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RESEARCH ARTICLE

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The Tale-Tell Heart: Evolutionary tetrapod shift from aquatic to terrestrial life-style reflected in heart changes in axolotl (*Ambystoma mexicanum*)

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Abstract

Background: During amphibian metamorphosis, the crucial moment lies in the rearrangement of the heart, reflecting the changes in circulatory demands. However, little is known about the exact shifts linked with this rearrangement. Here, we demonstrate such myocardial changes in axolotl (*Ambystoma mexicanum*) from the morphological and physiological point of view.

Results: Micro-CT and histological analysis showed changes in ventricular trabeculae organization, completion of the atrial septum and its connection to the atrioventricular valve. Based on Myosin Heavy Chain and Smooth Muscle Actin expression we distinguished metamorphosis-induced changes in myocardial differentiation at the ventricular trabeculae and atrioventricular canal. Using optical mapping, faster speed of conduction through the atrioventricular canal was demonstrated in metamorphic animals. No differences between the groups were observed in the heart rates, ventricular activation times, and activation patterns.

Conclusions: Transition from aquatic to terrestrial life-style is reflected in the heart morphology and function. Rebuilding of the axolotl heart during metamorphosis was connected with reorganization of ventricular trabeculae, completion of the atrial septum and its connection to the atrioventricular valve, and acceleration of AV conduction.

KEYWORDS

atrial septation, metamorphosis, micro-CT, optical mapping, trabeculae, ventricular septation

1 | INTRODUCTION

The immense interest in amphibian heart morphology and development has lasted for more than one century.[1-3] Diversity among amphibians is extremely high with respect to morphology, physiology, and life-histories.[4,5] This diversity is also reflected in amphibians hearts.[6,7] Amphibian heart generally consists of five compartments: the sinus venosus, the left and right atrium, the ventricle, and the conus arteriosus.[8] However, the heart divisions differ significantly among the amphibian orders (Gymnophiona, Caudata, Anura).[9]

Amphibian ontogeny is driven by metamorphosis, when the cardiovascular system goes through the complete rearrangement from larval stage to adult terrestrial form.[10-13] The metamorphosis itself can be induced by

the Thyroid Hormones (THs),[10,14] and it is connected with redesigned vessels caused by the conversion from gills to lungs/skin breathing[15-17] as well as with the changes at the heart level.[18] THs control of metamorphosis, broadly conserved across amphibians,[19] supports the idea that THs are necessary and sufficient to induce metamorphosis in anurans[10] and caudata.[20,21] Thus, amphibians could provide a good source of experimental models for different types of cardiovascular physiological questions[3,9] important to understand vertebrate evolution. By studying the ontogenetic changes during amphibian metamorphosis, we could obtain a valuable insight into how the evolution of the heart transformation is involved in tetrapodean cardiovascular system.

Here, we focused on the axolotl (*A. mexicanum*) as a model organism because it represents the paedomorphic (sexual maturity in larval stage[22]) salamander species with facultative metamorphosis.[23] Furthermore, it is easily bred in the laboratory[24-27] and it is commonly used in developmental,[28-30] regenerative,[31-34] and electrophysiological studies.[35-37] However, little is known about morphology and physiology of the axolotl heart in the context of heart changes connected with transition in breathing due to metamorphosis.[38] Most of the cardiovascular studies to date have been focused on the paedomorphic form of axolotls.[33,36,39-41]

The goals of the present study are focused on the developmental changes occurring during TH-induced metamorphosis, which should be involved in the redesign of axolotl heart from paedomorphic (larval) stage to adult (metamorphic) stage. Our aim was to correlate the changes in the heart signal spreading over the ventricle with the morphological changes of the individual heart compartments before and after metamorphosis.

2 | RESULTS

2.1 | Process of axolotl metamorphosis

During the 3 weeks of metamorphosis, the axolotls were losing their external gills, closing branchial slits, and reducing the caudal fin rim. The animals also molted their epidermis. Micro-CT clearly showed differences in the heart fine structures as well as the organization of the trabeculae in the ventricle of paedomorphic (P) and metamorphic (M) axolotl hearts (Figure 1). It was observed how the gills were disappearing and the heart of specimens sampled during the metamorphosis (Figure 1 image "during Metamorphosis") already resembled metamorphic axolotl heart.

It should be noticed that we did not find any differences between sexes regarding their hearts before, during, and after metamorphosis. So, from this point of view, we considered the hearts equal.

2.2 | Gross heart morphology changes related to the metamorphosis

As in other amphibians, the axolotl heart generally consists of five compartments: the sinus venosus, the left and right atrium, the ventricle, and the conus arteriosus.

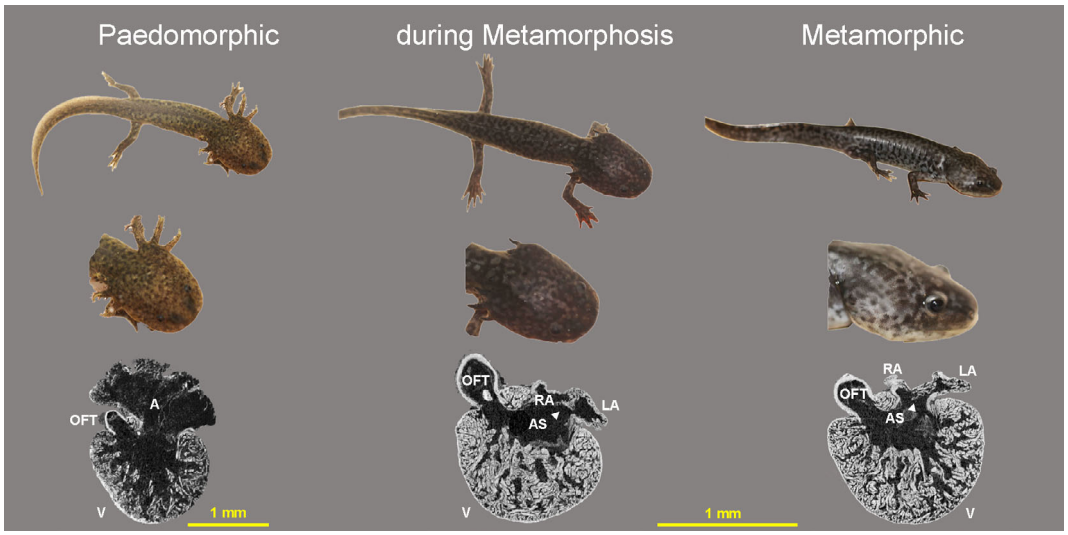

FIGURE 1 Metamorphosis and differences in the heart structures between paedomorphic (P) and metamorphic (M) axolotl. Heart structures and organization of the trabeculae among P, transitional stage, and M group. A, atrium; LA, left atrium; RA, right atrium; AS, atrial septum; OFT, outflow tract; V, ventricle; white arrowheads point to the atrial septum; scale bars - 1 mm

These structures were observed in both the P as well as in the M axolotls (Figures 1 and 2). By using micro-CT we were able to distinguish the fine structures within the cardiac chambers (Figure 1, Video S1 – paedomorphic and Video S2 – metamorphic axolotl heart). Higher magnification of the histological images (Figure 2) of the outflow tract (OFT) in both groups, in the P as well as in the M axolotls, showed partially septated OFT in the area of the conus arteriosus (Figure 2G,H).

2.3 | Metamorphosis-induced changes through the heart

The right atrium was notably bigger and with more extensive pectinate muscles (*musculi pectinati)* than the left atrium in both groups (Figure 2 part AB/H&E). In the P group, micro-CT showed a voluminous atrium with fine inner structures leading to the completion of the atrial septum (Figure 2A, B, E). It was observed that the atrial septum was connected to the atrioventricular valve (AVV) in the M group (Figure 2D, C′, D′). The P group revealed spongious meshwork of trabeculae in the ventricle without apparent organization or preferential direction. In the M group, the trabeculae were organized into several thick sheets (Figure 2C). These sheets revealed radial organization from the atrioventricular canal (AVC) toward the ventricular apex. Trabeculae were also more differentiated in thickness with thinner and smaller ones on the edges and progressively thicker ones in the center (Figure 2, part AB/H&E). However, in the center of the ventricle, no difference in cardiomyocyte size between the P and M groups was observed (to avoid underestimation, only cells with nucleus in transverse dimension were measured; 91 $\mu m^2 \pm 2$ N = 235 and 90 $\mu m^2 \pm 3$ N = 130 in the P and M animals, respectively). Based on the immunohistochemical examination, the AVC showed thicker musculature in the M group compared with the P group (Figures 3 and 4). These observations were confirmed by all used approaches

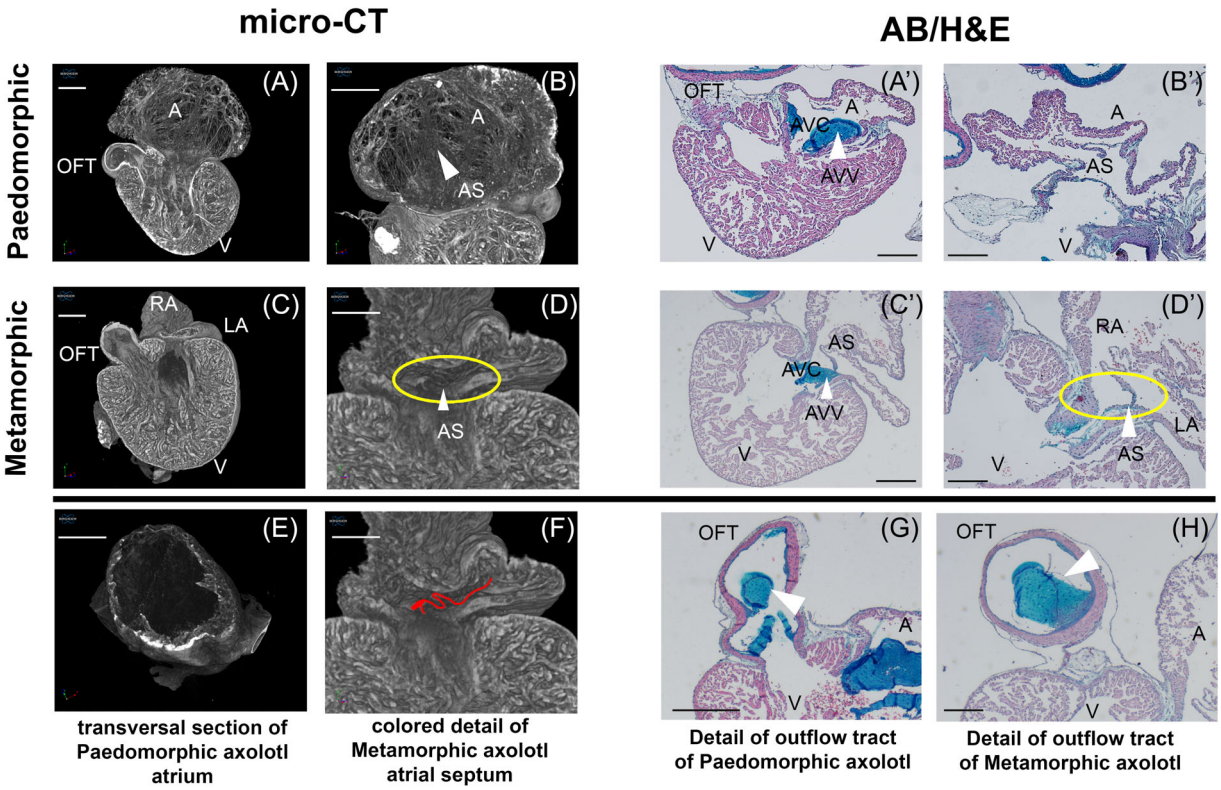

FIGURE 2 Micro-CT and histological sections (staining by Alcian Blue, Hematoxylin, and Eosin [AB/H&E]) of specific structures. Detailed section of the Outflow tract in both groups. Frontal section of paedomorphic axolotl heart in micro-CT (A). Detailed frontal section of paedomorphic atrium (B). Frontal section of the paedomorphic ventricle staining by AB/H&E (A′). White triangle points to valve in the atrioventricular canal. Detailed frontal section of paedomorphic atrium (B′). Frontal section of metamorphic axolotl heart in micro-CT (C). Detailed frontal section of metamorphic atrium (D). Note yellow circle and white arrowhead labeling the atrial septum. Frontal section of metamorphic ventricle (C′). Note the white triangle showing valve in the atrioventricular canal. Detailed frontal section of metamorphic atrium with yellow circle and white arrowheads showing atrial septum (D′). Transversal section of the atrium of paedomorphic axolotl (E). Detailed and colored atrial septum in metamorphic axolotl heart (F). Both pictures in micro-CT. Detailed section of the outflow tract with partially septated conus arteriosus (white arrowheads) (G,H). A, atrium; AS, atrial septum; AVC, atrioventricular canal; LA, left atrium; RA, right atrium; OFT, outflow tract; V, ventricle; AVV, atrioventricular valve. Scale bars - 1 mm

(micro-CT Figures 1, 2, Histology Figure 2, Immunohistochemistry, Figures 3 and 4).

2.4 | Metamorphosis-induced changes in the myocardial differentiation

In the detailed pictures of the atria in the P and M axolotls (Figure 3C,F), the atrial septum was positive for Smooth Muscle Actin (SMA), an early marker of the myocardium, in both groups of axolotls. Moreover, very distinct pectinate muscles were found in both images of P and M groups. SMA was distributed uniformly throughout the ventricular trabeculae in both groups (Figure 3A, D). In the M axolotls, however, staining was more intense in the proximity of the AVC (Figure 3D) and appeared less intense in the P group (A). The presence of the SMA marked also the coronary vessels and there were no differences between the P and M axolotl groups (Figure 3B, E).

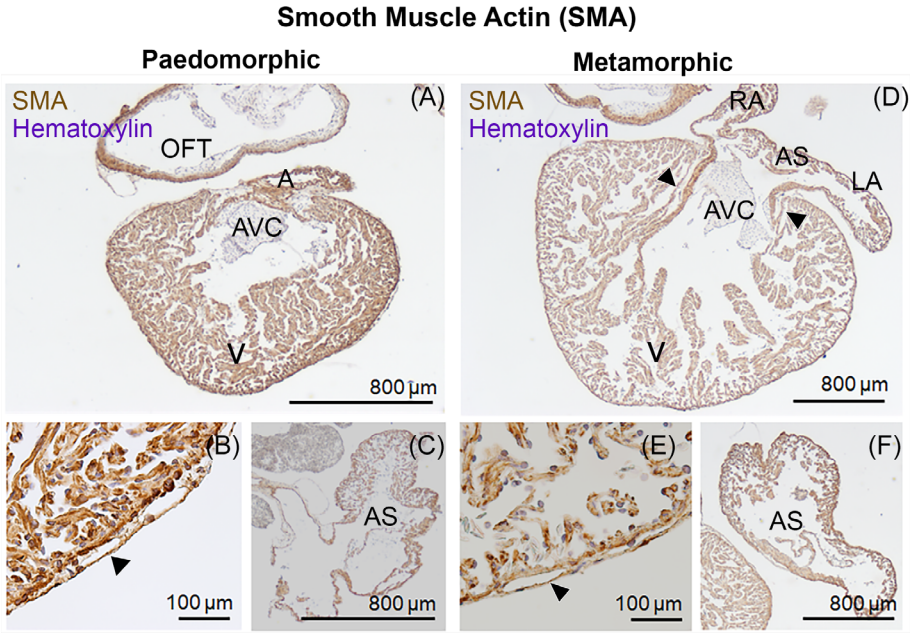

FIGURE 3 Smooth Muscle Actin (SMA) staining shows positivity in the ventricle and vessels in paedomorphic as well as in metamorphic axolotl heart. Paedomorphic axolotl ventricle (A) - black arrowheads point to the atrioventricular canal; metamorphic axolotl ventricle (D) - black arrowheads point to the atrioventricular canal. Detail of SMA positivity in vessels (B, E) - black arrowheads point to the vessel. Detail of the atria and atrial septum in paedomorphic (C) and metamorphic axolotl heart (F). A, atrium; AS, atrial septum; AVC, atrioventricular canal; LA, left atrium; RA, right atrium; OFT, outflow tract; V, ventricle. Scalebar (A, D, C, F) - 800 µm, scale bar (B, E) - 100 µm

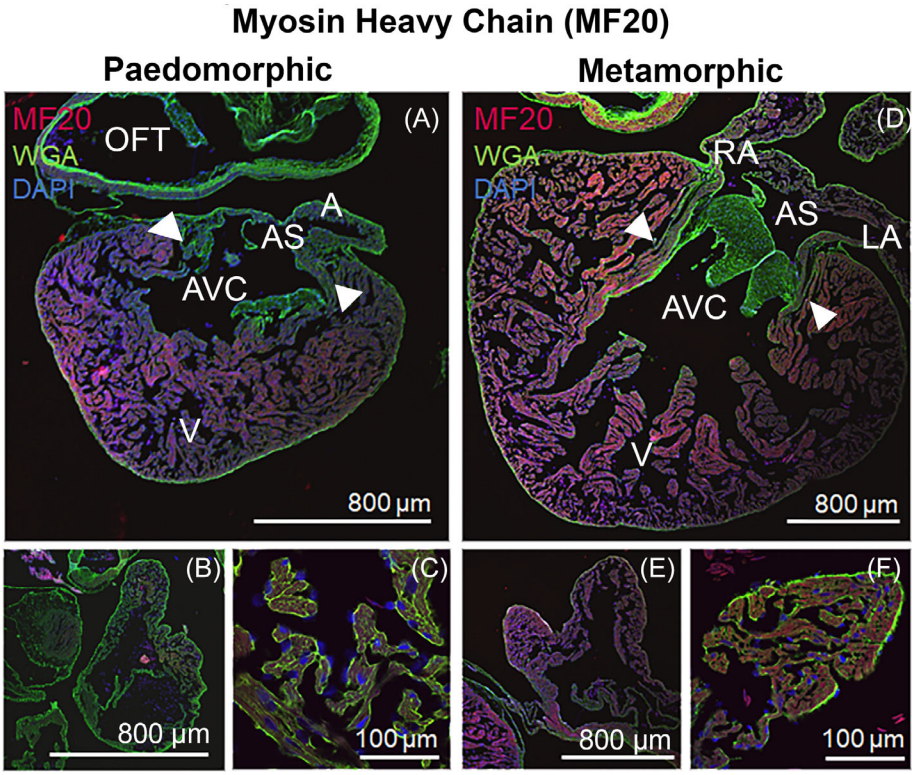

FIGURE 4 Myosin Heavy Chain (MF20) staining and counterstaining with Wheat Germ Agglutinin (WGA), which marks the outer as well as the inner cell membranes in axolotls hearts. Organization of the trabeculae in the atrioventricular canal (white arrowheads, A). Musculature in the atrioventricular canal (white arrowheads, D). Detail of the atria of pedomorphic (B) and metamorphic (E) hearts. Positivity for MF20 in ventricular trabeculae (panel C pedomorphic, and F metamorphic). A, atrium; AS, atrial septum; AVC, atrioventricular canal; LA, left atrium; RA, right atrium; OFT, outflow tract; V, ventricle. Scale bar - 800 µm (A, B, D, E); scale bar - 100 µm (C, F)

By the combination of myocardial marker Myosin Heavy Chain (MF20) and the plasma membrane marker Wheat Germ Agglutinin (WGA), different staining patterns in myocardium between the P and M groups (Figure 4) were observed. Specifically, there was a lower intensity of MF20 staining in the P group (Figure 4A). In the M group, there was a strong MF20 staining especially around the AVC (Figure 4A,D), the OFT, as well as in the thicker and more condensed trabeculae in the ventricle (Figure 4F). In both groups, the trabeculae in the ventricle were positive for MF20 (Figure 4C,F) while the outer compact layer was only faintly stained in the P group.

2.5 | Metamorphosis reveals changes in ventricular electrophysiology

Using optical mapping approach, we did not observe significant differences in the heart rate (50.4 ± 9.0 and 55.5 ± 5.7 for the P group and the M group, respectively; Figure 5) and total ventricular activation time (12.8 ± 2.7 ms and 12.7 ± 1.5 ms for the P group and the M group, respectively; Figure 5). The activation pattern was from base to apex direction in both groups. More specifically, the signal spread from the atrioventricular junction of the heart (orange asterisks) to its apex. The signal (in color bar) continued to the last regions activated (in yellow color), which were the heart margins. Each color band corresponded to the interval stated in initial panels (2 ms). Pattern of ventricular activation was similar before and after metamorphosis (Figure 5B, D). The significant difference was observed in the speed of the signal transduction through AVC with slower propagation in the P axolotls than in M group ($P < 0.05$; 404 ± 60 ms and 344 ± 31 ms for the P group and the M group, respectively; Figure 5E).

3 | DISCUSSION

3.1 | Amphibian metamorphosis and axolotl development

Evolution of the ability to breathe air goes together with the establishment of pulmonary circulation and the separation of the oxygen-rich and oxygen-poor blood within the heart.[42,43] This strategy formed the success of terrestrial life-style in ectothermic tetrapods.[44] Respiration in amphibians comes in several different modes: cutaneous respiration (skin breathing), gill respiration, and lung respiration, and even combinations of these.[45-47] All these variations of air breathing are connected with the rebuilding of the heart during the metamorphosis. [48] The heart transformation happening in air breathing salamanders is tied to metamorphosis, which has the enormous consequence to the life-history of this particular species.[10] Additionally, axolotls possess high regenerative potential. In this context, it was shown that during metamorphosis their regenerative potential decreases, life is shortened, and the metamorphosis reduces reproductive fitness as well.[49-51] In axolotls as well as in other

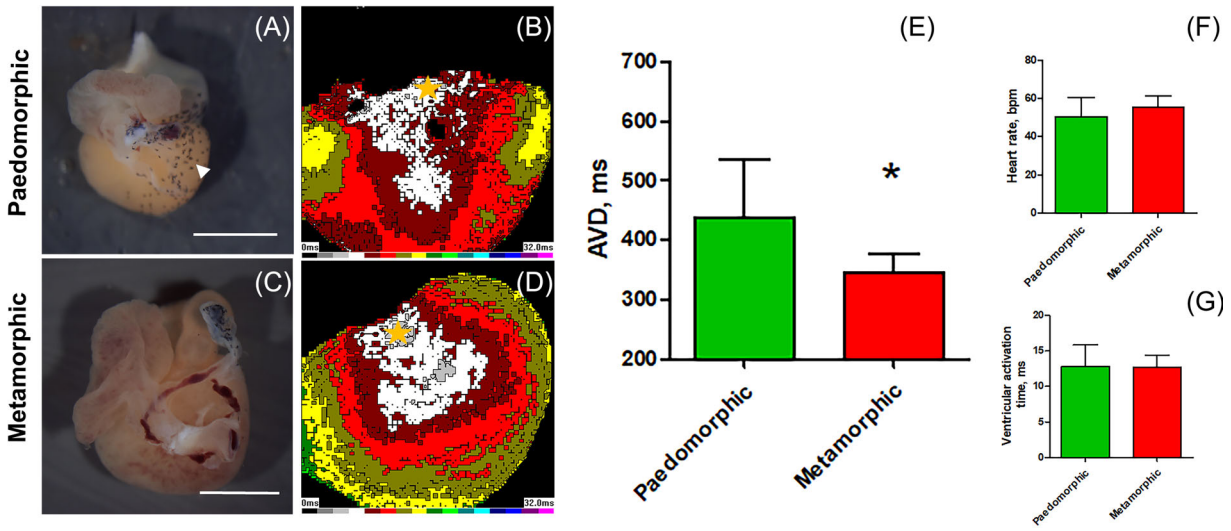

FIGURE 5 Spreading of electrical activation over the ventricle in paedomorphic and metamorphic axolotls with quantification of the electrophysiological parameters. The heart from dorsal side (A,C). Signal spreading pattern through the ventricle is similar between groups (B, D). Each color band corresponds to the interval of 2 ms. Note presence melanocytes in the heart in the paedomorphic axolotl (white arrow). AVD, atrioventricular delay; bpm, beats per minute; ms, milliseconds; scale bar - 2 mm

amphibians, metamorphosis is a conserved trait[11,19] and it is a well-studied example of a complex developmental process that is regulated by endocrine factors[10,52] and supports the idea that L-thyroxine (T4; relatively inactive form of TH) and 3,5,30-triiodothyronine (T3; more active form of TH) levels notably increase at metamorphic climax.[19] It was confirmed that THs hormones (T3 or T4) are necessary and sufficient to induce metamorphosis in Anura,[10,53,54] and Caudata.[19,39]

3.2 | Metamorphic changes in the axolotl heart

By optical mapping we showed that the signal spreading pattern within the ventricular wall was similar to base-to-apex pattern as it was observed also in the metamorphosed anurans, particularly in Common Toad (*Bufo bufo*),[55] and Common Frog (*Rana temporaria*).[56] In case of the heart rate, there is a different approach in our study compared to Bullfrog (*Lithobates catesbeianus*),[57] where changes in resting heart rate after metamorphosis were reported. While the study of measured the heart rate in vivo,[57] we used ex vivo approach, where the heart rates ex vivo reflects automaticity of the unregulated pacemaker.[58,59] In the amphibians, already at the larval stage, the resting heart rate is affected by vegetative nervous system.[57,60]

Morphological changes showed differences in trabeculation between the P and the M axolotl groups, which are more likely connected to terrestrial life-style. Similar results were described previously in Bullfrog before and after metamorphosis, where the heart mass was proportional to the particular stage.[61] However, unfortunately these findings of the Bullfrog heart mass were not further followed up by histology, so it is not clear how the extra myocardium is organized. Moreover, some prominent trabeculae were observed in the M group, which may be connected with the compartmentalization of the blood and the need of compensation of higher hemodynamic forces in these areas, particularly in part adjacent to the OFT.[62]

Additionally, the incomplete atrial septum was detected including the mesenchymal cap, which will eventually connect the valve to the AVC[63] in the P group. There was a connection of the atrial septum with the AVV in the AVC in the M group, which corresponds with the literature. [63]

Myosin Heavy Chain isoforms are important markers of differentiation of the striated muscles across vertebrate species.[64] Therefore, we chose MF20, which could be used as a marker of mature myocardium not only in mammals[65] but also in ectothermic species[66,67] such as amphibians.[68-70] The MF20 staining shows stronger signal in the specific area (predominantly ventricle) of the axolotl heart tissue. In this area, the metamorphic heart needs to resist higher blood pressure, which leads to maturation of cardiomyocytes in these regions manifesting as increased MF20 expression.[71] Based on MF20 staining, the atrium of the M group was more muscularized than in the P group because of the staining intensity in myocardium as it was also observed already in primitive vertebrates such as chimaeras (Holocephali).[66]

3.3 | Heart morphology in amphibians compared with axolotl

Amphibians evolved in ample amount during the Carboniferous period.[72] The ancestral lineage of Tetrapoda (*Ambystoma* is the representative species here) arose during the Paleozoic period (around 350 - 370mya).[72-74] Variability in amphibian heart structures can lead to the idea that the heart morphology[46,75] and high convergences in the amphibian phylogeny have something to do with possibly numerous paraphyletic genera.[76]

The life-history differences have to be taken into consideration as well, particularly between salamander and anuran species. Generally, axolotls protracted the larval phase to paedomorphosis and they reproduce in this stage,[23] they go to metamorphosis only when they urgently have to.[77] On the other hand, the anurans diversify in reproduction strategies from metamorphic strategy to direct development.[78] Moreover, axolotl is an ambush predator[79] in its aquatic stage as well as in the metamorphic stage and moves constantly and slowly.[80,81] Therefore axolotl does not need faster metabolism described in metamorphic anurans.[82]

Among amphibians (Gymnophiona, Caudata, Anura), there is a high heart structural variance.[9] However, some of these structures can be comparable to each other. In the axolotl hearts (paedomorphic and metamorphic groups), the atria contain pectinate muscles and the ventricular cavities are filled with trabeculations as it was described previously for Caecilians. [7] Nevertheless, in the context of atria, there is a tremendous variability in the development of atrial/interatrial septa and their numbers among amphibians. In specialized salamanders, such as the lungless salamanders (Plethodontidae),[73,83] the atrium is only partially separated.[46] We observed atrial septum both in the P and M axolotl groups. According to the literature, for example, in species *Siren*[84] (complete atrial septum), species *Necturus*[85] (incomplete atrial septum), or in *Cryptobranchus*, which breathes by skin (incomplete atrial septum),[86] most of

the findings tend to be in favor of incomplete atrial septum in the majority of Caudata.[87] In contrast to Caudata, in the order Anura, the atria are completely separated and therefore, the anurans have a common three-chambered heart—with two atria and one ventricle.[7] As a result of one undivided ventricle, the oxygenated and deoxygenated blood is being mixed in amphibians.[88] However, amphibians have good blood compartmentalization[75] and blood shunts are possible—intracardiac as well as vascular shunts.[89-91] These shunts enable amphibians to potentially maintain the arterial blood gas composition actively[92] as well as the blood distribution.[93] In amphibians, trabeculation combined with a spiral valve in the OFT allow separation the oxygenated blood directly to the systemic circulation.[88] Our observation corroborates that the ventricle contains mostly trabeculated myocardium, more developed in the M group, with internal ridges, also more pronounced in the M group, as was described for salamanders from genera *Siren*[94] and *Necturus*.[85] Particularly *Necturus* has a partially divided ventricle by organized trabeculae[85] as was observed in the metamorphic axolotls hearts, which could indicate an efficient blood separation also in the metamorphic axolotls.

3.4 | Similarities between axolotls and lungfish hearts

Amphibians could be somehow phylogenetically connected with lungfish (Dipnoi),[95] which had its evolutionary peak during the Devonian period.[96] However, the order Caudata accounts for multiple taxa lineages and therefore, the question of the particular origin of each lineage is still unresolved.[97] Yet, there are physiological, morphological, and ontogenetic similarities between these two lineages.[75,98,99] Among them belong air breathing, heart structures, and paedomorphosis[64,99] (eg, in *Neoceratodus forsteri*) with similar form as in amphibians.[95] Unfortunately, from the physiological point of view, the data about the signal propagation and its spreading across the AVC[98] in the lungfish are missing.

From our study, we observed that there are similarities in metamorphic axolotl group and lungfish in the extent and localization of atrial septation, massive trabeculations in M axolotls, which are organized in specific directions compared to the P group. We also noted the partially separated conus arteriosus segment of the heart in both groups. The differentiation of its myocardium followed the situation in the ventricle, that is, decrease of SMA staining intensity and increased MF20 positivity in the M group. Conus arteriosus is a massive structure in axolotls, and this characteristic is similar and also typical for lungfish.[99] However, there is no ventricular septum, as we know from lungfish, either in the M or in the P group.[94]

4 | CONCLUSION

Metamorphosis stimulated by THs changed heart morphology in axolotl (*A. mexicanum*). The modifications to air breathing corresponded with the organization of the ventricular trabeculae for more efficient blood compartmentalization after metamorphosis, differentiation of cardiomyocytes in AVC and trabecular regions as well as completion of the atrial septum, and faster signal propagation through the AVC. Taken together, our data support hypothesis of the heart adaptation to hemodynamic needs linked with the transformation to the terrestrial life-style in axolotl as we can observe in amphibian species such as *Siren*, *Necturus* or lungfish *Neoceratodus*.

5 | EXPERIMENTAL PROCEDURES

5.1 | Animal metamorphosis

We used 32 subadult (age 6-8 months, length approximately 10-12 cm) axolotls (*A. mexicanum*). Animals were divided into two groups, the experimental - metamorphic (M) group ($N = 18$; Males: 8. Females: 8. Unsexed: 2) and the control - paedomorphic (P) group ($N = 13$; Males: 5. Females: 4. Unsexed: 4). The hearts were divided according to the particular method – optical mapping, micro-CT, histology and immunohistochemistry. The treatment followed the protocol according to Kollros.[100] Both groups were kept in the same temperature 21°C ± 0.5°C with water column 7 cm with gradual decreasing of water surface to 5 cm. Only the experimental group was treated by L-Thyroxine (Sigma #BCBZ7522) in dose of 1 μg to 10 L H_2O in leave-to-stand water twice a week, when water was changed. Metamorphosis took 3 weeks to complete. In order to establish the particular metamorphic state, external morphological changes were followed in gills (losing external gills and closing branchial slits), caudal fin rim (its reduction), and the presence of lungs (buccal movements). The animals were deeply anaesthetized in 0.2% solution of MS 222 according to Mitchell[101] and sacrificed by quick decapitation. Only isolated hearts were analyzed in vitro.[102] After the sacrifice and extraction of the heart, the animals were also dissected for sexing.

5.2 | Micro-CT

The heart morphology of selected specimens (dissected heart in 70% ethanol in closed plastic tube, $N = 9$, N (P) = 3, N(M) = 6) was evaluated using micro-CT (SkyScan 1272, Bruker microCT, Belgium). Specimens were X-ray contrasted by phosphotungstic acid according to the protocol described by Metscher[103] and scanned under the following scanning parameters: 4 or 7 μm pixel size (based on specimen size), source voltage 70 kV, source current 142 μA, 0.5 mm Al filter, frame averaging = 2, 180° rotation, scanning time 1.5 to 2 hours for each specimen. The flat-field correction was updated prior to each acquisition. Projection images were reconstructed into cross-section images using NRecon software (Bruker). Standardized two-dimensional visualizations were achieved using DataViewer (Bruker).

5.3 | Histology and immunohistochemistry

The hearts were processed for paraffin embedding and then sectioned at 8 μm in the frontal plane for further histological and immunohistochemical analysis. Morphological changes in trabecular organization and levels of fibrous tissue in the hearts were assessed by histological staining – hematoxylin and eosin combined with Alcian blue (AB/H&E).

To address the changes in myocardial differentiation, immunohistochemical staining for two myocardial markers was applied. SMA mouse monoclonal IgG antibody (Sigma #A2547) was used in dilution 1:800 and visualized with DAB for the bright field imaging and analysis. The second myocardial marker – Myosin Heavy Chain (MF20 - DSHB #AB2147781[104]) was used to assess the shape and size of ventricular myocardial cells in dilution 1:20 in fluorescence, combined with plasma membrane marker – Wheat Germ Agglutinin (WGA) – Alexa 488 (Invitrogen #W11261) in dilution 1:50. Sections were mounted and analyzed in transmitted light - microscope Olympus BX51 with CCD cameras Olympus DP71 and DP80, and confocal microscope Olympus IX61 with FluoView system for the fluorescent staining. A further analysis of the trabecular shape and density as well as antibody staining intensity was performed by ImageJ software (NIH, USA). To avoid underestimation, myocyte width (maximum transverse diameter) was measured in the cells with clear nucleus only.

5.4 | Optical mapping

In order to determine the impact of lung circulation development on electrophysiological characteristics of the axolotl ventricle, the optical mapping was performed (M group $N = 11$, P group $N = 5$). The hearts were rapidly explanted from the axolotls and optically mapped in modified Tyrode's solution saturated with 100% O_2 (composition: NaCl 95 mmoL/L, KCl 2.5 mmoL/L, $CaCl_2$ 1.5 mmoL/L, NaH_2PO_4 1.2 mmoL/L, $MgSO_4$ 1 mmoL/L, glucose 5 mmoL/L, Tris 5 mmoL/L; pH = 7.5) at room temperature using voltage sensitive dye di-4-ANNEPS (Biota #61010). ULTIMA L camera and THT microscope with tandem Leica optics (both devices from Brain Vision, Tokyo, Japan) were used for imaging. The system was equipped with a LEX3 LED light source (Brain Vision, Tokyo, Japan) providing high intensity and high stability of illumination.[105] Epicardial activation maps were generated and ventricular activation pattern, heart rate and atrioventricular delay were analyzed by BV_Ana software (SciMedia Brain Vision, Tokyo, Japan) as described before.[106]

After optical mapping, the hearts were fixed overnight in 4% paraformaldehyde and processed by either micro-CT or histological examination.

5.5 | Statistical analysis

The GraphPad Prism6 was used for graphic presentation and statistical analysis. Data are presented as mean ± SD values. Differences between the groups in ventricular activation time, heart rate and atrioventricular delay were analyzed by unpaired two-tailed t-test and considered significant at $P < 0.05$.

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AUTHOR CONTRIBUTIONS

Veronika Olejnickova: Conceptualization; formal analysis; investigation; methodology; validation; visualization. **Hana Kolesova:** Formal analysis; investigation; methodology; validation; visualization. **Martin Bartos:** Data curation; formal analysis; methodology; validation; visualization. **David Sedmera:** Funding acquisition; investigation; validation. **Martina Gregorovicova:** Conceptualization; data curation; formal analysis; investigation; methodology; project administration; supervision; validation; visualization; writing - original draft.

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