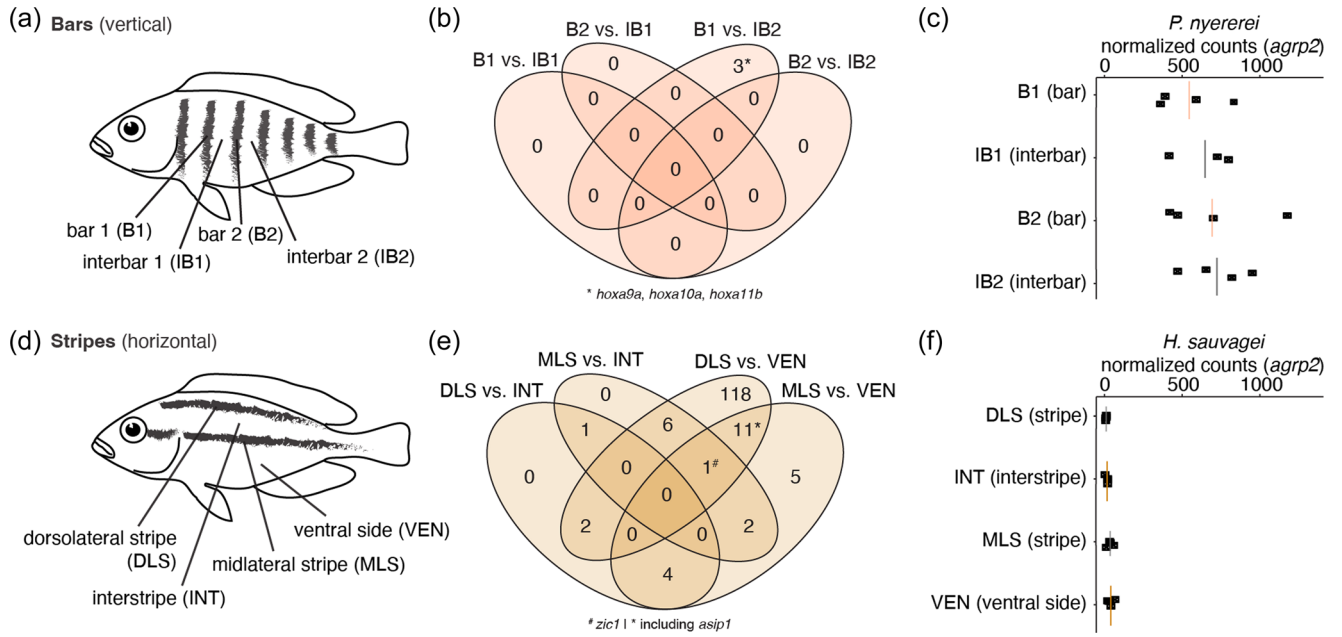


FIGURE 4 Comparisons between melanic and nonmelanic regions of species with bars (*P. nyererei*) and stripes (*H. sauvagei*). (a) Schematic of *P. nyererei* (photograph see Figure 1c) indicating the positions of the dissected bar and interbar regions. (b) We performed pairwise comparisons between each melanic and nonmelanic regions ($p < .05$). Only in one comparison three (*hox*) genes were differentially expressed (DE) that link to the anterior–posterior differences in *hox* gene expression between the most posterior (Interbar 2 [IB2]) and anterior (Bar 1 [B1]) region. (c) The gene *agrp2* shows consistently high expression across all regions (however, with a slight anterior–posterior gradient), indicating that there is no link between the pattern itself and *agrp2* expression. (d) Schematic of *H. sauvagei* (photograph see Figure 1c), indicating the positions of the dissected stripe and interstripe region. (e) In the pairwise comparisons between melanic and nonmelanic regions of *H. sauvagei*, we discovered more DE genes including genes that have been previously linked to dorso-ventral patterning (*zic1*, *asip1*). None however is consistently associated with pigmented and nonpigmented regions. (f) The expression of *agrp2* is consistently low in *H. sauvagei* with a slight dorso-ventral gradient (see also Supporting Information: Figure 3 for the same figure with a rescaled *y* axis).

the evolutionary loss and gains of stripe patterns (Kratochwil, 2019; Kratochwil et al., 2018, 2019; Urban et al., 2020) is the only gene that shows conserved differential expression between striped and nonstriped species. What was unknown so far is whether we can demonstrate the same striped versus nonstriped species differences in *agrp2* expression in the case of species with nonstereotypic stripe patterns, as the oblique stripe that can be found in *M. mola* (Figure 1c) and in several other non-mbuna cichlid species (e.g., *Aristochromis christyi*). Indeed, also here, we identify *agrp2* among the highly DE genes, suggesting that changes in *agrp2* expression are also contributing to the stripe loss and gain in non-mbuna cichlids with oblique stripes. Based on this discovery, it is interesting to speculate why certain melanic patterns (i.e., horizontal stripes and the oblique stripe) but not others (e.g., bar patterns) are affected by expression changes of the melanocortin signaling antagonist *agrp2*. Horizontal stripes and the oblique stripe are quite similar in the sense that the anterior portion of the oblique stripe is at the position of the DLS, whereas the posterior portion is at the same position as the MLS. Likely, the two pigmentation patterns are therefore homologous with the oblique stripe being formed through a fusion of DLS and MLS. However, this hypothesis must be tested through a careful evaluation of the development of both patterns. In the individual pairwise comparisons with the three lineages, we found—as expected—many DE genes including also a few previously known pigmentation genes, such as *atp6v0b*, *kitlga*, *mlana*, *mc5rb*, and *tyrp1b*, and which deserve further attention, as they might contribute to differences in stripe patterns or other coloration traits (e.g., differences in nuptial coloration Lake Victoria or genes involved in bar pattern formation in *P. demasoni*).

The second question we wanted to address here relates to those genes that might be regulated by *agrp2* and/or might interact with *agrp2*. The antagonists Agrp2 and Asip1 mainly bind to the melanocortin receptor Mc1r, but also to Mc5r (however, less potently [Zhang et al., 2010]). The agonist of the receptors is the α-melanocyte-stimulating hormone (α-MSH). There is also evidence that Mc1r and Mc5r can form dimers (Kobayashi et al., 2016), which affects α-MSH-mediated intracellular

signaling. Binding of Mc1r and/or Mc5r by α-MSH results in an intracellular increase in cyclic AMP levels, which can affect (a) proliferation of melanophores, (b) production of melanin, and (c) dispersion of melanosomes (Cal et al., 2017; Cortés et al., 2014). Low *agrp2* levels could therefore result in more melanophores, increased production of melanin, and higher dispersal of melanosomes. All three factors could contribute to the appearance of melanic stripes. The other antagonist *asip1*, as well as the receptors mc1r and *mc5r*, do not show substantial variation in expression between species. One exception is *mc5r* that has significantly lower expression in *P. demasoni* (Figure 2b). Although speculative, this might imply that less *mc5r*/*mc1r* heterodimerization occurs, which might affect the potency of *agrp2*. Additionally, it is also possible that receptor-binding properties of *agrp2* differ when comparing mc1r and mc1r/mc5r heterodimers. Regarding the downstream effects of *agrp2*, we cannot offer a conclusive answer. One reason for the lack of genes with strong signals of co-expression might be intrinsic problems of the transcriptomic approach (i.e., high heterogeneity of the tissue as pigment cells are only a small proportion of the skin). Another reason might however be that the dispersion/aggregation state of melanosomes is regulated by *agrp2*, which also would result in a darker/lighter appearance of skin regions. It would therefore not affect the number of melanophores or amount of melanin. Such a subcellular change would not necessarily lead to transcriptomic change.

The third hypothesis we wanted to test is whether we find gene expression differences that associate with melanic and nonmelanic regions of species with stripes and bars. Surprisingly, and contrary to results from other species (Liang et al., 2020), we could not identify the expected melanin synthesis and melanophore-related genes. In fact, there was not a single gene with consistently higher expression in melanic than nonmelanic regions—neither in the species with bars (*P. nyererei*) nor in the species with stripes (*H. sauvagei*). Thus, the interpretation of these results is similar as for the lack of genes being in a co-expression network with *agrp2*. First, the approach of performing transcriptomes of such a heterogeneous tissue might—at least in these species—not be powerful enough to detect differences in pigmentation genes. In fact, our imaging data show the patterns in the two Lake Victoria species are much less pronounced than in the Lake Malawi species (Figure 3), as well as *H. latifasciatus* (Liang et al., 2020). Alternatively, the main differences between bars and interbars, as well as stripes and INTs, are not due to spatial variation in the expression of genes but differences in tissue organization (i.e., organization of melanophores within the integument) or intracellular organization (i.e., aggregation and dispersal state of melanosomes) and can therefore not be detected by RNA-seq. Lastly, we can also show clearly, as suggested before (Kratochwil et al., 2018), that *agrp2*—at least in cichlids—does not contribute to shaping the stripe pattern (or bar patterns) itself, as it is ubiquitously expressed across the integument (alternatively *agrp2* could have shown higher expression in non-melanic regions).

Much remains to be investigated regarding the molecular mechanisms that shape stripe and bar formation (and the lack thereof). A first test of the developmental expression differences of *agrp2* does not support a developmental role of *agrp2*, but a more detailed analysis might be needed to investigate the onset of *agrp2* expression and how it relates to stripe pattern formation. An analysis of the exact cellular underpinnings of the patterns as previously done in other species (Liang et al., 2020) might provide more insights. An important insight of our work is how, compared with Lake Malawi cichlids, relatively late stripes form in Lake Victoria cichlids (40 dpf; Figure 3e–h) and how subtle the pattern is at the beginning (Figure 3e–h).

Our qPCR results suggest a lack of expression pattern differences in *agrp2* before the pattern is formed. If these results are confirmed, it would suggest a permanent, adult repression of stripe pattern formation by *agrp2*. An although similar adult roles of agouti family mem are known in vertebrates (e.g., during the seasonal coat color change of snow hares [Ferreira et al., 2020]), this would be certainly an interesting mechanism, as it would only act on the adult pattern without interfering with the develop.

In summary, using an unbiased (compared with target gene qPCR studies as previously conducted) comparative transcriptomic approach across three distantly related, but phenotypically convergent lineages of cichlid fishes, we confirm *agrp2* expression as robustly associated with stripe versus nonstripe divergence in East African cichlids. We discovered other genes that might—based on their differential expression—contribute to divergence in coloration and pigmentation. Furthermore, we increased the understanding of the development of these ubiquitous color patterns. Lastly, we suggest that *agrp2* might not act as strongly on melanophore number and melanin production as thought before, but rather contributes towards the spatial organization of melanophores within the integument as well as the dispersal state of melanosomes.

AUTHORS CONTRIBUTIONS

Yipeng Liang (gene expression data) and Jan Gerwin (ontogenetic data) collected data. Claudius F. Kratochwil and Paolo Franchini conducted the analysis. Claudius F. Kratochwil wrote the first draft of the manuscript. All

authors contributed to the writing. Claudius F. Kratochwil and Axel Meyer designed and coordinated the study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NCBI SRA at https://www.ncbi.nlm.nih.gov/sra?term=PRJNA635556, reference number PRJNA635556.

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SUPPORTING INFORMATION

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

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