Ambystoma mexicanum are able to mitigate teratogenesis in the chondrocranium

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Abstract

Retinoic acid is critical to development, yet toxic in excess. Existing in a concentration gradient across the anteroposterior axis in various regions of a developing vertebrate, retinoic acid acts as a posteriorizing morphogen. Its decrease causes anteriorization of pharyngeal arches while its increase truncates anterior regions and limits chondrification. In this study, cleavage-stage *Ambystoma mexicanum* embryos were treated with retinoic acid at 1 nM, 0.1 nM, or 0.01 nM, or with N,N-diethylaminobenzaldehyde, an antagonist of retinoic acid, at 10 μM, 1 μM, or 0.1 μM. Treated larvae were fixed zero, five, nine, eleven, and thirteen days post-hatching along with control larvae raised in either untreated media or 0.1% dimethyl sulfoxide. Until hatching, all embryos were incubated in the dark at 15°C. Measurements of the mandibular, ceratohyal, hypohyale, and quadrate cartilages were obtained using the Zeiss Pro® microscope and software program and were analyzed using ANCOVA, which revealed that retinoic acid has no significant effect on mandibular, ceratohyal, and quadrate cartilage size. The hypohyale, whose length significantly correlated with treatment type, was most sensitive to retinoic acid and a lack of retinoic acid seemingly caused further anteriorization of the premaxilla. Nevertheless, a concentration threshold in which *Ambystoma mexicanum* can tolerate exogenous retinoic acid without severe malformations in the chondrocranium exists between 1 nM and 50 nM. The role of CYP26 and other proteins in mitigating retinoic acid teratogenesis is postulated.

Introduction

Retinoic acid, more specifically all-*trans*-retinoic acid, is the most biologically active derivative of retinol, or vitamin A. Quite remarkably, the body uses this relatively simple, fat-soluble compound to achieve an overwhelming number of tasks[1,2,3,4]. It is a key regulator during chondrocyte differentiation and acts as a morphogen during chordate development[5,6,7]. However, exogenous retinoic acid in embryonic compartments requiring low concentrations causes teratogenesis and mortality[8]. Disease states such as cleft palate, macrocephaly, and a shortening of the mandible and maxilla[9] encourage an understanding of cellular response to retinoic acid and its relationship to chondrocranium development. The following details the general pathway of retinoic acid, its role in chondrification and teratogenesis, and its ability to posteriorize different regions during development.

General Pathway of Retinoic Acid

Retinoic acid is primarily obtained through ingestion and metabolism of retinol[10,11,12], which can be acquired directly from the diet or synthesized from β-carotene by its oxygenase[13,14]. Inside the intestinal lumen, retinol is absorbed and converted into retinyl ester, which acts as a storage molecule. The ester is then packaged and sent through lymphatic and circulatory vessels to target cells, especially those of the liver[12,15]. Inside the liver, enzymes reconvert retinyl ester into retinol. Through a series of highly regulated pathways, retinol is bound to its plasmatic carrier protein, RBP, and ejected into the bloodstream[16,17].

In embryos, this method of acquisition is different. Viviparous organisms cannot synthesize retinoids *de novo* and must obtain retinoic acid and its precursors through the placenta. Two common modes of delivery are simple diffusion of circulating retinol from the mother's bloodstream to the embryonic compartment and transfer of retinol from maternal binding

proteins to those of the embryo[18,19]. In oviparous organisms, retinoic acid is derived from the metabolism of carotenoids in vegetal cells[20].

Cells requiring retinol acquire the compound through specific receptors displayed on their surfaces. One mechanism requires a highly selective transmembrane receptor protein, STRA6[21,22]. The extracellular moiety of STRA6 is able to detect and bind to holo-RBP[23]. With the aid of intracellular proteins, STRA6 initiates retinol's release into the cell[24]. Studies have also demonstrated the ability of STRA6 to reload retinol onto RBP, helping alleviate high cellular concentrations of retinol[25].

During times of need, retinol can be converted into retinoic acid in several enzyme-dependent steps. It is first converted into retinaldehyde in a reversible process by retinol dehydrogenase. Retinaldehyde is then irreversibly converted into retinoic acid by retinal dehydrogenase. The irreversibility of this reaction has implications for retinoic acid homeostasis, as does the activity of cytochrome P450 family 26 (CYP26) enzymes, which aid in retinoic acid clearance[26].

Retinoic acid does not freely float in cytosol, but is bound to a cellular binding protein (CRABP)[27]. This is true for retinoic acid metabolized in the cell and obtained from plasma albumin[28]. The binding of retinoic acid to its cellular binding protein prevents non-specific oxidation of the substrate and regulates nuclear transport. Inside the nucleus, retinoic acid binds to retinoic acid receptors RARα, RARβ, and RARγ, which form complexes with receptors for other retinoids and initiates gene transcription. Full detail of the genetic mechanisms by which transcription occurs is beyond the scope of the present study and can be obtained from several papers[29,30]. It will be noted, though, that the interaction between retinoid receptors and retinoic acid response elements (RAREs) in promoter or enhancer sequences regulates gene

expression[31,32]. A number of genes activated by this general pathway have been documented[32]. This study focuses on the relatively few known to have a direct impact on cartilage development.

Retinoic Acid in Chondrification and Teratogenesis

The fact that retinoic acid is involved in the development and modeling of cartilage tissue is indicated by its ability to modify such cells. Addition of 1 nM or 10 nM retinoic acid to avian chondrocytes alters their shape, causes the cells to become fibroblastic, drastically reduces their ability to synthesize proteoglycans, and inhibits the initiation of type X collagen gene expression[33]. A decrease in translation and concentration of aggrecan, a protein crucial for proper functioning of articular cartilage[34], is linked to the inhibition of nuclear response to retinoic acid[35]. At 50 nM, formation of the second basibranchial cartilage in the *Ambystoma mexicanum* chondrocranium does not occur[36] and in cranial regions of frog embryos, retinoic acid causes anterior truncation, posterior or ventral character of mesoderm, and even acephaly[37].

Further evidence that suggests retinoic acid is involved in chondrification is the discovery of RAREs in genes responsible for chondrification, such as the *Col11a2* gene, which encodes for a collagen subunit critical to the extracellular matrix of chondral tissues[38]. All three retinoic acid receptors are involved in general cell growth and differentiation[41] and RARγ specifically aids in the formation of healthy cartilage[35]. What is more, retinoic acid induces the production of metalloproteinases that are involved in cartilage degradation[39,40].

Retinoic Acid as a Posteriorizing Agent

Retinoic acid acts as a posteriorizing agent in various regions of developing vertebrates, including the pharyngeal arches[41]. Compared to the anterior mandibular arch, branchial arches oriented more posteriorly have higher sensitivity to retinoic acid[42,43]. In frogs, exogenous

retinoic acid posteriorizes the neural plate. This causes the anterior neural plate border to transform into neural crest cells[44], which give rise to various cell types, including chondrocytes and chondroblasts[45]. In other regions, retinoic acid has been shown to form a concentration gradient culminating in the posterior domain[46]; however, it is currently unknown if retinoic acid forms a similar gradient across the anteroposterior axis of the skull.

Experimental Design and Focus

In light of retinoic acid's clear effect on chondrification, the present study aimed to document any cranial teratogenesis observed from exogenous retinoic acid at relatively low concentrations. Another aim was to determine a relative threshold concentration that cranial chondrocytes of *Ambystoma mexicanum* can tolerate before abnormalities are observed.

Because of the lower concentrations of retinoic acid utilized in this study relative to those previously mentioned[43], observation of such dramatic abnormalities such as missing cranial cartilages and anterior truncation was not expected. Because retinoic acid is crucial to development, it was expected that embryos treated with higher concentrations of its inhibitor, N,N-diethylaminobenzaldehyde (DEAB), would have cartilages positioned more anteriorly.

Methods

Cleavage-stage axolotl embryos were obtained from the Ambystoma Genetic Stock Center[47]. These were raised in 200 mL of either: 1 nM, 0.1 nM, or 0.01 nM retinoic acid; 10 μM, 1 μM, or 0.1 μM DEAB; untreated E3 media[48]; or 0.1% dimethyl sulfoxide (DMSO). Concentrations of retinoic acid and DEAB were attained using DMSO. Until hatching, both treated and control embryos were incubated in the dark at 15°C to prevent light-induced degradation of retinoic acid[49].

Upon their hatching, several treated and control larval axolotls were fixed in 10% formalin. Clearing was performed with 0.025% potassium hydroxide dissolved in glycerol and water, and staining with alcian blue (cartilage) and alizarin red (bone) dissolved in the same sort of solution. Cleared and stained axolotls were stored in 100% glycerol, which was also used for whole-mount imaging. Live brine shrimp were fed to remaining larvae each day until fixed. These were fixed five, nine, eleven, and thirteen days after hatching and prepared with the same protocol as were those fixed upon hatching.

Images of the skull and entire specimen were obtained at 5X magnification using the Zeiss Pro® microscope and software program, which facilitates the measuring of specimens by using a tracing tool that computes the total distance of several lines drawn on the captured image. The total body length of each axolotl was obtained by tracing the notochord from tail to tip, then from tip to the intermandibular ligament. Cartilages of the chondrocranium were measured using the procedure outlined below.

Mandibular (Meckel's) Cartilage

The summed length of the two mandibular cartilages was measured using the Zeiss Pro® software. Each recorded length of the mandible was the length of a single multiple-angled line drawn through the center of both cartilages from the beginning of the left cartilage to the end of the right, including the small ligament in between (*Figure 1*). For mandibles exhibiting cartilaginous ossification, only measurements of the cartilaginous tissues were taken.

Ceratohyal

Each of the two ceratohyal cartilages was measured and recorded separately. As was the case for the mandibular cartilage, each ceratohyal was measured from beginning to end, through its center (*Figure 1*). The ligament between the ceratohyal and hypohyale was not considered and was often indicated by a cessation of alcian blue staining or a pinching of the cartilage (*Figure 2*).

Hypohyale

Each of the two hypohyale cartilages was measured and recorded separately in the same manner as were the mandibular and ceratohyal cartilages (*Figure 1*). The ligament between the two hypohyale cartilages was not considered and was often indicated by the cessation of alcian blue staining or a thinning of the region (*Figure 2*).

Quadrate

Both length and width of each of the two quadrate cartilages were recorded. For the length, a straight line was drawn from the region in which the quadrate contacted the mandible to the flatter, longer portion of the quadrate (*Figure 1*). For older aged larvae, the quadrate often overlapped the mandible. Because focus constraints on the microscope prevented discernment of the quadrate's true end, the quadrate in these individuals was measured lengthwise from the

region of overlap to the flatter side (*Figure 2*). The width of the quadrate was measured from the upper edge of its stouter portion to the point at which its thinner arm overlapped the ethmoid cartilage (*Figures 1 and 2*). As an aside, the quadrate likely contacts the more ventrally positioned trabecular cartilage. However, because this contact was not always observed under the microscope, the quadrate was measured relative to its proximity to the ethmoid.

These measurements were analyzed using ANCOVA, in which the covariant was body length and individual ages were disregarded. A simple anatomical diagram of the axolotl chondrocranium and pharyngeal skeleton was created using literature[43,50] and stained individuals from the present study fixed five days after hatching and showing consensus in the layout and relative lengths of their cranial cartilages (*Figure 2*). Axolotls whose skulls considerably deviated from this diagram were selected for further analysis. Before an individual was classified as malformed, its stage in development as well as its angle and position on the wet-mount slide were taken into account, as explained below.

Seemingly deformed axolotls were remounted on glass slides and reimaged. These new images were compared to those of same-age control axolotls. If the apparent malformation was observed again in the recaptured image, the embryo was considered malformed and a note of the malformation was recorded. Posterior or anterior displacement of tissues, misshapen or missing cartilages, and oddly positioned structures were especially noted, if present.

Results

Sample Size

Ninety-one individuals were measured in this study. Of these, twelve were treated with 1 nM retinoic acid, thirteen with 0.1 nM retinoic acid, eighteen with 0.01 nM retinoic acid, seven with 10 µM DEAB, three with 1 µM DEAB, ten with 0.1 µM DEAB, fourteen with 0.1% DMSO, and fourteen were not treated at all (*Figure 3*).

Of the individuals treated with 1 nM retinoic acid, *one* was fixed upon hatching, *two* were fixed five days after hatching, *three* nine days after hatching, *three* eleven days after hatching, and *three* thirteen days after hatching. Of those treated with 0.1 nM retinoic acid, *one* was fixed upon hatching, *two* five days after hatching, *three* nine days after hatching, *four* eleven days after hatching, and *three* thirteen days after hatching. Of those treated with 0.01 nM retinoic acid, *one* was fixed upon hatching, *two* five days after hatching, *eight* nine days after hatching, *four* eleven days after hatching, and *three* thirteen days after hatching. Of those treated with 10 µM DEAB, *two* were fixed five days after hatching and *five* nine days after hatching. The *three* treated with 1 µM DEAB were all fixed nine days after hatching. Of those treated with 0.1 µM DEAB, *two* were fixed five days after hatching and *eight* nine days after hatching. Of those raised in 0.1% DMSO, *one* was fixed upon hatching, *two* five days after hatching, *seven* nine days after hatching, *two* eleven days after hatching, and *two* thirteen days after hatching. Of those untreated, *two* were fixed upon hatching, *two* five days after hatching, *four* nine days after hatching, *three* eleven days after hatching, and *three* thirteen days after hatching (*Figure 3*).

Malformations Not Observed

To summarize, malformed individuals were those who, after reimaging and comparison to same-age control axolotls, noticeably disaccorded with the skull diagram in shape, position, and spacing of cartilages (*Figure 2*). In this study, only individuals whose premaxillae were positioned anteriorly were considered malformed, as explained below. In all other instances where cartilage structure and arrangement appeared to vary between treatments, visual comparisons between individuals of the same age revealed that such variations were due to cranial growth typical during chondrification. For example, quadrate structure and position varied with age. In older individuals the quadrate extended from under the mandible to the trabecular cartilage located just ventral to the ethmoid. On the other hand, in younger individuals the quadrate was most commonly not overlapped by the mandible (*Figure 4*). Cranial spacing was also observed to vary with age. In younger axolotls, cartilages were more closely situated, giving the skull a more compact shape. In older axolotls, the skull appeared longer (*Figure 5*).

Premaxillae Potentially Anteriorized in the Absence of Retinoic Acid

The anteroposterior positioning of the premaxilla appears to vary across several individuals in a treatment-dependent manner. Premaxillae that did not fully cross the mandibular cartilage were considered anteriorized. Of the ten individuals with a noticeably anterior premaxilla, eight were treated with DEAB (four at 10 μM, three at 1 μM, and one at 0.1 μM DEAB), one was treated with 1 nM retinoic acid, and one was raised in 0.1% DMSO (*Figure 6*). In this group, the axolotl treated with 1 nM retinoic acid was fixed eleven days after hatching while the other nine were fixed nine days after hatching.

Apart from the Hypohyale, Treatment Has No Significant Effect on Cartilage Size

The ANCOVA using body length as a covariant revealed no significant difference between the type of treatment to which the axolotls were exposed and the measurements of their mandibular, ceratohyal, or quadrate cartilages (*Table 1*). With the exclusion of quadrate length, cartilage size correlated more significantly with body length than it did treatment type (*Table 1*). The length of the hypohyale was significantly affected by treatment [$p = 0.0007$] (*Table 1*). Regression lines obtained from ANCOVA demonstrate that the hypohyale was longest in those raised in 0.1% DMSO and shortest in those treated with 1 µM DEAB or 0.1 µM DEAB (*Figures 7 – 11*).

Discussion

Retinoic Acid Induces Teratogenesis in the Chondrocranium and Modification of the Hypohyale

Previous studies have demonstrated that exogenous retinoic acid causes abnormalities in cartilage tissues[33,34,35]. Molar concentrations comparable to those used in this study decrease cartilage production *in vitro*[33] and at slightly higher concentrations, retinoic acid induces anterior truncation and acephaly in frog embryos[36,37]. Studies using *Ambystoma mexicanum* show that retinoic acid removes only the second basibranchial cartilage at 50 nM, an ability attributed to dual embryonic origin and patterning of the pharyngeal skeleton[43]. Such findings reveal that cartilages of the same region have varying sensitivities to retinoic acid. This would explain why the mandibular but not the pharyngeal arches appeared unaffected by retinoic acid in the cited study and why only the hypohyale was significantly affected by retinoic acid in this study (*Table 1*). Nevertheless, considerably high concentrations of retinoic acid cause teratogenesis in all cartilages of the chondrocranium. If axolotls of the present study also were to have been treated with 100 nM retinoic acid, distortion and compaction of the chondrocranium[43] most probably would have been observed.

Retinoic Acid Potentially Posteriorizes the Axolotl Premaxilla

Studies have highlighted the role of retinoic acid as a posteriorizing agent in various regions of developing vertebrates[41,46]. Of specific interest in this study is its ability to posteriorize pharyngeal arches and the anterior neural plate border. Posteriorization of pharyngeal arches is suggested by knockout studies of RARα and RARβ in mice[41]. In frogs, retinoic acid posteriorizes the neural plate and leads to the production of neural crest cells, which are cartilage tissue cell lines. Inversely, quail embryos deficient in retinol experience neural crest cell apoptosis[42].

Although the limited parameters and methods of this study prevent analysis of posteriorization in areas known to be affected by retinoic acid such as those mentioned above, it may be conjectured that retinoic acid is able to posteriorize the premaxilla in *Ambystoma mexicanum* skulls. Of the relatively few individuals that exhibited anterior positioning of this structure, the majority were treated with DEAB (*Figure 6*). Is it possible, then, that inhibition of retinoic acid was responsible for the apparent anteriorization? To begin answering that question, it would be useful to know just how much inhibition of retinoic acid actually occurred. This can be achieved by monitoring cellular concentrations of retinoic acid and retinal dehydrogenase activity. Does the fact that the majority of individuals with anterior positioning of this structure were of the same age imply complications in the methods of treatment? Answers to these questions as well as replication of the study may elucidate underlying mechanisms of retinoic acid posteriorization.

Teratogenesis is Mitigated in the Ambystoma mexicanum *Chondrocranium*

Despite the apparent sensitivity of the hypohyale to retinoic acid, *Ambystoma mexicanum* overall appear quite effective at mitigating teratogenesis at 1 nM, 0.1 nM, and 0.01 nM retinoic acid. The present study reports no missing or visibly deformed cartilages, contrary to other papers[43] that linked exogenous retinoic acid to the absence of basibranchial 2 as well as to severe deformations in the chondrocranium and pharyngeal skeleton. This finding is not startling as the concentrations used here are orders of magnitude lower than those used in such studies. Moreover, the amount of retinoic acid actually taken up by CRABP is uncertain and can be at least partially responsible for the absence of malformations.

Threshold Concentration of Retinoic Acid

As gathered from this and the aforementioned study[43], it seems that the increase of retinoic acid from 1 nM to 50 nM is enough to induce observable teratogenesis in *Ambystoma mexicanum.* It is not yet clear how specific concentrations within this range affect development in the chondrocranium. Nevertheless, it can be said that relative to reported malformations caused by 50 nM and 100 nM, abnormalities caused by 0.01 nM to 1 nM retinoic acid are minor if not nonexistent and the overall structure of the chondrocranium and its tissues appear unaffected. Therefore, a retinoic acid threshold concentration may exist between 1 nM and 50 nM. Below this range, *Ambystoma mexicanum* are able to tolerate exogenous retinoic acid without experiencing dramatic teratogenesis in their chondrocranium, whereas above this range they cannot.

Retinoic Acid Clearance is Likely Involved in Mitigation of Teratogenesis

The fact that cells of the chondrocranium are able to tolerate certain concentrations of retinoic acid calls for analysis of retinoic acid cellular clearance. Although retinoic acid regulation at the cellular level is not fully understood, at least two proteins, CYP26 and CRABP, have been suggested to be direct influencers. Expression of CRABP is upregulated in regions of high retinoic acid concentrations and causes subsequent removal of the ligand as well as limited access to is nuclear receptors[51,52,53]. However, because CRABP has not yet been found in cartilage cells[54], the role of CYP26 in retinoic acid metabolism is more relevant to this study.

It is generally agreed that the binding of retinoic acid to its nuclear receptor prompts the production and activity of CYP26 enzymes. One study compared teratogenesis in $CYP26^{-/-}$ mutant embryos to that associated with excess retinoic acid[55], leading to the belief that retinoic acid is primarily cleared by CYP26. Thus, through activation of CYP26, retinoic acid induces its own metabolism. Nevertheless, not all cell types respond to retinoic acid by regulation of CYP26, and the presence and expression of CYP26 in cells of the axolotl chondrocranium have

not yet been confirmed. Could its activity, though, be connected to retinoic acid tolerance as observed in this study? If so, future projects, as later elaborated, may be useful in understanding the correlation between retinoic acid clearance and teratogenesis.

Of notable mention are two minor contributions to retinoic acid homeostasis— minor primarily due to limited research in these areas. STRA6 is capable of reloading retinol onto RBP and is also able to bind to retinoic acid[56]. Reduced cellular concentrations of retinol leave less of the substrate available for oxidation to retinoic acid. Perhaps a similar mechanism exists in which retinoic acid is exported from cells of oviparous embryos? Though this scheme is more imaginative than evidential, it is probable that cellular retinoic acid concentrations are regulated by cellular transport due to involvement of STRA6 in regulation. The irreversible conversion of retinaldehyde to retinoic acid hints at the concentration-dependent activity of retinal dehydrogenase. Indeed, a feedback loop involving STRA6 and retinal dehydrogenase helps maintains cellular retinoic acid homeostasis[57].

Future Studies

The limited parameters of this study encourage supplementary projects analyzing other aspects of retinoic acid control. For example, addition of retinoic acid to chondrocytes is known to cause structural and functional alteration.[33] Experiments measuring chondral tissue density, chondrocyte arrangement and configuration, and alkaline phosphatase activity as well as those monitoring collagen production after exposure to retinoic acid are thereby useful. Gene expression profiling may further illustrate cellular sensitivity to retinoic acid and perhaps reveal other genes important in chondrification. Returning to the role of CYP26 in retinoic acid clearance, the treatment of $CYP26^{-/-}$ mutants with the same concentrations of retinoic acid and its inhibitor used here would be an interesting follow-up study.

Final Thoughts

In conclusion, noticeable cranial teratogenesis was not produced in *Ambystoma mexicanum* by exogenous retinoic acid at concentrations of 1 nM, 0.1 nM, or 0.01 nM in this study. It seems that a retinoic acid concentration threshold, in which *Ambystoma mexicanum* can tolerate exogenous retinoic acid in the chondrocranium, exists between 1 nM and 50 nM.

Tables

	Cartilage Length				Cartilage Width
	Mandibular	**Ceratohyal**	**Hypohyale**	**Quadrate**	**Quadrate**
Whole Body Length	**<0.0001**	**<0.0001**	**<0.0001**	0.7622	**<0.0001**
Treatment	**0.0134**	**0.0005**	**0.0235**	**0.0162**	**<0.0001**
Whole Body Length*Treatment	0.1700	0.3629	**0.0007**	0.8023	0.1333

Table 1 P-values from ANCOVA highlighting the effect, if any, that treatment type has on cartilage size in axolotl (*Ambystoma mexicanum*) chondrocrania. The first row gives p-values for the correlation between whole body length and cartilage size. The second gives p-values for the correlation between treatment and cartilage size. The third gives p-values for the correlation between cartilage size and treatment using body length as a covariant. Bolded values are statistically significant ($\alpha = 0.05$). In-text ANCOVA results are also provided.

Figures

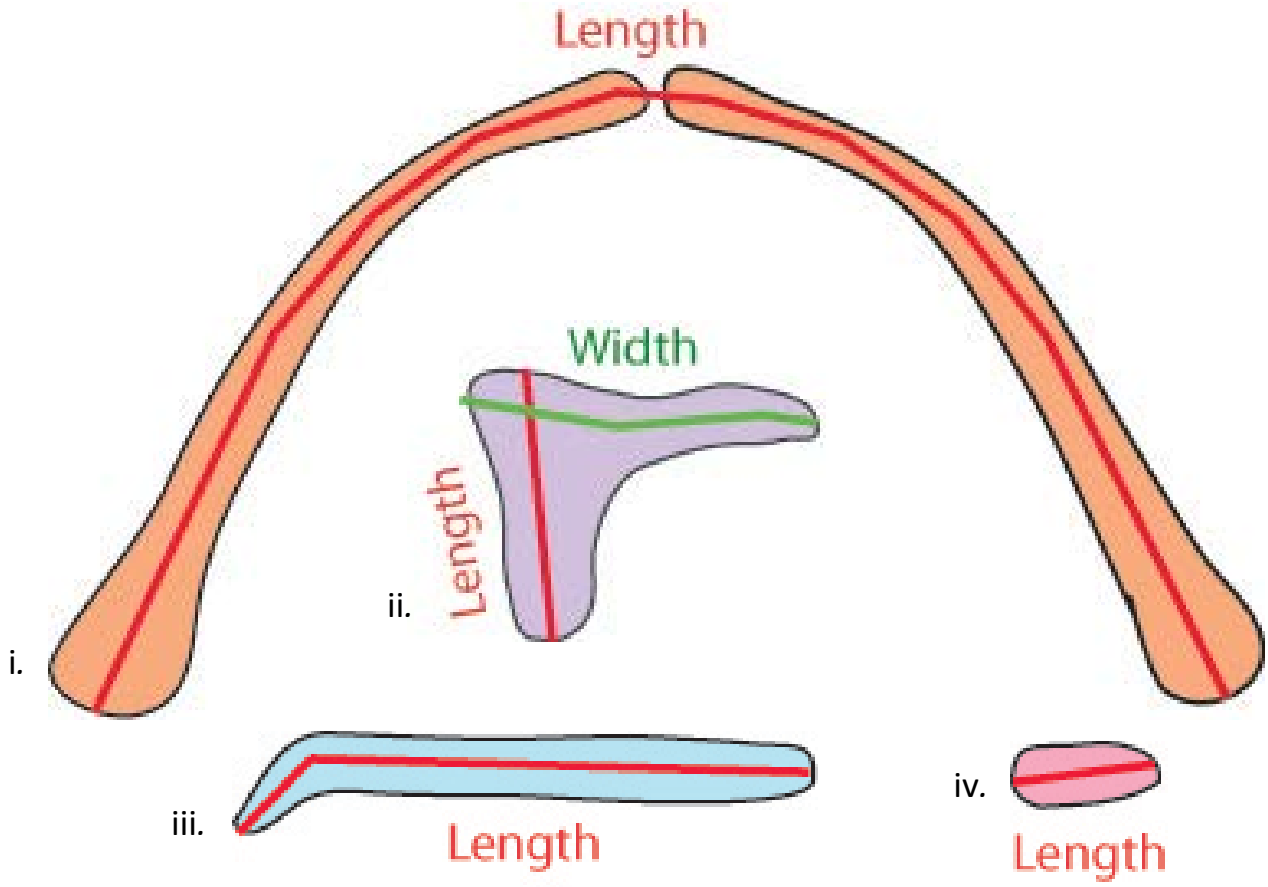


Figure 1 A schematic diagram illustrating the method used to measure cartilages in axolotls (*Ambystoma mexicanum*). Green and red lines mimic those of the tracing tool used in the Zeiss® microscope and software program, as explained in text. Compare to *Figure 2* for relative positions of each cartilage in the chondrocranium.

KEY*:* **i.** mandibular cartilage (**a.** in *Figure 2*); **ii.** quadrate cartilage (**c.** in *Figure 2*); **iii.** ceratohyal cartilage (**d.** in *Figure 2*); **iv.** hypohyale cartilage (**e.** in *Figure 2*).

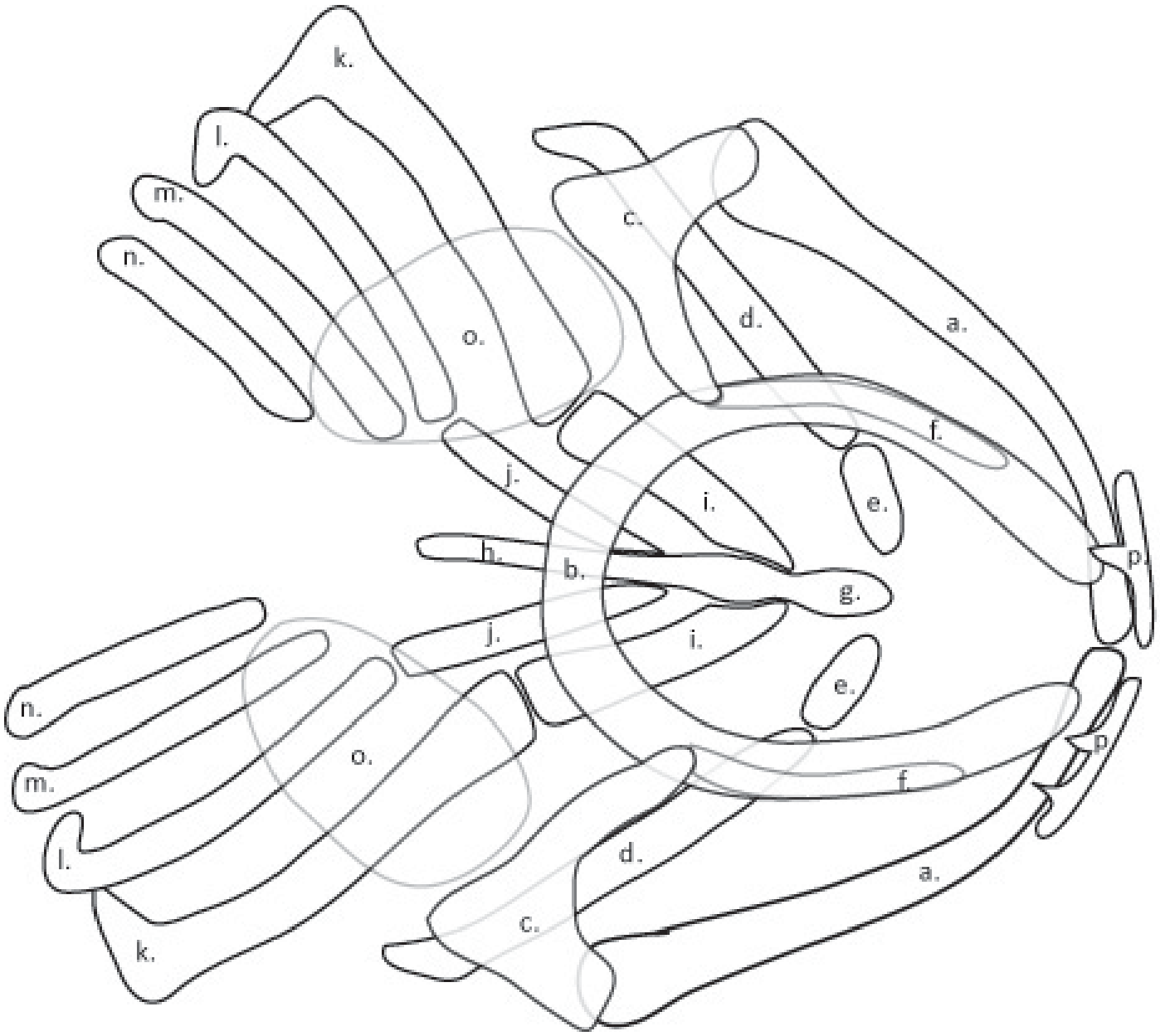


Figure 2 A simple anatomical diagram labeling known cartilages of the axolotl (*Ambystoma mexicanum*) chondrocranium and pharyngeal cartilages. Transparency indicates dorsal positioning. The relative positions of these cartilages are based on axolotl embryos fixed five days after hatching and literature, as explained in text. Unknown structures receive a red question mark after their name in the following key.

KEY*:* **a.** mandibular/Meckel's cartilage **b.** ethmoid cartilage? **c.** quadrate cartilages **d.** ceratohyal cartilages **e.** hypohyale cartilages **f.** trabecular cartilages? **g.** basibranchial 1 **h.** basibranchial 2 **i.** hypobranchial 1 **j.** hypobranchial 2 **k.** ceratobranchial 1 **l.** ceratobranchial 2 **m.** ceratobranchial 3 **n.** ceratobranchial 4 **o.** dorsal groove **p.** premaxilla

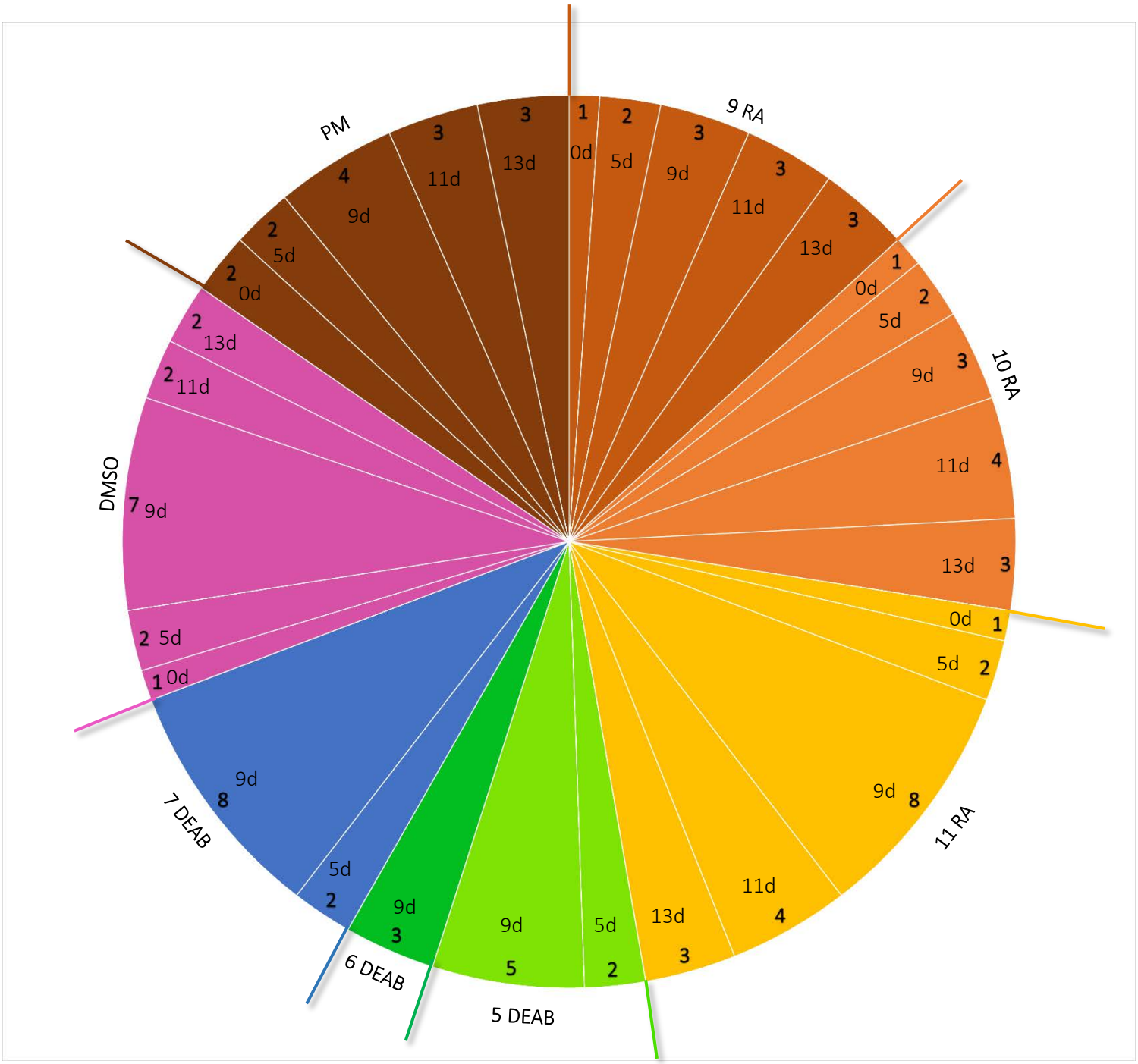


Figure 3 Sample sizes organized by treatment and labeled with age. Each color in the pie chart represents a different treatment, as labeled below each section. Numbers preceded by the letter "d," as in 0d, 5d, etc. refer to the number of days after hatching the individuals were fixed in each treatment. A total of 91 axolotls were measured.

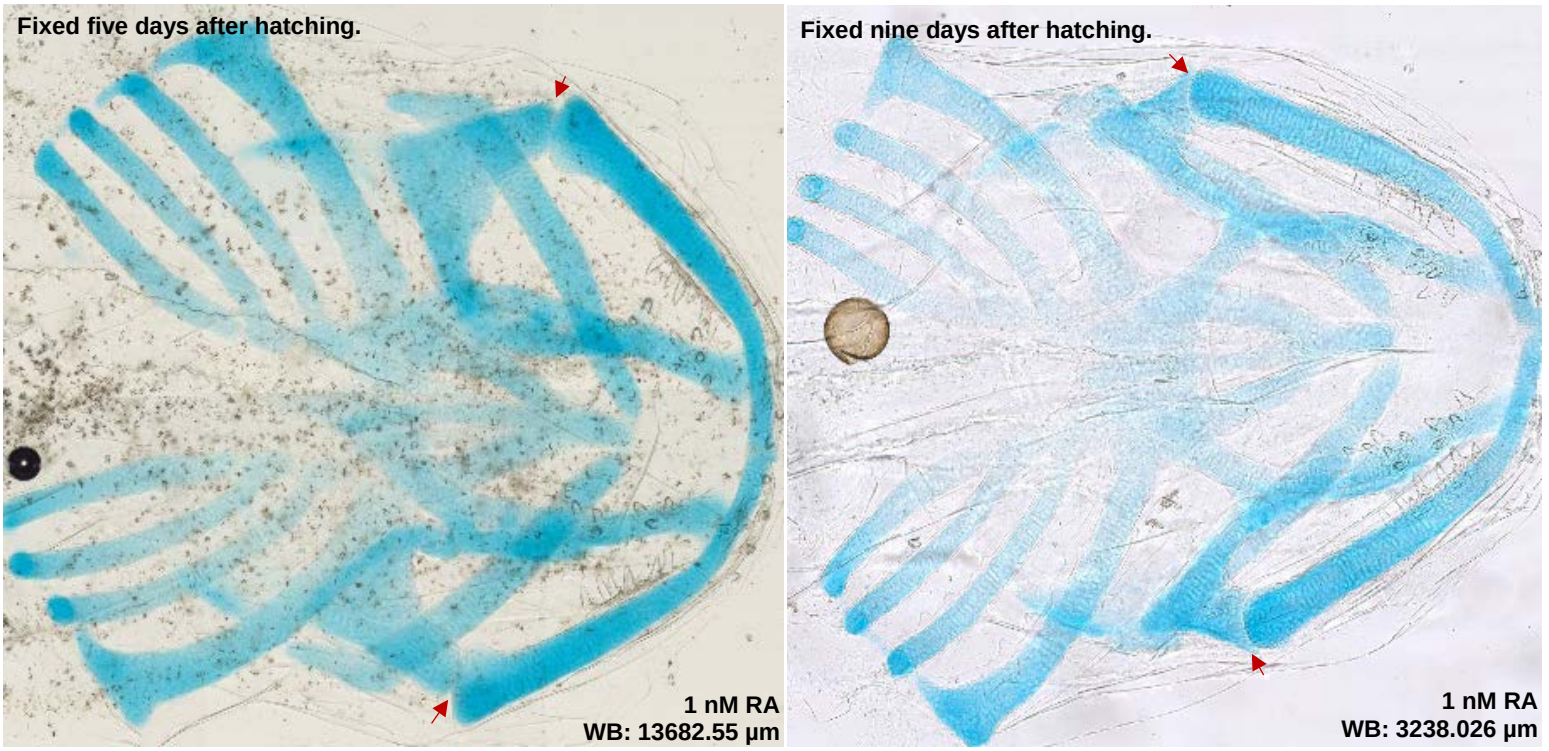


Figure 4 Images of two *Ambystoma mexicanum* chondrocrania, both treated with 1 nM retinoic acid, as displayed on the images. **WB** indicates the whole body length of the individual. *Left* Axolotl was fixed five days after hatching. *Right* Axolotl was fixed nine days after hatching. Red arrows note the different contact points of the quadrate cartilages.

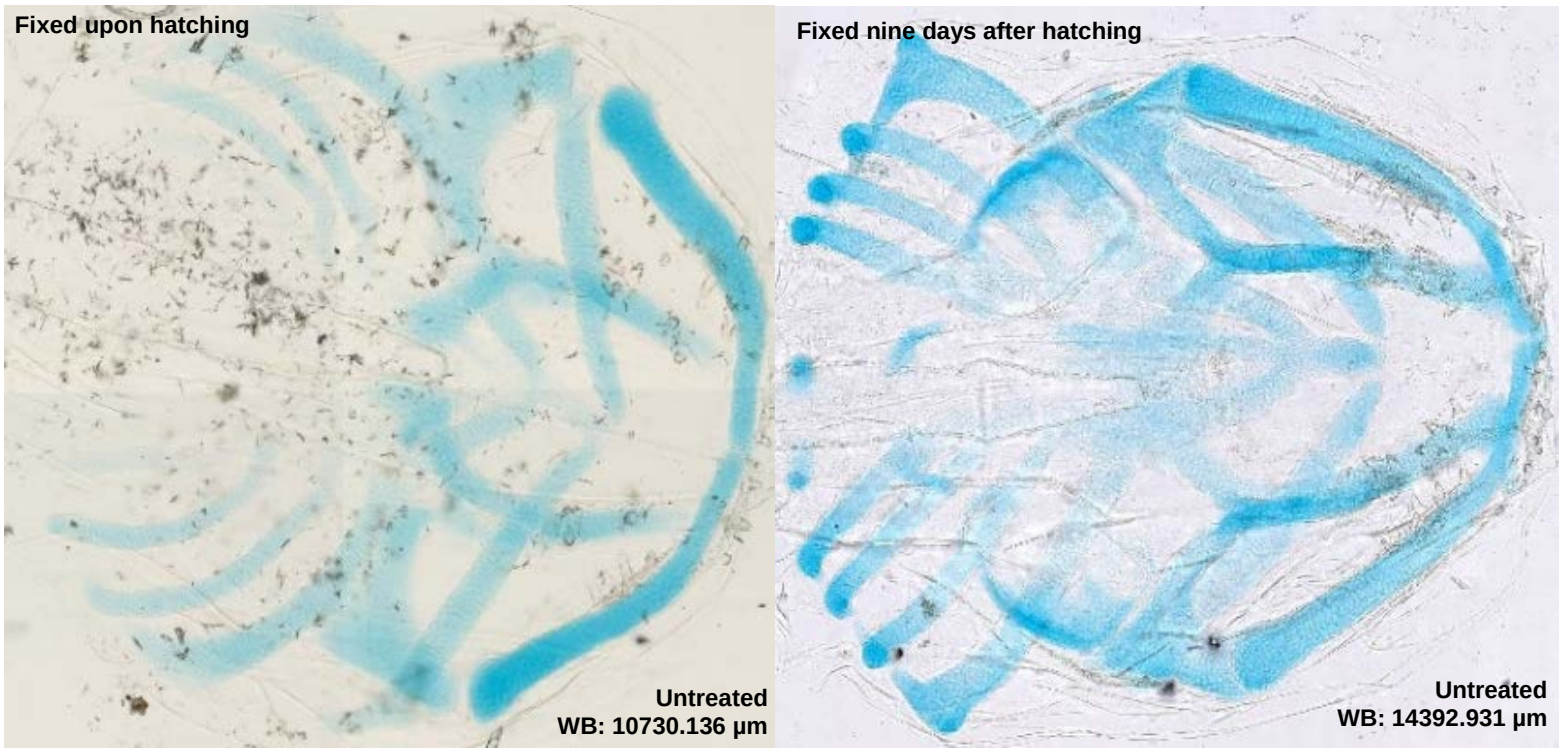
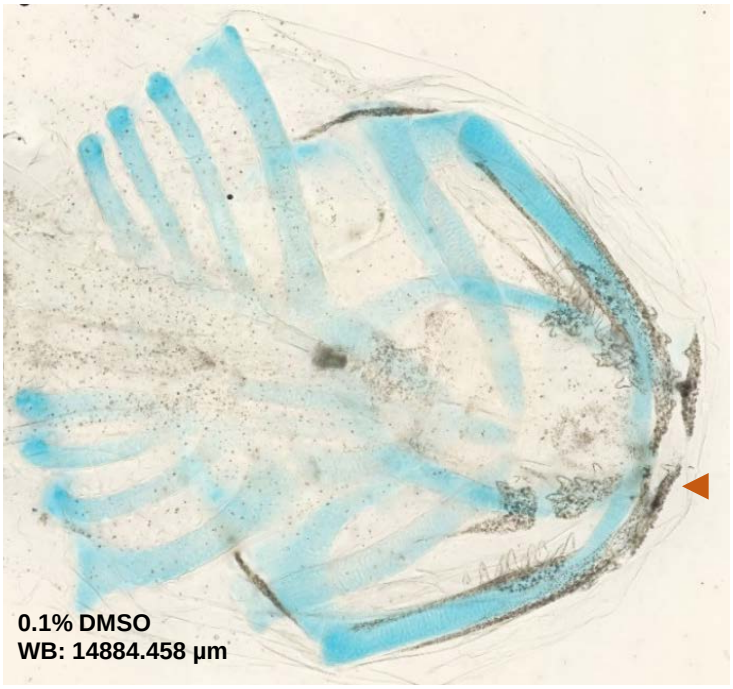
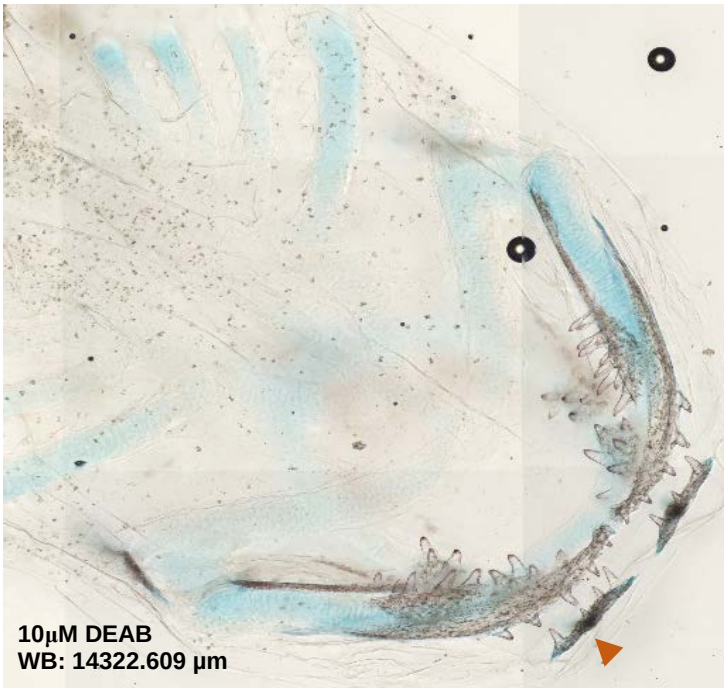
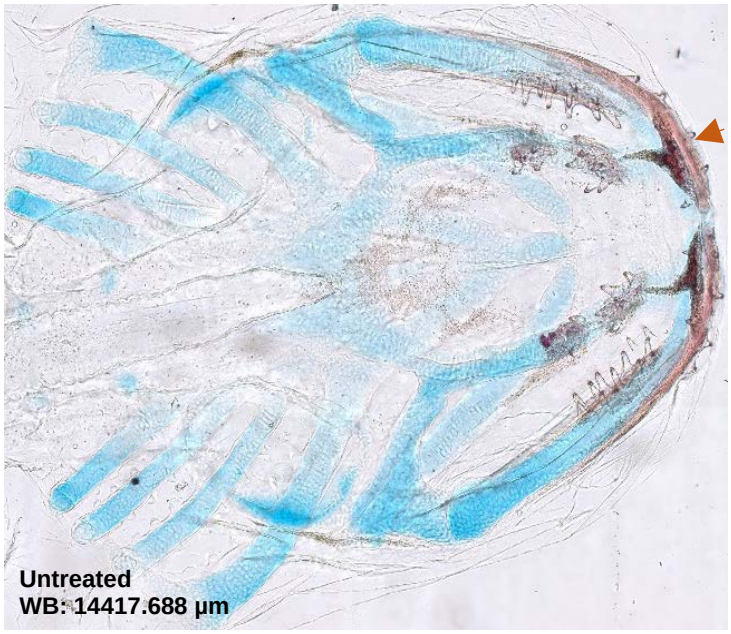


Figure 5 Images of two *Ambystoma mexicanum* chondrocrania, both untreated as is displayed on images.

Left Axolotl was fixed upon hatching. *Right* Axolotl was fixed nine days after hatching. **WB** indicates the whole body length of the individual. Note the difference in skull spatial arrangement between the two individuals, as explained in text.







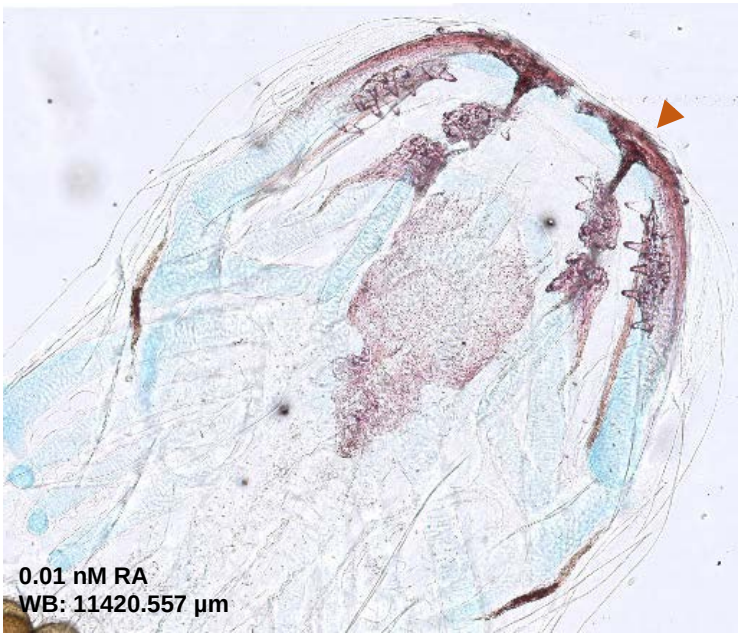


Figure 6 Images of axolotls (*Ambystoma mexicanum*) fixed nine days after hatching. Treatments and body lengths are indicated on the bottom left of each individual. Arrowheads indicate the premaxilla. As discussed in text, premaxillae in the untreated axolotl and in the axolotl treated with 0.01 nM retinoic acid are properly positioned in the chondrocranium. However, those raised in 0.1% DMSO, 0.1 µM DEAB, and 10 µM DEAB are positioned more anteriorly.

KEY: RA stands for retinoic acid, WB stands for whole body length.

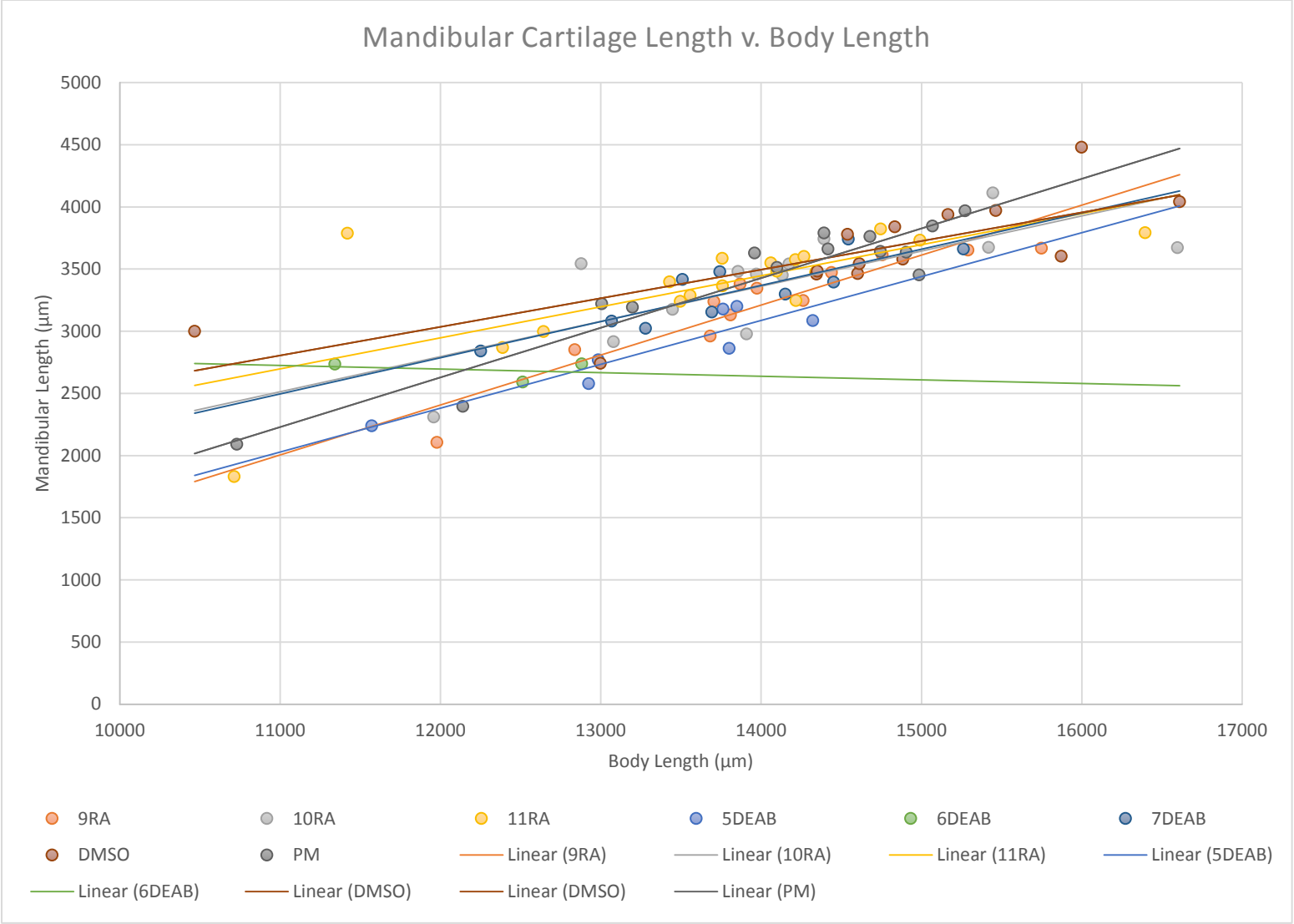


Figure 7 A plot of the regression between mandibular cartilage length and body length, as obtained from ANCOVA. Regression lines are indicated in the legend as "Linear" followed by the corresponding treatment.

KEY: 9RA, 10RA, and 11RA stand for axolotl embryos treated with 1 nM RA, 0.1 nM RA, and 0.01 nM RA, respectively. 5DE, 6DE, and 7DE stand for axolotl embryos treated with 10 µM DEAB, 0.1 µM DEAB, and 0.01 µM DEAB, respectively. DM and PM stand for axolotl embryos of the DMSO and plain (untreated) media controls, respectively.

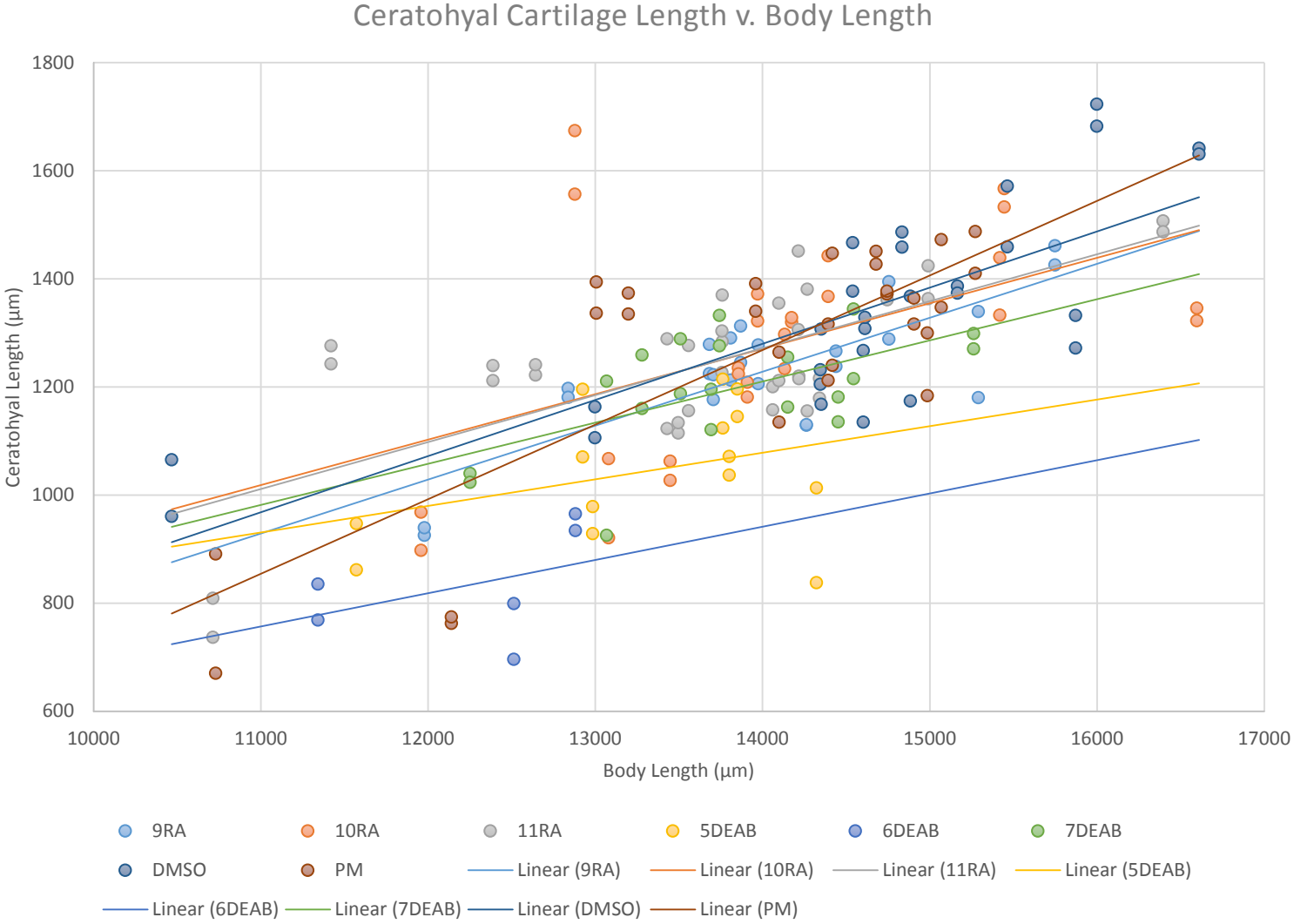


Figure 8 A plot of the regression between ceratohyal cartilage length and body length, as obtained from ANCOVA. Regression lines are indicated in the legend as "Linear" followed by the corresponding treatment.

KEY: 9RA, 10RA, and 11RA stand for axolotl embryos treated with 1 nM RA, 0.1 nM RA, and 0.01 nM RA, respectively. 5DE, 6DE, and 7DE stand for axolotl embryos treated with 10 µM DEAB, 0.1 µM DEAB, and 0.01 µM DEAB, respectively. DM and PM stand for axolotl embryos of the DMSO and plain (untreated) media controls, respectively.

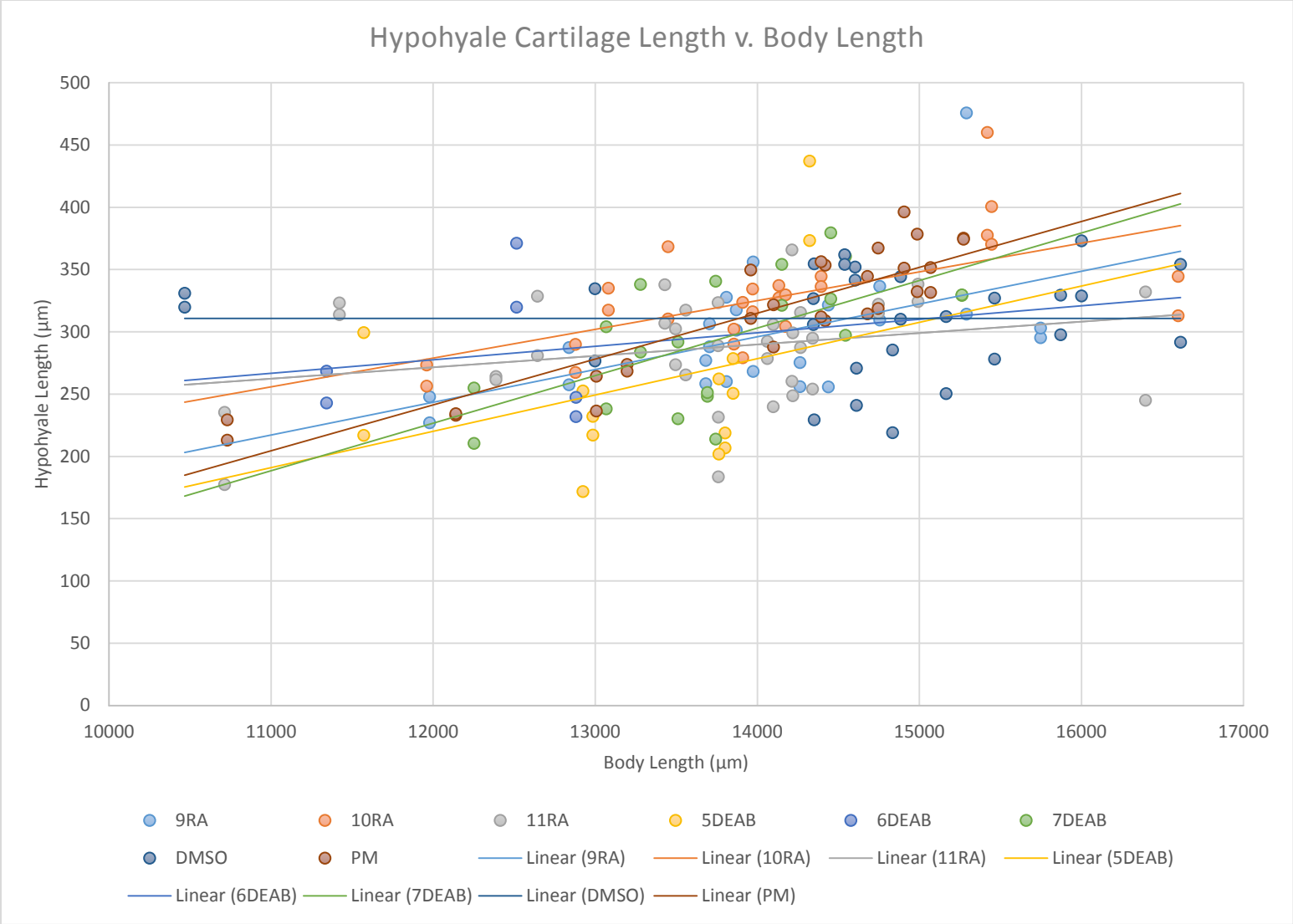


Figure 9 A plot of the regression between hypohyale cartilage length and body length, as obtained from ANCOVA. Regression lines are indicated in the legend as "Linear" followed by the corresponding treatment.

KEY: 9RA, 10RA, and 11RA stand for axolotl embryos treated with 1 nM RA, 0.1 nM RA, and 0.01 nM RA, respectively. 5DE, 6DE, and 7DE stand for axolotl embryos treated with 10 µM DEAB, 0.1 µM DEAB, and 0.01 µM DEAB, respectively. DM and PM stand for axolotl embryos of the DMSO and plain (untreated) media controls, respectively.

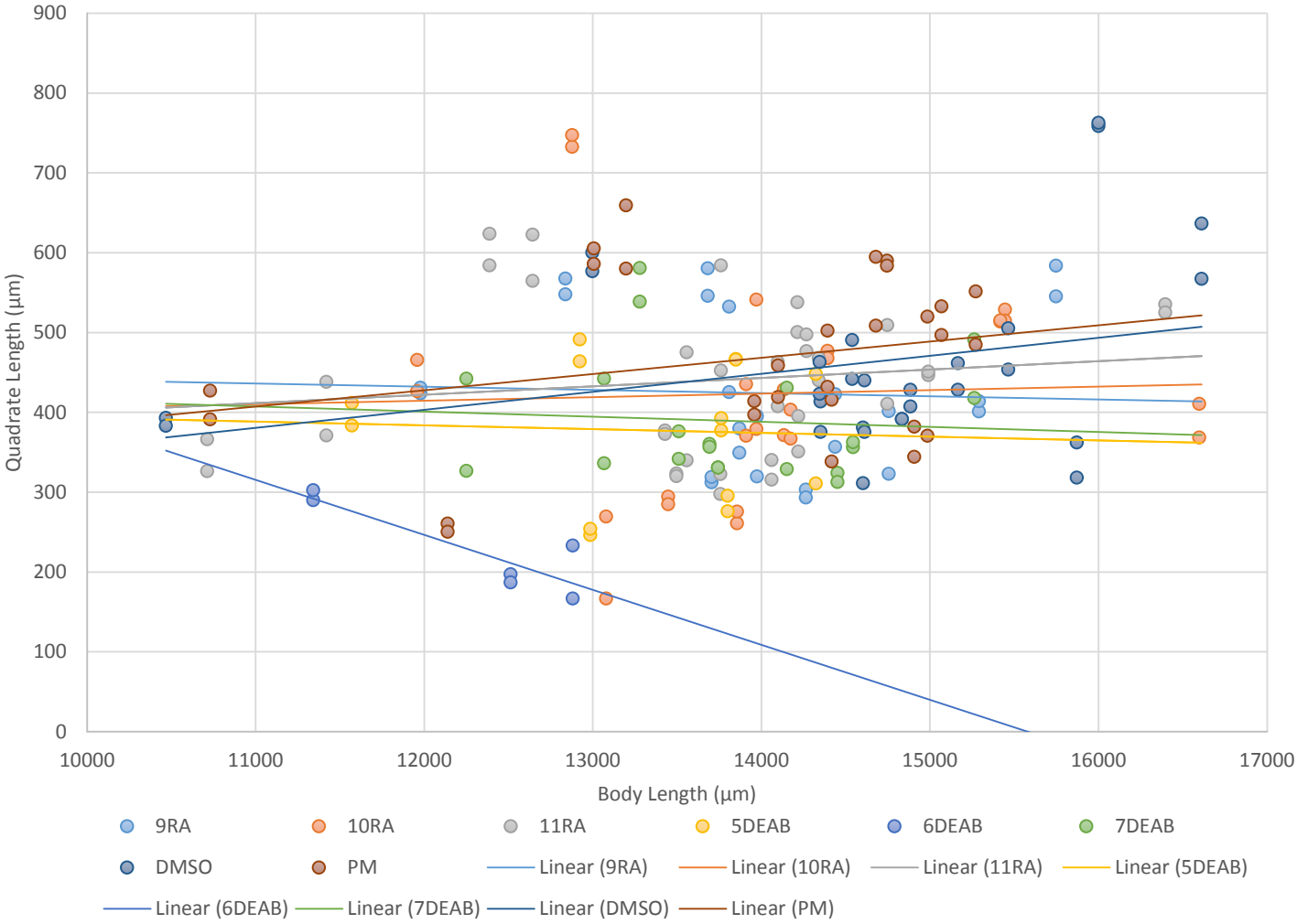


Figure 10 A plot of the regression between quadrate cartilage length and body length, as obtained from ANCOVA. Regression lines are indicated in the legend as "Linear" followed by the corresponding treatment.

KEY: 9RA, 10RA, and 11RA stand for axolotl embryos treated with 1 nM RA, 0.1 nM RA, and 0.01 nM RA, respectively. 5DE, 6DE, and 7DE stand for axolotl embryos treated with 10 µM DEAB, 0.1 µM DEAB, and 0.01 µM DEAB, respectively. DM and PM stand for axolotl embryos of the DMSO and plain (untreated) media controls, respectively.

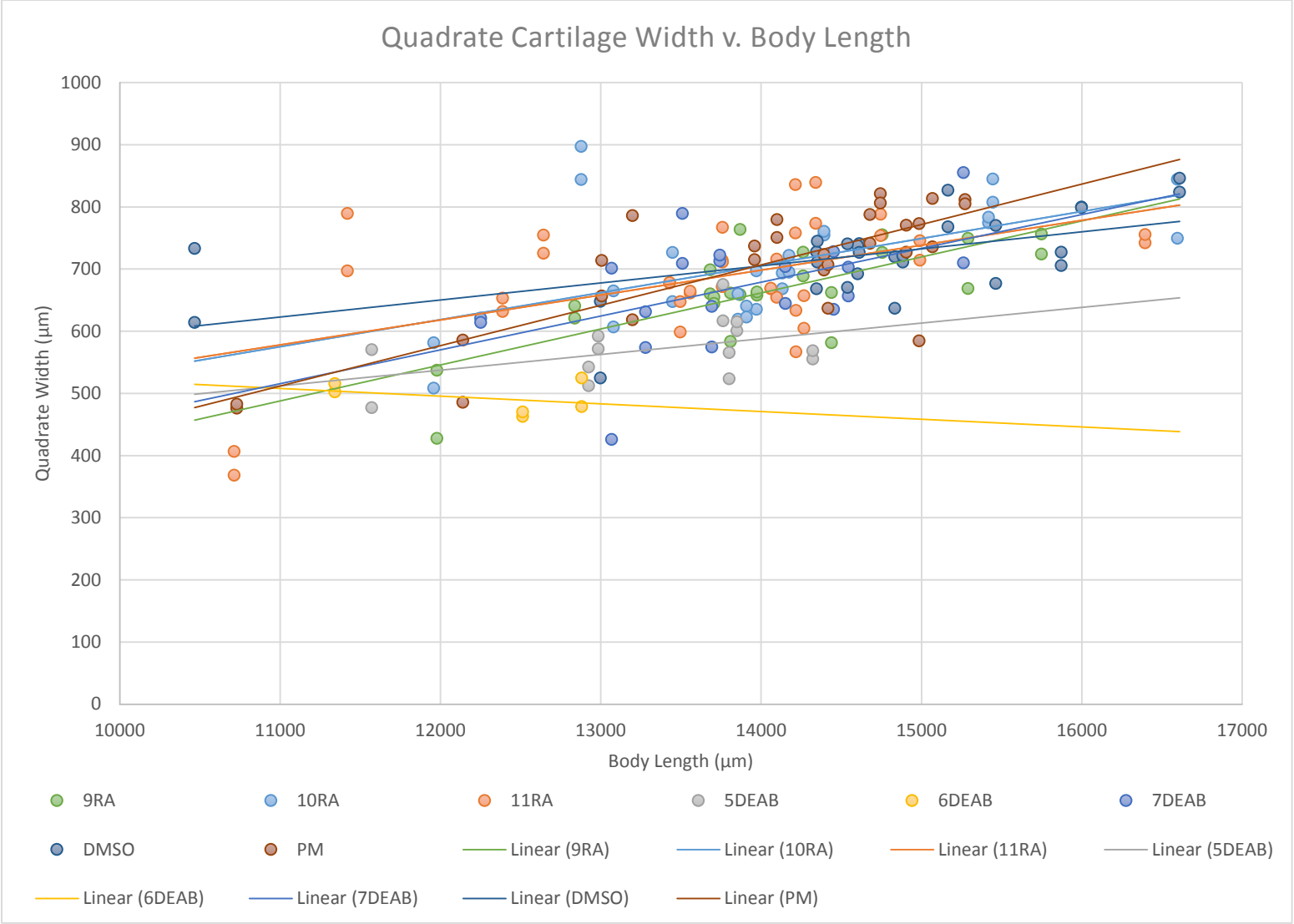


Figure 11 A plot of the regression between quadrate cartilage width and body length, as obtained from ANCOVA. Regression lines are indicated in the legend as “Linear” followed by the corresponding treatment.

KEY: 9RA, 10RA, and 11RA stand for axolotl embryos treated with 1 nM RA, 0.1 nM RA, and 0.01 nM RA, respectively. 5DE, 6DE, and 7DE stand for axolotl embryos treated with 10 µM DEAB, 0.1 µM DEAB, and 0.01 µM DEAB, respectively. DM and PM stand for axolotl embryos of the DMSO and plain (untreated) media controls, respectively.

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