

# THE HOMOLOGY OF SARCOPTERYGIAN GILLS

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## ABSTRACT

### THE HOMOLOGY OF SARCOPTERYGIAN GILLS

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Vertebrate gills may be either external (protruding from the body surface) or internal (enclosed in a chamber). Among living amphibians, external gills are found in salamander larvae and neotenes, early frog larvae, and caecilian embryos; internal gills are found only in later-stage frog larvae. Evidence for internal gills has also been found in stem tetrapods, and amphibian-like external gills have been found in some fossil temnospondyls and anthracosaurs. Gill homology among these groups and life stages has long been questioned. To address this, scanning electron microscopy, vascular casting, and paraffin sectioning were utilized to study the morphology of gills and associated vessels of four sarcopterygian species: the basal frog *Ascaphus truei*, the salamander *Dicamptodon tenebrosus*, and the lungfishes *Lepidosiren paradoxa* and *Protopterus* sp. In all studied species, blood flows from the heart through four pairs of afferent branchial arteries, through the gill lamellae (when present), and drains through efferent branchial arteries into the dorsal aorta. In *D. tenebrosus* and *A. truei* no gill lamellae are found on the fourth branchial arch; instead, the afferent branchial artery supplies blood to the lung. In the external gill of *D. tenebrosus* the afferent arteries travel posterolaterally within an elongation of the interbranchial septum and protrude dorsolaterally from the body, supplying blood to the paired, digit-like lamellae via a single vascular loop per lamella.



*Ascaphus truei*, unlike most anuran larvae, never develops external gills, but only internal gills. These extend directly from the ventral side of the branchial arches. Each unpaired lamella has multiple club-like branches, each housing a vascular loop. *Protopterus* sp. possesses internal gills on the hyoid arch and branchial arches III-V, with the hyoid and branchial arch V developing unpaired primary lamellae. The lamellae of all arches possess secondary lamellae. No gill lamellae were found in the studied larval *Lepidosiren paradoxa*. The external gills of *Dicamptodon tenebrosus* show some remarkable similarities to the internal gills of basal sarcopterygians, possessing paired primary lamellae (though they never develop any secondary lamellae), and provide further evidence that the external gills of amphibians are homologous to the internal gills of fishes. The evolutionary significance of the internal gills of frogs is less clear, but the morphology of the basal *Ascaphus truei* provides evidence suggesting that the internal gills of frogs are an independently evolved character, rather than a retained ancestral feature. These findings shed light on the morphology and evolution of gills within sarcopterygians.

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## INTRODUCTION

Gills are phylogenetically ancient organs that evolved early in the vertebrate tree (Gillis and Tidswell, 2017; Witschi, 1956). They develop in most primitively aquatic vertebrates and may be either external (protruding from the body surface) or internal (enclosed in a chamber). Some basal osteichthyans develop external gills (such as lepidosirenid lungfishes and the basal actinopterygians *Polypterus* spp.) and the lamellae of some cartilaginous fish and sturgeons sometimes protrude externally early in development (Laurent et al., 1978; Bartsch et al., 1997; Ballard et al., 1993; Park et al., 2013). External gills are also found in larvae of most extant groups of amphibians and internal gills are found in the larvae of frogs. The homology of the external and internal gills of amphibians to the internal gills of other primitive vertebrates has long been questioned (Noble, 1931; Schoch and Witzmann, 2010; Schmaulhausen, 1968). While they have the same general role throughout (respiration with some ion exchange functions), their morphology has some clear differences.

The purpose of this study was to provide an understanding of the homology of gills within vertebrates, specifically focused on the gills of sarcopterygian fishes and the question of their homology to the external and internal gills of amphibians. To answer this question, this project looked at the gill morphology of a basal frog, *Ascaphus truei*, a generalized salamander that has been previously used as a model for fossil amphibians, *Dicamptodon tenebrosus*, and lepidosirenid lungfishes as sarcopterygian outgroups. This study examined gill morphology using histological sectioning, scanning electron

microscopy, and vascular casting to examine the fine details of the vascular anatomy and was the first to use casting methods to examine the circulation of external gills in a salamander.

### General Gill Morphology

In gilled vertebrates, the gills develop on arch-like structures in the pharyngeal region of the animal called the branchial arches (Fig. 1 and 42; Mallatt, 1984; Saint-Aubain, 1985; Witschi, 1956). The branchial arches are supplied blood from the heart via an afferent branchial artery, which supply the gill lamellae of each arch, and are drained via an efferent branchial artery. Connective tissue partitions called gill interbranchial septa arise from the branchial arches, surround the supportive gill arch elements and vasculature, and separate slits between each arch that expose the pharynx to the external environment (Fig. 42). This allows water from the pharyngeal region of the animal to travel through the pharyngeal slits and into the external environment (Laurent et al., 1976; Wilson and Laurent, 2002).

### Elasmobranchii

In elasmobranchs, the well-developed interbranchial septa are supported by a series of cartilaginous rays, which allow the septa to protrude from the sides of the arches and fold posteriorly, covering the gills (Figs. 2, 3, and 42; Cooke, 1980). From the anterior and posterior sides of the septa develop long, thin gill lamellae. Additionally, along the length of each lamella paired rows of secondary lamellae develop – very thin, vascularized structures where most respiratory gas exchange takes place (Olson, 2002). The secondary lamellae are composed of two layers of flattened pavement cells that are

punctuated by pillar cells that span the two tissue layers and effectively create blood channels within the lamellae (Fig. 42; Bettex-Galland and Hughes, 1973; Cooke 1980).

Each branchial arch is supplied blood via one afferent branchial artery. Blood is collected from the lamellae by independent anterior and posterior efferent branchial arteries (one for each hemibranch) that drain into a single efferent branchial artery before leaving the arch (Hoar and Randall, 1984). Primary lamellae are supplied blood via an afferent branchial artery traveling along the gill arch. The afferent branchial artery gives off a series of afferent lamellar arteries that run through the length of the primary lamella and adjacent to each secondary lamella. There is no point in which the afferent lamellar artery and the efferent lamellar artery make contact – blood must travel through the secondary lamellae to get from the afferent lamellar artery to the efferent lamellar artery.

#### Actinopterygii

The most notable difference between the gills of actinopterygian fishes and those of Elasmobranchii is the extreme reduction of the interbranchial septa (Figs. 1, 3, and 42; Wilson and Laurent, 2002). They are highly reduced from the middle of each holobranch, allowing each primary lamella to hang somewhat independently from the branchial arches. Additionally, instead of the interbranchial septa being supported by a series of rays (as in Elasmobranchii), each lamella is supported by a ray (Fig. 42).

#### Sarcopterygii

The gills of the basal sarcopterygians, *Neoceratodus* and coelacanth, are superficially very similar to those of Elasmobranchii. The septum is very well developed and extends just short of the distal tips of the primary lamellae (Figs. 2 & 3; Hughes,

1972; Gannon et al., 1983). In the more derived lungfishes, *Protopterus* and *Lepidosiren*, the interbranchial septa are reduced and the lamellae (like Actinopterygii) hang somewhat independently from the branchial arch (Figs. 2 & 3; Laurent et al. 1978). *Protopterus* and *Lepidosiren* possess a hemibranch on the hyoid arch and holobranchs on all four branchial arches (though in some species the lamellae are sparse or absent on arches I and II) (Laurent, et al., 1978; Robertson, 1913). Additionally, *Protopterus aethiopicus* develops a set of external gills on branchial arches II-IV, while *Lepidosiren* develops external gills on all four branchial arches (Kerr, 1899; Robertson 1913) (Fig. 42). The gills of *Lepidosiren* are generally described as being less developed compared to other dipnoans and lack secondary lamellae altogether (de Moraes et al., 2005; Morgan and Wright, 1989).

#### Stem Tetrapods

Although the preservation of soft tissues, such as gills, is uncommon, there is some evidence of gills existing within stem tetrapods. In the primitive fishlike stem tetrapod, *Eusthenopteron*, a preserved internal gill has been described to look similar to that of the basal lungfish *Neoceratodus* (Schoch and Witzmann, 2011). Preserved external gills have also been found in some temnospondyl and anthracosaur larvae, such as *Isodectes* and *Discosauriscus*, though no evidence for gills of any kind have been found in fossil amniotes or lepospondyls (Schoch and Witzmann, 2010).

In many basal extant bony fishes (such as *Polypterus*, *Amia*, *Latimeria*, and *Neoceratodus*), grooves in the ceratobranchials, cartilaginous or bony elements that partly make up the gill arch, house the afferent and efferent branchial arteries of the internal

gills (Schoch and Witzmann, 2011). Grooved ceratobranchials are found in extinct tetrapod relatives such as *Tiktaalik*, *Acanthostega*, and *Ichthyostega*, indicating that they likely had internal gills (Coates and Clack, 1991; Daeschler, 2006; Janis, 1999, Schoch and Witzmann, 2010). Though they exhibit some terrestrial features, it is generally thought that these animals were primarily aquatic (Janis, 1999; Schoch and Witzmann, 2010).

### Extant Amphibians

Among living amphibians, external gills are found in salamander larvae and neotenes, early frog larvae, and intracapsular caecilian embryos; internal gills are found only in later-stage frog larvae (Darnell, 1949; Dünker, 2000; Noble, 1931; Nokhbatolfoghahai and Downie, 2008; Pérez, 2009) (Fig. 42). The external gills of frogs only develop for a short time in a frog's life, beginning to emerge just before hatching and then quickly disappearing as the gills regress and atrophy (Brunelli et al., 2004; Nokhbatolfoghahai and Downie, 2008; Schmalhausen, 1968). As the gills regress, a fold of skin called the operculum grows back from the hyoid arch and begins to cover the base of the gill; it moves caudally until it has completely covered the entirety of the external gill (McDiarmid and Altig, 2000). Eventually the external gills regress and atrophy fully and are replaced by another set of gills on the branchial arches (Lajmanovich et al., 1998; McIndoe and Smith, 1984; Nokhbatolfoghahai and Downie, 2008). These newly developed internal gills are morphologically similar to the external ones, forming vascular loops that connect the afferent and efferent branchial arteries.

This is unlike the condition that is found in salamanders, which retain their

external gills for a longer period of time (until metamorphosis) and never develop a set of frog-like internal gills (Brunelli et al., 2009; Kato and Kurihara, 1989; Saint-Aubain, 1985; Severinghaus, 1930). There are also species of salamanders that can retain their external gills and other larval characteristics into adulthood; a process called neoteny (Parker, 1994). Aside from these differences, the gills of salamanders and frogs remain morphologically similar, developing lamellae from the branchial arches and distribute blood via the afferent branchial artery.

Caecilians, the third major (and most basal) group of living amphibians, also exhibit three pairs of external gills as larvae, which detach from the body soon after hatching (Dünker et al, 2000; Pérez et al., 2009; Meng et al., 2016). They resemble the external gills of frogs and salamanders, though the lamellae appear to be thin and elongate in comparison.

It is not yet conclusive whether the external gills of amphibians are derived from the internal gills of fishes or whether they are an independently evolved trait. Furthermore, the evolutionary origin of the internal gills of frogs is also not clear. Therefore, this study had the goal of answering two main questions: 1) What structures are homologous between the internal gills of fishes and the external gills of amphibians?, and 2) What is the evolutionary origin of anuran internal gills? To help answer these questions, histological sectioning and electron microscopy were utilized to study the gill morphology of four sarcopterygian species.

## Study Species

### *Lepidosiren paradoxa*

*Lepidosiren paradoxa*, the South American lungfish, is the only extant species in its genus. It lives in freshwater lakes and is an obligate air-breather. Research on the gills in this species is relatively sparse but is known to possess underdeveloped lamellae and develop external gills as larvae (Kerr, 1899; Morgan and Wright, 1989). This species was chosen because it is a basal sarcopterygian that develops internal and external gills. It is of particular interest because it is reported to never develop secondary lamellae, which develop in other dipnoans, coelacanth, actinopterygians, and chondrichthyans

### *Protopterus* sp.

The genus *Protopterus* is composed of the African lungfishes — obligate air-breathers that live in freshwater habitats prone to periodical seasonal droughts. They develop external gills as larvae on the first three branchial arches and in some species can retain them into adulthood, as well as a set of internal gills (Longo et al., 2013; Smith, 1931). *Protopterus*, like *Lepidosiren*, was chosen due to being a basal sarcopterygian that develops internal and external gills. The vasculature has previously been described in *Protopterus aethiopicus*, and generally resemble the vasculature of extant amphibians (Laurent, 1978; Saint-Aubain, 1985).

### *Ascaphus truei*

The small Coastal Tailed Frog lives in cold, rocky streams in Northern California and other areas of the Pacific Northwest (Stebbins, 2003). It is a unique frog for a variety of reasons. Along with its sister group, *Leiopelma*, *Ascaphus* is the most basal lineage of



frogs, it lacks external gills as a tadpole and is the only genus to have internal fertilization (Brown, 1988; Gaige, 1920; Pyron and Wiens, 2011; Sokol, 1975). The tadpole has a large sucker-like mouth that allows it to feed while suctioned to the bottom of rocks – an adaptation that allows it to survive in fast flowing streams. *Ascaphus truei* was chosen because of its basal status within Anura in order to clarify the evolutionary origin of internal gills in frogs.

*Dicamptodon tenebrosus*

The Coastal Giant Salamander larvae can be found, as in *Ascaphus truei*, in cold, rocky streams in Northern California (Stebbins, 2003). Schoch and Witzmann (2010) observed that gills of salamanders in the family Dicamptodontidae “superficially resemble internal gills of fishes more than the bushy gills of other lissamphibians” due to the high number of paired secondary lamellae and the size and morphology of the gill septum. *Dicamptodon tenebrosus* was chosen for this study because it is a generalized salamander that develops external gills and has been previously used as a model for fossil amphibians. Additionally, they live in the same habitats (often in the same streams) as *Ascaphus truei* and may offer some insights on how cold, fast flowing streams affect the evolution of gills.

## METHODS

### Collection and Maintenance

Eleven larval *Dicamptodon tenebrosus* ranging from 8-13 cm total length (TL), 18 *Ascaphus truei* ranging from 4.5-6 cm TL, two *Lepidosiren paradoxa* both roughly 8 cm TL, and one adult *Protoperus* sp. were examined using light microscopy and scanning electron microscopy. Live animals were collected from streams in Humboldt County, California (California Department of Fish and Wildlife Collecting Permit # 0024438405-0). Larval *Lepidosiren paradoxa* were obtained through the pet trade and euthanized immediately upon acquisition. The adult *Protoperus* sp. specimen was obtained after it had died of natural causes in the Humboldt State University animal rooms. The amphibian larvae were maintained in the Humboldt State University animal rooms in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee (Protocol # 17/18.B.85-A). As both amphibians are located naturally in cold streams, they were maintained in water at 13 C. *Ascaphus truei* tadpoles were kept in a communal aquatic tank and were provided rocks with algae as a source of food. *Dicamptodon* were kept individually in tanks to avoid unwanted aggressive behaviors. They were fed chopped earthworm every other day.

### Light Microscopy

Animals were anesthetized in a 1:2000 solution of MS222 (3-aminobenzoic acid ethyl ester, Sigma) adjusted to pH 7.0-7.2. Measurements were taken while the animals were sedated. Tissue preparation was carried out as described by Humason (1972).

Animals were fixed in Bouin's solution (75 ml saturated aqueous picric acid, 25 ml concentrated formaldehyde, 5 ml glacial acetic acid) for 24 hours and rinsed in 50% ethanol. Animals were then decalcified in RDO RapidDecalcifier (Du Page Kinetic Laboratories, Inc. Naperville, IL) (100 ml RDO, 21.5ml 95% ethanol, 36.5 ml H<sub>2</sub>O) for 2 hours. Specimens were left in 70% ethanol for at least 1 hour, then dehydrated through a series of increasing ethanol concentrations, cleared in toluene, and embedded in Paraplast®. Transverse and frontal serial sections were cut with a steel knife on a rotary microtome at 10 µ thickness. The ribbons were then mounted on glass slides using Haupt's adhesive followed by 3% formaldehyde, dewaxed, stained using Delafield hematoxylin and counterstained with eosin. Sections were examined using a compound microscope (Nikon Eclipse E400) and selected images were taken using a digital camera (Nikon Coolpix 4500). Image manipulation included adjustment of brightness and contrast and the removal of artifacts in spaces using Adobe Photoshop 22.1.

### Scanning Electron Microscopy

Animals were euthanized as above and the gills exposed before placing in 3% glutaraldehyde fixative in 0.05M cacodylate buffer (pH 7.0). A secondary fix in 1.5% osmium tetroxide in distilled water was applied to reduce shrinking of the specimen. Specimens were passed through an ethanol dehydration series, followed by critical point drying. The specimens were mounted on stubs, sputter coated in gold, and examined using an FEI Quanta 250 SEM (Bozzola and Russell, 1999).

### Vascular Casting

Vascular casting methods followed previous work (Minnich and

Lametschwandtner, 2010). Briefly, animals were first euthanized as above. The ventral portions were dissected to expose the heart. Slits were made in the ventricle or at the base of the truncus arteriosus and a glass cannula was inserted through the ventricle and into the truncus arteriosus. After the cannula was ligatured in place, amphibian Ringer's solution was injected in order to remove the blood from the vessels. A slit in the atria allowed the solution to exit the system. After the Ringer's solution began to leave the atria, the corrosion casting medium Mercor II (manufactured by LADD Research Industries), mixed with the polymerizing catalyst, was injected using an Ismatec JPS 12 peristaltic pump. The specimen was let sit at room temperature to allow for initial polymerization, then placed in water at 60 degrees C for 12 hours. The soft tissues of the fully injected specimen were then dissolved by transferring the specimens into 7.5% KOH at 60 degrees C for 24 hours, resulting in a cast made only of the gills and associated vessels. The casts were placed on stubs, sputter coated in gold, and examined using scanning electron microscopy (SEM), as above.

## RESULTS

### *Dicamptodon tenebrosus*

#### The Gill System

*Dicamptodon tenebrosus* has three pairs of external gills – the fourth branchial arch bears vasculature within the septum but no gill lamellae are present. The gill septa develop internally off of the ventral edge of the ceratobranchials, then extend posteriorly past the gular fold to emerge externally (Figs. 4 and 5). The septa are exceptionally well developed, extending far ventrally and taking up the bulk of the subgular chamber. At their most distal, the septa taper into v-shaped points. The septum of branchial arch I is the broadest, extending very far into the subgular cavity, and it curves medially so that its most distal point is directly underneath the other three interbranchial septa. The septa of arches II-IV are roughly the same breadth but the septum of arch IV curves sharply medially, making it appear narrower. Branchial arch I is the first to attach posteriorly and the only one to merge laterally with the body wall. This is followed by arches II, III, and IV respectively as they attach dorsally to the body, though arch IV is always attached to the body medially, since there is no gill slit behind it. The gular fold covers most of the septa, leaving the lamellae exposed to the external environment. Roughly 20 paired (40 total), finger-like lamellae develop on the posterior and ventral ends of each septum of branchial arches I-III and protrude posteriorly (Fig. 6). The lamellae never are found internally, always extending into the external environment. Gill rakers extend medially off the dorsal-most region of the septa.

At the cellular level, most of the epithelial surface of the septum and lamellae consists of pavement cells. These cells are polygonal with well-defined borders and their surface is densely covered with microridges (Fig. 7). Another common cell type are the ciliary cells, which are distinguished by the presence of tufts of long cilia that protrude from the surface. Less common are the mitochondria-rich cells. These round cells are similar in appearance to the ciliary cells but instead of cilia possess short microvilli that protrude from the surface.

In cross sections, the lamellar arteries run along the medial and lateral margins of the lamellae and capillaries are found spanning the middle (Fig. 8). The lamellar arteries are usually not more than two cell layers from the external environment, often being only a single cell layer away. Capillaries can range from one to several cell layers away from the external environment, depending on their distance from the middle of the lamella. The lamellar arteries and capillaries take up about half of the volume of the lamellae, with connective tissue and epithelial cells taking up the other half.

### Vascular Morphology

#### General Blood Flow

Blood exits the heart via the truncus arteriosus. The four paired ABAs leave the truncus and supply blood to the gills and lungs (ABA IV becomes the pulmonary artery) (Fig. 9). The ABA of arch IV is the smallest in diameter, followed by ABA I, with ABAs II/III being roughly the same size. As noted above, each branchial arch runs posteriorly, laterally, and dorsally from its ventral origin. The ABAs travel posteriorly within the ventral portion of the septum through the gill arch, while the EBAs exit the septum of the

gill arch more posteriorly and dorsally (Fig. 10). The afferent branchial artery splits into a series of smaller afferent lamellar vessels that each supply the paired gill lamellae with blood. Each afferent lamellar artery bifurcates into a medial and lateral branch that supply the paired lateral and medial lamellae with blood (Figs. 11 and 12). Multiple lamellae drain into a common efferent lamellar artery before draining into the dorsal EBA (Figs. 10A and 13). The ABAs and the EBAs don't merge at the distal ends of the gills (Fig. 10B). The EBAs of gill arches II and III merge medially, then merge with the efferent branchial artery of gill arch I.

#### Lamellar Vessels

Blood travels distally through the afferent lamellar arteries, then loops back at the distal end of the lamella through the efferent lamellar arteries (Fig. 8A). Between the ala and ela vessels of the lamella are capillaries that bridge the gap between the two, allowing blood to travel from ala to ela without having to travel all the way through the afferent/efferent loop. The capillaries can extend from within the lamella, from proximal to the lamella (from the arteriole that supplies the lamella), or even from an arteriole that supplies a neighboring lamella. From the efferent lamellar artery, blood travels dorsally through vessels that drain into the efferent branchial artery.

#### Shunts

Shunts that bypass the gill lamellae and directly connect the ABAs with the EBAs exist in gill arches I-III, though the shunt of gill arch II is the most obvious (Fig. 14). These shunts branch off from the ABAs and continue to follow the ceratobranchials dorsally before merging with the EBAs. After giving off the shunt vessel, the ABA

continues posteriorly through the septum. These shunts exist at the anterior base of the gill arches, proximal to any gill lamellae – effectively allowing blood to bypass the respiratory elements entirely.

### *Ascaphus truei*

#### The Gill System

*Ascaphus truei* has four branchial arches, but only the first three ever develop any gills (Fig. 15). These gills are internal and are entirely enclosed inside of the gill chamber (Fig. 16). The arches run laterally and posteriorly so that the medial portions of the arch are more anterior. Roughly 20 gill lamellae develop directly from the septum on the ventral side of each branchial arch (Fig. 17). Each lamella develops a primary stalk that has multiple club-like lobes that branch distally, each club housing one or more vascular loops (Fig. 18). The arrangement of the lamellae on the arch appears haphazard and unpaired (Fig. 42).

At the cellular level, the entire epithelial surface of the gill lamellae consists of pavement cells, characterized by their obvious borders and microridges on the surface of the cells (Fig 19). In cross sections, the lamellar vessels take up the majority of the lamellae (Fig. 20). They are completely surrounded by epithelial tissue and are only one to two cell layers away from the external environment. Connective tissue is present but is sparse, with the vasculature taking up the bulk of the space.

On the dorsal portion of the branchial arches, *Ascaphus truei* develops the gill filters, running across the interbranchial septum in parallel rows (Fig. 21). Most rows span the entire width of the gill arch but other rows are incomplete. Like the gill lamellae,



the epithelial surface of the gill filters is formed primarily of pavement cells.

### Vascular Morphology

#### General Blood Flow

Blood exits the heart via the truncus arteriosus (Fig. 22). There are three paired vessels (afferent branchial arteries I-III) that leave the truncus arteriosus and supply blood to the gills – the paired afferent branchial artery IV never develops gills. ABA III and IV arise from a common trunk. ABA IV continues dorsally and meets the lateral dorsal aorta via the ductus arteriosus very close to where EBA III joins (Fig. 23). Close to the lateral dorsal aorta, another vessel of about the same diameter as ABA IV, the cutaneous artery, also emerges from the afferent branchial artery IV and continues laterally and anteriorly. The pulmonary artery was not found.

Blood from branchial arches I-III travels laterally through the afferent branchial arteries (Fig. 24). Along each of the afferent branchial arteries, roughly 20 smaller afferent lamellar arteries split off from the posterior side of the main ABA to supply each gill lamella with blood (Fig. 25). Many arterioles connect EBA I to the LDA, with many convoluted connections (Fig. 26).

The afferent and efferent branchial arteries run very close and parallel to each other, but almost never make direct contact with each other (Fig. 24B). The ABAs are more posterior than the EBAs and are blind-ending vessels – blood must travel through the lamellae to enter into the EBAs. In only one instance was a shunt vessel found, directly bridging the gap between ABA/EBA III (Fig. 27). Multiple efferent arterioles drain into a common vessel before draining into the efferent branchial artery. The EBAs

travel dorsally and medially and merge with the lateral dorsal aorta (Fig. 22A).

### Lamellar Vessels

The lamellar vessels of *A. truei* reside within the gill lamellae themselves. Each of the multiple club-like branches of the lamellae houses at least one vascular loop. There is no standard number of vascular loops per lamella and they generally appear haphazardly arranged (Fig. 28A). In the stalk of the lamellae are the main afferent and efferent lamellar vessels that directly connect to the afferent and efferent branchial arteries. Distally, the afferent vessels bifurcate, resulting in two opposing vessels which bifurcate again (Fig. 28B). The number of bifurcations can range from three to five or more in each lamella. These multiple bifurcated vessels then loop back at the very distal tip of the lamellae and merge with other vessels, eventually resulting in one efferent lamellar vessel connecting to the efferent branchial artery. Smaller capillary vessels can bridge the gap between the afferent and efferent lamellar vessels (Fig. 28B) but are rare.

### *Protopterus* sp.

In the studied adult *Protopterus* sp., functional internal gill primary lamellae were present on the hyoid arch and branchial arches III-V (with the hyoid arch and branchial arch V possessing hemibranchs) and there were no external gills (Fig. 29). No gill lamellae were found on branchial arches I and II (Fig. 30). Gills on branchial arches III and IV had paired lamellae and the lamellae on all arches developed secondary lamellae (Fig. 31). About 20 lamellae develop along each arch (about 10 on arch I).

The studied *Protopterus* sp. specimen was fixed in formalin after death and this caused some cell shrinkage, making it difficult to determine the cell types that make up

the lamellae, though it appears that the surface epithelia of the lamellae are mostly composed of pavement cells.

*Lepidosiren paradoxa*

Surprisingly, no gill lamellae at all were found in larval *Lepidosiren paradoxa*. Instead, the branchial arches form continuous loops that travel from the ventral aorta (truncus arteriosus) directly to the lateral dorsal aorta (Fig. 32). At this stage there are no obvious distinctions between afferent and efferent branchial arteries (Fig. 33). The interbranchial septa are also not developed in the gill arches (Fig. 34). The ceratobranchials are closely surrounded by epithelial tissue throughout the entirety of the arch as they travel dorsally.

At this stage there are three main vessels that emerge from the ventral aorta (afferent branchial arteries I-III), with the two anterior most vessels emerging from a common stem (Fig. 35). Afferent branchial artery III develops a posteriorly-travelling vessel just before reaching the lateral dorsal aorta and this is likely the pulmonary artery (Fig. 36). Afferent branchial artery III connects to the lateral dorsal aorta very close to the unpaired dorsal aorta. Afferent branchial artery I travels dorsally to meet the lateral dorsal aorta but also continues anteriorly, feeding blood to the head. Aortic arch III also gives rise to a series of convoluted capillary vessels that supply blood medially/dorsally.

On the right side of the organism, afferent branchial artery I gives rise to another vessel that continues posteriorly, contributing blood to a mesh of capillary vessels. This could possibly be the beginning developments of an efferent branchial artery being differentiated from the afferent branchial artery (Fig. 37). On afferent branchial artery II,

an unknown bulbous protuberance emerges – possibly being the beginning development of the lamellae (Fig. 38).

## DISCUSSION

### Interbranchial Septa

The interbranchial septa of basal vertebrates are highly variable structures. They are present in all primitively aquatic gnathostomes, and are likely homologous throughout (Cooke, 1980; Hoar and Randall, 1984). They are well developed in Chondrichthyes and Sarcopterygii, and in all groups (including Actinopterygii) surround the cartilaginous/osseous gill arches and vessels (Fig. 3; Wilson and Laurent, 2002).

As noted above in the introduction, in elasmobranchs the interbranchial septa extend laterally from the gill arch into the external environment and fold to act, functionally, as does the operculum in other fishes (Ballard et al., 1993; Cooke 1980). Well-developed interbranchial septa are retained into Sarcopterygii but are reduced from the elasmobranch condition. In coelacanth and the basal lungfish *Neoceratodus*, the septa extend close to the distal tips of the lamellae. In the more derived lungfishes, *Protopterus* and *Lepidosiren*, the septa are reduced, allowing much of the distal portions of each lamella to be independent from the other (Hoar and Randall, 1984; Hughes, 1972). The most extreme reduction of the interbranchial septa is seen in derived actinopterygians (teleosts), allowing the lamellae to hang independently from the branchial arch (Wilson and Laurent, 2002).

The interbranchial septa of *Dicamptodon tenebrosus* larvae are generally similar to other salamander species, though (unlike most species) the septa are not greatly elongated posteriorly and remain mostly enclosed by the gular fold, allowing only the

lamellae to emerge externally. Still, they are quite well developed - even rivaling the condition of Elasmobranchs (Fig. 39). The septa of all four branchial arches extend very far ventrally and posteriorly, taking up the entirety of the gill chamber and, in most species, extending out through the gular fold and far into the external environment. The paired lamellae emerge from the posterior and ventral sides of the distal-most region of the septum. This is comparable to the condition seen in Elasmobranchii in that the septa are elongated and make contact with the external environment. In *Dicamptodon tenebrosus*, however, the lamellae develop from the most posterior end of the septa, rather than along the entirety of the arch, and are external, rather than internal.

The extreme enlargement of the interbranchial septa along with lamellae developing on the distal-most margin of the septum (effectively removing them from the subgular chamber) dramatically changes the position of the vessels in the gill arch compared to other gilled vertebrates (Figs. 39 & 40). In most gilled vertebrates, both the afferent and efferent branchial arteries travel along the ventral side of the ceratobranchial. Due to the enlargement of the septa in *Dicamptodon tenebrosus*, the efferent branchial artery leaves the gill arch posterior to the ceratobranchial and never travels along its ventral side. The afferent branchial artery enters the septum anteriorly, close to the heart, and ventral to the ceratobranchials. As the afferent branchial artery continues posteriorly, it remains ventral and continues into the distal portion of the septum, but does not travel along the entirety of the ceratobranchial. Instead, the shunt vessel splits from the afferent branchial artery anteriorly and follows along the ceratobranchial ventrally before merging with the efferent branchial artery. This makes cross sections from different regions of the

arch look different (Fig. 40). Anteriorly, the afferent branchial artery is just ventral to the ceratobranchial. In the middle of the arch, the afferent branchial artery has moved ventrally and dorsally to it is the shunt vessel. In the posterior, external portion of the arch the lamellae develop and the ceratobranchial is absent. The efferent branchial artery is in the dorsal part of the arch and the afferent branchial artery is supplying blood to the lamellae ventrally.

In contrast to *Dicamptodon tenebrosus*, the interbranchial septa of *Ascapthus truei* are reduced and tightly surround the ceratobranchials and branchial arteries across the gill arches (Fig. 41). The stalks of the internal gill tufts (lamellae) develop directly from the posterior side of the arch, then travel ventrally to fill the gill chamber. Only the afferent and efferent branchial arteries are housed within the septa - the vasculature of the gill tufts is retained within the tuft itself. This forces the afferent and efferent branchial arteries to run very close to and roughly parallel with one another and the ceratobranchials as they run through the arch.

#### Lamellar Morphology

Unlike the interbranchial septa, the primary lamellae are quite conserved within gnathostomes, with few differences. Their morphology within Elasmobranchii, Actinopterygii, and the basal sarcopterygians *Neoceratodus* and coelacanths are essentially the same: the lamellae are thin outgrowths of tissue that develop in paired rows on both sides of the interbranchial septa (Cooke, 1980; Gannon et al., 1983; Olson, 2002; Wilson and Laurent, 2002). Each primary lamella develops paired rows of thin secondary lamellae. The secondary lamellae have column-like pillar cells that

functionally create the blood channels within them. In chondrichthyans, the septa are supported by gill rays, but in actinopterygians and coelacanth, each lamella is supported by a gill ray, and no gill rays are found within dipnoans or amphibians (Wilson and Laurent, 2002; Gannon et al., 1983) (Fig. 42). This likely makes the presence of gill rays a primitive condition in Osteichthyes that was secondarily lost in derived sarcopterygians.

Although they are related species, the lamellae of *Protopterus aethiopicus* are somewhat different to those of *Neoceratodus* (Laurent et al.; 1978). While the secondary lamellae of *Neoceratodus* have a distinctive flattened and rigid structure, the secondary lamellae of *Protopterus aethiopicus* have small blood vessels that are more capillary-like, and they lack pillar cells entirely (Fig. 42). Additionally, in *Protopterus aethiopicus*, the afferent and efferent lamellar arteries meet at the distal tip of the primary lamellae, whereas they remain independent in *Neoceratodus*.

In *Dicamptodon tenebrosus*, the lamellae contain a single vascular loop with capillaries spanning the center. They resemble the lamellae of dipnoans, especially *Protopterus aethiopicus*, in that they are long, tapered, paired, finger-like structures that attach to the septa by their bases only (Laurent et al. 1978). The vasculature of the gills is also similar, with each lamella housing a vascular loop that connects at the distal tip of the lamella (Laurent et al, 1978). The biggest difference between the primary lamellae of *Protopterus aethiopicus* and the lamellae of *Dicamptodon tenebrosus* is the lack of secondary lamellae on the latter (Fig. 42). The secondary lamellae of *Protopterus aethiopicus* lack the pillar-celled structure of ray-finned fishes and are more loosely arranged, with a mesh of capillaries within each secondary lamella. Unlike in *Protopterus*



*aethiopicus*, the lamellae of *Lepidosiren paradoxa* never develop secondary lamellae (de Moraes et al., 2005; Morgan and Wright, 1989). The lamellar vessels are composed of a vascular loop around the lamellae and an arrangement of capillaries and venules developing from them and spanning the center of the lamellae, resembling *Dicamptodon tenebrosus* even further.

In *Ascaphus truei*, each lamella is a multi-lobed structure with a vascular loop fitting inside of each club-like lobe. They differ from *Dicamptodon tenebrosus* in that they are unpaired, multi-lobed, have afferent lamellar vessels that bifurcate distally. The lamellae also differ by having almost the entire surface epithelium made up of pavement cells, while in *Dicamptodon tenebrosus* three cell types are commonly found. The general morphology is similar to that of other frog species (Brunelli et al.; McIndoe and Smith, 1984) in that they exist as stalks with club-like tufts. However, their vascular morphology is slightly different than in other frog species. In more derived species, such as *Litoria ewingii*, there is a primary afferent lamellar vessel that give rise to a series of smaller branches that connect via capillary loops or go through a more complex series of branching before looping into the efferent lamellar vessels (McIndoe and Smith, 1984). In *Ascaphus truei*, instead of a primary afferent vessel giving rise to several branches, the vessel bifurcates distally giving rise to a series of smaller distally bifurcating vessels that loop into the efferent lamellar vessels and to the efferent branchial artery.

The morphological similarities described above provide evidence that the primary lamellae of other gnathostomes and the external gill lamellae of salamanders are homologous. In basal vertebrates, such as *Neoceratodus forsteri* and elasmobranchs, the

lamellar arteries that supply the secondary lamellae remain independent from each other and never directly connect throughout the length of the lamella. The evolution from afferent and efferent lamellar arteries independent from each other to looping at the distal tip could have been achieved by a simple reduction of the distal ends of each lamellar artery and the reduction of the distal-most secondary lamellae. The loss of the pillar cell allows the vasculature of the secondary lamellae to become more loosely arranged. The reduction of secondary lamellar vessels into one or several main distal loop vessels would closely resemble the current condition seen in extant *Protopterus aethiopicus*. Reducing the vessels even further would result in a condition resembling *Dicamptodon tenebrosus*. The reduced condition of the lamellae of lepidosirenid lungfishes and of larval amphibians, however, is likely convergent and probably has more to do with the habitats they reside in, and their ability to utilize lungs and skin in addition to gills for respiration (de Moraes et al., 2005). It is unknown whether the lamellae of anuran tadpoles are homologous to the lamellae of other gilled vertebrates or if they are independently derived. The issue is complex and will be discussed further below.

### Branchial Shunts

Branchial shunts that span directly from afferent to efferent arteries, allowing blood to bypass the gill lamellae, arise in various species within Sarcopterygii – notably in the lepidosirenid lungfishes and larval frogs and salamanders (Laurent et al., 1978; Saint-Aubain, 1981). In the basal lungfish, *Neoceratodus forsteri*, no shunt vessels are found (Gannon et al., 1983). In *Protopterus aethiopicus*, however, shunts that connect the afferent and efferent branchial arteries develop at the base of each primary lamella,

resulting in multiple shunts in each gill arch (Laurent et al., 1978).

The vasculature within the gills of salamanders is generally conserved. Many species, such as *Siren lacertina*, *Ambystoma tigrinum*, and *Necturus maculosus*, develop shunt vessels at the base of branchial arches I-III, directly connecting the afferent and efferent branchial arteries (Darnell, 1949; Malvin and Dail, 1986; Saint-Aubain, 1985). Branchial arch I often has multiple, small diameter shunt vessels that connect the afferent/efferent branchial arteries. This condition is also seen in *Dicamptodon tenebrosus*, though the shunt on arch II is the largest.

Interestingly, the anatomical position of the shunt vessel of salamanders is more similar to the afferent and efferent branchial arteries of basal gnathostomes than are the branchial arteries of salamanders, since it runs closely along the ceratobranchial. It is possible that the shunt vessel of salamanders is a remnant of the double efferent system of basal sarcopterygians, with one efferent vessel moving laterally away from the arch to meet the afferent branchial artery looping through the external gill, with the other efferent remaining close to the arch and merging with the ventral afferent artery. Alternatively, it could also be a remnant of the continuous aortic arches found in the embryos of all vertebrates. Assessing the homology of this is difficult but could be clarified by either exceptional fossil evidence or examination of the development of the shunt vasculature of sarcopterygians (and possibly some basal actinopterygians). Additionally, shunts at the base of the gill, as in *Dicamptodon tenebrosus* and other salamanders, are not found in other described extant amphibians, likely making this condition derived in salamanders.

The shunt vessels of salamanders differ greatly from those of *Protopterus*

*aethiopicus* in that there is generally only one in each arch (branchial arch I which can have several) and they occur at the base of the gill, rather than at the base of each lamella (Laurent et al., 1978). The different locations of the shunt vessels in salamanders and *Protopterus aethiopicus* and the fact that no shunts have been found in the basal lungfish, *Neoceratodus forsteri*, show that they are not homologous between the groups.

Shunts that directly connect the afferent and efferent branchial arteries have been found in some derived species of anuran larvae, including *Rana temporaria*, *Bufo bufo*, and *Litoria ewingii* (McIndoe and Smith, 1984; Saint-Aubain, 1981). In the former two, the gill tuft vasculature emerges directly from the shunt vessel, meaning blood must travel into the shunt vessel to enter into the gill tuft vasculature (McIndoe and Smith, 1984; Saint-Aubain, 1981). In *Litoria ewingii*, blood can enter into the gill tuft vasculature without first entering into the shunt vessel.

In *Ascaphus truei*, the afferent and efferent branchial arteries run in very close proximity to each other, but the gills are almost entirely lacking the presence of any branchial shunts. Only in one individual was a single small shunt vessel found connecting the afferent and efferent branchial arteries at the base of branchial arch III. The varying shunt vascular morphologies in derived species and the lack of shunts in the basal anuran species *Ascaphus truei* suggest that shunts may be independently derived structures in anurans.

#### Surface Epithelial Cells

There are several gill surface epithelial cell types that are commonly found in vertebrates (Dunel-Erb and Laurent, 1980; Hoar and Randall, 1984; Hughes, 1979; Kato

and Kurihara, 1998; Kemp, 1996; Sturla et al., 2001). The most common type is the pavement cell, which is sometimes referred to as the respiratory cell (Fig. 42). This cell usually contains microridges on the surface and is found in the lamellae and covering the entire free surface of the secondary lamellae of fishes. It is also found in the lamellae of all amphibians and is likely homologous throughout.

Another common surface epithelial cell is the mitochondria-rich cell (termed the chloride cell in Chondrichthyes, Actinopterygii, coelacanth, and lungfishes) (Fig. 42). These are named for their high density of mitochondria and are found in the lamellae of these groups. They are thought to have an osmoregulatory function and the morphology differs depending on the salinity level in the habits and differs within phylogenetic groups. In Chondrichthyes and fishlike sarcopterygians, the mitochondria-rich cells possess basement membrane infoldings and in Actinopterygii they possess a complex intracellular tubular system. Mitochondria-rich cells are also found in the lamellae of amphibians, including *Dicamptodon tenebrosus* and *Rana cancrivora* (Uchiyama and Yoshizawa, 1992). These cells in amphibians differ from the mitochondria-rich cells of the other groups, lacking basement membrane infoldings. The mitochondria-rich cells of *Rana cancrivora* have been found to possess a vesiculo-tubular system in the apical portion of the cell, unlike the tubular systems that are found in actinopterygians (Uchiyama and Yoshizawa, 1991). More research on the morphology of the mitochondria-rich cells from sarcopterygians (especially amphibians and lungfish) must be done to understand the homology of these cells.

A third cell type, the ciliary cell, appears to have evolved early within

Sarcopterygii and is found in dipnoans and all three groups of extant amphibians (Fig. 42). The morphology is similar in these groups, with many, long cilia emerging from the top of the cell. They appear early in development, during neurulation in anurans - later in *Neoceratodus forsteri* after some organs have begun to develop and have varying patterns of arrangement and distribution (Kemp, 1996). Ciliary cells, however, are not exclusive to the gills of these species as they can be dispersed on much of the epithelial surfaces of the head and body. No ciliary cells were found in *Ascaphus truei* but they are common in the lamellae of *Dicamptodon tenebrosus*.

#### Habitat and Modes of Life

Habitat and modes of life likely plays a large role in the evolution of gill morphology. Even in closely related genera, gill morphology can be quite different. The gills of *Neoceratodus forsteri* are relatively conservative in morphology and greatly resemble the condition seen in Actinopterygii and Chondrichthyes, including the retention of pillar cells inside of the secondary lamellae (Fig.42). The lack of gill rays within the lamellae of *Neoceratodus forsteri* is the greatest difference from the other groups. This contrasts with the condition seen in *Protopterus aethiopicus*, in which the gills are generally less developed, and the vasculature arranged with vascular loops in the primary lamellae and capillaries in the secondary lamellae. This is even more exacerbated in *Lepidosiren paradoxa*, in which secondary lamellae never develop (de Moraes et al., 2005; Morgan and Wright, 1989). A major difference in these species is their modes of life. *Neoceratodus forsteri* has a set of paired lungs but it also has a well-developed set of gills, lives in areas of constant, lasting water and doesn't need to breathe air to survive.

*Protopterus aethiopicus* and *Lepidosiren paradoxa*, on the other hand, live in oxygen poor bodies of water that are prone to drying up and are obligate air-breathers, meaning the fishes must regularly come to the surface for air to survive. Additionally, some species of *Protopterus* are even capable of aestivating when their habitats dry up, burrowing into the mud and encasing themselves in a mucous cocoon until water has returned. The increased reliance on air-breathing compared to *Neoceratodus forsteri* likely had a large effect on the evolution of gills in these genera.

Similarly, despite being very closely related, the gill morphology of sister groups *Dicamptodon* and *Ambystoma* are different. The interbranchial septa of *Ambystoma* extend far posteriorly into the external environment, while in contrast, the septa of *Dicamptodon tenebrosus* are smaller and barely exit the gular fold (Valentine and Dennis, 1964). The difference is the habitat in which they reside. *Dicamptodon tenebrosus* resides in cold, fast-flowing creeks, while members of *Ambystoma* generally inhabit lakes and ponds. The species within *Rhyacotriton* resemble the condition seen in *Dicamptodon tenebrosus* and live in comparable habitats. This shows that reduction of the interbranchial septa and associated gills may represent a stream-adapted ecomorphotype (Valentine and Dennis, 1964). The higher oxygen content and potentially hazardous conditions of cold, fast flowing streams may have selected for the reduction of the interbranchial septa. It's important to note that *Ascapheus truei* also lives in cold, fast flowing streams – often in the very same place as *Dicamptodon* and are subjected to the same selection pressures.

The primitive condition of the gills in lissamphibians is difficult to establish for several reasons. One major reason is the lack of stem amphibian fossils with preserved gills or indirect evidence for gills. Another reason is the peculiar nature of caecilians. Even though caecilians are phylogenetically basal within Lissamphibia, they possess some incredibly derived characteristics – mostly evolving due to a fossorial lifestyle, including the complete lack of limbs and reduced eyes (Pyron and Wiens, 2011; Dünker, 2000). Caecilians develop intracapsular external gills that reduce or break off early in larval development, but they never develop internal gills. The uncertainty of the primitive lissamphibian condition makes the assessment of amphibian gill homology even more challenging. However, due to previous work and the results of this study, some conclusions can be made. In the following section I summarize the likely evolutionary history and homologies of lissamphibian gills.



## CONCLUSIONS

This study has provided examples of the gill morphology of four sarcopterygians with the goal of answering two main questions: 1) What structures are homologous between the internal gills of fishes and the external gills of amphibians and 2) What is the evolutionary origin of anuran internal gills?

### Homologies of Internal and External Gills

Some structures are undoubtedly homologous throughout gilled vertebrates, such as the cartilaginous ceratobranchials that support the gill arch, the interbranchial septa that hold the lamellae, as well as much of the vascular system, such as the branchial arteries (Fig. 42). The respiratory pavement cells and mitochondria-rich cells are also likely homologous throughout gilled vertebrates and the ciliary cell is likely homologous between dipnoans and amphibians.

The homology between the lamellae of amphibians and the lamellae of fishes is less conclusive, but is supported by some remarkable similarities in salamanders. The lamellae of *Dicamptodon tenebrosus* most closely resembles the lamellae of lepidosirenid lungfishes, in that they both have afferent and efferent arteries that form vascular loops around the periphery, with a network of capillaries spanning the middle. Although the increasingly underdeveloped nature of the lamellae in *Protopterus aethiopicus* and *Lepidosiren paradoxa* respectively is not homologous to those of amphibians, they provide a good example of how the lamellae of fishes could have evolved into amphibian-like lamellae.

The largest difference between the gills of *Protopterus aethiopicus* and those of *Dicamptodon tenebrosus* is that in *Protopterus aethiopicus*, the lamellae develop along the entire arch, as is typical in fishes. In *Dicamptodon tenebrosus* (and other salamanders), the lamellae are positioned only on the distal-most ventral and posterior sides of the arch. This unique positioning of the lamellae is made possible by the ventral and posterior elongation of the interbranchial septa, allowing the lamellae to emerge into the external environment. It has been shown that during suction feeding in *Ambystoma*, the branchial arches adduct and form a resistance to water flow, only allowing water to flow in from the mouth as the pharynx expands (Lauder and Shaffer, 1985). The increased development of the interbranchial septa may have been selected for to aid in a predaceous suction-feeding lifestyle by increasing the resistance to water flowing in posteriorly through the gill slits. For the gills to have well-developed septa for feeding and still be functional for respiration, the lamellae had to be positioned more posteriorly, out of the gill chamber and in the external environment. Thus, it is possible that lamellae inside of the gill chamber is a primitive condition for frogs and salamanders but that the increased development of the septa in salamanders selected for more posteriorly positioned lamellae.

This is likely similar to the evolution of external gills in Dipnoi as well. Only the derived lepidosirenid lungfishes, *Protopterus* and *Lepidosiren*, ever develop external gills. *Neoceratodus*, a basal species, never develops external gills and external gills have not been found in any fossil lungfishes. Although the external gills of lungfishes and amphibians are probably not homologous, they are both derived from the homologous

interbranchial septa, which could have simply elongated posteriorly in both groups independently.

The development of external gills is clearly the primitive condition in amphibians, as they are well developed in all three groups. Anurans raise only marginal doubts: the most basal genera, *Ascaphus* and *Leiopelma*, fail to develop any external gills, but other basal frogs have well-developed external gills (Brown, 1989; Stephenson, 1951; Nokhbatolfoghahai and Downie, 2008) (Fig. 42). Additionally, common cell types, such as mitochondria-rich cells, pavement cells, and ciliary cells are also well developed in the external gills of frogs (that possess them), salamanders, and caecilians (Kato and Kurihara, 1988; Nokhbatolfoghahai and Downie, 2008; Uchiyama and Yoshizawa, 1991). Although homologous, the morphology of external gills between the amphibian groups is somewhat different. The external gills of anurans are variable but usually develop on a primary rachis with finger or club-like lamellae developing off the distal-most end (Nokhbatolfoghahai and Downie, 2008). The external gills of caecilians are extremely long and filamentous and develop paired, finger-like lamellae along the entirety of the gill (Dünker, 2000). The external gills of salamanders are similar to those of caecilians but are more robust and generally shorter (Valentine and Dennis, 1964).

#### Evolutionary Origin of Anuran Internal Gills

The evolutionary origin of anuran internal gills is unknown and could be due to one of three possible scenarios: 1) The internal gills of anuran larvae are a derived character state of the amphibian external gill, 2) The internal gills are a derived character state that evolved from the internal gills of fishes (and were therefore lost in other

lissamphibians), or 3) They are a completely independently evolved trait - not derived from external or internal gills.

There is a mixture of available evidence that makes answering the question of homology of anuran gills difficult. The lamellae of *A. truei* are relatively simple in comparison to most anurans and possess very few epithelial cell types, with the vast majority being pavement cells. In more derived species, however, pavement cells, mitochondria-rich cells, and ciliary cells have been found. The vasculature of the lamellae in *Ascaphus truei* is also simpler when compared to the vasculature of more derived species.

Compared to the primary lamellae of chondrichthyans, actinopterygians, coelacanths, and lungfishes, the internal lamellae of frog larvae are quite different. The external morphology of the lamellae is made up of unpaired, club-like lobes that house vascular loops. This is unlike the primary lamellae of basal fishes, which are thin and elongate and develop paired secondary lamellae along the entire length of each lamella. The general vascular anatomy is similar in that afferent branchial arteries distribute blood to the lamellae and get drained by efferent branchial arteries that distribute blood to the rest of the body, though the vasculature of the internal lamellae of frogs is more like those of salamanders in that it exists as vascular loops that connect at the distal ends. *Ascaphus truei*, however, lacks the vast capillary network in the lamellae that is found in *Dicamptodon tenebrosus*.

The relatively simple lamellar morphology compared to other frog species and the differences compared to the lamellae of other gilled vertebrates support the idea that

internal gills of anurans are independently evolved (Schmalhausen, 1968). It is difficult to determine how the habitat of *Ascaphus truei* altered the evolution of the internal gill.

*Ascaphus truei* lives in cold, fast flowing, oxygenated mountain streams - the same habitat types as *Dicamptodon tenebrosus* and *Rhyacotriton* spp. (Valentine and Dennis, 1964). The gills of these stream-type salamanders are very much reduced in comparison to the external gills of other salamanders. It may be that living in a fast-flowing, oxygen-rich environment allows for the reduction of gills. However, it is not known whether this condition affects the internal gills of anurans as much as the external gills of salamanders. It is possible that the simpler gills of *A. truei* are, as in *Dicamptodon*, due to the habitat it resides in and not because of its basal phylogenetic status. More research must be done to conclusively determine the evolutionary origin of the anuran internal gills.

#### Future Studies

The gills of vertebrates are highly variable, especially in their embryonic and larval forms. Schoch and Witzmann (2010) have already pointed out the importance of embryonic developmental studies. They suggested tracing the fate of cells of embryonic lungfishes and salamanders in order to determine specifically what germ layer gives rise to the interbranchial septa, the lamellae, and arteries in the various groups. Similar studies should be performed on anuran embryos and other basal vertebrates to determine what germ layer gives rise to the internal and external gills, which may aid in establishing homologies.

Habitat clearly plays an important role in the evolution of gill morphology, as evidenced by the likely convergence in the development of external gills in several

groups (Dipnoi, *Polypterus* spp., extant amphibians). Gaining a better understanding of the morphological differences among gills of related species in different habitats, and similarities between unrelated species in the same habitats, will give a more robust perspective on how and why gills evolve.

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## TABLES

Table 1. Number of animals used for each method.

	Light Microscopy	External Morphology (SEM)	Vascular Casting (SEM)
<i>Ascaphus truei</i>	5	6	7
<i>Dicamptodon tene- brosus</i>	3	4	4
<i>Protopterus</i> sp.	0	1	0
<i>Lepidosiren para- doxa</i>	1	0	1

Table 2. List of abbreviations.

Abbreviation	Term
ABA	Afferent branchial artery
ala	Afferent lamellar artery
ACV	Anterior cardinal vein
BA	Branchial arches
Cap	Capillary
CC	Ciliary cell
CB	Ceratobranchial
Con	Connective tissue
Cut Art	Cutaneous artery
D Art	Ductus arteriosus
DA	Dorsal aorta
EBA	Efferent branchial artery
ECA	External carotid artery
ela	Efferent lamellar artery
GC	Gill chamber
GF	Gill filter
GR	Gill raker
GU	Gular fold
H	Heart

Abbreviation	Term
I	Intestine
ICA	Internal carotid artery
L	Lamella
LDA	Lateral dorsal aorta
Liv	Liver
MRC	Mitochondria-rich cell
OD	Oral disk
P	Pharynx
PVC	Pavement cell
RBC	Red blood cell
S	Septum
SGC	Subgular cavity
SL	Secondary lamellae
SV	Shunt vessel
TA	Truncus arteriosus

## FIGURES

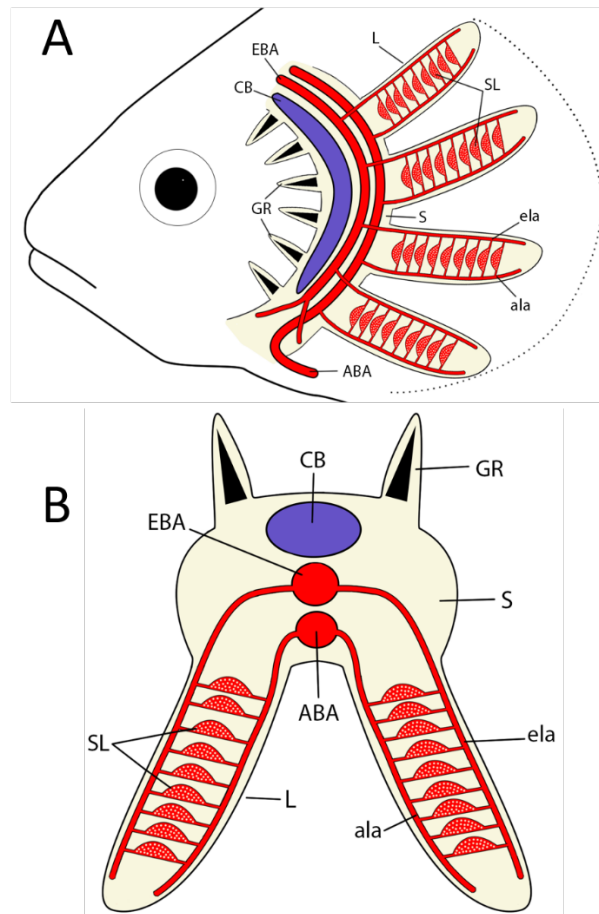


Figure 1. Diagrammatic drawings of a branchial arch and its associated vasculature in an actinopterygian fish. A) Lateral view with anterior to the left. Lamellae (L) are supplied blood via an afferent branchial artery (ABA) and drained of blood via an efferent branchial artery (EBA). The dotted line indicates the posterior edge of the operculum. B) Cross section of a branchial arch. The interbranchial septa (S) encompass the ceratobranchial (CB) and the branchial arteries. Notice the lamellae hang from the branchial arch independently.

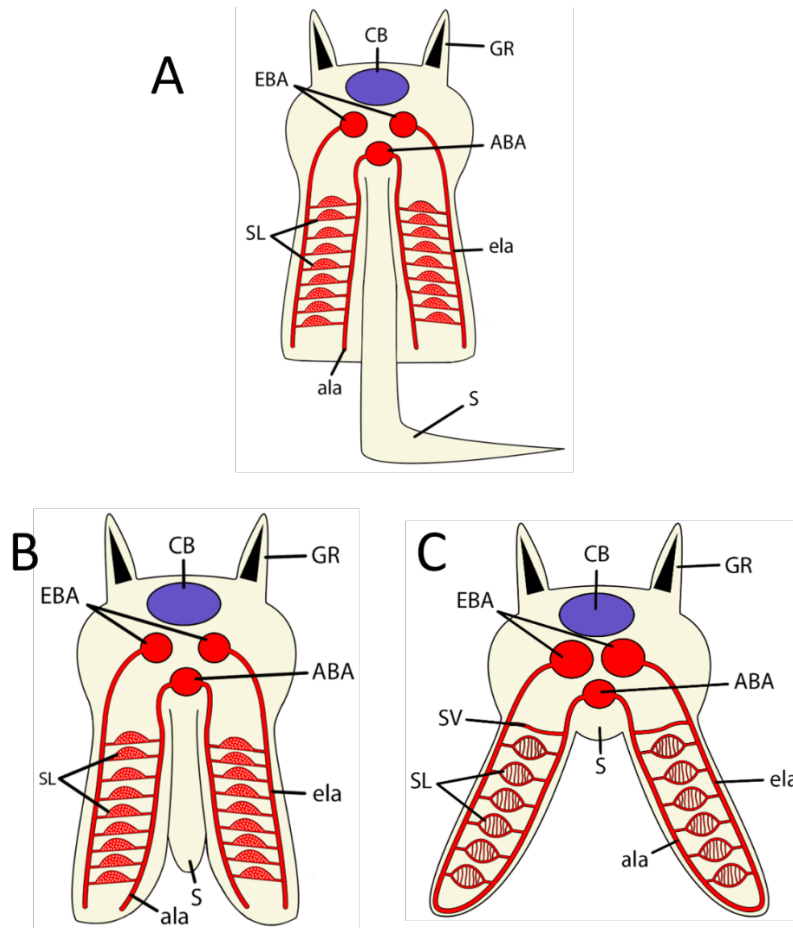


Figure 2. Diagrammatic cross sections of the branchial arches of Elasmobranchii, *Neoceratodus*, and *Protopterus*, showing the respiratory vasculature. A) Elasmobranchii. Notice the extreme length of the interbranchial septa (S), which connect to the lamellae across their entire medial edges. B) *Neoceratodus*. The interbranchial septa are somewhat reduced, but generally resemble the gills of Elasmobranchii. C) *Protopterus*. The interbranchial septa are further reduced, allowing the lamellae (which lack secondary lamellae) to hang independently. ABA, Afferent branchial artery; EBA, Efferent branchial artery; ala, Afferent lamellar artery; ela, Efferent lamellar artery; CB, Ceratobranchial; GR, Gill raker; SL, Secondary lamellae; SV, Shunt vessel

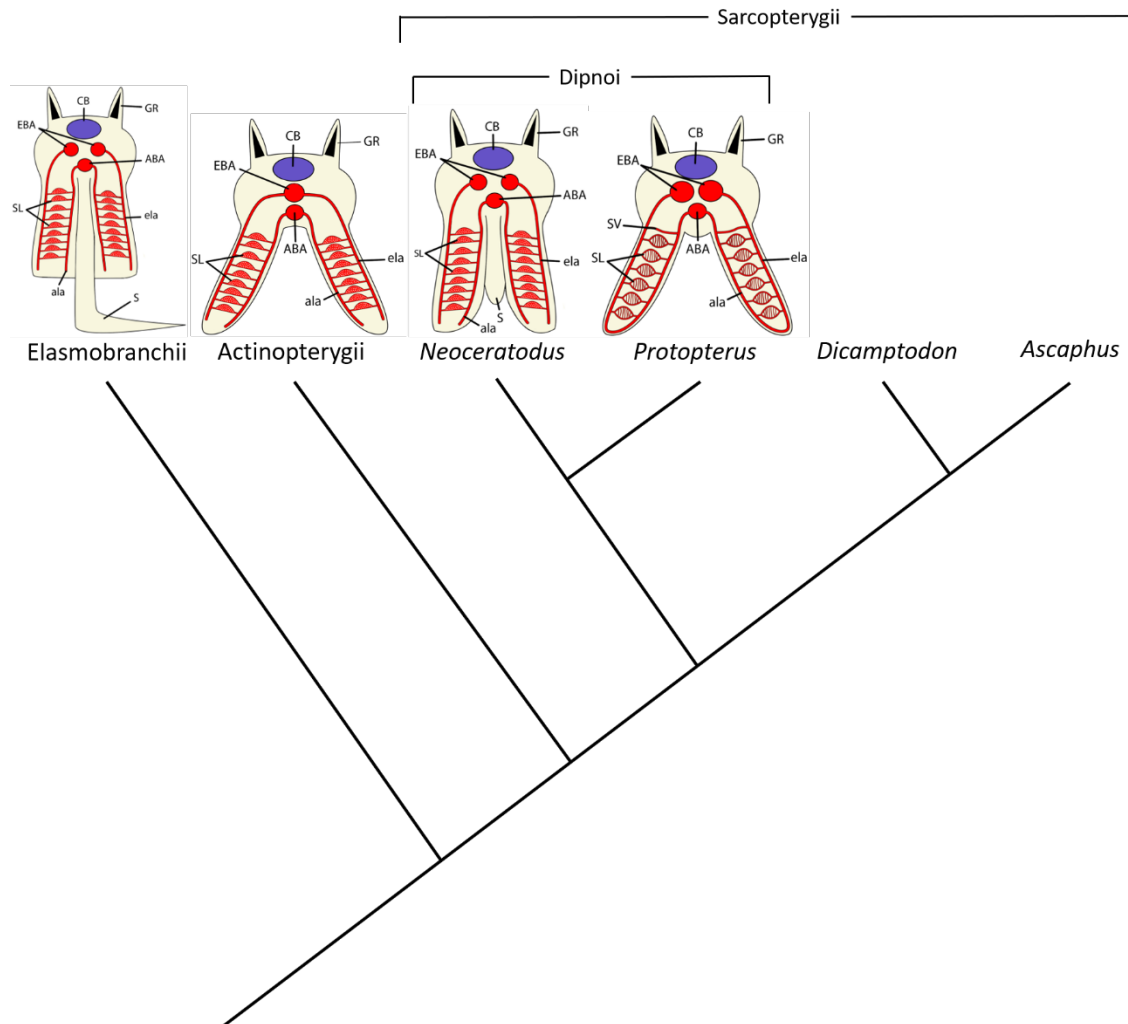


Figure 3. A phylogeny of vertebrates, showing the general gill morphology of Elasmobranchii, Actinopterygii, and the lungfishes *Neoceratodus* and *Protopterus*.

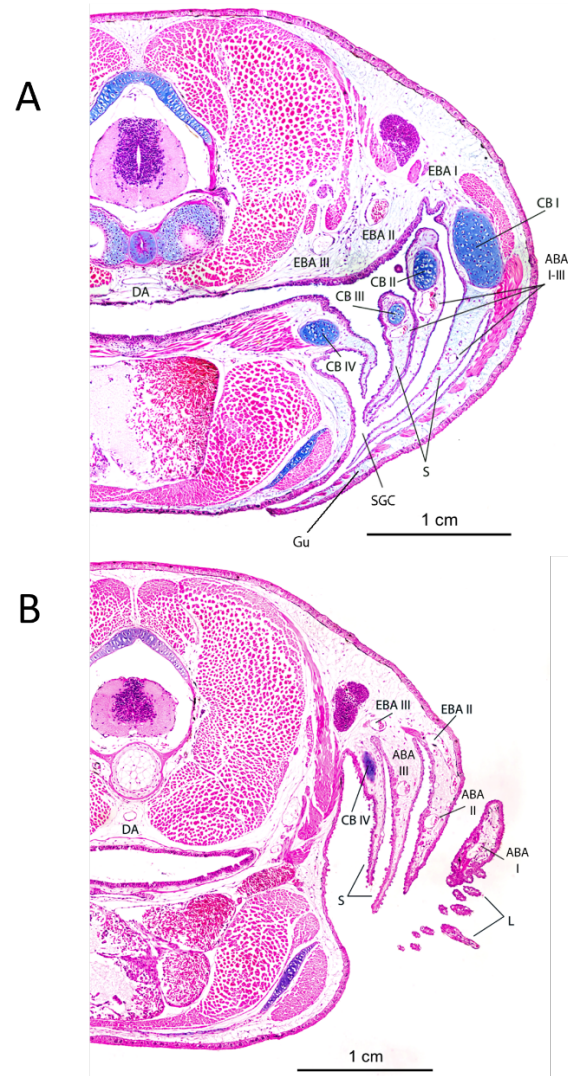


Figure 4. Histological cross sections of *Dicamptodon tenebrosus* showing the relative positions of the interbranchial septa in different regions of the body. A) Anterior cross section of the branchial arches (BA), including the supporting ceratobranchials (CB I-IV). Dorsal is top. The anterior portion of the gills bear no lamellae (L) and are enclosed inside the subgular cavity (SGC) by the gular fold (Gu). The interbranchial septa (S) develop around the ceratobranchials and extend very far ventrally. B) Posterior cross section of the branchial arches, dorsal top.



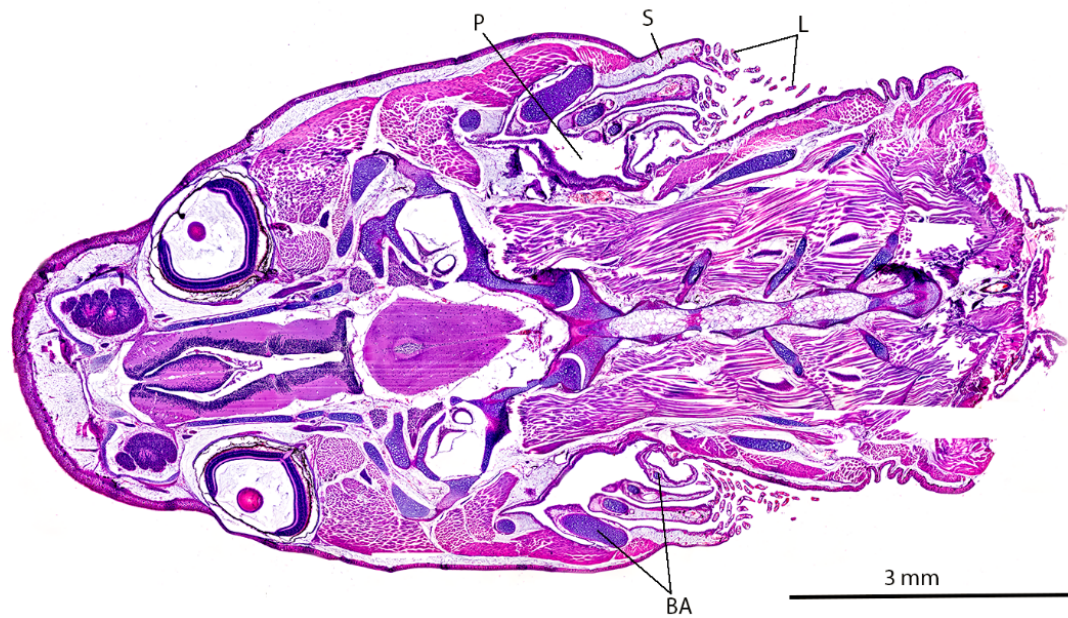


Figure 5. Frontal section of *Dicamptodon tenebrosus* showing the left and right branchial arches (BA), anterior to left. Notice that the interbranchial septa (S) extend posteriorly into the external environment. P, pharynx.

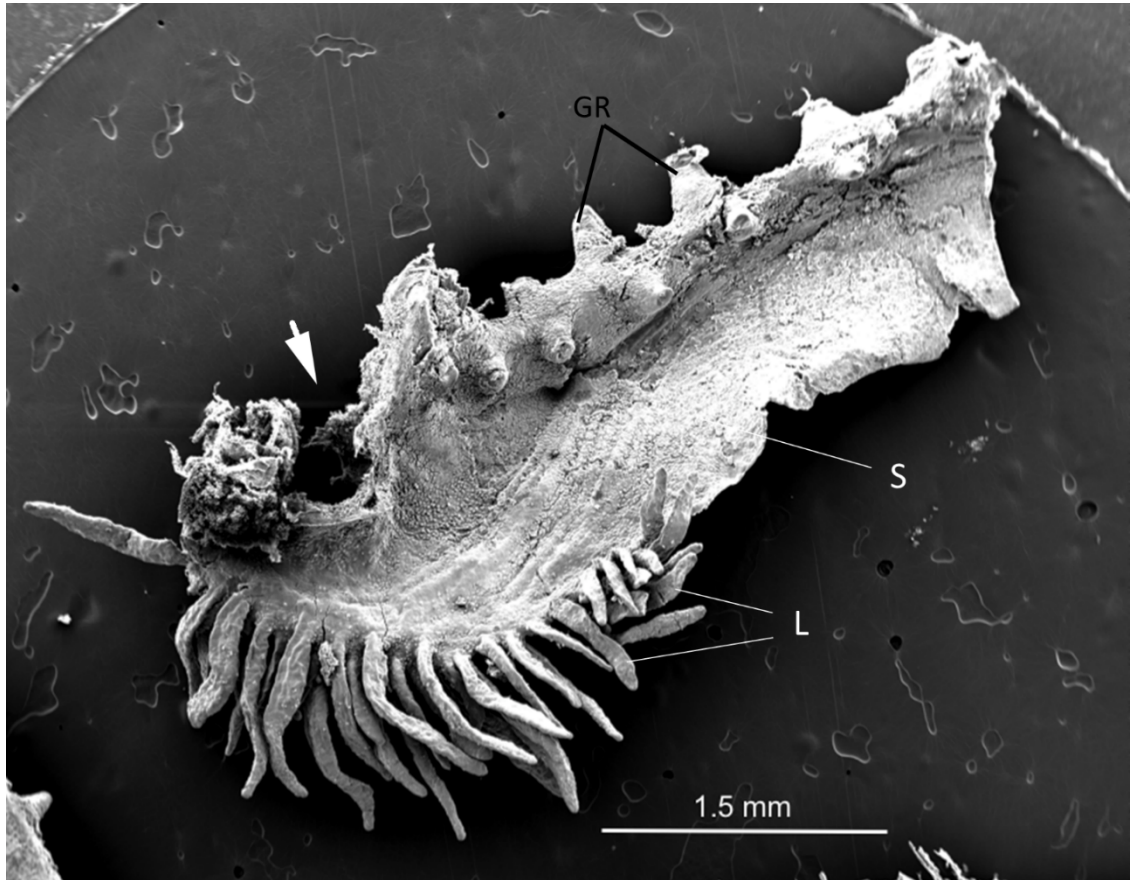


Figure 6. Scanning electron micrograph of the medial side of the left gill arch III of *Dicamptodon tenebrosus*. Anterior to the right. The lamellae (L) develop directly off the posterior/ventral portion of the septum (S). The inner region of the arch develops gill rakers (GR). The arrow indicates where the arch was dissected dorsally from the body wall.

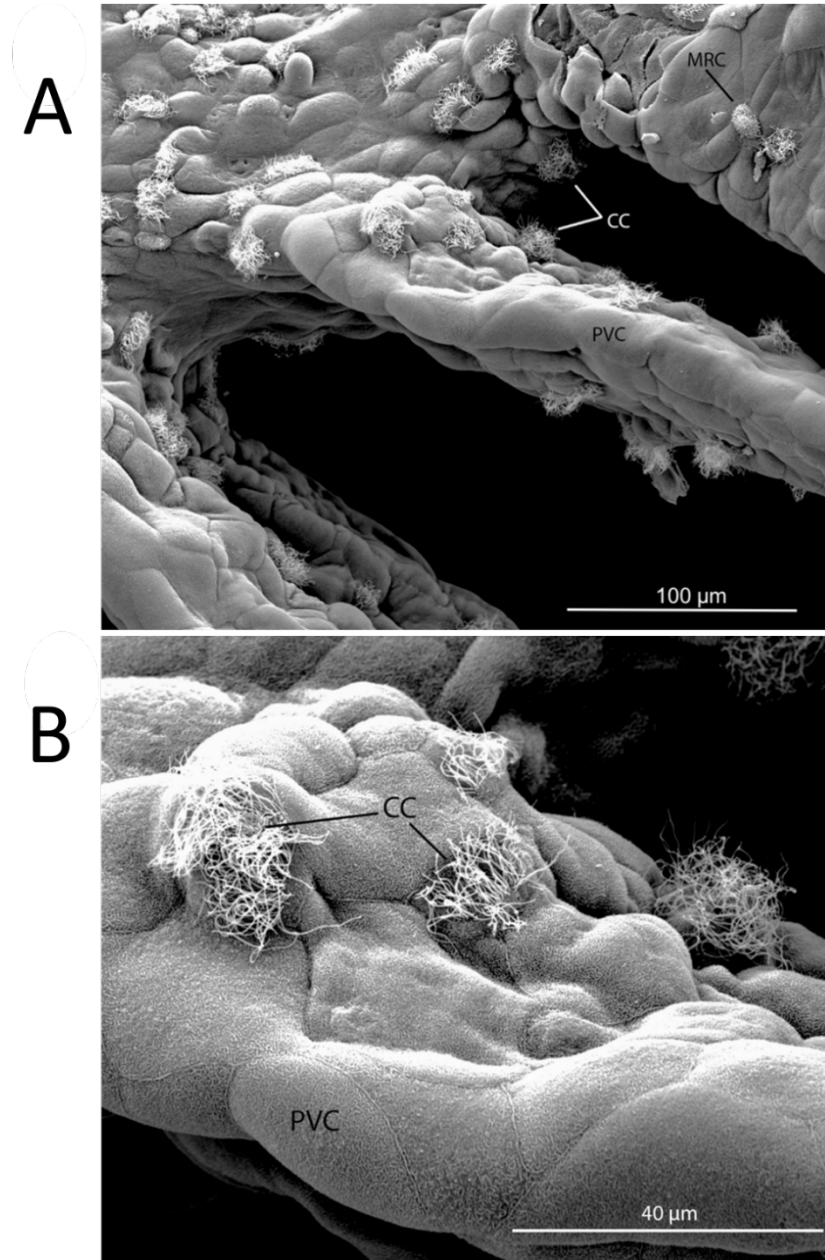


Figure 7. A) Scanning electron micrograph of *Dicamptodon tenebrosus*, showing a lateral view of the ventral portion of the septum where the lamellae extend ventrally, top is dorsal. Both the septum and lamellae share the same cell types – pavement cells (PVC), ciliary cells (CC), and mitochondria-rich cells (MRC). B) Scanning electron micrograph of a lamella, showing a closer view of the cell types.

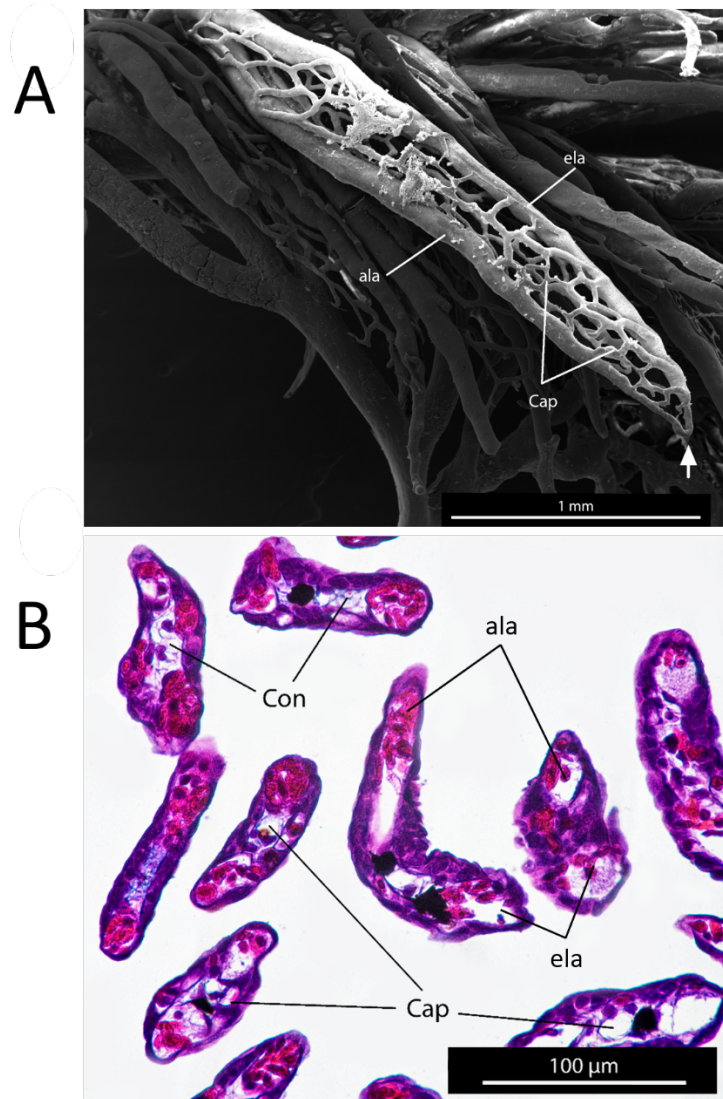


Figure 8. A) Scanning electron micrograph of the vasculature of a gill lamella in *Dicamptodon tenebrosus*. Blood enters each lamella via the afferent lamellar artery (ala) and exits the lamella via the efferent lamellar artery (ela). Spanning the efferent and afferent lamellar arteries is a network of capillaries (Cap) that runs through the middle of the lamellae. The white arrow shows the distal tip of the lamella which connects, creating a continuous vascular loop. B) Light microscopy cross section of gill lamellae. Spanning the middle of each lamella are capillary vessels (Cap) and connective tissue (Con).



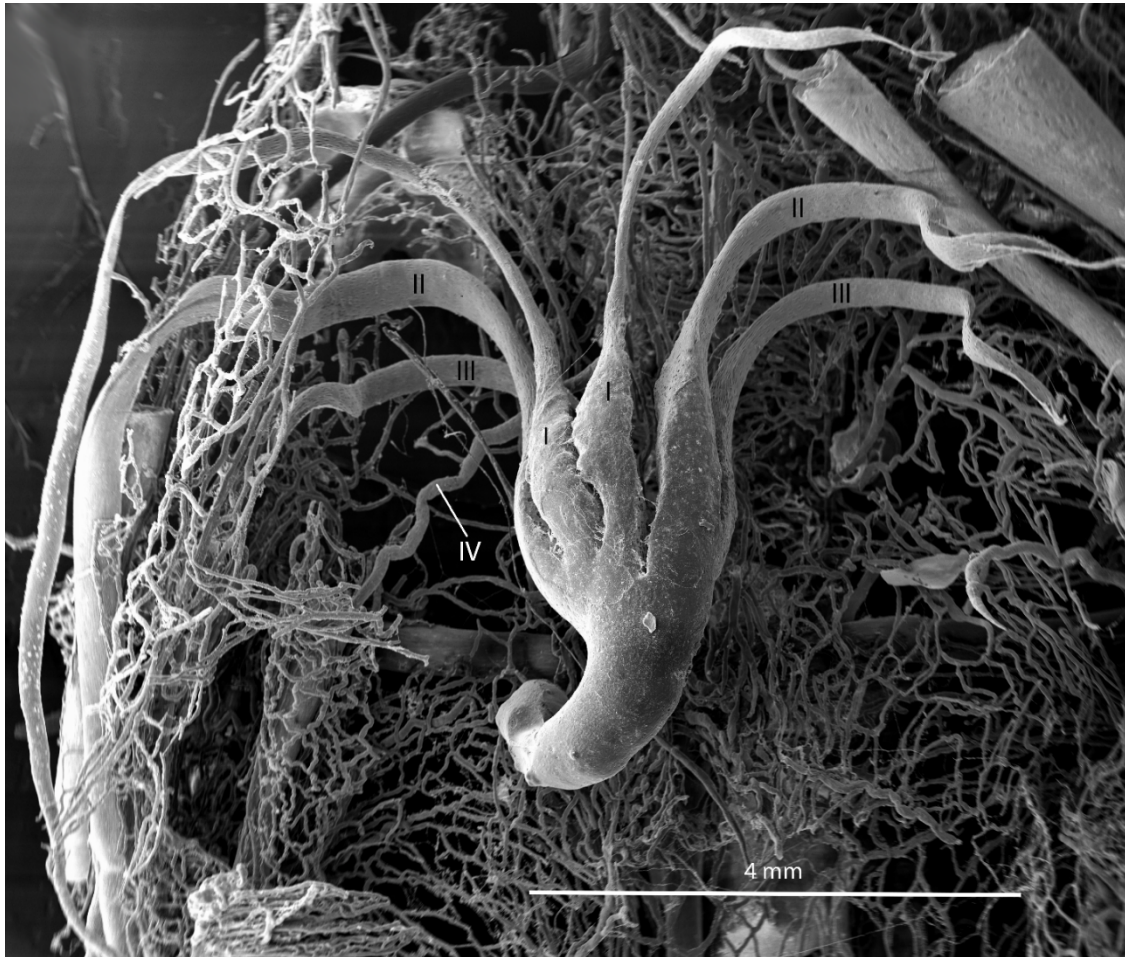


Figure 9. Scanning electron microscopic (SEM) image of a vascular cast of *Dicamptodon tenebrosus*, showing the truncus arteriosus and afferent branchial arteries (ventral view), anterior top. I-IV represent afferent arteries I-IV. The first three arteries supply branchial arches I-III with blood, while IV supplies blood to the lungs. Capillary networks seen are vessels supplying blood to the head and lower jaw.

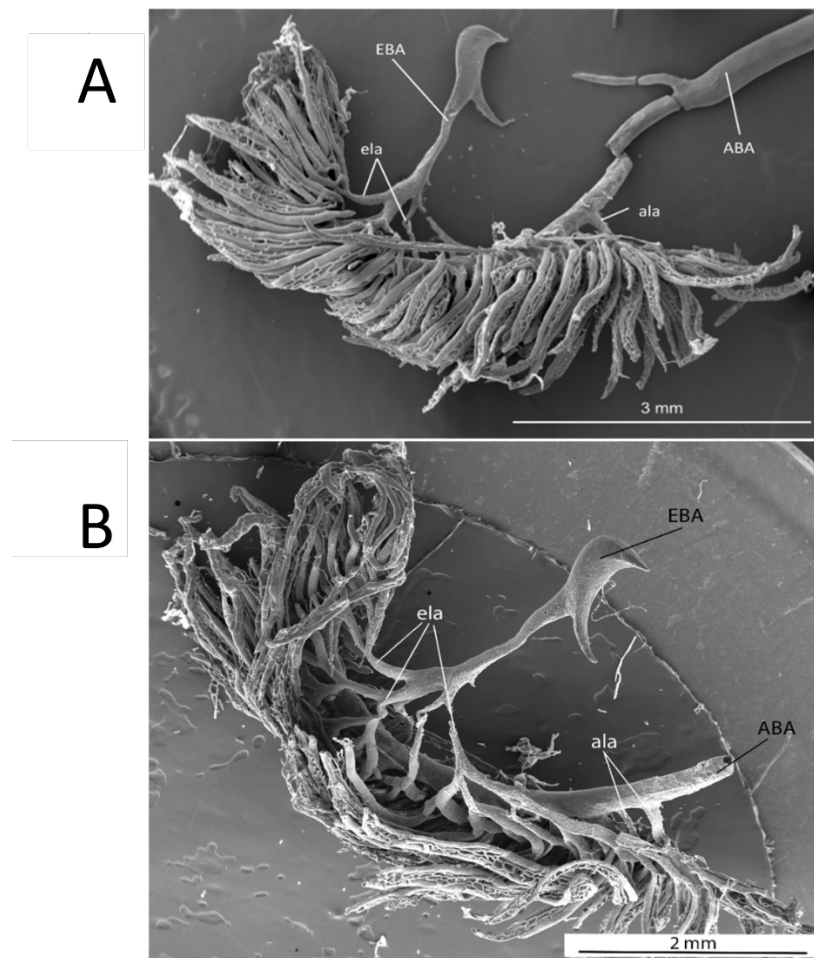


Figure 10. A) Medial view of branchial arch II vascular cast in *Dicamptodon tenebrosus*, dorsal at top and posterior left. Blood from the afferent branchial artery (ABA) travels into a series of smaller afferent lamellar arteries (ala) that feed one or more lamellae with blood. From the lamellae, blood is drained into a series of efferent lamellar arteries (ela) before draining into the efferent branchial artery (EBA). Some breakage occurred in the afferent branchial artery during mounting. B) A dorsal view of the same vascular cast, dorsal top. Note that the afferent branchial artery and efferent branchial artery never directly connect in the distal portion of the gill. Blood must travel from the afferent branchial artery (ABA) to the efferent branchial artery (EBA) via the gill lamellae.

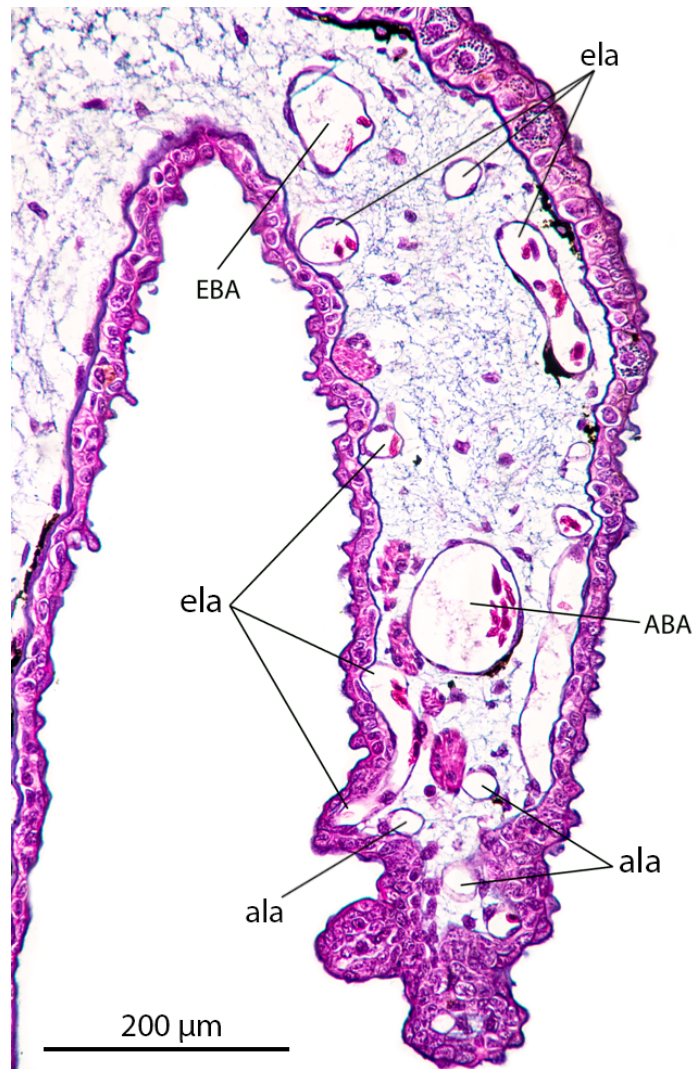


Figure 11. Light microscopy cross section of a gill arch in *Dicamptodon tenebrosus*, dorsal at top and medial to the left. From the afferent branchial artery (ABA), the afferent arterioles (ala) travel ventrally and to the lateral/medial edges of the septum, supplying the lamellae with blood. From the lamellae, efferent arterioles (ela) travel dorsally along the lateral/medial edge of the septum and drain into the efferent branchial artery (EBA).

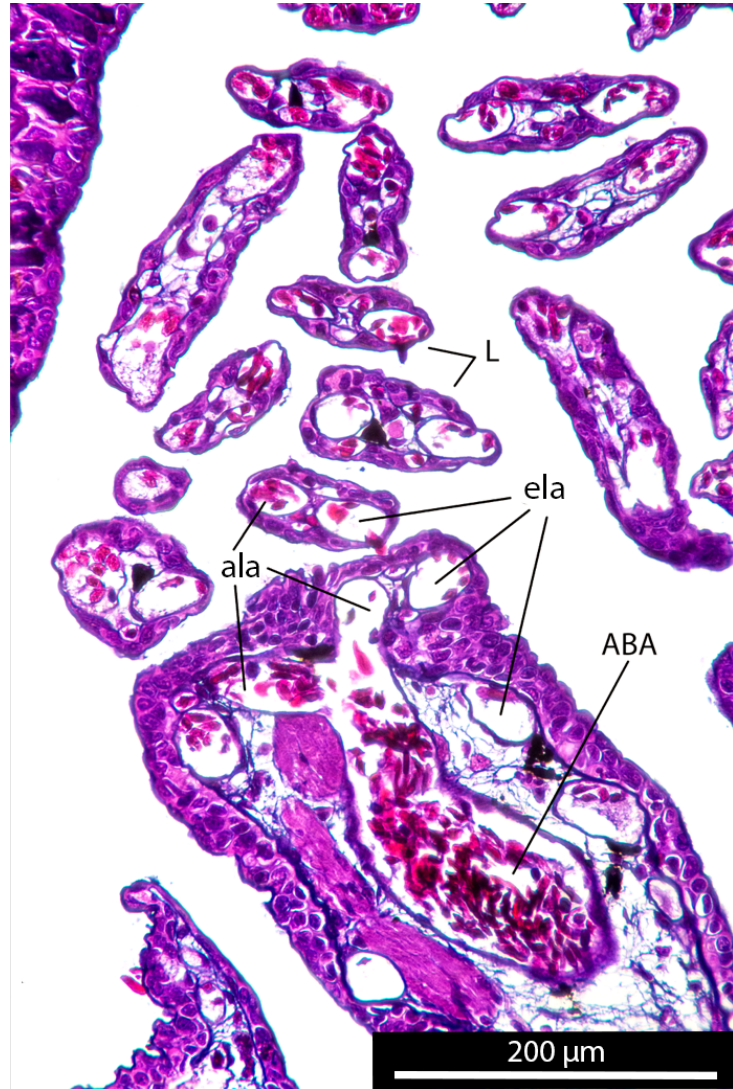


Figure 12. Light microscopy frontal section of *Dicamptodon tenebrosus*, looking at a distal portion of the interbranchial septum, with anterior at bottom and medial at the left. Notice that the afferent branchial artery (ABA) branches distally, supplying blood to the paired lamellae (L) on both the medial and lateral side of the septum via the afferent lamellar arteries (ala). Blood will loop through the lamellae and enter back into the medial and lateral sides of the septa via efferent lamellar arteries (ela) and eventually drain dorsally and anteriorly into the efferent branchial artery (not pictured).



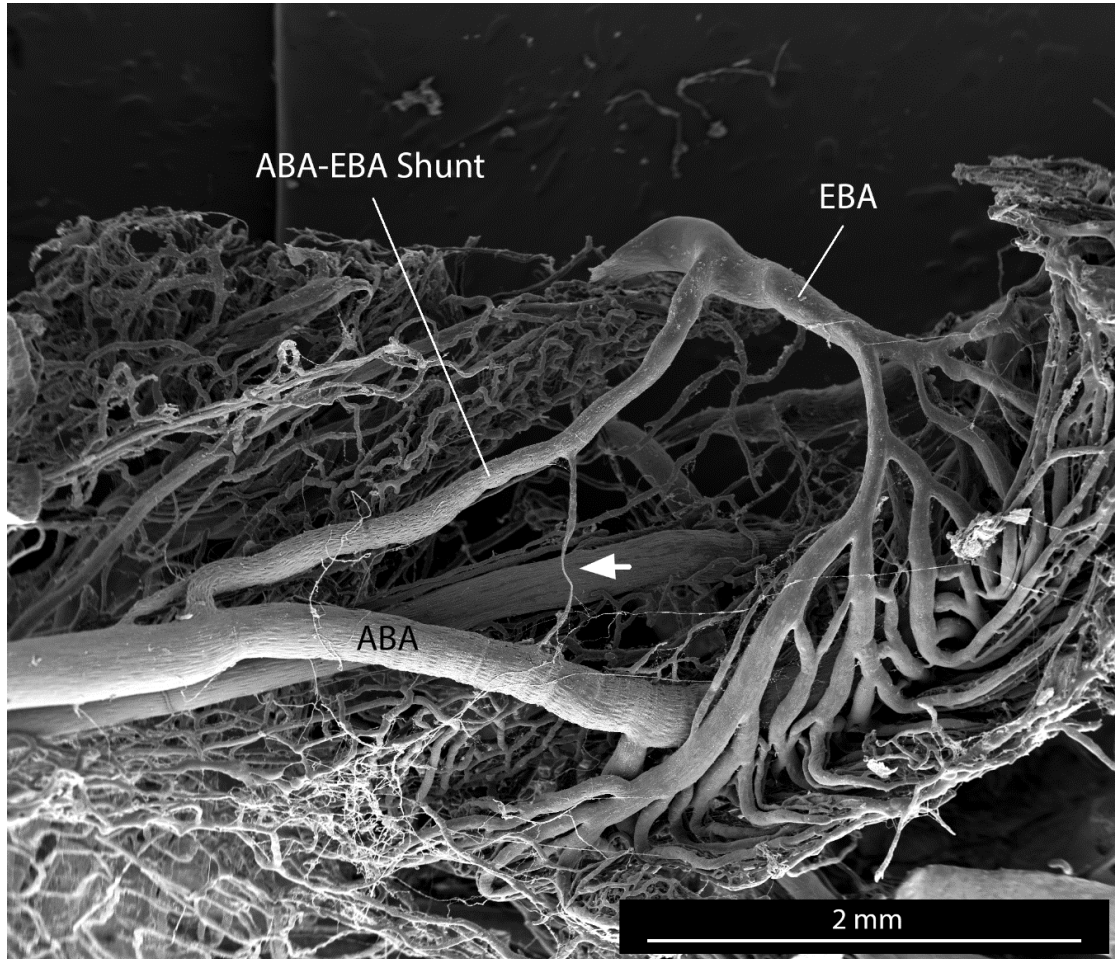


Figure 13. Scanning electron micrograph showing a lateral view of a vascular cast of branchial arch II in *Dicamptodon tenebrosus*. Anterior to the left and ventral down. Notice the ABA-EBA shunt at the base of the gill. This allows blood to travel dorsally from the afferent branchial artery (ABA) directly to the efferent branchial artery (EBA) without travelling through the lamellae. The white arrow is pointing to another vessel, spanning from the afferent branchial artery to the ABA-EBA shunt.

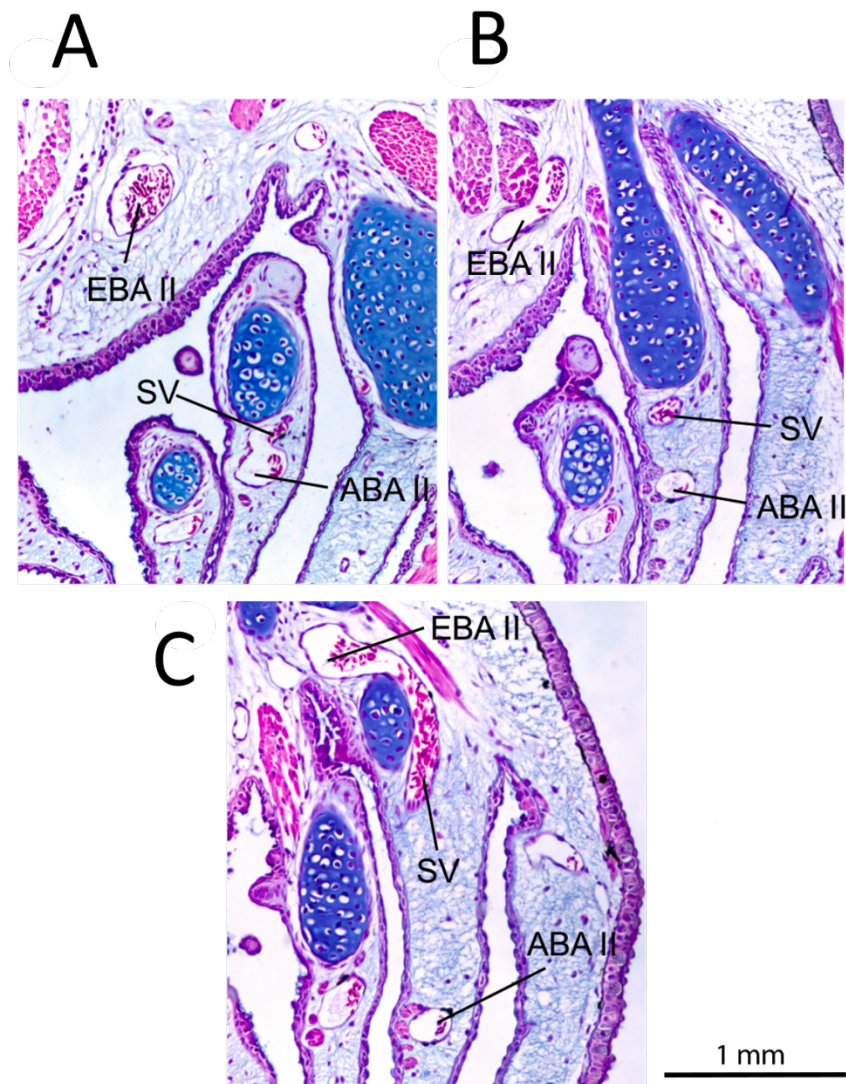


Figure 14. Light microscopy cross section images of branchial arch II shunt in *Dicamptodon tenebrosus*, from anterior to posterior. Dorsal is top and medial is left. A) Shunt vessel (SV) begins to split from ABA II. B) Shunt vessel has split from ABA II. C) Shunt vessel and EBA II are merging.

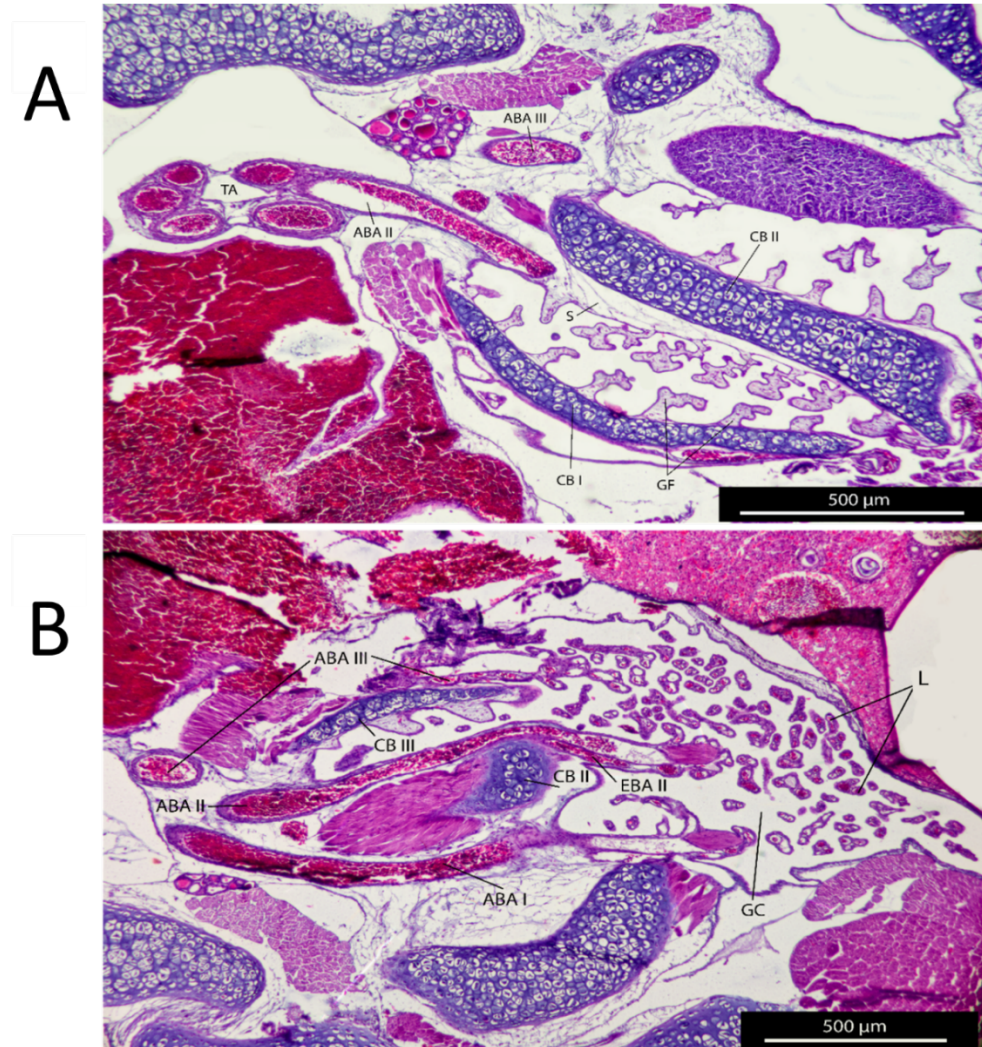


Figure 15. Frontal sections of the left branchial arches in *Ascaphus truei* from dorsal to ventral. In both pictures the bottom is anterior and left is medial. A) Seen are branchial arches I and II and the truncus arteriosus (TA) that supplies each arch with blood. The ceratobranchials (CB I, CB II) travel laterally and support the gill arch. B) The afferent branchial arteries (ABA I-III) and efferent branchial arteries (EBA I-III) run parallel to each other laterally. Some gill lamellae (L) can be seen laterally, in the gill chamber.



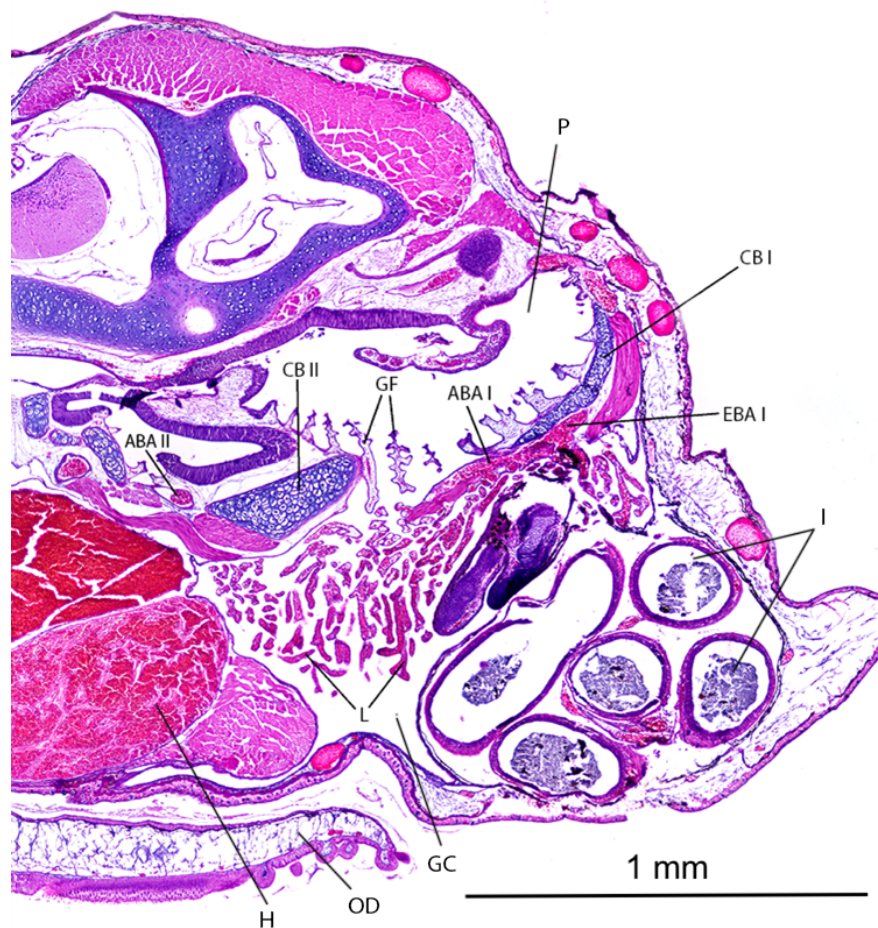


Figure 16. Light microscopy cross section of *Ascaphus truei* larva at level of otic capsules. Portions of branchial arches I and II are visible, including the supporting ceratobranchials (CB I, CBII). Note gill filters (GF) extending up from arches into the pharynx (P), and gill lamellae (L) extending down into the gill chamber (GC). The heart (H) is visible, as are the afferent branchial artery of arch II (ABA II), and the efferent branchial artery of arch I (EBA I)), lying ventral to the corresponding ceratobranchial. The intestine (I) extends forward in the body cavity lateral to the gill chamber. Note also the oral disc (OD) ventrally.

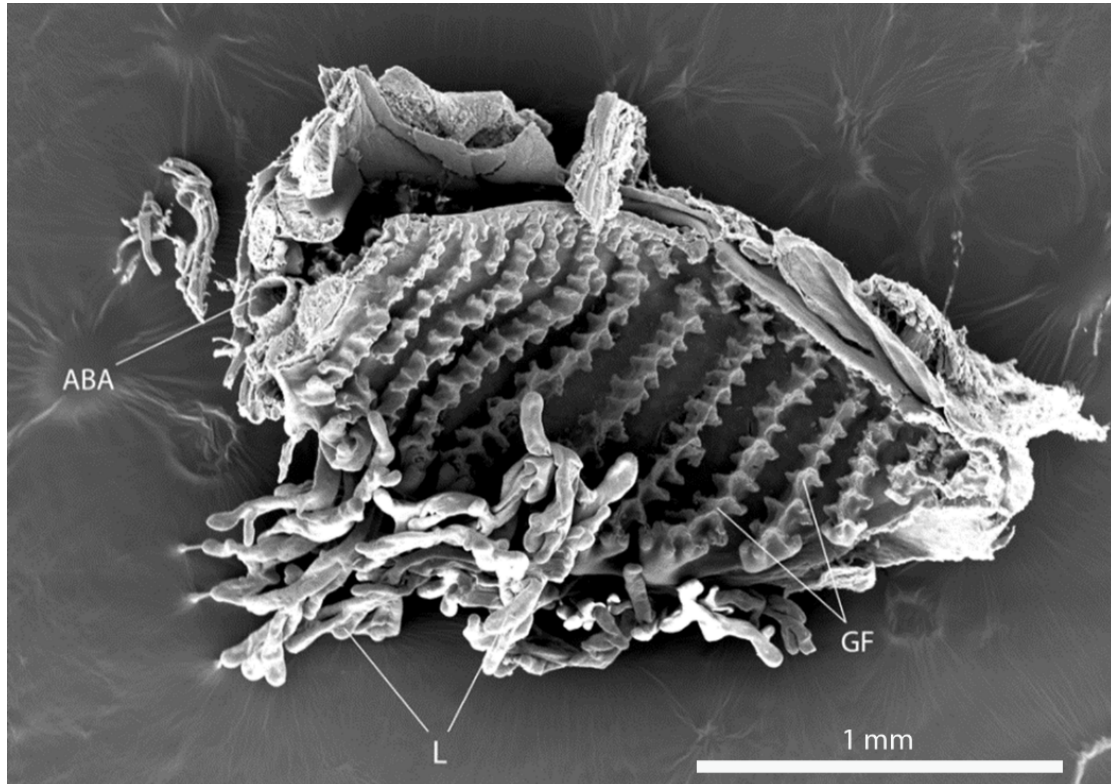


Figure 17. Scanning electron micrograph of dissected out gill arch II in *Ascapheus truei*. Top is dorsal and left is medial and anterior. The lamellae (L) develop off the arch ventrally and are supplied blood via the afferent branchial artery (ABA) travelling laterally through the gill arch. Also note the dorsally-located gill filters (GF).

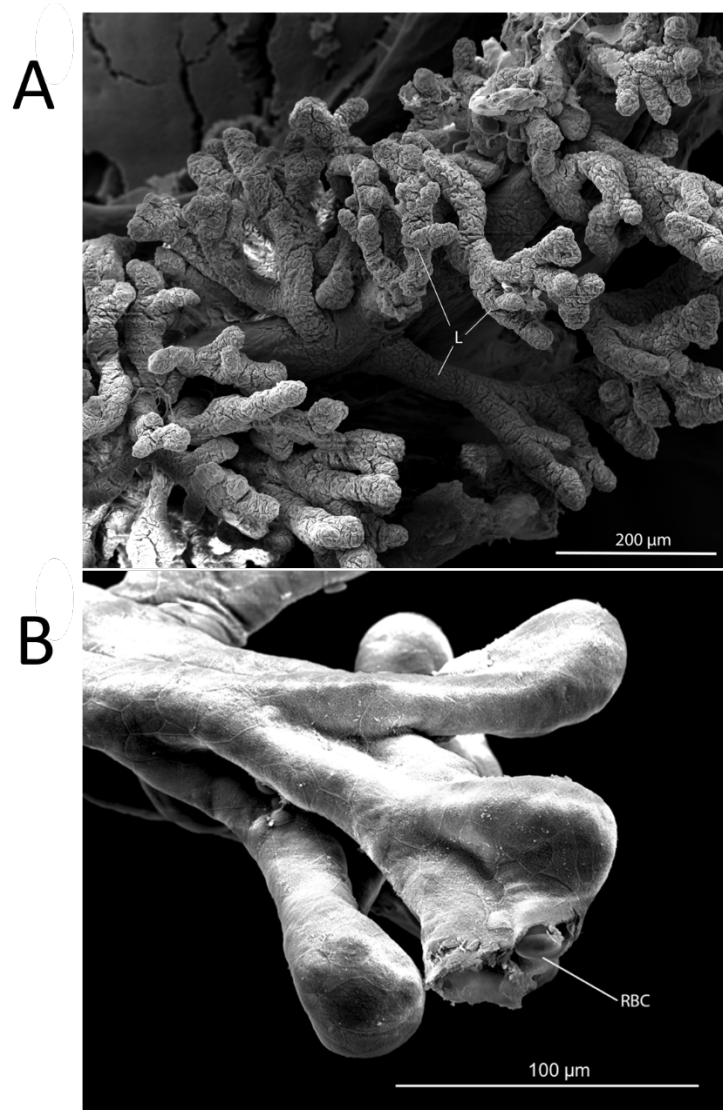


Figure 18. A) Scanning electron micrograph of the ventral view of branchial arch II in *Ascaphus truei*, with medial left. The lamellae (L) emerge from the branchial arch on a stalk and branch distally. Specimen was fixed in formalin and resulted in some cell shrinkage but allows for a clear view of the lamellar morphology. B) Scanning electron micrograph of the distal tip of a gill lamella. Ventral is right. The lamellae are branched with club-like lobes distally, each housing a vascular loop. The tip of one club is torn, allowing a red blood cell (RBC) to emerge.

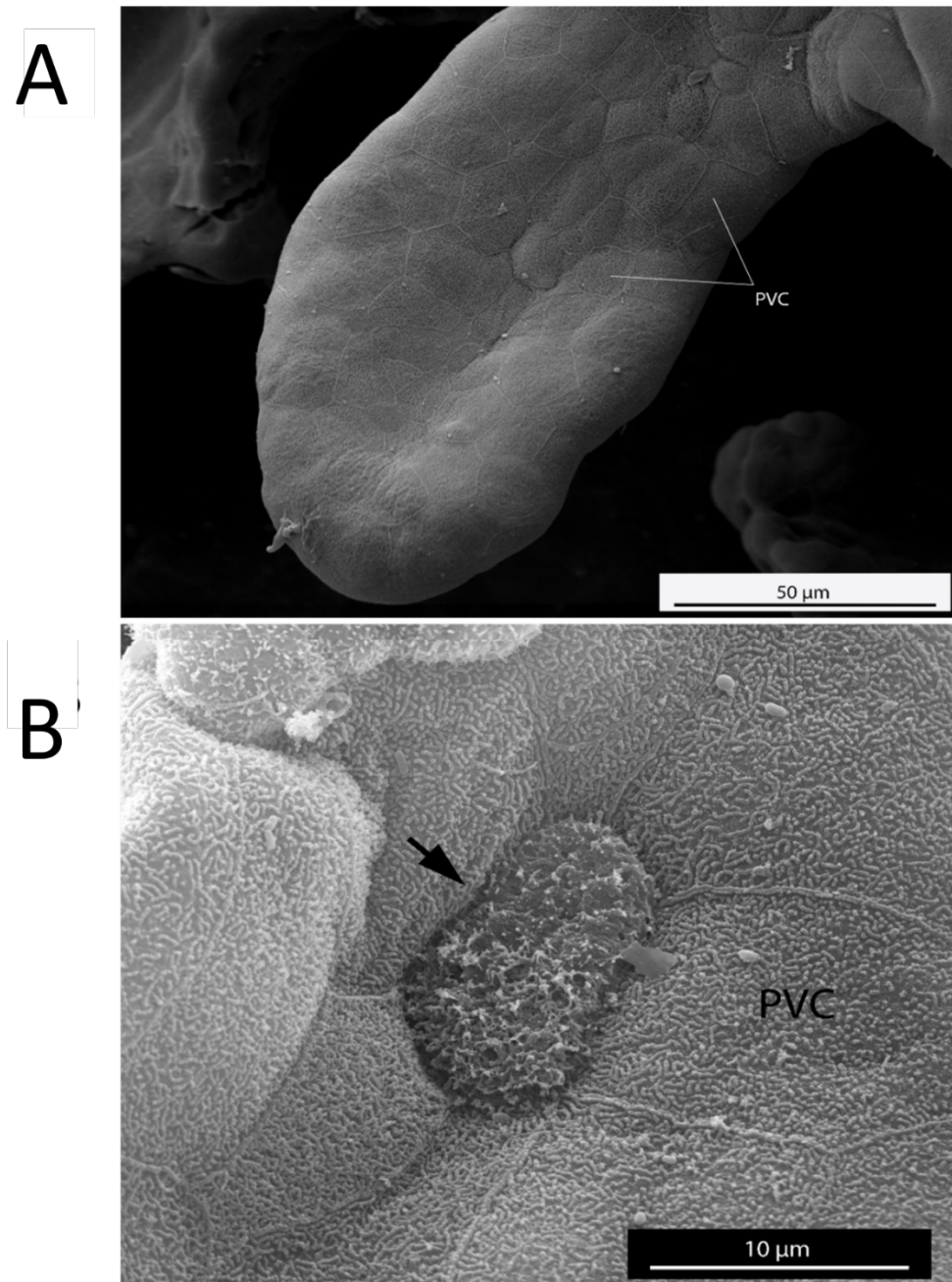


Figure 19. Scanning electron micrographs of the surface epithelium in *Ascapthus truei*. A) Seen is the distal tip of one lobe of a gill lamella. The vast majority of the epithelial surface of the gill lamellae are covered in pavement cells. B) A closer view of the surface epithelium of a gill lamella. The black arrow points to an unknown cell type.



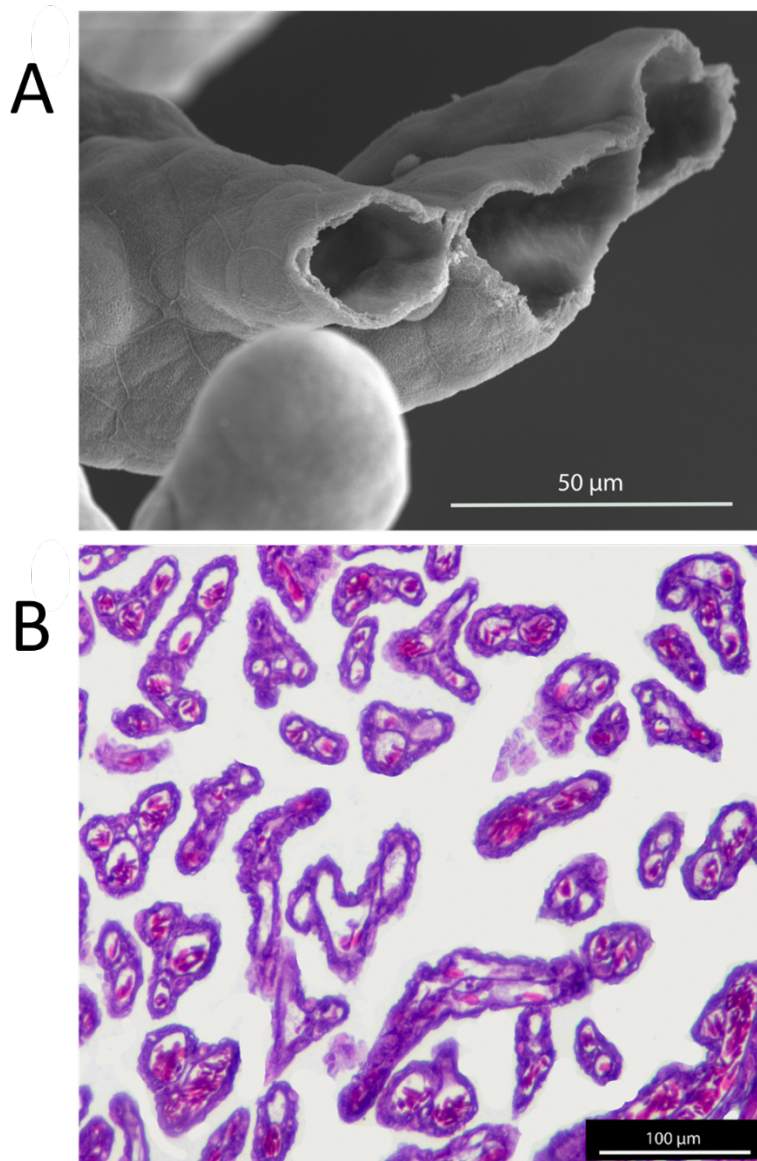


Figure 20. A) Scanning electron micrograph of the distal tip of a gill lamella in *Ascapthus truei*, with ventral to the right. The tips are torn, revealing the thin epithelial surface of the lamella. Note that most of the internal lamellae is made up of blood channels. B) Light microscopy frontal section of the ventral portion of gill arch II, showing the insides of the gill lamellae. Most of the lamellae consists of vasculature, which is only 1-2 cell layers away from the external environment.



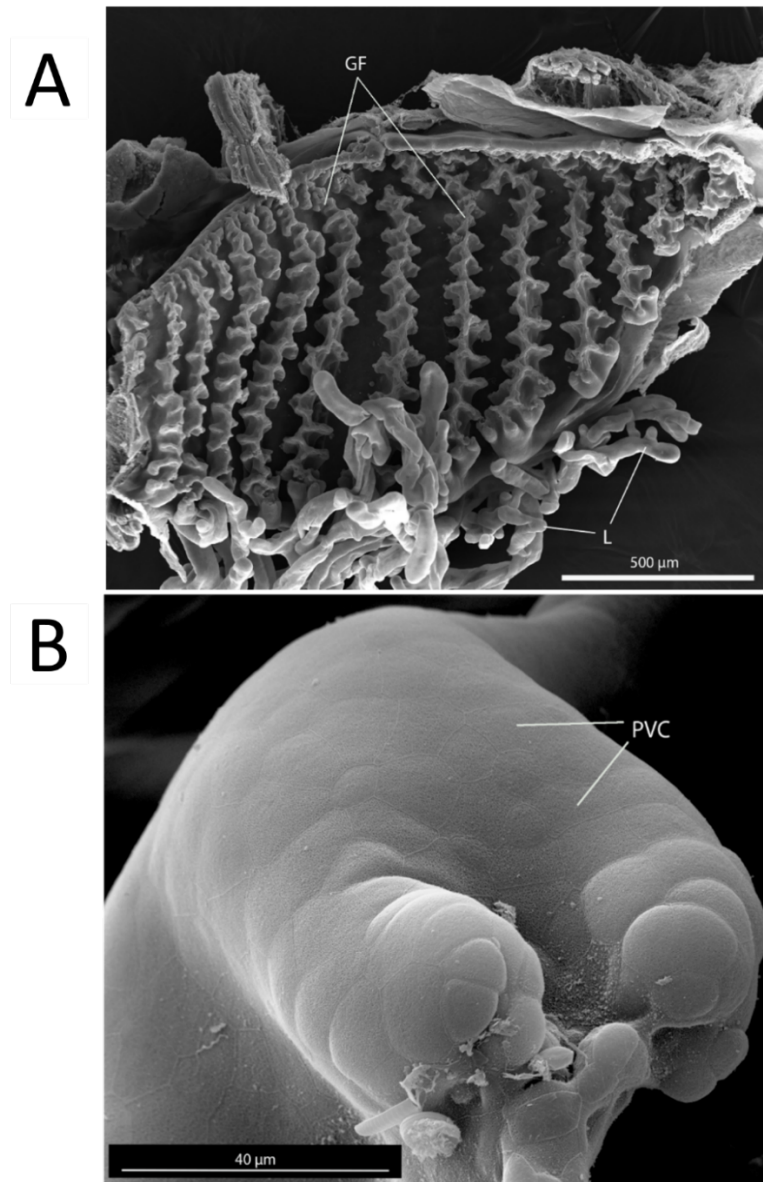


Figure 21. A) Scanning electron micrograph of a dissected-out gill arch in *Ascaphus truei*. Top is dorsal and left is medial. The gill filters (GF) run in parallel rows on the dorsal (bottom) part of the gill arch. They run in parallel rows, usually along the entire arch, though some are short. B) Scanning electron micrograph of the ventral end of a gill filter in *A. truei*, top is ventral. The entire surface epithelium is made up of pavement cells (PVC).

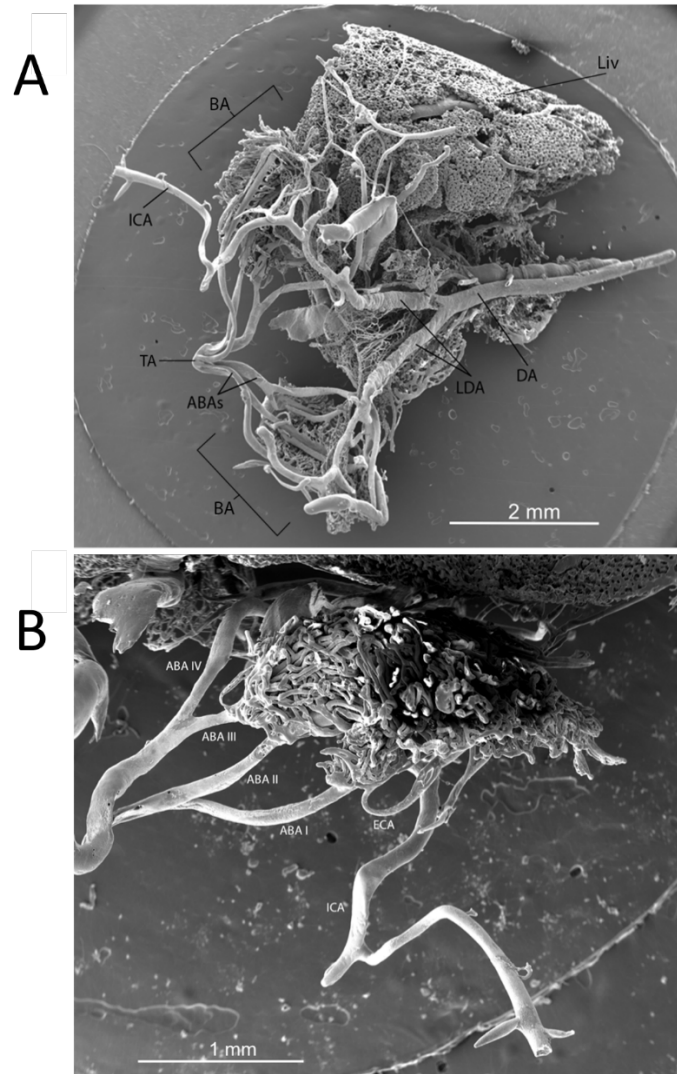


Figure 22. A) Dorsal view of a vascular cast in *Ascaphus truei* showing the vasculature of both the left and right branchial arches (BA). Anterior is left. Blood travels from the truncus arteriosus (TA) and through the afferent branchial arteries (ABA) and into the lateral dorsal aorta and dorsal aorta (LDA/DA). B) A ventral view of a vascular cast of the branchial arches (ABA I-IV). Bottom is anterior and medial is left. Note that only the first three branchial arches bear gills that extend ventrally from the branchial arches. Efferent branchial artery I supplies the external carotid artery (ECA).

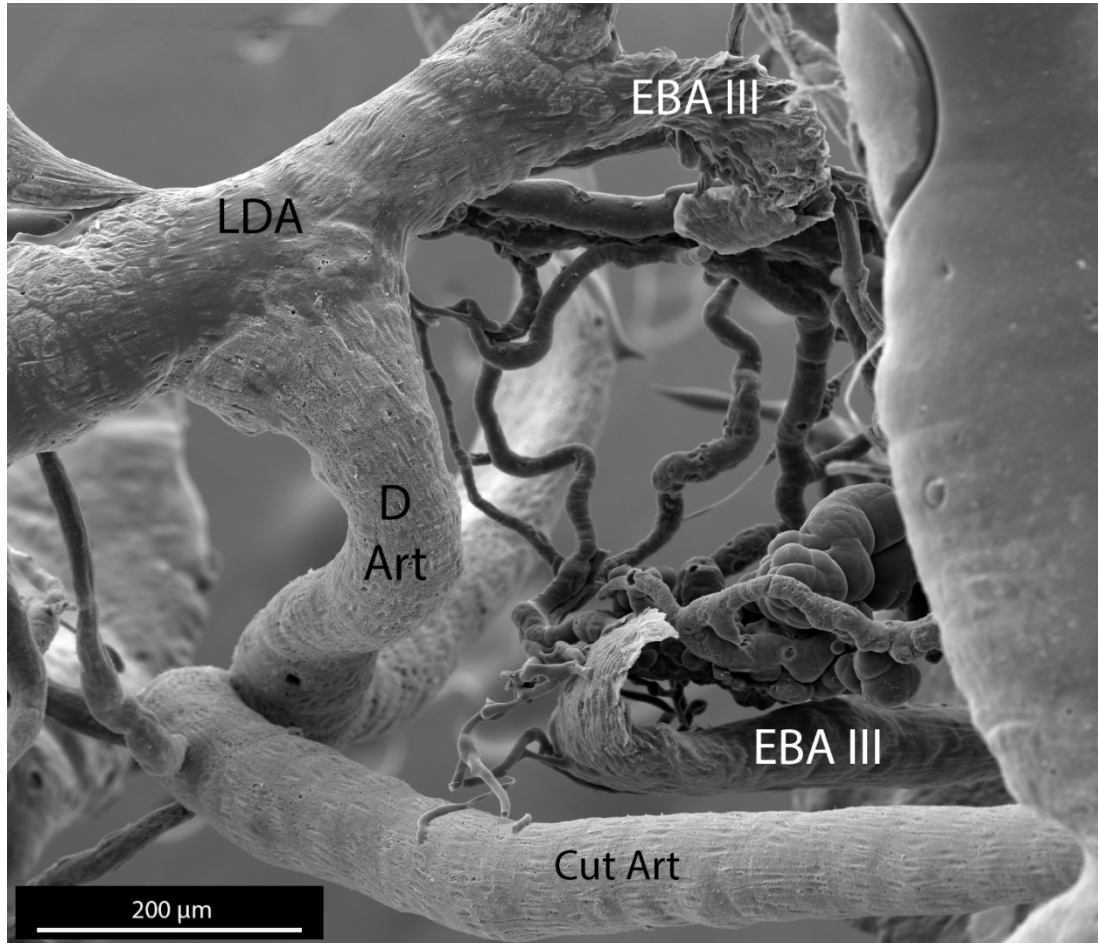


Figure 23. Scanning electron micrograph of the right branchial arch IV in *Ascapheus truei*. Dorsal is top and lateral is right. Afferent branchial artery IV meets the lateral dorsal aorta (LDA) via the ductus arteriosus (D Art). A large cutaneous artery (Cut Art) splits from afferent branchial artery IV and travels laterally and anteriorly. The vessel of efferent branchial artery III (EBA III) did not fill completely.

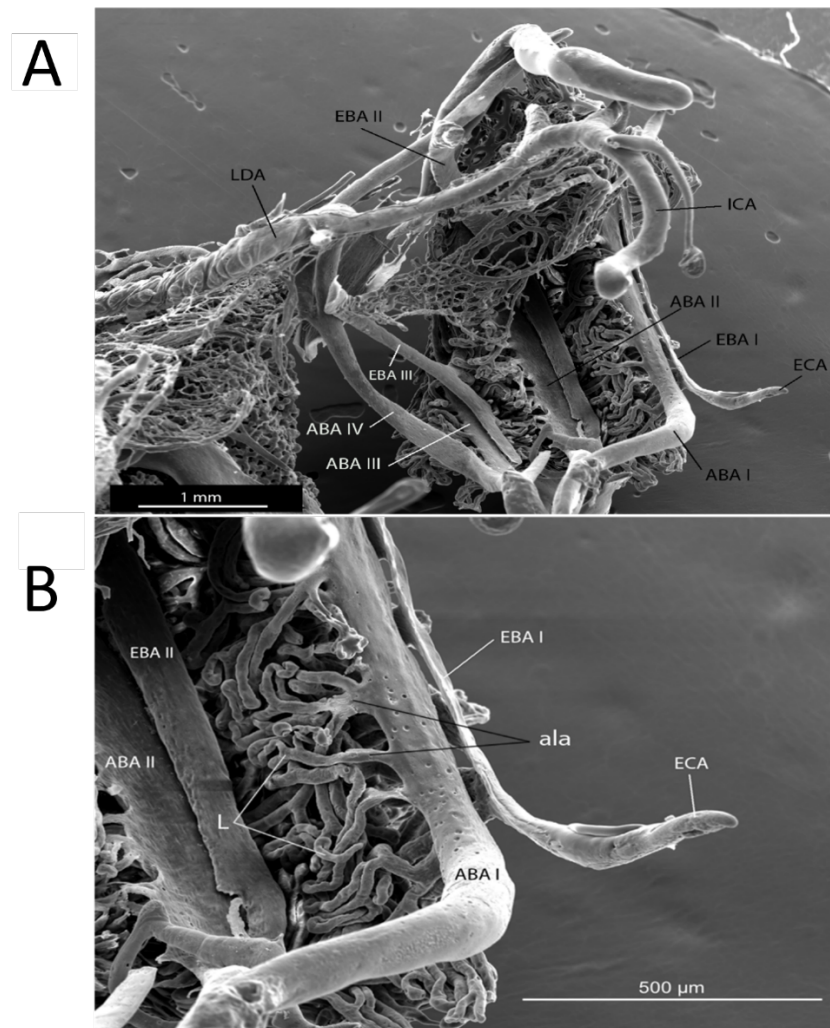


Figure 24. Scanning electron micrograph of a vascular cast in *Ascaphus truei*. A) Dorsal view of the left branchial arches. Anterior is right and lateral is top. The afferent branchial arteries (ABA I-IV) distribute blood laterally through the arches. Blood is drained from the arches via the EBAs and travel dorsally to meet drain into the lateral dorsal aorta (LDA). B) A closer view of ABA I. Note the afferent lamellar arteries branching posteriorly from ABA I and supplying the ventrally positioned lamellae (L) with blood. The external carotid artery (ECA) emerges medially off EBA I and travels anteriorly to supply the head with blood.

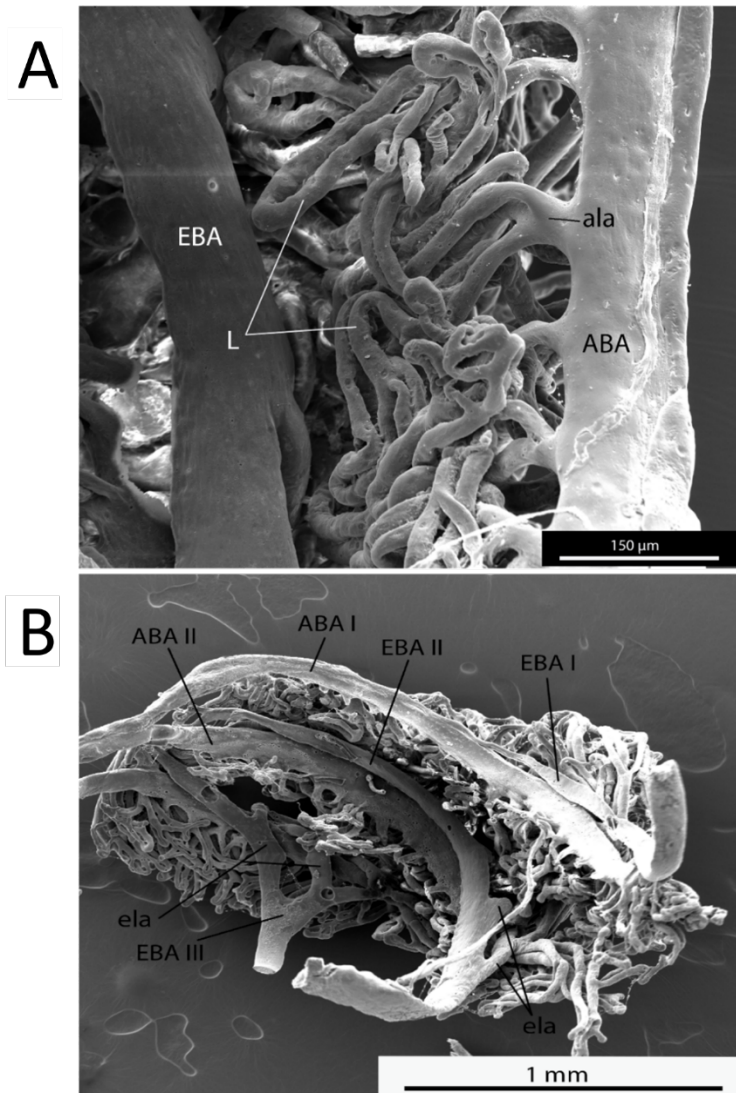


Figure 25. Scanning electron micrograph of a vascular casts in *Ascapthus truei*. A) Dorsal view of the vessels of branchial arch I. Anterior is right and dorsal is top. The afferent branchial artery (ABA) supplies a series of roughly 20 afferent lamellar arteries (ala) that emerge posteriorly. B) Dorsal view of the vessels of branchial arches I-III. Anterior is top and medial is left. The efferent lamellar arteries (ela) merge with each other before reaching the efferent branchial arteries, resulting in fewer efferent lamellar arteries than afferent lamellar arteries.



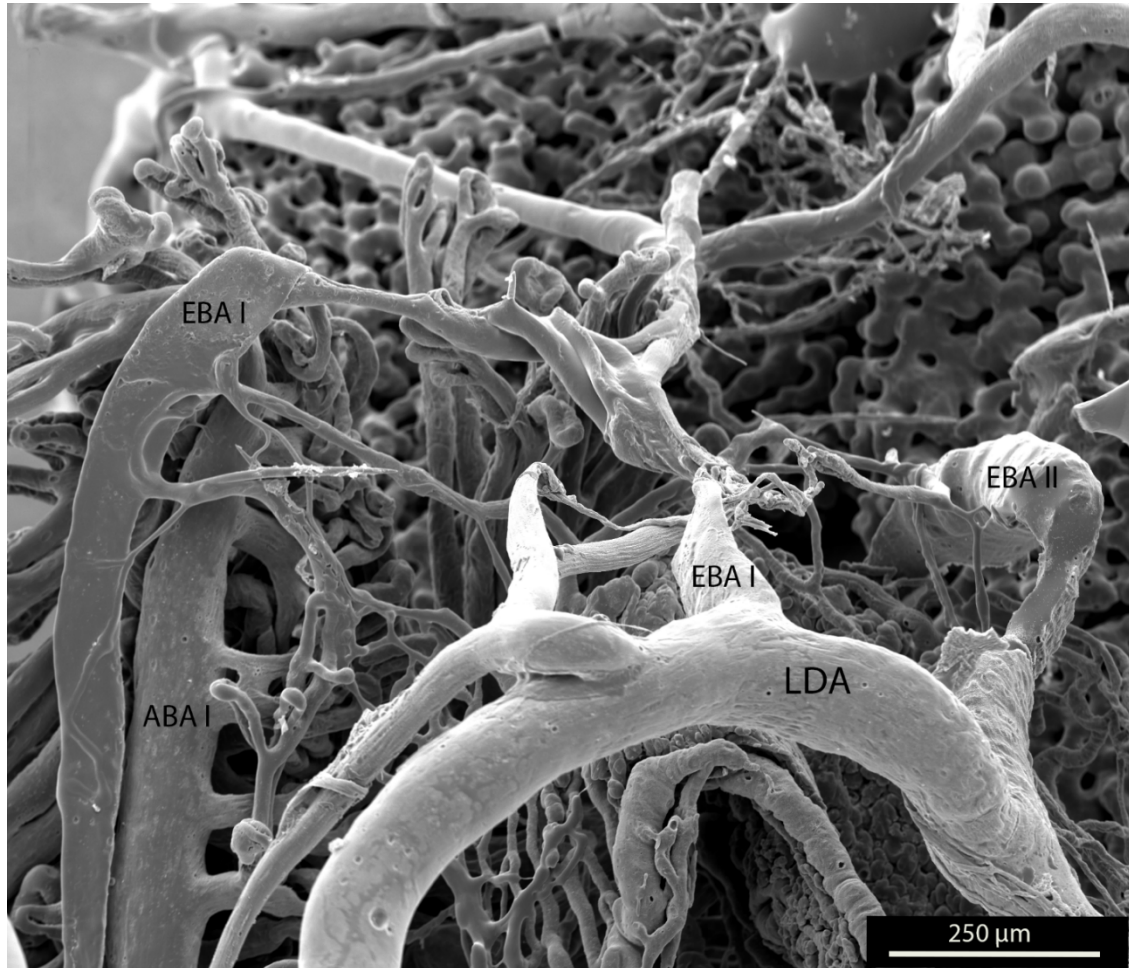


Figure 26. Scanning electron micrograph of the left efferent branchial artery I (EBA I) in *Ascaphus truei*. Anterior is to the left and medial is bottom. Many convoluted vessels leave efferent branchial artery I and eventually connect to the lateral dorsal aorta (LDA). Efferent branchial artery II connects to the lateral dorsal aorta just posteriorly to efferent branchial artery I.

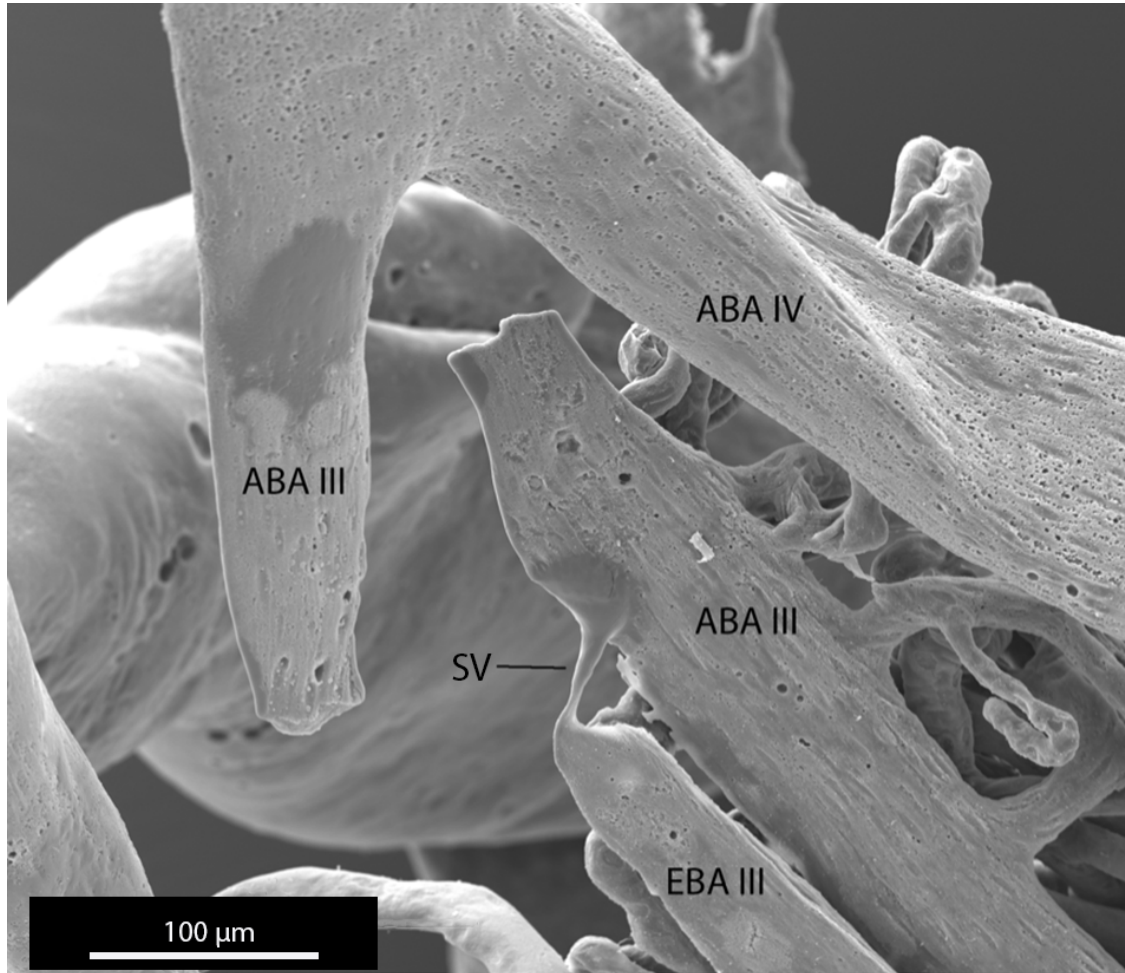


Figure 27. Scanning electron micrograph of the vasculature of branchial arches III and IV (ABA III and ABA IV) in *Ascapbus truei*. A single shunt vessel (SV) was found at the base of efferent branchial artery III, directly connecting afferent and efferent branchial arteries III (ABA III and EBA III). This is the only shunt vessel that was found, however, and is likely an artifact of the vascular casting process. Afferent branchial artery III is broken from the main branch.

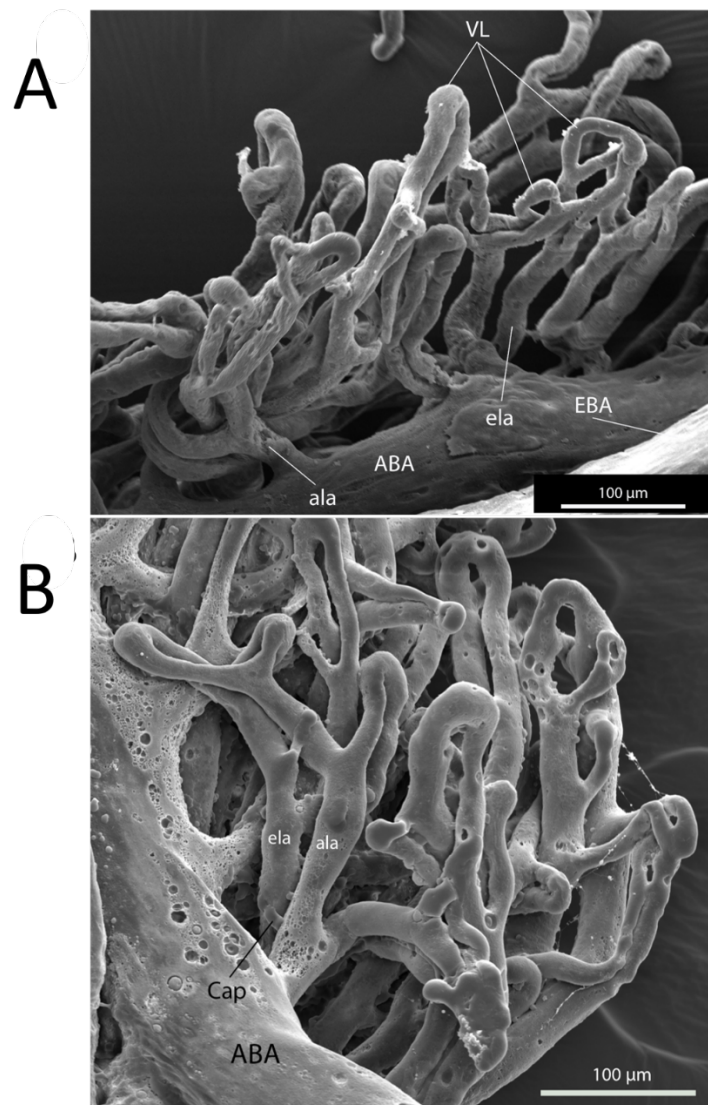


Figure 28. A) Scanning electron micrograph of the vasculature of branchial arch II in *Ascapthus truei*. Ventral is top and medial is right. Notice that at the distal end of the gill lamellae are multiple vascular loops. B) Scanning electron micrograph of the vasculature of a gill lamella. Anterior is left and lateral is top. The afferent branchial arteries (ABA) supply the gill lamella with blood. The afferent lamellar arteries (ala) are drained via the efferent lamellar arteries (ela). Note the small capillary vessel (Cap) connecting the efferent and afferent lamellar arteries.



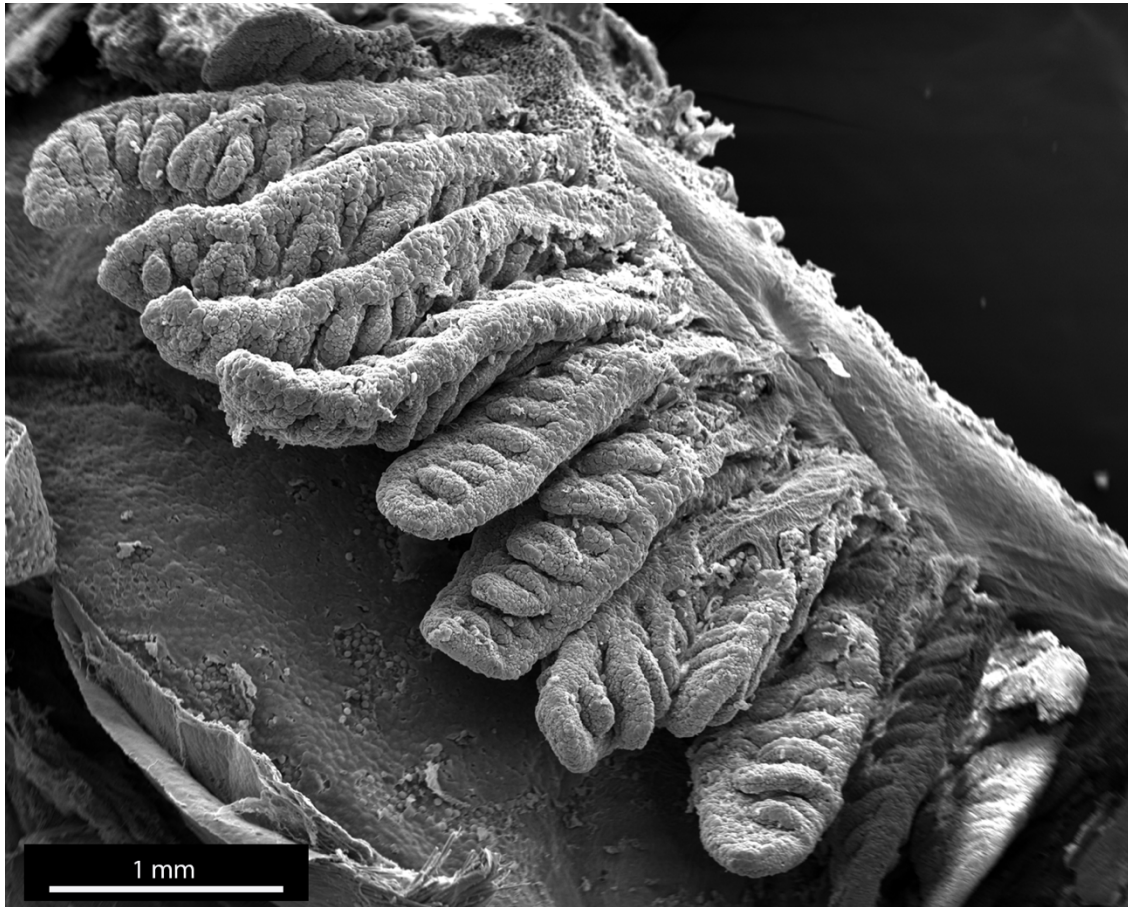


Figure 29. Scanning electron micrograph of the dissected out hyoidean gill in *Protopterus* sp. Notice that the lamellae on this arch are unpaired.

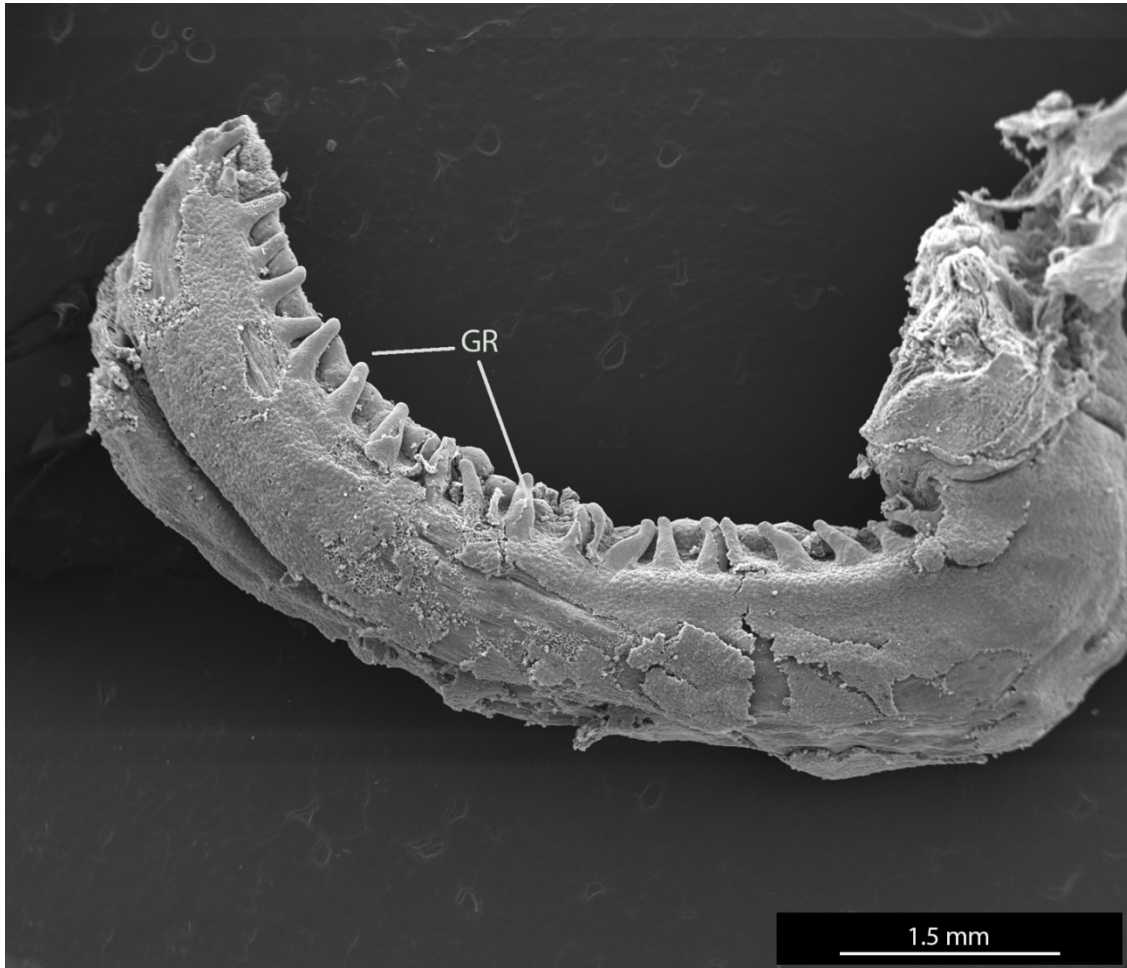


Figure 30. Scanning electron micrograph of dissected out branchial arch III in *Protopterus* sp. The arch possesses gill rakers (GR) but has no gill lamellae.

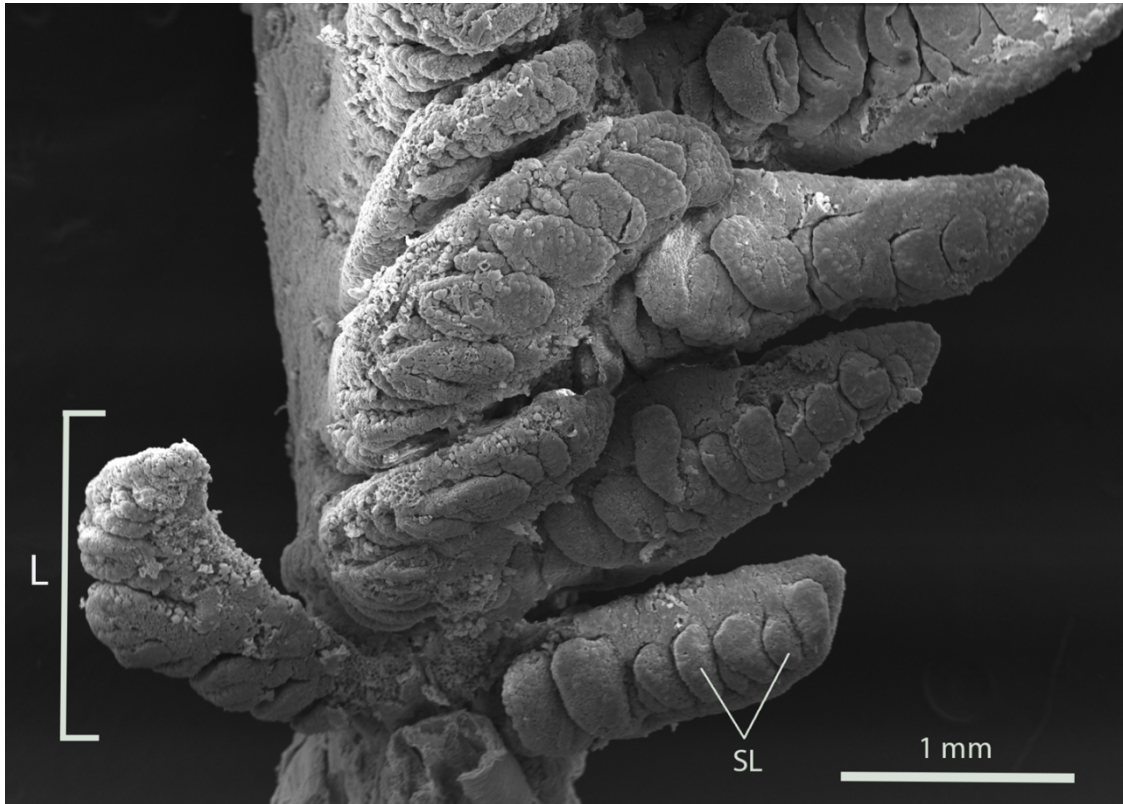


Figure 31. Scanning electron micrograph of dissected out gill arch IV, ventral view.

The gills of *Protopterus* sp. have paired lamellae (L) that develop rows of secondary lamellae (SL) along each lamella.



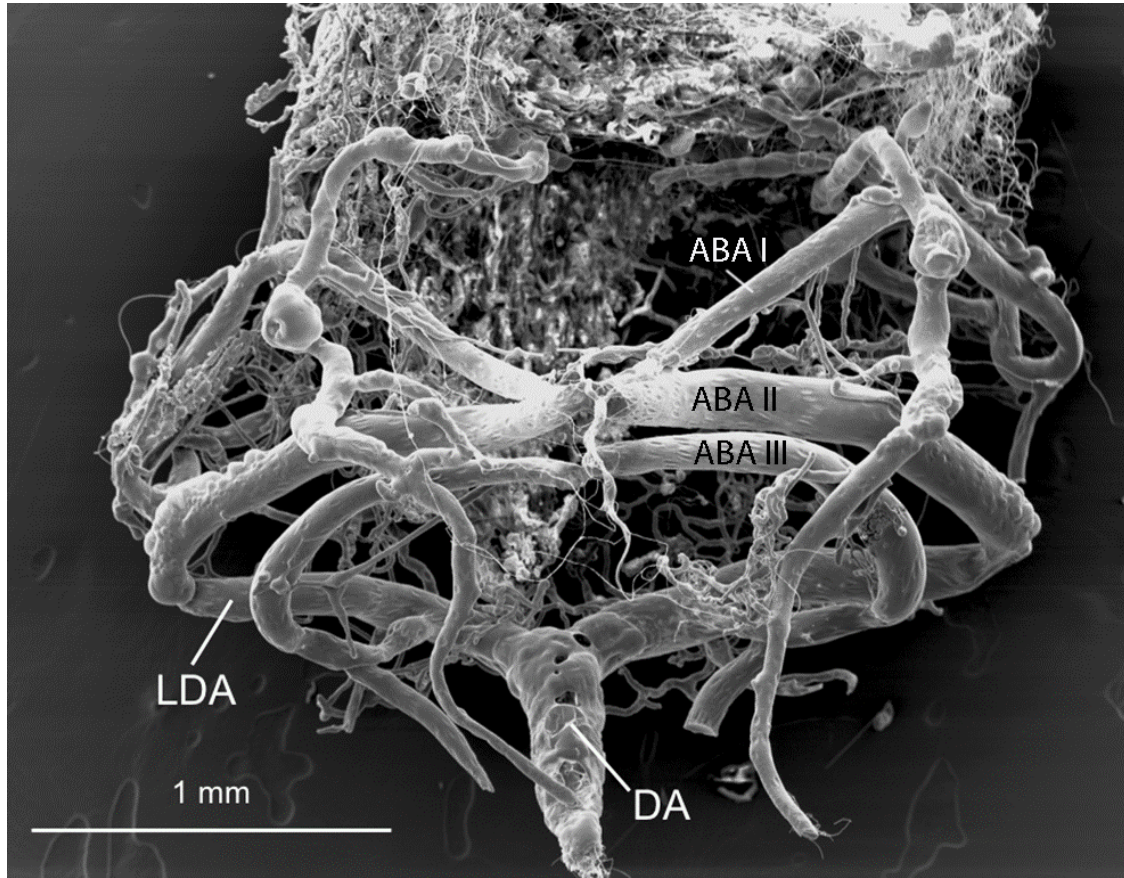


Figure 32. Scanning electron micrograph of vascular cast of the gill arches in *Lepidosiren paradoxa*, ventral view. Anterior is top. The afferent branchial arteries (ABA I-III) drain into the lateral dorsal aorta (LDA) and drain into the dorsal aorta (DA).

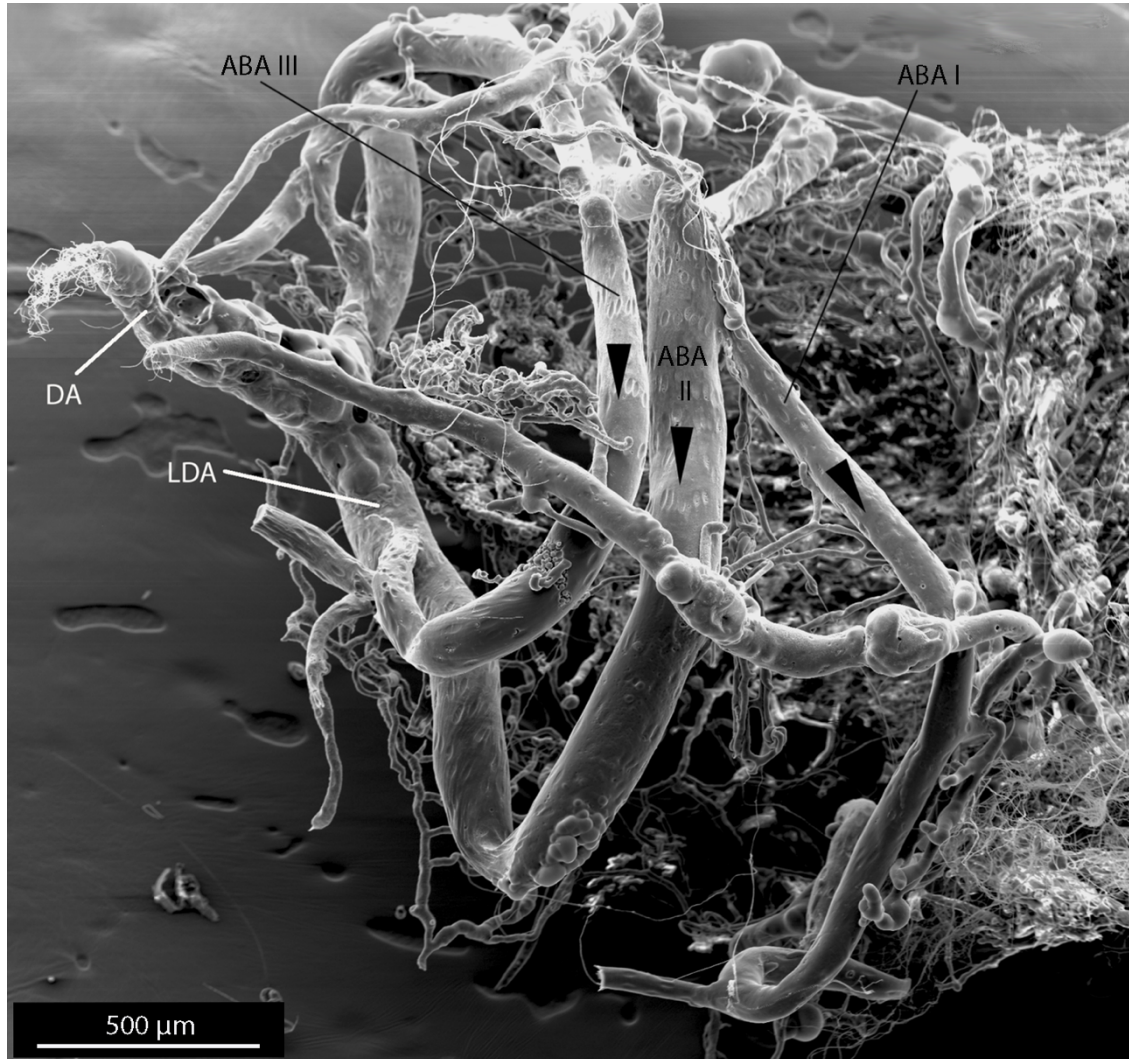


Figure 33. Scanning electron micrograph of vascular cast of a lateral view of the left gill arches in *Lepidosiren paradoxa*. Anterior is right and dorsal is down. The black arrows indicate the direction of blood flow in the aortic arches. Notice that the afferent branchial arteries (ABA I-III) are undifferentiated and don't have distinct efferent branchial arteries. Also visible are the lateral dorsal aorta (LDA) and the dorsal aorta (DA).





Figure 34. Light microscopy cross section of the anterior portion of the left gill arches in *Lepidosiren paradoxa*. Dorsal is top and medial is left. Notice the ceratobranchials (CB I-III) are entirely surrounded by epithelial tissue and never develop interbranchial septa. The heart (H) and afferent branchial arteries are also visible.

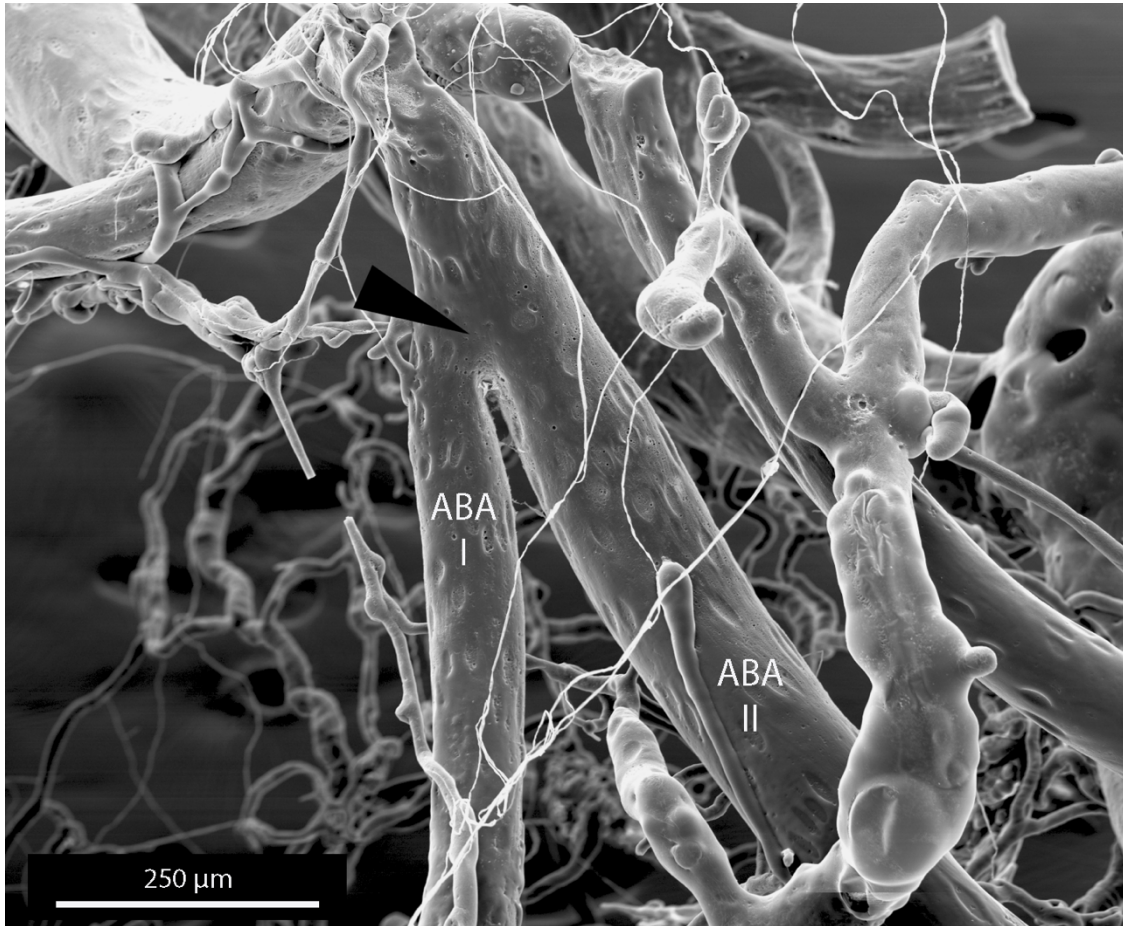


Figure 35. Scanning electron micrograph of the right afferent branchial arteries I and II (ABA I, ABA II) in *Lepidosiren paradoxa*. Ventral is top and anterior is left. The black arrowhead indicates the common stem the two vessels emerge from.

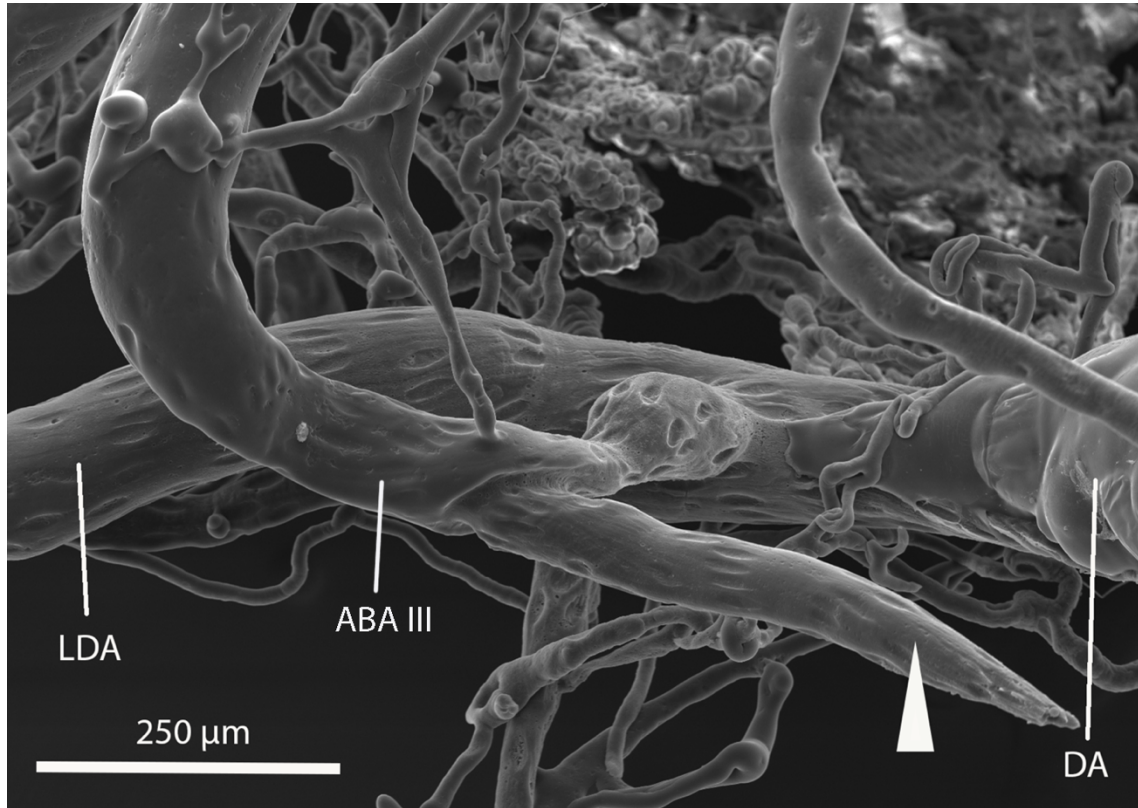


Figure 36. Scanning electron micrograph of afferent branchial artery III in *Lepidosiren paradoxa* (ABA III). Another vessel, indicated by the arrowhead, emerges from ABA III close to the lateral dorsal aorta (LDA) and continues posteriorly, likely being the pulmonary artery.



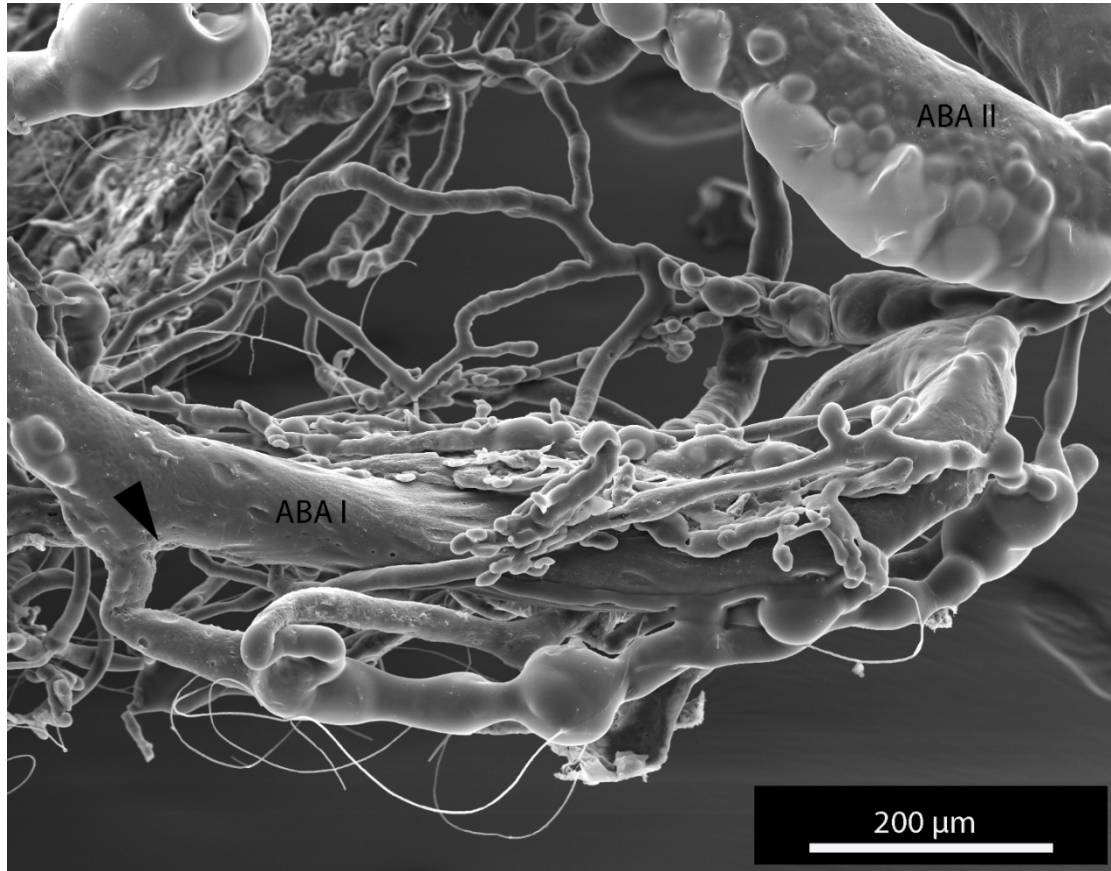


Figure 37. Scanning electron micrograph of afferent branchial arteries I and II (ABA I, ABA II) on the right side in *Lepidosiren paradoxa*. Ventral is top and anterior is left. The black arrow shows an unknown split from the aortic vessel – possibly being the beginning development of the efferent branchial artery.

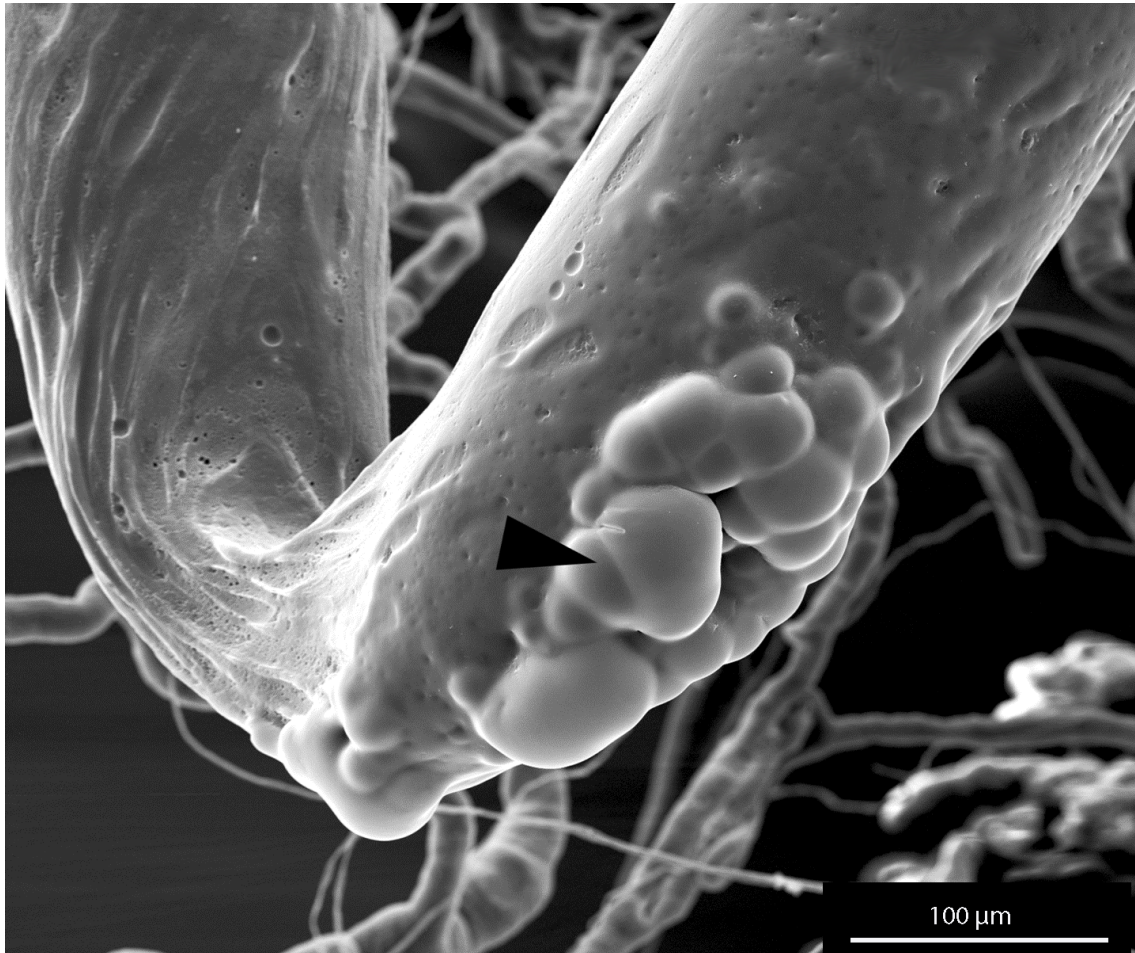


Figure 38. Scanning electron micrograph of the right afferent branchial artery II in *Lepidisoren paradoxus*. Ventral is top and anterior is left. The black arrowhead indicates unknown bulbous protuberances emerging from vessel.

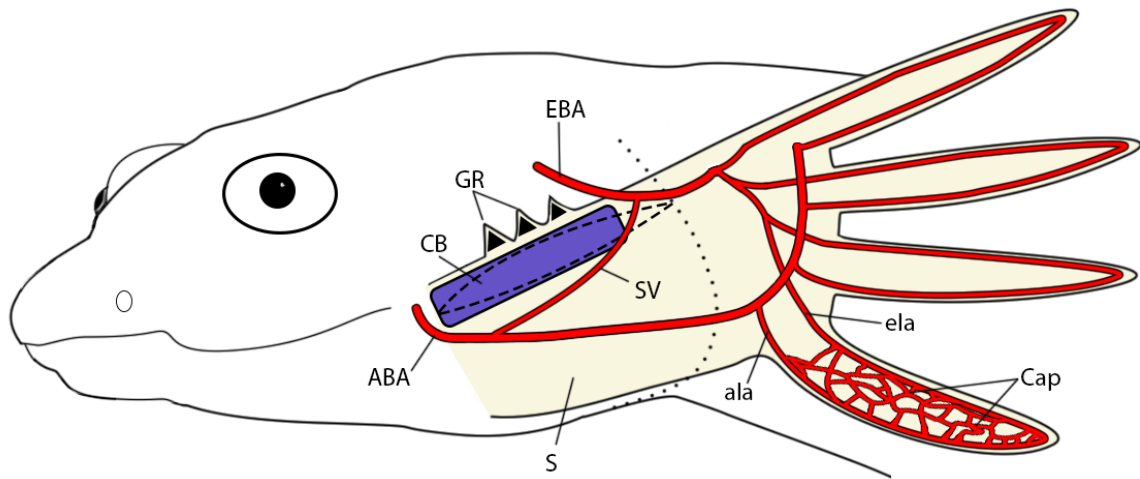


Figure 39. Diagrammatic lateral view of a gill arch and its associated vasculature in *Dicamptodon tenebrosus* (only four of about 20 lamellae are shown). The afferent branchial artery (ABA) enters the arch anteriorly, then travels posteriorly and supplies the ventral and posterior lamellae with blood. Several efferent lamellar arteries (ela) merge into a common vessel before draining into the efferent branchial artery (EBA). The efferent branchial artery is only in the posterior portion of the arch and drains the arch dorsally. Dashed lines indicate the position of the gill slit and the dotted line indicates the posterior edge of the gular fold.

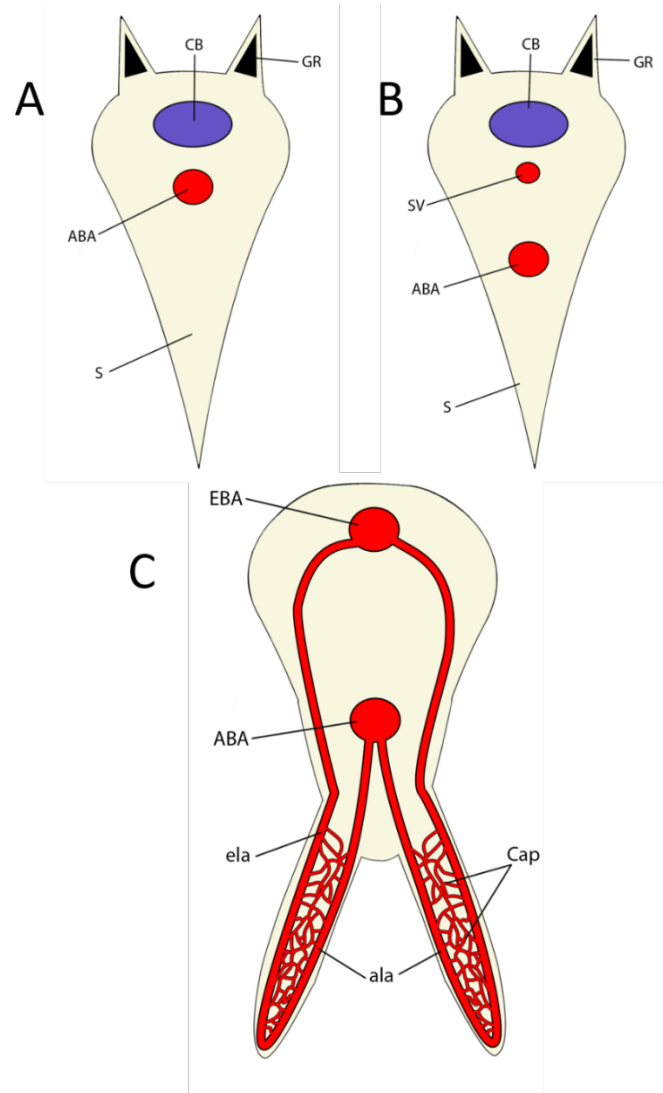


Figure 40. Diagrammatic cross sections of the anterior, middle, and posterior regions of a branchial arch in *Dicamptodon tenebrosus*. A) Anteriorly, interbranchial septum (S) is elongated ventrally. The afferent branchial artery (ABA) remains close to the ceratobranchial (CB). B) In the middle of the arch, the shunt vessel (SV) has split dorsally from the afferent branchial artery and travels along the ceratobranchial. C) The posterior portion of the arch is the only part of the arch that has lamellae; it sticks out into the external environment and lacks the ceratobranchial. EBA, Efferent branchial artery.

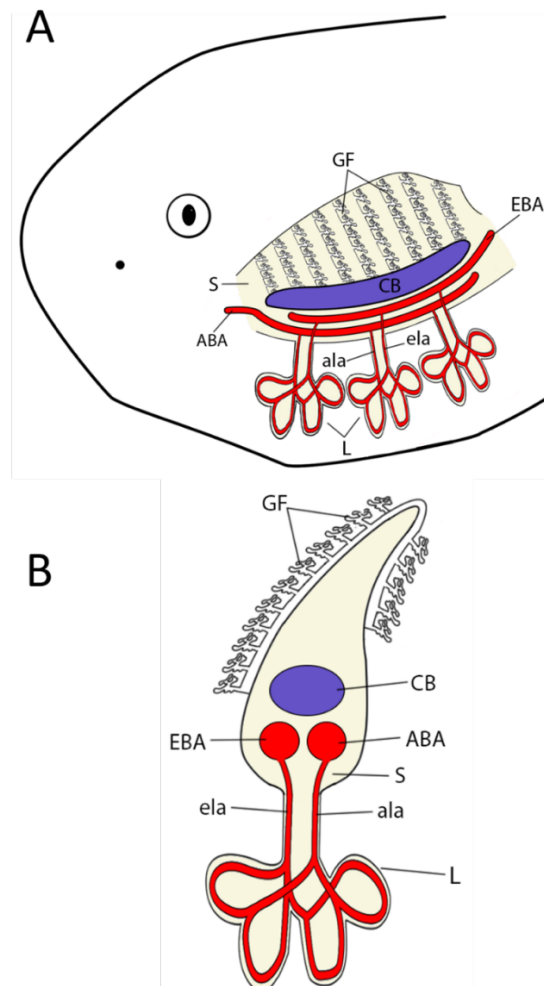


Figure 41. Diagrammatic drawings of a branchial arch in *Ascapthus truei*. A) Lateral view with anterior to the left. Only one gill arch and a few of the lamellae are shown. B) Cross section of a branchial arch. The interbranchial septum (S) is reduced, allowing the lamellae to develop directly off the ventral side of the arch. Dorsally, the arch contains rows of gill filters (GF). The afferent branchial artery (ABA) gives rise to a series of afferent lamellar arteries (ala) that bifurcate distally. Blood loops back into the branchial arch via efferent lamellar arteries (ela) and is collected by the efferent branchial artery (EBA).

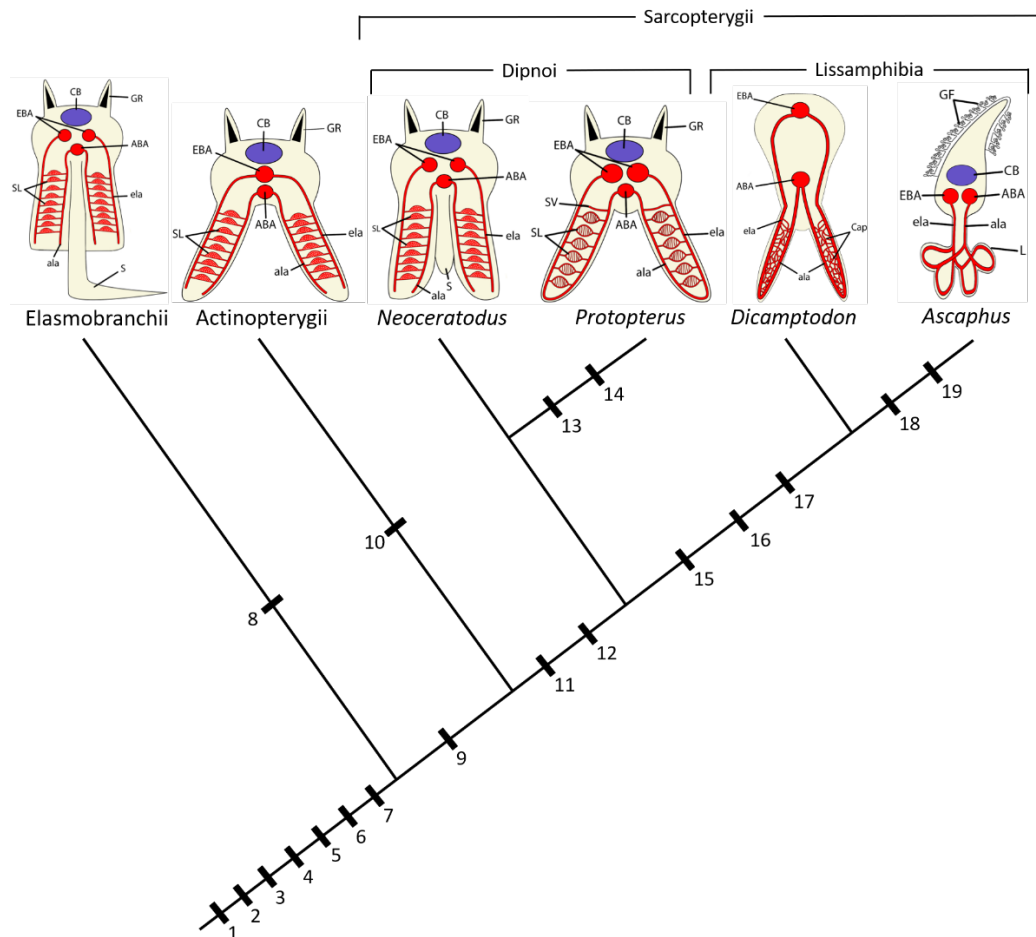


Figure 42. A general phylogeny of vertebrates, mapping the evolution of gills within Elasmobranchii, Actinopterygii, and Sarcopterygii. 1) Internal gills present. 2) Well-developed interbranchial septa. 3) Ceratobranchials present. 4) Primary lamellae paired. 5) Secondary lamellae with pillar cells. 6) Pavement cells present. 7) Mitochondria-rich cells present. 8) Interbranchial septa supported by gill rays. 9) Lamellae supported by gill rays. 10) Interbranchial septa reduced. 11) Ciliary cells present. 12) Loss of gill rays. 13) Loss of pillar cells. 14) External gills present. 15) Loss of secondary lamellae. 16) Loss of pillar cells. 17) External gills present. 18) Lamellae are unpaired. 19) External gills absent.