Radiographic anatomical description of the skeletal system of adult and subadult axolotls (*Ambystoma mexicanum*): a pilot study

Radiografische anatomische beschrijving van het skeletsysteem van adulte en subadulte axolotls (Ambystoma mexicanum): een pilootstudie

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ABSTRACT

In this study, the use of radiographic imaging to examine the skeletal structures of living axolotls (*Ambystoma mexicanum*) is described. Dorsoventral and left lateral radiographs were taken of both healthy adult and subadult axolotls without restraint, directly on the X-ray table. The aim was to comprehensively describe their skeletal anatomy using radiographic assessment. The method accurately depicted bone shapes and structures, identifying bone names based on comparisons with similar salamanders. However, internal organs within the coelomic cavity were not clearly visible except for the lungs. Interpreting subadult axolotl radiographs was harder, likely due to less developed skeletal ossification or X-ray parameter variations. Variations were observed in the number of certain bones and vertebrae types among individuals. This method offers a non-invasive way to understand healthy animal skeletal appearance, eliminating the need for autopsies. The radiographic approach contributes to better understand axolotl skeletal morphology, benefiting comparative anatomy, veterinary medicine and conservation efforts.

SAMENVATTING

In deze studie wordt het gebruik van radiografische beeldvorming beschreven voor het onderzoek naar skeletstructuren van gezonde axolots (*Ambystoma mexicanum*). Er werden dorsoventrale en links laterale radiografieën genomen van adulte and subadulte axolotls zonder sedatie. Het doel was om de skeletale anatomie van axolots zo uitgebreid mogelijk te beschrijven gebruikmakend van radiografische beeldvorming. Deze methode toonde nauwkeurig de vorm en structuur van de beenderen aan, waarbij de naam van beenderen (gebaseerd op een vergelijking met gelijkaardige salamanders) werd geïdentificeerd. Enkele interne organen binnen de coeloomholte waren echter niet duidelijk zichtbaar, behalve de longen. De interpretatie van de radiografieën van subadulte axolotls was moeilijker, waarschijnlijk door de minder ontwikkelde skeletale ossificatie of door variaties in de opnameparameters. Er werden individuele variaties waargenomen in de hoeveelheid van bepaalde beenderen en in de types vertebrae. Deze methode is een non-invasieve manier om inzicht te krijgen in het skeletale stelsel van gezonde dieren en zo autopsie te vermijden. De radiografische benadering draagt bij tot een beter inzicht in de skeletale morfologie en komt op die manier de vergelijkende anatomie, de diergeneeskunde en het soortbehoud ten goede.

INTRODUCTION

Ambystoma mexicanum, commonly known as the Mexican axolotl, is a critically endangered amphibian belonging to the *Ambystomatidae* family, native to the Xochimilco and Chalko canals of Mexico (Aceves et al., 1970; Contreras et al., 2009). Rapid urbanization, pollution and habitat degradation have led to a significant decline in its natural habitat (Contreras et al., 2009). Efforts to conserve and protect this remarkable species are urgently needed to ensure its survival and maintain the biodiversity of its ecosystem.

The axolotl has become increasingly popular as a pet in recent times (Contreras et al., 2009). Nevertheless, maintaining axolotls in captivity can present certain challenges, such as the risk of predation from other tankmates and potential health concerns if proper care is not provided. The husbandry conditions for axolotls involve providing a minimum of 30 square centimeters of space per animal, maintaining a twelve-hour day/night cycle, and feeding them a diet consisting of pellets, fish and earthworms. Proper management of water parameters is crucial, including maintaining specific levels of nitrates (50 mg/L), unionized ammonia (0.02 mg/L), nitrites (1 mg/L), carbon dioxide (5 mg/L), pH (7 - 8), and water temperature (18 - 20 °C) (Farkas and Monaghan, 2015; Khattak et al., 2014; K. Wright and Whitaker, 2001). Axolotls can be susceptible to various diseases, such as gastrointestinal issues (e.g. foreign bodies), osteo articular diseases (amputation), as well as neoplasia and buoyancy disorders (Takami and Une, 2017, 2018). In reptiles and other amphibians, these pathologies could be explored using radiography. As in other species, radiographic examination has the potential to provide valuable insights into the axolotl's skeletal system. However, to date, no data have been reported in the literature.

Although axolotls are well-known for their regenerative abilities, the diagnostic imaging of their anatomy is still not well-documented, and there is limited knowledge of the structure and radiographic appearance of their skeletal system.

The aim of this article is to explore the gaps in the understanding of the axolotl's skeletal system and the potential role of radiographic examination in furthering the knowledge in this area.

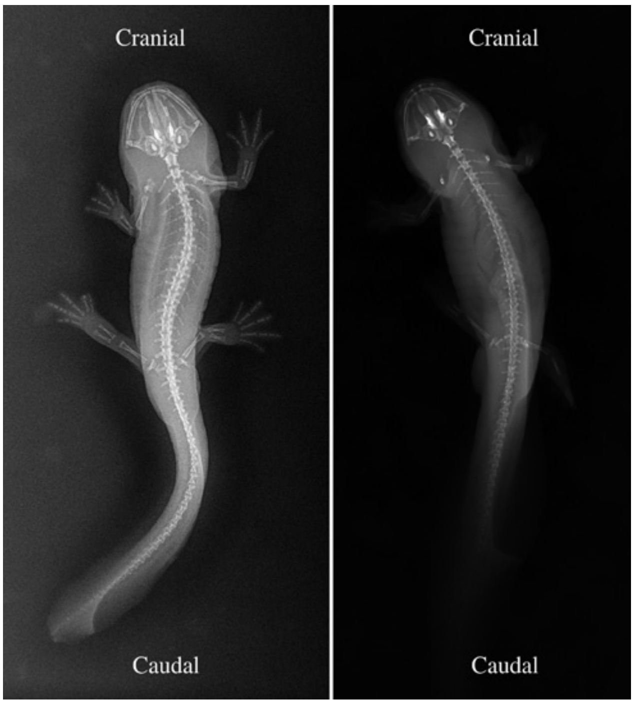

Figure 1. Comparison of two dorsoventral radiographic views of an adult (left) and a subadult (right) axolotl (*Ambystoma mexicanum*).

MATERIAL AND METHODS

This study was approved by the ethical review committee of Vetagro-Sup, Lyon, France (n° 2346).

Study subjects

X-rays were performed on seven live adult animals (males = 4 ; females = 3; age range: 2-4 years, weight 170-300 g) and seven live subadult animals (age range: 6 months - 1 year, weight 70-100 g). All subjects were captive-bred wild-type adults originating from a breeder and considered healthy. The animals were housed in groups of one, two or three, with separation based on sex (for adults only). Specific husbandry conditions were strictly followed, which included regular temperature monitoring, complete replacement of dechlorinated water every two days, and the use of air pumps. To minimize the interference of digesta in imaging, the animals were fasted for seven days prior to the examination. On the day of examination, all the animals were weighed. Physical examinations confirmed that the axolotls were in a healthy condition.

For the sake of sample homogeneity, adult axolotls weighing less than 170 g were excluded from the study, as well as subadults weighing over 100 g.

IMAGING

The radiographic description of the skeletal system was conducted by studying the skeletons of the seven adult and seven subadult axolotls.

The radiographs were captured using a X-DR Static Classic machine (generator: 32kW / max. 450 mA) (Examion GmbH,70736 Fellbach, Germany). The image capture and management software used was X-AQS (Version 3.06.04) (Examion GmbH,70736 Fellbach, Germany).

X-rays were taken with the axolotls placed directly on the X-ray table, including both dorsoventral and left lateral views. The dorsoventral view was selected due to the absence of a need for chemical or physical

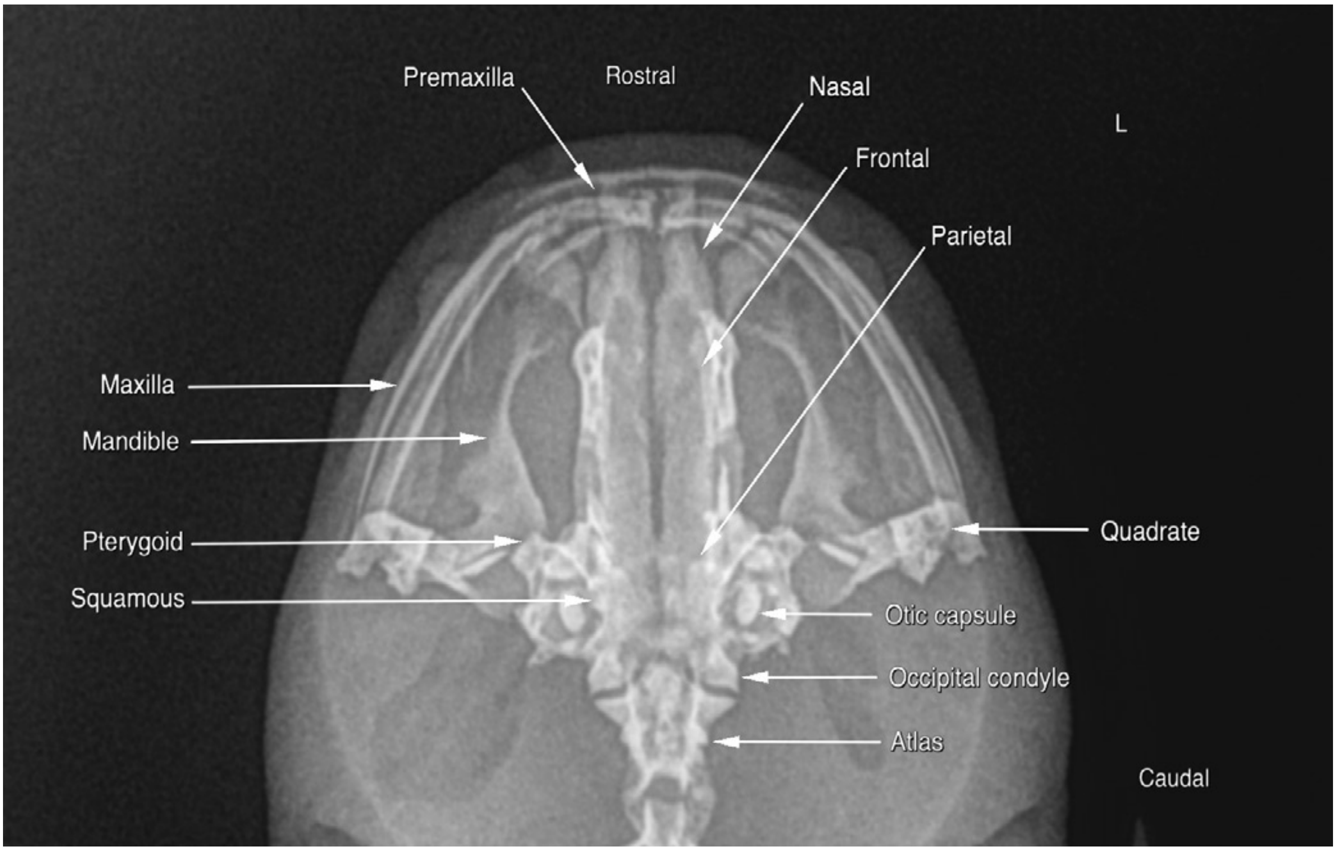

Figure 2. Skull bones observed on the dorsoventral radiographic view of an adult axolotl (*Ambystoma mexicanum*).

restraint of the animal, because radiographic examination is a routine procedure in veterinary practice, requiring quick and unprepared execution. The lateral views were introduced for study purposes, with the animals positioned in balance on their right side to aid in bone demarcation (especially vertebrae). However, the primary focus in the study was on the dorsoventral views, as they offer the clearest and most easily attainable results in practical application.

The thickness of the animals was measured using a calliper to choose the X-ray parameters (44 kV; 8 mAs).

The parameters were not changed between the animals. The axolotls were not restrained. X-rays were performed by the same operator (CF). The X-rays of each axolotl were studied by a European College of Veterinary Diagnostic Imaging diplomate (OE).

RESULTS

Haut du formulaire

All the structures described here were identified and delimitated on X-rays.

The names of the observed structures were based on studies conducted on other urodeles (*Ambystoma tigrinum, Ambystoma tshudi, Salamandrina spp.)* that are morphologically similar to the axolotl (Lauder and Shaffer, 1988; Reilly and Lauder, 1990; Wright, 2001; Macaluso et al., 2020; Ledesma et al., 2022). This parallel seamed necessary due to the lack of recent data on the description of the axolotl's skeletal system. For clarity's sake, figures were based on the X-rays of adult axolotls, because of the increased opacity of their bones (Figure1).

Axial skeleton

The skull bones were well-delineated and visualized in all axolotls. The best radiographic view for delineation of the skull bones was the dorsoventral radiographic view (Figure 2). The skull bones were composed of the mandible, premaxilla, nasal, maxilla, frontal, parietal, pterygoid, quadrate and squamous bones. The occipito-otic complex was composed of the otic capsules, which were completely ossified (except for the operculum) and the occipital condyles, which were well-delineated and visualized in all axolotls. The occiput was smooth, regular and concave to receive the odontoid process of the atlas, which was convex. The hyoid apparatus is formed by one bone, i. e. the urohyal bone (Figure 3).

The vertebral column was poorly differentiated into the atlas, the trunk vertebrae, the sacral vertebra, the caudosacral vertebrae and the caudal vertebrae (Figures 4 and 5).

The cervical region comprised only the atlas vertebra. The atlas was composed of an elongated spinous

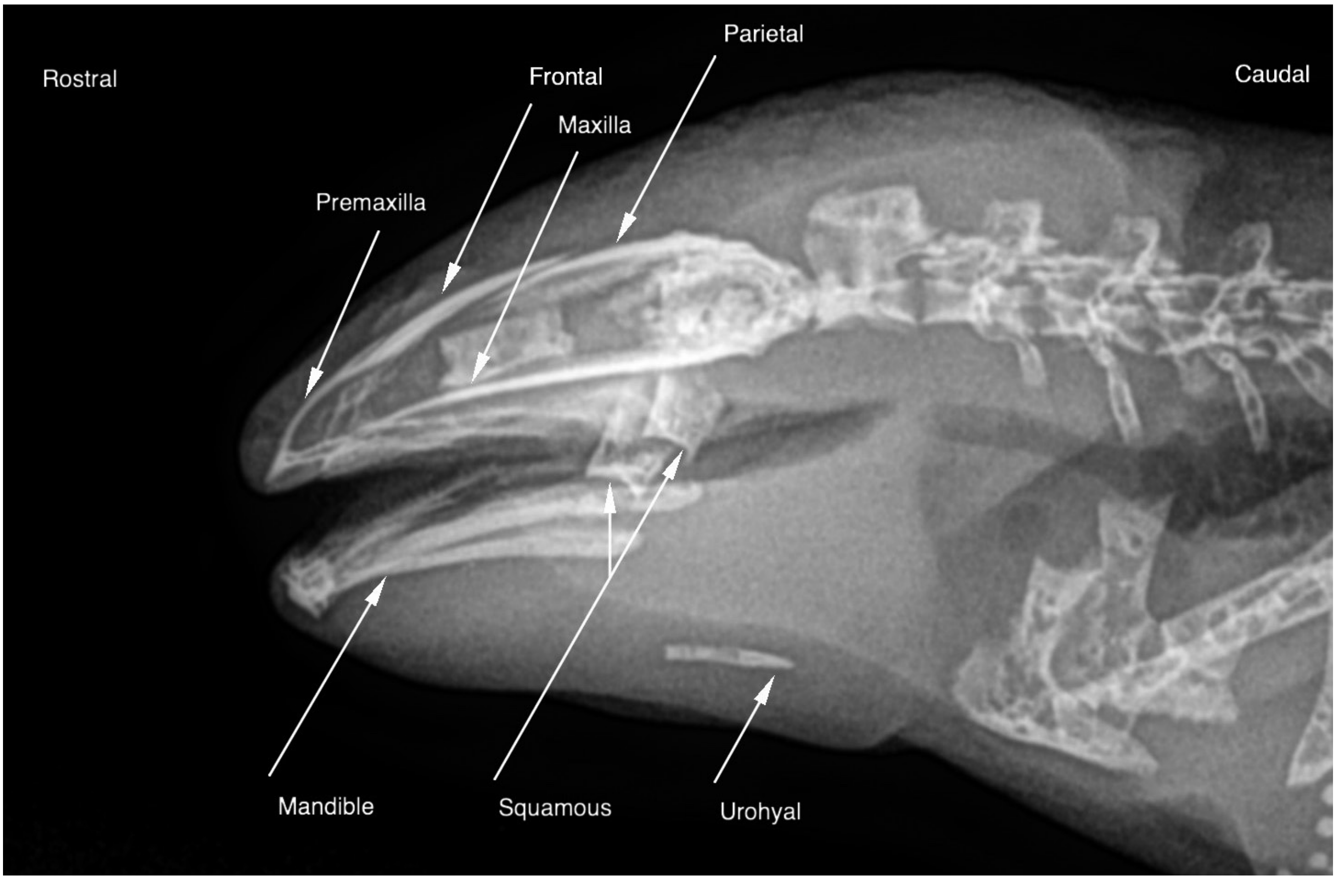

Figure 3. Skull and mandibular bones observed on the lateral radiographic view of an adult axolotl (*Ambystoma mexicanum*).

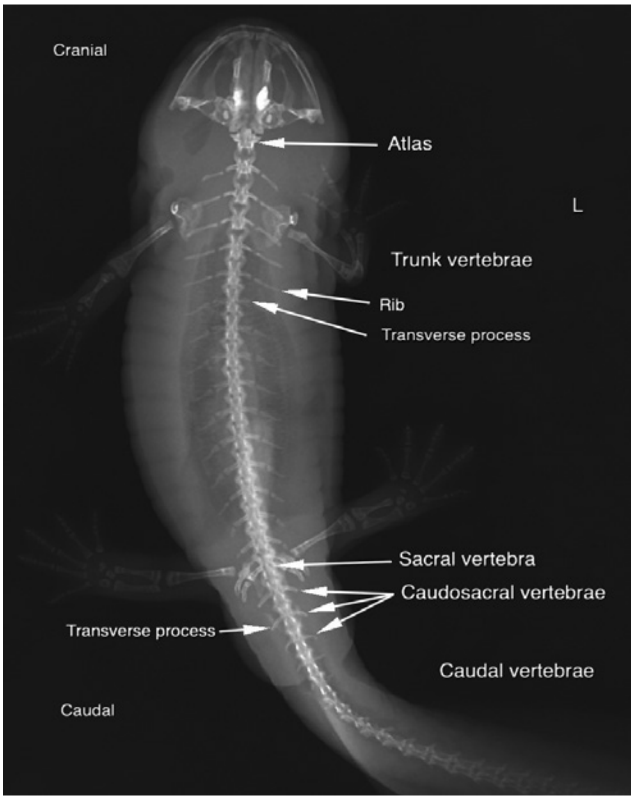

Figure 4. Vertebrae types observed on the dorsoventral radiographic view of an adult axolotl (*Ambystoma mexicanum*).

process, better visualized on the lateral radiographic view. The occipital condyles of the skull formed articulations with two cranial flat atlantal condyles, while an elongated convex odontoid process articulated with the occiput. These structures were visible in the images of every adult and subadult axolotl.

The 'thoracic' region consisted of the trunk vertebrae, which corresponded to the thoracic vertebrae. Notably, there were no lumbar vertebrae present. In the adult axolotls, 15 trunk vertebrae were found in six animals and 14 trunk vertebrae were found in one animal. In the subadults axolotls, five animals had 15 trunk vertebrae and two animals had 16 trunk vertebrae. The trunk vertebrae were tubular shaped, with paired short and prominent cranial and caudal articular processes. The transverse process was thin and elongated and articulated with a thin pair of ribs. The rib cage was composed of the trunk vertebrae, each of which articulated with one short rib on each side.

The sacral region was comprised of a single vertebra, in contrast to the fused three vertebrae observed in mammals. The sacral vertebra was represented by slightly different vertebra from the previous trunk vertebrae by a thicker transverse process and slightly larger vertebral body. The pelvis was formed solely by the sacral vertebra, which directly articulated with the femurs on each side.

Unlike mammals, axolotls have three caudosacral

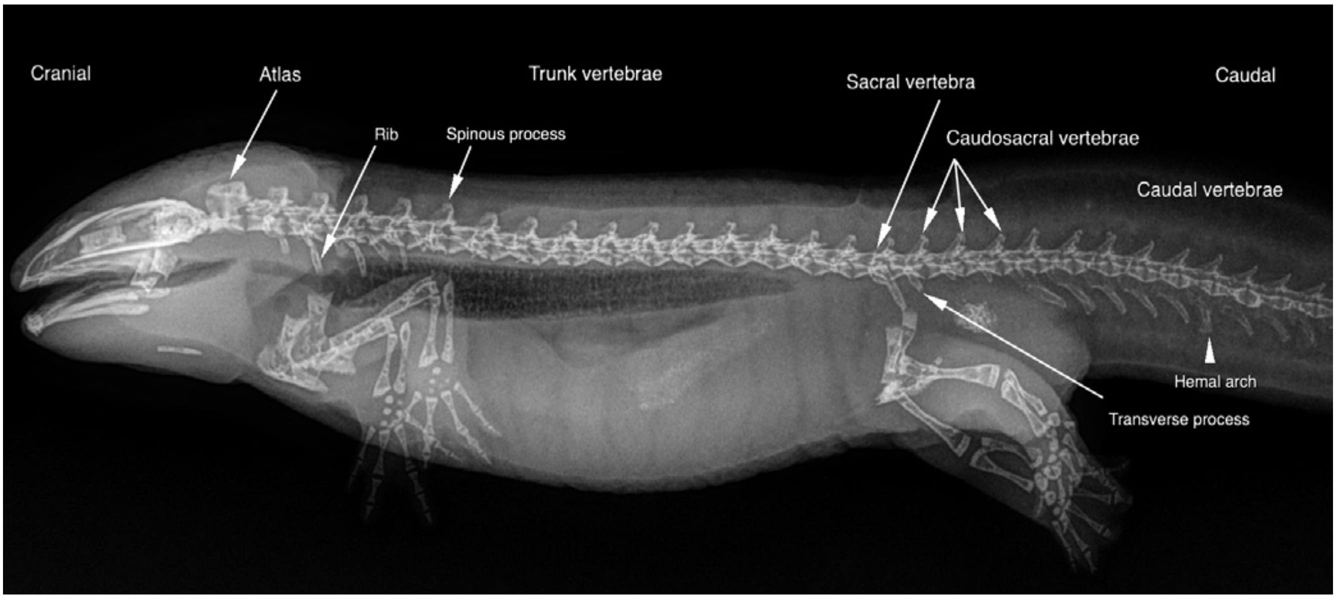

Figure 5. Vertebrae types observed on the lateral radiographic view of an adult axolotl (*Ambystoma mexicanum*). The lateral view helped to distinguish the different types of vertebrae.

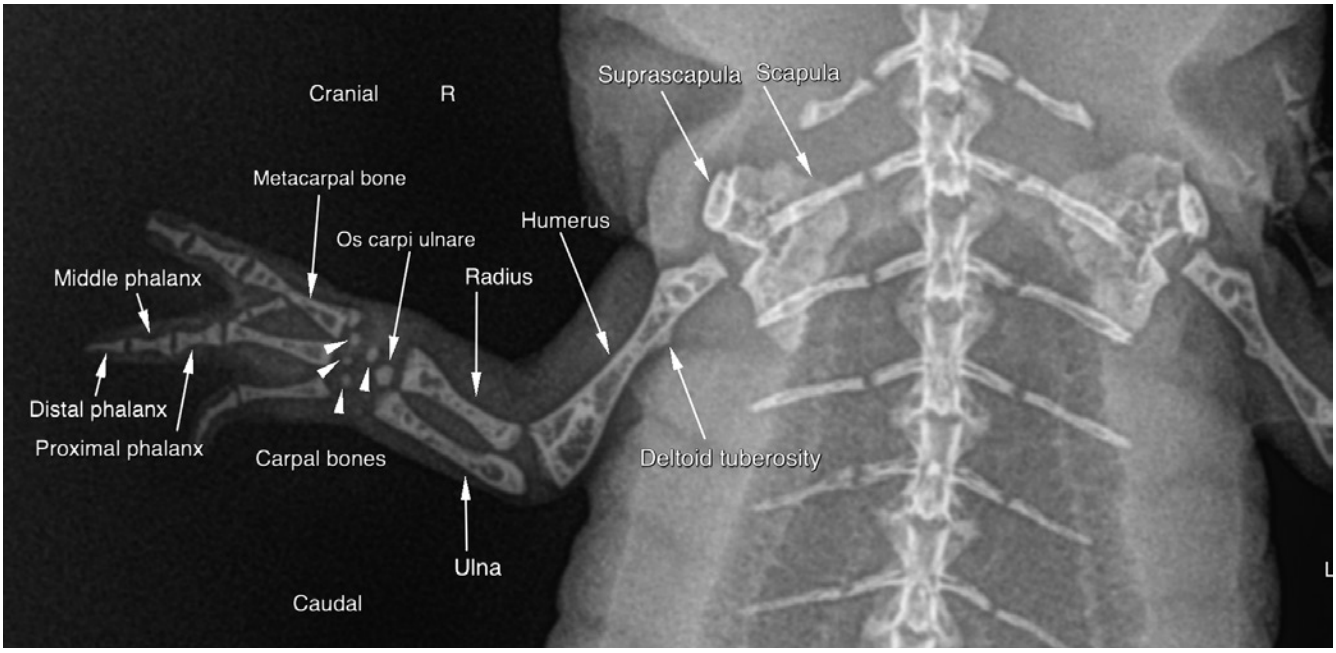

Figure 6. Focus on the lateral radiographic view of the thoracic limb of an adult axolotl (*Ambystoma mexicanum*). The left side of the figure corresponds to the caudal portion of the individual.

vertebrae, followed by multiple caudal vertebrae, corresponding to the coccygeal vertebrae and easily recognizable by their ventral hemal arches. The caudosacral vertebrae were at the number of three with a progressively shorter and thinner transverse process, associated with progressively shorter vertebral bodies.

The caudal vertebrae were easily recognized with their gradual reduction of size and the presence of ventral hemal arches. Their number varied between 30 and 45.

Appendicular skeleton

On each image, the axolotls had two thoracic limbs and two pelvis limbs. The thoracic limb was formed of the scapula, humerus, radius, ulna, carpal and metacarpal bones, and a variable number of phalanges (Figures 6 and 7).

The scapula was a well-delineated flat triangular-shaped bone with irregular margins, which was identified in all axolotls (Figure 6). The suprascapula represented a lateral bony protruberance arising from the distal aspect of the scapula and was best visualized on the dorsoventral radiographic views.

The humerus bone was a tubular-shaped long bone composed of a long shaft with proximal and distal extremities. The cortical was thin and well-delineated in all axolotls with good corticomedullary distinction.

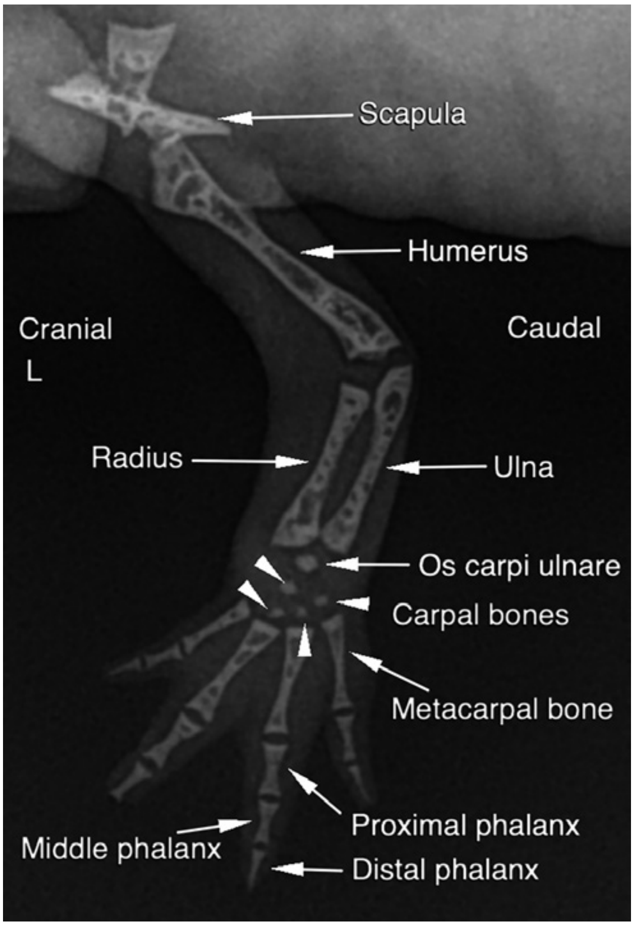

Figure 7. Focus on the dorsoventral radiographic view of the thoracic limb of an adult axolotl (*Ambystoma mexicanum*). A superimposition of two fingers is visible. The left side of the figure corresponds to the caudal portion of the individual.

The deltoid tuberosity was a large bony protuberance along the lateral aspect of the proximal humerus and was identified in all axolotls on the dorsoventral view. The radius and ulna were two slightly curved short bones, composed of a short diaphysis with proximal and distal extremities. The distal extremities of the radius and ulna were larger than the proximal extremities. The narrowest point of radius and ulna was located at the mid aspect of the diaphysis.

The carpus was formed of two rows of carpal bones. The proximal row of the carpus was constituted of three bones; according to the literature: os carpi radiale, os carpi ulnare and os carpi intermedium. Two bones, os carpi radiale and os carpi intermedium, were always identified in the proximal row of carpal bones. The os carpi radiale was not visible in some of the animals (adults and subadults). The distal row of carpal bones was formed of four carpal bones, which were identified in all axolotls.

The thoracic limbs had four digits in almost all adult axolotls, composed of four metacarpal bones, but with a variable number of phalanges. One adult axolotl had three digits on one side and another one had one digit on one side. In the subadult axolotls, the identification of the digits was difficult in two cases.

The pelvic limbs consisted of the femur, tibia, fibula, tarsal and metatarsal bones, and a varying number of digits (Figures 8 and 9). The femur was a tubular shaped, composed of a long diaphysis, proximal and distal flared extremities. A solitary trochanter was observed along the femur, manifesting as a bony protuberance. This trochanter was seen along the caudal aspect of the proximal shaft, visible in all axolotls on the dorsoventral view. The tibia and fibula were two

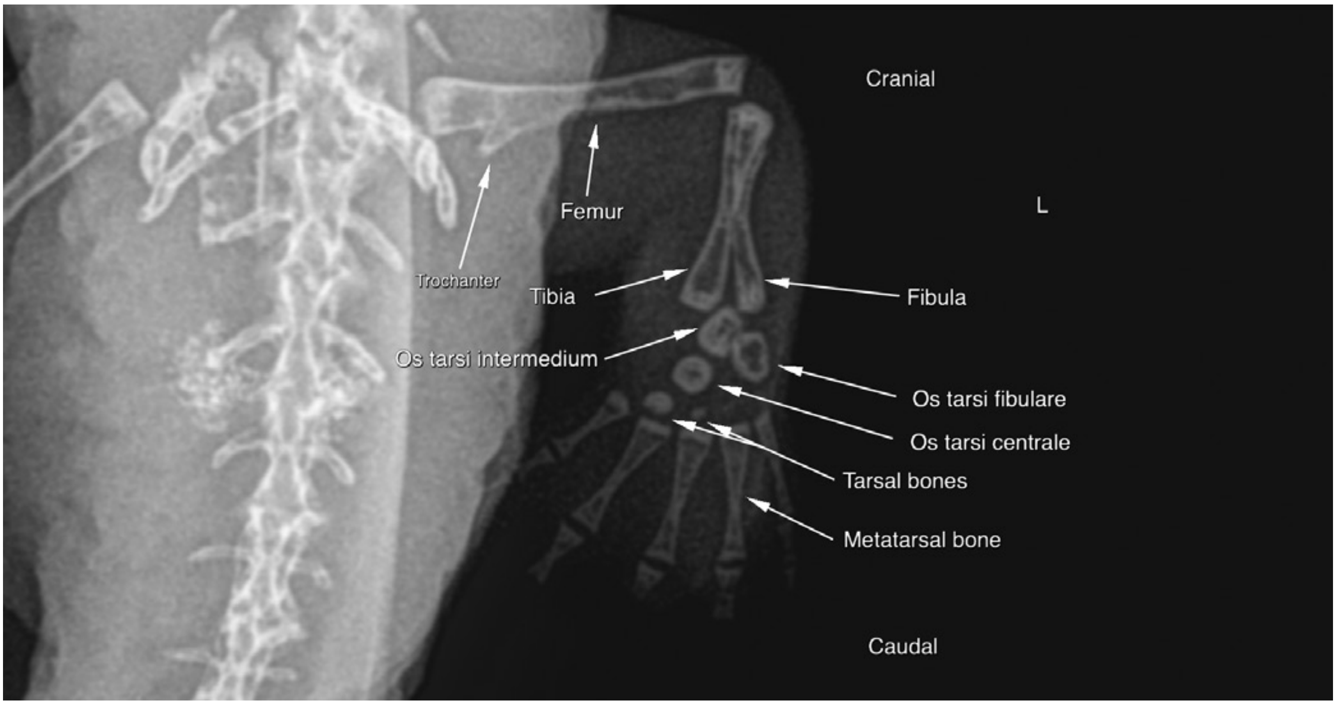

Figure 8. Focus on the lateral radiographic view of the pelvic limb of an adult axolotl (*Ambystoma mexicanum*). The left side of the figure corresponds to the caudal portion of the individual.

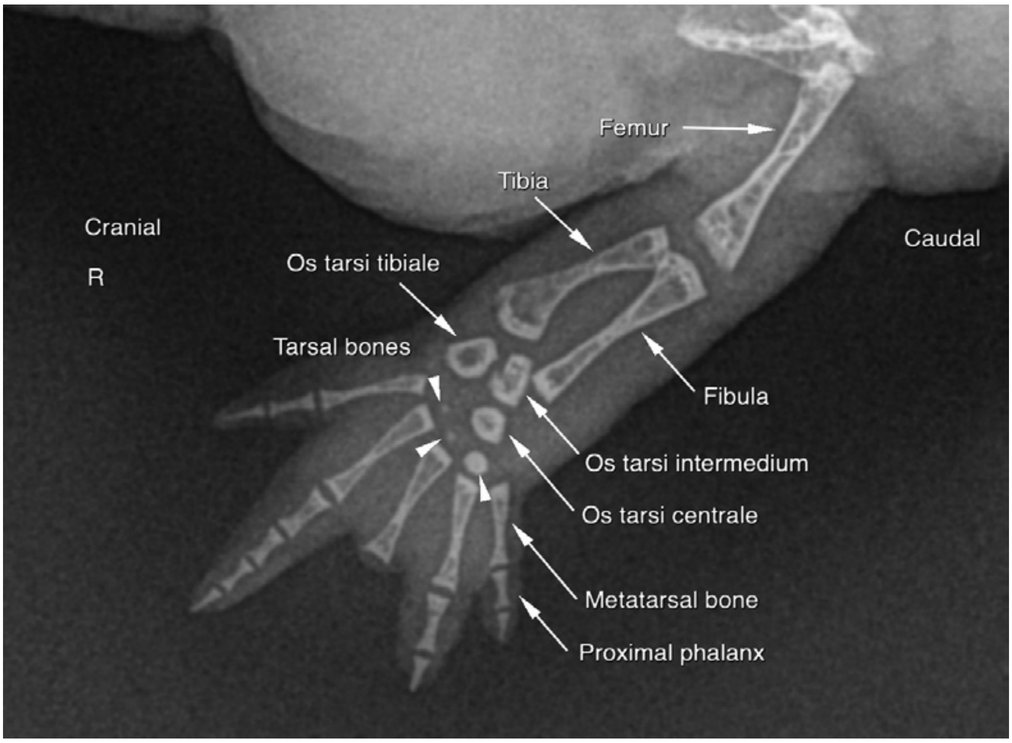

Figure 9. Focus on the lateral radiographic view of the pelvic limb of an adult axolotl (*Ambystoma mexicanum*). The limb shows a discrete rotation, which allows the identification of the trochanter. The left side of the figure corresponds to the caudal portion of the individual.

short, slightly curved and flared bones.

The cortex of the femur, tibia and fibula was thin and well-delineated in all axolotls, as well as the corticomedullary distinction.

According to the literature, the tarsal joint is composed of nine individual bones: the os tarsi tibiale, os tarsi intermedium, os tarsi prehallux, os tarsi centrale, os tarsi fibulare and four tarsal bones (2nd to 5th tarsal bones) (Macaluso et al., 2020).

The intermedium, fibulare and centrale bones were identified in all axolots. The prehallux and tibiale bones were not visualized. All tarsal bones were seen. There were five metatarsal bones in 7/8 adult axolotls and in 0/8 subadult axolotls. A variable number of phalanges were seen in all digits (2-4).

DISCUSSION

It is important to acknowledge that the limited number of individuals used in this study (seven axolotls) poses a limitation when drawing definitive conclusions about the validity of the method. While the results obtained from these individuals provide valuable preliminary insights, a larger sample size would enhance the statistical power and generalizability of the findings.

Obtaining lateral radiographs of unsedated axolotls presents challenges due to the difficulty in achieving optimal positioning. Axolotls cannot be readily immobilized in a standardized position by adjusting limb placement, making precise alignment challenging. This limitation is particularly relevant when attempting to capture clear images of the coelomic organs, as they may become partially obscured by limb superposition. The majority of studies involving axolotl imaging are conducted on sedated animals (Dittrich et al., 2018; Lazcano et al., 2021).

Moreover, the differences in thickness between the limbs and the body resulted in radiographs that were challenging to interpret using the same parameters, especially for the subadults. Adaptation of the X-ray parameters may result in a better readibility of the radiograph.

Other criteria, like the measurement of bone density, could have been a precise criterion to describe the X-rays and compare adults and subadults, because the assessment of bone opacity through conventional radiography is a subjective process, necessitating a significant loss of mineralization, often around 40-50%, before any noticeable changes in bone opacity can be identified (Zotti et al., 2004).

Variations in the number of phalanges among the axolotls of the present study might have been influenced by factors, such as intraspecific predation, which could potentially lead to the loss of digits. Despite the absence of visible injuries, this hypothesis cannot be entirely dismissed, particularly in cases where the animals were kept in close proximity. Moreover, the subadults displayed limited ossification, which occasionally complicated the counting of phalanges.

Differentiating between types of vertebrae in axolotls, especially the sacral and caudo-sacral vertebrae, proves challenging through the dorsoventral view alone. While some subtle differences like slightly elongated transverse processes might be discernible, a conclusive distinction is best achieved through lateral views, where ventral hemal arches were identified.

Differences in bone opacity between adult and subadult axolotls was subjectively noted. The axolotl (*Ambystoma mexicanum*) exhibits a remarkable ability for continuous bone growth throughout its adult life (Hutchison et al., 2007). Ossification continues to occur and progresses towards the epiphyses of long bones as axolotls age. This ossification process remains responsive to L-thyroxine, which enhances the rate of bone formation (Hutchison et al., 2007; Riquelme-Guzmán et al., 2022). The process of ossification in the appendicular skeleton initiates when animals reach a length of 10 cm (Hutchison et al., 2007). At this stage, cartilage cells are gradually replaced by a primary ossification center, which comprises cortical bone and a marrow cavity filled with adipocytes. As the bone continuously matures, it is accompanied by a simultaneous and continuous growth of the body (Hutchison et al., 2007). For this reason, it is probable that interpreting radiographs of subadults would be more complex, as their bones are less calcified and thus less radiodense.

The limb skeleton of anuran amphibians undergoes development from cartilaginous anlagen to bones. However, the skeletogenesis in anuran amphibians exhibits distinct characteristics when compared to that of mammals and birds: anuran amphibians experience growth with fewer bone trabeculae and underdeveloped epiphyseal growth plates (Miura et al., 2008). Additionally, the process of endochondral ossification in anuran amphibians demonstrates delayed progression in comparison to other species (Miura et al., 2008). This process has not been demonstrated in urodeles yet.

In axolotls, the regeneration of skeletal elements does not involve the direct participation of skeletal tissue. Instead, these structures are shaped and developed through cells originating from non-skeletal connective tissue (McCusker et al., 2016). Notwithstanding its unparalleled regenerative capacities, the axolotl seems to have no potential to regenerate non-union bone fractures. While it can successfully mend a non-stabilized union fracture akin to other vertebrates, it lacks the ability to heal a bone gap of critical size. The axolotl does not seem to rely on its regenerative mechanisms for mending bone fractures (Hutchison et al., 2007).

In captivity, skeletal ossification defects can arise due to inadequate maintenance practices. Vitamin D3 can be synthesized internally through endogenous processes involving UV-B radiation or obtained from the diet, subsequently undergoing metabolism to its biologically active hormonal form, calcitriol. Insufficient calcitriol levels in amphibians lead to nutritional metabolic bone disease, potentially affecting reproduction and immune function (Antwis and Browne, 2009).

In conclusion, gaining insights into the radiographic appearance of the skeletal structure of adult or subadult axolotls could enable non-invasive monitoring of fracture healing, tracking limb regeneration progress, and verifying appropriate skeletal calcification for a given age and size.

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