Developmental tinkering of gene regulation facilitated super rapid adaptive radiation

Anna Duenser*[1,2], Pooja Singh*[1,3,4], Ehsan Pashay Ahi[1,5], Marija Durdevic[6,7], Sylvia Schaeffer[1], Wolfgang Gessl[1], Ole Seehausen[3,4], Christian Sturmbauer[1]

[1]Institute of Biology, University of Graz, Graz, Austria

[2]Federal Agency for Water Management, Institute for Freshwater Ecology and Fisheries Management, Mondsee, Austria

[3]Aquatic Ecology Division, Institute of Ecology and Evolution, University of Bern, Bern, Switzerland

[4]EAWAG, Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland

[5]Organismal and Evolutionary Biology Research Programme, University of Helsinki, Helsinki, Finland

[6]Diagnostic & Research Center for Molecular BioMedicine, Institute of Pathology, Medical University of Graz, Graz, Austria

[7]Core Facility Computational Bioanalytics, Center for Medical Research, Medical University of Graz, Graz, Austria

*These authors contributed equally

Corresponding authors: Anna Duenser (anna.duenser@gmail.com), Pooja Singh (pooja.singh09@gmail.com) & Christian Sturmbauer (christian.sturmbauer@uni-graz.at)

Abstract

Developmental shifts in gene regulation underlying key innovations remain largely uncharacterised at short evolutionary timescales. Here we investigate the gene expression and alternative splicing landscape of trophic innovations in the fastest vertebrate adaptive radiation: cichlid fishes from Lake Victoria. Using whole-transcriptomes of the oral and pharyngeal jaws from two life stages of species adapted to divergent trophic niches, we show that gene and isoform expression were fine-tuned during development to generate specialised species-specific eco-morphologies in adults. This is a striking contrast to mammals where tissue-specific gene expression is conserved across species even after 90 million years of evolution and suggests that gene regulatory programs have evolved rapidly in Lake Victoria cichlids. We show that gene expression during development is highly modular and this developmental modularity may have facilitated trophic diversification. Furthermore, we discovered that retrotransposon mediated exonisation of a craniofacial development gene, KAZNB, has contributed to the evolution of a novel trophic niche. This advocates for the importance of exonisation and the splicing machinery in rapidly expanding the coding capacity of the genome <15,000 years. We also identified a major transcription factor of craniofacial remodelling in Darwin's finches, ALX3, to have contributed to jaw divergence in Lake Victoria cichlids. Overall, our results suggest that developmental tinkering of gene regulation is a major facilitator of key innovations and diversification during adaptive radiation.

Introduction

Since King and Wilson (1975) concluded that variation in protein sequence alone cannot explain the phenotypic differences between human and chimpanzees, transcriptional regulation has been shown to play a fundamental role in phenotypic evolution and diversification (Brawand et al. 2011; Barbosa-Morais et al. 2012; Merkin et al. 2012). However, few studies have focused on recently diverged species, where regulatory changes are hypothesised to dominate phenotypic divergence, as they can emerge more rapidly from standing genetic variation and relaxed selection than de novo

protein-coding variation (Singh et al. 2017; El Taher et al. 2020; Richards et al. 2021; Singh et al 2021; Singh and Ahi 2022; Carruthers et al. 2022). Even fewer studies have looked at regulatory changes during development in recent and rapid bursts of diversification (Cardoso-Moreira et al 2019).

Cases of adaptive radiation, where species diversify rapidly in tight connection with ecology, provide promising model systems to study the role of gene regulation in rapid evolutionary diversification (Abzhanov et al. 2006; Gillespie et al. 2020; De-Kayne et al. 2022). The radiations of cichlid fishes in the Great East African lakes are such model systems where rapid diversification proceeded along many ecological and morphological axes (reviewed in Kocher 2004). The Lake Victoria (LV) cichlid radiation comprising ~500 species is arguably one of the most spectacular of these radiations, having emerged after the complete desiccation of the lake <15,000 years ago (Johnson et al. 2000; Seehausen 2002). Lake Victoria cichlids evolved diverse trophic adaptations facilitated by the reshuffling of ancestral standing genetic variation as ecological opportunity arose (Meier et al. 2023). The functionally decoupled oral and pharyngeal jaws of cichlids is hypothesised to be a key innovation and basis of their evolutionary success that enabled rapid trophic diversification (Fryer and Iles 1972; Parsons et al. 2011). It was recently found that cichlids dominate the biomass of Lake Victoria not because they arrived first as the lake filled up, but because they had ecologically versatile trophic morphology (Ngoepe et al. 2023).

Many studies have investigated the genetic architecture and myriad signalling pathways underlying trophic morphologies in teleosts and more specifically in cichlids (Albertson et al. 2005; Albertson and Kocher 2006; Parsons and Albertson 2009; Roberts et al. 2011; Parsons et al. 2014; Ahi 2016; Singh et al. 2021). In addition to gene expression variation, alternative splicing of pre-mRNA is also thought to contribute to transcriptional diversity and act as substrate for rapid adaptive evolution (Singh et al. 2017). Through alternative splicing a single gene can be altered into many mRNA isoforms which can potentially be translated into different proteins, boosting transcriptomic and proteomic diversity (Kim et al. 2007; Nilsen and Graveley 2010). Alternative splicing can provide

rapid paths to the evolution of phenotypic diversity (Singh and Ahi 2022). Although it is debated to which extent AS events are contributing to proteomic and phenotypic diversity (Tress et al. 2017; Blencowe 2017). Splicing processes such as exonisation, where exons evolve from retained introns can also rapidly generate protein-coding and regulatory variation (Keren et al. 2010; Sorek 2007). While it is generally thought that intron retention is rare in animals compared to plants, Singh et al (2017) found higher intron retention in cichlid fishes than humans. The relationship of alternative splicing for adaptive diversification and speciation is a research topic of increasing interest in animals and plants (Singh et al. 2017; Bush et al 2017; Smith et al. 2018; Smith et al. 2020; Jacobs and Elmer 2021; Wright et al. 2022), but more research is needed to illuminate the role of alternative splicing during adaptive radiation, and speciation in general (Singh and Ahi 2022).

Recent work has shown that ontogenetic diet shifts, which are common in animals (Sánchez-Hernández et al. 2019), can alter the trajectory of adaptive radiations in the presence of ecological opportunity (Ten Brink and Seehausen 2022). Little attention has been directed towards the role of ontogenetic developmental changes during adaptive radiation. In cichlids, there is some evidence that key shape changes in cichlid jaw cartilage and bone occur during early development (Albertson and Kocher 2006; Fujimura and Okada 2008) and that specialised morphologies are already distinct between trophic niches at the onset of exogenous feeding (Singh et al. 2017). But most studies on the transcriptional basis of trophic divergence across multiple species have focused on one stage (El Taher et al. 2020), and not much is known about the transcriptional patterns and mechanisms underlying the larval development of alternative trophic morphologies and further ontogenetic changes towards adulthood.

Here we leveraged the comparative framework to investigate the differences and commonalities between two postembryonic key life stages in cichlids: the end of the larval stage: stage 26 (Fujimura and Okada 2007) and the adult stage. The end of the larval development (stage 26) is marked by the complete absorption of the yolk sac into the body cavity and denotes the onset of independent foraging as the larvae are released from the mouthbrooding mother's mouth. The adult stage (marked by the

onset of breeding and the emergence of male nuptial coloration) stands for full maturity of the entire phenotype. To identify developmental genes and regulatory mechanisms involved in species differentiation and trophic adaptation, we explored the transcriptional landscape underlying the trophic adaptations of the oral jaw apparatus (OJA) and pharyngeal jaw apparatus (PJA) (Fig. 1 B and C) in the exceptionally young radiation of Lake Victoria cichlids (Fig. 1).

Results

Genome-guided transcriptome assembly and annotation

To analyse the transcriptional landscape of LV cichlids adapted to different diets we generated whole mRNA transcriptomes from oral and pharyngeal jaws of five Lake Victoria species (*Haplochromis thereuterion* (Ht), *Gaurochromis hiatus* (Gh), *Mbipia lutea* (Ml), *Neochromis omnicaeruleus* (No) and *Paralabidochromis sauvagei* (Ps)) across two developmental stages (stage 26-yolk sac absorption into body cavity and adulthood-onset of male nuptial colouration) (Figure 1). Each stage for each species was represented by five biological replicates. Tissues from two individuals were pooled for each biological replicate due to the small size of the larvae. Our total sample set comprised 140 whole transcriptomes. After quality filtering, we retained an average of 3-9 million reads per sample. Of these reads 68%-82% could be mapped to the reference genome (*O. niloticus*; NCBI accession number: GCA_001858045.3) in the larval samples and 75%-82% in the adult samples (Supplementary File 1). We chose to map to *O. niloticus* because it is the best annotated reference genome and high quality transcript annotations were important for our analyses. The final merged annotation of all samples included 37,334 genes and the transcript annotation 59,849 transcripts.

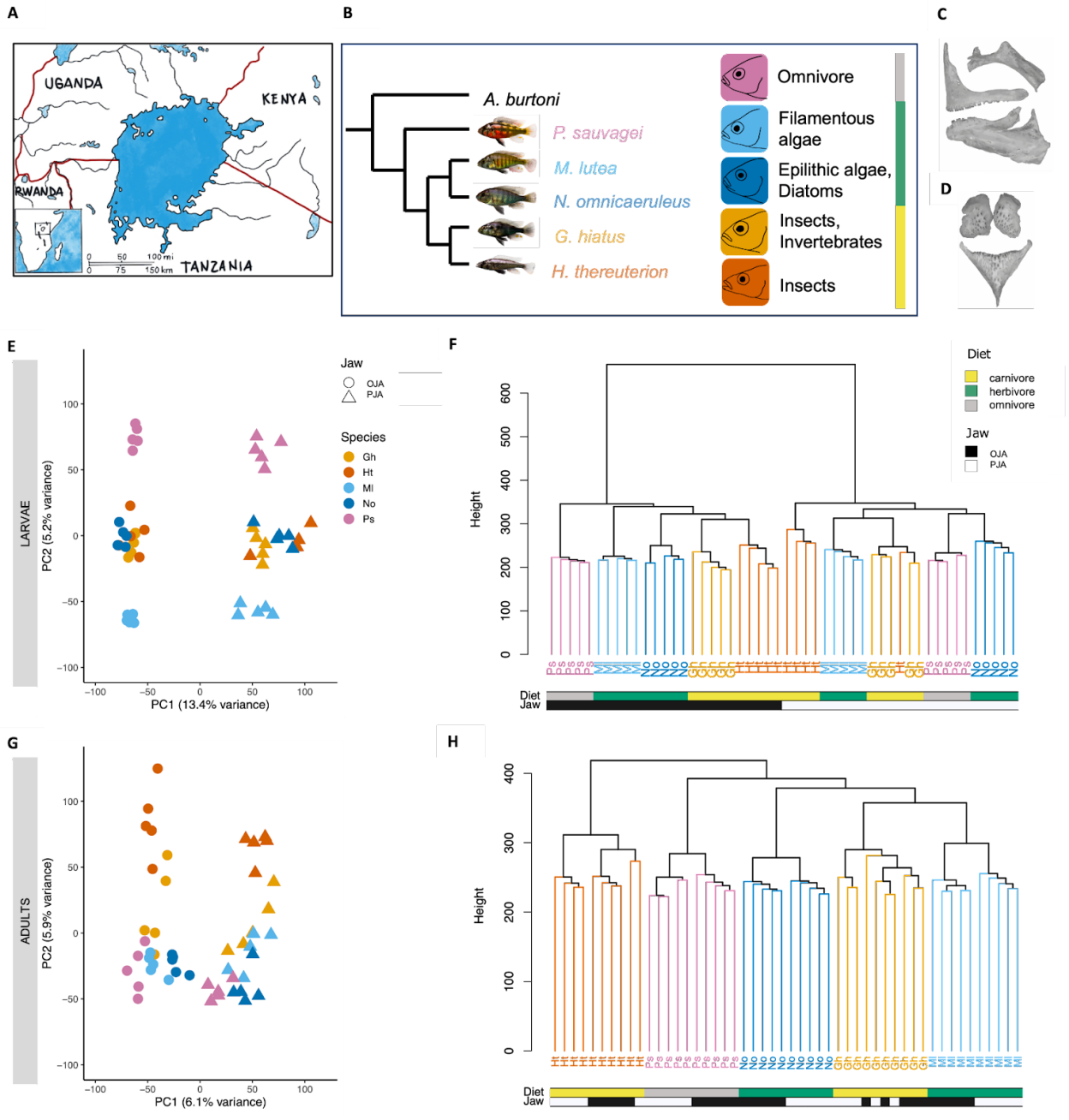


Figure 1: Overview of study design and broad patterns of gene expression. (A) Map of Lake Victoria (B) Study species and their respective trophic niches. Photographs of Oral jaw apparatus (OJA) (C) and pharyngeal jaw apparatus (PJA) (D). Normalised gene expression PCA plot for larvae (E) and adults (G) based on 37,334 genes. (F) and (H) show dendrograms of the Euclidean distance based on the gene expression matrix in. Oral jaw apparatus (OJA; black), pharyngeal jaw apparatus (PJA; white), H. thereuterion (Ht; dark orange), G. hiatus (Gh; bright orange), M. lutea (Ml; bright blue), N. omnicaeruleus (No; dark blue) and P. sauvagei (Ps; pink).

Transcriptome wide patterns of gene and isoform expression

We quantified the gene expression of 37,334 genes across all samples. To obtain an overview of global gene expression patterns we conducted a principal component analysis (PCA) based on normalised expression of 37,334 genes. The PCA shows a clear separation of expression profiles of the two jaw tissue sets for both life stages but the separation is less prominent in adults (Larvae: PC1 (13.4%), Adults: PC1 (6.1%))(Fig. 1 E and G). We also quantified expressions of 59,849 transcripts to see how isoform expression compared to the gene expression. The isoform expression PCA showed similar patterns as the larval gene expression profiles, with a clear distinction between the two jaw apparatuses but less differentiation among species (PC1 (11.4%)) (Fig. 2 A). Isoform expression clustering in adults is more species specific but less variation is explained by PC1 (5.6%) and PC2 (4.4%) when compared to the gene expression PCA (Fig. 2 C).

Additionally we analysed the expression data using hierarchical clustering analysis (Figure 1F-H). In the clustering analysis of the larvae, the expression of the two jaws is clearly distinct and the OJA clustering follows the trophic niche classification, while PJA is species specific (Fig. 1 F). The clustering of gene expression in adults is strictly species-specific, regardless of jaw tissue (Fig. 1 H). The hierarchical cluster analysis of the isoform expression matrix shows a slight difference in the species patterning of the larval PJA compared to the hierarchical clustering of the gene expression matrix (Fig. 2 B), while the adult patterning stays the same (Fig. 2 D).

To place the gene expression patterns into a wider phylogenetic context, we included data from individuals of *Tropheops tropheops* (Tt) and *Astatotilapia burtoni* (Ab) at both stages as outgroups to extend the comparison to two more divergent species from Lake Malawi and the rivers around Lake Tanganyika respectively. The PCA as well as the hierarchical cluster analysis based on Euclidean distance shows a clear separation of expression profiles of *A. burtoni* and *T. tropheops*, from the Lake Victoria species (Fig. S1).

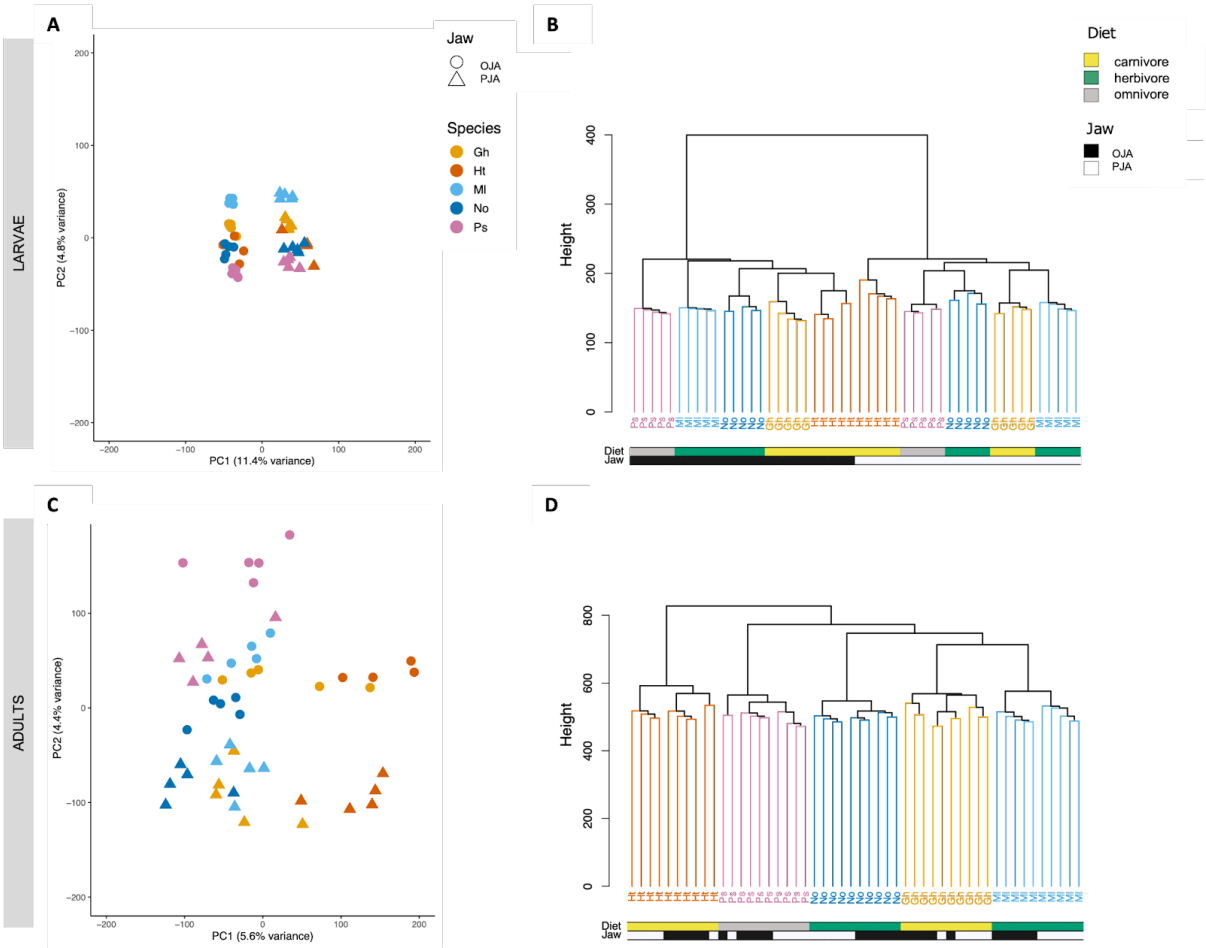


Figure 2: PCA plot for larvae (A) and adults (B) based on 59,849 normalised isoform expression counts. (B) and (D) show dendrograms of the Euclidean distance for larvae (B) and (D) adults. Oral jaw apparatus (OJA), pharyngeal jaw apparatus (PJA), H. thereuterion (Ht), G. hiatus (Gh), M. lutea (Ml), N. omnicaeruleus (No) and P. sauvagei (Ps).

Genes involved in species and trophic differentiation in Lake Victoria cichlids

To identify genes that contribute to species differences in oral and pharyngeal jaws of LV cichlids we conducted differential expression analysis for all pairwise species comparisons for each jaw and developmental stage. Of the 37,334 genes, 5,743 - 10,024 (15.4%-26.9%) showed significant (adjusted p-value < 0.05) differential expression (DE) between at least one pairwise species comparison in all tissues and at both developmental stages (Table S1). Between 591 - 1,664 (1.6% - 4.5%) genes were differentially expressed in more than 50% of the comparisons investigated. Interestingly, no gene was differentially expressed across all comparisons.

To identify genes that play a role in trophic adaptation of the jaw apparatuses we primarily focused on comparing the carnivorous species (Ht, Gh) to the herbivorous species (Ml, No). We identified the intersection set of genes that were differentially expressed across all four comparisons (Gh vs Ml, Gh vs No, Ht vs Ml, Ht vs No) (Fig. 3), and discovered 237 genes that were differentially expressed in larval OJA across all four herbivore vs carnivore comparisons and 159 genes were differentially expressed in the larval PJA of all four comparisons (Fig. 3 A and B respectively). In adult OJA comparisons we identified an overlap of 128 genes that were consistently differentially expressed and 107 genes were differentially expressed in the PJA (Fig. 3 C and D). Of these gene intersection sets between carnivores versus herbivores 79% - 96% shared the same direction of differential expression in all stages and jaw modules (Table S2). Such genes that were upregulated in carnivorous species and downregulated in herbivorous species or vice versa are interesting genes for further investigation in shaping divergent trophic morphologies.

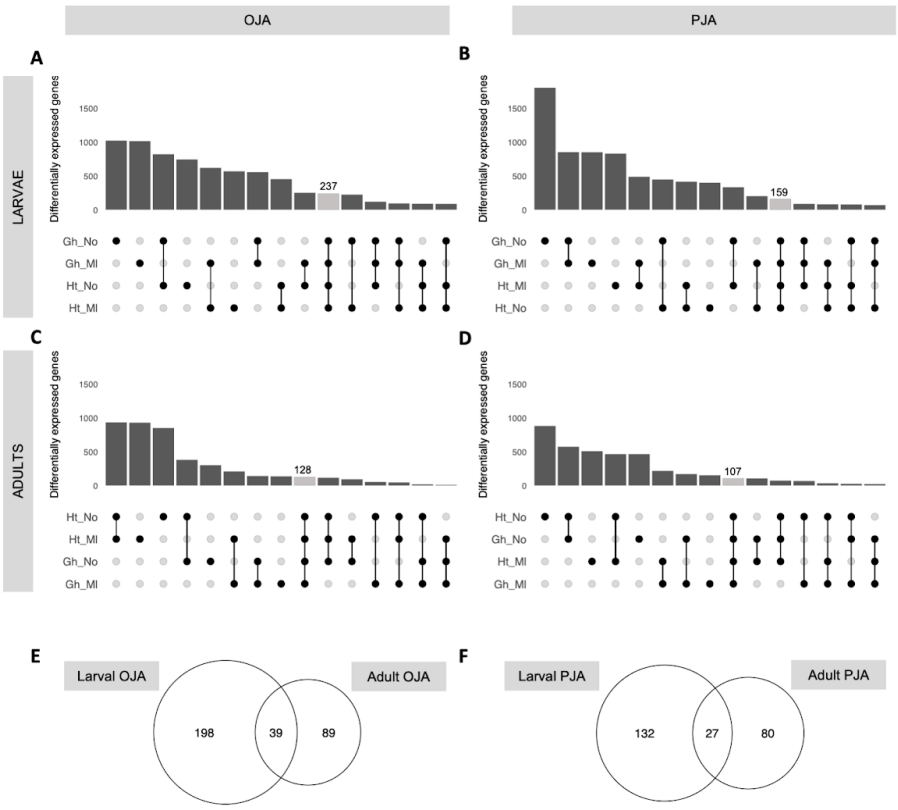


Figure 3: Differentially expressed genes between jaws of herbivorous and carnivorous Lake Victoria cichlids. Upset plots of overlapping differentially expressed genes for species comparisons including the carnivorous and herbivorous species. Points below bars show overlapping genes between indicated pairwise comparisons or unique sets of genes for single points. Light gray bars indicate an overlap between all pairwise comparisons. The total sum of differentially expressed genes for one pairwise comparison results from the sum of indicated bars. Upset plots are shown for larval OJA (A), larval PJA (B), adult OJA (C) and adult PJA (D). Venn diagrams show the overlap in larval and adult differentially expressed genes for OJA (E) and PJA (F). Oral jaw apparatus (OJA), pharyngeal jaw apparatus (PJA), H. thereuterion (Ht), G. hiatus (Gh), M. lutea (Ml) and N. omnicaeruleus (No).

We generated expression heatmaps with hierarchical clustering of all the aforementioned shared genes for each jaw tissue and developmental stage that were differentially expressed in all pairwise comparisons between carnivores and herbivores (Larvae: OJA: 273, PJA: 159; Adults: OJA: 128, PJA: 107; grey bars in Fig. 2 A - D). We also included the expression of these genes from omnivorous LV species *P. sauvagei*. The heatmap clustering placed *P. sauvagei* closer to the carnivorous *G. hiatus* and *H. thereuterion* for the oral and pharyngeal jaw gene expression at the larval (Fig. S2 A & B) and adult stages (Fig. S2 C and D). Additionally, we conducted Gene Ontology (GO) enrichment analysis to identify the biological processes that DE genes were involved in for these different stage and tissue comparisons (carnivorous versus herbivorous). We found that larval OJA genes were associated with jaw development related terms such as ‘roof of mouth development’ (*sox9b*) and ‘collagen catabolic process’ (*mmp2*); and also more general functions like ‘netrin-activated signalling pathway’ involved in axon extension and cell migration, ‘regulation of lipid catabolic process’ and ‘actin filament severing’ (Fig. 4 F, for full list see: Supplementary File 3). Genes differentially expressed in the larval PJA showed enrichment for GO terms involved in functions like ‘regulation of cell shape’ (*stat3*, *amotl2b*) and ‘skeletal muscle satellite cell migration’ (*rhocb*) (Supplementary File 3). In adult OJA, genes were significantly associated with the ‘the positive regulation of canonical wnt signalling pathway’ (*bambia*, *fam53b*) as well as ‘wnt signalling pathway’ (*bambia*, *bcl9l*, *fam53b*). Interestingly, genes differentially expressed in the adult PJA showed a significant enrichment in ‘regulation of alternative mRNA splicing, via spliceosome’ (*rbfox1*) (Full list in Supplementary File 3).

Differentially expressed genes spanning both life stages

Comparing the intersection sets of differentially expressed genes between carnivorous and herbivorous species at both life stages showed an overlap of 39 genes in the OJA (25 downregulated and 13 upregulated in carnivores compared to herbivores; initial DEG sets: larval OJA: 237; adult OJA: 128) (Fig. 3 E) and 27 genes in the PJA (18 downregulated and eight upregulated in carnivores compared to herbivores; initial DEG sets: larval PJA: 159; adult PJA: 107) (Fig. 3 F) (Supplementary File 2) across the life stages.

One gene that was consistently downregulated in the two carnivorous species as well as the omnivorous species in both tissues at both stages was *kaznb*, also called *Kazrin* or periplakin-interacting protein b (Fig. 4 A and D). We validated *kazbn* expression using qPCR analyses and found it supports the RNA-seq results (Fig. 4 B and E). A more detailed inspection of the *kaznb* gene found that the differentially expressed transcript of interest is absent in the reference genome's *kaznb* annotation. This transcript is spanning an intronic region in the *O. niloticus* reference and is not linked to any other annotated exons of *kaznb*. This observation suggests that exonization of introns is a mechanism through which both novel coding variation (new exon) and novel regulatory variation (new mRNA isoform) have evolved in the radiating modern haplochromine cichlids in Lake Victoria.

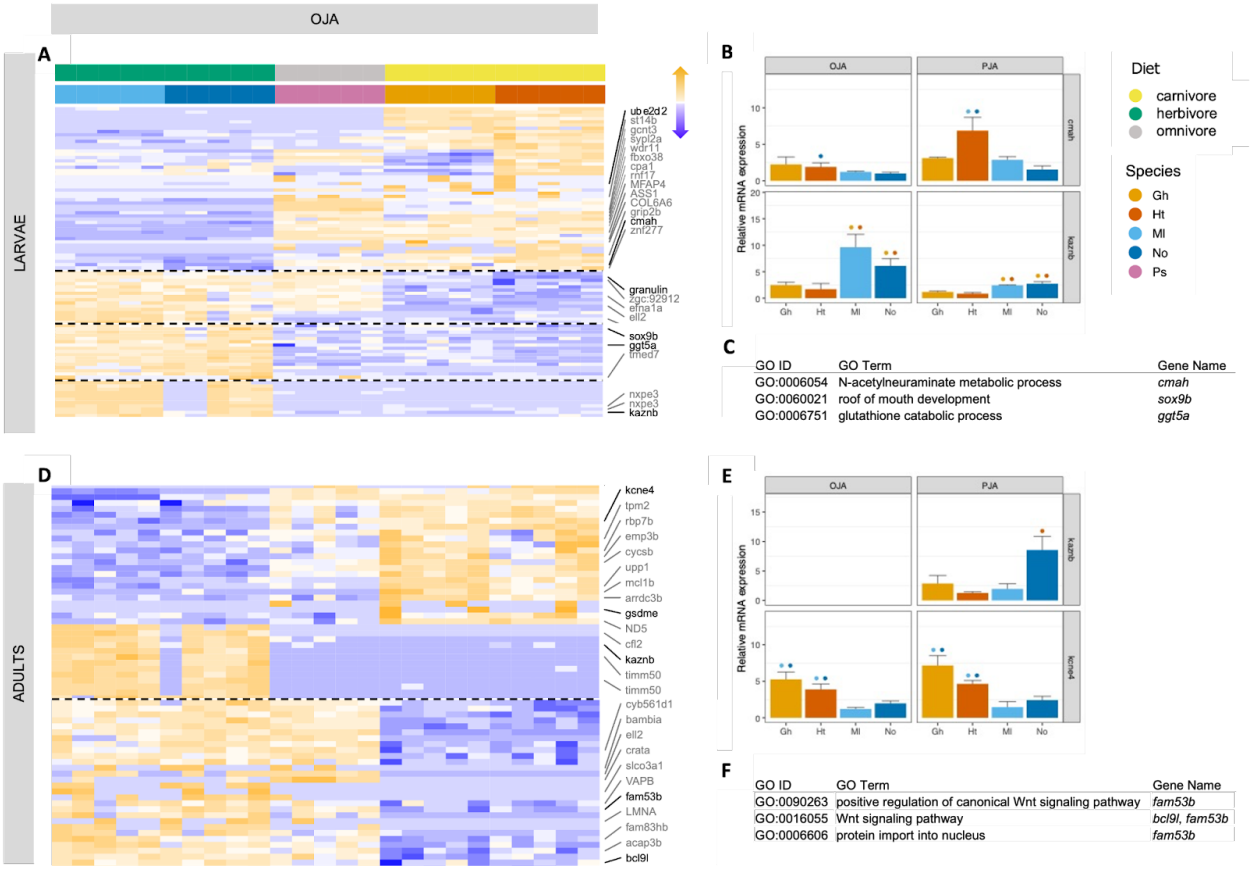


Figure 4: Genes involved in regulating herbivorous vs carnivorous jaw adaptations in Lake Victoria cichlids. Selected parts of heatmaps (dashed lines show cuts in heatmap) showing differentially expressed genes in comparisons including carnivorous versus herbivorous species for larval oral jaw apparatus (A) and adult oral jaw apparatus (D) respectively (Full heatmaps in the supplementary (Fig. S3 A - D)). Genes printed in black were either used in the qPCR validation analysis and/or have been significantly enriched for development and morphology related GO terms. Important jaw development associated GO terms are listed in (C) for larval OJA tissue and (F) for adult OJA tissue. The bar plots show qPCR validation of cmah and kaznb gene in larval (B) and kaznb and kcne4 in adult jaw tissues (E). Oral jaw apparatus (OJA), pharyngeal jaw apparatus (PJA), H. thereuterion (Ht), G. hiatus (Gh), M. lutea (Ml), N. omnicaeruleus (No) and P. sauvagei (Ps).

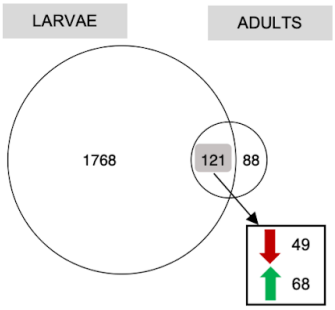
Gene expression modularity of the two jaws

Evolutionary modularity in gene regulation can contribute to morphological diversification and specialisation through the rewiring of gene regulatory networks (Wagner et al. 2007). We investigated gene expression differences between the oral and pharyngeal jaw apparatuses in herbivorous versus carnivorous species, to see whether the underlying gene regulatory architecture behind these functionally independent jaw modules differs between trophic niches. At stage 26 larvae, 1,889 (1768+121) genes were found to be differentially expressed between oral and

pharyngeal jaw apparatus in all pairwise species comparisons, while at the adult stage only 209 (121+88) genes were consistently differentially expressed between the two jaws (Fig. 5 A; Fig. S3 A and B). One hundred and twenty-one of these genes were shared between both developmental stages, of which 68 were upregulated and 49 downregulated in the OJA vs PJA across all species and stages (Fig. 5 A). Four of these genes showed different directions of expression between the larval and the adult stage. Three of those four genes *EPHB3*, *SLC9A3* and *tbx3* were upregulated in larval PJA and downregulated in adult PJA. One gene, with the opposite expression pattern was unannotated. Several genes of the *Hox* family (*hoxa4a*, *HOXA4*, *hoxa5a*, *hoxb3a*, *hoxc5a*, *hoxc4a*) show a decreased expression in the OJA at both stages.

GO enrichment of the 121 overlapping differentially expressed genes between the OJA and the PJA of all carnivores and herbivores for both life stages showed significant enrichment for genes involved in 'cranial skeletal system development', regulation of muscle contraction', 'multicellular organism development' and 'regulation of transcription, DNA-templated' (Fig. 5 B). Genes that were associated with the GO term 'cranial skeletal system development' include the genes *ALX3* aristaless-like homeobox 3, *prrx1a*, paired related homeobox 1a, *dlx4b*, distal-less homeobox 4b and *nkx3-2*, NK3 homeobox 2 (Fig. 5 B).

A



B

GO ID	GO Term	Gene Name
GO:0001501	skeletal system development	ALX3, dlx4b, foxc1a, bglap, nkx3-2
GO:1904888	cranial skeletal system development	ALX3, prrx1a, dlx4b, nkx3-2
GO:0007275	multicellular organism development	ALX3, hoxa4a, HOXA4, hoxa5a, alx4a, prrx1a, hoxd4a, LAMA3, sfrp2, igfbp2a, angpt1, Mb, dlx4b, foxc1a, desma, bglap, nkx3-2

Figure 5: The Venn diagram shows the overlap in larval and adult differentially expressed genes in the oral versus the pharyngeal jaw apparatus and their direction of expression (upregulated genes are indicated by the upward facing arrow in green, downregulated genes by the downward facing arrow in red; direction indicated for OJA vs PJA) (A). Selected GO terms are listed in (B).

Validation of expression patterns using qPCR

To validate our findings concerning differentially expressed genes between carnivores and herbivores we conducted qPCR analysis for five candidate genes for trophic divergence at the stage 26 larvae and four candidate genes for trophic divergence at the adult stage, which were found to be differentially expressed in both jaw apparatuses in our DGE analysis. For the larval stage these included *cmah*, *ggt5a*, *granulin*, *kaznb* and *mpc1* and for the adult stage *kaznb*, *kcne4*, *mcl1b*, *slc5a8*. All of these differentially expressed genes showed the same direction of expression between the carnivores and the herbivores in our DGE analysis. While not all of the qPCR differences were significant, they followed similar trends as our DGE analysis results (Fig. S4). *cmah* was consistently upregulated in the DGE analysis in both tissues for all carnivorous species at the larval stage (Fig. 4 A; Fig. S4 A), while the qPCR analysis for *cmah* in both larval tissues showed a significant difference in expression only between *N. omnicaeruleus* and *H. thereutherion* in the OJA and both herbivores compared to *H. thereuterion* in the PJA (Fig. 4 B). The differential expression analysis of *mpc1* showed an upregulation in larval herbivores in the PJA, which can be seen in *N. omnicaeruleus* in the qPCR analysis, but the difference is not significant (Fig. S4 A). The gene expression of *kaznb* was upregulated in herbivores versus carnivores in all tissues and all stages in the DGE analysis. The qPCR analysis confirmed this pattern in both larval tissues, but at the adult stage, relative mRNA expression in the OJA of *kaznb* could not be detected, while in the PJA, a significant difference was detected only between *H. thereuterion* and *N. omnicaeruleus* (Fig. 4 E). The gene *slc5a8* did also not show significant differences between carnivorous and herbivorous species in relative mRNA expression in the adult OJA, despite it being upregulated in herbivorous species in the differential gene expression analysis (Fig. S4 B).

Differential Alternative Splicing underlying adaptation

To investigate the role of alternative splicing in trophic adaptation in Lake Victoria herbivorous and carnivorous species we conducted pairwise species comparisons to detect differentially used isoforms. The numbers of differentially used isoforms ranged

330 from 671 to 980 across all comparisons and stages (Table S3). Further investigations
331 revealed quite small numbers of significantly alternatively used isoforms between the
332 two trophic niches at each stage within the OJA and PJA. In total we found 56 isoforms
333 to be differentially used between carnivorous and herbivorous species in the OJA at the
334 larval stage and 43 isoforms in the PJA at the larval stage. At the adult stage we found
335 75 isoforms in the OJA to be differentially used and 78 isoforms in the PJA at the adult
336 stage (Fig. S5 A, B, C, D). The intersection sets of differentially used isoforms between
337 the stages per tissue reveals even less overlap with 20 isoforms between the OJA of
338 larvae and adults and seven between the PJA (Fig. S5, E and F).

339 To visualise differential isoform usage, we chose the median PSI (percentage or
340 proportion spliced in) values, describing the relative abundance of each isoform, for
341 differentially spliced transcripts between our carnivorous and herbivorous comparisons
342 (Fig. S6; Supplementary File 4). Two isoforms of *fgfr2* (fibroblast growth factor receptor
343 2) were differentially used in the OJA (Fig. 6 A & D) at both stages and the PJA of
344 adults (Fig. S6) but showed a trophic signal only in the larval OJA tissue, with only
345 *fgfr2.1* being expressed in the herbivores while both isoforms were used in the
346 carnivores. In the adult OJA a gene called *frzb* (frizzled related protein) showed a clear
347 distinction between the carnivorous and herbivorous species, whereby in the herbivores
348 only one isoform is used (*frzb.1*), while in the adult carnivorous and the omnivorous OJA
349 tissues the other isoform (*frzb.2*) shows the higher median PSI value (Fig. 6 E). *frzb*
350 also is significantly differentially used in our comparisons of larval OJA tissue and
351 showed up in the GO enrichment, however the pattern is more species-specific, with
352 only *frzb.1* being abundant in larval Gh (carnivorous) and Ps (omnivorous) OJA tissue.
353 The PJA of larvae showed the most distinct isoforms to be differentially used compared
354 to the other three tissues (Fig. S6 B). Another gene that showed very similar patterns in
355 the OJA tissues of both stages and the adult PJA is *col6a1*, collagen type VI alpha 1
356 chain, associated with maintenance of tissue integrity. The isoforms are more equally
357 used in the carnivorous species, while in the herbivores *col6a1.1* shows a higher rate of
358 usage than *col6a1.2* (Fig. 6 C & F).

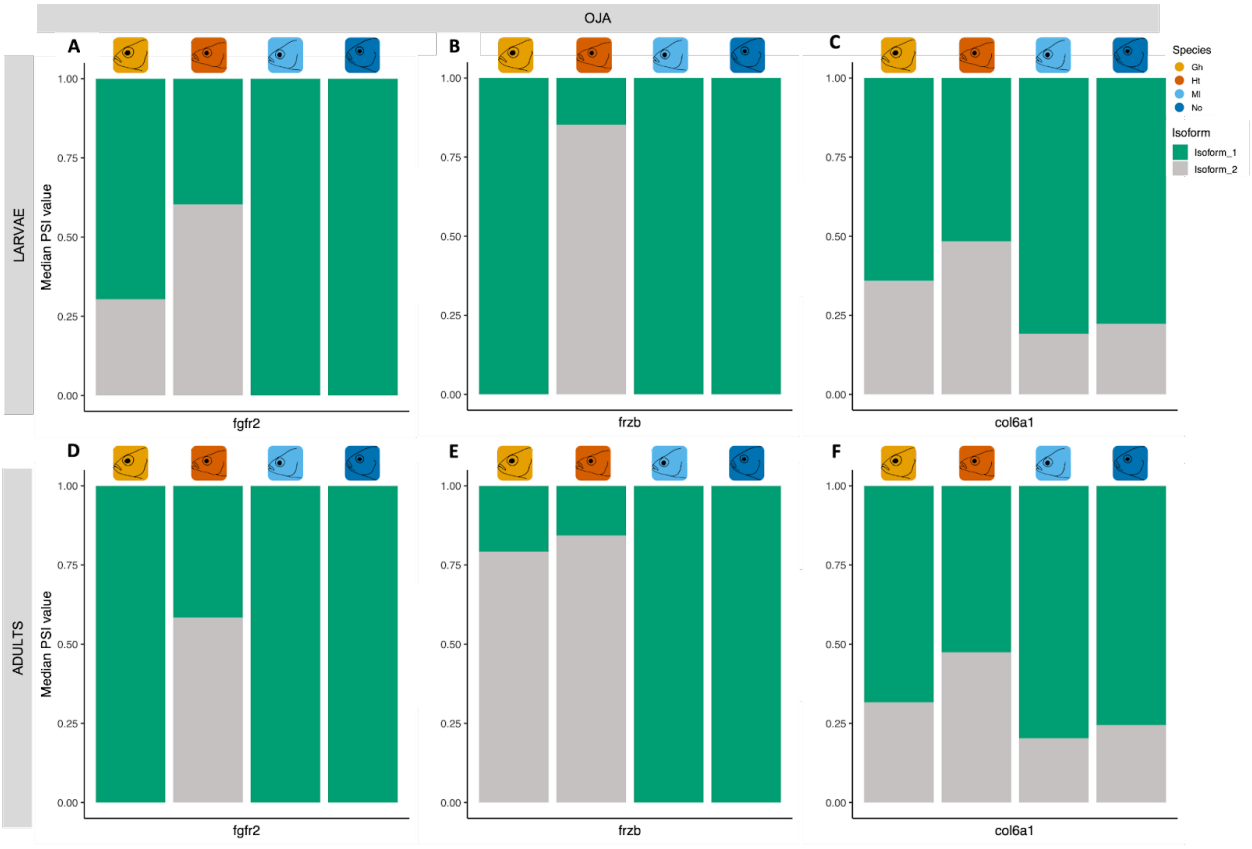


Figure 6: Median PSI values for isoforms, which were differentially used in carnivorous versus herbivorous comparisons in larval (A, B, C) or adult (D, E, F) OJA and PJA. Depicted are three selected genes (fgfr2, frzb and col6a1) and their respective median PSI values in the respective tissue. Oral jaw apparatus (OJA), H. thereuterion (Ht), G. hiatus (Gh), M. lutea (Ml), N. omnicaeruleus (No).

GO enrichment analysis of genes that were alternatively spliced showed *frzb* and *fgfr2* to be significantly enriched in the OJA at both stages, as well as the PJA of the adult stage (Supplementary File 5). *frzb*, was associated with 'chondrocyte proliferation' and *fgfr2* with 'fibroblast growth factor receptor signalling pathway' in larval and 'chondrocyte proliferation', 'peptidyl-tyrosine phosphorylation' and 'peptidyl-tyrosine modification' in the adult OJA as well as 'positive regulation of cell proliferation' and 'transmembrane receptor protein tyrosine' in adult PJA GO enrichment analysis.

Contrasting differential gene expression and alternative splicing

To investigate if genes that were differentially expressed were also alternatively spliced we looked at the intersection set between the two analyses. Interestingly, there was little to no overlap between differentially expressed and alternatively spliced genes between

herbivores and carnivores. Only one transcript was both, differentially expressed as well as differentially spliced in the OJA of larvae, but was not annotated. In larval OJA different transcripts associated with three genes (*col6a1*, *efna1a* and *si:ch211-219a4.3*) were differentially spliced and differentially expressed. This suggests that gene expression and alternative splicing perform non-redundant functions.

Discussion

Variation in gene regulation is associated with phenotypic diversity and adaptation (King and Wilson 1975) and it is more likely to be important during short evolutionary timescales because new beneficial protein-coding and structural mutations take more time to evolve and establish. While the role of gene regulation has been studied in the context of adaptation and speciation (Mack and Nachman 2017, Singh et al. 2017, El Taher et al. 2020, Pavey et al. 2010), its developmental and ontogenetic dynamics are not well understood. The extremely young and ecologically diverse species flock of haplochromine cichlids from Lake Victoria in East Africa represents a highly suitable model to test the relative importance of different levels of gene regulation during development across different species. Here we compared the gene regulatory landscape of Lake Victoria cichlid species adapted to divergent trophic niches at two post-embryonic life stages and found evidence for substantial transcriptional rewiring and modularity during development between species adapted to divergent trophic niches.

Developmental transcriptional dynamics of cichlid jaws

Studying natural populations that have undergone adaptive diversification can provide insights into how organisms adapt to changing environments or new ecological opportunities. It can help solve the evolutionary puzzle as to why some lineages diversify rapidly, while others do not, and which genetic and molecular mechanisms underlie these processes. We analysed the transcriptional landscape of oral and pharyngeal jaws in Lake Victoria cichlids and found a strong signal of gene and isoform expression conservation at the tissue level in larvae (Fig. 1 E & F, Fig. 2). Unlike the

403 pharyngeal jaws, which are used for processing food, oral jaw samples are clustered by
404 trophic niche specialisation at the larval stage. Oral jaws are used to capture food and
405 morphological differences in species-specific adult morphologies already arise during
406 early juvenile development (from stage 26 onwards) (Fujimura and Okada 2008). This
407 suggests that the gene regulatory pathways that are involved in trophic differentiation of
408 oral jaws are active early in development, in preparation for when the larvae leave their
409 mouthbrooding mother's buccal cavity to start feeding independently. In contrast to the
410 larvae, jaw gene and isoform expression in adults was species-specific (Fig. 1 G & H,
411 Fig. 2). So oral and pharyngeal jaws of one species were more similar to each other
412 than to the same tissue in a closely related species. This is different to what has been
413 observed in adult mammals where gene expression is conserved in deep evolutionary
414 time ~100 Ma (Merkin et al. 2012). It suggests that gene expression is evolving rapidly
415 in cichlid jaws, and this may have allowed this lineage to rapidly evolve versatile trophic
416 adaptations. Overall our results suggest that gene regulation was fine-tuned during
417 development in species adapted to different trophic niches from being tissue specific in
418 larvae and species-specific in adults.

419 **Cichlid oral and pharyngeal jaw expression decoupling versus conservation**

420 It has been proposed that evolutionary decoupling (i.e. modularity) of jaws played an
421 important role in cichlid diversification (Liem 1973). However, this hypothesis has mostly
422 been tested in form and function (Liem 1973; Hulsey et al. 2006; Conith and Albertson
423 2021), or gene regulation at one developmental stage (Singh et al 2021) and little is
424 known about the role of development and post-larval ontogeny underlying cichlid jaw
425 modularity. As pointed out by Conith and Albertson (2021) it is important to study
426 integration/modularity at different taxonomic and developmental levels. Our data found
427 that two jaw apparatuses are developmentally modular, in that the morphological
428 trajectories according to the trophic niche are set early in life and later the transcriptional
429 maintenance of the tissues, including response to environmental influences via gene
430 expression, becomes more species-specific (Fig. 1 F & H). The two jaw apparatuses
431 become more integrated (i.e. clustered by similarity) in gene expression towards

adulthood, pointing to a shift from modular to more integrated along the life history in Lake Victoria cichlids. Hence, ontogenetic fine-tuning of gene expression is important for building species-specific jaws in cichlids, at least at an early stage of adaptive radiation.

Developmentally changes in gene regulation were also reflected in the number of differentially expressed genes between OJA and PJA across the stages (Fig. S3 A & B) and in the ratio of genes shared across species (Fig. 5). A subset of genes was differentially expressed between the two jaws at both stages in all species (a total of 121 genes - 6,8% of the shared genes for larvae but 63.7% for adults) and showed the same direction of expression (except four genes). This points to a conserved function of these 121 genes at both life stages. Several *Hox* genes were part of this conserved gene set as *Hox* genes play an important role in the anteroposterior patterning of organisms and are known to be not present in the first pharyngeal arch, the mandibular arch (MA), forming the jaws in gnathostomes (Kuratani 2004; Ahi 2016). Recently it has also been shown that genes of the *Hox* family are not only expressed during development but also in adult tissues, contributing to bone regeneration (Rux and Wellik 2017; Bradaschia-Correa et al. 2019). Members of the ALX transcription factor family are major players in skeletogenesis in vertebrates (Khor and Ettensohn 2020). While the exact role of *ALX* genes during development is not completely understood, they are known for their association with cranial disorders (frontonasal dysplasia; FND). We found that alx3, which is known to be involved in cranial skeletal system development, was upregulated in oral jaws of LV cichlid fishes. In zebrafish (*Danio rerio*), *Alx* genes are enriched in frontonasal neural crest cells (NCC) at early stages of development, playing a role in shaping skeletal elements of the anterior neurocranium (Mitchell et al. 2021) . While *Alx3* has been repeatedly lost in chicken (*Gallus gallus*), a frog (*Xenopus tropicalis*) and lizard (*Anolis carolinensis*) species, its role might be compensated for by Alx1 and/or Alx4 (McGonnell et al. 2011). An alx1 haplotype has been associated with beak shape diversification across the adaptive radiation of Darwin's finches (Lamichhaney et al. 2015). Beak shape in finches has evolved in response to selection

462 pressures to adapt to different food sources. Our findings suggest the ALX transcription
463 factors may be key players in trophic diversification during adaptive radiation across
464 vertebrates.

465 **Gene expression divergence in herbivores vs carnivores**

466 Trophic diversification is a hallmark of adaptive radiation. While the transition from
467 carnivory to herbivory seems limited to only some fish genera, including cichlids and
468 carps, the repeated evolution of herbivory is even rarer but can be observed in modern
469 haplochromines (He et al. 2015; McGee 2020; Singh et al. 2022). Unravelling the
470 underpinnings of the evolution of herbivory and carnivory is key to understanding how
471 trophic diversity shaped cichlid radiations. Singh et al. (2022) found that in Tropheini
472 from Lake Tanganyika, the repeated trophic transitions between herbivory and carnivory
473 are probably based on repeated cases of separation and secondary contact, but the
474 molecular driver of these transitions may be regulatory variation rather than coding
475 mutations. As regulatory changes can evolve more rapidly than coding mutations,
476 investigating the role of gene regulation is especially interesting in very young adaptive
477 radiations.

478

479 To understand the role of gene expression in trophic adaptation we compared
480 expression patterns in pairwise species comparisons between herbivorous and
481 carnivorous species. A small number of shared differentially expressed genes across all
482 species pairs indicates highly species-specific gene regulatory programs in Lake
483 Victoria cichlids. The majority of genes (79% - 96%) that were differentially expressed
484 between all carnivores vs herbivores pairs has the same direction of expression,
485 suggesting a role of these genes in repeatedly contributing to carnivorous and
486 herbivorous trophic adaptations. Several of these genes were associated with Wnt
487 signalling, such as the *cmah* gene that was higher expressed in carnivores than
488 herbivores at the larval stage (Fig. 4 A & B). *Cmah* encodes an enzyme that regulates
489 Wnt signalling (Nystedt et al. 2009). The Wnt/β-catenin signalling pathway plays a
490 crucial role in skeletogenesis of trophic structures, especially in remodelling and growth

491 of fish jaws (reviewed in Ahi 2016). Higher expression of Wnt in Lake Malawi cichlids,
492 was also correlated with a more steeply descending profile and proposed to lead to
493 early variation and increased ossification in craniofacial development (Parsons et al.
494 2014). Herbivory vs carnivory genes in OJA at the larval stage were enriched for GO
495 terms like 'roof of mouth development' (Fig. 4 C) that was associated with genes *sox9b*,
496 which is downregulated in the carnivorous species. *Sox9b* is a transcription factor
497 involved in chondrogenesis and has been shown to be important for craniofacial
498 development (Yan et al. 2005). The repression of *sox9b* expression through activation
499 of the Ahr pathway resulted in the development of a shortened jaw (Xiong et al. 2008).
500 The differential regulation of the Ahr pathway has been shown to play a key role in the
501 trophic divergence of the OJA in Arctic charr (Ahi et al. 2015). In adults, *slc5a8* was
502 upregulated in the jaws of herbivores. The role of *slc5a8* in skeletal development and
503 morphogenesis is unclear, however, it has been shown to be a regulator of Wnt/β
504 -catenin signalling in mammals (Hu et al. 2016). In the OJA of adults, significant GO
505 enrichment terms included 'wnt signalling pathway' and 'positive regulation of canonical
506 Wnt signalling' (Fig. 4 F) associated with the genes *fam53b* and *bcl9l*. *Fam53b* plays a
507 pivotal role in regulating the Wnt pathway (Kizil et al. 2014); *bcl9l* has been linked to
508 tooth development in mice (Cantù et al. 2017) and craniofacial malformations in
509 zebrafish (Cantù et al. 2018). Both these genes showed a lower expression in
510 carnivores versus the herbivores.

511

512 Besides genes related to the canonical Wnt/β-catenin signalling pathway we found *ggt5*
513 to be downregulated in the jaw tissues of larval herbivores. *Ggt5* is a direct downstream
514 target of *runx2*, a major osteogenic transcription factor (Tarkkonen et al. 2017). It is
515 likely that increased expression of *ggt5* in skeletal tissue enhances bone resorption by
516 induction of osteoclastogenesis which has also been observed in another member of
517 the same gene family in mice (Hiramatsu et al. 2007). Thus, the lower expression of
518 *ggt5a* in both jaws of herbivorous species may indicate differences in bone resorption
519 level between the trophic niches. Interestingly, another gene with reduced expression in
520 both jaws of herbivores, *granulin*, is also known for its key role in the stimulation of

521 osteoclastogenesis through activation of RANK signalling (Oh et al. 2015). Taken
522 together expression results for *ggt5a* and *granulin* may imply a distinct bone resorption
523 process between carnivores and herbivores at the larval stage.

524

525 In adults, the most consistent expression differences in both jaws (validated by RNAseq
526 and qPCR) between the trophic niches were observed for *kcne4* showing reduced
527 expression in herbivores. The role of *kcne4* in skeletal development has remained
528 unclear, however, it is known that *kcne4* directly interacts with calmodulin (CaM)
529 (Ciampa et al. 2011), the ubiquitous Ca2+-transducing protein with an important role in
530 jaw skeletogenesis (reviewed by Ahi 2016). The signal mediated by CaM has been
531 already demonstrated as a key molecular mechanism contributing to variation in jaw
532 structures among closely related species of birds and fish (Abzhanov et al. 2006;
533 Parsons et al. 2009; Gunter et al. 2014). Interestingly, *kcne4* is identified as the only ion
534 channel gene which showed an upregulated expression after mechanical loading in rats
535 (Mantila Roosa et al. 2011) which is accompanied with increased bone formation. This
536 could imply an increased bone formation in herbivores as has also been suggested by
537 Albertson et al. (2012) when comparing the maxilla of a cichlid specialised in scraping
538 tough, filamentous algae to a closely related more pelagic planktivorous species. The
539 maxilla of the herbivore species is revealed to contain more bone and to be more
540 resistant to bending, compared to the planktivorous species (Albertson et al. 2012).

541

542 **Alternative splicing contributed to trophic divergence of jaws**

543 Similar to differentially expressed genes, alternatively spliced genes showed
544 stage-specific and tissue-specific patterns. Some genes were alternatively spliced in
545 more than one tissue and stage. One of those genes that was significantly enriched in
546 the GO analysis with the biological process term 'chondrocyte proliferation' (larval OJA
547 and adult OJA) was *frzb*, frizzled related protein. While the two isoforms of this gene
548 showed a species-specific usage pattern in the larval OJA, a trophic niche specific
549 pattern in usage in the adult OJA is apparent (Fig. 6 B & E). Kamel et al. (2013) showed

550 that *frbz* plays an important role in craniofacial development and palate morphogenesis,
551 with a knockdown of *frzb* in zebrafish causing the complete loss of the lower jaw. *frzb*
552 also enhances the diffusion of Wnt by blocking short-range effects and increasing
553 long-range gene responses (Rochard et al. 2016), making it a modulator of Wnt
554 signaling and playing a role in the convergent-extension of the jaw skeleton, meaning
555 that it might be an underlying molecular reason for the protrusion of the jaw in
556 carnivores. A gene with different isoform usage between the OJA of larval herbivores
557 and carnivores was *fgfr2*, fibroblast growth factor receptor 2 (Fig. 6 A & D). Mutations in
558 *fgfr2* have been associated with Crouzon syndrome, causing craniosynostosis (the
559 premature fusion of sutures) and is also linked, among other features, to a short
560 hypoplastic maxilla and mandibular prognathism (Reardon et al. 1994; Glaser et al.
561 2000), potentially linking differences in isoform usage to a steeper jaw profile in
562 herbivores. In cichlids *fgfr2* has also been found to be involved in tooth development
563 (Hulsey et al. 2016). Overall these results suggest that the alternative usage of spliced
564 isoforms played a role in the rapid evolution of jaws adapted to different food sources in
565 Lake Victoria cichlids. Alternative splicing has been shown to evolve faster than gene
566 expression in deeply divergent vertebrates (Barbosa-Morais et al. 2012). Since the long
567 evolutionary divergence time between those species is not comparable to the very
568 young Lake Victoria cichlids studied here, alternative splicing seems to already play an
569 important role in formation of adaptive morphologies at very early stages of evolutionary
570 divergence.

571 **Exonization generated novel variation for the evolution of herbivory**

572 Exonization, the process of non-coding intronic regions becoming protein-coding exons,
573 is a mechanism of genome evolution that can generate new gene isoforms (Sorek
574 2007). Although most exonization events are believed to be molecular noise, it provides
575 an efficient way for generating new evolutionary solutions from the original genome. As
576 long as the original isoform maintains functionality, the new isoform can avoid selection
577 and either evolve a novel function or be purged from the population (Schmitz and
578 Brosius 2011). Because exonization can rapidly evolve from standing variation, it has
579 the potential to be an important generator of adaptive isoforms during diversification.

580 When we compared the jaw transcriptomes of herbivorous and carnivorous cichlids in
581 our study, we found that only a small fraction of genes to be differentially expressed
582 between all carnivores and herbivores across both stages within each jaw and just a
583 few of those were annotated (Supplementary File 2). One of these was a transcript of
584 *kaznb*, also called Kazrin, encodes periplakin interacting protein b, and is involved in
585 several cellular functions such as cytoskeletal organisation, epidermal differentiation,
586 and cell adhesion (e.g. Sevilla et al. 2008; Cho et al. 2011). The expressed transcript
587 falls into an intronic region in the *O. niloticus* reference and was consistently expressed
588 in the same direction across all tissues and stages with lower expression in the
589 carnivorous (and the omnivorous) species and higher expression in herbivores. We
590 were able to validate the expression profile of the *kaznb* gene in the larval tissues via
591 qPCR analyses (Fig. 4 B) and to some extent in the pharyngeal jaws of the adult tissues
592 (Fig. 4 E). A study in *Xenopus laevis* larvae concentrating on the function of *kaznb*
593 showed that it plays an essential role in craniofacial development, through controlling
594 neural crest cell (NCC) establishment (Cho et al. 2011). When depleting *X. laevis*
595 embryos for *kaznb* in the developing head region with morpholino oligo injection that
596 blocked *kaznb* translation, Cho et al. (2011) detected eye and head defects, with
597 smaller ceratohyal and ceratobranchial cartilages and eyes on the treated sides,
598 suggesting an important role in craniofacial development. This seems to be an
599 interesting gene which has not been suggested so far to be relevant in shaping
600 divergent cichlid trophic morphologies, at least to our knowledge. Future studies should
601 address a possible link to size differences between herbivorous and carnivorous jaw
602 structures.

603 It has been previously shown that specialised herbivorous and carnivorous
604 haplochromine cichlids had significantly higher intron retention in their mRNA than
605 omnivorous species (Singh et al. 2017). Some retained introns may represent exonised
606 introns that are translated produce a novel protein with new domains. As exonization
607 provides a relatively rapid way to generate protein coding variation and potentially
608 evolutionary novelty, it could play a previously underappreciated role in adaptive
609 radiation.

610 **Distinct roles of gene expression and alternative splicing during adaptive**
611 **radiation**

612 A number of studies have empirically investigated the differing roles of differential gene
613 expression and alternative splicing in phenotypic adaptation and found them to play
614 non-redundant roles. For example, it was found that the overlap of differentially
615 expressed and differentially spliced genes was not greater than expected by chance in
616 *Drosophila melanogaster* adapted to extreme temperatures (Jakšić and Schlötterer
617 2016) and differentially spliced genes were involved in different processes compared to
618 differentially expressed genes in arctic charr (Jacobs and Elmer 2021). In the butterfly
619 *Bicyclus anynana* more than half of the genes that were differentially expressed were
620 also differentially spliced but none of the genes associated with seasonal plasticity were
621 differentially expressed and alternatively spliced (Steward et al. 2022). Similarly a recent
622 study on the Eda haplotype in sticklebacks found only a small overlap (six genes) of
623 gene expression and alternative splicing, but a lot of differentially spliced genes (no
624 significant enrichment) were involved in the same biological processes as the
625 differentially expressed ones (Rodríguez-Ramírez et al. 2023).

626

627 In our study, even though almost no genes that were both differentially expressed and
628 differentially spliced, we did find several GO terms involved in morphological structure
629 formation, even if not related to the exact same biological processes. The alternative
630 splicing GO enrichment showed involvement in 'chondrocyte proliferation' (L OJA, A
631 OJA and A PJA) while the differential expression GO enrichment was associated with
632 terms like 'roof of mouth development' (L OJA) and 'Wnt signalling pathway' (A OJA).
633 Even though different genes were regulated by these two gene regulatory mechanisms,
634 the same developmental pathways were being targeted by both alternative splicing and
635 differential gene expression to shape trophic morphologies. This is different to what has
636 been found in the much older Lake Tanganyika cichlids where differentially expressed
637 genes are involved in housekeeping processes while differentially spliced genes are
638 involved in trophic morphologies (Singh et al. 2017). Furthermore, the distinction
639 between which genes are regulated by alternative splicing or gene expression

640 divergence is likely affected by the genetic architecture (i.e. polygenic or simple) of the
641 adaptive phenotype and the evolutionary age of the species.

642 Conclusion

643 Little is known about how gene expression and alternative splicing evolve at very short
644 time scales as most studies published so far have focused on older lineages.
645 Additionally the developmental dynamics of these two mechanisms is not well
646 understood. Here we show that both differential expression and differential splicing are
647 important regulatory processes for driving rapid morphological change. We also find the
648 gene regulation at different life stages is distinct and future studies would benefit from
649 including multiple developmental stages in their study design. Remarkably, we find that
650 exonisation may be an important evolutionary mechanism for generating novel protein
651 variation during rapid adaptive radiation. However, more research is needed to establish
652 how common this mechanism is.

653 Material and Methods

654 Sampling

655 In this study we focus on five model species from Lake Victoria (*Gaurochromis hiatus*
656 (*Gh*), *Haplochromis thereuterion* (*Ht*), *Mbipia lutea* (*Ml*), *Neochromis omnicaeruleus*
657 (*No*) and *Paralabidochromis sauvagei* (*Ps*)). We picked those five species to try to
658 reflect the broad spectrum of trophic specialisations in Lake Victoria. *G. hiatus* feeds on
659 chironomid larvae as well as on bivalves and mollusks (Witte et al. 1982). *H.*
660 *thereuterion* mostly feeds on insects like chironomid larvae, but a substantial portion of
661 its nutrition is covered by collecting terrestrial insects from the water surface (Oijen et al.
662 1996). They both represent differentially specialised carnivores. *M. lutea* and *N.*
663 *omnicaeruleus* are both mainly herbivorous but with different modes of feeding. While
664 *M. lutea* feeds mainly on filamentous algae and also takes in some detritus , *N.*
665 *omnicaeruleus* grazes off epilithic algae and diatoms (Seehausen et al. 1998). *P.*

666 *sauvagei* is an omnivore, feeding on epilithic algae, associated insects and sponges
667 (Seehausen 1996). As an outgroup we included *Tropheops tropheops* (Tt), an
668 herbivorous Lake Malawi cichlid, which mainly feeds on algae and scratches it from
669 hard surfaces, but has also been reported to exclusively feed on plankton if abundant
670 (Konings 1990). As a second outgroup species we selected *Astatotilapia burtoni* (Ab), a
671 mostly riverine cichlid that is located in Lake Tanganyika and its surrounding waterways
672 and can be classified as an omnivore, feeding on plants, aufwuchs, insects and fish
673 remains (Muschick et al. 2012).

674 The *M. lutea* were collected from Makobe Island and the *G. hiatus* from the northern
675 Mwanza Gulf. Both were collected by Ole Seehausen. The other samples, *N.*
676 *omnicaeruleus*, *P. sauvagei* and *H. thereuterion* were obtained via aquarium trade. All
677 fish were reared in the fish facility at University of Graz under standardised aquarium
678 conditions regarding tank environment, diet and water parameters. Eggs were taken
679 from the mouth of their mother 24 hours after fertilisation, by applying slight pressure
680 onto their cheeks, and reared in a separate tank until the yolk sac was completely
681 absorbed (stage 26, (Fujimura and Okada 2007)). The larvae were euthanized with an
682 overdose of MS 222 and stored in RNAlater in a fridge for seven days and then moved
683 to a -80°C freezer. Fish raised to the adult life stage were fed with a standardised diet of
684 green spirulina flakes. When individuals started to show adult colouring and breeding
685 behavior males were sacrificed with MS 222 and dissected. Bones were stored in
686 RNAlater in a fridge for seven days and then frozen at -80°C.

687 **RNA extraction**

688 Per species and stage five biological replicates of oral and pharyngeal jaw apparatus
689 samples were taken. In the case of the larvae the area of the OJA and of the PJA were
690 sampled under a stereo microscope and submerged in a lysis buffer and 1-thioglycerol
691 according to the protocol of the Promega RNA Reliaprep kit. This kit includes filter
692 columns and a DNA digestion step. The sample in the lysis buffer was crushed with a
693 ceramic bullet in a homogenizer (FastPrep-24, MP Biomedicals, Santa Ana, CA, USA),
694 before further processing. Two individuals were pooled per sample to increase the

695 amount of RNA. For the adult species the bones were all cleaned from skin and muscle
696 tissue and stored in RNAlater. As the whole set of bones would have been too much
697 tissue input for the RNA extraction, only half of the bones were collected. The set of the
698 bones is the same for all samples, but if they were collected from the left or right side
699 was randomly decided by a simple randomization script in R (R Core Team 2020). RNA
700 for the adults was extracted using TRIzol reagent (Thermo Fisher Scientific), following
701 the protocol of the TRIzol protocol for RNA extraction with the inclusion of a DNAse step
702 at the end (invitrogen). RNA quality was tested at a TapeStation 2200 with RNA Screen
703 Tapes. It was tried to reach a RINe number of >= 7 for each sample.

704 **Library preparation and sequencing**

705 Library preparation was done with the TruSeq Stranded mRNA Sample Prep Kit using
706 24 indexing adapters and following the standard protocol. For the input RNA we tried to
707 reach 1000 ng for each sample. The quality of the cDNA was tested on a TapeStation
708 2200 (Agilent) with D1000 Screen Tapes from the Agilent RK6 ScreenTape System.
709 Sequencing was executed at the Vienna BioCenter Core Facilities on a HiSeq 2500 with
710 paired end 125 cycles per read (2 x 125 cycles). Demultiplexing was conducted by the
711 same facility.

712 **Transcriptome assembly**

713 For each of the samples we got an average of ten million paired-end reads. Sequence
714 reads are available at NCBI sequence read archive (SRA) under the accession number
715 PRJNA640176. After a quality check with Fastqc (v0.11.8) (FastQC 2015) and a
716 trimming step with Trimmomatic (v0.3.9) (Bolger et al. 2014), only reads with a phred >
717 28 and a minimum length of 70 bp were retained. For each sample a median of about 5
718 million reads were obtained. For read alignment we used RNAstar (v2.7.3.a) (Dobin et
719 al. 2013) with the *Oreochromis niloticus* genome reference from the University of
720 Maryland (Conte et al. 2017) as guide reference, as it is very well annotated and this is
721 important for accurate isoform reconstruction. After checking the mapping statistics with
722 samtools idxstats (v1.9) (Danecek et al. 2021) and merging the single files for the same

723 species and jaw type with picard (v2.21.7) (Picard toolkit 2019) we used StringTie
724 (v2.0.6) (Pertea et al. 2015) without a reference to assemble the RNA-seq alignments
725 into potential transcripts. This was done separately for the single files (per fish) and the
726 merged files (per species and jaw). The single files for each biological replicate were
727 then merged according to species, to subsequently merge all the files per species,
728 tissue and stage. These repeated merging steps were conducted to reduce the
729 probability of false positives. To estimate the accuracy of the produced annotation files
730 we compared them with gffcompare (v0.11.2) (Pertea and Pertea 2020) to our reference
731 annotation. We filtered for monoexonic transcripts not contained in the reference and a
732 class code assigned by gffcompare, indicating 'possible polymerase run-on' fragments.
733 The maximum intron length was reduced to 200000 bp, which is the maximum intron
734 length found in the *O.niloticus* reference. Based on these annotations, the expression
735 estimates were generated with StringTie allowing no multimapping. From these
736 expression estimates, count matrices were produced using the prepDE.py script that
737 comes with StringTie (v2.0.6) to extract raw count data from StringTie results. The code
738 used for the whole analysis is stored in a github repository
739 (https://github.com/annaduenser/LV_RNAseq).

740 **Differential gene expression analysis**

741 We were not able to directly compare jaws in adults and larvae as we were not able to
742 apply an identical dissection and extraction process at both life stages due to the
743 minimal size of the larvae. We counteracted this shortcoming with not directly
744 comparing the data sets, but conducting the same species-pairwise comparisons and
745 further investigating the intersection set between those. This method should reveal
746 identical patterns in gene expression and alternative splicing between trophic niches, if
747 present. When looking at the Euclidean cluster dendrogram at the larval stage, that also
748 includes our outgroup, we see a clear distinction between the LV species and the latter.
749 When including species from LM and LT, a jaw tissue specific pattern is still visible for
750 LV as well as for LM (clustering next to LV), but not for LT (Singh in preparation)
751 suggesting that this separation is not solely caused by the inclusion of surrounding
752 muscle tissue. If anything, the surrounding tissue could potentially add to the trophic

753 niche clustering in the larval oral jaw apparatus. We used DESeq2 (Love et al. 2014) in
754 (R Core Team 2020) using RStudio (RStudio Team 2019) to detect differentially
755 expressed genes running comparisons between all species and tissues as well as
756 comparisons between the tissues within a species. DESeq2 uses raw read counts and
757 estimates variance-mean dependence based on a model that utilizes the negative
758 binomial distribution (Love et al. 2014). The cutoff for differentially expressed genes was
759 chosen at a false discovery rate of ($p < 0.05$).

760 **GO term enrichment**

761 GO terms for *Oreochromis niloticus* were acquired in R (R Core Team 2020) using
762 RStudio (RStudio Team 2019) via the biomaRt package (v2.46.1)(Durinck et al. 2005;
763 Durinck et al. 2009) in R from the Ensembl database. Gene set enrichment analysis
764 was conducted with the R program topGO (v2.36.0)(Alexa and Rahnenfuhrer 2019)
765 using the method *weight* to account for GO topology and a Fisher's exact test for the
766 enrichment analysis. We used our annotation to build the gene universe, where we then
767 tested the list of genes, overlapping between carnivorous and herbivorous species in
768 gene expression or alternative splicing analysis for gene set enrichment.

769 **qPCR analysis**

770 From four replicates of RNA samples of each jaw apparatus, stage and species, we
771 used 500 ng of RNA to synthesise first strand cDNA using the High Capacity cDNA
772 Reverse Transcription kit (Applied Biosystems). The cDNA from each sample was
773 diluted 1:10 times in nuclease-free water to be used for qPCR. For the adult stage, we
774 selected five candidate target genes; *kcne4*, *kaznb*, *mcl1b*, *rnf114* and *slc5a8*, and for
775 the larval stage we also chose five genes; *ggt5a*, *kaznb*, *granulin*, *faah* and *cmah* (Fig.
776 S5). We used *rpl18* as a reference gene, which we have already validated as a stably
777 expressed gene in both cichlid jaw tissues across different trophic niches (Ahi et al.
778 2019), to normalise our expression data for qPCR. The primers were designed at
779 conserved sequence regions of the coding sequences from all species using CLC
780 Genomic Workbench, version 7.5 (CLC Bio, Aarhus, Denmark) for alignment and

781 delineation of exon boundaries using *A. burtoni* annotated genome in the Ensembl
782 database (<http://www.ensembl.org>) (Zerbino et al. 2018). Primer Express 3.0 (Applied
783 Biosystems, CA, USA) and OligoAnalyzer 3.1 (Integrated DNA Technology) were used
784 to design the primers as described in details in our previous studies (Ahi et al. 2018; Ahi
785 et al. 2019) (Supplementary data for primer info). Maxima SYBR Green/ROX qPCR
786 Master Mix (2X) (Thermo Fisher Scientific, Germany) was used to generate qPCR
787 reactions. The amplification steps were conducted in 96 well-PCR plates on ABI 7500
788 real-time PCR System (Applied Biosystems). The primer efficiency calculation, qPCR
789 program, plate set-up and a dissociation step were performed as described previously
790 (Ahi et al. 2018; Ahi et al. 2019). To normalise the expression levels, the Cq value of
791 *rpl18* was used as normalisation factor (Cq reference), and Cq of each target gene was
792 calculated accordingly (ΔCq target = Cq target - Cq reference). Relative expression
793 quantities (RQ) were calculated for the normalised values using E-ΔCq (Pfaffl 2001) and
794 then fold difference values were calculated by transformation of RQ values to
795 logarithmic values in order to conduct further statistical analysis (Bergkvist et al. 2010).
796 The statistical expression differences were identified using ANOVA statistical tests,
797 followed by Tukey's HSD post hoc tests.

798 **Alternative splicing analysis**

799 As the alternatively spliced isoforms can be a result of different types of splicing, it is not
800 as straightforward to assess but can be explored from different angles. Either from an
801 exon centric approach via differential exon usage (DEU), or with an isoform centric
802 approach, which can be subdivided by differential transcript expression (DTE) and
803 differential transcript usage (DTU). To get the most information about splicing patterns,
804 we use a combination of DEU and DTU analysis in this study. Alternative splicing
805 analysis was conducted with SUPPA2 (v2.3) (Trincado et al. 2018) to evaluate
806 differential transcript usage (DTU) between the samples. SUPPA2 takes the normalised
807 isoform counts of all replicates of a condition and calculates a relative abundance for
808 each transcript in each sample (PSI). To detect the difference in transcript usage
809 between conditions dPSI is computed (dPSI = PSI condition 2 - PSI condition 1)

(Trincado et al. 2018). By taking the dPSI comparisons of herbivore versus carnivore species and selecting the isoforms that were significantly used differently. We illustrated the difference in usage by computing the median PSI value over the five biological replicates for each species for each transcript. Visualisation of the analysis was conducted in R (R Core Team 2020) using RStudio (RStudio Team 2019).

Data availability

Sequence reads are available at NCBI sequence read archive (SRA) under the accession number PRJNA640176.

The code for the analysis is available at the following github repository (https://github.com/annaduenser/LV_RNAseq).

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