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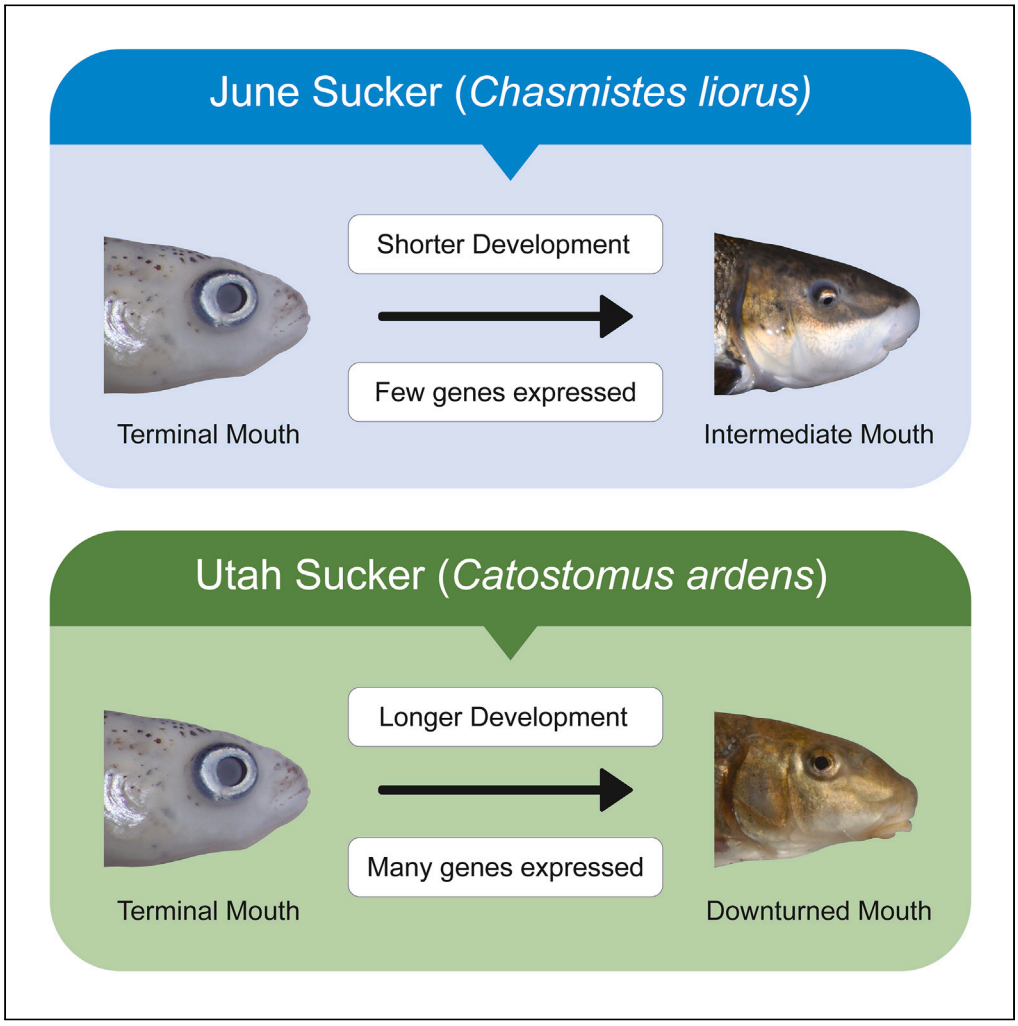

Peter C. Searle, Dennis K. Shiozawa, R. Paul Evans, Jonathon T. Hill, Arminda Suli, Michael R. Stark, Mark C. Belk

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Highlights

June and Utah sucker exhibit synchrony between gene expression and head development

Divergence in mouth morphology is consistent with paedomorphosis in June sucker

Incorporating ontogeny informs evolutionary change among closely related species

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Heterochronic shift in gene expression leads to ontogenetic morphological divergence between two closely related polyploid species

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SUMMARY

Heterochrony—alteration to the rate or timing of development—is an important mechanism of trait differentiation associated with speciation. Heterochrony may explain the morphological divergence between two polyploid species, June sucker (*Chasmistes liorus*) and Utah sucker (*Catostomus ardens*). The larvae of both species have terminal mouths; however, as adults, June sucker and Utah sucker develop subterminal and ventral mouths, respectively. We document a difference in the timing of shape development and a corresponding change in the timing of gene expression, suggesting the distinctive mouth morphology in June suckers may result from paedomorphosis. Specifically, adult June suckers exhibit an intermediate mouth morphology between the larval (terminal) and ancestral (ventral) states. Endemic and sympatric *Chasmistes*/*Catostomus* pairs in two other lakes also are morphologically divergent, but genetically similar. These species pairs could have resulted from the differential expression of genes and corresponding divergence in trait development. Paedomorphosis may lead to adaptive diversification in Catostomids.

INTRODUCTION

Understanding the evolution of biological diversity is a major goal in evolutionary developmental biology because developmental processes are tightly linked with morphological changes and corresponding evolutionary divergence.[1–3] Gene expression analyses coupled with descriptions of ontogenetic morphological change have recently gained attention as an important method for exploring evolutionary relationships. Changes in developmental gene regulation can lead to substantial morphological divergence as a consequence of altered developmental timing.[4] This process of alteration to the rate or timing of development in a species compared to its ancestor is termed heterochrony.[5,6] Slight changes to temporal (i.e., the onset or offset of expression, as well as the rate of expression) or spatial gene expression during periods of rapid ontogenetic change, can shorten or prolong development, significantly altering the appearance of a descendant from its ancestor.[6,7] Heterochrony has been identified as an important evolutionary mechanism across many taxa.[8–14] Because heterochrony is often the product of changes in gene regulation, it can result in species that are well differentiated morphologically but show only small genetic differentiation across the genome.[15] Such species are difficult to differentiate when only genetic data are used to determine levels of differentiation and corresponding phylogenetic relationships.[16] Thus, the explicit incorporation of ontogeny in both developmental morphological change and in gene expression derived from transcriptome analysis is critical for observing patterns of evolutionary change and divergence among such species.[2,3]

Heterochronic shifts in gene expression and corresponding morphological changes may explain the evolutionary divergence between June sucker (*Chasmistes liorus*) and its co-occurring sister species, the Utah sucker (*Catostomus ardens*). June sucker is a highly derived Lake sucker (genera: *Chasmistes*, *Deltistes*, and *Xyrauchen*, Pisces: Catostomidae) that is endemic to Utah Lake, Utah, USA. June sucker is adapted for planktivorous feeding and is characterized by a subterminal mouth, lower lip lobes separated by a wide central gap, thin lips with reduced papillation, and numerous, dendritic gill rakers.[17] Utah sucker is a typical benthic sucker (widely distributed in lakes and rivers of the intermountain western USA) adapted for the suction feeding and filtration of larger macroinvertebrates from muddy substrates.[18,19] Utah sucker is characterized by a ventral mouth, wide lower lip lobes with no central gap, heavy papillation on the lips, and unbranched gill rakers.[17] The same pattern of highly derived lake suckers (*Chasmistes*) co-occurring with closely related benthic suckers (*Catostomus*)

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is repeated multiple times in western North America.[17] In each case, the pattern of differentiation in mouth morphology is the same as described for the June sucker and the Utah sucker, and the mouth morphology of adults is the most distinctive trait that differentiates the co-occurring species pairs. In contrast to adults, the morphology of the head and mouth in larvae is extremely similar between lake suckers and benthic suckers. Larvae of both groups have terminal mouths, relatively large eyes and heads, and relatively small and narrow bodies.[20] Thus, the polarity of development from a similar larval shape to a differentiated adult shape is common to all sympatric lake sucker/benthic sucker pairs.

These morphological differences between co-occurring sucker species have been well documented in both extant and extinct species. Taxonomic characterizations based on the shape and position of the mouth formed the basis for the conclusion that *Chasmistes* and *Catostomus* represent ancient lineages, and that individuals that exhibited intermediate mouth morphology in Utah Lake were the result of recent introgression between June sucker and Utah sucker.[21] However, more recent work aimed at using genetic data to definitively differentiate the species has been unsuccessful at finding consistent patterns of genetic differences. Lack of genetic variation between the two putative species (June sucker and Utah sucker) in Utah Lake led Mock et al.[19] and Cole et al.[22] to propose that Utah sucker in Utah Lake recently differentiated into two forms: a planktivorous form (June sucker) and a benthivorous form (Utah sucker; See Smith et al.[17] for a discussion of an alternative hypothesis why June sucker and Utah sucker lack genetic markers). Thus, the presence of intermediates could represent a convergence toward or divergence away from the June sucker-like morphology, depending on environmental conditions. Cole et al.[22] further suggested that the sucker complex in Utah Lake could be the result of a long history of reticulated evolution (Reticulated evolution is often defined as gene transfer between species.[23] Here we define reticulated evolution specifically as described by Cole et al.[22]: "a long … history of genetically shallow but morphologically pronounced divergence and convergence, following fluctuating environmental conditions.") or could represent putative lineages that recently diverged (incipient species). However, Cole et al.[22] did not provide any explanations as to how reticulated evolution or recent divergence could occur in these suckers. The condition of little difference in genetic variation but consistent variation in adult morphology can be further resolved by reference to ontogenetic patterns of development in both gene expression and corresponding morphology.

In this study, we combine analyses of morphological change and gene expression to reveal differences in the ontogenetic developmental pattern between June sucker and Utah sucker (Utah sucker is represented by two lineages in our study because it is a widespread species). We hypothesized that the easily recognizable differences between adults resulted from differential timing, i.e., heterochrony, of development over the ontogenetic transition from larvae to juveniles. Specifically, we describe and test for synchrony between the expression of genes associated with the cranial development and development of distinctive head and mouth morphology between the two species. We used geometric morphometric analyses of head shape, as well as premaxilla shape and size to determine the critical developmental period during ontogeny when mouth position began to diverge between these species. We then used differential gene expression analyses to determine general gene expression patterns that coincided with morphological changes, as well as identify specific genes associated with these morphological changes. We document a difference in the timing of shape development and a corresponding change in the timing of gene expression between the June sucker and Utah sucker lineages, consistent with a paedomorphic heterochronic change in development in June sucker. This study provides an important example of the integration of ontogeny into the exploration of differences between closely related polyploid species.

RESULTS

Head shape undergoes a shorter developmental sequence in June sucker

We used landmark-based geometric morphometrics[24] to determine how head shape changed during the larval to juvenile transition in one June sucker and two Utah sucker lineages (Utah sucker from Utah Lake, UT, USA and Utah sucker from an introduced population of fish in Strawberry Reservoir, UT, USA; see STAR Methods for additional discussion). We found that head shape differed significantly among developmental weeks and among the three lineages, and that week and lineage exhibited a significant interaction (see two-way and three-way interactions with index variable; Table 1). Relative warp 1, which accounted for 64.59% of the variation in head shape, captured the shape change that occurred during larval development, including a shift from a larval stage with smaller heads and larger eyes to a juvenile stage with larger heads and smaller eyes, as well as lineage-specific variation including an elongation of the snout and more ventral positioning of the mouth in the Utah sucker lineages compared to June sucker (Figure 1A). Initially, on relative warp 1 each lineage exhibited common shape variation, including rapid shape change from weeks 0–6, followed by a decrease in the magnitude of shape change from weeks 6–12. After week 12, lineage-specific variation became more apparent, with Utah sucker lineages continuing to move further to the right along relative warp 1 compared to June sucker (Figure 1B). There was no clear separation among developmental weeks or lineages along relative warp 2, which accounted for an additional 9.57% of the variation in head shape (Figure 1B).

Week 14 represents the endpoint of the developmental sequence we explored in this study. At this endpoint, we found that the two Utah Sucker lineages exhibited similar shapes on all relative warps except relative warp 7. In contrast, shape differences between the June sucker and one or both Utah sucker lineages occurred on relative warps 1, 2, 4, 5, 6, 7, and 8 (Figure 1C). To visualize total shape differences among all three lineages, we compared shape differences at week 14 across all relative warps. Consistent with patterns in Figure 1C, shape was similar between the Utah sucker lineages, but was different between June sucker and each Utah sucker lineage (Figure 1D). Both Utah sucker lineages exhibited an elongated snout, down-turned mouth, and smaller eye compared to the June sucker lineage.

Table 1. Multivariate analysis of variance effects for head shape and premaxilla shape of June sucker and two lineages of Utah sucker

Source	Degrees of Freedom	F-Value	p Value
Head Shape			
Lineages (Ln)	2, 1809	2.4	0.0910
Week	14, 1809	30.23	<0.0001
Index	8, 1471	7.15	<0.0001
Ln*Index	16, 1982	16.27	<0.0001
Week*Index	112, 3079	13.01	<0.0001
Ln*Week*Index	243, 3198	1.72	<0.0001
Premaxilla Shape			
Lineages (Ln)	2, 558	2.69	0.0687
Week	5, 558	4.38	0.0006
Index	5, 510	0.29	0.9178
Ln*Index	10, 653	6.37	<0.0001
Week*Index	25, 831	7.53	<0.0001
Ln*Week*Index	60, 930	1.68	0.0012

Premaxilla shape is different between lineages after 14 weeks of development

The premaxilla is a diagnostic feature used for differentiating extant and extinct species in Catostomidae.[21] Specifically, the premaxilla in June sucker has a moderately obtuse angle between the premaxillary limbs, and the vertical and lateral limbs are relatively equal; whereas the premaxilla in Utah sucker has a right angle between the premaxillary limbs, and the vertical limb is longer than the lateral limb (Figure S1; also, see[21] Figure 10 for examples from adult suckers). We used landmark-based geometric morphometrics[24] of premaxilla shape, as well as measurements of premaxilla size to determine how shape and size changed during the larval to juvenile transition among these three lineages.

We found that premaxilla shape differed significantly among developmental weeks and among the three lineages and that both week and lineage exhibited a significant interaction (see two-way and three-way interactions with index variable; Table 1). Similar to head shape, relative warp 1 accounted for more than half (54.79%) of the shape variation in our specimens. Relative warp 1 captured the shape change that occurred during larval development, including a shift from a larval stage, with a relatively longer lateral premaxillary limb in comparison to the vertical limb, to a juvenile stage with a relative increase in the length of the vertical limb compared to the lateral limb (Figure 2A). On relative warp 1, each lineage initially underwent rapid shape changes from weeks 3–6, followed by a decrease in the magnitude of shape change from weeks 6–14 (Figure 2B). Relative warp 2 (22.57%) captured lineage-specific variation, including a slightly less obtuse angle between the premaxillary limbs in the Utah sucker lineages compared to the June sucker (Figure 2A). Specifically, Utah sucker lineages had slightly more positive values along relative warp 2 compared to June sucker at week 14 (Figure 2B).

We further explored premaxilla shape differences between lineages at the developmental endpoint represented by week 14. Similar to the analysis of head shape, we found that the shape of the premaxilla did not differ between the two Utah sucker lineages except on relative warp 6; whereas, the June sucker lineage differed from one or both Utah sucker lineages on relative warps 2, 3, 4, and 6 (Figure 2C). To visualize total shape differences among all three lineages, we compared shape differences at week 14 across all relative warps. Consistent with the analysis of head shape, we found that shape was different between the June sucker and both Utah sucker lineages (Figure 2D) at week 14. June suckers show a more obtuse angle between the two premaxillary limbs compared to both of the Utah sucker lineages. However, the comparison between the two Utah sucker lineages revealed that the shape of the premaxilla of the Utah Lake Utah sucker was intermediate between the June sucker and the Strawberry Reservoir Utah sucker.

Premaxilla size undergoes a shorter developmental sequence in the June sucker

We used linear measurements of the premaxilla to determine how the size of the premaxilla changed during the larval to juvenile transition. A plot of premaxilla size (defined as the sum of the length of the vertical and lateral limbs) revealed that growth rate and corresponding size were similar among all three lineages at 4, 6, 8, and 10 weeks. Growth of the premaxilla accelerated in the two Utah sucker lineages but slowed in the June sucker lineage from weeks 10–14 (Figure 3), consistent with changes in head shape among the three lineages. Unlike the head shape, but consistent with the shape of the premaxilla, the final size of the premaxilla in Utah Lake Utah sucker was intermediate between June sucker and Strawberry Reservoir Utah sucker.

De novo transcriptomes are comparable between lineages

We *de novo* assembled transcriptomes using PacBio sequences (560 gigabytes of sequence data with 5,691,721 reads) for each sucker lineage resulting in 36,866; 35,656; and 32,115 transcripts for June sucker, Utah Lake Utah sucker, and Strawberry Reservoir Utah sucker, respectively

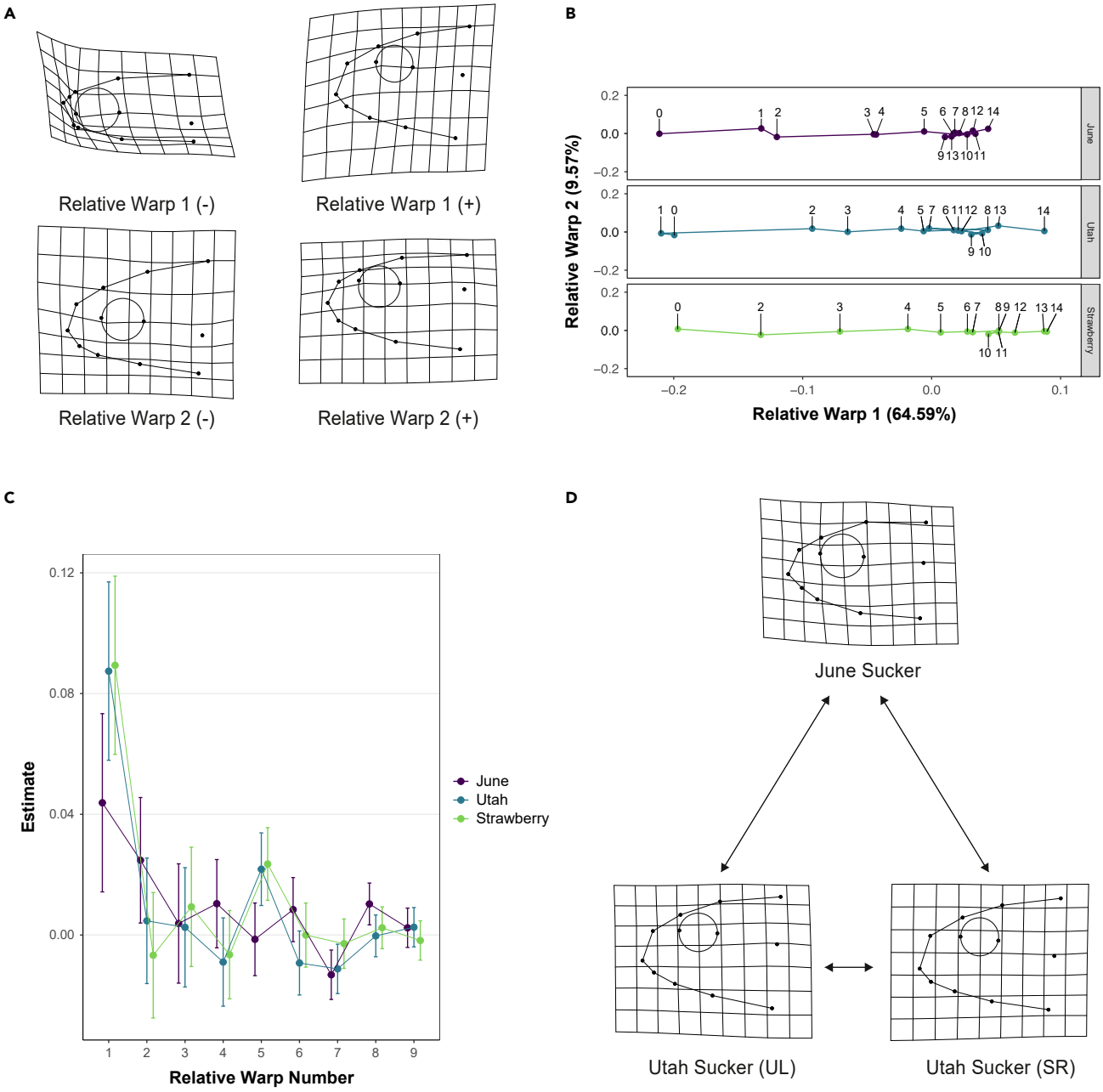

Figure 1. Head shape undergoes a shorter developmental sequence in June sucker

Results of geometric morphometric analysis of head shape.

(A) Thin plate spline deformations from positive and negative extremes of relative warps 1 and 2.

(B) Least squares means of head shape by week for June sucker, Utah Lake Utah sucker, and Strawberry Reservoir Utah sucker on relative warps 1 and 2.

(C) Least squares means (error bars represent 95% confidence intervals of the mean) for each sucker lineage on the 9 relative warps from the multivariate linear mixed model of head shape variation at week 14.

(D) Thin plate spline deformations of divergence in head shape between sucker lineages at week 14. UL = Utah Lake, SR = Strawberry Reservoir.

(Table S1). We validated our *de novo* transcriptomes by running BUSCO (v5.1.2)[25] and performing a functional annotation. BUSCO revealed that the transcriptomes contained ~77.5% complete BUSCOs (highly conserved single-copy orthologs) for all lineages, with a high number of duplicate BUSCOs (Figure S2). The high percentage of complete, duplicated BUSCOs is consistent with the tetraploid origin of suckers. In addition, the transcriptomes had ~27.5% missing BUSCOs. This is likely because we only sequenced the head of the lineages and would expect transcripts from other tissues to be missing from the transcriptomes.

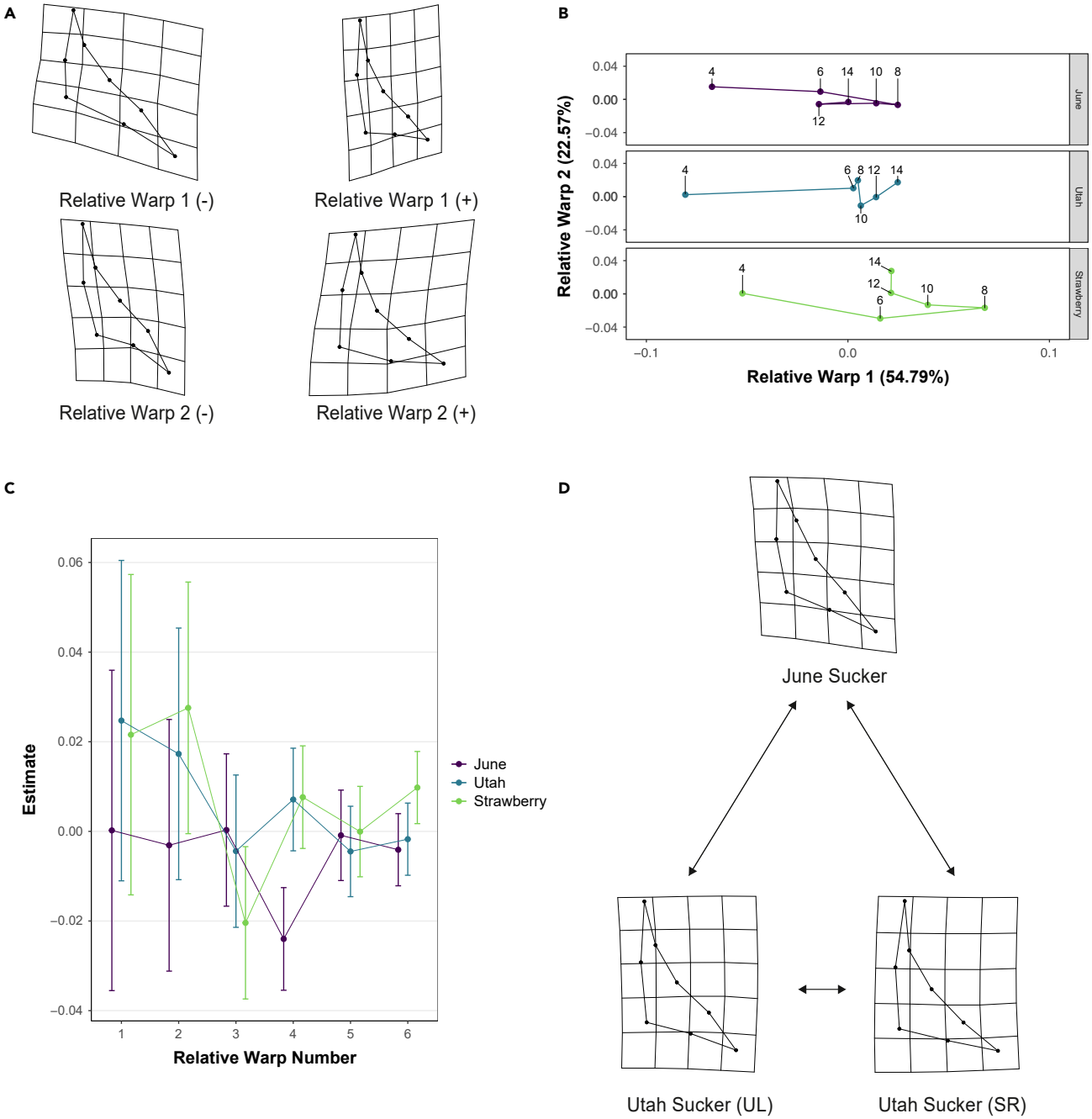

Figure 2. Premaxilla shape is different between lineages after 14 weeks of development

Results of geometric morphometric analysis of premaxilla shape.

(A) Thin plate splines deformations from positive and negative extremes of relative warps 1 and 2.

(B) Least squares means of premaxilla shape by week for June sucker, Utah Lake Utah sucker and Strawberry Reservoir Utah sucker on relative warps 1 and 2.

(C) Least squares means (error bars represent 95% confidence intervals of the mean) for each sucker lineage on the 6 relative warps from the multivariate linear mixed model of premaxilla shape variation at week 14.

(D) Thin plate spline deformations of divergence in premaxilla shape between lineages at week 14. UL = Utah Lake, SR = Strawberry Reservoir.

The functional annotation showed that the *de novo* transcriptomes were also similar across several metrics. A total of 31,538 (85.55%), 29,829 (83.66%), and 27,250 (84.85%) transcripts were annotated for June sucker, Utah Lake Utah sucker, and Strawberry Reservoir Utah sucker, respectively. Each transcriptome shared blast hits against the same top ten species, all from the order Cyrpiniformes (June sucker, 91.78%; Utah Lake Utah sucker, 90.89%; Strawberry Reservoir Utah sucker, 91.91%). The three top gene ontology categories (Level 2) for all lineages

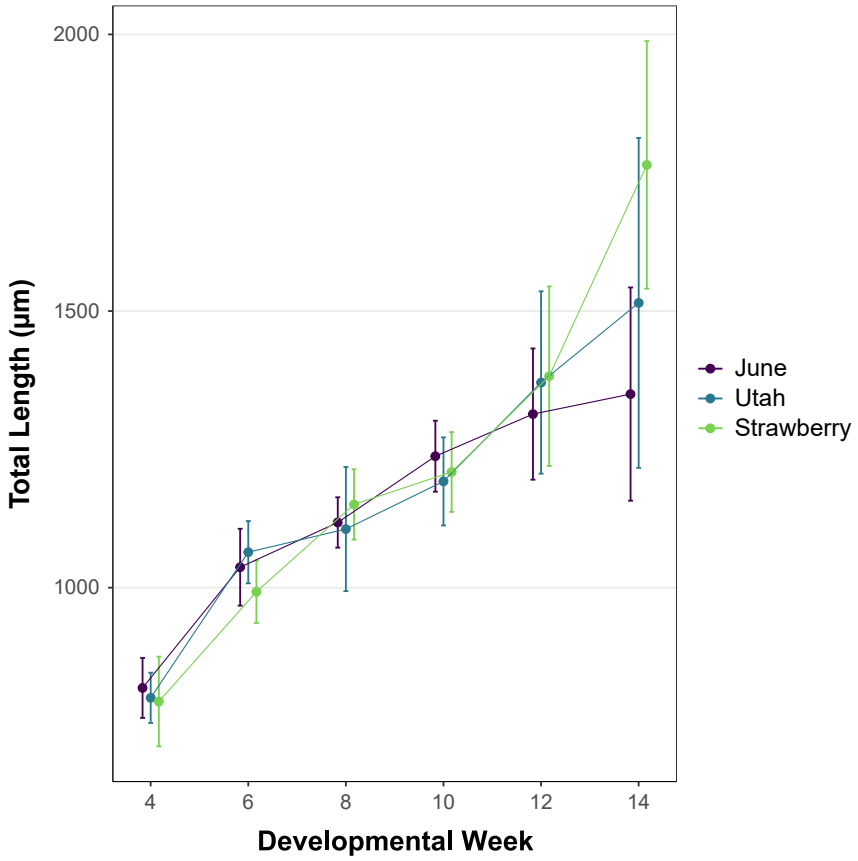

Figure 3. Premaxilla size undergoes a shorter developmental sequence in June sucker
Plot of mean total limb length of the premaxilla (error bars represent 95% confidence intervals of the mean).

were cellular anatomical entity (cellular component), cellular process (biological processes), and binding (molecular function). Transferases were the prominent enzyme category, with the top subclass being the phospho-transferase group for all lineages. Taken together, these analyses indicated that we had high-quality transcriptomes that were comparable between lineages.

General and specific gene expression patterns are consistent with shorter development in June sucker

We analyzed tissue-specific transcript expression patterns during periods of common (9–12) and lineage-specific (12–14) shape (head) and size (premaxilla) variation. We used tissue at the tip of the rostrum, directly in front of the eye, to avoid identifying differentially expressed transcripts associated with the development of other anatomical structures. Multi-species comparisons of gene expression are inherently challenging because of differences in transcript sequence and transcript length.[26] One approach to explore transcript expression across species is to generate lists of differentially expressed transcripts (DETs) within a species and then compare these lists between species.[27,28] We used this method to explore general transcript expression patterns in these lineages for each pairwise comparison of time points.

During periods of common shape and size variation (pairwise comparison of weeks 9 and 12), relatively few transcripts were differentially expressed in each lineage. Specifically, the Strawberry Reservoir Utah sucker had 5 DETs, June sucker had 18 DETs, and Utah Lake Utah sucker had 356 DETs. During weeks 9 and 12, only one DET was shared between the Utah sucker lineages. None of the remaining DETs were shared between lineages. During periods of lineage-specific shape and size variation (pairwise comparisons of weeks 9 and 14, as well as 12 and 14), June sucker had the fewest DETs (7 and 29), followed by Utah Lake Utah sucker (960 and 1030), and Strawberry Reservoir Utah sucker with the most DETs (2757 and 3364). During weeks 9 to 14 and 12 to 14, the Utah sucker lineages shared 212 and 260 common DETs, respectively. No DETs were shared across all lineages (see Figure 4 for comparisons between other lineages).

Of the 50 candidate transcripts (34 genes) identified—with known association with craniofacial development—the largest classification of transcripts was those that were unchanged during weeks 9 and 12 in all lineages and only differentially expressed in Strawberry Reservoir Utah sucker during weeks 12 and 14 (36 transcripts: 10 upregulated, 26 downregulated). The second largest classification of transcripts was those that were unchanged during weeks 9 and 12 in all lineages and only differentially expressed in Utah Lake Utah sucker during weeks 12 and 14 (9 transcripts: 6 upregulated, 3 downregulated). In addition, none of these 50 candidate transcripts were differentially expressed in the June sucker during any of the weeks (Table 2; Figures S7 and S8).

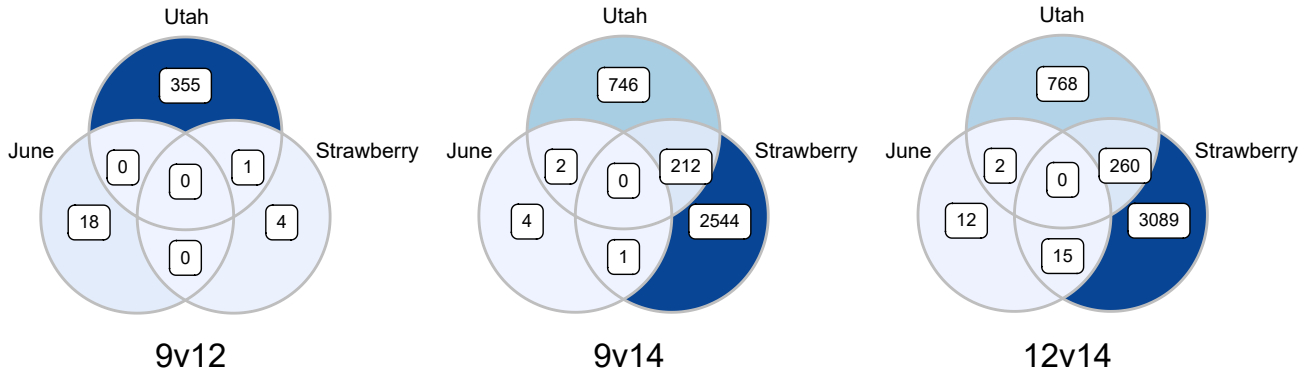

Figure 4. General transcript expression patterns are consistent with shorter development in June sucker

Venn diagrams of differentially expressed transcripts for all pairwise comparisons (9v12, 9v14, 12v14) for each lineage. Darker colors indicate a higher numbers of differentially expressed transcripts.

DISCUSSION

June sucker are paedomorphic

We aimed to explore how morphology and gene expression patterns change during the larval to juvenile transition in June sucker and two Utah sucker lineages. Using geometric morphometrics and RNA-seq analyses, we identified a difference in the timing of shape development and a corresponding change in the timing of gene expression. We add to the growing number of studies that have successfully used RNA-seq to unravel the complicated relationship between morphology and gene expression.[29–31] Specifically, our results suggest that the morphology in the June sucker may be the result of paedomorphosis, in which June sucker exhibits a less developed morphology compared to the ancestral adult with an intermediate mouth between that of the larval (terminal) and ancestral (ventral) states.

Our analyses of head shape and premaxilla size revealed that the restructuring of mouth position took less time to develop in the June sucker lineage compared to Utah sucker lineages. The June sucker's mouth reached a subterminal position and stopped changing around week 10–12, while the Utah sucker lineages' mouths continued to shift ventrally through week 14 (Figures 1 and 3). We used premaxilla shape to analyze how mouth shape changed between lineages. We found that although premaxilla shape began to diverge among lineages around week 14, these differences were subtle (Figure 2). During morphogenesis, different anatomical structures develop at different rates. Thus, mouth shape may take longer to develop in these species compared to mouth position. Alternatively, we were limited in the number of specimens we could stain and clear for the analysis of the premaxilla shape. Increasing our sample size for each week may have revealed a clearer pattern.

Our RNA-seq analyses were consistent with our geometric morphometric analyses. Each lineage experienced common expression patterns during weeks 9 to 12—a period of common shape variation—with few DETs in each lineage. During weeks 12 to 14, lineage-specific

Table 2. Classifications of 50 candidate transcripts for contrasts from weeks 9 to 12 and 12 to 14

Week 9 v 12			Week 12 v 14				
JS	UL	SR	JS	UL	SR	Total	Transcripts
0	0	0	0	0	–	26	*aldh1a2_1, aldh1a2_2, cldn5_1, crebbp_1, crebbp_2, crebbp_3, fgfr2_1, foxn3_1, foxn3_4, insig2_2, kat6a_1, map2k2_1, map2k2_2, nipbla_1, pax9_1, pax9_4, pitx2_2, plekha1_1, plekha1_3, rab23_1, rras_1, rras_2, ski_1, ski_2, tgfb1_1, wnt11_1*
0	0	0	0	0	+	10	*ankrd11_2, bbs4_1, cfdp1_1, chd7_2, map2k1_2, plekha1_2, sox3_1, sox3_2, tiparp_2, tiparp_3*
0	0	0	0	+	0	6	*mmp14_1, mmp2_1, mmp2_2, schip1_1, tgfb3_1, tgfb3_2*
0	0	0	0	–	0	3	*grhl3_1, grhl3_2, sgpl1_1*
0	0	0	0	+	–	2	*mmp14_2, mmp16_1*
0	+	0	0	–	0	2	*insig1_1, mapk3_1*
0	0	0	0	–	+	1	*tgfbr2_1*

Unchanged (0), Upregulated (+), and Downregulated (–). JS = June sucker, UL = Utah Lake Utah sucker, and SR = Strawberry Reservoir Utah sucker. Underscore followed by a number indicates which cluster a transcript came from when there were multiple transcripts annotated as the same gene.

global expression patterns became apparent between the lineages, with the number of DETs increasing dramatically in the Utah sucker lineages compared to the June sucker (Figure 4). These suites of genes that were being upregulated and downregulated were associated with the active anatomical restructuring in the Utah sucker lineages as the mouth continued to shift into a ventral position in the Utah sucker lineages. However, June sucker maintained the same expression patterns seen from weeks 9 to 12 (i.e., very few DETs), consistent with the shortened developmental sequence in these fish.

Utah sucker lineages exhibit differences during the development

Despite the Utah sucker lineages being more like each other than to the June sucker lineages, there were still noticeable differences between these lineages in our analyses. Specifically, we found that (1) Utah Lake Utah sucker premaxilla shape and size were intermediate between June sucker and Strawberry Reservoir Utah sucker at week 14 (Figures 2D and 3), (2) the total number of DETs was variable between these lineages, and (3) the number of shared DETs between each lineage was small compared to the total number of DETs in each lineage at weeks 12 to 14 (Figure 4). The differences between transcript expression in the Utah sucker lineages became more apparent when we analyzed the expression of individual transcripts. Of the 50 candidate transcripts we identified, the majority were only differentially expressed in Strawberry Reservoir Utah sucker during weeks 12 to 14 (36 transcripts), followed by transcripts that were only differentially expressed in Utah Lake Utah sucker during weeks 12 to 14 (9 transcripts). In fact, none of these transcripts shared common expression patterns in the Utah sucker lineages (Table 2; Figures S7 and S8).

Several potential genetic, ecological, and morphological differences between these lineages could account for this variation. Although Utah suckers in Strawberry Reservoir were introduced, we do not know the source population of these fish. Thus, we do not know how long this population has been separated from Utah Lake Utah sucker and what population-level genetic variation may exist between these lineages. In addition, these lineages were collected from systems that have different abiotic factors. Strawberry Reservoir is a man-made waterbody at an elevation of 2320 m, with an average depth of 14 m and a maximum depth of 70 m,[32] while Utah Lake is a natural lake at an elevation of 1368 m, with an average depth of 2.4 m and a maximum depth of 3.7 m[33] The variation between these lineages could be indicative of local adaptation to different growing seasons. Finally, the Utah Lake Utah sucker brood lot has been in captivity for some time (these fish represent at most an F2 generation as these fish are long-lived and fish were replaced using within-lot crosses), while Strawberry Reservoir Utah sucker was spawned at Strawberry Reservoir. Thus, the differences between the Utah sucker lineages could be the result of a plastic response in morphology to the captive environment.[34]

Differences between Utah sucker lineages could also be because Catostomids are allopolyploids.[35] Polyploidy is known to negatively impact the quality and accuracy of gene expression analyses.[36] This can be due to the difficulty of clustering assembled transcripts into putative genes,[36] as well as functional divergence and expression differences between paralogous genes.[37] In our transcriptomes, we often identified multiple transcripts annotated as the same gene in each lineage. Clustering these transcripts, revealed that transcripts within clusters were highly similar (comparing between lineages), but transcripts between these clusters (comparing within lineages) were highly dissimilar (see Table S3 for example from *mmp2*). These groups of homologous sequences could represent isoforms, alleles, orthologs, and/or paralogs. In addition, we found evidence of conserved (*aldh1a2*, *crebbp*, *foxn3*, *grhl3*, *map2k2*, *mmp2*, *pax9*, *rras*, *ski*, *sox3*, *tgfb3*, *tiparp*) and diverged (*mmp14*, *plekha1*) expression patterns between these homologous sequences (Figures S7 and S8). A recent assembly of the genome of the Chinese sucker (*Myxocyprinus asiaticus*), identified high synteny between homeologous chromosomes, suggesting that in Catostomids, both subgenomes retain some level of function.[35] The presence of many homologous sequences experiencing similar expression patterns is consistent with the presence of functionally redundant paralogs in these species. Additional analyses would be useful in order to identify which genes are experiencing relaxed selection, resulting in functionally redundant paralogs, and which genes are undergoing strong positive selection, resulting in more expression divergence.[37] Any number of these issues could have contributed to the variation seen between the Utah sucker lineages.

Mouth development genes are only differentially expressed in Utah sucker lineages

Although the 50 candidate transcripts we identified did not share common expression patterns between the Utah sucker lineages, none of these transcripts were differentially expressed in the June sucker. Because these transcripts are known to be associated with craniofacial development, these findings are consistent with a lack of anatomical restructuring in June sucker resulting from a shortened developmental sequence.

For example, *mmp2* is known to play roles in craniofacial skeletal morphogenesis, specifically the degradation of the extracellular matrix.[38–40] Ahi et al.[41] identified *mmp2* as an important gene associated with the development of Arctic char morphotypes (*Salvelinus*) in Lake Thingvallavatn, Iceland. Arctic char in this system has been differentiated into four phenotypic morphs, including a large benthivore, small benthivore, pelagic planktivore, and pelagic piscivore. Ahi et al.[41] found that the expression of *mmp2* was higher in the head of the benthic morphotypes compared to the pelagic morphotypes across all embryonic stages tested. Our plots of normalized counts for both transcripts annotated as *mmp2* show that expression levels of *mmp2* are similar between all lineages from weeks 9 to 12. However, during week 14, *mmp2* expression in the Strawberry Reservoir Utah sucker (benthic morph) was slightly elevated above that in the June sucker (pelagic morph; Figure 5).

Nipbl—in conjunction with the cohesin protein complex—is know to modify the expression of key developmental regulatory genes.[42] Inactivation of *Nipbl* in mice and *nipbla* in zebrafish leads to craniofacial dysmorphia, consistent with Cornelia De Lange Syndrome (down-turned lips, upturned nose, and compromised bone and cartilage development).[43,44] We found that *nipbla* (initially identified by eggNOG-mapper

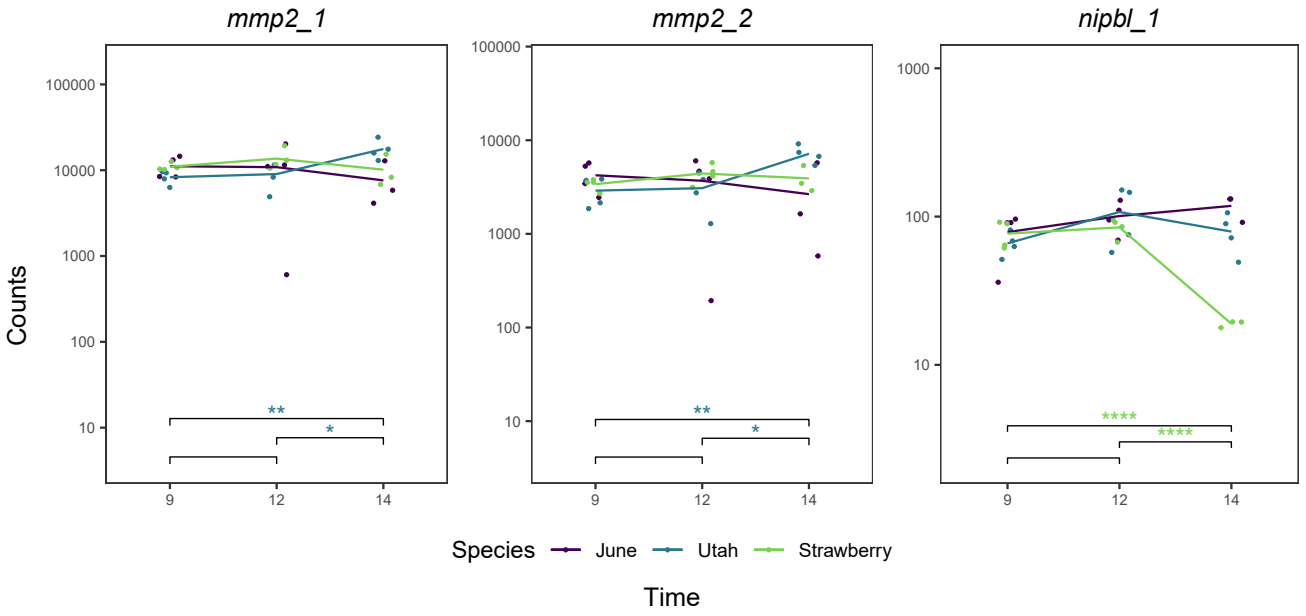

Figure 5. Mouth development genes are only differentially expressed in Utah sucker lineages
Plots of the transcript-level expression of *mmp2* (represented by two transcripts) and *nipbla*.

as *nipbl*, but later confirmed to be *nipbla* using BLAST,[45] see "Limitations of the study") expression was similar between all lineages from weeks 9 to 12. However, during week 14, *nipbla* expression in Strawberry Reservoir Utah sucker was downregulated below the June sucker and Utah Lake Utah sucker (Figure 5). Although the differences in expression level are small between these lineages, development has been shown to be extremely sensitive to slight changes in *Nipbl* activity.[42] Both *mmp2* and *nipbla* show promising expression patterns that need to be analyzed further.

June sucker and Utah sucker are in the "messy middle" of the speciation continuum

Speciation is best viewed as a continuous process (speciation continuum), rather than a discrete event.[46] However, drawing a line on this continuum often leads to conflicting results, especially in closely related, recently diverged species. It is possible that the June sucker and Utah sucker are reticulating or are incipient species in the "messy middle" (*sensu*[47]) of the speciation continuum. How speciation and ontogenetic morphological divergence proceeds in Utah Lake, may be influenced by long-term variation in water levels. Since its formation about 10,000 years ago as a remnant of Lake Bonneville,[48] Utah Lake has undergone subsequent expansions and contractions. Evidence suggests that during periods of drought, Utah Lake may have become significantly dessicated.[22] This pattern of alternating environmental conditions may contribute to the variation seen in the June sucker and Utah sucker along the benthic-limnnetic axis. During wet periods, increased depth would favor limnetic morphs due to the increased relative availability of zooplankton. During droughts, benthic feeding and associated benthic morphology would be favored. The fluctuating conditions may lead to the persistence of intermediates and a prolonged period of incomplete separation between the two species. Because of this incomplete separation, different lines of evidence typically used to define species produce conflicting results.[49] Although there is no universally accepted species concept, there is agreement that species delimitation should include multiple lines of evidence, such as morphology, ecology, and genetic patterns, considered in an ontogenetic developmental framework.[2,3] Our morphometric and gene expression analyses provide parallel lines of evidence that the June sucker and Utah sucker are genetically shallow, yet morphologically diverged species in Utah Lake.

Broader Implications: Paedomorphosis in other Chasmistes/Catostomus pairs

June sucker and Utah sucker are one of the four *Chasmistes* and *Catostomus* pairs whose taxonomic relationships have been challenging to discern. These pairs exhibit similar morphological (subterminal vs. ventral mouths) and genetic patterns (lack of genetic markers that consistently separate these species) as seen in the June sucker and Utah sucker. In addition, in all but *Chasmistes cujus* and *Catostomus funeiventris*, these species pairs are sympatric and fish exist with intermediate mouth morphology.[17,22,50,51] Paedomorphosis—resulting from the differential expression of genes in response to environmental cues—could be the mechanism that drove the convergent evolution of these *Chasmistes*/*Catostomus* pairs. These findings are consistent with studies across many taxa,[52–54] particularly amphibians,[55] which show that differential gene expression leads to paedomorphisis. Studies of similar designs will be required to determine if paedomoprhosis is general among these *Chasmistes*/*Catostomus* pairs.

Limitations of the study

Proper annotation of transcriptomes in polyploid species can be difficult. Transcripts annotated as the same gene—within and between species—can be isoforms, alleles, orthologs, and/or paralogs. Thus, it is critical to carefully determine the correct relationships between these transcripts for gene expression analyses. In addition, eggNOG-mapper sometimes predicted gene names, that were general, and not specific to fish. For example, eggNOG-mapper identified several transcripts as *nipbl* although two *nipbl* genes are present in zebrafish (*nipbla or nipblb*). Well-annotated, reference genomes from *Chasmistes* and *Catostomus* would be ideal for future analyses of gene expression in Catostomids.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109566.

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AUTHOR CONTRIBUTIONS

Conceptualization: D.K.S., R.P.E., and M.C.B; methodology: P.C.S., D.K.S., R.P.E., J.T.H., A.S., M.R.S., and M.C.B; validation: P.C.S.; formal analysis: P.C.S.; investigation: P.C.S.; resources: D.K.S., R.P.E., J.T.H., A.S., M.R.S., and M.C.B; data curation: P.C.S.; writing – original draft: P.C.S.; writing – reviewing and editing: P.C.S., D.K.S., R.P.E., J.T.H., A.S., M.R.S., and M.C.B; visualization: P.C.S.; supervision: M.C.B.; project administration: M.C.B.; funding acquisition: P.C.S., D.K.S., R.P.E., and M.C.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Chasmistes liorus	State of Utah Fisheries Experiment Station	See Table S1.
Catostomus ardens (2 lineages)	State of Utah Fisheries Experiment Station	See Table S1.
Chemicals, peptides, and recombinant proteins		
Tricaine Methanesulfonate	MilliporeSigma	Catalog #: E10521
RNALater	MilliporeSigma	Catalog #: R0901
Modified Carnoy's solution	N/A	N/A
Critical commercial assays		
Alizarin Red/Alcian Blue Staining Protocol	Gillis et al.,[56] Thisse and Thisse[57]	N/A
TRIzol Plus RNA Purification Kit	Thermo Fisher Scientific	Catalog #: 12183555
ProNex Size-Selective Purification System	Promega	Catalog #: NG2001
Iso-Seq Express 2.0 Workflow	Pacific Biosciences	N/A
RNeasy Plus Mini Kit	Qiagen	Catalog #: 74314
NEBNext Ultra II RNA with Poly-A selection	New England Biolabs	Catalog #: E7775
Deposited data		
Genetic Data	This Paper	NCBI: PRJNA818778
Morphometric Data	This Paper[58]	Zenodo: https://zenodo.org/records/10806437
Code	This Paper[58]	Zenodo: https://zenodo.org/records/10806437
Software and algorithms		
tpsUtil, tpsDig, tpsRelw	Rohlf[59]	https://life2.bio.sunysb.edu/ee/rohlf/software.html
SAS	SAS Institute Inc.	v9.4; https://www.sas.com/en_us/home.html
R	R Development Core Team	v4.1.1; https://www.r-project.org/
Python	N/A	v. 3.11.5
SMRT Link Run QC	Pacific Biosciences	v9.0; https://www.pacb.com/support/software-downloads/
IsoSeq3	Pacific Biosciences	v3.4.0; https://github.com/PacificBiosciences/IsoSeq
LoRDEC	Salmela and Rivals[60]	v0.9
CD-HIT-Est	Li et al.[61]	v4.8.1
DIAMOND	Buchfink et al.[62]	v2.0.11
BUSCO	Seppey et al.[25]	v5.1.2
OmicsBox	BioBam	v2.0.24; https://www.biobam.com/
Blast2GO	Götz et al.[63]	N/A
InterProScan	Jones et al.[64]	v86.0
eggNOG-mapper, eggNOG	Cantalapiedra et al.[65] Huerta-Cepas et al.,[66] Huerta-Cepas et al.,[67]	v2.1.0, v5.0.2
TrimGalore	Krueger[68]	v0.6.5
FastQC	Andrews[69]	v0.11.9
Salmon	Patro et al.[70]	v1.8.0
tximport	Soneson et al.[71]	v1.26.0
DESeq2	Love et al.[72]	v1.32.0

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Olympus stereo microscope	Olympus	N/A
PacBio Sequel II System	Pacific Biosciences	N/A
Illumina NovaSeq 6000 System	Illumina	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact: Peter C. Searle (pcs222@cornell.edu).

Materials availability

The study did not generate new unique reagents.

Data and code availability

- All morphometric data have been deposited at Zenodo[58] and are publicly available as of the date of publication. DOIs are listed in the key resources table. All genetic data associated with the publication are found at NCBI: PRJNA818778 and are publicly available as of the date of publication.
- All original code has been deposited at Zenodo[58] and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

June sucker and Utah sucker

June sucker—once abundant in Utah Lake—declined to a historical low of approximately 1,000 individuals in 1989. During the 1990's and early 2000's, captive brood lots were created from forced spawning of wild June sucker to augment natural reproduction while critical spawning habitat was restored.[73] Belk et al.[73] formally evaluated lower lip morphology of these brood lots and determined that lower lip morphology ranged between that of pure June sucker (crosses between wild June sucker from Utah Lake; thin, separated lower lip lobes with few papillae) and pure Utah sucker (crosses between wild Utah sucker from an introduced population in Strawberry Reservoir, Utah, USA; broad lower lip lobes with no gap between them, that are highly papillose), with most brood lots exhibiting intermediate phenotypes.[49] For our analysis, we acquired samples from two of these brood lots from Utah Lake: a lot identified as the most June-sucker-like (June sucker) and another lot identified as the most Utah-sucker-like (Utah Lake Utah sucker). We also obtained larval samples originating from wild Utah suckers from Strawberry Reservoir (Strawberry Reservoir Utah sucker). We included fish from Strawberry Reservoir so we could analyze development in Utah suckers that were not influenced by suckers from Utah Lake.

The three sucker lineages were hatched and reared in 2014 at the State of Utah Fisheries Experiment Station (Logan, UT, USA) in a common-garden environment. After hatching, larval samples were collected for 15-weeks: once weekly for weeks 0-2, twice weekly for weeks 3-11, and again once weekly for weeks 12-14 (n=416) following approved Brigham Young University Institutional Animal Care and Use Committee (IACUC) protocols (IACUC -approved protocol #15-0602, Utah Division of Wildlife Resources' scientific collection permit #1COLL5950). Specimens collected on week 0 were 0 days after hatching, whereas specimens collected on week 14 were 98 days after hatching. At each sampling date, twelve fish were collected from each sucker lineage and euthanized with tricaine methanesulfonate (MS-222, MilliporeSigma, St. Louis, MO, USA). Six specimens were stored in RNAlater (MilliporeSigma, St. Louis, MO, USA) at -20°C for RNA sequencing. Six specimens were stored in a modified Carnoy's solution (60 mL 100% ethanol, 30 mL 37% formaldehyde, 10 mL glacial acetic acid) at 4°C for 1 to 4 days for the geometric morphometric analysis. The fish stored in the modified Carnoy's solution were then dehydrated in 75% ethanol (75 mL 100% ethanol, 25 mL diH_2O; three 30 min intervals), 100% ethanol (3 one-hour intervals) and stored at -20°C. Because these specimens were in the larval stage of development, sex could not be determined and was not included in analyses.

METHOD DETAILS

Geometric morphometrics

We photographed the left lateral head of all specimens (n=416; 140 June sucker, 143 Utah Lake Utah sucker, 133 Strawberry Reservoir Utah sucker) using an Olympus stereo microscope (Olympus, Shinjuku City, Tokyo, Japan). Eighteen fish were misshapen and were removed from our analysis (4 June sucker, 9 Utah Lake Utah sucker, and 5 Strawberry Reservoir Utah sucker). Strawberry Reservoir Utah sucker collected

during week 2 were misplaced and thus, were not included in the analysis. After these steps, 398 fish remained including 136 June sucker, 134 Utah Lake Utah sucker, and 128 Strawberry Reservoir Utah sucker (Table S1).

To photograph the premaxilla, a subset of the formalin fixed fish (n=213) was cleared and stained using a modified Alizarin Red/Alcian Blue staining protocol.[56] Prior to clearing and staining, pigment was removed using a hydrogen peroxide treatment (.5g KOH, 90 mL 1x PBS, 10 mL 30% H_2O_2). Fish were incubated in the hydrogen peroxide solution at room temperature until pigment disappeared and then washed in series (1X PBS, 25%, 50% and 75% ethanol in 1X PBS, 10 min/wash).[57] Next, the fish were placed in 70% ethanol (30 mL diH_2O, 70 mL 100% ethanol, 2 h) and then acetic ethanol (30 mL glacial acetic acid, 70 mL 100% ethanol, 2 h). Cartilage was stained using Alcian Blue 8GX (MilliporeSigma, St. Louis, MO, USA; 20 mg Alcian Blue, 100 mL acetic ethanol, 24 h). Specimens were destained in acetic ethanol and rehydrated in series (70%, 50%, 25% ethanol in diH_2O and 100% diH_2O, 2 h). Tissue was cleared using a trypsin solution (Ward's Science, St. Catharines, ON, Canada; 1 g trypsin, 2 g sodium tetraborate, 97 mL diH_2O) until approximately 50% clear and then placed in an Alizarin Red S solution (MilliporeSigma, St. Louis, MO, USA; 0.1 g Alizarin Red S, 100 mL 1% KOH, 24 h) to stain bone. Specimens were again cleared in a trypsin solution until 100% clear and placed in a .5% KOH solution (75 mL .5% KOH, 25 mL glycerol, 24 h). Specimens were soaked in a .5% KOH/glycerol series (3:1, 1:1, 1:3, .5% KOH:glycerol, 24 h/wash) and stored in 80% glycerol (80 mL glycerol, 20 mL 1x PBS).

After staining, we photographed the premaxilla of 213 fish (72 June sucker, 72 Utah Lake Utah sucker and 69 Strawberry Reservoir Utah sucker) using an Olympus stereo microscope. The premaxilla for weeks 0-2 was not developed enough to photograph and was excluded from our analysis (n=24). The premaxilla of one fish was damaged and was excluded from our analysis (n=1). After these steps, 188 fish remained including 63 June sucker, 63 Utah Lake Utah sucker, and 62 Strawberry Reservoir Utah sucker (Table S1).

We used tpsDig to digitize landmarks.[74] To ensure consistent, homologous placement of landmarks, we had one researcher place landmarks and another researcher independently inspect the landmarks. For the head, we included 12 landmarks: (1) posterior maximum of operculum, (2) posterior maximum of eye, (3) anterior maximum of eye, (4) dorsal margin of upper lip, (5) anterior maximum of eye projected onto the dorsal outline, (6) posterior maximum of eye projected onto the dorsal outline, (7) posterior maximum of operculum projected onto the dorsal outline, (8) posterior maximum of operculum projected onto the ventral outline, (9) posterior maximum of eye projected onto the ventral outline, (10) anterior maximum of eye projected onto the ventral outline, (11) midpoint between landmarks 4 and 10 on the ventral outline, and (12) midpoint between landmarks 4 and 5 on the dorsal outline. Landmarks 11 and 12 were sliding semi-landmarks (Figure S3; *sensu*[75]). For the premaxilla, we included 8 landmarks: (1) maximum of vertical limb, (2) maximum of medial angle, (3) maximum of lateral limb, (4) midpoint between 1 and 3 on right lateral outline (5) midpoint between 1 and 4 on right lateral outline, (6) midpoint between 1 and 2 on medial outline, (7) midpoint between 2 and 3 on ventral outline, and (8) midpoint between 3 and 4 on right lateral outline. Landmarks 4 through 8 were sliding semi-landmarks (Figure S4). The premaxilla is a symmetrical structure, so we only landmarked the left premaxilla of each specimen.[76] See description of statistical analyses below.

Sequencing

We generated PacBio long-reads for *de novo* transcriptome assembly. For PacBio sequencing, specimens were thawed and placed in grooves on agar plates made with custom 3D molds, ensuring consistent placement of specimens for tissue removal. The entire head of each specimen was excised by cutting directly behind the operculum with a scalpel. A new groove and sanitized scalpel were used for each specimen. Three pooled samples consisting of 3 specimens per lineage—1 specimen from weeks 8, 11, and 13—were used for PacBio sequencing (Table S1). Weeks 9, 12, and 14 were identified as occurring during the critical developmental period from our geometric morphometric analyses (see Results) but specimens were used from weeks 8, 11, and 13 instead of weeks 9, 12, and 14 because not enough specimens were available from weeks 9, 12, and 14 for both PacBio and Illumina sequencing. We used the whole head of specimens from multiple weeks to generate a comprehensive transcriptome represented by multiple tissues. Tissues were frozen in RNAlater (MilliporeSigma, St. Louis, MO, USA) and delivered to Brigham Young Universities' DNA Sequencing Center (Provo, UT, USA) on dry ice for RNA extraction, library preparation and sequencing. Total RNA was extracted according to the manufacturer's instructions using the TRIzol Plus RNA Purification kit (Thermo Fisher Scientific, Waltham, MA, USA), secondary cleanup was performed using the ProNex Size-Selective Purification System (Promega, Madison, WI, USA) and total RNA was then pooled into a single sample for each lineage (as described above). Barcoded cDNA libraries were prepared according to the Iso-Seq Express 2.0 workflow system (Pacific Biosciences, Menlo Park, CA, USA) using polyA selection. Libraries were sequenced using a PacBio Sequel II System on a single SMRT cell 8M for a 24-h movie with insert size of 2,500 bp (Pacific Biosciences, Menlo Park, CA, USA).

We generated Illumina short-reads for error correction of our transcriptomes, as well as RNA-seq analyses. For Illumina sequencing, tissue at the tip of the rostrum, directly in front of the eye, was removed (as described above). Tissues were frozen in RNAlater and shipped to Admera Health, LLC (South Plainfield, NJ, USA) on dry ice for RNA extraction, library preparation, and sequencing. Total RNA was successfully extracted from 34 specimens from weeks 9 (n=12), 12 (n=12), and 14 (n=10) for Illumina sequencing. For week 14, total RNA was only successfully extracted from 10 specimens (June sucker, n=3; Utah Lake Utah sucker, n=4; Strawberry Reservoir Utah sucker, n=3, Table S1). Total RNA was extracted according to the manufacturer's instructions using a RNeasy Plus Mini kit (Qiagen, Hilden, Germany). Barcoded cDNA libraries were prepared from total RNA using the NEBNext Ultra II RNA with Poly-A selection kit (New England Biolabs, Ipswich, MA, USA) which selects for mRNA. Libraries were sequenced using an Illumina NovaSeq 6000 system with S4 flow cell and read length of 2x150 bp (Illumina, San Diego, CA, USA).

Transcriptome assembly

PacBio sequence quality was checked using SMRT Link Run QC (v9.0; (https://www.pacb.com/support/software-downloads/) before the sequences were processed using the IsoSeq3 (v3.4.0) protocol (https://github.com/PacificBiosciences/IsoSeq, See Figure S5 for diagram outlining key steps for assembly, annotation, and differential expression analyses). In brief, circular consensus sequences were generated using ccs (v6.0.0); primers were removed, and barcodes identified using lima (v2.0.0); poly (A) tails trimmed and concatemers identified and removed using IsoSeq3 refine (v3.4.0); and full-length non-concatemer reads clustered using IsoSeq3 cluster (v3.4.0). High- and low-quality transcripts were identified using a predicted accuracy cutoff of 0.99 and only high-quality transcripts were retained for subsequent analyses (Table S2A). High quality transcripts were error corrected using all Illumina sequences from each lineage (pre-processing of Illumina sequences described below) using LoRDEC (v0.9).[60] Finally, error-corrected transcripts were clustered using CD-HIT-Est (v4.8.1) to remove additional sequence redundancy (Table S2B).[61]

Transcripts were checked for contamination by blasting the transcripts against the nr database (NCBI non-redundant protein sequences, https://www.ncbi.nlm.nih.gov/) using DIAMOND (v2.0.11).[62] Top 10 hits using DIAMOND all came from fish in the order Cypriniformes (June sucker, 91.78%; Utah Lake Utah sucker, 90.89%; Strawberry Reservoir Utah sucker, 91.91%) indicating no significant contamination, so all transcripts were retained for future analyses. BUSCO (Benchmarking Universal Single-Copy Orthologs; v5.1.2; -l actinopterygii_odb10) was used to assess transcriptome assembly completeness.[25]

Functional annotation

We functionally annotated our transcriptomes to validate that the transcriptomes were similar between lineages. We used DIAMOND (as described above) to obtain the best blast hits for each transcript from the nr database (NCBI non-redundant protein sequences, https://www.ncbi.nlm.nih.gov/). Blast hits and transcripts were then imported into OmicsBox (v2.0.24, https://www.biobam.com/). Next, candidate gene ontology terms were retrieved for each transcript and assigned to the transcripts using Blast2GO.[63] We obtained additional gene ontology terms using InterProScan5 (v86.0), which searches the InterPro member databases using protein signature and protein domain information,[64] as well as eggNOG-mapper (v2.1.0) which uses precalculated orthologous groups from the eggNOG (v5.0.2) database.[65–67] These additional gene ontology terms were merged with the Blast2GO annotations.

We generated common transcriptomes that consisted only of shared transcripts among all lineages so we could compare differential expression patterns between lineages. We reran eggNOG-mapper (v2.1.12), this time restricting annotations to species within Actinopterygii to generate as fine-scale annotations as possible. We used the predicted gene names generated from this program to then filter the transcripts using R. EggNOG-mapper identified predicted gene names for 21,033 (57.1%), 19,679 (55.2%), and 18,255 (56.8%) transcripts for June sucker, Utah Lake Utah sucker, and Strawberry Reservoir Utah sucker, respectively. After filtering, 18,726, 17,849, and 16,787 transcripts—consisting of 8,392 common genes among all lineages—remained for June sucker, Utah Lake Utah sucker, and Strawberry Reservoir Utah sucker, respectively. Initially, when multiple transcripts shared the same gene name, we only retained the transcript with the lowest e-value for each lineage. However, we found that this final filtering step often led to the selection of transcripts that were dissimilar (< 97% identity) between lineages, despite lineages sharing highly similar transcripts (>99.5% identity) prior to filtering. To avoid using dissimilar transcripts between lineages we performed additional clustering. Specifically, we a) merged all the transcripts identified as each gene for all the sucker lineages, b) clustered these transcripts using CD-HIT-Est, and c) only retained clusters of transcripts that were represented by all lineages and had a cluster identity greater than or equal to 97%. Also, when multiple transcripts were present for a lineage in a cluster, we only retained the transcript with the highest cluster identity. This additional clustering step resulted in transcriptomes consisting of 7,195 genes and 10,426 transcripts for each lineage. In these transcriptomes, the number of transcripts associated with the genes ranged from 1 to 11 transcripts, with most genes only having one transcript.

Differential expression

We generated count tables using our assembled and annotated transcriptomes, as well as the Illumina sequences. Specifically, we pre-processed the Illumina sequences by performing read adapter and quality trimming with Trim Galore (v0.6.5) and then checked sequence quality using FastQC (v.0.11.9).[68,69] Transcript-level abundances were generated with Salmon (v1.8.0).[70,77] The overall alignment rate was 35.40%, 33.06%, and 33.41% for June sucker, Utah Lake Utah sucker, and Strawberry Reservoir Utah sucker, respectively. We used tximport (v1.26.0) to generate transcript-level count matrices.[71] We chose to analyze transcripts annotated as the same gene at the transcript-level so we could explore how expression changed between these divergent transcripts. All subsequent transcript expression analyses were performed using these count matrices with DESeq2 (v1.32.0) in R.[72]

The quality of the count tables were assessed prior to running differential expression analyses. Transcript-level counts were normalized and transformed using the regularized logarithm (rlog) in DESeq2 and analyzed using a separate principal components analysis for each lineage. The principal components analysis for each sucker lineage revealed two potential outliers for June sucker and Strawberry Reservoir Utah sucker, both in week 12 (Figure S6). Although the fish were raised in common garden conditions, differences in preservation and relative age (i.e., not all fish were the same length when sampled) could have contributed to these differences. Analyses were conducted including and excluding these samples. General patterns (as described in the Results) were consistent with and without these samples, so these two samples were included in all subsequent analyses. See description of statistical analyses below.

Candidate genes

An initial list of candidate genes was generated with AmiGO2[78] on Geneontology.org [79,80] using two separate searches ("development+face" and "development+craniofacial"). From these searches, 1059 gene annotations (332 unique genes) were identified, of which 79 transcripts were present in our filtered transcriptomes. Of these 79 candidate transcripts, four transcripts with low counts were removed because DESeq2 was unable to determine if the transcripts were differentially expressed. In addition, 25 transcripts were removed that were classified as being unchanged across all contrasts and lineages, resulting in 50 candidate transcripts (Table 2). Finally, we generated plots of transcript-level expression (normalized counts) for each candidate transcript using DESeq2 (Figures S7 and S8). The genetic data associated with these analyses are found at NCBI: PRJNA818778 and the original code associated with these analyses has been deposited at Zenodo.[58]

QUANTIFICATION AND STATISTICAL ANALYSIS

Geometric morphometrics

We used tpsRelw to generate shape variables from the landmark data.[81] We used the minimize d^2 option in tpsRelw for sliding semi-landmarks.[59] We used the first 9 of 20 relative warps for the head and the first 6 of 12 relative warps for the premaxilla in our analysis because they captured 98.98% and 96.77% of the shape variation in the head and premaxilla, respectively. We excluded relative warps to account for loss of dimensionality from the use of sliding semi-landmarks.[82] We also excluded relative warps that individually accounted for < 1% of shape variation to avoid inflating the real degrees of freedom associated with analysis of shape variation.[82–84]

We ran a multivariate linear mixed model to analyze shape change in the head and premaxilla during development in the sucker lineages.[82–88] We analyzed head and premaxilla shape separately, using relative warps as the response variable. The multivariate linear mixed model requires a univariate response variable, so we vectorized the matrix of relative warps so each individual specimen was represented by 9 rows (head) and 6 rows (premaxilla) of data. Thus, the response variable is transposed from a row in a matrix to a vector column of responses. We included an index variable as a discrete predictor variable to maintain the original ordering of the relative warps. The index variable preserved the order of the relative warps so that comparisons were made across matching relative warps between lineages.[82–85] Explanatory variables in our model included: lineage (discrete predictor; three values), week (discrete predictor; 15 values for head shape; 6 values for premaxilla shape, described below), the index variable, the interactions between the main effects and the index variable and the three-way interaction between lineage, week, and the index variable. We did not include centroid size (multivariate measure of shape derived from analysis in tpsRelW) as a covariate in our analyses because week acted as an index for stage of development and size. Because relative warps result from a principal components analysis, they have a mean of zero making interpretation of the main effects and index variable by themselves uninformative. Thus, we used the two-way interactions (lineage*index and week*index) and the three-way interaction (lineage*week*index) to test whether shape varied between lineages across ontogeny on at least some set of relative warps (Table 1).[82–85] In addition, we included individual ID as a random effect in our model because each specimen had multiple relative warps as response variables. Degrees of freedom were estimated using the Kenward-Roger method.[89] For the premaxilla, we combined adjacent weeks (in pairs) to increase our sample size, so our statistical analysis converged. We ran all analyses with Proc MIXED in SAS (SAS version 9.4, SAS Institute Inc., Cary NC, USA).

Relative warps 1 and 2 accounted for 64.59% and 9.57% of the shape variation in the head, respectively. Relative warps 1 and 2 accounted for 54.79% and 22.57% of the shape variation in the premaxilla, respectively. We used geomorph (v4.0.4)[90,91] in R (v4.1.1)[92] to generate thin-plate spline deformations of head and premaxilla shape for the positive and negative extremes of relative warps 1 and 2 to visualize the range of shape variation represented on each of the first two relative warps (Figures 1A and 2A).[81] To visualize change in head and premaxilla shape over ontogeny, we plotted least squares means for each lineage across all weeks on relative warps 1 and 2 (Figures 1B and 2B). We plotted least squares means and 95% confidence intervals for each relative warp for each lineage at week 14, to determine which relative warps differed between lineages (Figures 1C and 2C). We calculated divergence vectors to visualize shape variation between lineages at week 14.[84,93,94] Specifically, we ran a principal components analysis on the least-squares means for each relative warp for each pairwise comparison of sucker lineages. We generated divergence scores by summing the products of the first eigenvector and its associated relative warp score for each specimen. We regressed divergence score against shape (i.e., generalized Procrustes aligned specimens) using geomorph to generate thin-plate spline representations of the extremes of shape variation between lineages (Figure 1D).

To compare patterns of size change across the developmental sequence in the premaxilla, we measured absolute distances between landmarks 1 and 2 (vertical limb length), as well as 2 and 3 (lateral limb length) using geomorph. We summed both limb lengths to create an overall measure of size. We plotted the means and 95% confidence intervals of size across the developmental sequence (Figure 3) for comparison among lineages. The code and morphometric data associated with these analyses have been deposited at Zenodo.[58]

Differential expression

We identified differentially expressed transcripts (DETs) by running the standard DESeq2 pipeline for each lineage using pairwise comparisons (contrasts) of weeks 9 and 12, 9 and 14, and 12 and 14 with a Wald test. Transcripts were considered to be differentially expressed if they had a Benjamini-Hochberg adjusted p-value < .05. Transcripts were considered to be upregulated if they had a log2 fold change greater than 0 and downregulated if they had a log2 fold change less than 0.[72] Venn diagrams were created to compare DETs between lineages for each pairwise comparison.