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PATTERNS & PHENOTYPES

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Normal development in *Ambystoma mexicanum*: A complementary staging table for the skull based on Alizarin red S staining

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Abstract

Background: As the role of *Ambystoma mexicanum*, or the Mexican axolotl, expands in research applications beyond its traditional use in studies of limb regeneration, a staging table that is more anatomically extensive is required. Here, we describe axolotl skull development as it relates to previously established developmental stages that were based on limb development.

Results: We find that most key developmental events in the skull correspond to these previously established stages, creating easily recognizable stages of axolotl throughout skull morphogenesis.

Conclusions: With this complementary staging table in hand, researchers can stage axolotl larvae when limb data are missing or incomplete, or when cranial data alone is available.

KEYWORDS

Ambystoma mexicanum, axolotl, development, skull, staging

1 | INTRODUCTION

Ambystoma mexicanum, commonly referred to as the axolotl, is a widely used model organism particularly in studies of vertebrate development and limb regeneration.[1-8] Early development in the axolotl (i.e., from fertilization to hatching and prior to forelimb bud formation) has been described extensively[9-12]; however, the only staging table that describes post-hatching development pertains to stages defined by events in limb development.[13] While the Nye et al.[13] staging table is a valuable resource for axolotl researchers, the role of axolotl in research is ever expanding beyond its historic use in limb regeneration studies.[14-17] As such, researchers studying the cranial anatomy of axolotl are currently unable to quickly and accurately assign stages to material consisting of skulls only, and researchers lack the means to assign a stage to specimens artifactually or pathologically missing limbs. Description of skull development in axolotl, complementing current stages based on limbs, would solve these and additional limitations presented by current staging tables.

Here, we provide such a table in an attempt to assist researchers studying aspects of axolotl skull development. Using our staging table, researchers can confidently assign their specimens to the stages of Nye et al.[13] by means of cranial anatomy rather than strictly limb anatomy. Our staging table therefore complements that of Nye et al.[13] We find, in most cases, key events in skull development are represented by existing stages in the table of Nye et al.[13] However, the earliest Nye et al.[13] stages (i.e., 44 and 45) did not have any substantial ossifications; therefore, ossification events in the skull of axolotl start at stage 46. This new complementary staging table for the skull of axolotl will hopefully be a useful tool in aiding researchers synthesizing anatomical data from a whole body perspective.

2 | RESULTS

2.1 | Stages 44 and 45

No ossifications are evident at this stage. Only cartilaginous chondrocranial and hyobranchial elements are present. A couple of tooth crowns are faintly stained in the future coronoid region in at least one specimen at stage 45 (Figure 1A,B,C). In another one of the larger specimens, approaching stage 46, a faintly ossified coronoid bone was observed on the medial surface on both sides of the lower jaw.

2.2 | Stage 46

In this stage, the first obvious ossifications appear. Here, four paired elements are present: vomers, palatines, coronoids, and dentaries. The tiny vomer occurs in the anterior portion of the future palate, anterior to the eyes (Figure 1D,E,F). It is a small, circular element with at least one tooth present. Posterior to the vomer is the first sign of the palatine. It is a small, similarly-sized, circular element. In one specimen, only the palatine appears, suggesting the palatines appear before the vomers. On the dorsomedial portion of Meckel's cartilage, lateral to the level of vomer and palatine, the coronoid is present as a thin ossification (Figure 1D,E,F). In one specimen, the dentary is present as a very small and thin ossification on the anterior margin of Meckel's cartilage. Left and right dentaries are separated from one another medially by a substantial gap (the presumptive symphysis).

2.3 | Stage 47

The vomers and palatines are still roughly the same size as one another, and each bears about four to five teeth. In one specimen, the parasphenoid has started to ossify. It is represented by a faint patch of stained bone in the midline region just anterior to the otic capsules.

All specimens at stage 47 now have consistently present dentaries. The dentary is present as a thin sliver of bone on the anterior margin of Meckel's cartilage. The gap between dentaries (the future symphysis) is wide, as in the one specimen, at stage 46, with dentaries present. The dentary is slightly bowed with minimal ossification progressing posterolaterally onto the lateral surface of the lower jaw (Figure 1G,H,I). There is no overlap with the coronoid at this stage. At least one specimen has two to three teeth in the dentary. The coronoid ossification is growing and it now fills the space between the end of the dentary and the anterior margin of the eye (Figure 1G,H,I). There are five to seven teeth in each coronoid.

2.4 | Stage 48

The premaxilla has appeared as a thin strip of bone at the anterior margin of the skull. A weakly-developed point on its dorsal margin represents the future alary process in one specimen (Figure 1J,K,L). There are no teeth on the premaxilla at this stage. The vomer and palatine have increased in size slightly. The vomer now appears more oval-shaped than the circular palatine (Figure 1J,K,L). The parasphenoid is now present consistently as a midline patch of triangular- to diamond-shaped bone, just anterior to the otic capsules. In one specimen, the parasphenoid has a small patch of ossification extending posteriorly between the otic capsules (Figure 1J,K,L).

In the lower jaw, the dentary is increasing in anteroposterior extent. It now curves onto the lateral surface and occupies approximately one-third of the length of Meckel's cartilage. At its posterior extent, it is now overlapping half of the coronoid laterally (Figure 1J,K,L). There are teeth in the anterior portion of the dentary of one specimen. Posterior and medial to the dentary, the coronoid is getting larger. It now bears at least seven teeth and appears as a more oval-shaped bone.

2.5 | Stage 49

The premaxilla now has a definitive T-shape, thanks to a thin, dorsally directed alary process. The alary process is still relatively short, extending to about the level of the anterior portion of the vomer (Figure 1M,N,O). In one specimen, the frontals are present as thin slivers of bone, just medial to the eyes. The squamosal is now present as a thin sliver of ossification in the posterodorsal portion of the future element (Figure 1M,N,O). The vomer and palatine are overall similar to those of stage 48. The vomer is slightly larger and more elongate or bean-shaped than in earlier stages (Figure 1). The pterygoid is now present as a thin process extending posteriolaterally from the palatine, ventral to the eyes. No suture can be seen between the palatine and pterygoid, and so together form the compound palatopterygoid element. The parasphenoid is now an extensive, rectangular-shaped element with a consistently present triangular-shaped posterior portion between the otic capsules (Figure 1M,N,O). In smaller specimens, the parasphenoid terminates anteriorly at the level of the palatine portion of the palatopterygoid; however, in one specimen, it extends a bit further anteriorly

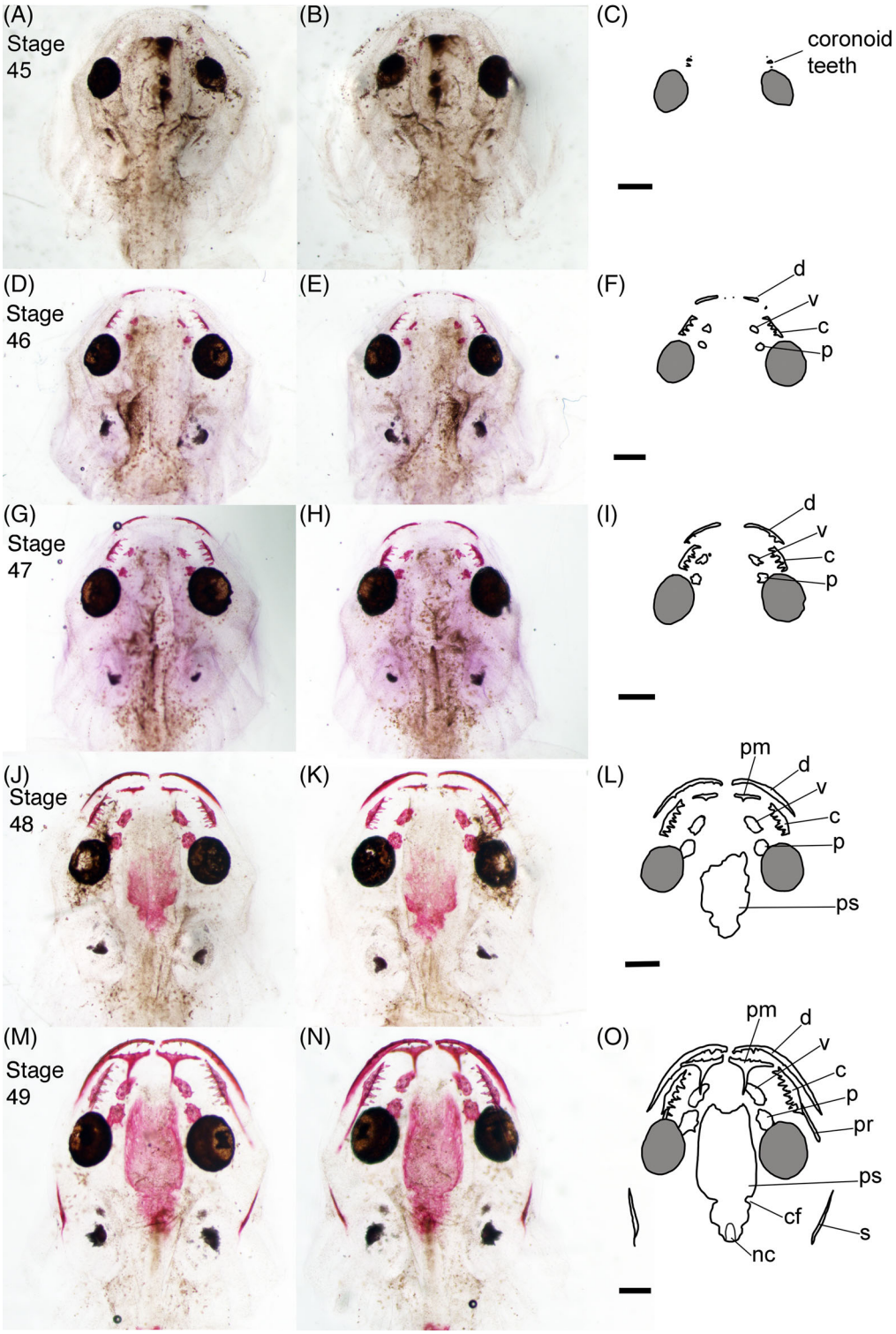

FIGURE 1 Stages 45 to 49. From left to right, each row consists of a photograph in dorsal and ventral view, and an illustration in dorsal view. A-C, stage 45; D-F, stage 46; G-I stage 47; J-L, stage 48; and M-O, stage 49. Scale bars equal 1 mm. Abbreviations: c, coronoid; cf, carotid foramen; d, dentary; nc, notochord; pm, premaxilla; p, palatine; pr, prearticular; ps, parasphenoid; s, squamosal; v, vomer

to reach the level of the gap between the vomer and palatine. At the level of the anterior margin of the otic capsule, the lateral margins of the parasphenoid are incised by the carotid foramina (Figure 1O).

In the lower jaw, the dentaries now closely approach one another at the midline symphysis. There are also teeth present in the dentaries for the anterior half of its length. Additionally, the dentary has grown posteriorly to

occupy two-thirds of the length of Meckel's cartilage. The prearticular has begun ossifying on the medioventral surface of Meckel's cartilage (Figure 1M). This element has been described as a fusion of the prearticular and angular in *Ambystoma texanum*[18]; however, separate ossifications indicating a fusion were not observed here in the specimens of axolotl. The dentary overlaps the prearcticular ossification for about three quarters of the length of the latter. The coronoid is slightly larger with up to 10 teeth.

2.6 | Stage 50

The premaxilla is now a strong T-shape with tall, thin alary process terminating closer to the anterior to the level of the eye than that in stage 49 (Figure 2A,B,C). The alary process is now longer than the ventral tooth bearing portion which is wide, the latter of which now has five to six teeth present. The frontal is present in four out of six specimens. Where present, it is a thin sliver of bone representing the lateral margin of the element (Figure 2A,B,C). Anteroposteriorly, the frontal ossifications span the space between where the alary process of the premaxilla terminates and the midpoint of the eye. The squamosal now extends more ventrally as a thin strip of bone that approaches the jaw articulation and is beginning to widen dorsally (Figure 2A,B,C).

On the palate, the vomers and palatines are slightly larger than in stage 49. The pterygoid is now much longer, reaching the level of just posterior to the eye. The parasphenoid now consistently extends anteriorly to the level of the gap between the vomer and palatine. The posterior expansion of the parasphenoid, located dorsal to the indentations for the carotid foramina, is now slightly larger and more posteriorly expanded. There is a deep incision in the posterior portion of the parasphenoid that receives the notochord (Figure 2A,B,C).

In the lower jaw, the dentary is longer than in stage 49. The prearticular is also now more extensive, where its anterior extent reaches the level of the dentition in the dentary and its posterior extent approaches the terminus of Meckel's cartilage (Figure 2A,B,C). The prearticular is also becoming a wider element posteriorly, giving it a slight wedge shape. The coronoid is slightly larger as well, where it now extends posteriorly beyond the level of the anterior edge of the eye. There are upwards of 12 teeth on the coronoid.

2.7 | Stage 51

All specimens at this stage have frontals. Although the anterior limit of the frontal remains roughly the same as that observed in stage 50, the posterior limit of the frontal now extends to the level of the posterior one-third of the eye (Figure 2D,E,F). In the most advanced specimens, the frontal has started to ossify medially toward the midline, where the two elements will eventually meet. Additionally, ossification of the squamosal is expanding anteriorly in its dorsal portion, giving the anterior margin of this element a pointed outline and generally triangular appearance in this region (Figure 2D,E,F).

The pterygoid portion of the palatopterygoid is now a more robust element than that observed in stage 50, although the amount of ossification in this posterior process differs between specimens (Figure 2D,E,F). The vomer is slightly more oval, where its anterior margin is growing anteromedially. The palatine appears the same as in stage 50. In three specimens, the parasphenoid has extended anteriorly and now terminates at a level between the vomers.

The dentary now terminates almost at the level of the termination of the prearticular (Figure 2D,E,F). The prearticular has increased in ossification and appears more robust, especially at its posterior end. The coronoid is also slightly bigger, as it has increased in size along its anterior-posterior axis.

2.8 | Stage 52

Stage 52 is variable with respect to ossification in the skull. Two of the four specimens observed appear identical to specimens described in stage 51, whereas one of the four specimens has larger frontals and the squamosals have a more extensive anterior growth in the dorsal region (Figure 2G,H). The fourth specimen is slightly more advanced yet. There, the alary process of the premaxilla is longer again and now overlaps the frontal for half of the length of the latter. The frontal has also extended posteriorly and now reaches the posterior margin of the eye. The parietal has begun ossification at its lateral margins in this advanced specimen. The thin strip of bone representing the parietal extends from the level of the midpoint of the eye to almost the midpoint of the otic capsule.

On the palate, the vomer is similar in shape and size to that in stage 51. The palatine is now slightly more oval-shaped than before. The pterygoid is wider than it was in stage 51. The parasphenoid now consistently terminates at the level in between the vomers.

The elements of the lower jaw appear similar in degree of ossification to those in stage 51.

2.9 | Stage 53

The premaxilla is similar in appearance to that in stage 52. The frontal is now more extensive, reaching the level

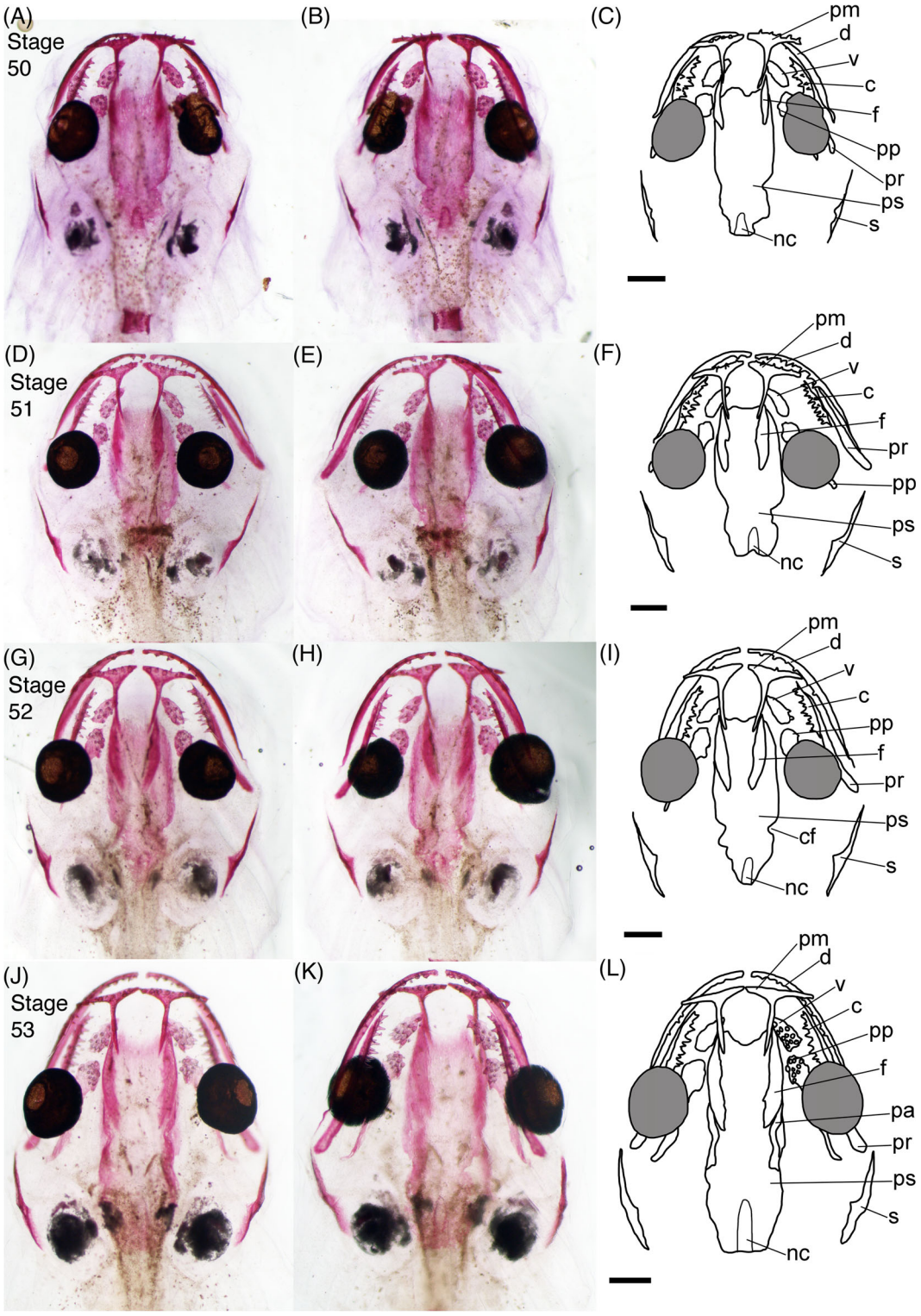

FIGURE 2 Stages 50 to 53. From left to right, each row consists of a photograph in dorsal and ventral view, and an illustration in dorsal view. A-C, stage 50; D-F, stage 51; G-I stage 52; and J-L, stage 53. Scale bars equal 1 mm. Abbreviations: c, coronoid; cf, carotid foramen; d, dentary; f, frontal; nc, notochord; pa, parietal; pm, premaxilla; pp, palatopterygoid; pr, prearticular; ps, parasphenoid; q, quadrate; s, squamosal; v, vomer

of the posterior limit of the eye and with more ossification medially (Figure 2J,K,L). The maxilla is variably present as a thin strip of bone on either one side of the skull only or on both sides. When present, it is a small, narrow element that occurs posterolateral to the lateral most extent of the premaxilla, in the space between the

TABLE 1 Comparison of the ossification sequence of species of *Ambystoma* to that of *A. mexicanum* obtained in the current study

Ambystoma texicanum[18]			*Ambystoma talpoideum*[21]			*Ambystoma maculatum*[23]			*Ambystoma mexicanum* (this study)		
Stage	**Bone**	**Number**	**Stage**	**Bone**	**Number**	**Stage**	**Bone**	**Number**	**Stage**	**Bone**	**Number**
I	[Coronoid, vomer, palatine, dentary]	10	**I**	[Premaxilla, vomer, palatine, dentary, prearticular, squamosal]	4	**I**	[Vomer, coronoid, dentary, palatine]	3	**46**	[Vomer, palatine, coronoid]	10
									47	Dentary	2
									48	[Premaxilla, parasphenoid]	12
II	Premaxilla	1	**II**	[Frontal, parietal, parasphenoid]	1	**II**	Premaxilla	11	**49**	[Squamosal, prearticular, pterygoid]	7
	Prearticular	1					Parasphenoid	13			
	Squamosal	7					Squamosal	8			
	Parasphenoid	6					Prearticular	8			
III	Frontal [parietal, pterygoid]	2	**III**	[Prefrontal, opisthotic, quadrate, pterygoid, maxilla, stapes, exoccipital, prootic]	7	**III**	Pterygoid frontal parietal	2			
								1			
		9						6	**50-51**	Frontal	12
									52	Parietal	7
									53	maxilla	10
IV	Exoccipital	9	**IV**	Orbitosphenoid	2	**IV**	Exoccipital	58			
V	Maxilla	2	**V**	Septomaxilla	1	**V**	Quadrate	22	**54-56**	[Exoccipital, quadrate]	24
	Quadrate	5					Maxilla	40			
VI	Opisthotic [prefrontal, prootic]	3	**VI**	Nasal	13	**VI**	Prootic	1	**57**	Nasal	2
							Opisthotic	17			
		11					Prefrontal	5			
	Stapes	11					Operculum	12			
	Orbitosphenoid	4									
VII	[Nasal, septomaxilla]	13	**VII**			**VII**	Septomaxilla	13			
							Orbitosphenoid	2			
							Nasal	16			

Note: "Number" refers to the number of specimens observed at each stage.

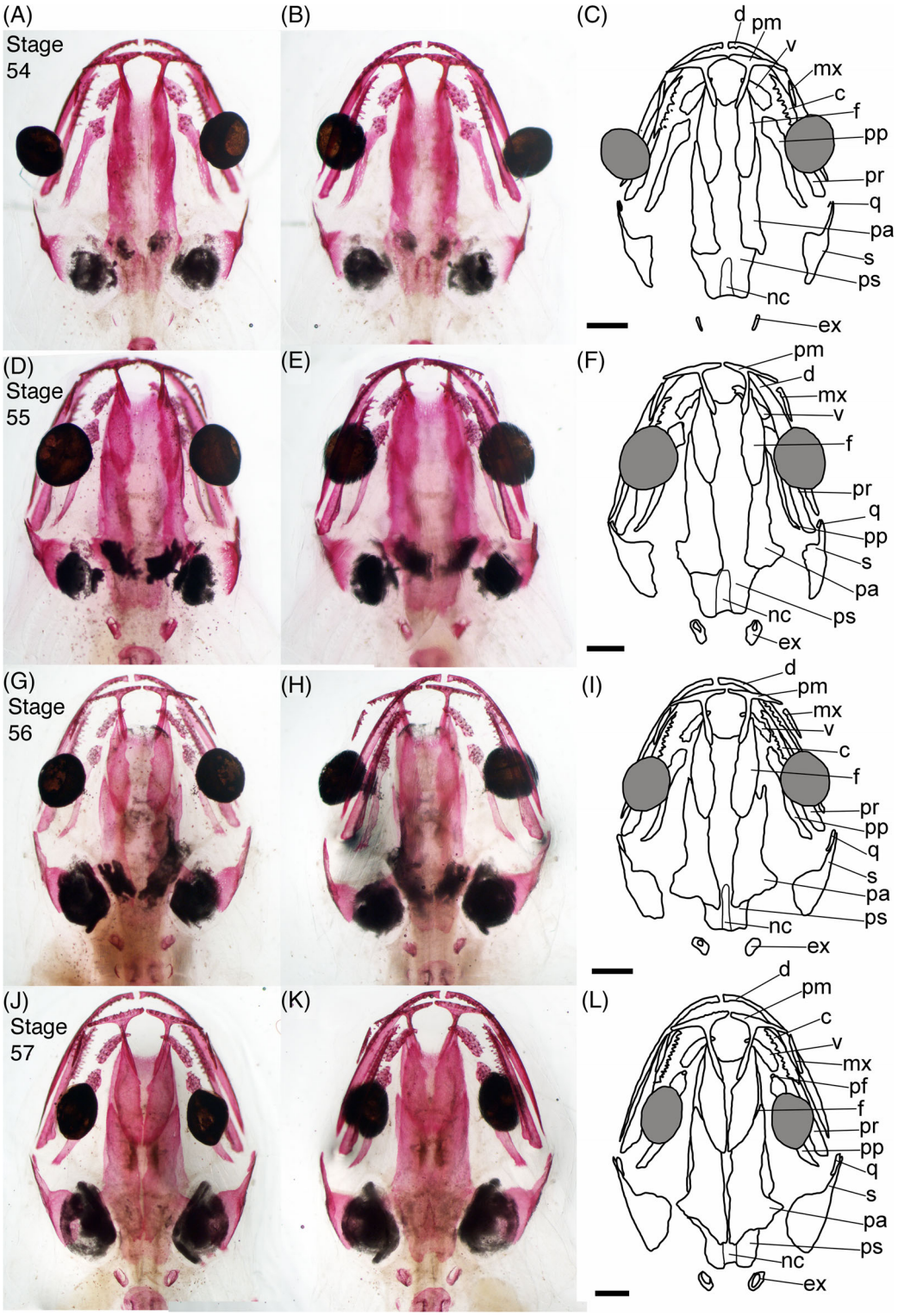

FIGURE 3 Stages 54 to 57. From left to right, each row consists of a photograph in dorsal and ventral view, and an illustration in dorsal view. A-C, stage 54; D-F, stage 55; G-I stage 56; and J-L, stage 57. Scale bars equal 1 mm. Abbreviations: c, coronoid; d, dentary; ex, exoccipital; f, frontal; mx, maxilla; nc, notochord; pa, parietal; pf, prefrontal; pm, premaxilla; pp, palatopterygoid; pr, prearticular; ps, parasphenoid; q, quadrate; s, squamosal; v, vomer

premaxilla and eye. By stage 53, the parietals are consistently present as ossifications of their lateral margins in all specimens (Figure 2J). They span the space from the midpoint of the eye to the anterior portion of the otic capsule, slightly overlapping the frontals laterally. In three specimens, the dorsal portion of the squamosal is more

triangular in shape than in earlier stages, as it expands ossification anteriorly over the lateral surface of the otic capsule. In one specimen, a tiny sliver of bone on the anterior margin of the ventral tip of the squamosal represents the first signs of quadrate ossification.

On the palate, the posterior process of the palatopterygoid continues to ossify posteriorly, and has now reached beyond the level as the posterior terminus of the lower jaw (Figure 2J,K,L). It is also much more robust now posteriorly. The parasphenoid extends anteriorly to occupy much of the space between the vomers. Posteriorly, ossification of the parasphenoid reaches the posterior half of the otic capsules. It is still deeply incised by the notochord.

The elements of the lower jaw are much more robust than in stage 52. The dentary now overlaps most of the prearticular and tapers rapidly to a blunter posterior terminus than before (Figure 2K). The prearticular is also more robust with a deeper posterior terminus at the jaw articulation.

2.10 | Stage 54

The maxilla is now consistently present on both sides of the skull, and has between one and four teeth. Both the frontal and the parietal have expanded ossification medially, resulting in wider elements than observed in earlier stages. The parietal has grown anteriorly to extensively underlap the posterior portion of the frontal to the level just anterior to the midpoint of the eye (Figure 3C). There is still a modest gap between paired right and left frontals and parietals. The squamosal continues to expand into a broad sheet of bone covering the lateral surface of the otic capsule with ossification more extensive than that observed in stage 53. The squamosal is also much broader anteroposteriorly than previously. Both quadrates are consistently present at this stage. The element is still represented by a tiny sliver of bone on the anterior margin of the ventralmost tip of the squamosal. In the occipital region, the paired exoccipitals have begun ossifying (Figure 3A,B,C). They appear as thin ossifications covering the lateral surface of the cartilage of the occipital condyles.

The vomers have become even more elongated elements on the palate. The palatopterygoid now consistently extends posteriorly beyond the limit of the eye and the posterior portion is wider than that observed in previous stages. The lateral margins of the anterior portion of the parasphenoid have grown to a level close to the anterior extent of the vomers. Posteriorly, the parasphenoid is slightly more extensive than that in stage 53.

In the lower jaw, the coronoid appears to be more elongated and the prearticular is becoming more distinctly wedge-shaped as it grows in height at its posterior end.

2.11 | Stage 55

The frontals now taper to fine points anteriorly, laterally overlapping the alary processes of the premaxillae. The maxillae have grown slightly in length and consistently have three or more teeth (Figure 3E). The frontals and parietals are more extensive medially but still do not meet at the midline. The squamosals are no longer triangular in shape in their dorsal region owing to expanded ossification of the entire element (Figure 3D,E,F). The exoccipitals are more extensive, and have now ossified medially to encircle the entire occipital condyle cartilage with bone.

On the palatal surface, the palatine portion of the palatopterygoid is now more diamond-shaped in outline. The pterygoid portion of the palatopterygoid has continued to extend posteriorly, and in two specimens, it extends slightly beyond the posterior limit of the prearticular (Figure 3E). The posterior portion of the parasphenoid, which is positioned between the otic capsules, is now more rectangular in shape than the diamond-like shape observed in previous stages. The entire element is also now more robust overall, and in most specimens, completely encloses the carotid foramina.

In the lower jaw, the prearticular is a stronger wedge shaped, with a greater posterodorsal expansion and it is pierced by a tiny foramen posterodorsally.

2.12 | Stage 56

Stages 55 and 56 are very similar. Some elements appear less porous than before, suggesting more extensive ossification (Figure 3D,G). The quadrate is slightly larger with a taller dorsoventral extent. The posterior limit of the parasphenoid has also expanded. In the lower jaw, the coronoid is now positioned in a trough that is in the dorsal margin of the prearticular.

2.13 | Stage 57

By stage 57, the frontal and parietal elements have almost met along the midline of the skull (Figure 3J,L). The frontal elements are more closely converging at the posterior portion of the element, whereas the parietals are converging from their midpoint moving posteriorly. The parietals also have a medial extension that extends posteriorly and a lateral process that is approaching the posterior portion of the squamosal (Figure 3L). The bone on the dorsal portion of the squamosal is less porous than in previous stages, indicating further ossification. On the

ventral surface of the parasphenoid, the posterior portion is notched (Figure 3K).

In the largest stage 57 specimen, the prefrontals are present. They are represented by tiny dots of ossification at this stage (Figure 3L).

3 | DISCUSSION

The present study represents the first descriptive staging table for the skull of the model species *A. mexicanum*, known as the Mexican axolotl. The staging table of Nye et al.[13] has served as a foundational tool for axolotl researchers primarily working on limb regeneration. The complementary staging table of the skull provided here will further assist researchers studying many additional aspects of axolotl development, as their full potential as a model system is realized.

With this staging table of the skull in hand, some initial comparisons of skull development can be made between *A. mexicanum* and other *Ambystoma* species. The staging table of *A. mexicanum* presented here captures the majority of events in skull morphogenesis. However, the table of Nye et al.[13] ends before late ossifying elements, such as the nasal, prootic, orbitosphenoid, and septomaxilla, appear. These elements do eventually ossify in *A. mexicanum*, but they appear well after limb differentiation is complete, between 63 and 153 days post hatching.[19,20]

For the earlier ossifying skull elements, our results seem to generally corroborate previous ossification sequences for *A. mexicanum*.[19,20] One important distinction is the presence and ossification of a basioccipital. Rose[19] lists an ossified basioccipital developing after the pterygoid and parasphenoid, but prior to the frontals. However, neither we find a basioccipital (cartilaginous nor ossified) in any of our specimens, nor do Smirnov and Vassilieva[20] describe or figure a basioccipital. In comparison to other closely related species, the ossification sequence of the skull of *A. mexicanum* progresses in a near identical sequence to that of close relatives *A. maculatum*, *A. tigrinum*, *A. texanum*, and *A. talpoideum* prior to metamorphosis (Table 1).[18,19,21,22] This is an interesting result given the deviation in developmental trajectory, and further supports its use as a generalized model species in this regard.

4 | EXPERIMENTAL PROCEDURES

4.1 | Axolotl embryo and larva husbandry

Adult axolotl breeding pairs are maintained as part of the breeding colony in the Maddin Lab at Carleton University, Ottawa, Canada. All axolotls are housed and cared for in accordance with the Canadian Council on Animal Care approved animal use protocols (protocol #102951). Clutches of axolotl embryos were obtained from a natural breeding event between male and female leucistic mature adults (source: Ambystoma Stock Center, Kentucky). Embryos were transferred to small, glass bowls filled with 40% Holtfreter's solution and kept in an incubator at 18°C. At hatching, larvae were transferred to 20% Holtfreter's solution and maintained at 19°C on a 12-h day and 12-h night light cycle.

Once able to feed, hatched larvae were fed a diet of baby brine shrimp once per day. Once the larvae started forming forelimb buds, they were transferred into individual cups for further rearing to avoid the common problem of injury due to biting. This also ensured all limbs remained intact, and on their original development trajectory (not regenerating) for accurate staging according to Nye et al.[13] Shortly after this, larvae were switched to a diet of blood worms fed once daily.

4.2 | Staging and fixation

The axolotl larvae were observed daily and staged according to Bordzilovskaya et al.[11] for stages up to 44 and Nye et al.[13] for stages 44 to 57 (Table 1). Once larvae reached the desired stage, they were anesthetized using a 4% MS-222 solution (Sigma-Aldrich E10521, St. Louis, MO) and fixed overnight in 10% neutral-buffered formalin (Thermo Fisher Scientific SF1004, Waltham, MA). After fixation, larvae were washed in distilled water and transferred through an ethanol dehydration series (approximately 1 h in each: 30% and 50%) before final storage in 70% ethanol.

4.3 | Bone staining

Prior to bone staining, specimens were rehydrated to 30% ethanol. The specimens were then placed in a 1% Alizarin red S (Sigma-Aldrich A5533) in a 1% potassium hydroxide solution (KOH) for 1 hour. After bone staining, specimens were moved into a 30% saturated borax solution with 1% trypsin, and incubated at 37°C, until they were almost completely clear. For storage and to finish clearing, the specimens were transferred through a series of 1% KOH plus glycerol solutions (approximately 1 h in each: 3:1, 1:1, and 1:3 KOH to glycerol) and stored in 100% glycerol.

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