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Genetic mapping of craniofacial traits in the Mexican tetra reveals loci associated with bite differences between cave and surface fish

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Abstract

Background The Mexican tetra, *Astyanax mexicanus,* includes interfertile surface-dwelling and cave-dwelling morphs, enabling powerful studies aimed at uncovering genes involved in the evolution of cave-associated traits. Compared to surface fish, cavefish harbor several extreme traits within their skull, such as a protruding lower jaw, a wider gape, and an increase in tooth number. These features are highly variable between individual cavefish and even across different cavefish populations.

Results To investigate these traits, we created a novel feeding behavior assay wherein bite impressions could be obtained. We determined that fish with an underbite leave larger bite impressions with an increase in the number of tooth marks. Capitalizing on the ability to produce hybrids from surface and cavefish crosses, we investigated genes underlying these segregating orofacial traits by performing Quantitative Trait Loci (QTL) analysis with F_2 hybrids. We discovered significant QTL for bite (underbite vs. overbite) that mapped to a single region of the *Astyanax* genome. Within this genomic region, multiple genes exhibit coding region mutations, some with known roles in bone development. Further, we determined that there is evidence that this genomic region is under natural selection.

Conclusions This work highlights cavefish as a valuable genetic model for orofacial patterning and will provide insight into the genetic regulators of jaw and tooth development.

Keywords Jaws, Teeth, Feeding behavior, Quantitative trait loci, Cavefish

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Background

One of the hallmarks of early vertebrate evolution is the biting jaw [1, 2]. Because the mandibular arch can be found in jawless fishes such as lamprey and hagfish, it is likely that the morphological identity of lower jaw components (i.e. pharyngeal arches) was present in a common ancestor to the jawless cyclostomes and jawed gnathostomes [3]. Among other cranial bones, the lower jaw is highly conserved across vertebrates from extinct armored placoderms to living tetrapods [4], suggesting conserved genetic networks govern jaw development.

The emergence of diversity in jaw morphology is linked to feeding ecology [5]. Classic examples of adaptive radiations, such as beak shape in Darwin's finches [6] and jaw diversity in cichlids [7], occur through the expansion into new feeding niches, leading to extreme changes in morphology and in some cases speciation events. For example, cichlids exhibit a spectrum of variation in their oral jaws, from short jaws amenable to biting hard surfaces to elongated jaws for suction feeding [8]. The emergence of these morphological changes is integrated in response to environmental and ecological pressures.

Perhaps one of the most extreme environmental pressures an organism can face is the subterranean habitat. Obligate cave-dwellers face perpetual darkness, scarce food sources and isolation from other ecosystems. Despite these challenges, cave organisms thrive in this environment. For example, *Astyanax mexicanus* cavefish have evolved physiological and morphological traits suited for life in complete darkness, such as starvation resistance [9–11], enhancement of sensory systems [12–14], sleep loss/constant foraging [15], and changes to their immune system [16], relative to extant surface-dwelling fish. In addition to these changes, cavefish harbor extreme changes in morphology, including several craniofacial traits, such as cranial bone fragmentations and spontaneous fusions, as well as fluctuating and directional asymmetries [17]. These craniofacial features are highly variable across both individual cavefish, as well as the ~ 30 known *Astyanax* cavefish populations found in northeastern Mexico. Within their oral jaws, adult cavefish exhibit an increase in both upper and lower jaw dentition (tooth number) compared to surface fish [18]. Further, larval cavefish have wider and more protruding lower jaws [12, 19].

An elongation of the lower jaw is not unique to the blind Mexican cavefish, however. Protruding lower jaws have been characterized in cavefish across the globe including the Chinese cavefish (*Sinocyclocheilus*) [20], the cavefish of the Ozarks (*Amblyopsis rosae*) [21], and an Australian cavefish (*Milyeringa brooksi*) [22]. This parallel evolution of changes in the lower jaw suggests a possible adaptive significance.

Toward that end, we set out to characterize changes in lower jaw morphology in adult *Astyanax* cavefish using morphological, behavioral, and genetic analyses. We discovered that the wider, protruding lower jaws observed in larval cavefish persist in the adult cranium, resulting in an underbite compared to the slight overbite or normal occlusion found in surface fish. To determine if the underbite is of functional importance, we assessed the maximum gape (mouth opening) and feeding behavior using a novel feeding assay. Further, we capitalized on the ability to generate viable hybrids from surface x cavefish crosses and employed a genetic association study to illustrate that bite differences are under genetic control in *A. mexicanus*. Next, we were able to pinpoint an associated region in the genome and generated a subsequent list of candidate genes for this trait. Together, our analyses reveal a novel role for differences in jaw morphology and tooth patterning in cavefish that likely evolved as an alternative feeding strategy in nutrient poor caves.

Results

Cavefish exhibit differences in jaw morphology compared to surface fish

Cavefish harbor an underbite, compared to an overbite or even occlusion in surface fish, which manifests as an elongated lower jaw and a wider mouth opening or "gape" (Fig. 1A, D). Gape was measured by taking the maximum angle of the maxillary bone to the lateral mandible. Cavefish averaged a significantly higher gape angle ranging from 130°-139° (mean = 136°), compared to a range of 96°-116° in surface fish (mean = 106°; Fig. 1B). Surface x cavefish F_2 hybrids were separated into "overbite" and "underbite" groups. F_2 hybrids scored as having an overbite had an average gape angle of 116°, compared to an average angle of 130° in F_2 hybrids with an underbite (Fig. 1B). An ANOVA revealed significant variation in gape angle across populations ($F = 18.83$; $p < 0.001$). A post hoc Tukey test showed significant differences between overbite and underbite groups at $p < 0.05$ (See Table S1). In addition to a larger gape angle, F_2 hybrids with an underbite have significantly longer lower jaws (normalized mandible length) compared to overbite F_2 hybrids ($p < 0.05$; Fig. 2I). No sex differences were observed for any of the jaw morphology metrics analysed. Further, we found no significant difference in body size (standard length) between fish exhibiting an overbite (average length of 4.86 cm) vs. underbite (4.65 cm; $p = 0.07$).

A novel behavioral assay illustrates that fish with an underbite feed differently on substrate compared to fish with an overbite

Cavefish display a difference in feeding posture compared to surface fish [23, 24]. To determine if F_2 hybrids with an underbite feed at a similar angle to cavefish (and

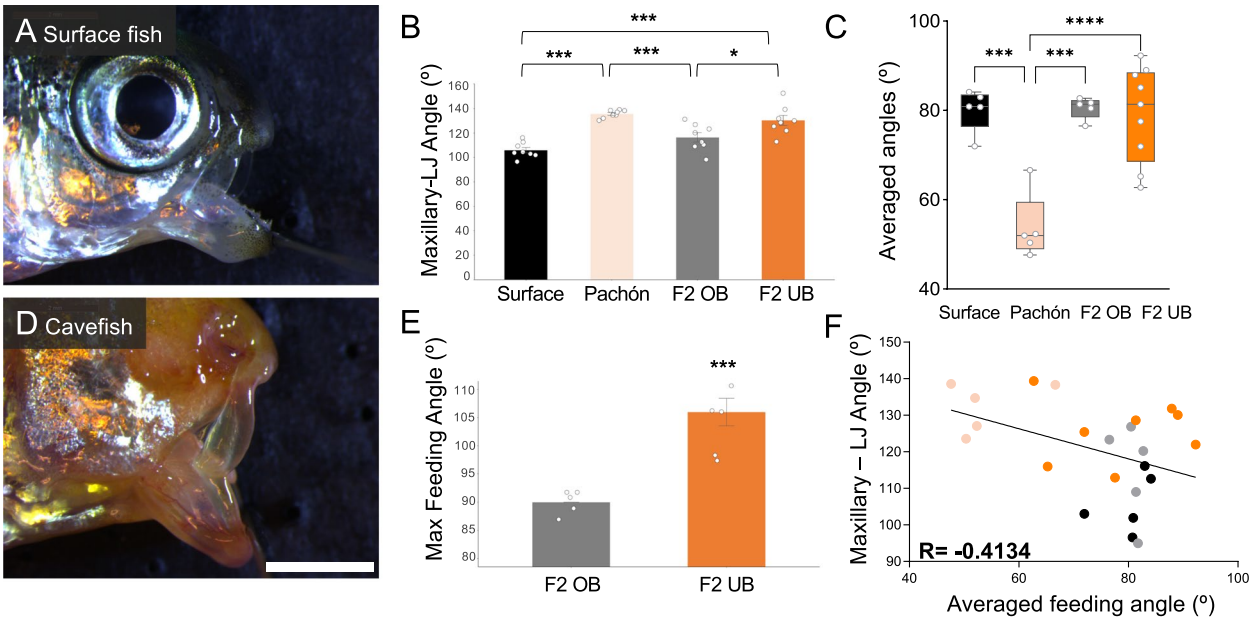

Fig. 1 Fish with an underbite exhibit a larger maxillary – lower jaw angle (gape), which negatively correlates with feeding angle. Adult surface fish (**A**) have a smaller maxillary – lower jaw angle (mean = 106°) compared to cavefish (**D**; mean = 136°; $p < 0.001$) (**B**). F_2 hybrids scored as having an overbite (OB) were not significantly different than surface fish (mean = 116.5°; $p = 0.1$) (**B**). Additionally, F_2 hybrids scored as having an underbite (UB) were not significantly different from cavefish (mean = 130°; $p = 0.62$) (**B**). In agreement with data from Kowalko et al. [23], we determined that surface fish feed at an average of ~ 80–90° angle compared to cavefish that feed at ~ 45°. While F_2 hybrids with an overbite feed at a similar angle to surface fish, F_2s with an underbite feed within a wide averaged range between 65–95° (**C**). Compared to F_2s with an overbite that have a maximum feeding angle of 90°, underbite F_2s had a significantly higher maximum feeding angle at 110° (**E**). There is a negative correlation (R = -0.4134; $p < 0.04$) between a higher maxillary – lower jaw angle and feeding posture angle (**F**). White scale bar set at 2 mm

if hybrids with an overbite feed like surface fish), we co-opted the feeding behavior assay used by Kowalko et al. [23]. Consistent with previous findings, cavefish fed at the expected posture with an average of 54°, compared to surface fish that fed at an average angle of 80° (Figs. 1C and 2A, B, Fig. S1A, B). F_2 hybrids with an overbite displayed a similar feeding angle to surface fish, with individual trial averages ranging from 76°-81° (post hoc Tukey $p > 0.05$; Fig. 2C). Surprisingly, F_2 hybrids with an underbite did not recapitulate cavefish feeding posture, feeding at a wider range of 62°-90° (Figs. 1C and 2D). An ANOVA revealed significant variation in feeding angle across populations ($F = 19.01$; $p < 0.001$). Further, feeding angle is negatively correlated with gape angle (Pearson correlation: R = -0.4134; $p < 0.04$; Fig. 1F), suggesting that fish with a larger gape feed at more acute angles.

Despite differing from cavefish feeding posture, F_2 hybrids with an underbite do display interesting feeding behaviors. Compared to hybrids with an overbite (maximum feeding angle at 90°), F_2 hybrids with an underbite had a maximum feeding angle of 110°, extending their lower jaws and feeding at an almost upside-down posture ($p < 0.001$; Fig. 1E and Fig. S1E). Cavefish also exhibit an increase in the number of teeth on both the upper and lower jaws [18]. Tooth number was counted for F_2 hybrids post behavior assay. There was no significant difference in tooth number in the upper jaw between overbite and underbite hybrids ($p > 0.05$; Fig. 2J). However, F_2 hybrids with an underbite have significantly more teeth in their lower jaw compared to F_2 hybrids with an overbite ($p < 0.001$; Fig. 2K). Taken together, F_2 hybrids with an underbite have similar morphology to cavefish, with an elongated lower jaw and an increase in tooth number.

We were able to take a closer look at feeding behavior by designing a method for extracting bite impressions during behavior trials. Food carpets (see Methods) were used to visualize the number of tooth marks made by a fish during a feeding strike (Fig. 2E-H). Surface fish, feeding at ~ 90°, were observed using their upper jaw to bite into the food carpet, leaving smaller bite impressions with an average of 6.7 tooth marks (Fig. 2E, L). In contrast, cavefish were observed using their lower jaws to make larger bite impressions, averaging 10 tooth marks per bite (Fig. 2F, L). F_2 hybrids with an overbite displayed similar biting behavior to surface fish, averaging 5.6 tooth marks per bite (Fig. 2G, L). Like cavefish, F_2 hybrids with an underbite made large bite impressions, averaging 8.6 tooth marks per bite (Fig. 2H, L). An ANOVA revealed significant variation in the number of tooth marks across populations ($F = 10.71$; $p < 0.001$). A post hoc Tukey test

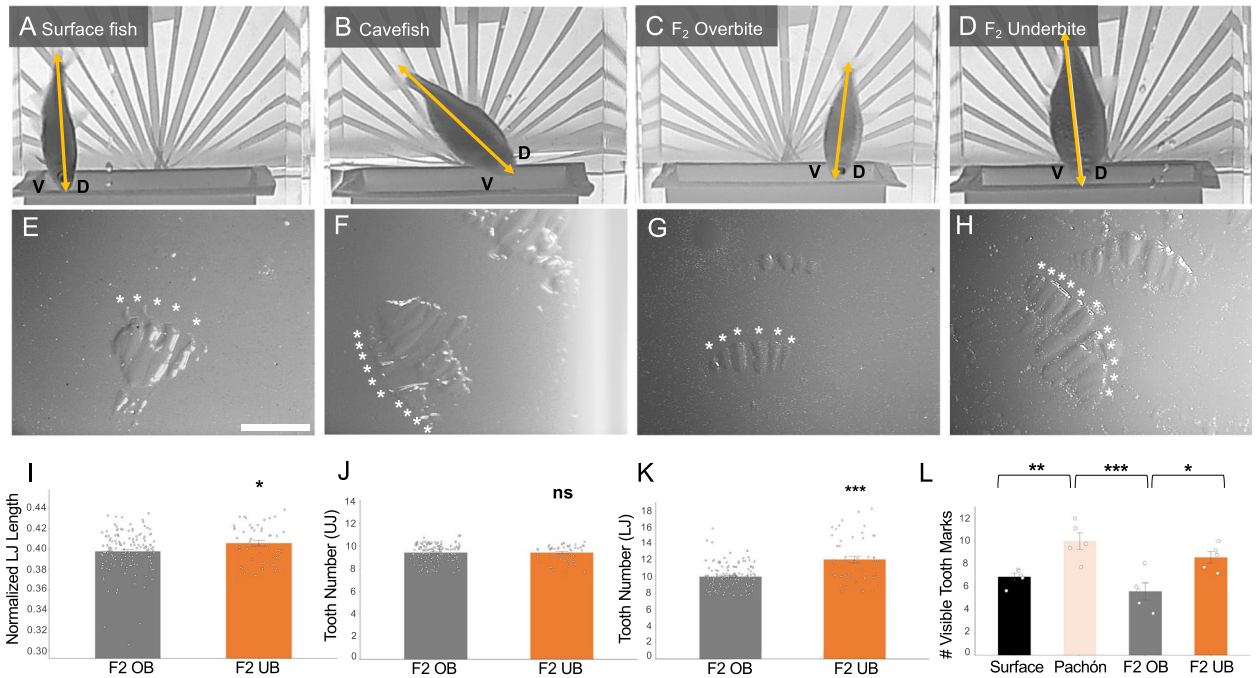

Fig. 2 Fish with an underbite leave a greater number of tooth marks in bite impressions compared to fish with an overbite. Surface fish (**A**, **E**) make bite impressions with fewer tooth marks (mean = 6.7) compared to cavefish (mean = 10; $p < 0.01$) (**B**, **F**, **L**). Accordingly, F_2 hybrids with an overbite (**C**, **G**) left fewer tooth marks (mean = 5.6) than F_2 hybrids with an underbite (mean = 8.6; $p < 0.05$) (**D**, **H**, **L**). F_2 hybrids with an underbite have significantly longer lower jaws (LJ) (normalized length; $p < 0.05$) (**I**) and an increase in lower jaw tooth number ($p < 0.001$) compared to F_2 hybrids with an overbite (**K**). There was no significant difference between upper jaw (UJ) tooth number between the two hybrid groups (**J**). White scale bar set at 1 mm

showed significant differences between overbite and underbite groups at $p < 0.05$ (See Table S2).

Bite differences are under genetic control in *Astyanax*

Quantitative trait loci (QTL) analysis was performed to assess whether bite differences in cavefish are associated with genetic loci. Within our F_2 mapping pedigree, ~ 25% of individuals were scored as having an underbite (Fig. 3B). A significant QTL peak was recovered for the bite phenotype that rose above the significance threshold ($p < 0.05$ LOD is 4.01) with a LOD score of 4.708 on linkage group (LG) 1 (Fig. 3C, D). The percent variance (PVE) explained by the bite phenotype is 9.4%. Seven genetic markers reside under the QTL peak with LOD scores ranging from 4.032 to 4.708 along a ~ 5 cM region on linkage group 1 (Table S3). The phenotypic effect for flanking genetic markers revealed that the homozygous cavefish genotype is associated with the underbite phenotype, while the heterozygous and homozygous surface fish genotypes are associated with an overbite (Fig. 3E).

The critical QTL region at the end of LG 1 was anchored to four Pachón cavefish annotated genome scaffolds (AstMex102; Table S3) [25]. The analogous scaffold regions mapped to an ~ 8 Mb region of chromosome (Chr.) 7 on the surface fish genome (Fig. 4) [26]. We used the surface fish annotation (v2.0) to identify 82 genes within the interval of 4 to 12 Mb on Chr. 7 (Fig. 4; Table S3).

Candidate genes for bite differences exhibit genetic alterations

To determine if cavefish harbor genetic alterations in candidate genes within the QTL interval, genomic sequences from wild-caught fish from multiple populations were assessed. We discovered three genes that had sequence alterations in Pachón cavefish, and in two other populations (Molino and Tinaja; Table 1). The gene *RAB19*, a member of the RAS oncogene family, is predicted to have a nonsynonymous single nucleotide polymorphism resulting in a single amino acid substitution (H164G) in all three cavefish populations compared to cDNA sequences in both Rascón and Choy surface fish populations. Next, we discovered a predicted single amino acid substitution (P412H) only present in the Pachón population for the gene *arfgap3*, known as ADP ribosylation factor GTPase activating protein 3, compared to surface fish.

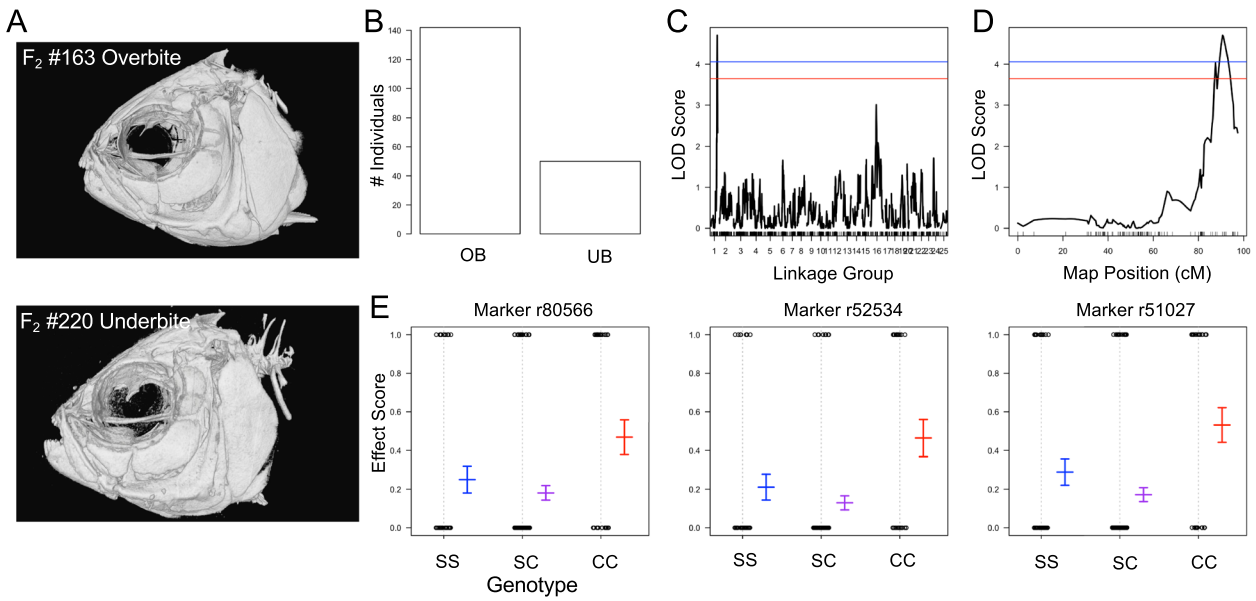

Fig. 3 Quantitative Trait Loci (QTL) analysis reveals a genetic basis for bite differences. Representative F_2 hybrid microCT images demonstrate bite differences scored as a binary trait for overbite (#163) and underbite (#220) (**A**). The frequency of F_2 individuals exhibiting an overbite was ~ 75%, while ~ 25% of pedigree was scored as having an underbite (**B**). A single QTL peak was recorded for the bite phenotype rising about the significance threshold (blue line $p < 0.05$; red line $p < 0.1$) (**C**). The QTL peak resides on linkage group 1 between map positions 86–95 cM (**D**). Genetic marker r52534 had the peak LOD score (4.708) and the effect plot indicates that the homozygous cavefish genotype is associated with an underbite, while the homozygous surface fish and heterozygous genotypes are associated with an overbite (**E**). Flanking genetic markers r80566 and r51027 illustrate the same phenotypic effect (**E**)

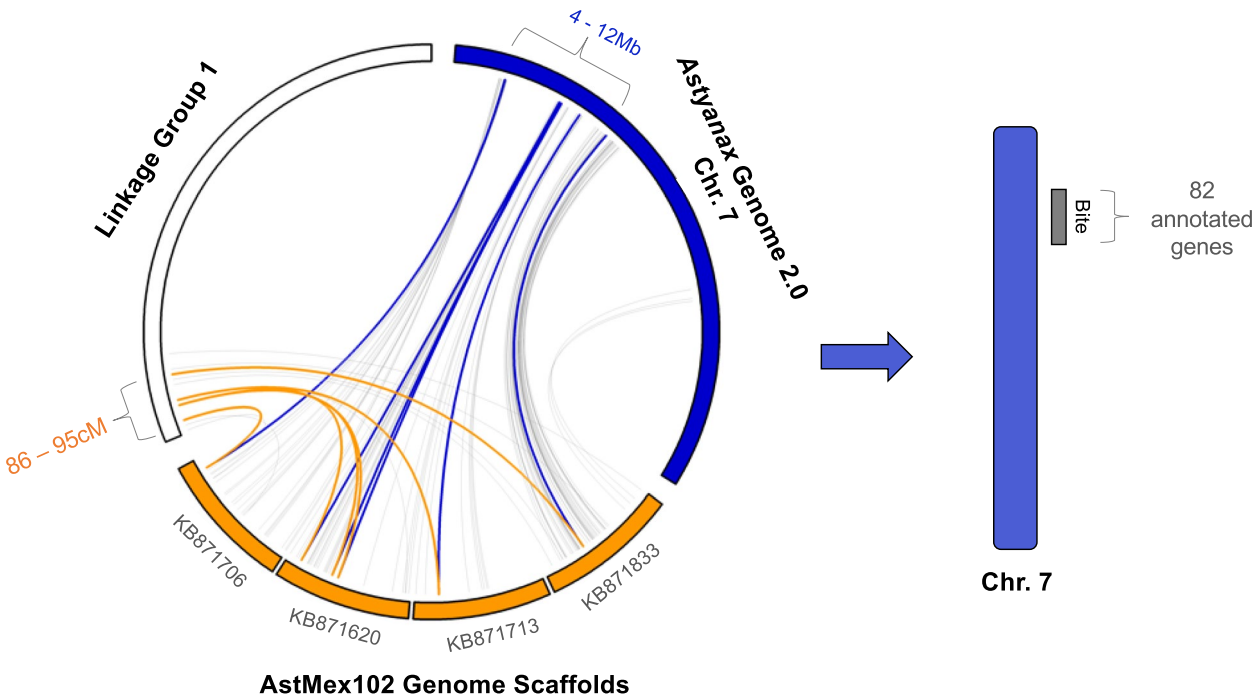

Fig. 4 The peak QTL region maps to both the Pachón cavefish and surface fish genomes. Six genetic markers on linkage group 1 (86–95 cM) were anchored to four Pachón cavefish genome (AstMex102) scaffolds (KB871706, KB871620, KB871713, and KB871833). These four cavefish genome scaffolds map to an 8 Mb region on *Astyanax* surface fish chromosome 7 (Table S3). Within this 8 Mb region on Chr. 7 resides 82 annotated genes. Three of the genetic markers map to another cavefish genome scaffold (KB871620) and to a ~ 1 Mb region on Chr. 7 with 24 annotated genes

Table 1 Genetic alterations identified in candidate genes associated with bite differences

Gene	Name	Location	Genetic Alteration	Amino Acid Change	Cavefish Population affected
RAB19	*RAB19, member RAS oncogene family*	7:84044128414687	Nonsynonymous SNP	Histidine -> Glutamine	Pachón, Molino, and Tinaja
arfgap3	*ADP ribosylation factor GTPase activating protein 3*	7:84467928462735	Nonsynonymous SNP	Proline -> Histidine	Pachón
pacsin2	*Protein kinase C and casein kinase substrate in neurons protein 2*	7:84640678496854	Nonsynonymous SNP	Phenylalanine -> Leucine	Pachón and Molino
large1	*LARGE xylosyl- and glucuronyltransferase 1*	7:90767099165670	Nonsynonymous SNP	Aspartic acid -> Asparagine	Pachón, Molino, and Tinaja
USP15	*ubiquitin specific peptidase 15*	7:99288599967471	1-13 bp deletions	-	Pachón, Molino, and Tinaja

Three genes with identified genetic alterations have known roles in bone development and homeostasis. A single amino acid substitution (F310L) was predicted for the gene *pacsin2*, known as protein kinase C and casein kinase substrate in neurons protein 2, in the Pachón and Molino populations compared to surface fish. Based on phenotypic annotations extracted from Ensembl's BioMart (v104), alterations in the *pacsin2* gene result in abnormal bone mineralization in mice. This gene also shows evidence of being under selection in the three cavefish populations based on diploS/HIC (hard selective sweeps in all three caves with neutral evolution in surface populations) and HapFLK (0.959; $p < 0.05$, indicating positive selection) selection analyses. Next, a single amino acid substitution (D721N) was predicted for the gene *LARGE1*, known as large xylosyl- and glucuronyltransferase 1 in all three cavefish populations. Annotations for the *LARGE1* gene suggest that mutations result in abnormal tongue morphology and bone structure in mice. By employing a diploS/HIC analysis, we determined that *LARGE1* shows evidence of hard selective sweeps in all populations, indicating that the ecotype-specific variation observed in this gene is likely driven by selection.

Finally, we discovered a putative deletion, ranging from 1–13 base pairs (potentially impacting amino acid positions 413–417) depending on the individual cavefish and population, in the gene *USP15*, known as ubiquitin specific peptidase 15. According to Biomart phenotypic annotations, alterations to *USP15* result in increased bone mineral density in mice. Further, *USP15* has been shown to enhance bone morphogenetic protein signaling by targeting ALK3/BMPR1A [27]. This gene also shows evidence of being under selection based on HapFLK rating (0.974; $p < 0.05$) and diploS/HIC, with hard selective sweeps in Tinaja and Molino, but not Pachón cavefish. While it is presently unclear how these alternations impact jaw growth in cavefish, these are candidates worth pursuing in future studies.

Discussion

Bite morphology is of functional importance for feeding, communicating, and breathing. As humans evolved smaller jaws, issues of malocclusion, tooth crowding, and facial pain arose [28]. Despite the increased in frequency of these aberrations, the precise genetic mechanisms controlling jaw size remain unclear. Here, we capitalize on the natural variation of jaw size and bite differences in divergent forms of teleost fish. We discovered that bite differences are indeed under genetic control in cavefish.

While the majority of previously studied craniofacial traits appear to be under complex genetic control in cavefish [29], we discovered a single QTL peak for the bite phenotype, with the frequency near a 3:1 (overbite: underbite) ratio in the F_2 pedigree, suggesting a Mendelian pattern of inheritance with the surface fish alleles being dominant. However, the QTL explains < 10% of the variance so this is unlikely a monogenic trait and it is possible that multiple genes or networks may be impacted. Five of the genes in the QTL region (*RAB19*, *arfgap3*, *pacsin2*, *LARGE1*, and *USP15*) exhibit fixed nonsynonymous mutations in cavefish compared to surface fish. We found that some mutations (*RAB19*, *LARGE1*, and *USP15*) were present in all three populations of the cavefish we investigated (Pachón, Molino and Tinaja). However, other mutations were only present in one or two populations compared to the surface fish. A potential explanation for this is that different cavefish populations may employ different genetic mechanisms to converge on similar phenotypes. An example of this is a mutation in the insulin receptor (*insra*) governing glucose intolerance in Pachón and Tinaja populations, but not Molino [11]. While the genes *pacsin2*, *LARGE1*, and *USP15* have been previously implicated in altered bone mineralization and may play novel roles in controlling bone size differences, none of the candidates have been specifically linked to changes in jaw morphology. Future functional analysis studies are needed to uncover the precise role of these genes in jaw development.

It is also possible that bite differences may not be mediated solely by a genetic mutation affecting the amino acid sequence, but rather a change in temporal or spatial gene expression during development. Protruding lower jaws have been observed in larval cavefish [12, 19], suggesting that lower jaw cartilage (Meckel's cartilage) may lay down the foundation for jaw size differences observed in adult skulls. Bone morphogenetic protein (BMP) signaling is a key regulator of endochondral ossification and has been shown to stimulate cell differentiation during cartilage development [30]. Allelic variation and expression of *bmp4* have been implicated in differences in cichlid jaw shape [31]. One candidate gene exhibiting sequence deletions in cavefish, *USP15*, is a known regulator of BMP signaling and may play a role in chondrogenesis of the jaw [27]. Another gene within the QTL region is *wnt7ba,* which together with ortholog *wnt7bb* are expressed in the developing zebrafish head as early as 24 h post fertilization [32] and wnt/beta-catenin signaling has been shown to induce cartilaginous matrix remodeling [33]. Together, *USP15* and *wnt7ba* should be further investigated across jaw development to determine if changes in expression result in an increase in lower jaw cartilage in cavefish.

While jaw size and dentition differences have been previously characterized in cavefish, the evolutionary mechanism underlying these changes remains unclear. Varying degrees of eye degeneration, shown through lens ablation studies, does not affect the length of the lower jaw [34] and we did not find any correlations with eye size and any jaw metrics presented here. However, there is overlap between bite and eye size QTL on chromosome 7 [23, 35]. This suggests a possible pleiotropic genetic mechanism underlying eye and jaw size differences.

Because it was necessary to perform behavioral analysis in live fish, we were not able to include feeding angle, tooth mark number and gape angle traits in our QTL analysis of the bite phenotype. However, previous studies provide insight to the relationship between feeding behavior and jaw morphology. Kowalko et al. [23]) determined that cavefish feed at a more acute angle compared to surface fish, but we did not find that F_2 hybrids exhibiting an underbite feed at the same posture as cavefish. Further, multiple QTL for feeding angle were discovered [23], but do not overlap with the bite phenotype. This suggests that feeding angle is controlled by a different genetic mechanism than jaw morphology in cavefish. A previously discovered QTL for jaw angle (ventral jaw width) does map to chromosome 7, but not at the same genomic position and a different scaffold than the bite QTL. Another previously characterized QTL for lower jaw size [35] maps to Chr. 14 near the gene *ghrb* [36]. From these studies we can infer that the size of the adult lower jaw is likely controlled by different loci than lower jaw protrusion or bite. Besides bone and cartilage, other features within the cranium may contribute to bite differences, such as potential muscle or joint differences.

While bite differences do not correlate with feeding angle, we did discover that an underbite is associated with differences in feeding strategy, such that fish with an underbite used their lower jaws, exposing more teeth in each strike compared to fish with an overbite. This is consistent with findings in cichlids, wherein fish with shorter, stout jaws feed on hard substrate, while fish with elongated jaws can range from suction feeders to predators [37]. Further, fish exhibiting a short dentary, with long distances between the quadrate joint of the jaw and opening/closing ligaments feed on attached foods, such as algae and microinvertebrates, requiring a greater force to remove from surfaces [7]. This is consistent with what surface fish likely encounter in terms of feeding ecology, although further behavioral studies are required in the natural environment.

In the caves, however, there is no photosynthetic input and few available prey options. Why then would cavefish need to evolve wider, longer jaws with more teeth? Espinasa et al. [38] analysed gut contents from wild-caught cavefish from the Pachón cave during both the rainy and dry seasons and determined adult cavefish mainly subside on a diet of bat guano and detritus. This suggests that cavefish use their larger jaws and increased tooth number to sift through detritus on the muddy cave pool floor. Additionally, cavefish have an increase in tastebud number, both extraorally and specifically within the lower jaw extending along the lingual epithelium toward the posterior part of the jaw [39]. Cavefish may have evolved an increase in jaw size and wider gape to expose more tastebuds, thus increasing taste sensitivity in a nutrient poor environment. Alternatively, tooth and jaw differences may have evolved as a consequence of indirect selection [40], in which case, sensory enhancements such as increased cranial innervation [41] and taste bud number were under selection causing pleiotropic changes that resulted cranial modifications.

Conclusions

Astyanax mexicanus cavefish have evolved morphological and behavioral adaptations in response to the extreme pressures of the cave environment. By coupling behavioral analyses together with QTL mapping, we show that bite differences in cavefish are under genetic control and confer differences in feeding strategy. Further studies using genetic perturbations will uncover the precise mechanisms governing these changes. Taken together, we have established cavefish as a powerful genetic model

for understanding evolutionary changes in morphology and behavior, particularly in the context of jaw evolution.

Materials and methods

Fish husbandry and specimens

Fish were bred and maintained in the laboratory of Dr. Clifford Tabin at Harvard Medical School on a custom recirculating system (Temperature: 23 °C, pH: 7–7.5, and Conductivity: 1200-1400μS) under a 10:14-h light/dark cycle. F_1 hybrids were generated from a paired mating of male *Astyanax mexicanus* surface fish (derived from the Río Choy river) and female Pachón cavefish. The genetic mapping pedigree was made up of F_2 hybrids ($n=219$; three clutches) from paired F_1 surface x Pachón hybrid siblings [42]. For behavioral analysis a second F_2 population ($n=30$) was generated from a single cross of F_1 siblings. It has been previously determined that there is no maternal effect on jaw morphology for hybrid crosses by looking at reciprocal hybrids [43]. All procedures were approved under the Institutional Animal Care and Use Committee IACUC protocol (#IS00001612).

Feeding behavior assay

We created "food carpet" molds that were placed at the bottom of assay tanks, from which we could recover bite impressions. Solidified gelatinous food carpets were made using comestible gelatin (Knox). Gelatin powder was melted in boiled, filtered reverse osmosis water and mixed with a solution base of infused fish pellets (New Life Spectrum Thera + A) using an electric kettle (Muller) in a ratio of 1:1. The warm liquid mix was poured into silicone molds, chilled at room temperature and stored overnight at 4 °C for solidification. Molds with solidified gelatin were placed at the bottom of recording tanks filled with water, occupying the entire bottom of the tank as a "food carpet" (Fig. 2A-D).

The feeding behavior assay was performed on surface fish ($n=5$), Pachón cavefish ($n=5$), and F_2 hybrids ($n=20$) recorded in 1.7L tanks. Each fish was recorded under a condition of complete darkness for 1 h (h) with a high definition infrared camera (Grundig Pro, Germany). All assays were watched live to control for actual feeding episodes; feeding episodes are described here as active mouth-picking on the "food carpet". After 1 h of trial, fish were returned to their housing tank and food carpets were extracted from recording tanks. Food carpets were dried for ~ 12 h in a low humidity room and imaged under a light stereomicroscope (Leica M165FC) at 32 × magnification (Fig. 2E-H).

Tanks were filmed via a front-facing camera and videos were acquired through Open Broadcaster Software (OBS) studio in ".avi" format. Videos were manually analysed with Odrec software (S. Pean, IFREMER, France) to quantify the average and maximum body angles adopted by the fish over 1 h periods for each feeding episode (Fig. S1). Accurate measurements of the body angle were facilitated with a protractor overlayed directly on the tank in 10° quadrants (Fig. 2A-D).

Phenotypic analysis

To assess the maximum mouth opening ("gape"), specimens ($n=8$ from each group) were sacrificed using a lethal dose (400 ppm) of tricaine (MS-222; Sigma) and immediately imaged under light microscopy at 20 × before rigor mortis set in to maintain flexibility in the jaw joints. Upper and lower jaws were pinned using Styrofoam backing at the maximum gape (Fig. 1A, D). Gape was measured as the angle at the intersect of the maxillary and dentary (lower jaw) bones using the angle tool in ImageJ software (v2.0.0-rc-69). An analysis of variance (ANOVA) and post hoc Tukey's HSD were performed using R studio software (v2022.07.2; Table S1). Lower jaw length was measured in F_2 hybrids ($n=186$) using the line tool in ImageJ and normalized to fish standard length (Fig. 2I). For pairwise comparisons, a t-test comparison of means (StatPlus:mac LE v6.2.21) was used to test for statistical significance.

For three-dimensional analysis of the bite phenotype, high resolution micro-computed tomography (MicroCT) imaging was performed at the Center for Advanced Orthopaedic Studies at the Beth Israel Deaconess Medical Center (Boston, MA). MicroCT scans were performed on Pachón cavefish ($n=5$), surface fish ($n=5$), and F_2 hybrids ($n=219$) at 15uM resolution producing ~ 500 DICOM formatted images per specimen that were reconstructed into a single three-dimensional volume rendered file using Amira software (v6.0; FEI Company, Hillsboro, OR) according to methods outlined in Powers et al. [44].

Quantitative Trait Loci (QTL) analysis

The program R/qtl (v1.46–2) [45] was used to perform QTL analysis. A linkage map comprising of genetic loci identified from genotyping-by-sequencing (GBS) technology was previously assembled by Riddle et al. [46]. The linkage map consisted of 1,839 GBS markers from 219 F_2 individuals assembled into 25 linkage groups. Bite was scored as a binary trait; overbite was scored as 0 and underbite was scored as 1 (Fig. 3A). A genome-wide logarithm of odds (LOD) score was calculated for the bite phenotype by performing a permutation test (1000 random permutations). The Haley-Knott regression model for a binary trait was used and peak markers rising above the significant LOD threshold (4.01; $p<0.05$) were extracted and phenotypic effect plots were generated to

determine which genotypes were associated with bite differences (Fig. 3E).

Markers within the critical QTL region, defined by genetic markers exceeding the LOD $p < 0.05$ threshold (Table S3), were mapped to the Pachón cavefish genome (AstMex102) scaffolds [46] and the surface fish genome (*A. mexicanus* genome 2.0) chromosomes using the BLAST algorithm (Ensembl v108). The associated regions between the linkage map (LG1), cavefish genome scaffolds, and surface fish genome were visualized by generating a Circos plot (Fig. 4) [47]. We used the annotated *A. mexicanus* surface fish genome (v2.0) to identify genes within the QTL region.

Sequence analysis

A population genomic analysis was performed using DNA from wild-caught specimens from Río Gallinas (surface fish Rascón population), Río Choy (surface fish Choy population from which laboratory surface fish are derived), as well as Pachón, Tinaja and Molino caves in Mexico. We used a 10 individuals per population for sequence assessment. Population genomic metrics and analysis procedures are outlined in Riddle et al. [42]. cDNA sequences were aligned using SnapGene (v6.1.2), from which fixed coding sequence changes were noted (Table 1). We identified known zebrafish, mouse and human phenotypes associated with candidate genes using the BioMart tool in Ensembl (v104). To determine if genes within the QTL region are under selection, we performed genome scans using hapFLK and diploS/HIC analyses to show evidence of selective sweeps [48, 49]. Briefly, diploS/HIC employs multiple population genetic statistics to infer selection, classifying genomic windows as either containing a hard or soft sweep, whether they are linked to a genomic region under selection, or under neutral evolution. HapFLK is an Fst-based analysis of selection in a hierarchical tree and haplotype structure.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12862-023-02149-3.

Additional file 1: Supplemental Table 1. Gape angle statistics.

Additional file 2: Supplemental Table 2. Tooth mark statistics.

Additional file 3: Supplemental Table 3. Genetic marker alignment to the Pachón and surface fish genomes.

Additional file 4.

Additional file 5.

Additional file 6: Supplemental Figure 1. Ethograms illustrate feeding posture differences between surface, cavefish and hybrids. Consistent with findings from Kowalko et al. [23], we determined that surface fish have a near perpendicular feeding posture with an average angle between 80°-90° (A) and cavefish feed at a lower angle of 40°-60° (B). Surface x Pachón F_2 hybrids demonstrated three feeding posture categories: surface-like F_2 hybrids with an average feeding angle between 80°-90° (C), a mix of surface- and cave-like feeding postures with angles ranging from 40°-90° (D), and an extreme obtuse posture with angles up to 110° (E).

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Authors' contributions

AKP, CH, and CJT designed the study. AKP, CH, MRR, YKK, KT, AA, TEB, EF, BM and JBG collected, analyzed data and created figures. AKP wrote the manuscript draft. RLM and SEM performed population genetic analysis. CH, SEM, RLM, MRR, JBG and CJT edited the manuscript. All authors read and approved the content of the manuscript.

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Availability of data and materials

The phenotypic and genotypic raw data used in this study is available in Supplementary file Table S4. The sequencing datasets analysed during the current study were submitted to SRA and the accession numbers are listed in Supplementary file Table S5.

Declarations

Ethics approval and consent to participate

All methods were performed in accordance with relevant guidelines and regulations. Animal protocols for this study were approved by the Institutional Animal Care and Use Committees (IACUC; protocol #IS00001612) and the Harvard Medical Area Standing Committee on Animals at Harvard Medical School (Boston, MA, USA).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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