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O verdadeiro segredo do coração das rãs: análise comparativa do sistema cardiorrespiratório de quatro espécies de Brachycephaloidea (Amphibia, Anura), com ênfase em *Brachycephalus rotenbergae*

The real secret of the frogs' hearts: comparative analysis of the cardiorespiratory system of four Brachycephaloidea species (Amphibia, Anura), with emphasis in *Brachycephalus rotenbergae*

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Orientador: Dr. Raoni Rebouças Santos

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RESUMO

Anuros do gênero *Brachycephalus* estão entre os menores vertebrados do mundo, como consequência do processo de miniaturização pela qual o grupo passou. Por esse processo, estruturas e órgãos internos também são reduzidos em tamanho, o que pode resultar em alterações estruturais e funcionais em vários sistemas corpóreos. Como exemplo desse processo, as espécies do gênero *Brachycephalus* apresentam características como perda do ouvido médio e hiperossificação do crânio e das vértebras. Portanto, também é possível que esses organismos apresentem outras diferenças anátomo-fisiológicas devido à miniaturização. Assim, este estudo visou observar e descrever o sistema cardiorrespiratório através de dissecações de indivíduos da espécie *B. rotenbergae*, e compará-los com outras espécies congêneres, como *B. pitanga*, e com outras de tamanho semelhante, de outros gêneros, mas ainda dentro dos Brachycephaloidea, como *Eleutherodactylus johnstonei* e *Ischnocnema parva*. Com as dissecações realizadas observamos que *B. rotenbergae* e *B. pitanga* apresentam uma redução do septo atrial e ausência do corpo carotídeo. Em *Ischnocnema*, apesar de ser um grupo próximo a *Brachycephalus*, os indivíduos de *I. parva* apresentam coração com septo completo e presença do corpo carotídeo como em *E. johnstonei*. Partindo das observações feitas em *B. rotenbergae* e *B. pitanga*, levantamos a hipótese de que a respiração, nessas espécies, ocorre principalmente pela pele, apesar de serem organismos eminentemente terrestres.

Palavras-chave: Anuros, Coração, Morfologia, Anatomia Comparada, Miniaturização

ABSTRACT

Anurans of the genus *Brachycephalus* are among the smallest vertebrates in the world, as a consequence of the miniaturization these species underwent. By this process, internal structures and organs are also reduced in size, which can result in structural and functional changes in various systems. As an example of this process, species of the genus *Brachycephalus* exhibit features such as loss of the middle ear and hyperossification of the skull and vertebrae. Therefore, it is possible that these organisms show more differences due to miniaturization. Thus, this study aims to compare, observe and describe the cardiorespiratory system through dissections of individuals of the species *B. rotenbergae*, and compare it with other congeneric species, such as *B. pitanga*, and with others of similar size, from other genera, but still within the Brachycephaloidea, such as *Eleutherodactylus johnstonei* and *Ischnocnema parva*. With the performed dissections we observed that *B. rotenbergae* and *B. pitanga* present the reduction of the atrial septum and the absence of the carotid body. *Ischnocnema* despite being the next group to *Brachycephalus*, the individuals of the species *I. parva* present a heart with a complete septum and the presence of the carotid body as in *E. johnstonei*. With those observations made in *B. rotenbergae* and *B. pitanga*, we hypothesize that the individuals of these species breathe mainly through their skin despite being such a terrestrial organism.

Keywords: Anurans, Heart, Morphology, Comparative Anatomy, Miniaturization

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1 Introduction

1.1 Blood circulation in anurans

The generalized heart model for anurans presents five cavities, from which the arteries that transport blood to the body originate, and where the veins arrive to return blood to the heart (Foxon, 1947; Sharma, 1957; Hoffman, 1931; Marshall, 1916). In this model, the *sinus venosus* is dorsal to the ventricle and has a semi-triangular shape. The atria are in the anterior region of the ventricle and the *sinus venosus* posterior to the latter. The *bulbus cordis* originates in the right dorso-anterior region of the ventricle and, finally, the *truncus arteriosus* is located immediately after the *bulbus cordis* (Foxon, 1947; de Graaf, 1957; Sharma, 1957, 1961; Hoffman, 1931).

To date, morphologists consider that the two anuran atria are divided by an atrial septum (Rau, 1924; Foxon, 1947; Sharma, 1957; Iuliis & Pulerà, 2006). However, different patterns can be observed for distinct amphibian orders. In Caecilians, it was reported that the heart presents a single or two atrial septa, one septum dividing the right atrium from the left and another septum dividing the right atrium into two, the former named the right atrial septum (de Bakker *et al.* 2015). In lungless salamanders of the Plethodontidae family the heart has a single atrium, with an incomplete septum (Putnam & Dunn, 1978). The absence of a complete septum is due to the absence or reduction of the lungs, which probably indicates that lungs are not relevant for respiration (Lewis & Hanken, 2017, Putnam & Dunn, 1978).

Concerning the blood circulation paths described so far for anurans (Foxon, 1947; de Graaf, 1957; Sharma, 1961; Simons, 1959), the oxygenated blood coming from the pulmonary veins returns to the left atrium, while the deoxygenated blood, and part of the oxygenated blood coming from the musculocutaneous vein, returns through the *sinus venosus* to the right atrium. In species where respiration occurs primarily through the skin (e.g., Putnam & Dunn, 1978; Putnam & Kelly, 1978; Lewis & Hanken, 2017), blood mixing begins in the *sinus venosus*, as the oxygen

captured by the skin is carried from the capillaries to the musculocutaneous veins, which carry the blood towards the *sinus venosus*. Therefore, deoxygenated blood reaches the *sinus venosus* through the innominate veins, through the external jugular veins while oxygenated blood runs through the musculocutaneous veins (Saint-Aubain & Wingstrand, 1979). In species where respiration occurs primarily through the lungs, once the blood is in the atria, both the oxygenated and deoxygenated blood streams are completely separated by a complete atrial septum (Haberich, 1965; Foxon, 1947; de Graaf, 1957). After the atrial systole, the blood occupies the space of the ventricle, with the oxygenated blood occupying the left side and the deoxygenated blood occupying the right side. Thus, a partial mixture of oxygenated and deoxygenated blood still occurs in the middle region of the ventricle (de Graaf, 1957; Simons, 1959). As the blood from the left atrium extends to more than half of the ventricle, it mixes with the blood leaving the right atrium, which only extends to the middle region of the ventricle (de Graaf, 1957; Simons, 1959). After ventricular systole, blood is sent to the *bulbus cordis*, which has a septum dividing it into a *cavum pulmocutaneum* and *cavum aorticum* (Foxon, 1947; Sharma, 1957; Kraus & Metscher, 2021). With the help of the spiral valve, the blood is kept separated, preventing a complete mixing of the two blood streams, sending deoxygenated blood to the *cavum pulmocutaneum* and oxygenated blood to the *cavum aorticum* (Nigam, 1983). However, the percentage of mixing between oxygenated and deoxygenated blood that occurred in the ventricle is still maintained (Foxon, 1947; de Graaf, 1957). Therefore, a percentage of oxygenated blood is sent to all the arterial arches, but the blood that reaches the pulmocutaneous arteries is mainly deoxygenated. Finally, with the contraction of the *bulbus cordis*, the blood is sent to the main arteries, and the cycle is repeated as blood returns to the *sinus venosus* and the left atrium via the pulmonary veins (Foxon, 1947; de Graaf, 1957; Sharma, 1957; Simons, 1959).

1.2 Miniaturization

Miniaturization is a phylogenetic concept defined as the evolution of an unusually small adult size in a lineage that promote dramatic changes in morphology, physiology, and ecology (Hanken & Wake, 1993; Yeh, 2002; Pérez-Ben *et al.* 2018). Small sizes can present several advantages for animals, such as predator avoidance and the differential use of food and microhabitat resources (Yeh, 2002). Generally, the first aspects recognized in miniaturized vertebrates are reduction of external morphological traits and new skeleton arrangements (Pérez-Ben *et al.* 2018). Thus, internal organs anatomy is the least reported in these cases and represents a feature that has received the least attention (Duellman & Trueb, 1994; Vitt & Caldwell, 2014; Silva *et al.* 2007; Campos *et al.* 2010, Goutte *et al.* 2017), although they may indicate novel organ functions.

In this context, several amphibian species are highlighted as cases of miniaturization. One of these amphibian groups, is the species of the genus *Brachycephalus*, which is endemic to the Atlantic Forest, usually found in the coast of Brazil (Condez *et al.* 2020). The almost 40 species of this genus (Frost, 2022) are represented by toadlets, mostly diurnal, with colored skin (mostly yellow, orange, red, green or brown), and some of them presenting tetrodotoxin in their skin (Vitt & Caldwell, 2014; Pires *et al.* 2005; Sebben *et al.* 1986). Species of the *B. ephippium* group are characterized by a bufoniform morphology and aposematic coloration (Rebouças *et al.* 2019; Lyra *et al.* 2021) and are among the smallest vertebrates in the world (Reis *et al.* 2021). The miniaturization of these frogs resulted in hyperossification of the skull, caused by the increase in mineralization that can vary in different species (Campos *et al.* 2010). These structures can be observed in parotid plates, which are found in the upper part of the squamosal bone, and in paravertebral plates, which form a dermal bone shield that dorsally ossifies the vertebrae in some species (Campos *et al.* 2010; Clemente-Carvalho *et al.* 2008; Clemente-Carvalho *et al.* 2009;

Reis *et al.* 2021; Haddad *et al.* 2010). Other examples of miniaturization in this group are the reduced number of digits (Duellman & Trueb, 1994; Vitt & Caldwell, 2014; Silva *et al.* 2007), the absence of tympanum and middle ear bones (Silva *et al.* 2007), and the lack of innervation associated with higher frequencies hearing, which impairs individuals to listen to the sounds of their own call (Goutte *et al.* 2017).

In smaller anuran species, skin respiration is generally more efficient compared to larger anurans as they have more capillaries and a thinner epidermal layer (Tattersall, 2007). For that reason, we hypothesize that as *Brachycephalus rotenbergae* and *B. pitanga* are miniaturized, with a body size of less than 2 cm in length, skin respiration is probably the main source of oxygen intake. As a consequence, we also hypothesize that if *Brachycephalus* breathes mainly through the skin, pulmonary respiration will be reduced, which could imply in modifications of their lungs and, consequently, of their hearts. These hypotheses are supported by previous studies, where it was observed that the species that present a greater respiration through the skin or present a reduction in the lung also present a modification in the internal morphology of the heart (Noble, 1925; Lewis & Hanken, 2017). There is also a record of an anuran species, *Barbourula kalimantanensis* (Iskandar, 1978), which has no lungs (Bickford *et al.* 2008) and therefore also presents variation in the cardio-respiratory system.

Therefore, we aimed to compare morphological aspects of the cardiorespiratory system in *B. rotenbergae*, *B. pitanga*, *E. johnstonei* and *I. parva*, allowing comparisons between closely related species. Furthermore, we provided novel descriptions for the internal anatomy of these four species.

2 Methods

2.1 Individuals sampling

We collected 13 specimens of *B. rotenbergae* (snout-vent length [SVL] = 0.8 – 1.8 cm), 27 of *B. pitanga* (SVL = 1.1 – 1.4 cm), 24 of *E. johnstonei* (SVL = 1.1 – 2.2 cm), and 30 individuals of *I. parva* (SVL = 1.1 – 2.2 cm) to perform dissections for photography and histology. Individuals of *B. rotenbergae* were collected in São Francisco Xavier, municipality of São José dos Campos (22° 54' 31" S, 45° 57' 04" W), *Ischnocnema parva* were obtained at the Estação Ecológica Bananal, municipality of Bananal (22° 40' 51" S, 44 ° 19' 25" W), *B. pitanga* individuals were collected in the Parque Estadual Serra do Mar, núcleo Santa Virgínia, municipality of São Luiz do Paraitinga (23° 54' 26" S, 46° 10' 50" W), and the specimens of *E. johnstonei* were collected in the Brooklin neighbourhood, municipality of São Paulo (23° 36' 56" S, 46° 40' 50" W), all in the state of São Paulo (SP), Brazil. After histomorphology processing, all material was conserved and deposited in the Museu de Diversidade Biológica (MDBio), Universidade Estadual de Campinas (Unicamp), Campinas, SP, Brazil. All pictures and videos were deposited according recommendations (Dena *et al.* 2020, 2018) in MDBio (ZUEC-PIC 703–785; ZUEC-VID 1000–1042). Samplings were approved by national institutions (SISBio #79186, COTEC #004767/2020-42, #005121/2021-37, CEUA #5903-1/2021).

2.2 Fresh preparations and photography

Specimens were submitted to a dose of lidocaine hydrochloride 2% ointment. This anesthetic gel solution was applied through direct contact to the skin of individuals. We performed reflex tests on all four limbs and eyes of all individuals before starting the dissection to ensure that the lidocaine had taken effect (Sebben, 2007). Once the individuals were anesthetized, a ventral abdominal dissection was immediately performed to expose the visceral

organs and heart according to Carman (1955), Iuliis & Pulerà (2006), and Sebben (2007). Then, they were immersed in an isotonic saline solution (sodium chloride - NaCl - 0.65 % solution), which allowed us to better record the contractions of the heart and see the blood flow. The saline solution guarantees the maintenance of the morphophysiological characteristics of the organs and prevents light reflections in the organs for correct visualization; in addition, this solution prevents blood coagulation. Photographs and videos were taken by implementing an Asus A001D cell phone and a Canon 5D Mark III camera with a Sigma 105 mm macro lens to a stereoscopic microscope (Leica S9). We were able to observe the following structures: *sinus venosus*, left and right atria, ventricle, *conus arteriosus*, *truncus arteriosus*, as well as arteries and veins, skin capillaries, and right and left lungs. A Pasteur pipette was used to remove blood, skin, or any artifacts to avoid image interference (Sebben, 2007). The sex of the individuals was assigned during the dissection through the observation of the gonads and in the same way we observed if they were adult individuals. The nomenclature for the heart, veins, and arteries follows Iuliis & Pulerà (2006).

2.3 Histology

We removed the heart, lungs and skin from the abdomen of six individuals from each species (three males and three females) for histological analysis. With histological sections of the lung, we observed the blood supply through the capillaries of the lung wall (Guangming *et al.* 2020), and through skin sections we observed and compared the epidermal layers.

We fixed the heart, lung and skin in paraformaldehyde buffer solution (4% with phosphate buffer, pH 7.2) for 24 h at 4 °C. Subsequently, the samples were dehydrated in increasing concentration of ethanol (15, 30, 50, 70, 85, 90 and 100 %) at 4 °C, with a bath of 40 min. After dehydration, the material was immersed in xylene + alcohol (1:1) and subjected to two xylene

baths for 40 min each. To finalize the preparation of the frameworks, they were embedded in glycolmetacrylate resin (Historesin Leica), according to the manufacturer's recommendations. Histological sections with a thickness of 3 μm were made with a Leica microtome (RM 2245) (Salla et al., 2020). For each organ, we made serial microtome sections and stained 20 slides (4 to 5 cuts per slide) with hematoxylin (40 minutes) and eosin (5 minutes) (H&E; Merck), which is the staining technique for histological examination. from animal tissues (Salla et al. 2020).

The results were presented first by describing what was observed in *B. rotenbergae* and then by comparing it with the data obtained from the following species *B. pitanga*, *I. parva* and *E. johnstonei*. Beginning with what is observed in the heart, later the description of arteries, veins, capillaries, skin, lungs and finally blood circulation.

3 Results

As *Brachycephalus rotenbergae* was the most different species and in which it was already known that it presents miniaturization, we focused on this species and compared it with the others.

Brachycephalus rotenbergae's heart

In systole, the *sinus venosus* in *B. rotenbergae*, was cylindrically shaped, with the anterior region slightly wider than the posterior portion. During diastole, in the *sinus venosus* we observed a widening portion with a semicircular appearance near the junction of the left and right *venae cavae* (Fig. 1A).

The atrial morphology was globular (Fig. 1A), since the heart of individuals of this species has an incomplete uvula-shaped septum that emerges from the dorsal wall of the atrium (Fig. 2A). The atrium has a remarkably thinner muscular wall than that of the ventricle. The atrioventricular region is concave, and the anterior terminal part of the atrium is convex. The

ventricle, ventral to the *sinus venosus* and posterior to the atrium, has an ovoid morphology, which is maintained during systole and diastole (Fig. 1).

The *bulbus cordis* is located between the atrium and the ventricle, extending into the anterior region of the atrium, is originates at the right anterior dorsal region of the ventricle (Fig. 1A). This structure is cylindrical-shaped with a slight widening in the right posterior-ventral region. The *truncus arteriosus* is found after the *bulbus cordis*, in a smaller size, and seems to emerge from it, immediately after detached the three aortic arches (Fig. 1A). With the histology we were able to observe the lumen through where the blood passes towards the ventricle during its diastole to the *bulbus cordis* valve, and we were able to observe the presence of the spiral valve inside it (Fig. 3A).

The carotid, the systemic and the pulmocutaneous arteries detach from the *truncus arteriosus*, and bifurcate both to the left and to the right sides, forming an "u", leaving a space in the middle spatial portion free of arteries and veins. The three main arteries (carotid, systemic, and pulmocutaneous) arise independently directly from the *truncus arteriosus* in *B. rotenbergae* (Fig. 1A).

Comparison of heart anatomy among species

Out of the three species, *B. pitanga* was the most anatomically similar to *B. rotenbergae*. The morphology of the *sinus venosus*, the atrium and the ventricle were analogous in individuals of both species (Figs. 1A and 1B). However, in *I. parva* and *E. johnstonei* only the *sinus venosus* was similar to *Brachycephalus* spp. In addition, in *I. parva* and *E. johnstonei* it was possible to observe, during systole and diastole, that the two atria were present, while in *Brachycephalus* spp. only one atrial sac was present (ZUEC-VID 1000–1042). The ventricle in *I. parva* and *E. johnstonei* during systole and diastole was triangular-shaped (Fig. 1C and 1D), while in

Brachycephalus spp. the ventricle was circular-shaped. The *bulbus cordis* and *truncus arteriosus* were similar in individuals of all species. Also, all species had a spiral valve (Fig. 3), but its position varied, i.e., as it extended spirally within the *bulbus cordis*, and each histological section revealed a different position of the spiral valve.

***Brachycephalus rotenbergae*'s arteries, veins, and capillaries**

The carotid arteries were more ventral in relation to the other arches, and they were oriented towards the cranial region of the body (Fig. 1A). In *B. rotenbergae*, we did not observe a carotid body, so the external carotid arises directly from the carotid artery as a bifurcation, and it extended towards the cranial region. The internal carotid artery was a continuation of the carotid artery (Fig. 4A).

The pulmocutaneous artery in *B. rotenbergae* at its origin was similar to the pattern described for other anuran species (e.g., Saint-Aubain & Wingstrand, 1979; Hoffman, 1931). However, we observed that some individuals, mainly females, showed a reduction in blood flow to the lungs when compared to males (Fig. 5A). In one male, we observed that a second branch originated from the pulmonary artery in the anterior region of the lung, which was observed to be very small and reduced with respect to the artery from where it originated, which was larger and had a greater diameter (Fig. 5A). In females despite the presence of a pulmonary artery, we did not observe blood circulation in the lungs.

The systemic artery in *B. rotenbergae* presented a larger caliber than the carotid and the pulmocutaneous arteries (Fig. 1A). This artery headed towards the cranial region, curved towards the dorsal region, and was directed to the posterior region of the body. Its first branches were the occipital-vertebral arteries and the subclavian artery immediately after (Fig. 6A). In the dorsal region near the two kidneys, the systemic arteries joined to form the dorsal aorta, and the union of

celiac-mesenteric artery originated at the same point, which was formed as an extension of the right systemic artery (Fig. 7A). Therefore, the dorsal aorta was formed by the union of the two systemic arteries. This occurs through an anastomosis between the right and left systemic arteries, which was a small branch that connected both sides. The urogenital arteries originated near the origin of the dorsal aorta and connect to the kidneys, but we observed variations in this connection between conspecific individuals. The number of urogenital arteries observed was five to four for both kidneys. For a single kidney, the minimum number of supplying arteries was two (Fig. 8). In some individuals, we observed bifurcations in the urogenital arteries, before reaching the kidneys, and in other individuals it was a simple branch that went from the dorsal aorta to the kidney (Fig. 8).

Posterior to the kidneys, as a continuation of the dorsal aorta, we could observe the arise of the left and right common iliac arteries. As the iliac arteries continued toward the hind limbs, the hypogastric arteries arose from them, ventral to the ilium and near the pelvic region. Heading towards the outer region of the thigh, the femoral artery originated from the posterior part of the hypogastric and the anterior part of the sciatic arteries, which were located on the posterior part of the femur, supplying the hind limbs (Fig. 9A).

On the antero-lateral parts of the heart, we observed: i) the external jugular veins, with their two ramifications towards the anterior region; ii) the subclavian veins in the most posterior region, from which the brachial and musculocutaneous veins arose (these veins joined to form the subclavian vein, and consequently joined with the main branch of the jugular to form a single vein); and iii) in the middle of the two anterior veins, the innominate vein originates the internal jugular and the subscapular veins. The union of external jugular and subclavian formed a new vein, which joined with the innominate veins to form the left and right anterior *venae cavae* (Fig. 10), which merged with the *sinus venosus*, dorsal to the ventricle and the atrium.

In the pelvic patch, located in the posterior-ventral region, we observed that pelvic veins joined to form the ventral abdominal vein, which runs ventrally through body wall until bifurcate and connect to both sides of the liver. The two veins originating from the ventral abdominal vein seem to function as the hepatic portal system, since the branch that drains blood from mesenteric circulation was not found (hepatic portal vein) (Fig. 11A). Thus, the gastric vein and the intestinal vein (mesenteric vein) joined to form the hepatic portal vein, which joined the ventral abdominal vein and received the blood from the mesenteric circulation. Finally, two hepatic veins originated in the anterior region of each lobe of the liver and merged with the *sinus venosus*.

We also found evidence that the ovarian vein could originate directly from the posterior *venae cavae* or from one of the renal veins. The renal veins were observed in the anterior region of the kidneys, not extending to its middle region. In some individuals, we observed simple branches, while in others it could be a simple branch in one kidney and two in the other, which come together before forming the posterior *venae cavae*, or even two veins on each side of each kidney (Fig. 8).

In the ventral view of the posterior region, we observed that the femoral vein runs through the leg, and, the iliac vein originated as a bifurcation of the femoral vein, near the region where the femur ends. The renal portal vein extended to the external region of the kidneys, which received blood from the hind limbs. The pelvic veins originated from the femoral vein and joined with their counterpart to form the ventral abdominal vein. The sciatic vein also originated from the femoral vein, close to where the renal portal vein originated, but courses toward the inner thigh (Fig 12A).

Comparing arteries among species

Although we observed that the carotid, systemic, and pulmocutaneous arteries were ramifications from the *truncus arteriosus* in all studied species the initial portion of emergence of these arteries was different among species. In *B. rotenbergae* the carotid and systemic arteries arose independently, going directly straight out of the *truncus arteriosus*, but in *B. pitanga*, *I. parva* and *E. johnstonei* they were originally together from the *truncus arteriosus* and posteriorly divided as they continued. The pulmocutaneous arteries lied dorsally to the carotid and systemic arteries (Fig. 1).

The carotid arteries were found in the internal region of the *truncus arteriosus*, the pulmocutaneous artery was found in the external region, and the systemic artery was located in the middle region between the other two arteries. In the carotid artery, the carotid body originates, which we only observed in *I. parva* and *E. johnstonei*. In *Brachycephalus* spp. we did not observe the carotid body in any individual, and the internal and external carotid was a bifurcation of the carotid artery (Fig. 4). In *E. johnstonei* the external carotid originated adjacent to the carotid body (Fig. 4D), and in *I. parva* it originated from the carotid artery, in a short distance from the base to the carotid body (Fig. 4C). In all four species, this artery extended anteriorly to supply blood for the tongue and lower jaw. In *I. parva* and *E. johnstonei* the internal carotid artery arises directly from the carotid body.

In *I. parva* and *E. johnstonei* the pulmocutaneous artery in was like a regular artery, as described in previous studies (e.g., Saint-Aubain & Wingstrand, 1979; Hoffman, 1931). However, in *Brachycephalus* spp. There was a variation of the pulmonary artery between males and females, while lungs were similar in both species and sexes. The lungs of *I. parva* and *E. johnstonei* presented a greater blood supply from arteries when compared to *Brachycephalus* spp. (Fig. 5).

In all studied species the systemic artery presented a larger caliber compared to the carotid and pulmocutaneous arteries and it was equally oriented to the posterior region of the body. Also, the first branches that originated from the systemic artery were the occipital-vertebral arteries and the subclavian arteries immediately after. However, in *B. pitanga* and *I. parva*, a branch emerged from the subclavian artery, which was initially oriented towards the cranial region of the body. We could not identify which vein this branch was and we did not observe it in any other species (Fig. 6, 14, 15). In *I. parva* the occipital-vertebral and subclavian arteries either arised from the same branch or independently. In *Brachycephalus* spp. and *E. johnstonei* the arteries were separated, with the subclavian artery lying a short distance below the occipital-vertebral artery. At the origin point of the mesenteric and celiac arteries, individuals of *Brachycephalus* spp. and *E. johnstonei* had the same configuration. However, in *I. parva* there was an additional branch, so the celiac-mesenteric artery originated three arteries, and not only two as previously observed in the other three species. This additional branch bifurcated near the base of the mesenteric artery.

We have also found evidence of inter and intraspecific variations among the four species in relation to the arteries in the connection between the aorta and urogenital arteries with the kidneys (Fig. 8, 14, 15, 16). As observed in *B. rotenbergae*, there were some variations in the arrangement of arteries, which could be simple or present a bifurcation.

Also, the femoral artery presented a bifurcation in the hind limbs of *E. johnstonei* and *I. parva*, where one branch went towards the internal part of the hind thigh and the other branch run towards the pelvic region (Fig. 9C and 9D). While, in *Brachycephalus* spp. we could only observe a single branch (Fig. 9A and 9B).

Comparison of the veins among species

In *Brachycephalus* spp. and *I. parva* the subclavian vein joined to the main branch of the external jugular vein, and posteriorly merged with the innominate vein to form the anterior *venae cavae* (Fig. 10A, 10B and 10C). In *E. johnstonei* the external jugular vein, subclavian vein, and innominate vein joined at the same point to form the anterior *venae cavae* (Fig. 10D). In the *sinus venosus*, in addition to the left and right *venae cavae*, we observed the pulmonary veins behind the *sinus venosus*, which joined to form a single vein, called common pulmonary vein, and connected to the left atrium. We were unable to photograph this structure in *I. parva* and we did not see it in *Brachycephalus* spp.

The hepatic portal vein, which was absent in *B. rotenbergae*, was also absent in the other three species (Fig. 11). This vein is found in other anurans in the left lobe of the liver. Concerning the renal veins of our dissections, such as reported for arteries, we found variations in the four species in the number, shape, length, and in the point from which they ramified to the kidney to join with the opposing renal veins and form the posterior *venae cavae* (Fig. 8, 14, 15, 16). Only in *I. parva* we observed no variation among individuals, which presented only one vein on each side of the kidney (Fig 15).

The arrangement of the veins of hind limbs (femoral, sciatic, renal, and pelvic veins) were similar in *B. rotenbergae* and *I. parva*. In *B. rotenbergae* and *I. parva* the sciatic vein was closer to the renal portal vein than in *B. pitanga*. Also, we observed that the femoral vein, the renal portal vein and the pelvic vein presented a similar arrangement in all species (Fig. 12A and 12C). In *B. pitanga* we observed that two more veins originated from the sciatic vein and extended towards the inner face of the thigh (Fig. 12B). In *E. johnstonei* the femoral vein runs through the leg until it reaches the point of origin of the renal portal vein, which was extended towards the end of kidneys. In this region, the pelvic vein originated between the femoral vein and the iliac

vein, and the pelvic vein joined its counterpart to form the ventral abdominal vein. Finally, the sciatic vein arose from the iliac vein (Fig. 12D).

***Brachycephalus rotenbergae* skin**

The ventral skin of *B. rotenbergae* was composed of an outermost thin *stratum corneum* layer, followed by a germ layer, which was constituted by 1 or 2 semicircular-shaped cells layers. More deeply, the dermis can be found under the germ layer, which was divided into two layers. The first was the spongy layer, in which we observed the presence of mucous and granular glands. The basement membrane was under the spongy layer, and below it we observed the compact layer (Fig. 13A).

Comparison of the skin among species

The ventral skin of *B. pitanga* was similar to that observed in *B. rotenbergae*. In both species we observed a layer of *stratum corneum* and the *stratum germinativum* with 1 to 2 layers thick. In *I. parva* the difference we observed was the presence of 3 to 4 cells thick in the *stratum germinativum*. The dermis in the *stratum spongiosum* did not present granular glands, but we observed a large number of mucous glands (Fig 13C). Due to the thin epidermis that we observed in *Brachycephalus* spp. it was possible to observe the blood circulation in the vocal sac and in the abdomen with a slight zoom with the stereoscope (ZUEC-VID 1000–1023).

***Brachycephalus rotenbergae* lung**

There was a variation between males and females in the arteries that perfused the lung. It was easier to observe irrigation in the lungs of males when compared to females, which may

indicate greater blood supply in males. However, in all individuals, including males and females, the lungs presented a smooth surface (Fig. 5A).

In some males, in which the lungs contained more air, the pulmonary artery extended below the middle or shortly after the middle of the lung. In lungs with less air, there was as a network of thin arteries that surrounded the lung or its entire surface. In two *B. rotenbergae* males we observed that another artery branched off from the pulmonary artery, which was oriented towards the right portion of the lung in ventral view. In the lungs of females, it was not possible to observe blood supply (Fig. 17).

Comparison of the lungs among species

The lungs of *B. pitanga* had the same plain appearance as that of *B. rotenbergae* (Fig. 5A and 5B), and presented a reduced blood supply when compared to the lungs of *I. parva* and *E. johnstonei* (Fig. 5C and 5D). We also observed that the lung of *I. parva* was smaller in size when compared to the lung of the other three species (Fig. 5). In all four species we made lung histological sections but only in *I. parva* and *E. johnstonei* we observed blood vessels (Fig. 18).

***Brachycephalus rotenbergae*'s circulation**

In individuals of *B. rotenbergae*, the main circulation paths consisted in: (i) the blood in the *sinus venosus* had a portion of oxygenated and deoxygenated blood due to the cutaneous artery that returned oxygenated blood to the *sinus venosus*. This mixed blood is sent to the single atrium; (ii) in the atrium, blood mixing occurs once more, where the blood coming from the pulmonary vein mixes with the mixed blood coming from the *sinus venosus*. During systole, the blood is sent to the ventricle and the *bulbus cordis*, than in the ventricle the blood continues to mix; (iii) once the blood is in the ventricle, ventricular systole sends blood to the *bulbus cordis*;

(iv) after the blood reaches the *bulbus cordis*, the contraction of this same structure sends the blood towards the *truncus arteriosus* and the three main arches (ZUEC-VID 1003–1022).

Once the blood is in the arteries, the circulation occurs as follows: (i) the blood in the carotid arch is distributed to the head; (ii) the systemic artery supplies blood to all the viscera and extremities through the arteries that originate from it. Posteriorly, it joins with its counterpart to form the dorsal aorta; (iii) the blood carried by the pulmocutaneous artery is directed to the capillaries that perfuse the skin, so that it is oxygenated; (iv) when returning, the blood reaches the posterior *venae cavae*, which joins the *sinus venosus* and returns through the anterior *venae cavae*; (v) once the blood is in the *sinus venosus*, it empties into the single atrium. This configuration of circulation in *B. rotenbergae* was similar to *B. pitanga* because the heart of both species presents a reduction of the atrial septum. *Ischnocnema parva* and *E. johnstonei*, in turn, showed a typical anuran circulation, as already described for other species (e.g., de Graaf, 1957; Sharma, 1961).

4. Discussion

Heart

Due to the reduction of the interatrial septum, both *Brachycephalus* species presented only four cavities in the heart, and not five as widely known for other anurans (Hoffman, 1931; Sharma, 1957). Although a similar condition of reduced atrial septum has already been reported for lungless salamanders (plethodontids), which breathe mainly through their skin (Lewis & Hanken, 2017), this is a novel anatomic feature for anurans. Apparently, the absence of lungs and pulmonary veins could have enabled the reduction of the interatrial septum during the evolution of plethodontid salamanders (Lewis & Hanken, 2017). Similarly, the absence of lungs and

pulmonary veins and the occurrence of a complete blood mixture within the bloodstream of the *Brachycephalus* species, the atrial division was also no longer necessary.

Concerning lungless salamanders, Lewis and Hanken (2017) proposed that the reduction of the interatrial septum occurs in organisms without lungs, such as those with reduced pulmonary return, as observed in *Brachycephalus* spp. Thus, the absence of the interatrial septum is probably the result of differential selective pressure, even in individuals where pulmonary veins are present due to the narrowing of the pulmonary artery and the reduced amount of blood returning to the left atrium. Hypothetically, if there was a complete atrial division in *B. rotenbergae* and *B. pitanga* the right atrium would be exposed to a high blood pressure (compared to the left atrium) due to the higher blood volume and the reduced blood return, which could affect their cardiac output.

Studies have also reported for species of the *Cryptobranchus* and *Rhyacotriton* genus, that their heart may have a different morphology in individuals that did not use the lungs as the main mode of oxygen acquisition, including a reduction of the left atrium and fenestration, or reduction of the interatrial septum (Noble, 1925). Considering that these two species present cutaneous respiration as the main form of oxygen intake, with a highly vascularized skin, probably the hypothesis proposed for lungless salamanders can also be applied for *Brachycephalus* spp. Some evidences for this proposal are: (i) reduction in the caliber of the pulmonary artery; (ii) change in the morphology of the lungs; (iii) presence of one or two epidermal layers, which makes comparatively the skin thinner than those of the other species; and (iv) the poor blood supply from the pulmonary artery to the lung compared to *I. parva* and *E. johnstonei*.

Our analyses have raised evidence that *Brachycephalus* spp. present an incomplete atrial septum, which represent a novelty for anuran morphology and anatomy. Although a similar morphology has already been observed for other species, the reports seem confused, and studies

differ in relation to their results. Sedmera *et al.* (2003) and Corno *et al.* (2022), for example, reported an incomplete atrial septum in *Xenopus laevis*, while Mohun *et al.* (2000) reported that it is incomplete in the larval stage of 19 and 36 according to Nieuwkoop and Faber. Also, de Graaf (1957) reported not only a complete atrial septum, but also that the septum extends to the ventricle, forming an incomplete ventricular septum. Thus, it is not possible to state if the heart of *Xenopus laevis* presents an atrial septum or not, considering the divergences between the studies. Thus, even considering some of the previous works (de Graaf 1957; Mohun *et al.* 2000; Sedmera *et al.* 2003; Corno *et al.* 2022), there is no doubt that *Brachycephalus* spp presents a unique morphology within the anurans. Also, it is possible that this reduction of the atrial septum is present in more species of the genus *Brachycephalus*, and it could be a generalized pattern for the group.

Blood circulation

The absence of atrial division in individuals of *Brachycephalus* spp. results in an additional blood mixture in the heart. Considering the absence of atrial septum, deoxygenated and oxygenated blood mixes for the first time in the *sinus venosus*, but mixes for a second time in the atrium. Thus, the complete dual circulation is lost in *Brachycephalus* spp., as seen in lungless salamanders (Lewis & Hanken, 2017). In addition, respiration in *Brachycephalus* spp. possibly occurs almost exclusively through the skin, since the lungs have less blood perfusion, and the cutaneous artery has a greater amount of blood than the pulmonary artery. Comparatively, *I. parva* and *E. johnstonei* showed a greater blood perfusion in the lungs.

Through histology, we observed the present of spiral valve in *Brachycephalus* spp., which means that the blood is still separated into two circuits, even with the mixture in the heart. Our observations in *Brachycephalus* spp. do not agree with what was established by Noble (1925),

which states that there is a correlation between the distinction of arterial and venous flows, the presence of a functional atrial septum and the presence of a spiral valve. Thus, if one of these features would be missing, the other two cannot be present. We observed hereby that *Brachycephalus* spp. does not present an auricular division, but the spiral valve is still present.

Arteries and veins

There were two variations for the origin of occipital-vertebral artery. In one of the variations, the occipito-vertebral artery originates independently from the subclavian artery, which was already described (Rau, 1924; Iuliis & Pulerà, 2006; Hoffman, 1931). However, the second variation, where the subclavian artery originates at the same point as the occipito-vertebral artery, is described here for the first time. In *I. parva* we observe these two variations even in the same individual (Fig. 6C).

The renal veins in the four species studied are oriented towards the anterior region of the kidneys, thus the formation of the posterior *venae cavae* does not extend towards the middle region of the kidneys. This is a characteristic of small-sized anurans (Taboada *et al.* 2013) and although this characteristic is already known, the different variations that can be found within individuals of the same species have not been described (Fig. 8, 14, 15 and 16).

The morphology observed in the renal veins could be explained as follows: the renal portal veins produce a network of vessels that cover the dorsal surface of the organs and form the peritubular capillaries as they cross the kidneys. The network of capillaries is formed around the renal tubules, the efferent veins arising from this capillary network of the renal portal vein collect most of the renal blood from the peritubular capillaries. Finally, the efferent ducts, which emerge from the glomeruli, together with the efferent veins, will form the beginning of the renal veins that carry blood from the kidney to the posterior *venae cavae* (Holmes, 1916; Killorn & Toews,

2001). Thus, the cranial location in the kidney of the renal veins could be associated with the orientation of the efferent ducts and the efferent veins, which could be oriented towards the anterior region of the kidneys and give rise to the renal veins. These renal veins will join only in the anterior region of the kidney to form the posterior *venae cavae*. Histological sections of the kidneys and comparison with other individuals with different configurations could corroborate this hypothesis.

The absence of the hepatic portal vein that perfuses the right lobe of the liver is absent in all species examined here (Fig. 11). However, the presence of this vein has been reported for other species, such as *Aquarana catesbeiana*, *Rana temporaria*, and *Rana esculenta* (Haberich, 1965; Iuliis & Pulerà, 2006; Marshall, 1916). There are no studies related to the consequences of the lack of this structure, and further physiological studies are necessary to observe if there is a difference between individuals who present the hepatic portal vein that perfuses the right lobe of the liver and those who do not present it.

Lung

In *Brachycephalus* spp. the reduction of the pulmonary artery, and consequently a cutaneous artery of larger caliber, probably is a consequence of an improved cutaneous oxygenation. This modification can be related to the size of the animals. *Brachycephalus* spp. have a snout-vent length between 0.8 cm and 1.7 cm (Giaretta and Sawaya, 1998; Nunes *et al.* 2021), and they are among the smallest vertebrates on earth. Smaller anurans present a higher abundance of capillaries in the skin than larger ones, which improve the cutaneous respiration (Szarski, 1964; Tattersall, 2007). Therefore, the reduced size of *Brachycephalus* spp. could allow them to obtain most of their oxygen through the skin because miniaturization could have led them to the extreme of having highly vascularized skin and a thinner epidermis compared to *I. parva*

and *E. johnstonei*, which would facilitate the obtaining of oxygen. With histology we observe in *Brachycephalus* spp. a thin layer of *stratum corneum* and below it can be seen the germinal layer that is 1-2 cells thick, and in *I. parva* and *E. johnstonei* an epidermal layer with 3 to 4 cells thick in the germ layer (Fig. 13). Therefore, the individuals of *Brachycephalus* spp. present a greater efficiency for the uptake of oxygen through the capillaries of the skin, due to the thin germinal layer that they present.

The arteries that perfuse the lungs have a reduced diameter when compared to the cutaneous artery. Consequently, the skin receives more blood than the lungs, since the diameter of the cutaneous artery is given by the amount of blood it transports. Therefore, considering the morphology of the arteries of the lungs, probably this organ may not be the main mechanism for blood oxygenation.

Individuals of *Brachycephalus* spp. inhabit the litter of montane forests (Lima *et al.* 2013; Bornschein *et al.* 2016) and the accumulation of leaves on the ground forms an important microenvironment with constant temperature and humidity (Olivier *et al.* 2015). *Brachycephalus rotenbergae* and *B. pitanga* have high population densities in these microhabitats, when the environmental conditions are suitable for their establishment, but they have little or no occurrence in other habitats that differ in some of these conditions, which is not observed for the others two species examined here. Therefore, these humidity and temperature factors are probably more limiting for *Brachycephalus* individuals than for other species with similar occurrence. Skin respiration is normally more efficient when amphibians are submerged in water (Tattersall, 2007), as was observed for *Xenopus laevis* (Emilio & Shelton, 1974) and *Telmatobius culeus* (Hutchison *et al.* 1976). However, these environments, although terrestrial, could provide a microhabitat where the skin respiration could be more effective than other terrestrial ones. In this way, the individuals of *Brachycephalus* spp. observed here, despite being terrestrial anurans,

could present almost total cutaneous respiration and survive in these environments due to the humidity of the habitat.

The observed general appearance of the lungs in *Brachycephalus* spp. could be due to the absence or reduction in thickness of some of the septa present in the lungs of anurans (primary, secondary and tertiary) (Smith & Rapson, 1977; Guangming *et al.* 2020). It was observed that the thickness of the septa and the lung wall is related to a more powerful respiratory function (Guangming *et al.* 2020). In the newt *Triturus carnifex*, a lung similar to that seen in *Brachycephalus* spp. was observed, also consisting of smooth sacs without septa, probably as a consequence of improved cutaneous respiration (Goniakowska-Witalińska, 1980).

According to Goutte *et al.* (2017), *B. rotenbergae* and *B. pitanga* are insensitive to high sound frequencies due to the reduced or lack of innervation of the basilar papilla. Probably the lack of hearing could have additional consequences for the animal, including the pattern observed for the lungs. The morphology that we observed in the lungs in *B. rotenbergae* and *B. pitanga* could be more efficient for internal sound resonance than the lungs observed in individuals of *I. parva* and *E. johnstonei*, since larger areas resonate more than the smaller ones. Lungs in *B. rotenbergae* and *B. pitanga* probably lack lung septa in the same way as the lungs of *Triturus carnifex* (Goniakowska-Witalińska, 1980). In *I. parva* and *E. johnstonei*, the presence of these septa can form different chambers within the lung and reduce resonance efficiency. Goutte *et al.* (2017) observed that individuals of *B. rotenbergae* and *B. pitanga* exhibit a clear vibratory response of the lungs to the airborne sound. Thus, the lack of high frequency audition, probably as a consequence of miniaturization, could lead to an improved mechanism of internal sound resonance, which probably modified the structure of lungs to be a resonance structure.

Carotid body

We observed a variation in the point of origin of the external carotid artery. The differences observed were already described for *Xenopus laevis*, *Aquarana catesbeiana*, *Pelophylax nigromaculatus*, and *Hyla japonica* (Kusakabe, 2002). The external carotid artery may arise directly from the carotid body or from the common carotid artery (Kusakabe, 2002). However, these differences in the point of origin of the external and internal carotid arteries had not been reported in previous studies for *I. parva* and *E. johnstonei*. (e.g., Carman, 1955; Ishii *et al.* 1966; Kusakabe, 1990, 2002) and for the first time we report the absence of the carotid body for anurans in individuals of species *B. rotenbergae* and *B. pitanga* (Fig. 4). As a consequence, it can be seen that the internal and external carotid arteries are formed as a prolongation of the common carotid artery, and not from an anastomoses of blood vessels, as observed in individuals with carotid bodies (Kusakabe, 1990).

The carotid body has a chemoreceptor and a baroreceptor function, which is related to the regulation of breathing in anurans and regulation of blood pressure, respectively. When the body has an optimal supply of oxygen, the chemoreceptors cease their activity (Ishii *et al.* 1966; Kusakabe, 1990, 2002). In addition to these two functions, the carotid body plays an important role in the control of vascular tone, as well as maintenance of homeostasis and adequate blood supply to the cephalic region (Kusakabe *et al.* 1987; Kusakabe, 2002). However, these functions can still be maintained through glomus cells, for example, in individuals that present no carotid body which can be located at the bifurcation region of the external and internal carotid arteries (Kusakabe *et al.* 1987). Glomus cells are present in bullfrogs in the initial stages of development, before the complete formation of the carotid body (Kusakabe, 2002). In addition, by not presenting a carotid body, the carotid nerve may probably be absent. This structure innervates the carotid body, and is activated by fluctuating blood pressure or by chemoreceptor activity (Ishii *et*

al. 1966), and it is originated from sympathetic and vagus nerve (Kusakabe, 2002). Considering that *B. rotenbergae* and *B. pitanga* have glomus cells, they will have a direct relationship with the sympathetic nerve and the vagus nerve. However, we did not observe the carotid nerve, and we have no evidence of glomus cells in individuals of these species. The absence of the carotid body could also modify respiration in *Brachycephalus rotenbergae* and *B. pitanga*, since the stimulation of the chemoreceptors causes an excitation of respiration (Ishii *et al.* 1966). Therefore, the absence of the carotid body could be related to the possible reduction of lung function in *B. rotenbergae* and *B. pitanga* due to their morphology.

One of the possible consequences of the limited distribution of *B. rotenbergae* and *B. pitanga* could be the absence of the carotid body. In environments with higher temperature variation, individuals of *B. rotenbergae* and *B. pitanga* cannot regulate blood pressure caused by these temperature changes, and consequently surviving individuals are restricted to the microhabitats with stable temperature. In *I. parva* and *E. johnstonei* we observed the presence of a carotid body (Fig. 4). Therefore, individuals of this species could regulate their blood pressure more efficiently, and consequently can inhabit more variable environments, compared to individuals of the *Brachycephalus* species that do not have a carotid body and are found in restricted areas of lowlands or mountains (Bornschein *et al.* 2016; Nunes *et al.* 2021)

We also propose for future work to investigate how efficient the lungs are in *B. rotenbergae* and *B. pitanga* and how these animals compensate for the lack of a carotid body. These studies will help on understanding how these reductions, or morphological variations, affect the life of the individuals of the other species of the genus *Brachycephalus*. In addition, there is a lack of research on the discoveries shown in this work to know if they are the cause of miniaturization or not.

Conclusions

Miniaturization is a fascinating process in nature that contains complex characteristics, which makes it difficult to define which morphological variations can be involved in this phenomenon. In this study, we observed in *B. rotenbergae* and *B. pitanga* unique morphologies within Anura in the heart, lungs, and skin. However, more work is needed to explain why these features occur only in individuals of the genus *Brachycephalus*, whether it is restricted to this group, and how they affect the organisms of the two species. We confirm our hypothesis, since we obtained positive results by observing morphological variations in the cardiorespiratory system, which lead us to effort that *B. rotenbergae* and *B. pitanga* breathe mainly through the skin. We also observed shared morphological differences between *E. johnstonei*, *I. parva*, *B. rotenbergae* and *B. pitanga*, which could be characteristic of small anuran species less than 3 cm. In this way, we realize that there is still much to be discovered about the internal morphology of anurans, and descriptive studies are the first step to understand the functioning and behavior of organisms.

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Author contributions

ASC, AS, RR and LFT designed this study; the dissection process was performed by ASC with the help of AS; the analysis of the internal morphology was carried out by ASC, AS and HRS; RFS, FDP and ASC performed the histology processes and analysis; and LFT provided all the necessary resources for the realization of the study.

References

- Bickford D, Iskandar D, Barlian A (2008) 'A lungless frog discovered on Borneo', *Curr. Biol* 18, R374–R375.
- Bornschein MR, Firkowski CR, Belmonte-Lopes R, et al. (2016) Geographical and altitudinal distribution of *Brachycephalus* (Anura: Brachycephalidae) endemic to the Brazilian Atlantic Rainforest. *PeerJ* 4, e2490.
- Campos LA, Da Silva HR, Sebben A (2010) Morphology and development of additional bony elements in the genus *Brachycephalus* (Anura: Brachycephalidae). *Biol J Linn Soc* 99, 752–767.
- Carman JB (1955) The carotid labyrinth in *Hyla aurea*, with a note on that in *Leiopelma hochstetteri*. *J Anat* 89, 503–525.
- Clemente-Carvalho RBG, Antoniazzi MM, Jared C et al. (2009) Hyperossification in miniaturized toadlets of the genus *Brachycephalus* (Amphibia: Anura: Brachycephalidae): Microscopic structure and macroscopic patterns of variation. *J Morphol* 270, 1285–1295.
- Clemente-Carvalho RBG, Monteiro LR, Bonato V et al. (2008) Geographic Variation in Cranial Shape in the Pumpkin Toadlet (*Brachycephalus Ehippium*): A Geometric Analysis. *J Herpetol* 42, 176–185.
- Condez HT, Haddad BFC, Zamudio RK (2020) Historical biogeography and multi-trait evolution in miniature toadlets of the genus *Brachycephalus* (Anura: Brachycephalidae). *Biol. J. Linn. Soc*, 129, 664–686.
- Corno AF, Zhou Z, Uppu SC et a. (2022) The secrets of the frogs heart. *Pediatr Cardiol*, 43, 1471–1480.

- de Bakker DM, Wilkinson M, Jensen B (2015) Extreme variation in the atrial septation of caecilians (Amphibia: Gymnophiona). *J Anat* 226, 1–12.
- de Graaf AR (1957) Investigations into the distribution of blood in the heart and aortic arches of *Xenopus laevis* (Daud.). *J Exp Biol* 34, 143–172.
- de Saint-Aubain ML (1982) The morphology of amphibian skin vascularization before and after metamorphosis. *Zoomorphology* 100, 55–63.
- Dena S, Rebouças R, Augusto-Alves G et al. (2018) Lessons from recordings lost in Brazil fire: deposit and back up. *Nature* 563, 473–474.
- Dena S, Rebouças, R, Augusto-Alves G. (2020) How much are we losing in not depositing anuran sound recordings in scientific collections? *Bioacoustics* 29, 590–601.
- Duellman WE, Trueb L (1994) Biology of amphibians. Johns Hopkins University Press, Baltimore.
- Emilio MG, Shelton G (1974) Gas exchange and its effect on blood gas concentrations in the amphibian, *Xenopus Laevis*. *J Exp Biol* 60, 567–579.
- Foxon GEH (1947) The mode of action of the heart of the frog. *Proc Zool Soc Lond* 116, 565–574.
- Giarretta AA, Sawaya RJ (1998) Second Species of *Psyllophryne* (Anura: Brachycephalidae). *Copeia* 1998, 985–987.
- Goniakowska-Witalińska (1980) Ultrastructural and morphometric changes in the lung of newt, *Triturus cristatus carnifex* Laur. during ontogeny. *J Anat* 130, 571–583.
- Goutte S, Mason MJ, Christensen-Dalsgaard J et al. (2017) Evidence of auditory insensitivity to vocalization frequencies in two frogs. *Sci Rep* 7, 12121.
- Guangming G, Zhe Y, Mei Z et al. (2020) Comparative morphology of the lungs and skin of two anura, *Pelophylax nigromaculatus* and *Bufo gargarizans*. *Sci Rep* 10, 11420.

- Haberich FJ (1965) The functional separation of venous and arterial blood in the univentricular frog heart. *Ann N Y Acad Sci* 127:459–476.
- Hanken J, Wake WD (1993) Miniaturization of Body Size: Organismal Consequences and Evolutionary Significance. *Annu Rev Ecol Evol* 24:501–19.
- Haddad CFB, Alves ACR, Clemente-Carvalho RBG et al. (2010) A new species of *Brachycephalus* from the Atlantic Rain Forest in São Paulo State, Southeastern Brazil (Amphibia: Anura: Brachycephalidae). *Copeia* 2010, 410–420.
- Hoffman AC (1931). On variations in the arterial system of *Bufo*, *Pyxicephalus*, and *Heleophryne*. *S Afr J Sci* 7, 410–420.
- Holmes S J (1916) The biology of the frog. 3rd ed. Macmillan, New York.
- Hutchison VH, Haines HB, Engbretson G (1976) Aquatic life at high altitude: Respiratory adaptations in the lake titicaca frog, *Telmatobius coleus*. *Respir Physiol*, 27, 115–129.
- Ishii K, Honda K, Ishii K (1966) The function of the carotid labyrinth in the toad. *Tohoku J Exp Med* 88, 103–116.
- Iskandar DT (1978). A new species of *Barbourula*: First record of a Discoglossid Anuran in Borneo. *Cope* 1978, 564–566
- Iuliis GD, Pulerà D (2006) The dissection of vertebrates: A laboratory manual. Elsevier
- Killorn EE, Toews DP (2001) The dynamics of venous return and response to hypervolemia in the toad, *Bufo marinus* (L.). *BMC Physiol* 1:13.
- Kraus N, Metscher B (2021) Anuran heart metamorphosis: Anatomical support for pulmonary blood separation in the early aquatic phase. bioRxiv 2021.04.06.438645. DOI: <https://doi.org/10.1101/2021.04.06.438645>

- Kusakabe T (1990) Comparative studies on the vascular organization of carotid labyrinths of anurans and caudates. *J Morphol* 204, 47–55
- Kusakabe T (2002) Carotid labyrinth of amphibians. *Microsc Res Tech* 59, 207–226.
- Kusakabe T, Ishii K, Ishii K (1987) A possible role of the glomus cell in controlling vascular tone of the carotid labyrinth of *Xenopus laevis*. *Tohoku J Exp Med* 151, 395–408.
- Lewis ZR, Hanken J (2017) Convergent evolutionary reduction of atrial septation in lungless salamanders. *J Anat* 230, 16–29.
- Lima M, Pederassi J, Souza C (2013) Habitat use by *Brachycephalus ephippium*. *Bol Asoc Herpetol Esp* 24:2.
- Lyra ML, Monteiro JPC, Rancilhac L (2021) Initial phylotranscriptomic confirmation of homoplastic evolution of the conspicuous coloration and bufoniform morphology of pumpkin-toadlets in the genus *Brachycephalus*. *Toxins*, 13, 816.
- Malvin GM (1988) Microvascular regulation of cutaneous gas exchange in amphibians. *Am Zool* 28, 999–1007.
- Marshall AM (1916) The frog; an introduction to anatomy, histology and embryology. ed. by F.W. Gamble. Ed. 11. MacMillan, London.
- Mohun TJ, Leong LM, Weninger WJ (2000) The Morphology of Heart Development in *Xenopus laevis*. *Dev Biol* 218, 74–88.
- Nigam H.C (1983) Structure and function of the truncus arteriosus of the Indian frog *Rana tigerina* (Daud), Proc. Indian Acad, Sci 92, 55–63.
- Noble GK (1925) The integumentary, pulmonary, and cardiac modifications correlated with increased cutaneous respiration in the amphibia: A solution of the ‘hairy frog’ problem. *J Morphol* 40, 341–416.

- Nunes Ivan, Guimarães CS, Moura PHAG et al. (2021) Hidden by the name: A new fluorescent pumpkin toadlet from the *Brachycephalus ephippium* group (Anura: Brachycephalidae). *Plos one* 16. 4, e0244812.
- Olivier R, Aranda R, Godoi M et al. (2015) Habitat structure in the composition of leaf-litter insects in mosaic environment. *Acta Biol Parana* 44, 71–87.
- Pérez-Ben CM, Schoch RR, Báez, AM (2018) Miniaturization and morphological evolution in Paleozoic relatives of living amphibians: a quantitative approach. *Paleobiology* 44, 58–75.
- Pires OR, Sebben A, Schwartz EF (2005) Further report of the occurrence of tetrodotoxin and new analogues in the Anuran family Brachycephalidae. *Toxicon* 45, 73–79.
- Putnam JL, Dunn J.F (1978) Septation in the ventricle of the heart of *Necturus maculosus*. *Herpetologica* 34, 292–297.
- Putnam JL, Kelly DL (1978) A new interpretation of interatrial septation in the lungless salamander, *Plethodon glutinosus*. *Cope* 1978, 251–254.
- Rau AS (1924) Observations on the anatomy of the heart, lungs and related parts of *Ceratophrys*. *J Anat* 58, 306–327.
- Rebouças R, Lima AB, Freitas M (2019). Is the conspicuous dorsal coloration of the Atlantic forest pumpkin toadlets aposematic? *Salamandra* 55, 39–47.
- Reis SF, Clemente-Carvalho RBG, Santos CMSFF (2021) Skull diversity and evolution in miniaturized amphibians, genus *Brachycephalus* (Anura: Brachycephalidae). *Anat Rec* 304, 1329–1343.
- Reis SF, Clemente-Carvalho RBG, Santos CMSFF et al. (2021) Skull diversity and evolution in miniaturized amphibians, genus *Brachycephalus* (Anura: Brachycephalidae). *Anat Rec* 304, 1329–1343.

- Saint-Aubain, ML, Wingstrand KG (1979). A sphincter in the pulmonary artery of the frog *Rana temporaria* and its influence on blood flow in skin and lungs. *Acta Zool* 60, 163–172.
- Salla R, Jones-Costa M, Abdalla F (2020) Differential liver histopathological responses to amphibian chytrid infection. *Dis Aquat Org* 142, 177–187.
- Sebben A (2007) Microdissecação fisiológica a fresco: uma nova visão sobre a anatomia de anfíbios e répteis. 14.
- Sebben A, Schwartz CA, Valente, D (1986) A tetrodotoxin-like substance found in the Brazilian frog *Brachycephalus ephippium*. *Toxicon* 24, 799–806.
- Sedmera D, Reckova M, de Almeida A (2003) Functional and morphological evidence for a ventricular conduction system in zebrafish and *Xenopus* hearts. *Am J Physiol Heart Circ Physiol*, 284, H1152–H1160.
- Sharma HL (1957) The anatomy and mode of action of the heart of the frog, *Rana tigrina* daud. *J Morphol* 100, 313–343.
- Sharma HL (1961) The circulatory mechanism and anatomy of the heart of the frog, *Rana pipiens*. *J Morphol* 109, 323–349.
- Silva HRD, Campos LA, Sebben A (2007) The auditory region of *Brachycephalus* and its bearing on the monophyly of the genus (Anura: Brachycephalidae). *Zootaxa* 1422, 59–68.
- Simons S (1959) The distribution of the blood from the heart in some Amphibia. *Proc Zool Soc Lond* 132, 51–64.
- Smith DG, Rapson L (1977) Differences in pulmonary microvascular anatomy between *Bufo marinus* and *Xenopus laevis*. *Cell Tissue Res* 178, 1–15.
- Stephenson A, Adams JW, Vaccarezza M (2017) The vertebrate heart: an evolutionary perspective. *J Anat* 231, 787–797.

- Szarski H (1964) The structure of respiratory organs in relation to body size in amphibia. *Evolution* 18, 118-126.
- Taboada C, Grant T, Lynch JD (2013) New Morphological synapomorphies for the new world direct-developing frogs (Amphibia: Anura: Terrarana). *Herpetologica* 69, 342.
- Tattersall G (2007) Skin Breathing in Amphibians. In *Arid. Endothelial Biomedicine* (pp. 85 – 91). Cambridge: Cambridge University Press.
- Vitt LJ, Caldwell JP (2014) *Herpetology: an introductory biology of amphibians and reptiles*. Fourth edition. Elsevier, AP, Academic Press is an imprint of Elsevier, Amsterdam; Boston.
- Yeh J (2002) The effect of miniaturized body size on skeletal morphology in frogs. *Evolution* 56, 628–641.

ANEXO 1: Fotografias

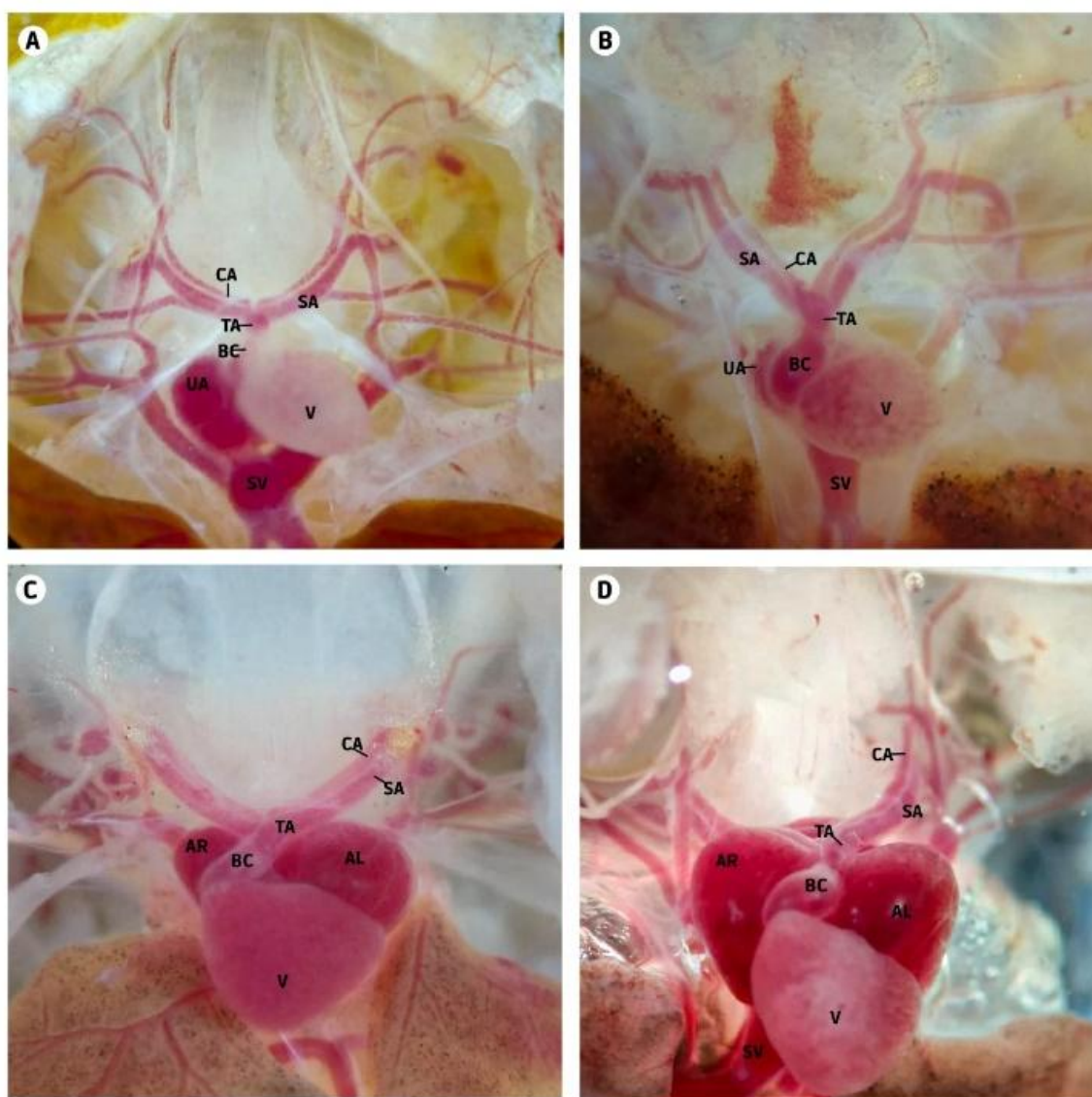


Fig. 1 Ventral view of the heart of the *Brachycephalus rotenbergae* (A), *B. pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D): carotid artery (CA), systemic artery (SA), *Truncus arteriosus* (TA), bulbus cordis (BC), unique atrium (UA), ventricle (V), sinus venosus (SV).

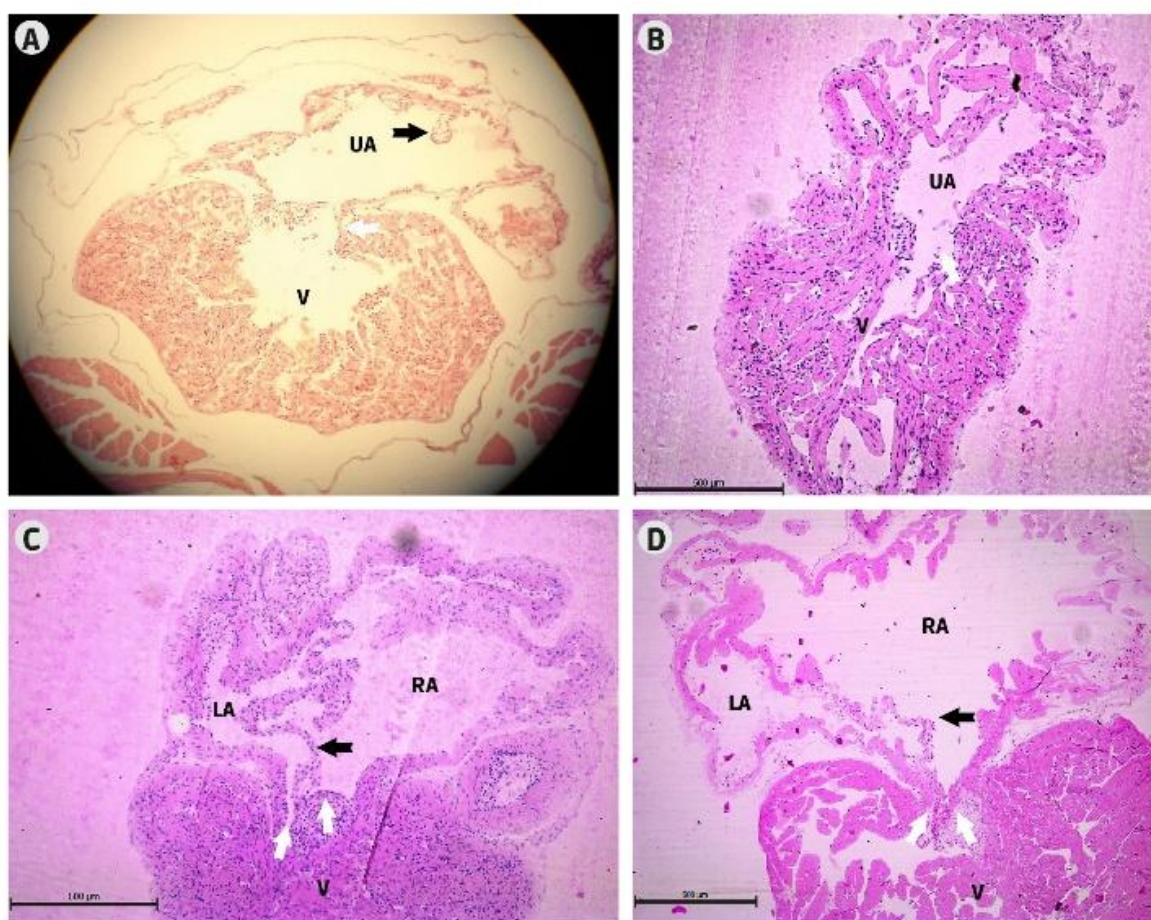


Fig. 2 Histology of the heart of *Brachycephalus rotenbergae* (A), *B. pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D): unique atrium (UA), right atrium (RA), left atrium (LA), ventricle (V), septum auricular (black arrow), atrioventricular valves (white arrow).

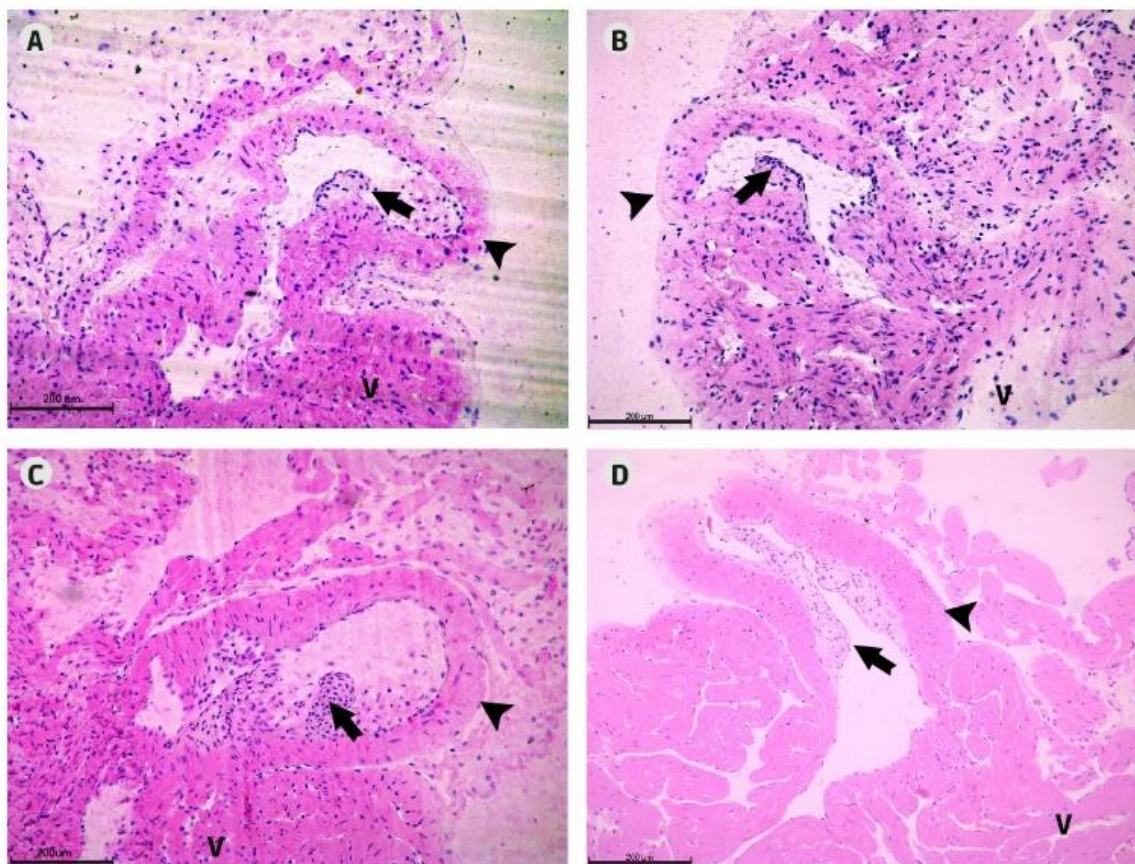


Fig. 3 Histology of the bulbus cordis of *Brachycephalus rotenbergae* (A), *B. pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D): bulbus cordis (black arrowhead), spiral valve (black arrow), ventricle (V).

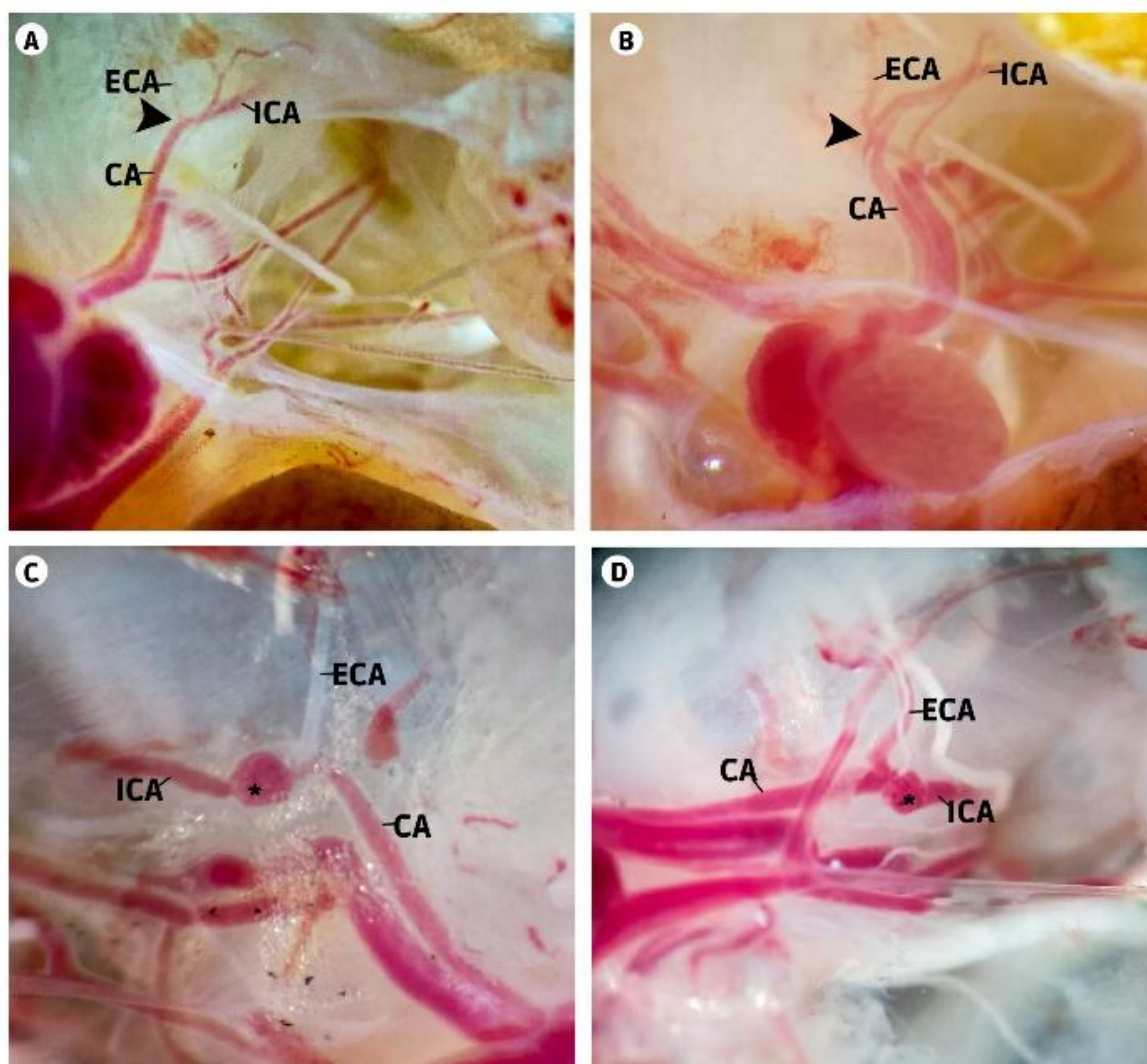


Fig. 4 Left lateral region of the heart, absence and presence of carotid body of *Brachycephalus rotenbergae* (A), *B. pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D): carotid artery (CA), external carotid artery (ECA), internal carotid artery (ICA), carotid body (back asterisk), absence of carotid body (black arrowhead).

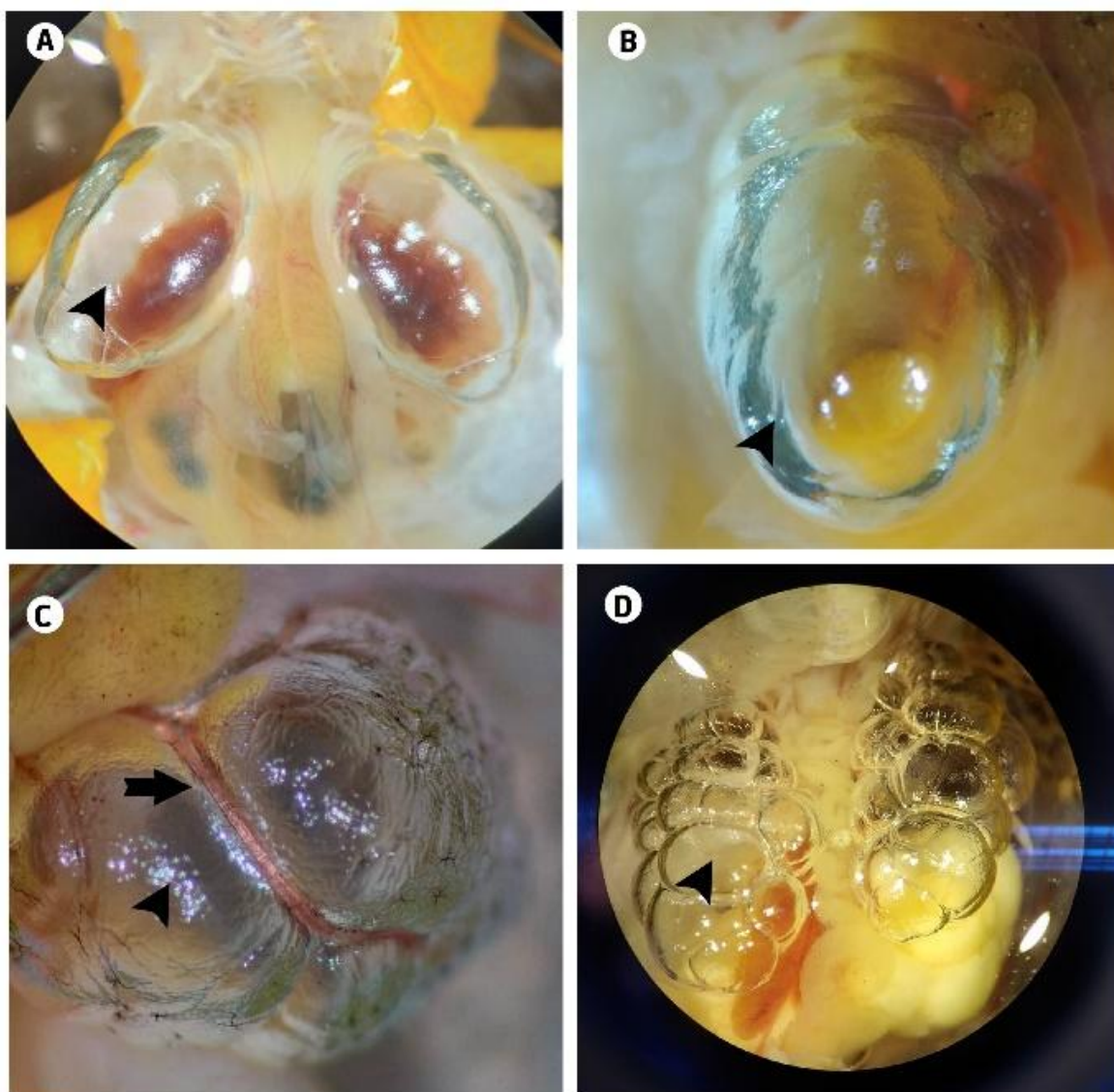


Fig. 5 Lungs of *Brachycephalus rotenbergae* (A), *B. pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D): lung (black arrowhead), artery with obvious blood present (black arrow).

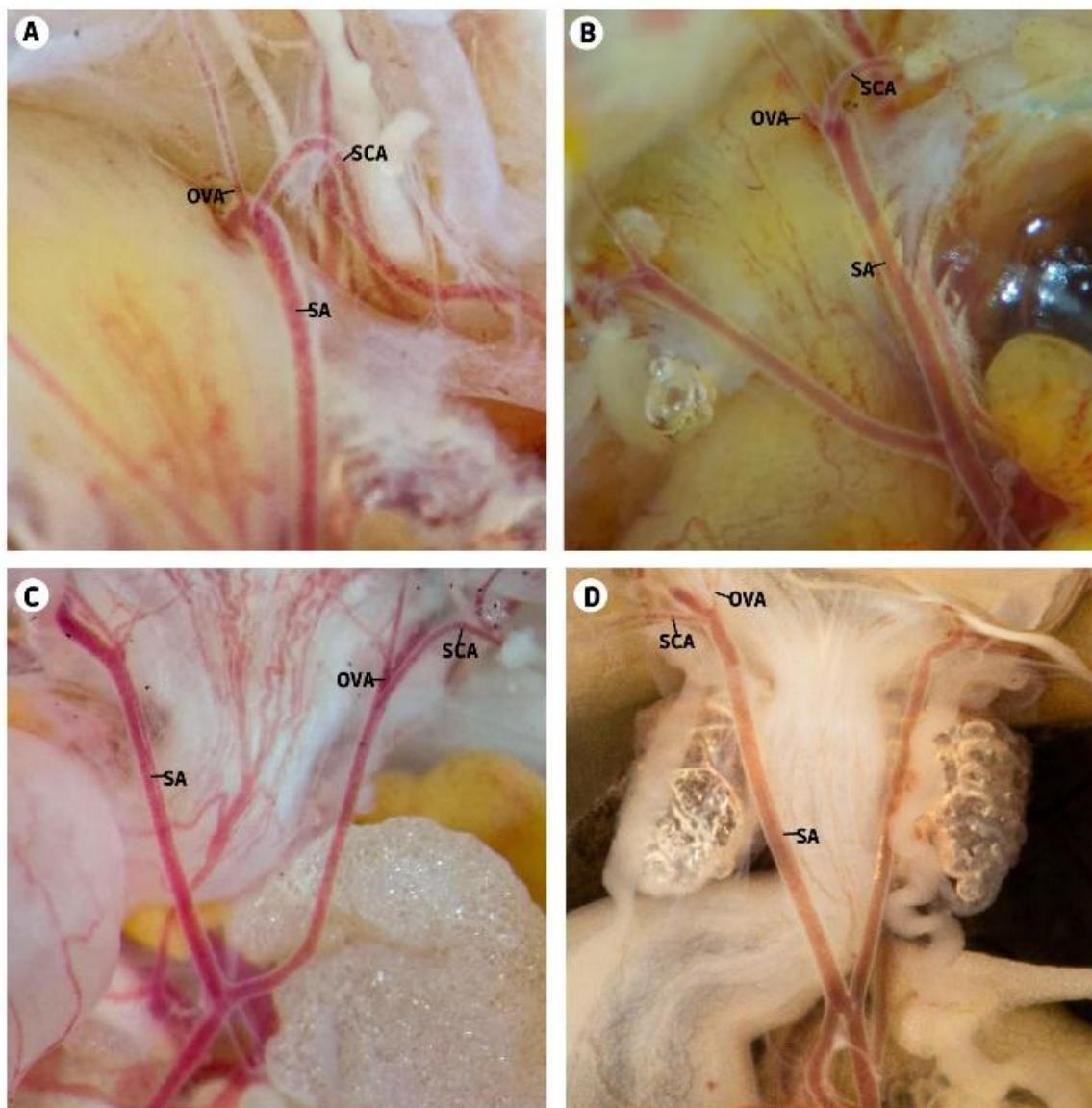


Fig. 6 Dorsal view dissection of the artery occipito-vertebral of the *Brachycephalus rotenbergae* (A), *B. pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D): occipito-vertebral arteri (OVA), subclavian artery (SCA), systemic artery (SA).

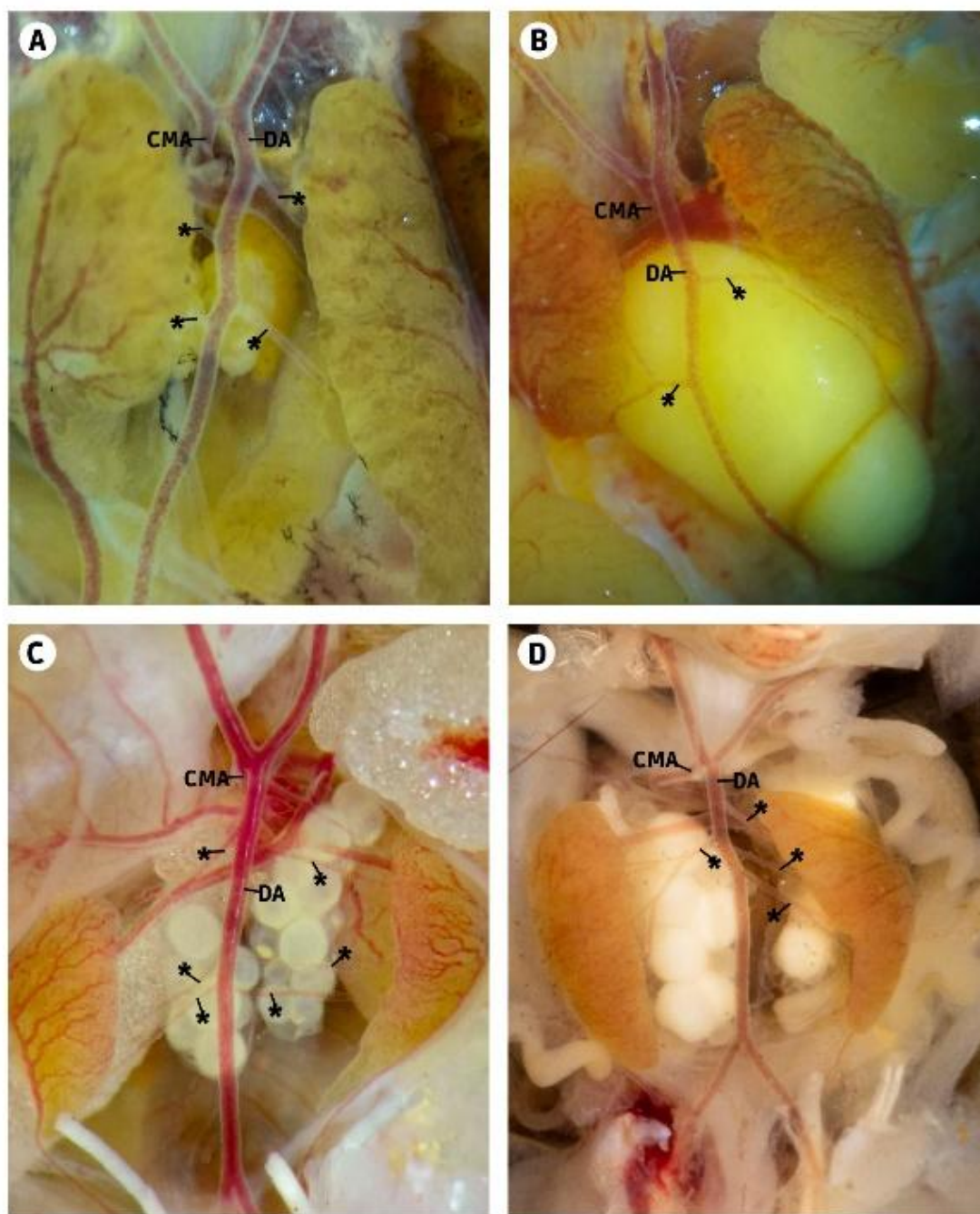


Fig. 7 Dorsal view dissection of the aorta dorsal of the *Brachycephalus rotenbergae* (A), *B. pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D): ciliaco-mesenterica artery (CMA), dorsal aorta (DA), urogenital arteries (back asterisk).

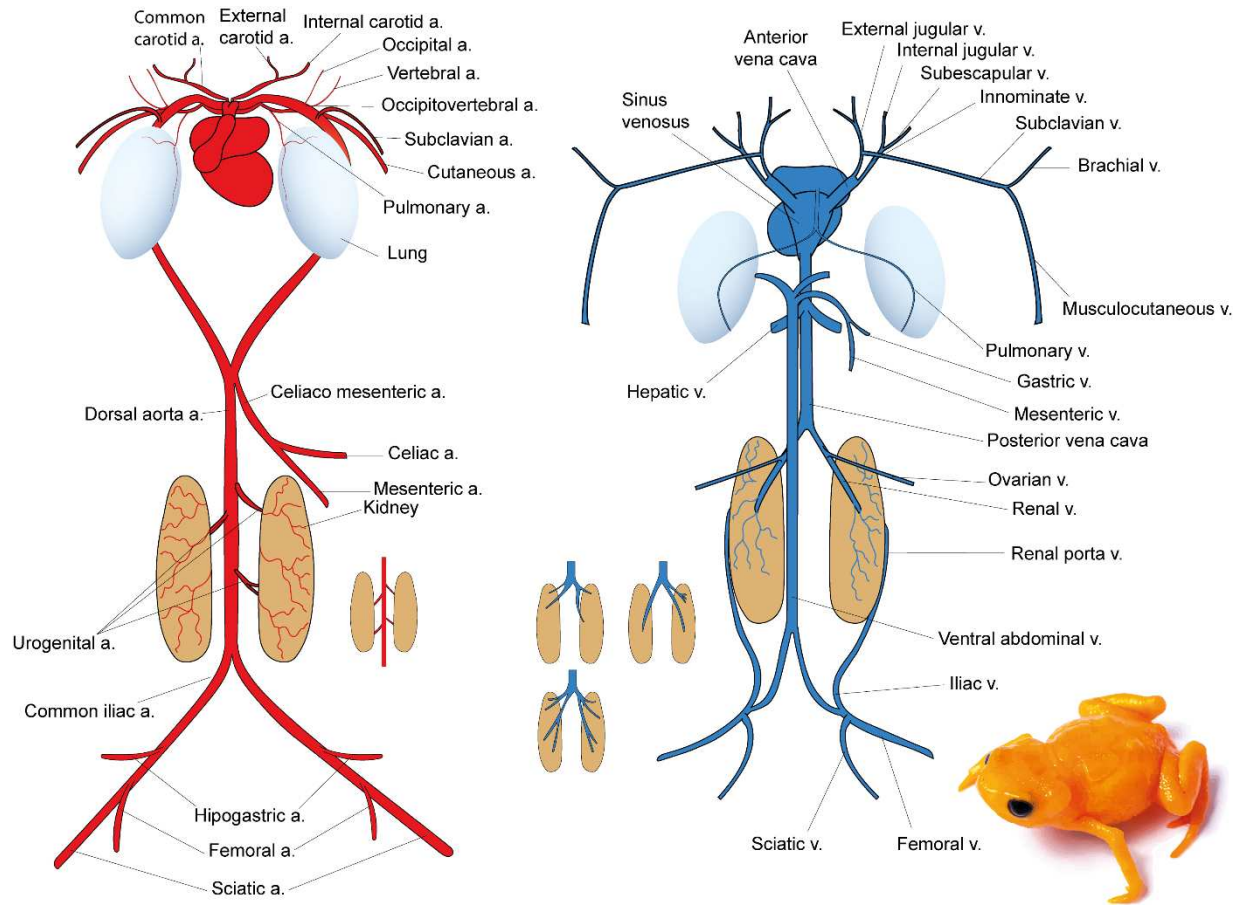


Fig. 8: Circulatory system of the *Brachycephalus rotenbergae*, in this picture we can see the variation in the urogenital arteria and renal veins.

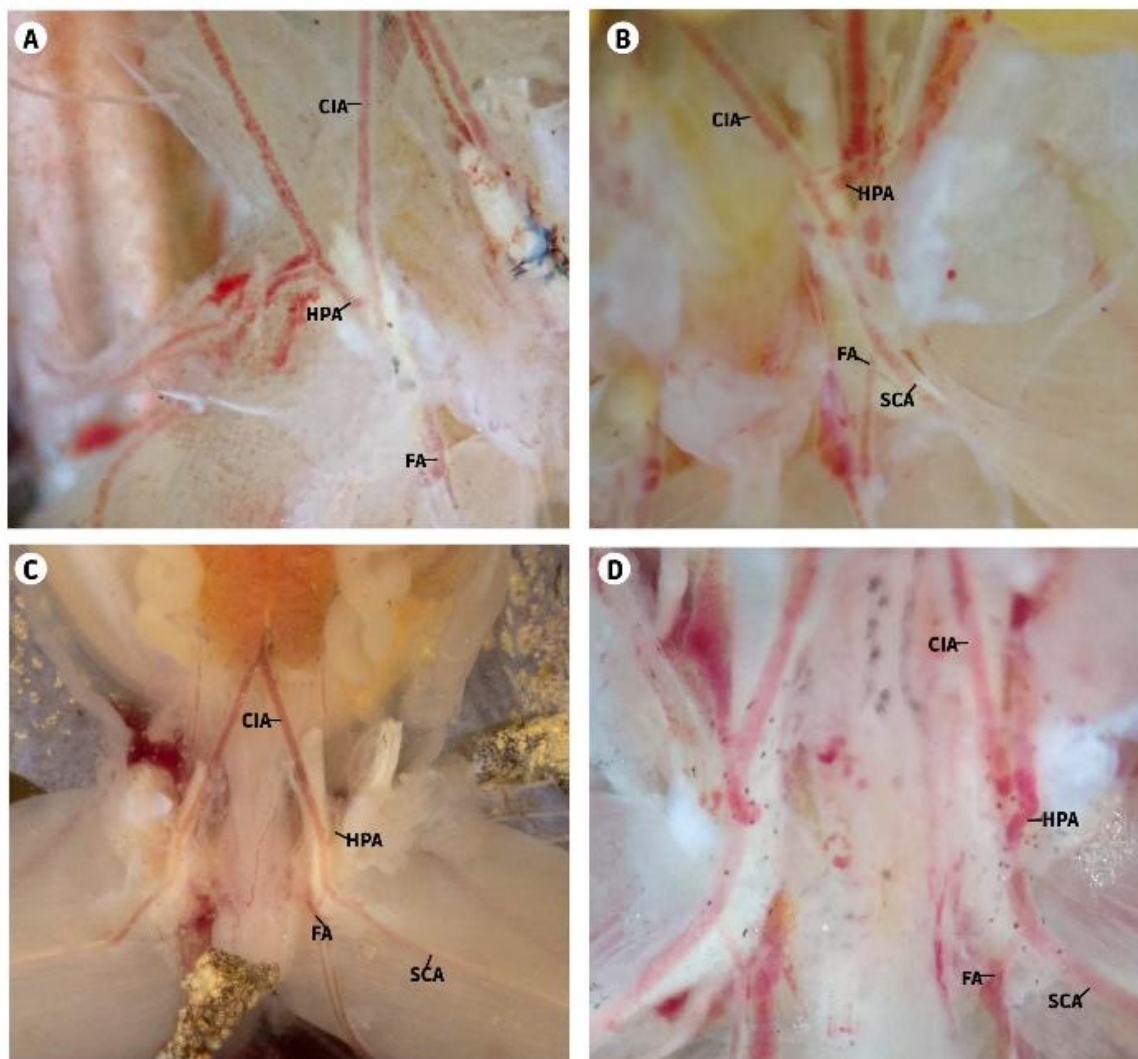


Fig. 9 Hind limb arteries of the *Brachycephalus rotenbergae* (A), *B. pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D): common iliac artery (CIA), hypogastric artery (HPA), femoral artery (FA), sciatic artery (SCA).

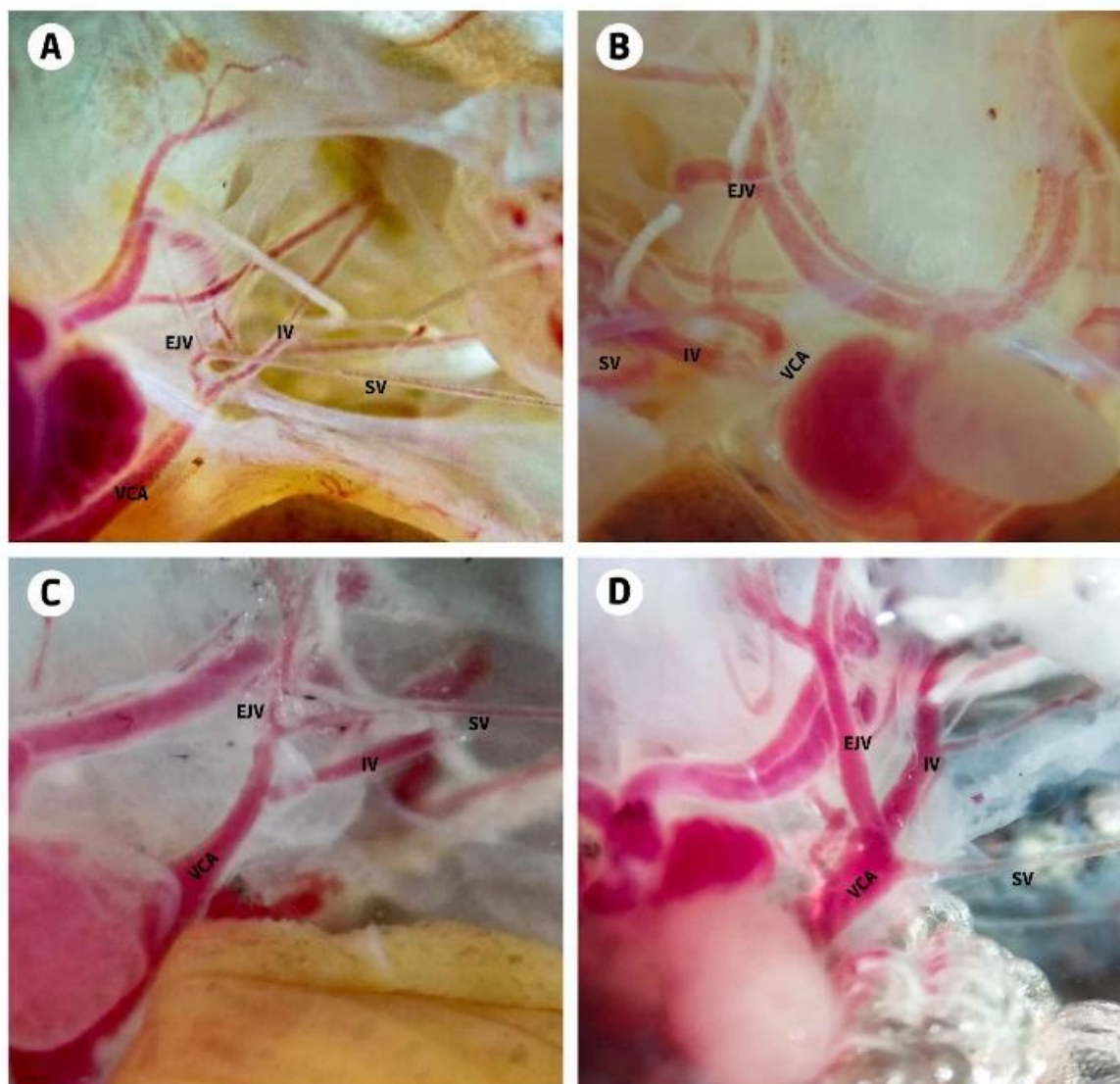


Fig. 10 Left lateral view of the heart of the *Brachycephalus rotenbergae* (A), *Brachycephalus pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D): external jugular vein (EJV), subclavian vein (SV), innominate vein (IV), anterior vena cavae (VCA).

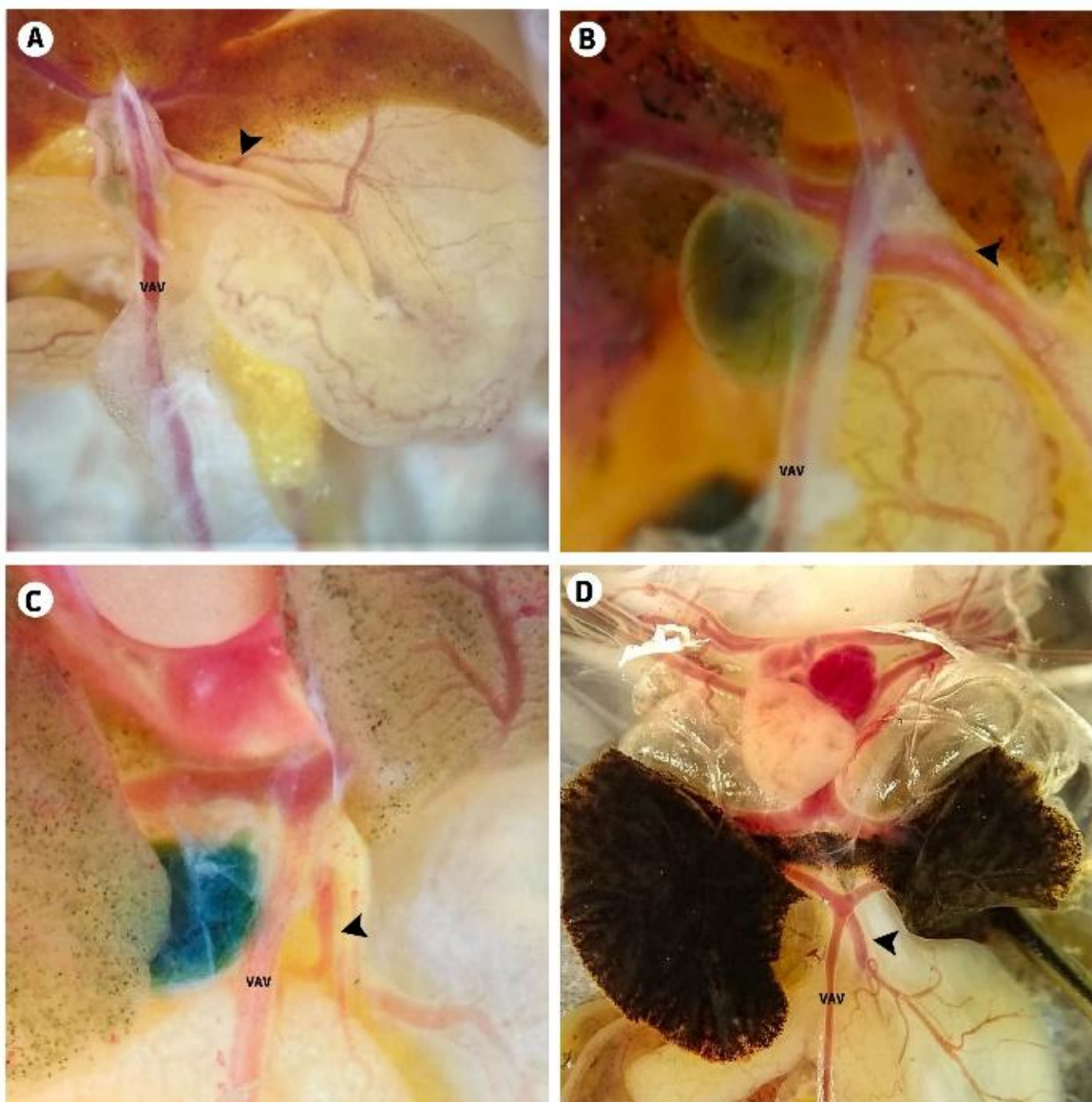


Fig. 11 Absence of the hepatic portal artery of the *Brachycephalus rotenbergae* (A), *Brachycephalus pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D): ventral abdominal vein (VAV), absence of hepatic portal vein (black arrowhead).

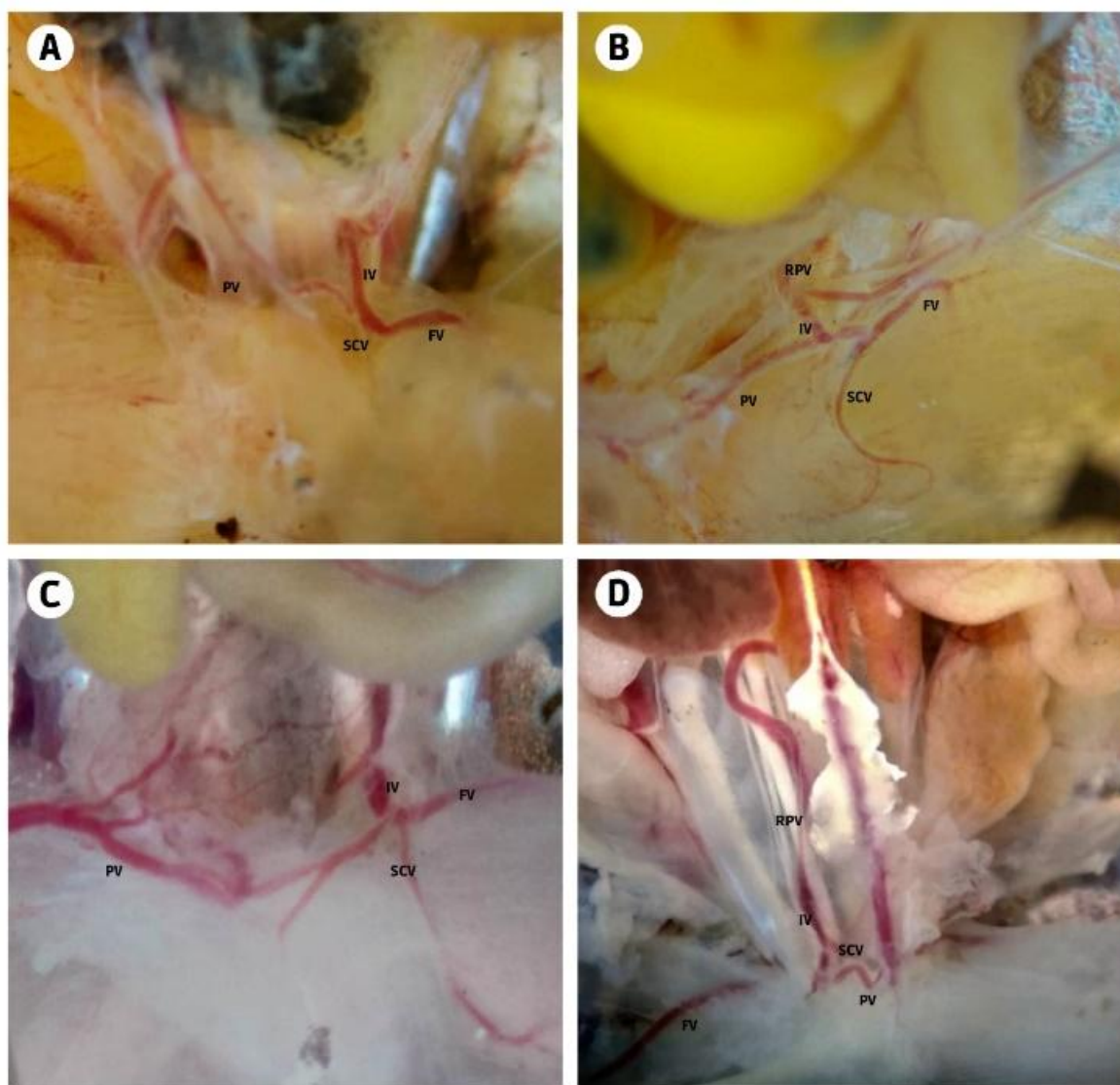


Fig. 12 Veins of the posterior region of *Brachycephalus rotenbergae* (A), *Brachycephalus pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D): femoral vein (FV), sciatic vein (SCV), iliac vein (IV), renal portal vein (RPV), pelvic vein (PV).

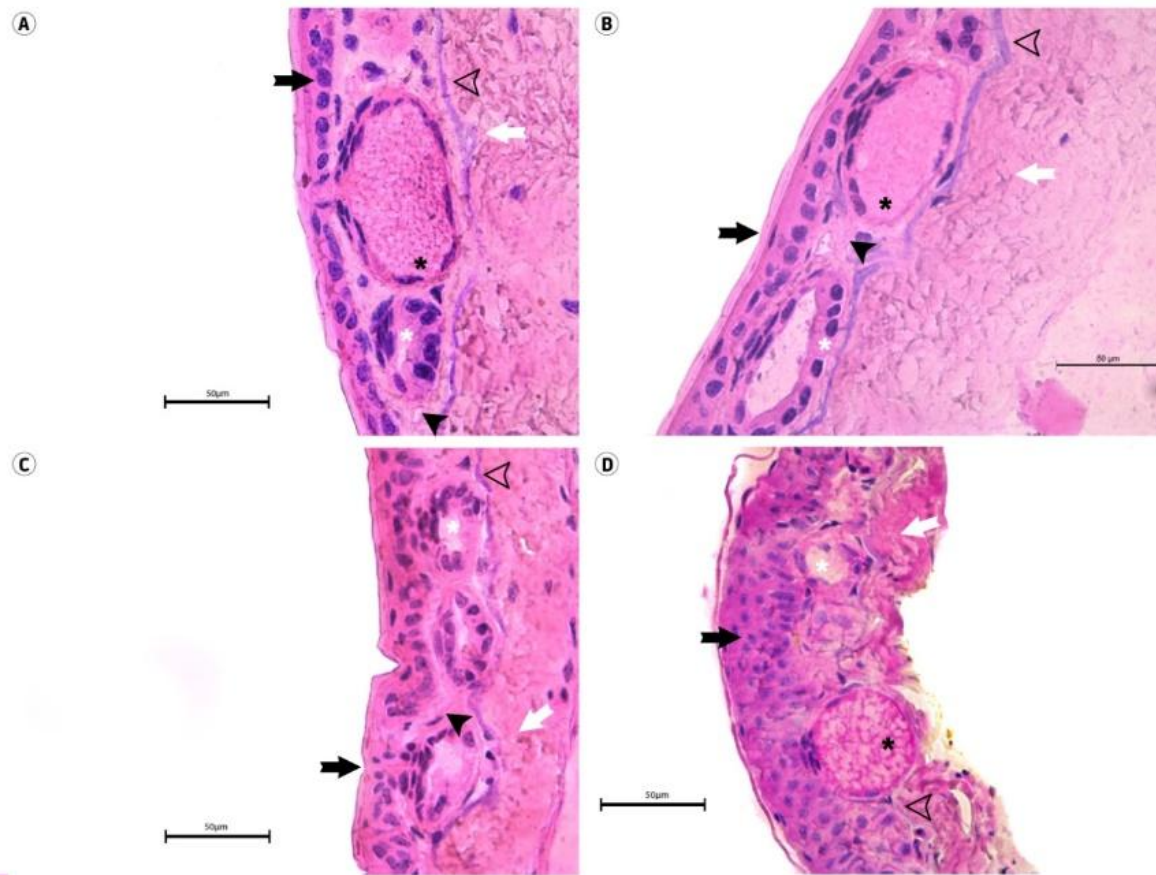


Fig. 13 Histological section of abdominal skin of the *Brachycephalus rotenbergae* (A), *Brachycephalus pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D): *stratum germinativum* with 1 to 2 cells thick (black arrow), in the *stratum spongiosum*, presence of mucous (white asterisk) and granular glands (black asterisk), below is the basement membrane (transparent arrowhead), and *stratum compactum* (white arrow).

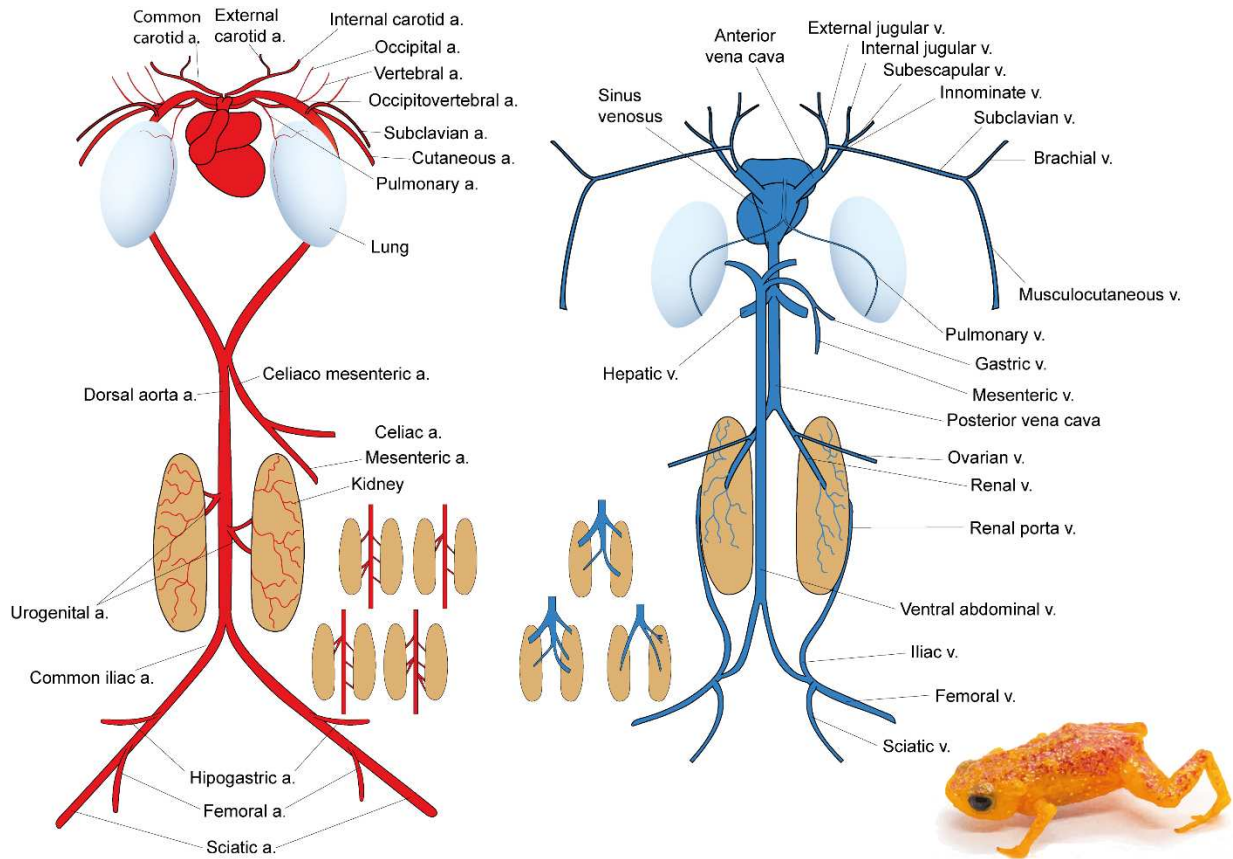


Fig. 14 Circulatory systems of the of the *Brachycephalus pitanga* in this picture we can see the variation in the urogenital arteria and renal veins.

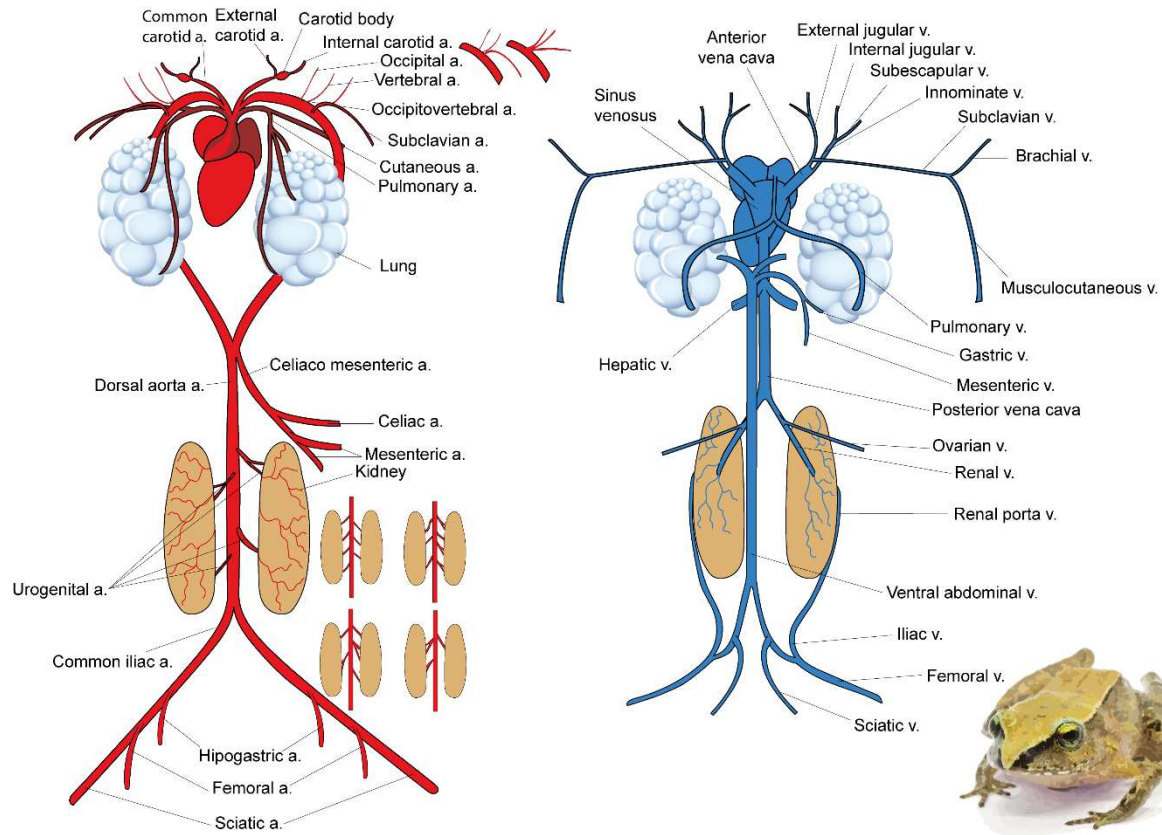


Fig. 15 Circulatory system of the of the *Ischnocnema parva* in this picture we can see the variation in the urogenital arteria and renal veins.

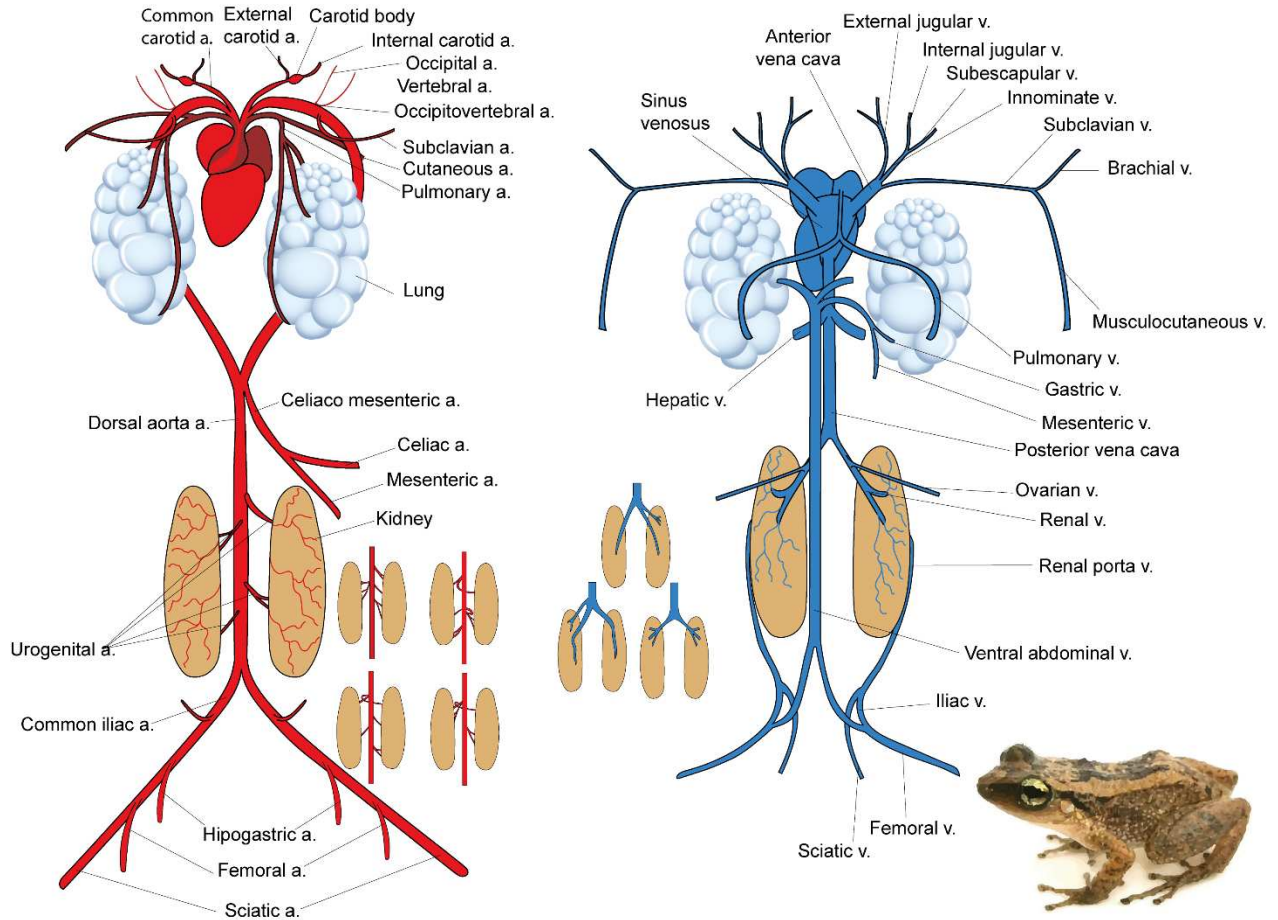


Fig. 16 Circulatory system of the of the *Eleutherodactylus johnstonei* in this picture we can see the variation in the urogenital arteria and renal veins.

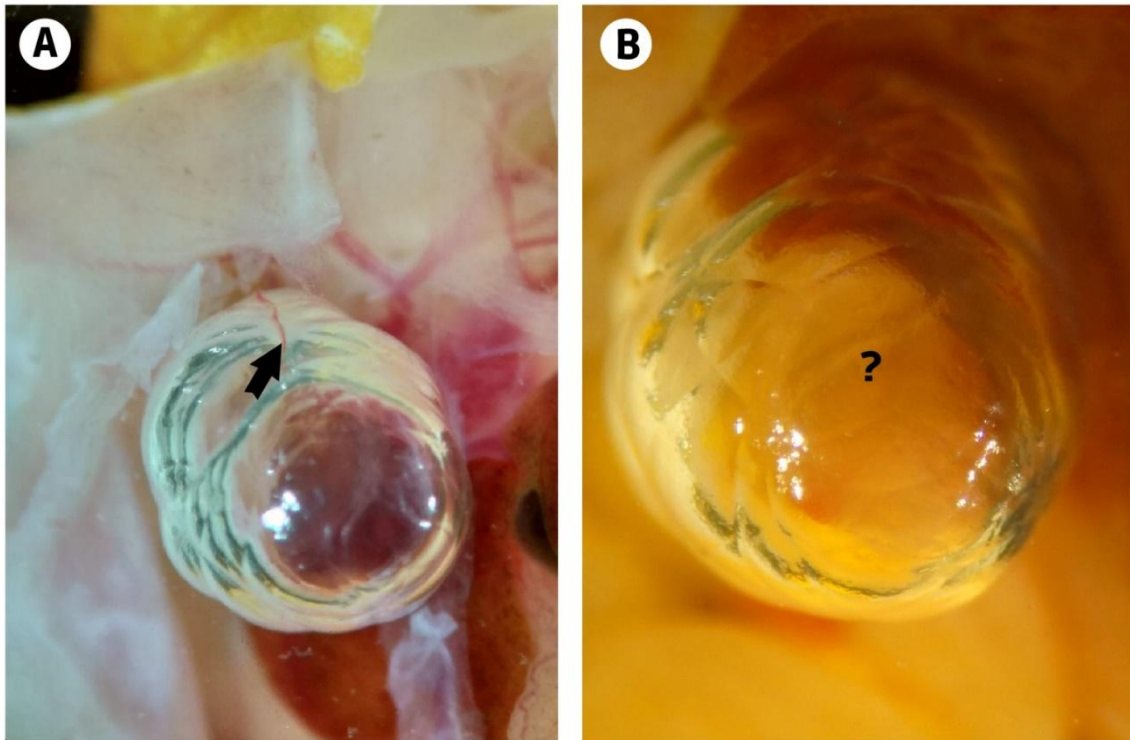


Fig. 17 Lungs of male (A) and female (B) of *B. rotenbergae*: In image A we can see the pulmonary artery supplying the lung in a male divide, however in image B, this artery is indistinct.

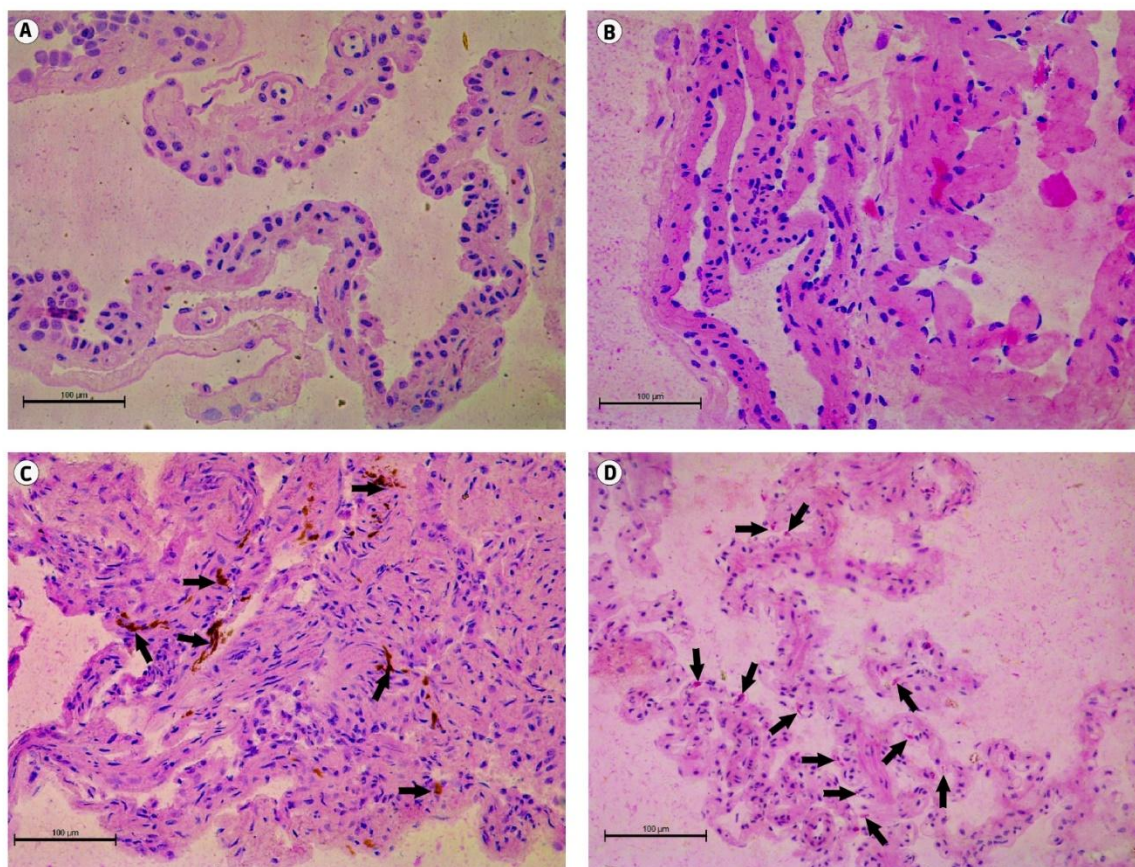


Fig. 18 Histological sections of the lung of *B. rotenbergae* (A), *B. pitanga* (B), *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei* (D). Blood capillaries indicated by arrows were only observed in *Ischnocnema parva* (C) and *Eleutherodactylus johnstonei*.

ANEXO 2: Certificado do comitê de ética

CERTIFICADO CEUA nº 21/2022



CERTIFICADO

Certificamos que a proposta intitulada **SISTEMA CARDIORRESPIRATÓRIO DE BRACHYCEPHALUS ROTENBERGAE**, registrada com o nº **5903-1/2021**, sob a responsabilidade de **Prof. Dr. Luis Felipe de Toledo Ramos Pereira e Andres Santiago Carrasco Medina**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA)**, tendo sido aprovada pela **Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP**, em reunião de **18/11/2021**.

Finalidade:	<input type="checkbox"/> Ensino <input checked="" type="checkbox"/> Pesquisa Científica
Vigência do projeto:	01/10/2021 a 25/07/2023
Vigência da autorização para manipulação animal:	18/11/2021 a 25/07/2023
Espécie / linhagem/ raça:	Anfíbio** / <i>Brachycephalus rotembergae</i>
No. de animais:	20
Idade/Peso:	1.00 Anos / 1.00 Gramas
Sexo:	20 Machos
Espécie / linhagem/ raça:	Anfíbio** / <i>Brachycephalus pitanga</i>
No. de animais:	20
Idade/Peso:	1.00 Anos / 1.00 Gramas
Sexo:	20 Machos
Espécie / linhagem/ raça:	Anfíbio** / <i>Eleutherodactylus jhonstonei</i>
No. de animais:	20
Idade/Peso:	1.00 Anos / 1.00 Gramas
Sexo:	20 Machos
Espécie / linhagem/ raça:	Anfíbio** / <i>Ischnocnema parva</i>
No. de animais:	20
Idade/Peso:	1.00 Anos / 1.00 Gramas
Sexo:	20 Machos
Origem:	Ambiente Natural
Biotério onde serão mantidos os animais:	LaDiVert, DBA/IB/UNICAMP

A aprovação pela CEUA/UNICAMP não dispensa autorização e junto ao **IBAMA SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, **03 de fevereiro de 2022**.

Prof. Dr. Wagner José Favaro
Presidente

Rosângela dos Santos
Secretária Executiva

NOTAS: Pedir a alteração no prazo para envio de relatório final de atividades referente a este protocolo até 30 dias após o encerramento de sua vigência. O formulário deverá ser via digitalizado no sistema da CEUA/UNICAMP, área de pesquisadores responsáveis. A não apresentação do relatório no prazo estabelecido levará a que o novo protocolo seja submetido.

Documento assinado. Verificar autenticidade em sigad.unicamp.br/verifica
Informar código 41EA212A 99C04C4A A0D1F46A F0A2AFF4

Documento assinado eletronicamente por **WAGNER JOSE FAVARO, PRESIDENTE DA CEUA/UNICAMP**, em 03/02/2022, às 19:28 horas, conforme Art. 10 § 2º da MP 2.200/2001 e Art. 1º da Resolução GR 54/2017.

Documento assinado eletronicamente por **ROSANGELA DOS SANTOS, SECRETÁRIA EXECUTIVA DA CEUA/UNICAMP**, em 03/02/2022, às 16:56 horas, conforme Art. 10 § 2º da MP 2.200/2001 e Art. 1º da Resolução GR 54/2017.



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ANEXO 3: Certificado do comitê de ética



UNIVERSIDADE ESTADUAL DE CAMPINAS

Comissão de Ética no Uso de Animais



CEUA/UNICAMP

Certificamos que **ANDRES SANTIAGO CARRASCO MEDINA** concluiu o curso online **Legislação e procedimentos para utilização de animais de laboratório**, oferecido pelo Instituto de Biologia da UNICAMP e pela Comissão de Ética no Uso de Animais de Laboratório - CEUA/UNICAMP.

Este certificado tem validade de 02 (dois) anos a partir da data de emissão.

Campinas, 25 junho 2021.

A handwritten signature in blue ink, reading "Wagner José Fávaro".

Prof. Dr. Wagner José Fávaro
Professor Assistente Doutor
Presidente da CEUA/UNICAMP