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Article

Minimizing the damage: a methodological proposal to remove the brains of anurans and squamates

Andressa M. Bezerra[1], Nathalie Citeli[2], Ana Galvão[3] & Sergio P. de Carvalho-e-Silva[1]

1. Laboratório de Anfíbios e Répteis, Departamento de Zoologia, Universidade Federal do Rio de Janeiro, RJ, Brazil. (andressademellobezerra@gmail.com)
2. Laboratório de Fauna e Unidades de Conservação, Departamento de Engenharia Florestal, Universidade de Brasília, Brasília, DF, Brazil.
3. Laboratórios de Ornitologia e de Bioacústica, Departamento de Zoologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

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ABSTRACT. The brain is one of the most important organs of vertebrates. Over the years, several studies have investigated brain features under different approaches, such as comparative morphology. Although many recent studies use non-invasive methods, such as micro-CT scan, some methods require access to the brain, such as histological analyses and cell count methods. In addition, several researchers do not have access to those expensive devices and rely on the traditional dissection to conduct their studies. Still, for most vertebrates, very few protocols are available for removing the brain, especially those committed to minimizing the damage to the specimen for further examinations. Here we describe in detail a method to dissect the brains of anurans and squamates maintaining the specimen's external morphology as undamaged as possible. This simple method can be performed using few tools and can be achieved in the first trials, representing an incentive for more research on vertebrate's brains. This method contributes to the maximum utilization of each animal collected, a positive practice from both ethical and practical perspectives.

KEYWORDS. Comparative anatomy, herpetology, histology, neuroanatomy, soft anatomy

The brain has a vital role in understanding and shaping the perception of events in living organisms (Legendre *et al*., 1994; Ito *et al*., 2007; Gonda *et al*., 2013). For this reason, it has always been the subject of interest in different fields in biology, such as evolution, developmental biology, ecology, and behaviour (*e.g*., Martin, 1981; Kiecker & Lumsden, 2005; Sugahara *et al*., 2017).

Vertebrates have complex brains, which influence ecological and behavioural features of their lives (Amiel *et al*., 2011; Powell *et al*., 2017). Within vertebrates, amphibians exhibit a high diversity (8458 living species; Frost, 2022) and several specializations that allowed them to pass from an aquatic to a terrestrial environment (Manzano *et al*., 2017; Frost, 2022), such as tetrapod-limb movement (Manzano *et al*., 2017). Additionally, anurans have high diversity of life histories and reproductive strategies, making it an interesting group to study the brain under a comparative approach (*e.g*., Taylor *et al*., 1995; Amiel *et al*., 2011; Liao *et al*., 2015).

Squamates also have high diversity in the number of species (ca. 11,000 species; Uetz *et al*., 2021) and lifestyles, including a wide range of behaviours, niche occupation, and locomotion modes (Pianka & Vitt, 2003; De Meester *et al*., 2019). Therefore, the study of squamate brain morphology can shed light on their ecological and behavioral adaptations (*e.g*., De Meester *et al*., 2019; Macrí *et al*., 2019) and contributes to a better understanding of the evolution of the brain over the evolutionary history of amniotes (Naumann *et al*., 2015).

Studies regarding anuran and squamates nervous system increased in the last years, and we can easily find the undergoing process of brain dissection illustrated in some of their pictures. However, most of these works do not provide a detailed description of the process, especially how to maintain the specimen in good shape for further examinations (*e.g*., Mancera *et al*., 1991; Crews *et al*., 1993; Northcutt, 2013; Mai *et al*., 2016; Zeng *et al*., 2016). This is also true in general reference books to study vertebrate comparative neuroanatomy (*e.g*., ten Donkelaar & Nicholson, 1998; ten Donkelaar 1998a,b). This lack of protocol availability might increase the anatomical damage inflicted by researchers while learning dissection through a "trial and error process", delaying their research and using more individuals to dissect than needed.

Another relevant issue is the infliction of widespread destruction to the specimen during brain removal, which precludes further studies such as the elucidation of taxonomic questions after the dissection. Thus, a method committed to minimizing the damage to the specimen would contribute to the maximum utilization of each animal collected, an extremely positive practice both from an ethical and practical perspective, optimizing the material available in zoological collections (Winker, 2000). Here we describe a method to dissect anuran and squamate brains, focusing on maintaining the specimens as preserved as possible for taxonomic purposes and/or any further studies.

MATERIAL AND METHODS

We dissected 25 specimens from two Brazilian zoological collections (Appendix I), *Coleção de Anfíbios do Laboratório de Anfíbios e Répteis da Universidade Federal do Rio de Janeiro* (ZUFRJ) and *Coleção Didática do Laboratório de Anatomia Comparada de Vertebrados da Universidade de Brasília* (UnB). We identified the snakes following Peters & Orejas-Miranda (1970), Campbell & Lamar (2004), and Passos & Fernandes (2008), whereas Harvey & Gutberlet (1998) and Harvey *et al*. (2012) were used to identifying the lizards. The amphibians were identified following the recent literature on each group, according to Frost (2022). We measured the snout-vent length (SVL) of all specimens of anurans and lizards with a digital caliper to the nearest 0.01 mm, while snakes were measured with a flexible ruler to the nearest 1.0 mm.

We dissected 18 anurans representatives of the families Bufonidae, Craugastoridae, Cycloramphidae, Hemiphractidae, Hylidae, Hylodidae, Leptodactylidae, Microhylidae, and Odontophrynidae (Appendix I). The anurans' snout-vent length (SVL) varied from 13.5 mm to 67.2 mm. We also dissected four species of snakes: *Bothrops moojeni* Hoge, 1966 (Viperidae), *Epicrates crassus* Cope, 1862 (Boidae), *Philodryas patagoniensis* (Girard, 1858), and *Philodryas olfersii* (Lichtenstein, 1823) (Dipsadidae), which varied from 500 mm to 1,070 mm SVL; and two lizards, one *Ameiva ameiva* (Linnaeus, 1758) (Teiidae), SVL = 129.88 mm, and one *Tropidrurus* **aff**. *torquatus* (Wied-Neuwied, 1820) (Tropiduridae), SVL = 103.5 mm (Appendix I).

All specimens were ultimately preserved in ethanol 70%. However, since we could not access the steps before being soaked in 70% ethanol, all individuals were fully immersed in 10% formaldehyde solution for 24 hours before the dissection, ensuring that the brain would be appropriately fixed, i.e., harder to damage and consistent enough to manipulate. For applying the proposed method, the following items and solutions are required: 10% formaldehyde, 4% formaldehyde, scalpel, microsurgery scissors, and a small clamp.

The anatomical nomenclature of brain parts follows Butler & Hodos (2005) for anurans and Platel (1976) for squamates. The anatomical terminology of bone structures follows Trueb *et al.* (1993) for anurans and Rieppel & Zaher (2001) and Evans (2008) for squamates.

RESULTS

Dissection protocol for anurans

1) Immerge the individual in 10% formaldehyde solution for 24 hours before the dissection.

2) After 24 hours immersed in the solution, put the frog in dorsal view, and cut its skin using a scalpel in cross orientation. The sagittal cut of the cross should start at the level of the arms toward the tip of the nostrils. The transversal cut must be done from the posterior border of one tympanum toward the other tympanum (Figs 1, 2). If the specimen has no external tympanum, the transversal cut must be done in the medial region between the eyes and the arms.

3) Carefully lift the two anterior portions, i.e., those closest to the nostrils, almost completely detaching the skin from the muscles but leaving it attached to the region close to the maxilla (Fig. 3). With the same purpose, slightly separate the skin from the muscles of the posterior portion of the sagittal section. The released skin can be folded laterally between the muscles and the skin itself (Fig. 3). In most species, when this step is completed, it is possible to see the brain through the bones of the top of the head (Fig. 3).

4) Using microsurgery scissors, make an incision in the posterior portion of the medulla oblongata, piercing the bones (Fig. 4). If one cannot precisely determine its position, make the incision in the posterior region of the head, at the most posterior level of arms insertion.

5) Cut the bones surrounding the brain from the incision made at the previous step following the sagittal/medial plane (Fig. 4). Since the anterior portion of the brain, i.e., the olfactory bulb, is more difficult to visualize through the bones, continue to cut until the tip of the nostrils. If one cannot precisely determine the brain contours, cut the bones from the incision made in the previous step in a straight line toward the nostrils (Fig. 5). In both cases, the scissors must be constantly pointed up, i.e., to the outside, to prevent damage to the brain. After that, gently remove the loose bones, exposing the brain (Fig. 6).

6) Cut the spinal cord at the posterior portion of the medulla oblongata (Fig. 6), separating the brain from the rest of the medulla. After that, finish the release by gently passing a tweezer between the brain and the bones of the bottom of the braincase, cutting the brain nerves attached to it. This step requires special attention when releasing the olfactory bulb due to its fragility. Finally, smoothly remove the brain with the tweezer.

7) When the dissection is completed, return the skin to its original position, covering the empty braincase.

Dissection protocol for Squamata (Lizards and Snakes)

1) Immerge the individual in 10% formaldehyde solution for 24 hours before the dissection.

2) After 24 hours immersed in the solution, put the specimen in dorsal view and cut its skin using a scalpel in cross-orientation. Start by making a sagittal section from the upper edge of the rostral scale toward the occipital scales (Fig. 7). The cut should extend to the back of the head, at the level of the quadrate pivot point. The transversal cut must be done at the level of the posterior edge of the orbits, extending throughout the dorsum of the head (Fig. 8).

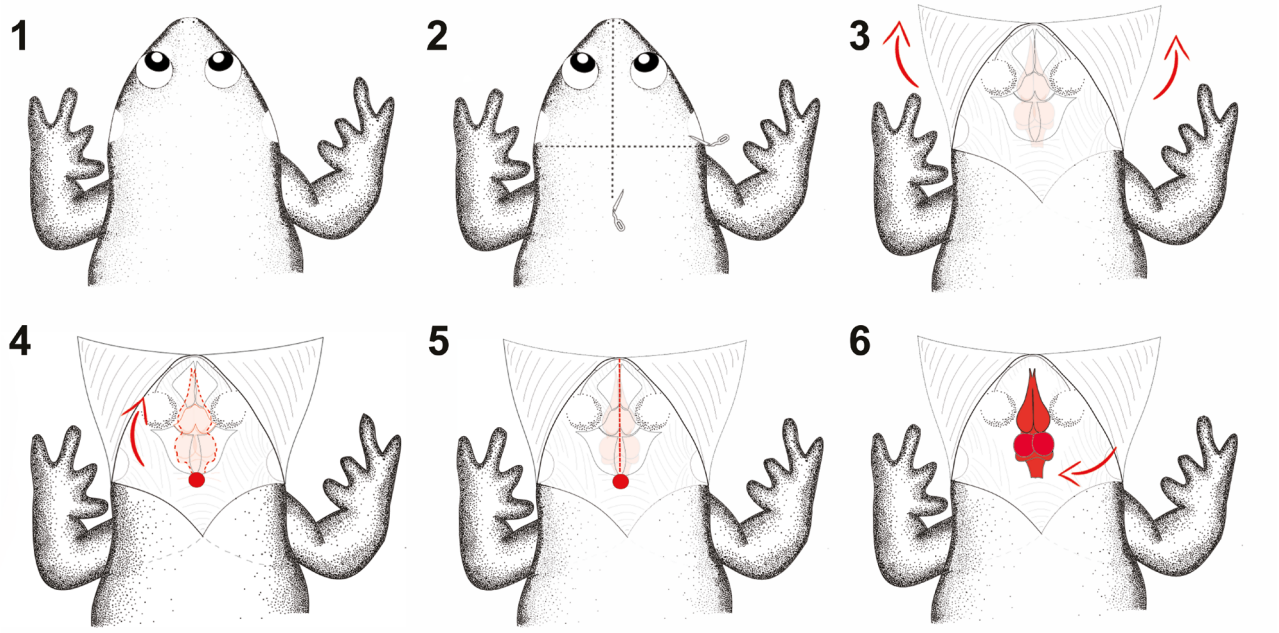
3) With the aid of a surgical spatula, carefully release the skin from the musculature, avoiding damaging the scales. Then, loosen all visible muscles with the assistance of a tweezer, exposing the bones (Fig. 9). One can now visualize the nasal, prefrontal, frontal, parietal, and supratemporal bones. In specimens with no very dense ossification, one can notice the brain through the bones (*e.g.*, snake genera *Bothrops* and *Philodryas*).

4) Using the microsurgery scissors, make an incision starting on the superior part of the orbit, at the junction of the frontal bone with the parietal (Fig. 9).

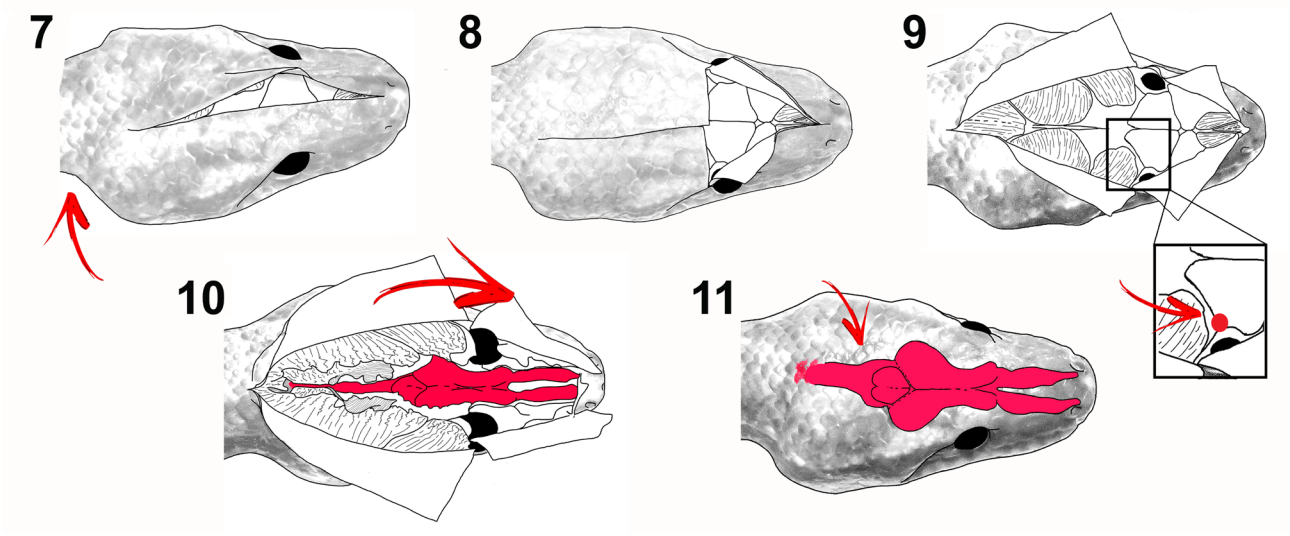
5) With scissors facing upwards, cut the bones mentioned in the second step by the previously done incision, following the sagittal/medial plane (Fig. 10). In species with dense ossification (e.g., Boidae), pliers can be used, except to cut the nasal bone, which should be done using scissors because of its proximity to the fragile olfactory bulbs. When the brain is finally exposed, remove the loose bone portions.

6) Cut the spinal cord at the posterior portion of the medulla oblongata, separating the brain from the rest of the medulla (Fig. 10). Then, finish the release by gently passing a tweezer between the brain and the bones of the bottom of the braincase, cutting the brain nerves attached to it. This step also requires special attention when releasing the olfactory bulb due to its fragility. Finally, smoothly remove the brain (Fig. 11) with the tweezer.

7) When the dissection is completed, return the skin to its original position, covering the empty braincase.



Figs 1-6, Step-by-step dissection of an anuran brain: 1, specimen condition before the dissection; 2, cross-section cuts; 3, lifting the skin; 4, bones cut off surrounding the brain; 5, bones cut off straightly toward the nostrils; 6, exposed brain. The red dots represent the points of insertion of the scissors. The arrows in figures 3 and 4 represent the direction of the movement. The arrow in figure 6 represents the medulla oblongata.



Figs 7-11, Step-by-step dissection of a snake brain: 7, sagittal cut from the upper edge of the rostral scale toward the occipital scales; 8, transversal cut from the posterior edge of the supraocular scale toward the other supraocular scale; 9, lifting the skin. The junction of the frontal bone with the parietal is highlighted, and the red dot represents the point of the insertion of the scissors; 10, bones cut off straightly toward following the sagittal/medial plane; 11, exposed brain. The arrow in figure 7 is pointing to the square bone. The arrow in figure 10 represents the direction of the movement. The arrow in figure 11 represents the medulla oblongata.

DISCUSSION

Special cases. Many anuran species have co-ossified bones (*sensu* Trueb, 1973), with the skin fused to the bones of the head, such as *Brachycephalus ephippium* (Spix, 1824), *Itapotihyla langsdorffii* (Duméril & Bibron, 1841), and *Nyctimantis brunoi* (Miranda-Ribeiro, 1920). For these species, this method has a limitation caused by the impossibility of detaching the skin from the bones, preventing the visualization of the brain as described in the third and fourth steps. The impossibility of seeing the brain through the bones muddles the precise incision of the scissors posteriorly to the medulla oblongata. Therefore, dissecting these species' brains requires a more invasive approach, starting the cutting directly from the back of their head toward the nostrils. Besides, since they have thicker bones, dissection demands more robust scissors, like those used to cut nails or pliers. In these specimens, it is also not possible to keep the skin covering the empty braincase at the end of the process, leaving a small hole at the top of their head.

For squamates, the disposition of the head scales can be used for identification at the level of family and genus (*e.g.*, Campbell & Lamar, 2004; Giri *et al*., 2019; Peter & Orejas-Miranda, 1970), while the head shape may provide morphometric data to detect sexual dimorphism, ontogenetic and static allometry (*e.g.,* Abegg *et al*. 2020; Murta-Fonseca & Fernandes 2016). Although our method maintains the external morphology well preserved, we suggest researchers evaluate each case before the dissection. Some lizards also have their head skin close or fused to the bones, such as *Ameiva ameiva* (Linnaeus, 1758), or scales underlain by bony plates (osteoderms), such as the families Anguidae, Diploglossidae, Scincidae, and others (Williams *et al*., 2022). In those cases, the squamate protocol shows the same limitations and solutions as those aforementioned for anurans with co-ossified skulls.

Conclusions. We describe a dissection protocol of brain extraction in anurans and squamates for the first time, preserving the specimen as undamaged as possible. This protocol has been successfully performed for comparative studies and can be conducted using simple tools, usually available in zoological museums and scientific collections. Our method is a suitable alternative for institutions that do not have access to image processing equipment, such as computed tomography (CT and micro-CT scan), and to approaches that depend on direct access to the organ and cannot be replaced by digital endocasts, such as histological information (*e.g.*, Palci *et al*., 2019), cell counting methods (Herculano-Houzel & Lent, 2007), and other researches involving specific internal morphological features of the brain (de Schotten *et al*., 2019).

By following our method, the brain can be removed in good shape, and the overall condition of the specimen will still be useful for most studies based on external morphology. This new protocol allows reconciling invasive studies with the maximum preservation of the specimen, maximizing the usefulness of each animal collected. Such a perspective can make material already deposited in the herpetological collections more accessible, significantly increasing the diversity of species available for future studies of the brain of amphibians and reptiles.

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Appendix I. Specimens examined

Anurans. *Aplastodiscus leucopygius* (ZUFRJ 2106); *Crossodacthylodes izecksohni* (ZUFRJ 14331); *Chiasmocleis carvalhoi* (ZUFRJ 4435); *Dendropsophus anceps* (ZUFRJ 14269); *Dendropsophus elegans* (ZUFRJ 3809); *Fritziana goeldii* (ZUFRJ 16797); *Haddadus binotatus* (ZUFRJ 1081); *Hylodes nasus* (ZUFRJ 6252); *Itapothyla langsdorfii* (ZUFRJ 6801); *Leptodactylus fuscus* (ZUFRJ 11945); *Proceratophrys boiei* (ZUFRJ 14106); *Phyllodytes luteolus* (ZUFRJ 4656); *Phyllomedusa burmeisteri* (ZUFRJ 13564); *Physalaemus signifer* (ZUFRJ 997); *Rhinella pygmea* (ZUFRJ 2150); *Stereocyclops parkeri* (ZUFRJ 13277); *Thoropa miliaris* (ZUFRJ 181).

Squamates. *Bothrops moojeni* (LACV 3834); *Bothrops moojeni* (LACV 3835); *Epicrates crassus* (LACV 3836); *Philodryas olfersii* (LACV 3837).