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Growth dynamics and molecular bases of evolutionary novel jaw extensions in halfbeaks and needlefishes (Beloniformes)

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Abstract

Evolutionary novelties—derived traits without clear homology found in the ancestors of a lineage—may promote ecological specialization and facilitate adaptive radiations. Examples for such novelties include the wings of bats, pharyngeal jaws of cichlids and flowers of angiosperms. Belonoid fishes (flying fishes, halfbeaks and needlefishes) feature an astonishing diversity of extremely elongated jaw phenotypes with undetermined evolutionary origins. We investigate the development of elongated jaws in a halfbeak (*Dermogenys pusilla*) and a needlefish (*Xenentodon cancila*) using morphometrics, transcriptomics and in situ hybridization. We confirm that these fishes' elongated jaws are composed of distinct base and novel 'extension' portions. These extensions are morphologically unique to belonoids, and we describe the growth dynamics of both bases and extensions throughout early development in both studied species. From transcriptomic profiling, we deduce that jaw extension outgrowth is guided by populations of multipotent cells originating from the anterior tip of the dentary. These cells are shielded from differentiation, but proliferate and migrate anteriorly during the extension's allometric growth phase. Cells left behind at the tip leave the shielded zone and undergo differentiation into osteoblast-like cells, which deposit extracellular matrix with both bone and cartilage characteristics that mineralizes and thereby provides rigidity. Such bone has characteristics akin to histological observations on the elongated 'kype' process on lower jaws of male salmon, which may hint at common conserved regulatory underpinnings. Future studies will evaluate the molecular pathways that govern the anterior migration and proliferation of these multipotent cells underlying the belonoids' evolutionary novel jaw extensions.

KEYWORDS
evolutionary novelty, halfbeak, heterochrony, jaw development, needlefish, transcriptomics

1 | INTRODUCTION

The term 'evolutionary novelty' (or 'evolutionary innovation') typically describes a derived unique (apomorphic) trait that might have arisen via an evolutionary novel and distinct developmental trajectory or pathway (i.e. without apparent homologue). Evolutionary novelties often opened up a new ecological niche to a lineage, thereby possibly facilitating an adaptive radiation (Pigliucci, 2008). Understanding how often complex traits can evolve seemingly from scratch will require the characterization of the developmental mechanisms by which they form. Surprisingly, in some cases novel features arose through relatively few evolutionary steps by co-opting the regulatory genetic circuitry from other, evolutionarily older traits. Such examples include the turtle carapace, certain genital structures in fruit flies, 'gin-trap' beetle pupae or rhinoceros beetle horns (e.g. Glassford et al., 2015; Hu et al., 2018; Kuraku et al., 2005; Ohde et al., 2018). It however remains to be tested how prevalent this mechanism is during novel trait evolution for it can be expected that many routes can produce evolutionary novelty.

Teleost fishes are the most diverse lineage of vertebrates and across their phylogeny some traits can be considered 'evolutionary novelties'. Examples include the pharyngeal jaws in cichlid fishes (Liem, 1973; Schneider et al., 2014), bony spines in the acanthomorpha (Höch et al., 2021; Near et al., 2013) and male pregnancy in syngnathids (pipefish and seahorses; Lin et al., 2016; Whittington & Friesen, 2020; Schneider et al., 2022). Another example is the distinctly elongated upper and/or lower jaws of the Belonoidei ('needlefishes' and allies, comprising 248 species; Froese & Pauly, 2010). Their jaw elongation likely contributed to their ecological diversification and speciation, as suggested by the considerably less species-rich sister taxon, the suborder Adrianichthyoidei (ricefishes, including Medaka), which lack elongated jaws (only 37 Adrianichthyoidei species; Froese & Pauly, 2010).

Belonoid fishes feature diverse jaw phenotypes, and jaw lengths vary, both throughout their phylogeny and their development (Boughton et al., 1991; Kobayashi et al., 2020; Lovejoy et al., 2004). Two elongated jaw types are distinguished: the 'halfbeak' type, which has a lower jaw that extends far anterior to the upper jaw, and the 'needlefish' type, with both upper and lower jaws being elongated. Developmental diversity is also observed, because needlefish larvae possess a 'halfbeak' phenotype, while some halfbeaks have larvae with jaws that resemble the ancestral state (i.e. not elongated). Furthermore, in flying fishes (Exocoetidae), a family within the Belonoidei, either normal or halfbeak phenotypes dominate in both larvae and adults, while in some species the larvae have a halfbeak phenotype, but adults have seemingly normal, nonelongated jaws (Fahay, 2007; Lovejoy et al., 2004).

Elongated jaws have evolved repeatedly across fishes. For example, lineages within the poeciliids and characins, as well as gars and pikes, have superficially similar phenotypes to needlefish that evolved independently of the belonoids (e.g. Enny et al., 2021). These are interpreted as being 'developmentally stretched out' through proliferative processes that act along the entire embryonal jaw axis (Hilton et al., 2014; Kammerer et al., 2006). However, belonoid jaw development appears to be different to gars or pikes (Gunter et al., 2014; Hilton et al., 2014). For example, in the wrestling halfbeak (*Dermogenys pusilla*) jaw elongation is initiated during early postnatal development by skeletal elements resembling a pair of bar-like protrusions at the tip of the lower jaw. These appear to scaffold the incipient jaw extensions (Boughton et al., 1991) and have been referred to as 'toothless extensions' sensu Gunter et al. (2014). Furthermore, these skeletal elements have been proposed to form part of Meckel's cartilage (a structure that scaffolds the dentary; Clemen et al., 1997). However, their histological characteristics in the wresting halfbeak (Gunter et al., 2014) as well as needlefish (Hilton et al., 2014) cast doubts on this interpretation homology assignment (Gunter et al., 2014). Rather, it appears that in belonoids Meckel's cartilage does not contribute to the elongated part of the lower jaw but instead remains restricted to the jaw base (Gunter et al., 2014; Hilton et al., 2014). Therefore, outgrowth of the belonoid lower jaw appears to occur through a unique and lineage-specific mechanism, classifying the extension as an evolutionary novelty of this lineage of teleost fishes (Gunter et al., 2014). The mechanism for jaw extension in the upper jaw in needlefishes has not yet been characterized. Comparisons of candidate genes between the upper and lower (extension-bearing) jaw in the wrestling halfbeak *D. pusilla* suggested a potential role of *calm1* during jaw extension outgrowth (Gunter et al., 2014). However, we lack a more detailed understanding of the similarities and differences in the genetic and developmental mechanisms underlying the upper and lower jaw elongation in belonoids.

Here, we further investigated the evolution and development of elongated jaws in belonoids. Using the wrestling halfbeak (*Dermogenys pusilla*) and the freshwater 'garfish' (*Xenentodon cancila*—a needlefish), we performed linear morphometrics on stained and cleared specimens to assess the growth dynamics of the 'jaw base' and the 'jaw extension'. We described their relative contributions to the extended jaw phenotype and investigated how this elongation is mediated on a molecular level using RNA sequencing and protein class analysis. Spatial gene expression analysis was conducted using in situ hybridization in the examined needlefish species, permitting the comparison of spatial regions within jaw parts. Using these results, we identify a developmental pathway that may orchestrate the jaw elongation in belonoids.

2 | METHODS

2.1 | Fish husbandry

Oryzias latipes, *Dermogenys pusilla* and *Xenentodon cancila* individuals were obtained from the commercial pet trade and the laboratory of Prof. Manfred Schartl (University of Würzburg) and were bred for at least one generation at the University of Konstanz animal care facility. *D. pusilla* were kept in groups of 5–20 individuals in ~100 L tanks. To obtain newborn fish, pregnant females

(*D. pusilla* is an internally fertilizing and live-bearing species) were kept individually until they gave birth, after which the female was returned to the stock tank. Newborns were raised in E3 water and fed with *Artemia* nauplii. *X. cancila* were kept in shallow 600 L tanks in groups of three to ten individuals, which were fed with live zebrafish, poeciliids or goldfish. Eggs were laid daily and attached to plants or rocks. Eggs were collected whenever needed and incubated in large petri dishes in E3 with added Methylene Blue. After hatching, the larvae do not feed for 2 to 3 days, after which they were fed with zebrafish larvae. All experiments were conducted in accordance with local ethics standards (Regierungspräsidium Freiburg, G16/12, Tierversuchsanlage Universität Konstanz T15/04 and T18/01).

2.2 | Morphological analyses of jaw development

Morphometric measurements of distinct jaw parts were recorded throughout the early development of both species. To do this, fish were euthanized with an overdose of MS222, fixed with 4% PFA (in PBS, pH = 7.4) and stained with Alcian Blue and Alizarin Red to visualize cartilage and ossified tissues, respectively (protocol of Gunter et al., 2014). These stained specimens were photographed, including one lateral shot of the whole specimen and one of the head, and measurements were from the photographs (see Figure S1). These were as follows: fish length (FL; from the centre of the eye to the tip of the caudal peduncle), length of the 'upper jaw base' (UJB; from the most posterior point of Meckel's cartilage to the most anterior tip of the ethmoid plate), length of the 'upper jaw extension' (UJE; from the most anterior tip of the ethmoid plate to the upper jaw tip), length of the lower jaw base (LJB; from the most posterior point of Meckel's cartilage to its anterior tip) and length of the 'lower jaw extension' (LJE; from the most anterior tip of Meckel's cartilage to the lower jaw tip). These measurements were taken for needlefish on specimens euthanized within 24 h after hatching and also after 1, 2, 3, 4, 7 and 10 additional days. For halfbeaks, the same measurements were taken on specimens euthanized 2, 4, 7, 10, 15 and 30 days after birth (Data S1).

2.3 | Transcriptome analysis

To explore gene expression patterns associated with the different jaw parts, we conducted a pilot RNA-seq experiment. Eight *Dermogenys pusilla* individuals were sampled in total and were terminally sedated using an overdose of MS222 (0.04%). Of these, four were sampled at the day of birth (0 days post-birth [dpb]), when no recognizable jaw extensions were detected. Four individuals were sampled when jaw extensions started to form, which was in two individuals after 12dpb and in two others after 16dpb. From the four 0dpb samples, the upper and lower jaws were dissected and stored in RNA later at −20°C. From the older samples, the lower jaw extension (LJE) was dissected, and also the upper jaw (UJ) and the remaining lower jaw base (LJB) were dissected off the body and stored in RNAlater at −20°C.

RNA extraction was performed for these 20 tissue samples using a Qiagen RNA Mini kit according to the manufacturer's recommendations and including an on-column DNase digestion. RNA quality and quantity were determined using an Agilent Bioanalyzer and an Invitrogen Qubit. cDNA libraries were synthesized using an Illumina TruSeq kit and these were sequenced at the University of Konstanz, on a GAIIx sequencer (Illumina). After de-multiplexing, reads were trimmed and adapters were removed using the Trimmomatic software (v 0.34; Bolger et al., 2014). An average of 11.3 million reads per sample (forward and reverse reads) were obtained, with the lowest read number among samples quantitatively analysed being 8 million.

A de novo transcriptome was assembled using the Trinity pipeline (v.2.4.0; Haas et al., 2013) on these 227 million reads, resulting in 86,722 Trinity transcripts. To filter and annotate transcripts, a reference protein sequence database was created comprising all protein sequences known from medaka (*Oryzias latipes*) downloaded from Ensembl (v92). The Blastx algorithm was used to query the Trinity transcripts to this database using an e-value of 1e-8 and transcripts with a hit were retained (44,340 transcripts). To improve our annotation, transcripts without an assigned gene name were blasted against a transcript database derived from other selected teleosts (stickleback, Tilapia, Fugu, Amazon molly, zebrafish, cave fish, cod, platyfish, *Tetraodon*, downloaded from Ensembl), which had previously been filtered to only contain annotated sequences (e-value of 1e-8). Any annotations that were added in this way were marked with '*', while for any transcripts with hits in the Ensembl Medaka Protein database, retained their Medaka annotation (Data S2).

The Salmon software (v. 0.8.1; Patro et al., 2017) was used to quantify gene expression per transcript and sample, using the filtered Trinity transcript library as a reference Subsequently, gene counts (for differential expression) or TPM (for PCA) of transcripts with the same Ensembl Protein ID were summed (15,446 final unique protein IDs—referred to as 'gene' subsequently). For our analyses, only the older individuals with a recognizable jaw extension were considered (i.e. four individuals, each lower jaw base, lower jaw extension and upper jaw; Data S2).

Gene expression was analysed using principal component analysis (PCA) based on centred and scaled TPM (transcripts per million reads) expression data. Additionally, raw p-values for pairwise comparisons of gene expression between jaw structures were calculated using count data and DEseq2 for datasets containing always two focal jaw parts (e.g. 'lower jaw extension' vs. 'upper jaw'; with the design ~individual + jawpart to account for nonfocal differences among individuals; Love et al., 2014). Comparisons were only computed when a given gene (i) was expressed in all individuals of at least one of the two compared groups and (ii) had an average TPM expression value of >0.1 across all samples of the two groups, and obtained *p*-values were corrected for multiple testing using false discovery rate. As the sample size is rather low and sample selection

cannot exclude nested effects, which putatively biasing the data set, our data analyses were focussed on exploratory methods and results derived from *t*-tests should be treated with caution, as analysed data do not fulfil all data requirements for this test. Moreover, we conducted further investigations of the spatiotemporal dynamics of gene expression as a means of confirming the results of our RNA-seq analyses.

In order to identify genes specifically associated with the lower jaw extension, we selected 1% of genes with the most negative loading on PC2, as PC2 discriminated best between jaw extension samples (negative values) and the remaining samples (positive values, see Results section). To compare the gene composition between tissue types, also the 1% most positively loaded genes were selected, reflecting the genes most enriched in the other tissues (lower jaw base and upper jaw). The Panther gene ontology webtool (https://www.pantherdb.org/, v14.0) was used to annotate gene ontogeny terms using *Oryzias latipes* as reference. For 199 and 230 of these genes from the lower jaw extension and other parts, respectively, a GO protein class could be assigned. The 1% threshold was chosen so that enough genes would be included in the focal set to have reasonably broad representation of protein types without diluting possible differences between focal gene sets too much.

2.4 | In situ hybridization

Needlefish larvae were prepared for whole-mount in situ hybridization of different ages. Euthanized fish were fixed in 4% paraformaldehyde overnight, transferred into 100% methanol via a gradient and then stored at −20°C until usage. For the full protocol, see Woltering et al., 2009; Höch et al., 2021. Briefly, samples were transferred to PBS, bleached and cleared using KOH and H_2O_2, acetylation was performed and the permeability was increased via a proteinase K treatment, prehybridization and hybridization was then performed with the respective digoxigenin (Dig)-labelled RNA probe overnight at ~70°C (temperature were adjusted according to the probes binding properties). Target genes were selected based on preliminary transcriptome analyses and literature research. Primers for in situ probes were designed based on transcriptome read sequences, DNA targets amplified via PCR, fragments then cloned and Dig-labelled RNA probes synthesized. After hybridization, remaining probes were washed out and samples were blocked using blocking reagent and then treated with anti-Dig antibodies for several hours. After unbound antibodies were washed out over several days, BM-Purple (Roche) was used as a substrate for colour development.

3 | RESULTS

Using both morphometric measurements and *RNA-seq*, we analysed the morphological characteristics, growth dynamics and (for the halfbeak) gene expression patterns underlying their distinct jaw development, with a particular focus on the evolutionarily novel jaw extension (Figure 1).

3.1 | Morphological characteristics of jaw outgrowth in halfbeak and needlefish

Growth was considered for the jaw base and extension individually. At birth, halfbeak jaw morphology resembled that of fish without jaw extension, such as medaka (Figure 1b;G). In most larvae, two anlagen of the bar-like protrusions scaffolding the lower jaw extension anteriorly to the Meckel's cartilage arch were present at birth. Less mature embryos, born with an external yolk sac did not show any detectable anlagen, suggesting that these normally develop around the time of birth. Throughout subsequent development, the bar-like protrusions continued to grow terminally, and a thickening of the jaw extension was often observed at the distal tip. Ossification of the outgrowing jaw extension scaffolds started proximally and effectively likely reinforced the junction of the scaffolds and the jaw base (i.e. dentary bone), and these two segments became increasingly less distinguishable. The upper jaw also increased slightly in length, but did not show any indication of bar-like protrusions and generally elongation was less pronounced than in the lower jaw. Importantly, this slight extension could be attributed to an elongation of the premaxillary bones rather than to developing the bar-like protrusions characteristic of the lower jaw.

Needlefish larvae at hatching stage typically showed bar-like scaffolds in the lower jaw extension that were similar to the halfbeak, but notable outgrowth and ossification at its base commenced earlier (Figure 1k). Anlagen for bar-like protrusions could also be detected at the anterior tip of the upper jaw; however, outgrowth was lagging behind that of the lower jaw during the first days post-hatch. In needlefish manually removed from their chorion 1–2 days before their natural hatching time, anlagen for lower or upper jaw scaffolds could not be detected, suggesting that they develop around the hatching stage. Subsequent development in needlefish jaws differed from that of halfbeaks, most notably in that it proceeded much faster (Figures 1, 2) and that both upper and lower jaws developed tooth-bearing extensions. In needlefish, the upper jaws also lack the terminal thickening often present in the lower jaw. Alizarin Red fluorescence photography of the needlefish's lower jaw at ~7dph (Figure S2) showed that initially woven bone was formed at the base of the lower jaw extension, which stands in stark contrast to the lamellar bone of the dentary. This corresponds to the particularly fast type of bone formation (Gorski, 1998) observed in this region during early postembryonic development. Sections of the distal-most region of the lower jaws of needlefish (Figure S3) illustrated that the tip of the extension is spearheaded by the two bar-like growth zones, while slightly more proximate sections reveal that these merge to form a zone with a more homogeneous appearance, which then transitions into a

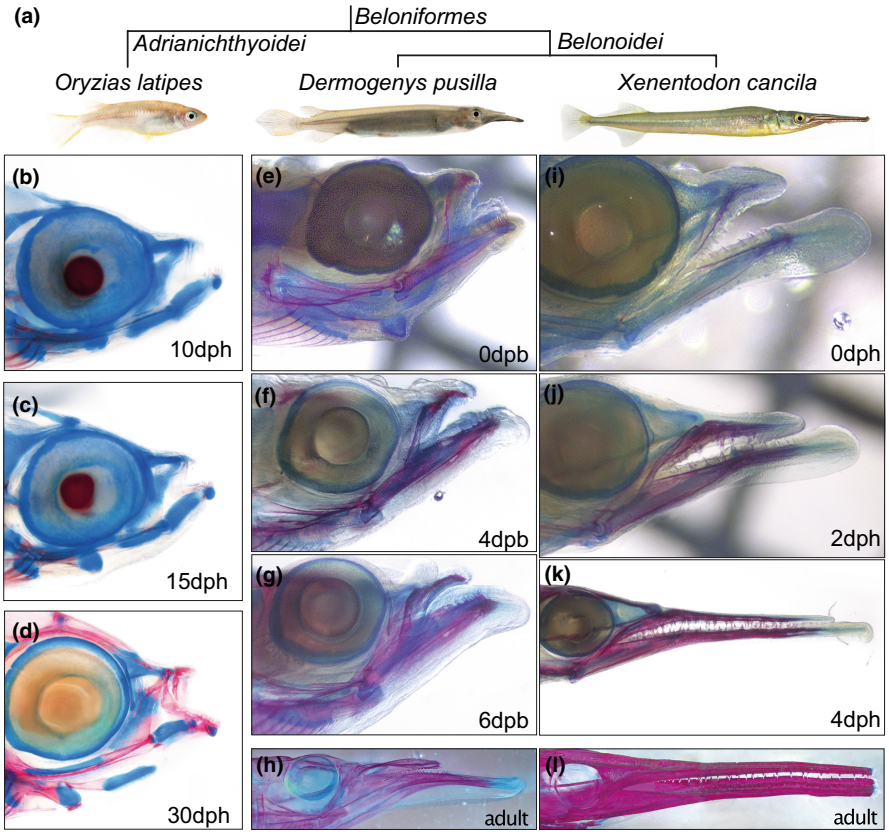

FIGURE 1 Early postembryonic head development in three Beloniformes. (a) Simplified cladogram of Beloniformes with *Oryzias latipes* (Medaka, a rice fish) representing the suborder Adrianichthyoidei, and *Dermogenys pusilla* (wrestling halfbeak, a halfbeak) *Xenentodon cancila* (freshwater garfish, a needlefish) representing the Belonoidei (=Exocoetoidei). (b–m) Alizarin Red (stains ossified bone) and Alcian Blue (stains cartilage) stainings of heads from a lateral perspective. (b–d) Medaka postembryonic jaw development. Notably, while the adult jaw does not show any jaw extension, a cartilaginous joint connects the two halves of the lower jaw anteriorly, which does not ossify in adults. (e–h) halfbeak neonates show similarities to medaka embryos, but rod-like anlagen emerge at the anterior tip of the lower jaw, which within a few weeks form the adult lower jaw extension. Adult lower jaws are equipped with prominent sensory membranes running ventrally and laterally along the lower jaw. (i–l) Needlefish neonates' lower jaw morphology resembles that of few-day-old halfbeaks, as growth buds emerge 1–2 days prior hatching. Growth buds on the upper jaw emerge 1–2 days after those on the lower jaw. Early outgrowth of the jaw extension is much faster than in halfbeak. Adult jaws are heavily ossified.

flattened bone upon ossification. In the upper jaws, interestingly, this homogeneous zone is missing.

3.2 | Morphological spatiotemporal dynamics of jaw development of halfbeak and needlefish

Halfbeak larvae grew relatively steadily and reached an average standard length of ~17 mm after 30 days (Figure 2a). Morphometric measurements confirmed that jaw extension outgrowth was considerably slower compared with the needlefish (see below). Throughout the observed 30 days post-birth, the total jaw length contributed increasingly to total body length, and while isometric growth of the lower jaw base was reached after approximately seven to ten days post-birth, the growth of the lower jaw extension remained allometric, at least until 30 dpb (Figure 2b).

In the needlefish *X. cancila*, the allometric phase of jaw growth occurred during seven to ten days post-hatching and is thus also considerably faster than in *Belone belone*, the needlefish species studied by Gunter et al. (2014) and Rosenthal and Fonds (1973). In *X. cancila*, overall body growth was particularly fast in the first four days post-hatching, followed by a pause and then by continued slower growth after the start of feeding (Figure 2c). In the first four (to seven) days post-hatching, both upper and lower jaw lengths grew allometrically faster than the rest of the body: while the jaws measured about 12%–15% of the FL on hatching day, the relative size of lower and upper jaw increased to about 32% and 28%, respectively, after 7 days post-hatching (Figure 2d). Afterwards, relative jaw size remained stable, indicating isometric growth until adult stages (values from an adult: 33% and 32%, respectively). When the lower jaw (Figure 2e) and upper jaw (Figure 2f) bases and extensions are considered separately, it is apparent that the allometric growth was mostly a result of allometric extension growth, as the jaw base to extension ratio decreases from ~1.3 and 2 to 0.65 and 0.5 in the lower and upper jaw, respectively, from hatching to 10dph. With ongoing development,

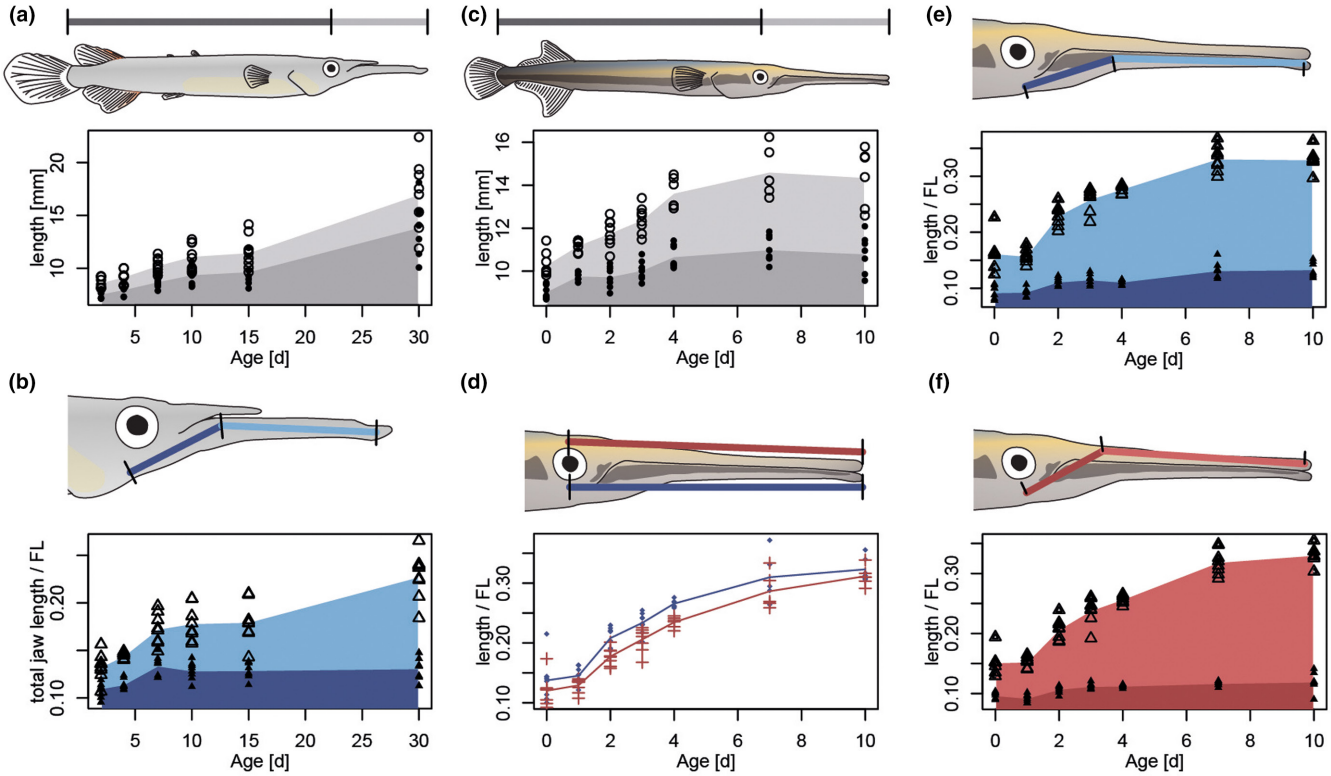

FIGURE 2 Jaw size development in a halfbeak and needlefish throughout early postembryonic development. (a) Halfbeak body and jaw growth throughout early development. The lower jaw grew allometrically throughout the first 30 days after birth and increases in relative length. (b) Relative length of the lower jaw base (dark blue) and extension (light blue) considered separately. The lower jaw base showed allometric growth only during the first ~5dpb (then the growth line slope is ~0), while the extension continues to grow allometrically throughout the observed 30dpb. (c) Absolute needlefish body and jaw lengths throughout early development. Allometric growth of both jaws was concluded after ~7 days post-hatch. (d) Relative total jaw lengths in needlefish. The jaw length was always slightly greater in the lower than in the upper jaw. The absolute length difference between jaws appeared to remain constant throughout development. (e–f) Relative length of the jaw base (darker colour tone) and stacked on top the relative length of jaw extension (lighter colour tone) for the lower and upper needlefish jaw, respectively. Plots illustrate that primarily the jaw extension contributed to the allometric growth, while the base showed much less allometric growth. Note that minimum *y*-axis values are >0.

the angle between the lower jaw base and extension increased and approached 180°, that is, the lower jaw base and its extension become parallel, and their individual lengths summed up to approximately the total jaw length of an adult, which thus increases the relative contribution of jaw base lengths to overall jaw lengths (as measured in Figure 2c).

Jaw growth dynamics in investigated halfbeak and needlefish were thus divergent in that halfbeaks go through a several week-long phase of slow allometric growth until the final jaw-to-body length ratio is reached, while the needlefish *X. cancila* has a brief phase of rapid allometric growth, after which the final jaw-to-body length ratio is reached.

3.3 | Transcriptomic divergence among jaw sections

Transcriptome analyses were performed on halfbeak upper jaws, lower jaw bases and lower jaw extensions to identify gene expression patterns for the extension and to compare them to the other jaw parts. We first examined the (on average) most highly expressed genes in the sampled lower jaw extensions. The mitochondrial *nd5* gene was the most highly expressed gene, suggesting an abundance of mitochondria in this tissue, followed by structural genes, *krt4* (and *krt1* and *krt8* slightly later), *col10a1a*, and soon after *col1*, *col1a* and *col1a1b* (these collagen genes made up ~75% of all collagen reads, Figure 3). The expression of many ribosome-associated genes (e.g. *eef1a*, *rpl7*, *rpl7a*, *rpl4*, *pabpc1a* and *rpl9*) suggests pronounced translational activity. With the exception of the collagens, these genes were similarly high expressed in the lower jaw base.

Next, we explored the global transcription patterns in halfbeak lower jaw bases, extensions and upper jaws, using a principal component analysis (PCA). We detected distinct clustering according to tissue identity across PC1 (~42% of total variation) and PC2 (~22%; Figure 4, Figure S4). Specifically, PC1 separated the upper jaw from the lower jaw base (with the lower jaw extension samples having intermediate scores), while PC2 separated the lower jaw extension samples from the others. The composition and identity of particularly heavily loaded genes on the PC axes were investigated firstly

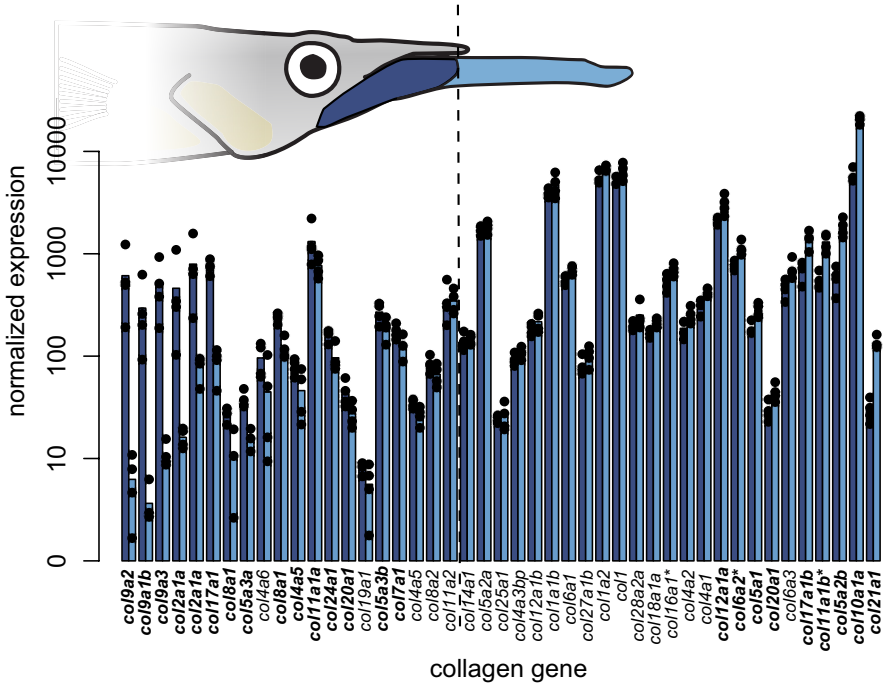

FIGURE 3 Collagen gene expression in needlefish lower jaw base and extension. Genes are sorted along the *x*-axis according to fold-change between lower jaw extension and the lower jaw base. Significantly differentially expressed genes between lower jaw parts after correction for multiple testing are indicated in bold. Note the log-TPM scale of the *y*-axis.

to delineate the tissue transcriptional characteristics (note that substantial loading does not necessarily imply significant difference; Figure 4, Data S2). Among the genes showing a strongly negative loading on PC1 (i.e. genes expressed more in the lower jaw than the upper jaw base) were several calmodulin-like genes, such as *pvalb4* and *pvalb1/pvalb1.1*, as well as other ion transporters (e.g. *atp2a1l*), which regulate cytosolic calcium ion concentrations and are involved in bone formation. Several (actin-) cytoskeletal protein genes also loaded very positively on PC1, including *mylz3*, *myl1*, *myl13*, *smyhc2* and actin-binding proteins, such as *tnni2.1*, *tnni2a.1*, *tnni4b.2* and *tnni1d*. These genes suggest a more pronounced cytoskeletal reorganization in the lower jaw than upper jaw. Also, numerous muscle-related genes were enriched in the lower jaw samples, such as *tnnc2*, *eno3* or *myhz1.1*, most likely due to the inclusion of higher amounts of muscle tissue in these samples (e.g. the tongue). Genes positively loaded on PC1, that is genes associated with the upper jaw, notably include many G-protein coupled receptors (e.g. *olfcg6*, *olfcj1*, *v2rl1*, *v2rx3*,...) and *otx1* and *neurod4*. These genes are likely to function in the development and function of the sensory tissues located on the upper jaw (e.g. neuromasts; Hirota et al., 2015). Among the most positively loaded genes was also *alx3*, a major developmental transcription factor gene specific to the upper jaw. Finally, we also note that other calmodulin-like/Ca^{2+}-ion binding genes were positively loaded on PC1, such as *s100z*, *pvalb5* and *icn*.

PC2 separates the halfbeaks' lower jaw extension (negative loadings and scores) from the lower jaw base and upper jaw. Most negatively loaded genes (most neg. 1%; i.e. genes highly expressed in the lower jaw extension but not the jaw bases) included many transcription factors, such as *hand1*, *alx1*, *alx4b*, *msx1* (and *msx1a*), *msx2*, *msx3*, *lhx9*; and genes associated with the extracellular matrix, such as *has1*, *cryaa*, *zpld1b*, *emilin2b*. Additionally, several genes involved in neuronal development and function (*nap1l1.1*, *cpa6*, *lgi1b*, *mab21l2*, *crhr1*, *galr1a*, *ppef1* and *neto2b*) loaded negatively as well as genes associated with cell proliferation, such as *rfc5*, *s100a1* and the aforementioned *nap1l1*. Also, strongly negatively loaded was the smooth muscle development inhibitor *prdm6* and the craniofacial cartilage-associated gene *ucmaa* (a downstream target of Runx2 and OSX; Lee et al., 2015). We also identified several genes involved in WNT signalling, for example *rspo2* (WNT signalling inhibitor), *wnt2ba*, *wnt2bb* and *wnt5b*. In contrast, most positively loaded gene included matrilin genes 1 & 2 (*matn1*: ENSORLP00000013999 & ENSORLP00000014004; *matn2*: ENSORLP00000003614 & ENSORLP00000003617), as their expression was virtually absent in the jaw extension but they were highly expressed in both upper jaw and lower jaw base. These matrilin genes are involved in cartilage formation, as are *acanb*, *epyc* and *chadlb*. Additionally, muscle-related genes were positively loaded, such as *tnni1d*, *tnnt1*, *mybpc1* and *mypc2b*, suggesting that the extension did not contain considerable amounts of muscle tissue at the stage investigated. Finally, many genes related to sensory perception were positively loaded, such as *otos*, *v2rl1*, *olfcg6* and *olfcj1*, reflecting likely that some sensory organs (e.g. nose, lateral line organ) were not present at the extension at the stage investigated.

3.4 | Protein class analysis of candidate genes

Protein class analysis of 1% of the most positively and negatively loaded genes, respectively, on PC2 was conducted using Panther (Figure 5, Data S3). While differences in protein class composition between genes with negative and positive loading were not formally tested, the proportion of genes whose expression was found to be positively associated with the lower jaw extension is considerably lower (~>2-fold) in the protein classes 'transporter',

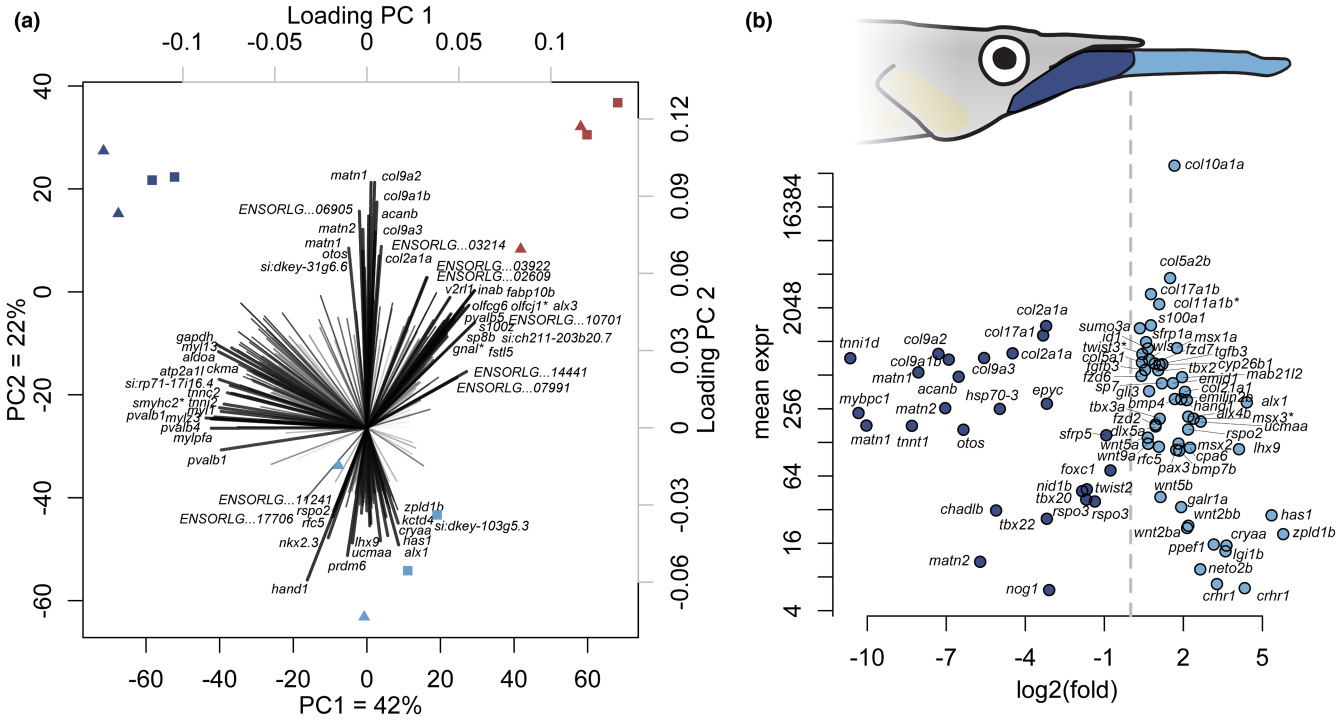

FIGURE 4 Plot of PCA scores & loadings and differential gene expression between lower jaw parts. (a) For PCA scores (lower *x*-axis and left *y*-axis for PC1 and PC2, respectively), dark blue (upper left cluster) are all samples of the lower jaw base, dark red are all samples of the upper jaw (upper right cluster) and light blue are all samples of the lower jaw extensions (lower cluster). Squares reflect 12dpb samples while triangles are 16dpb samples. PC1, reflecting 42% of total variation, separates upper jaw samples for lower jaw samples, while PC2, reflecting 22% of total variation, separates the lower jaw extension from the other samples. Loadings on PC1 and PC2 and PCA loadings (upper *x*-axis and right *y*-axis for PC1 and PC2, respectively) for ~1% of the most positively and negatively loaded genes are shown, and ~0.1% are labelled. Very negatively loaded genes on PC1 (e.g. *pvalb1*) reflect genes whose expression is associated with lower jaw bases, while the expression of very positively loaded genes (e.g. s100z) is associated with upper jaws. On PC2, most negatively loaded genes (e.g. hand1) are highest expressed in lower jaw extension, while expression of most positively loaded genes (e.g. matn1) is atypical for lower jaw extension. (b) Plot illustrating the fold-change (*x*-axis) and mean expression (*y*-axis) of significantly differentially expressed genes between lower jaw base and extension mentioned in the manuscript (for full results, see Data S2).

'cytoskeletal protein' and 'transfer/carrier protein', while it was considerably higher (~>2-fold) in the protein classes 'hydrolase', 'nucleic acid binding', 'signalling molecule', 'transcription factor' and 'oxidoreductase'. These results suggest that the jaw extension showed increased levels of gene expression regulation and metabolic rate compared with the jaw base.

3.5 | Differential gene expression between the lower jaw base and extension

Pairwise comparisons of gene expression between jaw structures revealed 1759, 1761 and 1724 differentially expressed genes (DEGs) for the comparisons lower jaw extension vs. lower jaw base, lower jaw base versus upper jaw and lower jaw extension vs. upper jaw, respectively (Data S2). Here, we focussed on the comparison of the lower jaw extension vs. lower jaw base and focussed primarily on genes involved in cartilage and bone formation.

In order to identify the molecular pathways underlying bone growth in the lower jaw extension, we first contrasted collagen gene expression in jaw base versus extension (Figure 3). Some collagen genes showed significantly reduced expression in the jaw extension compared with the jaw base. These include *col9a2*, *col9a1b*, *col9a3*, *col2a1a* (ENSORLP00000015981 & ENSORLP00000006442), *col17a1* and others (Figure 3), many of which also loaded positively on PC2 (Figure 4), indicating that they are typically expressed in the jaw base. Other collagen genes showed higher expression in the extension, including *col21a1*, *col10a1*, *col5a1* & *col5a2b*, *col11a1b* and *col17a1b*, and others (Figure 3).

Moreover, our analysis indicated that several major chondrogenic and osteogenic signalling pathways were differentially expressed between the lower jaw base and extension: the gene *sp7* (*osterix*) and *dlx5*, which alongside *runx2* play crucial roles in osteoblast differentiation and the repression of chondrocyte differentiation (Sinha & Zhou, 2013), were upregulated in the lower jaw extension, as was *tgfb3* (Wu et al., 2016). The osteoblast marker gene *runx2* was not detected in our transcriptome as no Trinity transcript blasted to the medaka gene, possibly due to strong divergence. Nonetheless, our transcriptome analysis revealed several upstream regulators in the BMP signalling pathway whose expression levels differ between the lower jaw base and its extension. For example, both *bmp7b* and *bmp4* were upregulated in the jaw extension.

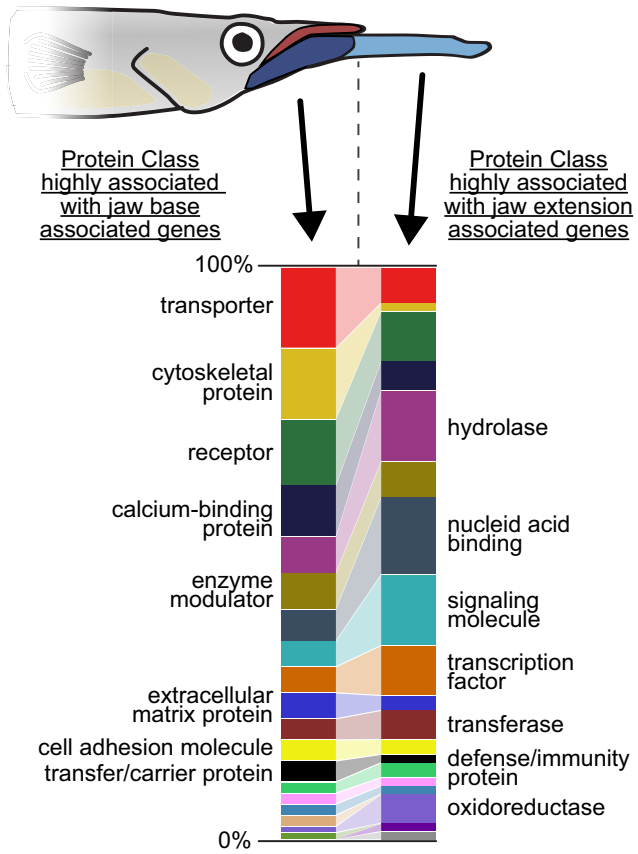

FIGURE 5 Analysis of over-represented protein classes of the 1% most negatively and positively loaded genes of PC2 from the PCA analysis. Protein classes associated with most genes are 'transporter', 'cytoskeletal protein', 'receptor' and 'calcium binding' in the lower jaw base of halfbeaks, suggesting pronounced bone formation in this morphological portion of the jaw. In the jaw extension, most dominant protein classes are instead 'hydrolases', 'nucleic acid binding', 'signalling molecule', 'receptor' and 'transcription factor', suggesting an increased metabolic rate and cell proliferation and differentiation.

Our results suggest the upregulation of osteoblast differentiation pathways in the jaw extension, relative to the base for both needlefish and halfbeaks. In situ hybridization confirmed strong *bmp7b* expression in the upper and lower jaw extensions of needlefish throughout its outgrowth, but not in the jaw bases (Figure 6). Both BMP transcription factors activate *msx1a* (Oxburgh et al., 2005), whose expression was also found to be highly upregulated in the jaw extension compared with the base in halfbeaks (Data S2), as shown by in situ hybridization and transcriptomics (Figure 6). In addition, the likely osteoclast-specific BMP signalling receptor *bmp1rba* (Nobutaka et al., 2020) was downregulated in the jaw extension, suggesting downregulation of bone degradation. The BMP antagonist noggin1 (*nog1*) was also found to be significantly downregulated in the jaw extension, indicating potentially increased BMP signalling and alongside the upregulation of BMP targets *tbx2* and *tbx3a* (both positively regulate osteoblast proliferation; Govoni et al., 2006). Finally, *foxc1* was downregulated in the jaw extension, and while earlier studies suggested a positive role of this gene on BMP signalling (Rice et al., 2003), more recent studies suggest a negative regulatory relationship, where this signalling pathway blocks osteoblast differentiation (Caddy et al., 2020). In line with the latter study, we also found upregulation of the *id1* gene, a positively regulated target of BMP signalling (Caddy et al., 2020).

Further evidence of likely osteoblast activation in the jaw extension was observed in the halfbeak RNA-seq data. As already indicated by the second PC axis's loadings, several genes involved in WNT signalling were differentially expressed between the jaw bases and extensions. All differentially expressed *wnt* genes (wnt2ba, *wnt2bb*, *wnt5a*, *wnt5b* and *wnt9a*) were upregulated in the jaw extension, as was the WNT target gene *axin2* (Green et al., 2017) and the WNT-associated chaperone *wls* (Zhong et al., 2012). The WNT signalling activators *rspo2* and *rspo3* were up- and downregulated in the jaw extension, respectively. Similarly, the WNT signalling suppressor *sfrp1a* and *sfrp5* were up- and downregulated, respectively. The retinoic acid pathway (which inhibits osteoblast differentiation) is likely to be downregulated in the jaw extension, as *cyp26b1*, which causes retinoic acid degradation (Laue et al., 2008), was upregulated in the extension. Finally, we identified the upregulation of *gli3* in the jaw extension, an antagonist of the chondrocyte inducing transcription factor *ihh* (Hilton et al., 2005).

Although the majority of the genes detected in the jaw extension have roles in the development and function of bones, we identified some genes involved in cartilage development. For example, we detected considerable expression of *sox9* and *col2a1a* in the jaw extension, although it was lower than the expression in the base (Figure 6 for *col2a1a*). Additionally, the *ucmaa* gene, which is thought to be involved in cartilage matrix formation (Bar Oz et al., 2016; Neacsu et al., 2011), was associated with the jaw extension according to PC loadings. Although we detected the downregulation of the antiosteogenic transcription factor *twist2* in the jaw extension, an inverse pattern was found for *twist3*, a member of the twist gene class without homologue in mammals and in which to date no function during bone formation is known (Germanguz et al., 2007). Finally, *pax3*, a transcription factor expressed in multipotent neural crest precursor cells that likely shields these cells from BMP-induced differentiation (Wu et al., 2008), was found to be upregulated in the jaw extension. *Pax3* expression may have been upregulated by the strongly expressed *sumo3a* genes (Gill, 2005).

Our transcriptome data suggest the presence of migrating neural crest cells in the jaw extension, which contribute to its outgrowth. This notion is supported by the upregulation of *alx1*, a gene involved in neural crest cell migration (Dee et al., 2013), as well as by the upregulation of several frizzled genes, a WNT receptor, such as *fzd2*, *fzd6* and *fzd7*, which potentially contribute to neural crest cell migration. The differential migration and division of these neural crest cells in different taxa may contribute to the observed differences in the development and evolution of the jaws in the Belonoidei.

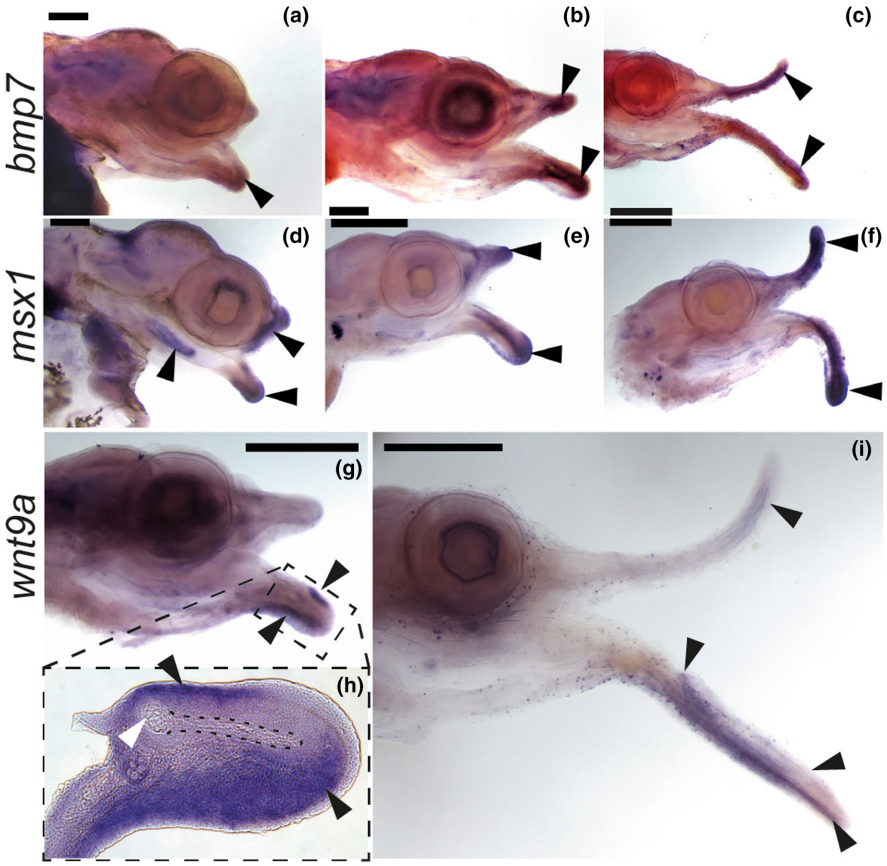

FIGURE 6 Photographs of colour in situ hybridization in needlefish at different stages of jaw outgrowth. (a–c) *Bmp7* is expressed in both the upper and lower jaw extensions, but in contrast to *msx1*, not in the tip of the upper or lower jaw extensions (d–f). (g–i) *Wnt9a* showed expression patterns comparable to *bmp7*; however, expression in the upper jaw extension was much lower than in the lower jaw extension. Notably, *wnt9a* (and also *msx1*, data not shown) expression is absent from the scaffold of the jaw extension. Scale bar is 200 μm (a, b, d) or 500 μm (c, e, f, g, i).

4 | DISCUSSION

Belonoids have evolved a unique jaw extension that allows them to efficiently localize and collect food items from their environment, which likely facilitated their global diversification (Gunter et al., 2014; Hirota et al., 2015; Montgomery & Saunders, 1985). Here, we provided the first comprehensive description of the development of the elongated jaws of two species of this group, the halfbeak *Dermogenys pusilla* and the needlefish *Xenentodon cancila*. We confirmed previous studies, indicating that extended jaws are not simply evolutionarily 'stretched-out' jaws but consist of two morphologically distinct portions, which we refer to as the jaw base and the jaw extension. The jaw base corresponds to the mandible of other fishes that forms surrounding the anterior part of Meckel's cartilage, while the jaw extension is a more anterior structure only found in belonoid fish.

Our observations of belonoid jaw growth show that the jaw base and the jaw extension contribute differently to their extended jaw phenotypes. First, we observed positive allometric growth of belonoid jaws in comparison with their overall body length during early larval development. While the jaw bases in both upper and lower jaws contributed to this effect, their extensions (if present) contributed more substantially (Figure 2). Interestingly, elongated jaw bases appear to be a conserved trait across the belonoid phylogeny, whereas jaw extension lengths are flexible and vary strongly during ontogeny (Lovejoy et al., 2004). Together, this suggests that independent regulatory circuitries govern the growth of the jaw base and jaw extension. This notion is supported by the recent description of an extensionless 'halfbeak' *Nomorhamphus aenigma* (Kobayashi et al., 2020) that has stretched jaw bases comparable to other species of the genus with jaw extensions.

Our study of the transcriptional characteristics of the wrestling halfbeak's lower jaw revealed the regulatory mechanisms orchestrating jaw extension outgrowth and details of its cellular composition. Pronounced differences were observed in gene expression between the jaw base and jaw extension. Notably, we observed a lower expression of muscle-related, neuron-related and sensory-related genes in the extension. This suggests that the jaw extension lacks muscle tissue and sensory organs at the examined stage, although older (adult) stages have a well-developed lateral line in the lower jaw extension (pers. obs., Montgomery & Saunders, 1985; Hirota et al., 2015).

Our transcriptome data also shed light on the nature of the hard tissues that comprise the jaw extension of halfbeaks. We interpret our results in light of the three main types of bone formation observed in teleost fish (Apschner et al., 2011). These include (i) endochondral ossification, which involves the secondary ossification of a cartilage scaffold, and (ii) intramembranous and (iii) perichondral ossification, where bone is deposited without direct replacement of a cartilage scaffold. Further bone types that only occur transiently in mammals are found as permanent components of the skeleton in teleosts, such as chondroid bone, where

a fibrous layer gives rise to osteoblasts-like cells that deposit osteoid but later transdifferentiate into cells with chondrocytes-like morphologies and characteristics (type I, specifically, see Witten et al., 2010). Our transcriptome dataset provides strong evidence for osteoblast activity in the jaw extension and weak evidence for chondrocyte activity. This includes expression of cartilage-related genes *sox9*, *col2* or *col9a*, *acanb* (Witten & Hall, 2002; Figure 6) and the BMP receptor *bmpr1bb* (Yoon et al., 2005). The transcriptome data are consistent with previous histological data that suggest the jaw extension is not formed by anterior growth of Meckel's cartilage (see Figure S2; Gunter et al., 2014). Instead, gene expression patterns observed in halfbeaks indicate pronounced osteoblast differentiation in line with intramembranous, perichondral or chondroid ossification (Witten et al., 2010), but traces of chondrocyte characteristics favour perichondral or chondroid ossification as likely mechanisms. For instance, the jaw extension displays strong expression of *msx1*, *msx2* and *msx3*, which are involved in dentary development in other fish. Specifically, *msx1* leads to mesenchymal stem cell proliferation, while *msx2* appears to impair chondrogenic differentiation in favour of osteogenesis (Alappat et al., 2003; Figures 4, 6).

The results of our transcriptome and morphological analyses suggest that lower jaw extension growth is driven by an anterior growth zone likely containing a population of multipotent proliferating cells that are shielded from differentiation and migrate continuously anteriorly during jaw development. This interpretation is based, in part, on the expression of *alx1* in the lower jaw extension at approximately 12dpb in *D. pusilla* (but not base; log2fold of 4.2). ALX1 is a major regulator of craniofacial bone development, which suppresses the differentiation of neural crest cells (which form the craniofacial bones and cartilages) and facilitates their migration (Hu et al., 2019). We propose two possible developmental origins of these undifferentiated cell populations: either (i) they might be derived from Meckel's cartilage's perichondrium and their anterior migration is initiated before the cell condensations of the dentary bone form, or (ii) they may originate from early dentary intramembranous cell condensations. In teleosts, both cell lineages may differentiate into osteoblast(–like) cells. We thus propose that in belonoids, this cell population shows an increased proliferation and migrates anteriorly as the result of prolonged *alx1* expression. This process might thereby initiate the formation of the bar-like protrusions observed in halfbeaks and needlefish. *Pax3* expression in the jaw extension further suggests that this population might be shielded from osteogenic signals, such as BMP and WNT, which are highly expressed in the surrounding tissue. Cells generated by this population that are left behind by the migrating tip of the jaw extension likely leave the *pax3* expression zone and differentiate to osteoblast(–like) progenitor cells, eventually differentiating to osteoid-depositing osteoblast(–like) cells. Identifying the molecular mechanism that maintains this population of multipotent cells is a pertinent question that should be addressed by further research. This is likely to involve noncanonical roles of developmental regulators such as *alx1*, as its mutation in humans causes 'frontonasal dysplasia', a dramatic shortening of the jaw bones (Uz et al., 2010).

Uniquely, we detected the expression of multiple cartilage-associated genes in the outgrowing jaw extension, which is surprising given the apparent lack of a cartilage template in this structure. This property may be relatively common in the osteoblasts of teleosts. For example, teleost periosteal osteoblasts derived from a perichondrium can show low levels of cartilage gene expression, such as *sox9* (as discussed in Paul & Crump, 2016). In this case, *sox9* might repress *pax3* and its target *sostdc1* (both were upregulated in the jaw extension), which repress BMP signalling and bone formation (Cairns et al., 2012; Wu et al., 2008), maintaining the patency of the tip of the extension. Alternatively, it may orchestrate neural crest cell migration in the jaw extension (Simões-Costa et al., 2014), which is likely to be of central importance during its morphogenesis and outgrowth.

The pattern of bony outgrowth arising from the tip of the dentary is also observed in other teleosts displaying rapidly growing mandibular ornaments. Specifically, Witten and Hall (2002) investigated the microstructure of the male salmons' hook-like extension of the lower jaw (the 'kype') during the mating season, describing it as '…"making bone as fast as possible and with as little material as possible." Unlike the normal compact bone of the dentary, the new skeletal tissue contains chondrocytes and cartilaginous extracellular matrix' (known as chondroid bone; Apschner et al., 2011). This description resembles, at least superficially, the development of the jaw extension in belonoids: rapid bone formation with a distal osteoid zone, and some involvement of chondrocytes (or genes associated with them; Gillis et al., 2006), that add cartilage matrix to the base matrix of the structure, all of which happens at the anterior tip of the lower jaw. The developmental process underlying the belonoids' jaw extensions may therefore share some homology with those underlying kype growth in salmon, suggesting the presence of a deeply conserved or convergently evolved bone formation mechanisms in teleosts that are typically used for temporary structures (such as in many flying fish and some halfbeaks; Fahay, 2007). While in our view transcriptional profiles suggest that bone matrix is likely the result of intramembranous or perichondral bone formation, it is possible that chondroid bone formation does contribute to some extent (as in the case of the salmon kype), especially if chondrocyte(–like) cells feature transcriptional similarity to osteoblasts. Comparative analyses of the kype and jaw extension ultrastructure may provide further insights into the histological similarities of the two structures.

The belonoids have evolved a unique developmental mechanism to extend their jaws, which likely allowed them to diversify into new ecological niches. We reveal that the jaw elongation in the lower (and upper) jaw is primarily (but not exclusively) due to a novel structural element, the jaw extension, that rapidly grows during early postnatal development. We propose a model where growth of the jaw extension involves distal bone formation, initiated by a population of multipotent cells at the anterior tip of the jaw, which continuously differentiate into osteoblasts that deposit bone matrix. Additionally, our observation of cartilage-associated genes suggests

minor cartilage characteristics in the jaw extension. Teleost skeletal structures sharing characteristics of both bone and cartilage have been described before, such as in the rapidly growing salmon kype formed from chondroid bone, suggesting that the potential for rapid bone formation at the anterior tip of the jaws may be a deeply conserved mechanism in teleosts that was evolutionarily co-opted to form extended jaws in belonoids. Understanding the molecular bases of rapid bone deposition in teleosts will promote a better understanding of jaw diversifications among the Belonoidei and of the origins of teleost skeletal novelties more generally.

AUTHOR CONTRIBUTIONS

Helen M. Gunter, Axel Meyer, Joost M. Woltering and Ralf F. Schneider conceived the study. Helen M. Gunter, Inken Salewski and Ralf F. Schneider conducted experiments. Ralf F. Schneider, Inken Salewski and Joost M. Woltering analysed the data and wrote the draft of the manuscript. All authors revised and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The raw reads supporting the conclusions of this article are available in the NCBI SRA repository, PRJNA1000498. Other datasets supporting the conclusions of this article are included within the article supplementary data files.

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SUPPORTING INFORMATION

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

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